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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCES IN ESOMEPRAZOLE BY RP-HPLC IN SOLID DOSAGE FORM

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Abstract:

A simple rapid, specific and selective reversed phase high performance liquid chromatographic (RP-HPLC) method for estimation of related substances in esomeprazole tablet was developed and validated. A gradient separation was achieved from Zorbax SB C-8, (150 x 4.6) mm, 5µm with mobile phase consisting of HPLC grade acetonitrile and phosphate buffer solution at a flow rate of 1ml/min with a UV detection at 280nm.The injection volume was 40µl.With stabilities studies for analytical solutions it was concluded that freshly prepared solution should be used for each injection. The method was specific as the peaks were homogeneous and there was no interference of Esomeprazole related substances with Esomeprazole peak and with each other. The Limit of Detection and Quantitation for all known related substances and Esomeprazole were within the limit. From the linearity studies, the correlation coefficient was found to be 0.990,which indicates that method was linear over 10% to 120%. The method was found to be precise and robust at each variable condition. Hence,the proposed method can be used for routine analysis of drug on account of related substances.

Keywords: *Esomeprazole, Method validation, Related substances, Proton pump inhibitor*

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INTRODUCTION:

Esomeprazole is a proton pump inhibitor, belonging to the benzimidazole group of drugs. It was developed as the S-isomer of Omeprazole and latest PPIs approved by United States Food and Drug Administration and developed by Astra Zeneca in 2001[1].

Esomeprazole Mg has a chemical name as bis(5-methoxy-2-[(S)-[(4-methoxy-3,5-dimethyl2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole-1-yl) magnesium trihydrate.[2][3](Fig.1)

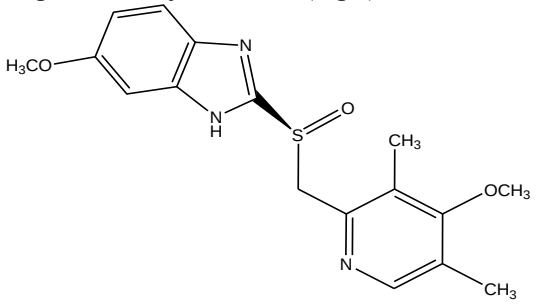

Fig. 1: Structure of Esomeprazole

In comparison to omeprazole and other drugs of proton pump inhibitors,ESO a single optical S-isomer of omeprazole provides better acid control and more convenient pharmacokinetic profile[4]. For the estimation of ESO alone and in combination with other drugs,many UV and RP-HPLC methods have been conducted.[5][6]

From both regulatory and quality perspective method validation is an indispensible necessity.[7]

The main objective of the stability indicating HPLC method is to estimate the related substances of Esomeprazole in the presence of degraded impurities, employed by forced degradation method and also to optimize the method according to different parameters by taking different individual conditions to avoid error, unreliability and contamination[8][9].

Material and Method

Reagents and Chemicals

ExcelaR grade Disodium hydrogen phosphate anhydrous, Orthophosphoric Acid (OPA), Sodium Hydroxide, Hydrochloric Acid, Hydrogen Peroxide (30%w/v) from Qualigens; HPLC grade Acetonitrile (ACN) from sd fine-chem. Esomeprazole Magnesium working standard was procured from jubilant generics limited. For analysis purified water was used.

Instrumentation

In this HPLC method Waters 2695 separations module with 2487 dual wavelength absorbance detector Empower 2 was used.Waters empower software was used for processing and gathering of data. Analytical balance XP 205 from Mettler Toledo, microbalance UMX2 from Mettler Toledo, Photo stability chamber from Thermo lab, Vacuum oven 530 from thermolab were used.

Method development

Method development involves many critical steps. Following are the steps that involve a sequence of events that were taken into consideration during the method development of esomeprazole in solid dosage form :

1. **Information about sample:** This step involves the study of raw materials and the intermediate products which was found necessary for identifying impurities and developing the Analytical Method for Esomeprazole.

Various related substances that were to be taken into account including raw materials and intermediates were benzimidazole(Impurity A),Desmethoxy (Impurity-B) ,Sulfide (Impurity-C), Sulfone(Impurity-D), N-oxide (Impurity-E), R-isomer (Impurity-F**)**

Chemical structure: Esomeprazole's chemical structure shows that it is a slightly acidic compound. pKa value of Esomeprazole is 3.9.

