S.S.Toranmal et al /J. Pharm. Sci. & Res. Vol. 11(9), 2019, 3108-3115

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Development and Validation of Stability Indicating HPLC Method for Simultaneous Estimation of Milbemycin Oxime and Praziquantel from Bulk and Marketed Formulation

S.S.Toranmal*, R.S.Buchade, S. D. Tandale, V.H.Wagh, P.P. Chaure.
Department of Quality Assurance Techniques Allard Charitable Trust's Allard College of Pharmacy Marunje Hinjewadi Pune- 411057

Abstract

Aim:Upon literature survey it was found that on Milbemycin Oxime and on Praziquantel separate method were developed but on combination HPLC method was not developed, for its determination in bulk and pharmaceutical dosage forms. In view of the need for a suitable method for routine analysis, attempts are being made to develop simple, precise and accurate analytical methods to determine Milbemycin Oxime and Praziquantel ingredient in bulk and formulation and validation of the developed method was performed.

Method: C_{18} (AGILENT) 4.6 x 150 mm were used as stationary phase. MeOH: 0. 05 % TEA Ph-3.0 with OPA was delivered best separation at 254 nm.

Results: In linearity study value of correlation coefficient was 0.9997 for MELB and 0.9995 for PRAZI, LOD and LOQ was found to be 0.1114 and 0.3376 µg/mL for MELB and 0.3872 and 1.1733 µg/mL for PRAZI, respectively, In accuracy % drug recovery of drug at each level was in between 98.0 to 102 % and % RSD was not more than 2.0, In Precision %RSD was not more than 2.0, In robustness % RSD was not more than 2.0.

Conclusion: The Proposed developed and validated HPLC method was found to be more sensitive, simple, precise, accurate, cost effective and robust. This method could be applied for analysis of bulk drug and tablet formulation.

Keywords: Milbemycin oxime, Praziquantel, MeOH, RP-HPLC

INTRODUCTION:

Milbemycin oxime chemically is UNII-0502PUN0GT; Milbemycin A, 5-oxime; Milbemycin, oxime; Interceptor flavor tabs; 0502PUN0GT; CGA-179246 (Figure No. 1). Milbemycin oxime have a similar mechanism of action, but a longer half life than avermectins. They open glutamate sensitive chloride channels in neurons and myocytes of invertebrates, leading to hyperpolarisation of these cells and blocking of signal transfer. Praziquantel chemically is Praziquantel; 55268-74-1; Droncit; Biltricide; Pyquiton; Cesol (Figure No. 2). Praziquantel effects the permeability of the cell membrane which causes in the contraction of schistosomes. The drug further causes vacuolization and disintegration of schistosome tegument. MELB and PRAZI is recently introduced in the market for the treatment of heartworms in dogs and cat. Multidrug administration is often associated with clinically significant interaction, especially of narrow therapeutic index drugs, either at pre-absorption or post-absorption stage. This can limit the desired therapeutic effect of either of drug molecules. Upon literature survey it was found that on Milbemycin Oxime LS-MS method, HPLC method was developed and on Praziquantel combination HPLC method, were developed, however but no work has been done on this particular drug combination of Milbemycin Oxime and Praziquantel, for its determination in bulk and pharmaceutical dosage forms using HPLC method. In view of the need for a suitable method for routine analysis, attempts are being made to develop simple, precise and accurate analytical methods to determine Milbemycin Oxime and Praziquantel ingredient in bulk and formulation. Analytical validation is the corner stone of process validation. Without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purport to confirm whether the manufacturing process has done what it purport to do. Hence there is a need to validate the new methods developed.

MATERIALS AND METHODS

Equipments

UV- Visible Spectrophotometer

LabindiaUV 3200 double beam spectrophotometer with 1 cm path length was used for spectral measurements.

High Performance Liquid Chromatography (HPLC)

Younglin (S.K) Gradient system with UV 730 detector and pump number SF930 D, software used was autochro 3000. C_{18} (AGILENT) 4.6 x 150 mm were used as stationary phase.

Materials

Acetonitrile, Ammonium acetate, Methanol, Triethylamine, Orthophosphoric acid, Acetone and Water all the chemicals and reagents used were of HPLC and analytical grade.

