BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ARMODAFINIL IN RABBIT PLASMA USING REVERSE PHASE -HPLC

Swetha Vegesna[1*], Chennu MM Prasada Rao[2]

Article History: Received: 22.02.2023 **Revised:** 08.04.2023 **Accepted:** 25.05.2023

Abstract

A simple, Accurate, precise method was developed for the simultaneous estimation of Armodafinil in human plasma was developed and validated. By using Protein Precipitation, the sample preparation was prepared. Chromatogram was run through Agilent C_{18} (150x 4.6 mm, 5µ) Mobile phase containing Buffer KH_2PO_4: Methanol taken in the ratio 55:45 was pumped through column at a flow rate of 0.2ml/min. Buffer used Potassium Phosphate Buffer in this method was buffer. For the separation of Armodafinil Internal Standard [IS] used is Dolutegravir. The Temperature was maintained at 30°C. Optimized wavelength selected was 260.0nm. Retention time of Armodafinil and Internal Standard were found to be 2.728 min and 3.159 min. The standard curve was linear (R2 >0.995) over the concentration range of 0.065-2.6 ng/ml. According to ICH guidelines, each analytical validation parameter was determined. As accuracy, precision, recovery, and other validation parameters were all within the guidelines' constraints, the bioanalytical technique created approach was selective, robust, and reliable. Without any interference from plasma, the peaks generated for the target substance and the internal standard were adequately separated from one another and had a sufficient tailing factor. Therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biological investigations might all greatly benefit from the technique.

Keywords: Armodafinil, RP-HPLC, Rabbit Plasma, Internal Standard, Bio-Analytical method, Validation.

[1*]Research scholar, School of Pharmacy, Raffles university, Neemrana, Rajasthan-301705
[2]Professor & Head, School of Pharmacy, Raffles university, Neemrana, Rajasthan -301705

Email id: [1*]sweth.analysis@gmail.com, [2]chennuprasad12@gmail.com

DOI: 10.31838/ecb/2023.12.s3.389

1. INTRODUCTION

Bioanalytical techniques, employed for the quantitative determination of drugs and their metabolites in biological fluids and creates a specific procedure to enable a coalesce of interest to be identified and at the same time to be quantified in a matrix. A coalesce is measured by several procedures. The choice of analytical procedures involve many considerations, such as: concentration levels, chemical properties of the analyte, specimen matrix, cost of the analysis, experimental speed, quantitative or qualitative measurement, required precision and necessary equipment[2]. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability.

Armodafinil is a 2-[(diphenylmethyl)sulfinyl] acetamide that has R configuration at the sulfur atom. Like its racemate, modafinil, it is used for the treatment of sleeping disorders such as narcolepsy, obstructive sleep apnoea, and shift-work sleep disorder. Peak concentration in the blood later occurs later following administration than with modafinil, so it is thought that armodafinil may be more effective than modafinil in treating people with excessive daytime sleepiness. It has a role as a central nervous system stimulant and a eugeroic. It is an enantiomer of a (S)-modafinil.

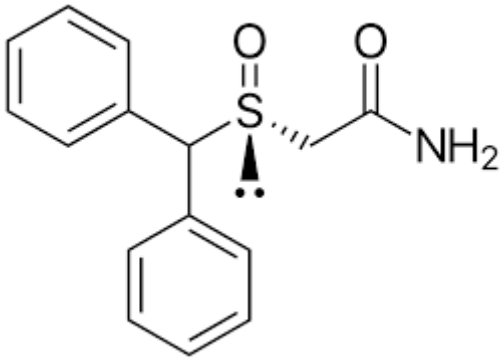

Figure1: Chemical structure of Armodafinil

Experimental Work:

2. MATERIALS AND METHODS:

Armodafinil API was obtained as a gift sample from Jai Ram Biosciences, Kukatpally, Hyderabad, Internal Standard from Akrivis Pharma pvt Ltd. K_2 EDTA control plasmaDeccan Pathological labs, Hyderabad. Water, Acetonitrile, Phosphate buffer, Methanol, Sodium dihydrogen phosphate, Ortho-phosphoric acid was purchased form Rankem, Avantor performance material India limited.

Method Development

Diluent: Based up on the solubility of the drugs, diluent was selected, 0.01N Potassium dihydrogen phosphate and acetonitrile taken in the ratio of 55:45.

Extraction procedure

Take 750µl of plasma and 0.5µl of internal standard, 0.25µl of Armodafinil from the spiking solutions of both into a centrifuging tube and add 1 ml of Acetonitrile go for cyclomixer for 15 sec. Then vertex for 2 min and finally centrifuge for 5 min at 3200 rpm speed. After the centrifugation collect the sample and filter it directly inject 10 µL into HPLC.

