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"Analytical Method Development and Validation of Simultaneous Estimation of Ambrisentan and Tadalafil in Bulk and Pharmaceutical Dosage Form by HPLC"

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Abstract: *Aim of this present work is to develop and validate a simple, efficient and economical method for simultaneous determination of Ambrisentan and Tadalafil in bulk and pharmaceuticals dosage form has been developed. Chromatographic separations of both drugs were achieved on Inertsil C_{18} (250×4.6 mm) 5µ using a mobile phase consisting of buffer (PH 3): methanol (50:50) with flow rate of 1 ml/min and detection was carried out at 260 nm and 20 µl injection volumes were selected. The linear dynamic response was found to be in concentration range of 20-60 µg /ml for Tadalafil and 5-15 µg /ml for Ambrisentan and show of correlation co-efficient (R^2) of 0.999. Accuracy was determined by recovery studies ranges from 100.898-99.45145 for Tadalafil and 100.482 – 99.6716 for Ambrisentan.*

Key words: Ambrisentan, Tadalafil, HPLC, Method Development.

INTRODUCTION

Ambrisentan (AMB) is an anti-hypertensive drug chemically referred to as (2S)-2-[(4,6-dimethylprimidin-2-yl) oxy]-3-methoxy-3,3-diphenyl-propanoic acid and its chemical formula is $C_{22}H_{22}N_2O_4$. Ambrisentan (AMB) is an endothelin receptor antagonist that's selective to endothelin type-A (ET-A). AMB belongs to the antihypertensive class of medicine and use in the treatment of pulmonary atrial hypertension in patient with WHO class II or III symptoms. Endothelin is a peptide that constricts blood vessels and elevates pressure of blood. AMB decreases blood pressure with in the lungs by restricts the effects of endothelin-1. The thickening of blood vessels in the lungs and heart is additionally inhibited by AMB **Fig-1.** [1, 2]

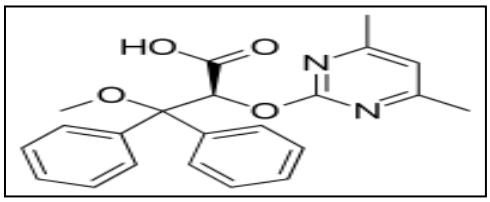

Fig-1: Molecular Structure of Ambrisentan

Tadalafil (TADA) chemically known as(6*R*,12a*R*)-6-(1,3-benzodioxol-5-yl)-2-methyl 2,3,6,7,12,12a-hexahydropyrazino[1',2':1,6] pyrido[3,4-b]indole-1,4-dione and its empirical formula is $C_{22}H_{19}N_3O_4$. TADA is mainly used to treat erectile dysfunction and pulmonary arterial hypertensionand it is selective inhibitor of cyclic guanosine monophosphet (CGMP) and enzyme phosphodiesterase type 5 (PDE 5) **Fig-2** [3, 4]

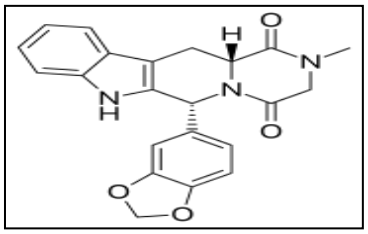

Fig-2: Molecular Structure of Tadalafil

Several HPLC methods have been reported in the literature for the estimation of Tadalafil available individually and in combined with other drug. An attempt has been made to developed simple and reliable HPLC method for the estimation of Tadalafil and Ambrisentan in bulk and pharmaceutical formulation. The results of analysis were validated in accordance with ICH guidelines [5-13]

Material and Methods

Standard of AMB and TADA were obtained from. Tablets were purchase from the local medical store. Buffer and

methanol were obtained from Rankem. All solvent and reagent were of analytical reagent.

Instrumentation: Gradient system HPLC equipped with aligned UV detector was used throughout the analysis. The analysis column inertsil C18 250mm x 4.6mm, 5µ thermo scientific was used as a stationary phase. The instrumental settings were a flow of 1.0 ml/min and injection volume 20µL column oven temperature was ambient.

Buffer preparation: 6.8 gm Potassium dihydrogen phosphate buffer was transferred to 1000ml beaker and 800 ml water was added shacked to dissolve and volume was made up with water, pH 3.0 was adjusted with diluted o-Phosphoric acid.

AMB Standard stock solution (100 µg/ml): Accurately weighed 10mg of AMBRS and Transferred to 100ml volumetric flask and volume was made up with the Diluent.

TAD Standard stock solution (400 µg/ml): Accurately weighed 40mg of TAD RS and Transferred to 100ml volumetric flask and volume was made up with the Diluent.

Standard Working solution (AMB 100µg/ml, TAD 40µg/ml): 1ml of standard stock solution was transferred to 10ml volumetric flask and volume was made up with the Diluent.