Chromatographic Conditions

The sample separation was achieved on a C_{18} (AGILENT) 4.6 x 150 mm, aided by mobile phase mixture of Methanol: 0. 05 % Triethylamine Ph-3.0 with Orthophosphoric acid (75:25). The flow rate was 1.0 ml/ minute and ultra violet detector at 254 nm that was filtered and degassed prior to use, Injection volume is 20µl and ambient temperatures.

Preparation of Solutions

Standard Solution

Std. MELB 2 mg and Std. PRAZI 5 mg in 100ml of MeOH was taken which is 2000µg/mL MELB and 5000µg/mL PRAZI- STOCK –I.

Sample Solution

Twenty containing MELB (10 mg) and PRAZI (25 mg) were weighed accurately to determine average weight and then the tablets were crushed to fine powder. The powder equivalent 10 mg was weighed for MELB and 25 mg for PRAZI and transferred to a 10 mL volumetric flask and dissolved in methanol. The content was sonicated for 30 min and then the volume was made up to the mark with methanol further dilution were made with the mobile phase. The solution was filtered through 0.2 µm membrane filter paper and injected.

Mobile phase preparation

The Methanol and 0.05 % Triethylamine Ph-3.0 with Orthophosphoric acid was selected as mobile phase in ratio of 75:25 and was filtered on membrane filter (0.45µ) to remove dissolve gases solvents were sonicated for 15-30 min.

Method Validation

Linearity

Take 0.1 mL from stock I was taken and make volume with mobile Phase10 mL it gives 2 µg/ mL MELB and 5 µg/ mL PRAZI. Take 0.2 mL from stock I and make volume with mobile Phase10 mL it gives 4 µg/ mL MELB and 10 µg/ mL PRAZI. Take 0.3 mL from stock I and make volume with mobile Phase10 mL it gives 6 µg/ mL MELB and 15 µg/ mL PRAZI Take 0.4 mL from stock I and make volume with mobile Phase10 mL it gives 8 µg/ mL MELB and 20 µg/ mL PRAZI. Take 0.5 mL from stock I and make volume with mobile Phase10 mL it gives 10 µg/ mL MELB and 25 µg/ mL PRAZI.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were separately determined based on the calibration curves. The standard deviation of the y-intercept and slope of the regression line was used.

The LOD and LOQ were calculated using the formulas,

$LOD = 3.3 \times D / S$

$LOQ = 10 \times D / S$

Where,

S = Slope of regression line

D = Standard deviation of y-intercept on the regression line.

Accuracy (Recovery Studies)

The accuracy of an analytical method is closeness of test result obtained to the true value. The accuracy of an analytical method established across its range. A known amount of standard solution of pure drug (MELB and PRAZI) was added to pre-analyzed sample solution and these solutions were subjected for analysis. The lower the value of relative standard deviation (RSD) indicates that the method is accurate. To check the degree of accuracy of the developed method, recovery studies were performed at 80%, 100% and 120% of the label claim. The solutions were analyzed by RP-HPLC method as described above. At each level, three determinations were performed.

Precision

The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple samplings of homogeneous sample.

a) Method Repeatability:

Degree of repeatability of the method and suitable statistical evaluation was carried out. Six samples of tablet formulation were analyzed as per the procedure given under tablet assay. The percentage mean content, its S.D and % RSD were calculated from the obtained readings.

b) Interday and Intraday Precision:

Variations of results within the same day (Intra), variation of results between days (Inter) were analyzed. Intraday precision was determined by analyzing tablet sample solutions at different time intervals on the same day while inter day precision was determined on different day. The results were expressed as %RSD for mean content of MELB and PRAZI by repeated analysis (n=6)

Robustness of Method:

Robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of change in flow rate, mobile phase ratio and wavelength on the retention time, theoretical plates, area under curve and percentage content of MELB and PRAZI were studied. The solution containing MELB and PRAZI was injected into sample injector of RP- HPLC six times under the varied conditions.

Forced degradation studies

Acidic/Basic Hydrolytic degradation

The hydrolytic degradation of a new drug in acidic and alkaline conditions can be studied by refluxing the drug in 0.1 N HCl/NaOH. If reasonable degradation is seen, testing can be stopped at that point. However, in case no degradation is seen under these conditions, the drug should be refluxed in acid/alkali of higher strengths and for longer duration. Alternatively, if total degradation is seen after subjecting the drug to initial conditions, acid/alkali strength can be decreased along with decrease in the reaction temperature.