Preparation of Armodafinil Spiking Solutions:

From the above Armodafinil stock solution 0.05ml, 0.1ml, 0.15ml, 0.6ml, 1.0ml, 1.2ml, 1.6ml and 2.0 ml was pipette and transferred to 8 individual 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.065 µg/ml, 0.13 µg/ml, 0.195µg/ml, 0.52 µg/ml, 1.3 µg/ml, 1.56 µg/ml, 2.08 µg/ml and 2.6µg/ml.

quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.065 µg/ml, 0.195µg/ml, 1.3µg/ml, 2.08 µg/ml, 2.6 µg/ml, 3.6 µg/ml,

Final concentration: From the above solution, take 0.5ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 10µg/ml ISD concentration

Validation Methodology in bioanalytical method

System Suitability Parameter

System Suitability test are performed that the test mixture is essential to check the specifications of a liquid chromatographic system. the System suitability testing limits are acceptance criteria that must be prior to sample analysis**.** The test is carried out by injecting six samples of quality control samples of MQC and check the criteria acceptance accordingly as the % CV of the retention time (RT) should be ≤ 2.00 %.

Auto Sampler Carryover

Carry-over is an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument, during validation carry-over should be assessed by analysing blank samples after the calibration standard at the ULOQ. Carry-over in the blank samples following the highest calibration standard should not be greater than 20% of the analyte response at the LLOQ and 5% of the response for the IS.

Specificity and Screening of Biological matrix

Specificity is the ability of a bioanalytical method to detect and differentiate the analyte from other substances, including its related substances (e.g., substances that are structurally similar to the analyte, metabolites, isomer, impurities, degradation products formed during sample preparation or concomitant medications that are expected to be used in the treatment of patients with the intended indication). Specificity is determined by the injecting six samples of standard solution and the LLOQC sample solution and check the % Interference Response of interfering peaks in STD Blk at the retention time of analyte should be ≤20.00 % of that in LLOQ and At least 80 % of the matrix lots (Biological Sample) with intended anticoagulant should be within the acceptance criteria.

Sensitivity

Sensitivity is often interpreted as related to the detection/determination ability, LLOQ based on precision and accuracy (bias) data, this is probably the most practical approach and defines the LLOQ as the lowest concentration of a sample that can still be quantified with acceptable Limit. the sensitivity is performed by injecting six injections of lower concentration of sample (LLOQ) the acceptance criteria of sensitivity of LLOQ are At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %.

Matrix Factor evaluation

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation it is necessary to evaluate the matrix effect between different independent sources/lots. The matrix effect should be evaluated by analysing at least 3 replicates of low and high QCs (LQC and HQC), each prepared using matrix from at least 6 different sources/lots. The accuracy should be within ±15% of the nominal concentration and the precision (per cent coefficient of variation (%CV)) should not be greater than 15% in all individual matrix sources/lots.

Linearity

The relationship between the nominal analyte concentration and the response of the analytical platform to the analyte, Calibration standards, prepared by spiking matrix with a known quantity of analyte, span the calibration range and comprise the calibration curve. Calibration standards should be prepared in the same biological matrix as the study samples. The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method and the % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %. The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Rugged Linearity

Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method and The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %.The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Precision and Accuracy (Intra-day)

Accuracy and precision should be determined by analysing the QCs within each run (within-run) and in different runs (between-run). Accuracy and precision should be evaluated using the same runs and data. The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run the overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Rugged Precision and Accuracy (Inter-Day)

Accuracy and precision should be evaluated using the same runs and data. The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run the overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Recovery

Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Drug The recoveries for Armodafinil at LQC, MQC and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the six samples of LQC, MQC and HQC with the main drug and check the interference with unextracted and extracted, The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Recovery of Internal Standard

The measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with Internal Standards containing the same area with known amount of Drug, the recoveries for IS at 6 replicates the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the six samples and check the interference with unextracted and extracted, The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Reinjection Reproducibility

Reproducibility of the method is assessed by replicate measurements of the QCs and

is usually included in the assessment of precision and accuracy. However, if samples could be reinjected (e.g., in the case of instrument interruptions or other reasons such as equipment failure), reinjection reproducibility should be evaluated and included in the Validation Report or provided in the Bioanalytical Report of the study where it was conducted. The reproducibility was performed by injecting the qc samples in 6 replicates and check the acceptance limits the % mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Stabilities

Stability evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte. The stability is assessed by long term stock solution stability and Matrix samples stability at -28±5 °C for 37 days & -80±5 ^{0}C, stability testing is performed by injecting the QC samples of high and low concentrations (HQC and LQC) with taken biological matrix The mean concentration at each QC level should be within ±15% of the nominal

3. RESULTS AND DISCUSSIONS

Bioanalytical Method Development

Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Armodafinil as per current ICH guidelines