Molecular weight: Molecular weight of Esomeprazole is 767.2.

Solubility: Esomeprazole is slightly soluble in water, soluble in methanol and DMF, sparingly soluble in ethanol.

Inference: As per this data and literature, reverse phase chromatography has been chosen.

2. Choose detector: Since Esomeprazole is a UV active compound, UV detector has been chosen for detection.λ_{max} of Esomeprazole is 302nm and the absorbance of impurities was observed at 280nm.

3. Choose LC methods, preliminary run, estimate best separation conditions:

LC methods: As discussed previously RP-HPLC method was used.

Preliminary run: In mobile phase preparation following solvent and buffer were taken:

Acetonitrile was used as it is having the UV-cut off 190nm and is commonly used solvent. Phosphate buffer was selected as buffer and triethylamine was used as peak modifier. Slightly polar column was taken in order to decrease the run time and resolution.

Estimate best separation conditions: Best separation conditions were determined by attempting various method development trials(6 trials) by varying composition of mobile phase, column change, gradient change, by varying run time, or with the help of peak modifiers etc. given in table 1 and 2.

Table: 1 Method development trials 1-3

Conditions	Trial-1	Trial-2	Trial-3
Column	Phenomenex Luna C-8 (150×4.6) mm,5 µm	Zorbax SB C-8 (150×4.6) mm,5 µm	Zorbax SB C-8 (150×4.6) mm, 5 µm
Mobile Phase	H_2O: ACN (50:50), pH - 7.0 with TEA	1.4 g Na_2HPO_4 Buffer : ACN (50:50), pH - 7.0 with OPA	1.4 g Na_2HPO_4 Buffer : ACN (70:30), pH - 7.6 with OPA
Isocratic	Time / MP-A 0.01 / 100 50 / 100	Time / MP-A 0.01 / 100 50 / 100	Time / MP-A 0.0 / 100 50 / 100
Injection Volume	20 µL	20 µL	40 µL
Flow Rate	1.0 ml/min.	1.0 ml/min.	1.0 ml/min.
Run Time	50 min.	50 min.	50 min.
Wavelength & Temperature	280 nm 25 ºC	280 nm 25 ºC	280 nm 25 ºC
Trial's observations & corrections to next trial	• Base line is good but the retention of the sample in the column was too much. • Too much fronting is seen. • No resolution between the peaks. • Column needs to be changed. Stable bond column is needed.	• Column was changed to stable bonded C8. • Reduction in fronting is seen. • Base line is good. • Still no peak resolution. • Buffer used to stabilize the pH. • Peak elution is still too early.	• Base line is good. • Resolution between the sulfone and esomeprazole peak is more than 2.

Table: 2 Method development trials 4-6

Conditions	Trial-4	Trial-5	Trial-6
Column	Zorbax SB C-8(150×4.6) mm, 5 µm	Zorbax SB C-8 (150×4.6) mm, 5 µm	Zorbax SB C-8 (150×4.6) mm, 5 µm
Mobile Phase **MP -A** **MP - B**	1.4 g Na_2HPO_4 pH - 7.6 with OPA Buffer:ACN::80:20 Buffer:ACN::30:70	1.4 g Na_2HPO_4 pH - 7.6 with OPA Buffer:ACN::80:20 Buffer:ACN::30:70	1.4 g Na_2HPO_4 pH - 7.6 with OPA Buffer:ACN::80:20 Buffer:ACN::30:70
Gradient	Time / MP-A / MP-B 0.01 / 80 / 20 10 / 80 / 20 20 / 55 / 45 40 / 55 / 45 45 / 80 / 20 50 / 80 / 20	Time / MP-A / MP-B 0.01 / 90 / 10 10 / 90 / 10 20 / 55 / 45 40 / 55 / 45 45 / 90 / 10 50 / 90 / 10	Time / MP-A / MP-B 0.01 / 87 / 13 10 / 87 / 13 20 / 55 / 45 40 / 55 / 45 45 / 87 / 13 50 / 87 / 13
Injection Volume	40 µL	40 µL	40 µL
Flow Rate	1.0 ml/min.	1.0 ml/min.	1.0 ml/min.
Wavelength, Temperature	280 nm 25 ºC	280 nm 25 ºC	280 nm 25 ºC

Trial's observations & corrections to next trial	• Baseline is good. • Changed the buffer composition and also undergone the gradient flow to increase the resolution between the peaks. • Still no resolution between desmethoxy and Esomeprazole.	• Base line is good. • Peak resolution between MBT, N-oxide, Esomeprazole and sulfide are not good.	• Blank interference in the main peaks is not seen. • No interference of any peak with the main peak and to each other. • Resolution between the sulfone and Esomeprazole peak is more than 2.