Preparation of Acid and Base Induced Degradation Product:

Weigh 4 mg MELB and 10 mg PRAZI into 10 mL of volumetric flask, to which each of hydrochloric acid (2 mL, 0.1 N) and sodium hydroxide (2 mL, 0.1 N) was added separately and refluxed at 60°C for 60 min. Then the mixtures were cooled and neutralized using sodium hydroxide (0.1 N) and hydrochloric acid (0.1 N) respectively. Before carrying out the HPLC analysis, samples were suitably diluted and 20 µL injected into the system and the chromatograms recorded to assess the stability of sample. As there was no degradation observed with 0.1N hydrochloric acid and sodium hydroxide concentration was gradually increased and HPLC analysis was been done for the same. Finally degradation was observed with 1 N hydrochloric acid and sodium hydroxide. Similarly degradation study were also carried out for marketed formulation

Preparation of Hydrogen Peroxide Induced Degradation Product:

Weigh 4 mg MELB and 10 mg PRAZI into 10 mL of volumetric flask, to which hydrogen Peroxide (2 mL) (3% v/v) was added and heat the solution at 60°C for 60 min. Before carrying out the HPLC analysis, samples were suitably diluted and 20 □L injected into the system and the chromatograms were recorded to assess the stability of sample. As there was no degradation observed with 3 % v/v hydrogen peroxide concentration was gradually increased and HPLC analysis was been done for the same. Finally degradation was observed with 10 % hydrogen peroxide after 60 min. Similarly degradation study were also carried out for marketed formulation

Neutral Hydrolysis Induced Degradation Product:

Weigh 4 mg MELB and 10 mg PRAZI into 10 mL of volumetric flask, to which water (2 mL) was added and heat the solution at 60°C for 60 min. Before carrying out the HPLC analysis, samples were suitably diluted and 20 □L injected into the system and the chromatograms were recorded to assess the stability of sample. Similarly degradation study were also carried out for marketed formulation

Stress testing should induce not more than 5-15 % degradation of the main compound. A stress test should be stopped when this percentage of degradation is achieved. It is not desirable to generate samples with extensive degradation because of their limited relevance and the formation of secondary degradation products, which would lead to complicated degradation pattern.

RESULTS

Preliminary tests

Melting point

167-169°C for Milbemycin oxime and and 136 °C for Praziquantel.

Solubility

Solubility study was carried out for both MELB and PRAZI using different solvents and the results revealed that both drugs are soluble in DMSO and ethanol.

Linearity

For calibration curve of MELB and PRAZI (n=6). MELB and PRAZI both follow linearity in range 2 to 10 and 5 to 15 µg/mL respectively. The regression equation for was found to be Y = 25932 X – 3150.4 and Y = 41762 X - 12287, for MELB and PRAZI with correlation coefficient (r2) 0.9999 and 0.999 respectively are shown in **Figure No. 3 and 4** respectively.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOQ and LOD values were found to be 0.1114 and 0.3376 µg/mL for MELB and 0.3872 and 1.1733 µg/mL for PRAZI, respectively shown in **Table No 1.**

Accuracy (Recovery Studies)

Three replicate injections, each of three different test concentrations in the range of 80, 100 and 120 % of labeled claim of tablet under study has % recovery within 98.72 to 101.62 % of true concentration of each drug. These results indicate that the method is accurate shown in **Table No 2 and 3.**

Precision

Precision was performed by injecting six replicate injections of working standard solution % RSD was less than 2 in intraday, interday precision. So the proposed method is more precise as shown in **Table No** 4, 5, 6, 7 and 8

Robustness of Method

Robustness studies were carried out using different parameters i.e. change in flow rate, wavelength and mobile phase concentration, results revels that developed method was robust are as shown in **Table No** 9, 10 and 11.