Chromatographic conditions

Mobile phase : KH_2PO_4: Methanol 55:45
Flow rate : 1.0 ml/min
Column : Agilent C_{18} (150mm x 4.6 mm, 5µ)
Detector wavelength : 260nm
Column temperature : 30^{0}C
Injection volume : 10µL
Run time : 6 min

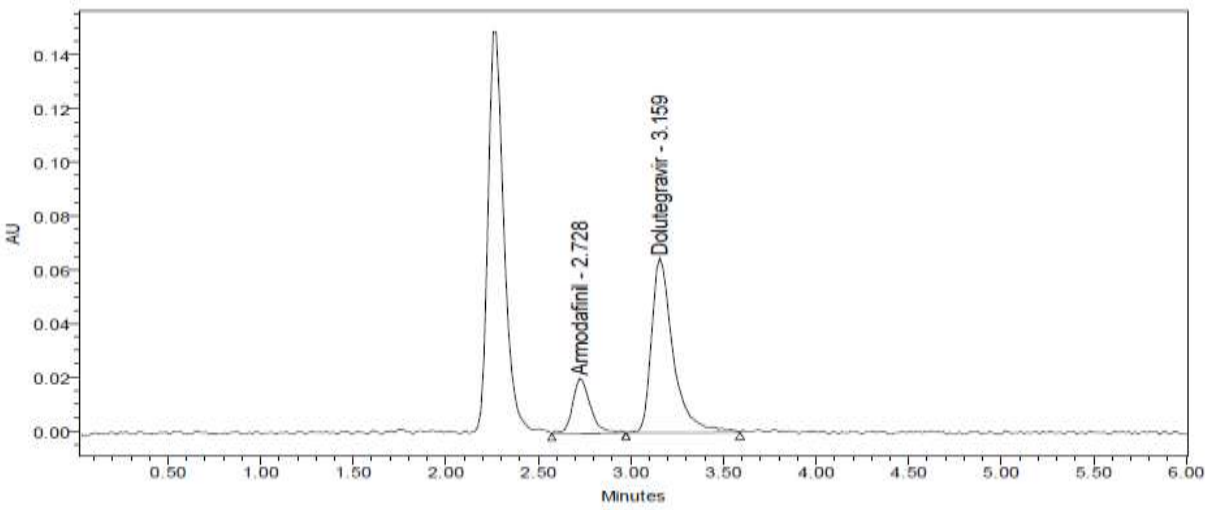

Figure2: optimised method chromatogram

S.No	Peak Name	Rt	Area	USP Plate Number	USP Resolution	USP Tailing	& Area
1	Armodafinil	2.728	642051	3983		1.0	20.66
2	Dolutegravir	3.159	936656	3811	2.2	1.6	79.34

Armodafinil and Internal Standard were eluted at 2.728 min, 3.159min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits

METHOD VALIDATION

System suitability of Armodafinil

This system suitability method is intended to guarantee that the HPLC system is working in such a way that correct and reproducible data may be submitted to regulatory agencies with confidence. This procedure includes signal stability, carryover, and instrument response tests.

S.No	Armodafinil Area	Armodafinil Rt	Dolutegravir (ISTD) Area	Dolutegravir (ISTD) Rt	Area Ratio
1	400513	3.84	935219	3.027	0.4283
2	404733	3.84	931849	3.035	0.4343
3	405496	3.84	933336	3.032	0.4345
4	408754	3.85	936445	3.038	0.4365
5	404962	3.86	934394	3.036	0.4334
6	409608	3.85	932517	3.053	0.4392
Mean		3.848		3.037	0.43436
SD		0.0083		0.0088	0.003652
%CV		0.22		0.29	0.84

Table 1: System suitability of Armodafinil

System Suitability plate count, tailing factor, resolution of Armodafinil was According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The % CV of the retention time (RT) should be ≤ 2.00 %.

Auto sampler carryover of Armodafinil

The carryover was tracked back to the injection valve and eradicated by converting from a partial loop injection to a full loop injection, which allowed more effective cleansing of the sample flow channel. The HPLC system's susceptibility to carryover was shown to be dependent on the detection method's absolute sensitivity and the mass of analyte injected at the assay's lower limit of quantitation (LLOQ).

Sample ID	Peak Area		% Carryover	
	Armodafinil	ISTD	Drug	ISTD
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	178134	487965	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	4765	487652	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	176354	486523	**0.00**	**0.00**
STD Blk	0	0		

LLOQ	4736	486521	N/A	N/A

Table2: Auto sampler carryover of Armodafinil

Specificity and Screening of Biological Matrix

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present

S.No.	Sample ID	Response		% Interference	
		Drug	ISTD	Drug	ISTD
1	STD Blk1	0	0	0.00	0.00
2	LLOQ1	4756	483685		
3	STD Blk2	0	0	0.00	0.00
4	LLOQ2	4763	487632		
5	STD Blk3	0	0	0.00	0.00
6	LLOQ3	4796	487632		
7	STD Blk4	0	0	0.00	0.00
8	LLOQ4	4746	487632		
9	STD Blk5	0	0	0.00	0.00
10	LLOQ5	4738	487632		
11	STD Blk6	0	0	0.00	0.00
12	LLOQ6	4796	487632		