Optimization Chromatographic Conditions: various combination of mobile phase was screened with relevancy to resolution, theoretical plate, capacity factor and other system suitability parameters. Finally the separation was performed with freshly prepared mobile phase comprises of Buffer (pH 3): Methanol (50:50) at rate of flow of 1.0ml/min. 260nm wavelength, injection volume of 20 µL temperature was maintained during the whole process to get symmetric peak of AMB and TADA.

Method validation (14, 15)

The developed HPLC method was validated by determining the following parameter.

System suitability: system suitability test are a fundamental part of liquid chromatography it ensure that system is working correctly. Standard solution of AMB and TADA was injected into the chromatographic system and recorded the chromatograms. System suitability parameter such as number of theoretical plate, retention time, and telling factor were evaluated.

Linearity: The linearity of method was performed by analyzing a standard solution of AMB and TADA to get a solution in a concentration range is 20-60 µg/ml 05-15 µg/ml for TADA and AMB respectively. The area at which level was calculated and the therefore the graph of area versus concentration was plotted. The correlation coefficient was calculated in linearity plot.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD and LOQ of AMB and TADA were determined by calibration curve used to determine method linearity.it may be calculated as

$$LOD = 3.3 \times (SD/slop)$$

$$LOQ = 10 \times (SD/slop)$$

Where

SD= the standard deviation of response (peak area)

SLOP= mean slop of the calibration curve

Precision: precision of the proposed method was determined by injection six replicates of unknown concentration of AMB and TADA are analyzed by injecting them into a HPLC column on the identical day. The intermediate precision was estimated by injecting sample prepared at the identical concentration on three different days. The %RSD for the AMB and TADA WAS calculated.

Accuracy: the accuracy of this method was performed at three different levels (80%, 100% and 120%) by the adding of unknown amount of standard to the sample at each level. Each sample was injected thrice.

Robustness: robustness is that the measure of optimized method capacity to stay unaffected by small but deliberate variation in method parameters like mobile phase flow rate. (±0.2 ml/min), P.H (±0.2) and PH ±2S.

Results

Optimization of chromatographic conditions:

Optimized chromatographic condition for estimation of AMB and TAD are finalized shown in below. A representative chromatogram is shown in **Fig-3.**

- ✓ **Column**: Inertsil C18 (250x4.6 mm)
- ✓ **Mobile Phase**: Buffer (pH 3.0): Methanol (50:50)
- ✓ **Flow Rate**: 1.0 ml/min
- ✓ **Detection Wavelength**: 260 nm
- ✓ **Runtime**: 6 min
- ✓ **Injection volume**: 20.0µl

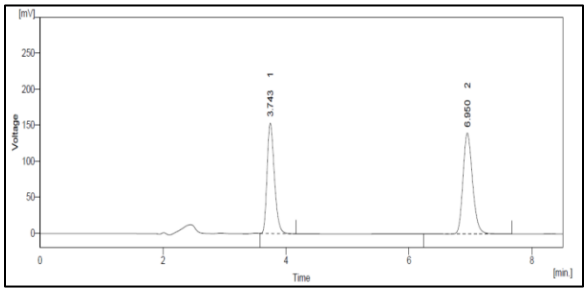

Fig-3: Chromatogram of Standard AMB and TADA

System suitability

The system suitability was performed by injecting mix standard solution containing 400µg /ml Tadalafil and 100µg /ml Ambrisentan in six replicates. For two of them the peak asymmetric were< 1.5 and the theoretical plate number is > 2000and % RSD of Ambrisentan and Tadalafil was less than 2. The result indicates that the system suitability parameter is within the acceptable limit. The results are shows in T**able 1.**

Table 1: Results for System Suitability Test

Parameters	**AMB**	**TADA**
Theoretical plates per column	9600	4516
Symmetry factor/tailing factor	1.341	1.467
Retention time (min)	7.077	3.807
Resolution	-	-

Linearity

The linearity of the method was established by determining the constructing calibration graph between tested calibration level and corresponding peak area for AMB and TADA in triplicate. Over a range of 5 – 15µg / ml and 20 – 60µg /ml respectively. The correlation coefficient was > 0.999 for all two drugs. The results are given in **Table-2** and **Fig-6 (a and b).**

Table 2: Linearity Data for AMB and TADA

Sr. No	**Tadalafil**		**Ambrisentan**	
	Conc. (Mcg/Ml)	**Area**	**Conc. (Mcg/ Ml)**	**Area**
1	20	704.631	5	738.555
2	30	1024.673	7.5	1075.825
3	40	1372.279	10	1438.651
4	50	1731.691	12.5	1822.059
5	60	2052.149	15	2139.902
	Co-Efficient Regression	0.999796426		0.999623758

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were found to be 0.117 and 0.357µg /ml for TADA and 0.0096 and 0.00088µg /ml for AMB. THE results are given in **Table 3.**