Assay

Twenty micro liters of pure mixed standard solution (n = 6) were injected separately to an injector of HPLC and chromatogram was recorded are shown below. Equal volume (20 µL) of standard and sample solutions was injected separately after the equilibrium of stationary phase. The chromatograms were recorded and the response i.e. AUC of major peaks is measured. The content of MELB and PRAZI was calculated by comparing the sample peak with that of standard as shown in **Table No** 12 and **figure** 5 and 6.

Forced degradation studies

Degradation of MELB and PRAZI in 1 N HCL AFTER 1 hr (4+10mg).

DEG 1 was of MELB which was having area of 3135.293 and RT was 2.6667, DEG 2 was of PRAZI with area 22.3805 with RT 5.35 as shown in Figure 7 and Table 13.

Degradation of MELB and PRAZI in 1 N NAOH AFTER 1 hr (4+10mg).

There was no degradation observed for MELB with 1N NAOH after 1 hr. DEG 1 was observed for PRAZI with RT 2.7667 and area 458.7625 as shown in Figure 8 and Table 14.

Degradation of MELB and PRAZI in 10 % H_20_2 AFTER 1 hr (4+10mg).

DEG1 and 2 was observed with MELB at area 99.1148 and 2884.1609 respectively with RT 2.6 and 2.75, DEG 3 was observed with PRAZI at area 13.0787 with RT 5.2 as shown in Figure 9 and Table 15.

Degradation of MELB and PRAZI in water AFTER 1 hr (4+10mg).

There was no degradation for MELB observed in the study, DEG 1 was observed with area 22.3564 and RT 5.15 as shown in Figure 10 and Table 16.

TABLE NO.1: LOD AND LOQ DATA OF MELB AND PRAZI.

Sample	LOD (µg/mL)	LOQ (µg/mL)
MELB	0.1114	0.3376
PRAZI	0.3872	1.1733

TABLE NO.2: RECOVERY STUDY FOR MELB

80%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	2	1.6	540.22	3.61	1.61	100.69
2	2	1.6	538.28	3.59	1.59	99.87
			Mean	3.6	1.6	100.28
			SD	0.01	0.01	0.58
			%RSD	0.39	0.88	0.58
100%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	2	2	593.91	3.97	1.97	98.79
2	2	2	599.89	4.02	2.01	100.82
			Mean	3.99	20.58	99.81
			SD	0.03	0.03	1.44
			%RSD	0.81	0.14	1.44
120%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	2	2.4	653.61	4.38	2.38	99.22
2	2	2.4	652.2	4.37	2.37	98.83
			Mean	4.38	2.38	99.97
			SD	0.01	0.01	0.28
			%RSD	0.16	0.3	0.28

TABLE NO.3: RECOVERY STUDY FOR PRAZI

80%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	5	4	1197.07	8.95	3.95	98.96
2	5	4	1199.32	8.97	3.97	99.25
Mean	5	4	1198.2			
SD	0	0	1.59			
%RSD	0	0	0.13			
100%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	5	5	1329.45	10.09	5.09	101.89
2	5	5	1323.34	10.04	5.04	100.84
Mean	5	5	1326.4			
SD	0	0	4.32			
%RSD	0	0	0.33			
120%						
Sr no.	**TAB Sol Taken**	**STD Sol Added**	**Area**	**Amt found**	**Amt recovered**	**% Recovered**
1	5	6	1442.04	11.06	6.06	100.41
2	5	6	1444.34	11.08	6.08	101.62
Mean	5	6	99.97			
SD	0	0	1.63			
%RSD	0	0	1.63			

TABLE NO.4: METHOD REPEATABILITY ANALYSIS

Sr No.	**Concentrations**	**SD**	**%RSD**
1	2	0.96	0.16
2	6	2.02	0.22
3	10	1.32	0.09

TABLE NO.5: INTRA-DAY PRECISION DATA FOR MELB.

Components	Concentrations	SD	%RSD
MELB	4	2.03	0.34
PRAZI	10	2.68	0.2

TABLE NO.6: INTER-DAY PRECISION DATA FOR MELB.

Sr No.	Concentrations	SD	%RSD
1	2	0.96	0.31
2	6	2.04	0.23
3	10	4.16	0.28

TABLE NO.7: INTRA-DAY PRECISION DATA FOR PRAZI.