Table 3: Specificity and Screening of Biological Matrix of Armodafinil

We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

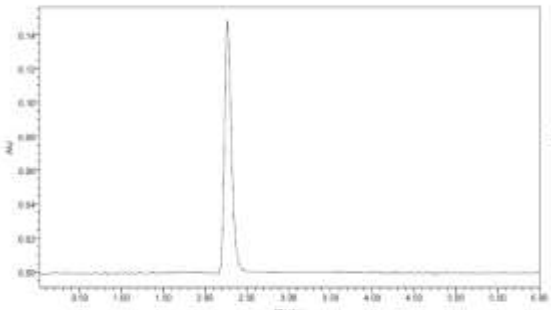

Figure 3 Blank Plasma Sample Chroamtogram

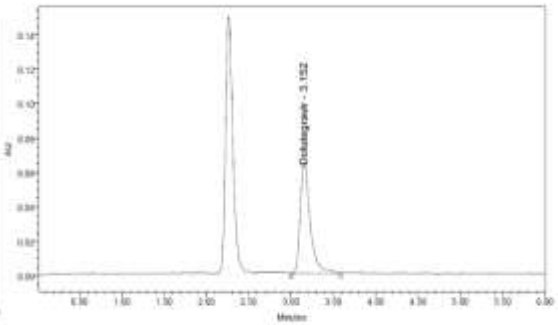

Figure4 Blank Plasma with Internal Standard Sample

The response areas obtained of analyte and internal standard are less than 20% and 5 % of LLOQ Area. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific

Sensitivity

A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ

Replicate No.	**LLOQ**
	Nominal Concentration (ng/mL)
	0.150
	Nominal Concentration Range (ng/mL)
	(0.120-0.180)
	Calculated Concentration (ng/mL)
1	0.154
2	0.139
3	0.163
4	0.174
5	0.126
6	0.144
Mean	0.1500
SD	0.01729
% CV	**11.52**
% Mean Accuracy	**100.00**

Table 4: Sensitivity of Armodafinil

Matrix factor evolution

P&A ID		**HQC**	**MQC1**	**LQC**	**LLOQ QC**
		Nominal Concentration (µg/mL)			
		2.080	**1.300**	**0.195**	**0.065**
		Nominal Concentration Range (µg/mL)			
		(1.768-2.392)	**(1.105-1.495)**	**(0.166-0.224)**	**(0.052-0.078)**
		Calculated Concentration (µg/mL)			
Different Column		1.795	1.152	0.169	0.056
		1.895	1.199	0.172	0.060
		1.925	1.254	0.185	0.063
		2.126	1.295	0.195	0.068
		2.192	1.312	0.214	0.070
		2.310	1.471	0.221	0.072
Mean		**2.0405**	**1.2805**	**0.1927**	**0.0648**
SD		**0.19883**	**0.11077**	**0.02149**	**0.00621**
% CV		**9.74**	**8.65**	**11.15**	**9.58**
% Mean Accuracy		**98.10**	**98.50**	**98.80**	**99.74**
Different Analyst		1.785	1.185	0.169	0.053
		1.895	1.196	0.175	0.058
		1.912	1.235	0.181	0.063
		1.974	1.281	0.189	0.066
		2.288	1.312	0.215	0.071
		2.391	1.492	0.221	0.074

Mean	**2.0408**	**1.2835**	**0.1917**	**0.0642**
SD	**0.24145**	**0.11314**	**0.02153**	**0.00788**
% CV	**11.83**	**8.81**	**11.23**	**12.29**
% Mean Accuracy	**98.12**	**98.73**	**98.29**	**98.72**

Table no 6: Matrix factor evaluation (absence of matrix factor)

Linearity:

Acquisition Batch ID	**STD1**	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	0.065	0.130	0.195	0.520	1.300	1.560	2.080	2.600
	Nominal Concentration Range (ng/mL)							
	(0.052-0.078)	(0.111-0.150)	(0.166-0.224)	(0.442-0.598)	(1.105-1.495)	(1.326-1.794)	(1.768-2.392)	(2.210-2.990)
	Back Calculated Concentration (ng/mL)							
P&A1	0.058	0.115	0.185	0.482	1.115	1.336	1.825	2.374
P&A2	0.063	0.133	0.192	0.518	1.357	1.521	2.078	2.510
P&A3	0.071	0.140	0.205	0.549	1.425	1.783	2.312	2.902
n	3	3	3	3	3	3	3	3
Mean	0.0640	0.1293	0.1940	0.5163	1.2990	1.5467	2.0717	2.5953
SD	0.00656	0.01290	0.01015	0.03353	0.16294	0.22460	0.24356	0.27415
%CV	10.25	9.97	5.23	6.49	12.54	14.52	11.76	10.56
% Mean Accuracy	98.46	99.49	99.49	99.29	99.92	99.15	99.60	99.82