4. Optimize separation conditions and check for problems

After the above trials, it was concluded that the last trial method need to be optimized.Method developed at Waters 2695 separations module with 2487 dual wavelength absorbance detector Empower 2.

Optimized chromatographic condition is as follow:

a) **Column** - Zorbax SB C-8, (150 x 4.6) mm, 5µm

b) **Column Temperature** - 25° C

c) **Flow Rate** - 1.0 mL per minute

d) **Gradient Program -(Table-3)**

Time (Min)	M P-A, %	M P-B, %
0.0	87	13
10	87	13
20	55	45
40	55	45
45	87	13
50	87	13

e) Injection Volume - 40 µL

f) Detector Wavelength - 280 nm

g) Run Time - 50 minutes

h) Relative Retention Time - Impurity-A, RRT about 0.36
Impurity-E, RRT about 0.43
Impurity-D, RRT about 0.66
Impurity-B, RRT about 0.89
Esomeprazole Magnesium, RRT 1.00
Impurity-C, RRT about 2.06

Method Validation

Preparation of various solutions

Preparation of Buffer

Weighed accurately about 1.4 gm of disodium hydrogen phosphate anhydrous and transfer the material into a 1.0 L bottle. 1.0 L of HPLC grade water was added. Solution was mixed well using a magnetic stir bar until the material was completely dissolved and adjustedpH to 7.6 ± 0.05 with dilute orthophosphoric acid (10%v/v). Solution was filtered through a 0.45 µm nylon membrane filter.

Preparation of Mobile Phase-A

Buffer and acetonitrile in the ratio of 80:20 was mixed and sonicated for 5 minutes.

Preparation of Mobile Phase-B

Buffer and acetonitrile in the ratio of 30:70 was mixed and sonicated for 5 minutes.

Preparation of Diluent

Mobile phase-A was used as Diluent.

Preparation of Reference Solution (a)

About 1.0 mg of Impurity-D and 1.0 mg of Esomeprazole magnesium reference/working standard was taken into a 10.0 mL volumetric flask.Dissolved and diluted to volume with diluent.

Preparation of Reference Solution (b)

14 mg of the Esomeprazole magnesium reference/working standard was taken into 100.0 mL volumetric flask. Dissolved and diluted to volume with diluent.1.0 mL of above solution was diluted to 100.0 mL with diluent. Further 1.0 mL of this solution was diluted to 10.0 mL with diluent.

Preparation of Test Solution

14 mg of the sample was weighed into 100.0 mL volumetric flask. Dissolved and diluted to volume with diluent. Use freshly prepared solution for injection

1) System suitability studies :System suitability solution was prepared as per method developed. Blank, system suitability solution was injected as per injection sequence and checked the acceptance criteria for system suitability.Using the system suitability software, Resolution (R) between Impurity D (Sulfone) and Esomeprazole peak was calculated, which should be greater than 3.0.

2) Stability in analytical solution:A sample solution of Esomeprazole Magnesium API was prepared and kept at 25°C in autosampler. Sample solution was analyzed initially and at different time intervals, at

25°C. Difference in area for each impurity with respect to initial peak area should not be more than 10.0%.

3) Specificity : Known related substances, Esomeprazole Magnesium API and diluent were analyzed individually as per the methodology, to examine interference, if any, of known related substances with Esomeprazole peak and with each other. Further, the sample solution of Esomeprazole Magnesium API was spiked with all known related substances of Esomeprazole to check their interference, if any, with Esomeprazole peak.