Sr No.	Concentrations	SD	%RSD
1	5	0.96	0.16
2	15	3.03	0.16
3	25	4.35	0.14

TABLE NO.8: INTER-DAY PRECISION DATA FOR PRAZI.

Sr No.	Concentrations	SD	%RSD
1	5	0.96	0.16
2	15	3.03	0.16
3	25	4.35	0.14

TABLE NO.9: FLOW RATE DATA OF MELB AND PRAZI.

Drug	µgm/ml	Flow rate	Area	SD	%RSD
MELB	4	0.9	577.96	3.43	0.59
	4	1.1	550.54	3.28	0.6
PRAZI	10	0.9	1276.16	4.95	0.39
	10	1.1	1310.77	8.9	0.68

TABLE NO.10: WAVELENGTH DATA OF MELB AND PRAZI.

Drug	µgm/ml	Wavelength (nm)	Area	SD	%RSD
MELB	4	255	541.1	9.45	1.75
	4	253	502.7	7.6	1.512
PRAZI	10	255	1225.8	4.28	0.35
	10	253	1105.36	1.48	0.134

TABLE NO.11: MOBILE PHASE DATA OF MELB AND PRAZI.

Drug	µgm/ml	Mobile Phase	Area	SD	%RSD
MELB	4	76:24:00	437	5.74	1.31
	4	74:26:00	400.42	1.43	0.36
PRAZI	10	76:24:00	717.8	3.66	0.51
	10	74:26:00	715.72	1.63	0.23

TABLE NO 12. ANALYSIS OF TABLET FORMULATION TABLET (ASSAY)

Drug	Amount present (mg/tab)	Area of Standard	Area of marketed formulation	Amount Found	% of drug found
MELB	10	1485.01	1485.88	10.14	100.92
PRAZI	25	1834.53	1834.97	25.01	100.3

TABLE NO 13. ACIDIC DEGRADATION OF MELB AND PRAZI IN 1 N HCL AFTER 1 HR (4+10MG)

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	DEG-01	2.6667	3135.293	67.76	5678.3	4.75	0
2	DEG-02	5.35	22.3805	0.48	4722.2	2.0714	10.0625
3	MELB	6.5333	453.064	9.79	10519.7	1.25	3.55
4	PRAZI	9.35	1016.1136	21.96	12119.4	1.1364	8.0476

TABLE NO 14. BASIC DEGRADATION OF MELB AND PRAZI IN 1 N NAOH AFTER 1 HR (4+10MG)

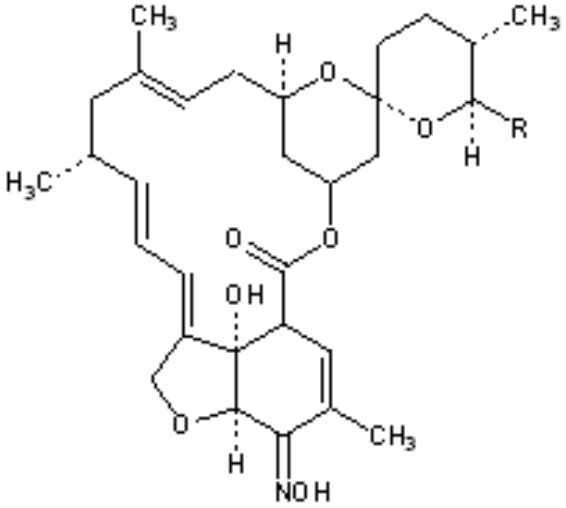
No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	DEG	2.7667	458.7625	28.47	779.6	1.375	0
2	MELB	6.3833	348.1325	21.61	8134.2	1.25	9.0417
3	PRZI	9.1333	804.4045	49.92	11564.2	1.0909	7.5

TABLE NO 15. OXIDATIVE DEGRADATION OF MELB AND PRAZI IN 10 % H202 AFTER 1 HR (4+10MG).

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	DEG-01	2.6	99.1148	2.59	3748.6	1	0
2	DEG-02	2.75	2884.1609	75.28	1509.7	3.75	0.5625
3	DEG-03	5.2	13.0787	0.34	5397.9	1.25	7.35
4	MELB	6.3333	251.3465	6.56	8007.3	1.25	3.4
5	PRZI	9.0333	583.6875	15.23	11312.4	1.2	7.3636

TABLE NO 16. WATER DEGRADATION OF MELB AND PRAZI AFTER 1HR (4+10MG).