Table 7: Linearity of Armodafinil

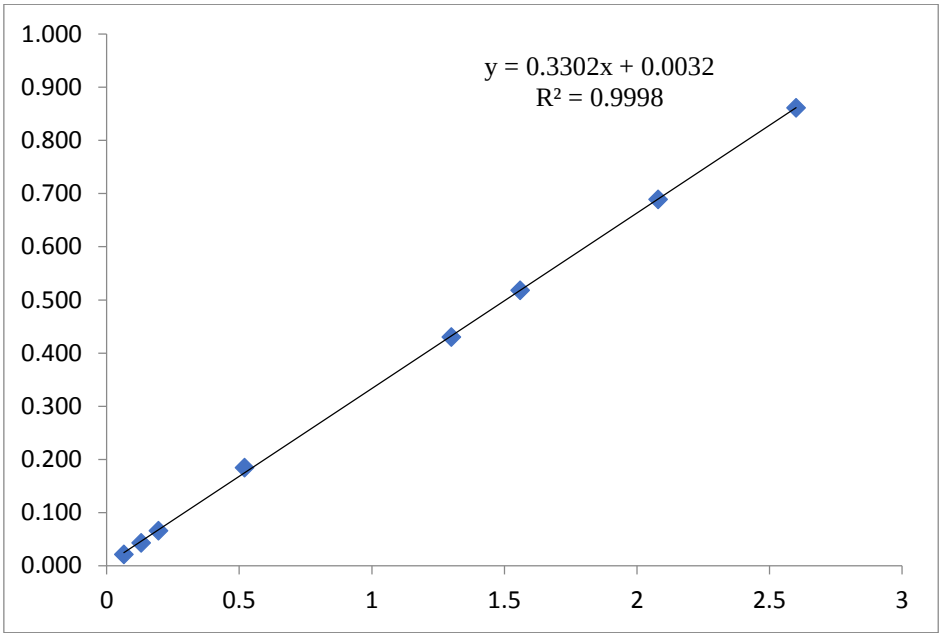

Figure. 5 Representative Calibration Curve for Regression Analysis

Precision and accuracy (intra-day runs of Armodafinil)

Acquisition Batch ID	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	2.080	**1.300**	**0.195**	**0.065**
	Nominal Concentration Range (ng/mL)			
	(1.768-2.392)	(1.105-1.495)	(0.166-0.224)	(0.052-0.078)
	Back Calculated Concentration (ng/mL)			
	1.795	1.154	0.169	0.055
	1.892	1.195	0.172	0.059
	1.962	1.212	0.187	0.060
	2.125	1.264	0.198	0.062
	2.214	1.398	0.210	0.075
	2.321	1.483	0.223	0.078
Mean	2.0515	1.2843	0.1932	0.0648
SD	0.20183	0.12884	0.02129	0.00937

%CV	9.84	10.03	11.02	14.45
% Mean Accuracy	98.63	98.79	99.06	99.74
	1.768	1.136	0.177	0.053
	1.786	1.145	0.185	0.059
	1.952	1.268	0.189	0.061
	2.139	1.385	0.192	0.066
	2.244	1.391	0.201	0.072
	2.315	1.465	0.219	0.078
n	**6**	**6**	**6**	**6**
Mean	2.0339	1.2983	0.1938	0.0648
SD	0.23365	0.13759	0.01465	0.00911
%CV	11.49	10.60	7.56	14.05
% Mean Accuracy	97.78	99.87	99.40	99.74
	1.796	1.129	0.169	0.055
	1.865	1.265	0.172	0.059
	1.994	1.295	0.185	0.063
	2.085	1.318	0.192	0.065
	2.115	1.365	0.214	0.069
	2.385	1.412	0.221	0.072
Mean	2.0400	1.2973	0.1922	0.0638
SD	0.20926	0.09751	0.02146	0.00627
%CV	10.26	7.52	11.17	9.83
% Mean Accuracy	98.08	99.79	98.55	98.21
Between Batch Precision and Accuracy				
Mean	18	18	18	18
SD	2.0418	1.2933	0.1931	0.0645

%CV	0.20242	0.11528	0.01823	0.00788
% Mean Accuracy	9.91	8.91	9.44	12.21

Table no8: precision data for intra-day runs of Armodafinil

Rugged Precision and Accuracy (inter-day runs of Armodafinil)