Table-3: LOD and LOQ

Drug	**LOD**	**LOQ**
Tadalafil	0.117	0.357
Ambrisentan	0.0096	0.00088

Precision

Method precision / repeatability

The % RSD value for six replicate injection of a unknown concentration of ambrisentan 100µg /ml and tadalafil 400µg /ml. carried out on the same day was found to be < 2 % which indicate that the method repeatable. The results for method precision are given in **Table-4.**

Table 4: Intraday Precision Data for Estimation of AMB and TADA

TADALAFIL				
Sr no	**Conc. ug/ml**	**Area**	**SD***	**%RSD****
1	50%	697.467	5.0523	0.7217
		696.751		
		705.838		
2	100%	1357.95	6.5282	0.4817
		1347.69		
		1359.82		
3	150%	2073.5	7.8532	0.3793
		2060		
		2075.46		

AMBRISENTAN				
Sr no	**Conc.ug /ml**	**Area**	**SD***	**%RSD****
1	50%	731.064	3.0708	0.4192
		730.327		
		735.976		
2	100%	1430.07	8.8767	0.6238
		1412.99		
		1425.73		
3	150%	2174.31	15.0594	0.69511
		2149.1		
		2175.97		

***standard deviation ** % relative standard deviation**

System precision / intermediate precision

Intermediate precision was determined by measuring the peak area of six replicate was inject into HPLC system and was analyzed and the were found within the acceptable limit (%RSD) intermediate precision given in **Table-5.**

Table 5: Interday Precision Data for Estimation of AMB and TADA

AMRISENTAN				
Sr No	**Conc. µg/ml**	**Area**	**SD***	**%RSD****
1	50%	731.064	3.0708	0.4192
		730.327		
		735.976		
2	100%	1430.07	8.8767	0.6238
		1412.99		
		1425.73		
3	150%	2174.31	15.0594	0.69511
		2149.1		
		2175.97		

TADALAFIL				
Sr.no	**Conc. µg/ml**	**Area**	**SD***	**%RSD****
1	50%	731.064	3.0708	0.4192
		730.327		
		735.976		
2	100%	1430.07	8.8767	0.6238
		1412.99		
		1425.73		
3	150%	2174.31	15.0594	0.69511
		2149.1		
		2175.97		

Accuracy

The percentage recovery was calculated by preparing standard concentration of AMB and TADA with concentration level of 80%, 100% and 120%. The percentage recovery obtained was found to be in the range of 100.89, 100.71, 99.451% for TADA and100.482, 99.814, and 99.671% for AMB. The acceptable limits of mean recovery are 100% - 102%. Good recovery of the spiked drugs was obtained at each added concentration.

Robustness

The method was found to be robust when minor changes were made in optimized chromatographic condition such as mobile phase flow rate (+ 0.2 ml/ min), M.P (+ 0.2), and pH (+ 0.2).it was observed that there was no marketing change in the analytical data of the drug which indicate good reliability during normal usage. The results are given in **Table-6 and 7.**

Table-6: Robustness Data for TADA

Sr. No.	Flow rate +2	Flow rate -2	M.P. +2	M.P. -2	pH +2	pH -2
1	1178.407	1545.962	1230.075	1517.092	1452.527	1270.067
2	1179.558	1535.143	1230.415	1509.492	1445.259	1241.154
3	1173.621	1532.095	1247.379	1518.645	1425.965	1253.56
avg. area	1177.195	1537.733	1235.956	1515.076	1441.25	1254.927
SD	3.148507	7.287372	9.89378	4.898116	13.72724	14.50489
%RSD	0.267458	0.473904	0.800496	0.323292	0.952453	1.155836

***MP** = mobile phase

Table-7: Robustness Data for AMB

Sr. no.	Flow rate +2	Flow rate -2	MP*. +2	MP. -2	pH +2	pH -2
1	1234.479	1620.637	1288.591	1590.368	1522.679	1309.338
2	1235.705	1609.288	1295.027	1582.383	1515.034	1300.182
3	1223.479	1606.067	1285.961	1598.218	1504.417	1313.194
avg. area	1231.221	1611.997	1289.86	1590.323	1514.043	1307.571
SD	6.732733	7.653534	4.66425	7.917596	9.171217	6.683477
%RSD	0.546834	0.474786	0.361609	0.497861	0.605743	0.511137

Conclusion: The proposed method was found to be simple, accurate, precise and rapid HPLC method suitable for the estimation of Ambrisentan and Tadalafil in bulk and tablet dosage form. All the parameters meet the standard of ICH guidelines for method validation and found to be simple, sensitive, accurate and precise. It can be concluded that the reported method is more economical and can find practical application & may be recommended for routine and QC analysis of the investigated drugs to provide simple, accurate and reproducible quantitative analysis for the determination of Ambrisentan and Tadalafil in bulk and tablet dosage form.

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