No.	Name	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	DEG-01	5.15	22.3564	5.75	5294.6	1.5	0
2	MELB	6.3	92.587	23.81	9781.7	1.3125	3.6316
3	PRZI	9	273.9927	70.45	11229	1.15	7.7143



Component A_3 R = CH_3

Component A_4 R = CH_2CH_3

Figure No.1: Structure of Milbemycin Oxime

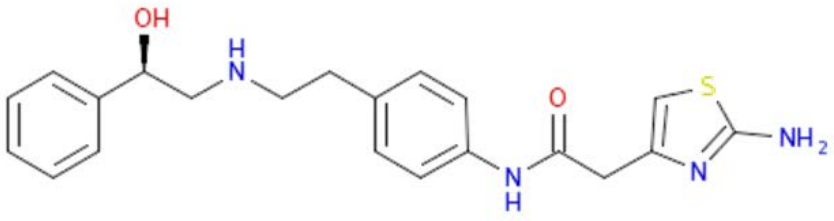


Figure No.2: Structure of Praziquantel

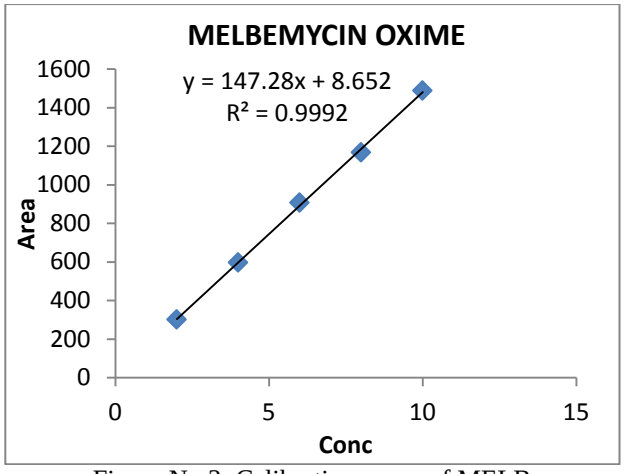


Figure No.3: Calibration curve of MELB

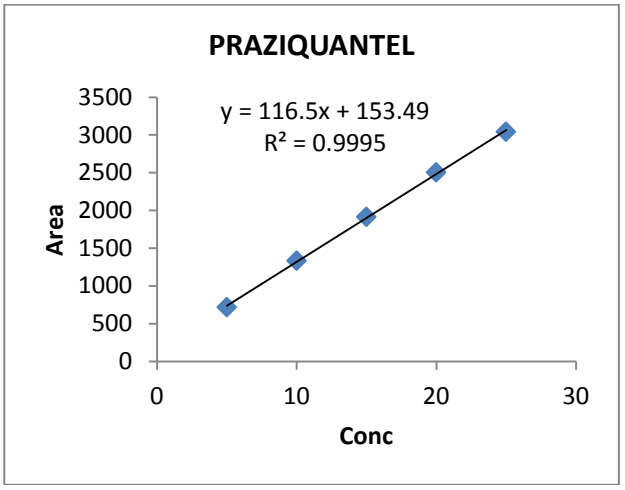


Figure No.4: Calibration curve of PRAZI

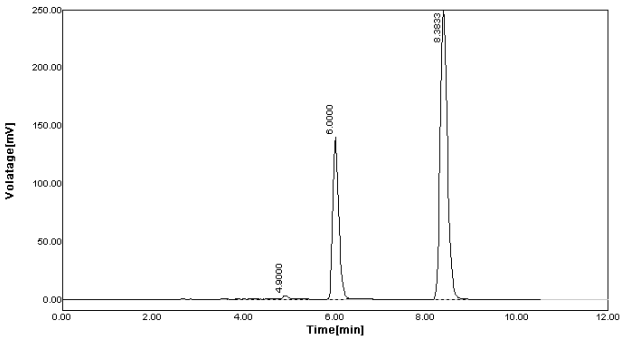


Figure No.5 Standard drug chromatogram