P&A ID		**HQC**	**MQC1**	**LQC**	**LLOQ QC**
		Nominal Concentration (ng/mL)			
		4.800	**3.000**	**0.450**	**0.150**
		Nominal Concentration Range (ng/mL)			
		(4.080-5.520)	(2.550-3.450)	(0.383-0.518)	(0.120-0.180)
		Calculated Concentration (ng/mL)			
Different Column		4.183	2.623	0.395	0.129
		4.452	2.952	0.415	0.132
		4.632	2.573	0.426	0.145
		4.852	3.152	0.470	0.152
		5.126	3.251	0.475	0.162
		5.365	3.321	0.512	0.178
Mean		**4.7683**	**2.9787**	**0.4488**	**0.1497**
SD		**0.43640**	**0.32037**	**0.04402**	**0.01855**
% CV		**9.15**	**10.76**	**9.81**	**12.40**
% Mean Accuracy		**99.34**	**99.29**	**99.74**	**99.78**
Different Analyst		4.187	2.552	0.392	0.132
		4.456	2.877	0.399	0.136
		4.623	2.937	0.413	0.139
		4.825	3.120	0.445	0.148
		5.162	3.228	0.489	0.156
		5.325	3.181	0.515	0.174
Mean		**4.7630**	**2.9825**	**0.4422**	**0.1475**
SD		**0.43000**	**0.25184**	**0.05047**	**0.01562**
% CV		**9.03**	**8.44**	**11.41**	**10.59**
% Mean Accuracy		**99.23**	**99.42**	**98.26**	**98.33**

Table no 9: precision data for inter-day runs of Armodafinil

Recovery of Armodafinil-

Acquisition Batch ID						
Replicate No.	**HQC**		**MQC1**		**LQC**	
	Un extracted Response	**Extracted Response**	**Un extracted Response**	**Extracted Response**	**Un extracted Response**	**Extracted Response**
1	147733	144077	91409	90396	13921	13869
2	148405	148841	91176	90379	13845	13804

3	143539	142713	91727	90872	13758	13870
4	147115	147096	91322	90120	13868	13756
5	145983	142915	90972	90745	14037	13983
6	142321	146531	90871	90208	14040	13997
n	**6**	**6**	**6**	**6**	**6**	**6**
Mean	145849	145362	91246	90453	13912	13880
SD	2428.42	2495.32	311.23	296.80	111.56	95.57
% CV	**1.67**	**1.72**	**0.34**	**0.33**	**0.80**	**0.69**
% Mean Recovery	**99.67**		**99.13**		**99.77**	
Overall % Mean Recovery	**99.523**					
Overall SD	**0.3437**					
Overall % CV	**0.35**					

Table no 10: Recovery of Armodafinil

Recovery - Internal standard

Acquisition Batch ID	**Date**	
S.No.	**Un extracted Area Ratio**	**Extracted Area Ratio**
1	556372	554111
2	559636	550373
3	556452	555743
4	559592	552130
5	558084	557544
6	554347	555372
n	6	6
Mean	557413.8	554212.2
SD	2075.94	2602.87
% CV	0.37	0.47
% Mean Recovery	**99.43**	

Table no 11: Recovery of Dolutegravir (IS)

Rugged Linearity:

Ruggedness Linearity								
Analyte	Armodafinil					**ISTD**	Dolutegravir	
P&A ID	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	0.150	0.300	0.450	1.200	3.000	3.600	4.800	6.000
	Nominal Concentration Range (ng/mL)							
Different column	(0.120 - 0.180)	(0.255 - 0.345)	(0.383 - 0.518)	(1.020 - 1.380)	(2.550-3.450)	(3.060-4.140)	(4.080-5.520)	(5.100-6.900)

	Calculated Concentration (ng/mL)							
	0.158	0.310	0.465	1.225	3.032	3.621	4.823	6.123
Different Analyst	Acquisition Batch ID						Date	
	0.179	0.322	0.479	1.834	3.214	3.963	4.935	6.724

Table no 12: Rugged Linearity of Armodafinil

Reinjection Reproducibility

P&A ID	**HQC**	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (µg/mL)			
	2.080	1.300	0.195	0.065
	Nominal Concentration Range (µg/mL)			
	(1.768-2.392)	(1.105-1.495)	(0.166-0.224)	(0.052-0.078)
	Calculated Concentration (µg/mL)			
P&A01	1.799	1.115	0.169	0.056
	1.821	1.169	0.174	0.059
	1.862	1.268	0.189	0.063
	2.193	1.285	0.191	0.066
	2.298	1.398	0.214	0.071
	2.390	1.482	0.220	0.073
n	**6**	**6**	**6**	**6**
Mean	**2.0605**	**1.2862**	**0.1928**	**0.0647**
SD	**0.26370**	**0.13727**	**0.02062**	**0.00665**
% CV	**12.80**	**10.67**	**10.70**	**10.29**
% Mean Accuracy	**99.06**	**98.94**	**98.89**	**99.49**