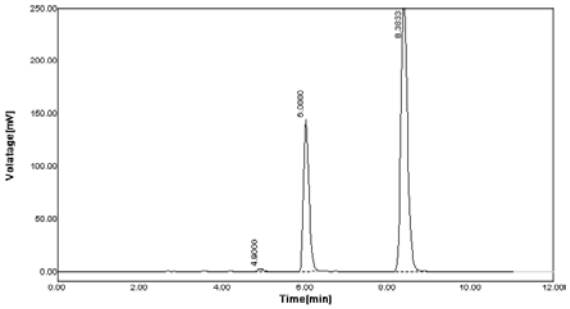


Figure No.6 Marketed formulation chromatogram

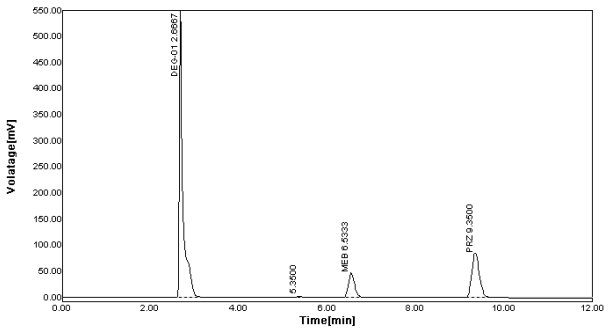


Figure No.7 Acidic degradation of MELB and PRAZI in 1 N HCL AFTER 1 hr (4+10mg).

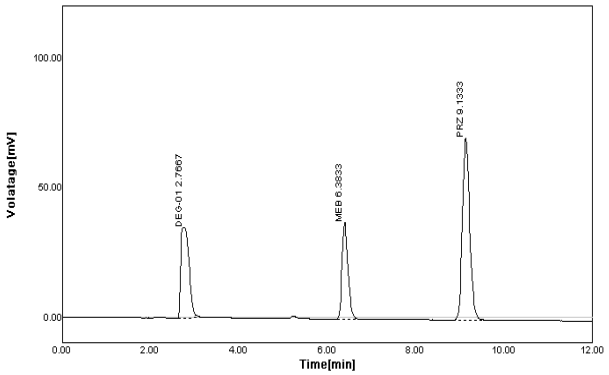


Figure No.8 Basic degradation study of MELB and PRAZI in 1 N NAOH AFTER 1 hr (4+10mg)

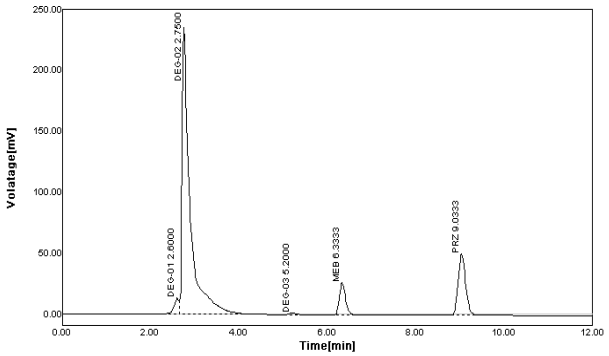


Figure No.9 Oxidative degradation of MELB and PRAZI in 10 % H202 after 1 hr (4+10mg).

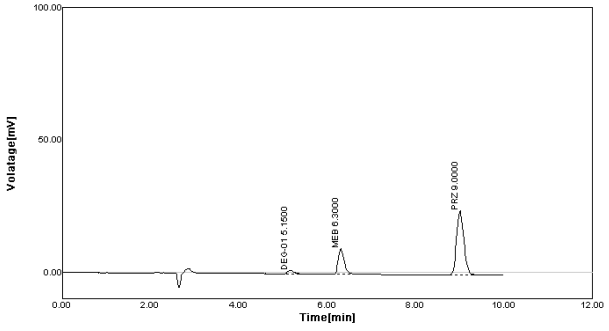


Figure No.10 Degradation study of MELB and PRAZI in water after 1hr (4+10mg).