Table no 13: Reinjection Reproducibility of Armodafinil

Stock solution Stability study

Long term stock solution stability

Replicate No.	**HQC**	**LQC**
	Nominal Concentration (ng/mL)	
	4.800	**0.450**
	Nominal Concentration Range (ng/mL)	
	(4.080-5.520)	**(0.383-0.518)**
	Calculated Concentration (ng/mL)	
1	4.125	0.383
2	4.362	0.422
3	4.481	0.438
4	4.936	0.464
5	5.126	0.481
6	5.329	0.505
n	6	6

Mean	4.7265	0.4488
SD	0.47359	0.04380
% CV	**10.02**	**9.76**
% Mean Accuracy	**98.47**	**99.74**

Table no 14: stability of Armodafinil (zero days)

Matrix samples stability at -28±5 °C for 37 days

Replicate No.	**HQC**		**LQC**	
	Nominal Concentration (µg/mL)			
	2.080	2.080	0.195	0.195
	Nominal Concentration Range (µg/mL)			
	(1.768-2.392)	(1.768-2.392)	(0.166-0.224)	(0.166-0.224)
	Calculated Concentration (µg/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	1.785	1.795	0.169	0.167
2	1.824	1.836	0.172	0.175
3	1.915	1.929	0.183	0.188
4	2.181	2.126	0.195	0.191
5	2.297	2.221	0.210	0.218
6	2.364	2.341	0.223	0.221
n	**6**	**6**	**6**	**6**
Mean	**2.0610**	**2.0413**	**0.1920**	**0.1933**
SD	**0.25122**	**0.22123**	**0.02147**	**0.02208**
% CV	**12.19**	**10.84**	**11.18**	**11.42**
%Mean Accuracy	**99.09**	**98.14**	**98.46**	**99.15**
% Mean Stability	**99.05**		**100.69**	

Table no 15: Matrix samples stability at -28±5 °C for 37 days

Matrix samples stability at -80±5 °C for 37days

Replicate No.	**HQC**		**LQC**	
	Nominal Concentration (µg/mL)			
	2.080	2.080	0.195	0.195
	Nominal Concentration Range (µg/mL)			
	(1.768-2.392)	(1.768-2.392)	(0.166-0.224)	(0.166-0.224)
	Calculated Concentration (µg/mL)			
	Comparison Samples	**Stability Samples**	**Comparison Samples**	**Stability Samples**
1	1.77	1.80	0.169	0.170
2	1.81	1.84	0.175	0.176
3	1.94	1.92	0.179	0.182

4	2.16	2.13	0.185	0.195
5	2.29	2.28	0.218	0.205
6	2.30	2.31	0.224	0.219
n	**6**	**6**	**6**	**6**
Mean	**2.0452**	**2.0458**	**0.1917**	**0.1912**
SD	**0.23612**	**0.22549**	**0.02339**	**0.01867**
% CV	**11.55**	**11.02**	**12.20**	**9.77**
%Mean Accuracy	**98.33**	**98.36**	**98.29**	**98.03**
% Mean Stability	**100.03**		**99.74**	

Table no 12: Matrix samples stability at -80±5 °C for 37 days

4. DISCUSSION:

Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Armodafinil as per current ICH guidelines. Armodafinil and Internal Standard were eluted at 2.728 min, 3.159min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. the plate count, tailing factor, resolution of Armodafinil was According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The % CV of the retention time (RT) should be ≤ 2.00 %. Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits. Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific. The response areas obtained of analyte and internal standard are less than 20% and 5 % of LLOQ Area. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific. A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ. - The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 11.52% and mean of 6 injections was found to be 100.00 % within the acceptance limits. As the limit of Sensitivity % CV was less than "20%" the system Sensitivity was passed in this method. The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 98.12% and 99.29% of HQC and LQC and % CV obtained are 11.83% and 11.23% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method. Calibration was found to be linear over the concentration range of 0.065 to 2.6 µg /ml. The coefficient correlation (r^2) value was found consistently greater than 0.999 in all the cases. This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes. The intraday and inter day accuracy and precision was assessed by analysing six replicates at five different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for Armodafinil at four concentration levels, i.e.,0.065µg/ml(LLOQ), 0.195 µg/ml

(LQC), 1.3 µg/ml (MQC) and 2.8 µg/ml HQC The intra-day and inter day accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 99.96-100.35%, and 98.99%-99.93 % for intraday and 99.06%-100.02 and 98.91%-100.24 for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.38-11.54% and 0.76%-13.49% for intraday and 0.66%-14.23% and 0.77 %-13.16% for inter day respectively. Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Armodafinil and . The overall % mean recovery for was found to be 99.52% at LQC, MQC and HQC levels and % CV ranged from 0.32- 0.13 for IS, 1.67,1.72, 0.34, 0.33,0.80,0.69 LQC, MQC and HQC(Extracted & UnExtracted). The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency. Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations (0.065 ng/ml-2.6ng/ml) of calibration standards not including blank and zero samples and establishing, the calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Armodafinil. In bench-top stability, six replicates of LQC & HQC samples (0.195 and 2.09 µg/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 98.47% for LQC and 99.74% for HQC respectively. Long term stock solution stability for the Armodafinil was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator. The % mean stability of the Armodafinil was found to be 99.05, 100.69% at 28 ± 5°C and 101.31%, 99.89% at 80 ± 5°C respectively. Long term stock solution stability for the was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator. The % mean stability of the was found to be 100.03%, 99.74% at 28 ± 5°C .