CONCLUSION

Chromatographic separation achieved isocratically on a C18 (AGILENT) 4.6 x 150 mm with particle size 5 µm column utilizing a mobile phase of MEOH: 0. 05 % triethylamine (TEA) pH -3.0 with orthophosphoric acid (OPA) (75:25) at a flow rate of 1.0 mL / min. Statistical analysis proves that the method is reproducible and selective for the simultaneous estimation of MELB and PRAZI. As the method could effectively separate the drugs from their degradation products, can be employed as stability indicating method. The developed method was validated as per ICH guidelines in terms of accuracy, precision, linearity and specificity. Thus the study aimed at developing and validating new HPLC method, being simple, accurate, selective, and sensitive and can be applied for the estimation of these drugs in combined dosage forms.

In linearity study value of correlation coefficient was 0.9997 for MELB and 0.9995 for PRAZI, LOD and LOQ was found to be 0.1114 and 0.3376 µg/mL for MELB and 0.3872 and 1.1733 µg/mL for PRAZI, respectively, In accuracy % drug recovery of drug at each level was in between 98.0 to 102 % and % RSD was not more than 2.0, In Precision %RSD was not more than 2.0, In robustness % RSD was not more than 2.0.

These results indicate that the method is linear, accurate, precise and robust. From the result all peak for standard degradant was well resolved which concludes that developed method was stability indication. Thus, the developed method can be easily used for the routine quality control of bulk and tablet form.

REFERENCES

[1] Kenkel J. *Analytical Chemistry for Technicians.* 3rd ed. Lewis Publishers; 2003; 1-16.

[2] Skoog D. A, Nieman TA, Holler FJ. *Principles of instrumental analysis.* 5th ed. London: Stanford University, Saunders College Publications; 1998; 1-4.

[3] Ahuja S, Jespersen N. *Comprehensive analytical chemistry*. Elsevier B.V; 2006; 1- Murray R. W. Anal Chem.1991;63(1): 271.

[4] Christian G. D. *Analytical Chemistry.* 5th ed. John Wiley and Sons; 2001; 1-3.

[5] Ohannesian L. *Handbook of Pharmaceutical Analysis*. New York: Marcel Dekker; 2002.

[6] Beckett A. H, *Stenlake J. B. Practical Pharmaceutical Chemistry*. 4th ed. New Delhi: CBS Publishers and Distributors; 2002.

[7] Kemp W. *Organic spectroscopy. 3rd ed. Kundali*: Replica Press Private Ltd; 1991.

[8] Chatwal G. R, Ananand S. K. Instrumental method of chemical analysis, 5th ed. Mumbai: Himalaya Publishing House; 2003.

[9] SkoogDA, Holler FJ, Nieman TA. *Principles of Instrumental Analysis*. 6th ed., Thomson Brooks/Cole. 2007; 762-63,816-827.

[10] Raynord P. *Liquid Chromatography for the Analyst.* Marcel Dekker Inc. New York; 1994; 2.

[11] Scott P. *Principles and Practice of Chromatography, Chrome-ED Book Series*; 1-2, 12-14.

[12] Snyder L. R, Stadalius M. A. High-*Performance Liquid Chromatography: Advances and Perspectives*. C. Horvath, ed., Academic Press, San Diego, CA, 1983; 3, 157-158.

[13] Willard M, Dean S. *Instrumental method of analysis 7thed*., New Delhi CBS publication and distributors 1986; 3-5, 684-689.

[14] Gennaro A. R, Remington: *The Science and Practice of Practice of Pharmacy*, twenty first edition volume II Lippincort, Williams and welkins Mack Publishing Company, page No 1680-1682.

[15] Lodder R. A. *Analytical Spectroscopy Research Group, Advanced Science & Technology Center*, University of Kentucky available from site: www. pharm.uky.edu/ ASRG/HPLC/Histry.html.

[16] Sethi P. D. *HPLC Quantitative Analysis of Pharmaceutical Formulations*.1st ed., CBS Publishers and Distributors 1996; 38-43.

[17] Lindsay S. *High Performance Liquid Chromatography. John Wiley and Sons Publication*; 1997; 14.

[18] Munson J. W. *Pharmaceutical Analysis modern methods.* 1st ed., Marcel Dekker series of books 2001;30-50.

[19] Dong M. W. *Modern HPLC for Practicing Scientist*, John Wiley and sons, 2006;1-13

[20] Ahuja S, Rasmussen H. *HPLC Method Development for Pharmaceuticals.* 1st ed.