5. CONCLUSION:

A simple, accurate, precise method was developed for the estimation of the Armodafinil in Rabbit plasma using the Dolutegravir as internal standard. Retention time of Armodafinil was found to be 3.159min., which reach the level of both drugs possibly found in Rabbit plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range. The proposed method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories

6. REFERENCES:

1. Lalit v sonawane, bhagwat n poul, sharad v usnale, pradeepkumar v waghmare and laxman h surwase , Bioanalytical Method Validation and Its Pharmaceutical Application, Pharmaceutical Analytical Acta,2014 vol.5,pg no:1-7.
2. Sachin, L.Darkunde, Rupali,N. Borhade, Bioanalytical Method Validation: A Quality Assurance Auditor View Point asian journal of pharmaceutical technology and innovation.2017.Vol.5. pgno:59-60
3. https://pubchem.ncbi.nlm.nih.gov/compound/Armodafinil
4. Tijare lk, rangari nt, mahajanun, A review on bioanalytical method development and validation, asian journal of pharmaceutical clinical research.2016 vol.9.pgno:1-5

5. Method development and validation skills and tricks .2019.pgno:3
6. Pushpa Latha E, and Sailaja B, Bioanalytical Method Development and Validation by HPLC journal of medical and pharmaceutical innovation.2015 vol.1.pgno:1-9
7. Kirthi1, R. Shanmugam, M. Shanti Prathyusha , D. Jamal Basha, a review on bioanalytical method development and validation by rp - hplc Journal of Global Trends in Pharmaceutical Sciences.2014 vol.5.
8. Gurdeep R.Chatwal , Sham K .Anand, Instrumental Methods of Chemical Analysis , Pg 2.566-2.638 (2007)
9. Kambham Venkateswarlu et al; A validated stability indicating RP-HPLC method for estimation of Armodafinil in pharmaceutical dosage forms and characterization of its base hydrolytic product: Pak J Pharm Sci 2017 Jan;30(1):23-28.
10. P. VIVEK SAGAR et al; Stability Indicating RP HPLC Method For The Estimation Of Armodafinil In Tablet Dosage Form: Int J Pharm Pharm Sci, Vol 6, Issue 8, 604-609.
11. Ramesh d, habibuddin m. Application of validated RP-HPLC method for the determination of armodafinil in bulk and formulation. Int j curr pharm res.;9(5):158-61.
12. Rao CP, Rahaman SA, Prasad YR, Reddy PG. RP-HPLC method of simultaneous estimation of amlodipine besylate and metoprolol in combined dosage form. International Journal of Pharmaceutical Research and Development. 2010 Nov;2(9):69-76.
13. Rao CM, Konda R, Rao RN, Ramanjeneeyulu S, Reddy PG. Estimation of Nevirapine Anhydrous Bulk Formulation by Using IR, RP-HPLC, GC Methods. Research Journal of Pharmacy and Technology. 2010;3(4):1088-92.
14. Kumar KR, Rao CH, Rao CB, Sekhar KB, Reddy PG. RP-HPLC Method development and validation for estimation of Capecitabine in capsules. Int. J. Chem. Tech. Res. 2010;2:307-11.
15. Rasheed SH, Ramakotaiah M, Kumar KR, Nagabhushanam CH, Rao CH. Estimation of rabeprazole sodium and itopride hydrochloride in tablet dosage form using reverse phase high performance liquid chromatography. E-Journal of Chemistry. 2011 Jan 1;8(1):37-42
16. Somisetty VS, Dhachinamoorthi D, Rao CM, Balaiah CM, Swetha RV, Chaitanya AS, Shankar LS. Development and validation of new derivative uv-spectrophotometric methods for quantitative estimation of abacavir sulphate in bulk and pharmaceutical dosage form. International Journal of Biological & Pharmaceutical Research. 2013; 4(8): 608-613
17. Subbarao sommisetty, CH.M.M.Prasada Rao et.al. "Development and Validation of Newer Analytical Methods for the Estimation of Deferasirox in Bulk and In Tablet Dosage Form By Calorimetric Method " International Journal of Pharmacy and Pharmaceutical Sciences 2013; 5(3): 512-525.