4) Forced Degradation Studies : Forced degradation studies were carried out on Esomeprazole Magnesium API. The sample was degraded in presence of acidic, basic, oxidative, thermal and photolytic conditions

Parameters and conditions selected for forced degradation study:

Degradation with 0.03N hydrochloric acid at room temperature for 2.0 minutes

Degradation with 1.0% hydrogen peroxide at room temperature for 1.0 minute

Degradation with 5 N NaOH at 80 °C for 1 hour

Thermal degradation of Esomeprazole (Dry heating at 105°C) for 24 hours

Photolytic degradation for 480 hours (exposed)

Photolytic degradation for 480 hours (unexposed)

Acidic Degradation

Transferred about 14.0 mg of esomeprazole sample into 100 ml volumetric flask, added about 10 ml of 0.03Nhydrochloric acid. Kept it at room temperature for 1.0 minute andneutralized with the same quantity and concentration of NaOH, added 60 ml diluent and sonicated to dissolved and diluted to volume with diluent.

Oxidative Degradation

Transferred about 14 mg of esomeprazole sample into 100 ml volumetric flask, added about 10 ml of 1.0 % hydrogen peroxide solution at room temperature, immediately added diluent and sonicated to dissolve and diluted to volume with diluent.

Alkaline Degradation

About 14 mg of sample was weighed accurately into a 100 mL volumetric flask, 5N sodium hydroxide was added and kept the volumetric flask at 80°C for 1 hourand neutralized with the same quantity and concentration of HCl. Alkaline degradation blank was prepared in the same way without using sample.

Thermal Degradation

About 500 mg of sample was weighed transferred in a LOD bottle and kept it at 105°C for about 24 hours in oven.

Weigh accurately about 14 mg of thermal degradation sample into a 100 mL volumetric flask, dissolve and make up to volume with diluent.

Photolytic Degradation

About 500 mg of sample was weighed and transferred into two separate LOD bottle, one LOD bottle was covered with lid and then with aluminium foil (dark control) and another LOD bottle (photolytic exposed sample) was placed as such into the photolytic chamber by covering with lid in such a way to get the minimum exposure of 1.2 million lux hours.

About 14 mg of dark control sample and photolytic exposed sample was weighed accurately into two separate 100 mL volumetric flasks. Sample was dissolved and diluted each to volume with the diluent.

Single injection of blank and sample solutions was injected of all degradation conditions. The peak purity and % degradation of esomeprazole peak in all the degradation conditions was checked by using following formula:

$$\% \text{ Degradation} = \frac{\%\text{Purity in control sample} - \%\text{Purity in degradation sample}}{\%\text{ Purity in control sample}} \times 100$$

5) Limit of Detection and Quantitation : The Limit of Detection and Quantitation for all known related substances and Esomeprazole was determined by showing precision of six replicate injections of predicted concentration of all known related substances and Esomeprazole. %RSD should not be more than 10 for LOQ and should not be more than 33 for LOD.

6) Linearity or Range : The linearity of response for Esomeprazole and all related substances were determined in the range from LOQ to 120% of specification levels. Response factor for impurities has been calculated

7) Accuracy :Esomeprazole Magnesium API was spiked with known quantities of all known related substances at LOQ, 100% and 120% of specification level, in triplicate. The samples were analyzed by the proposed method and the amount of all known related substances recovered was calculated.

8) Precision

i) System Precision

Six replicate injections of reference Solution (a) were given in the HPLC system and Resolution (R) between impurity-D and Esomeprazole peak has been calculated.

ii) Method Precision

Six samples of a single batch of Esomeprazole Magnesium API were prepared and analyzed by the proposed method. Data is shown in table: 40(Acceptance criterion: %RSD should not be more than 10.0 of individual and total impurity).

iii) Intermediate Precision

Preparing and analyzing six times the samples of a single batch of Esomeprazole Magnesium API were prepared and analyzed, by two different analysts on two different instruments and columns on different days verified Method ruggedness.

9)Robustness: Robustness of the method was

investigated by varying the instrumental conditions such as flow rate (± 10 %), Mobile phase composition by ± 2 % absolute, wavelength of detection (±5 nm), pH of buffer (± 0.2 pH units) and column oven temperature (± 5°C). The Resolution between Impurity-D (Sulfone) and Esomeprazole peak for each set of data was calculated.

RESULTS AND DISCUSSION:

METHOD DEVELOPMENT

While developing method, after six trials it was concluded that trail-6 need to be optimized and the optimized method was further proceeded for method validation.

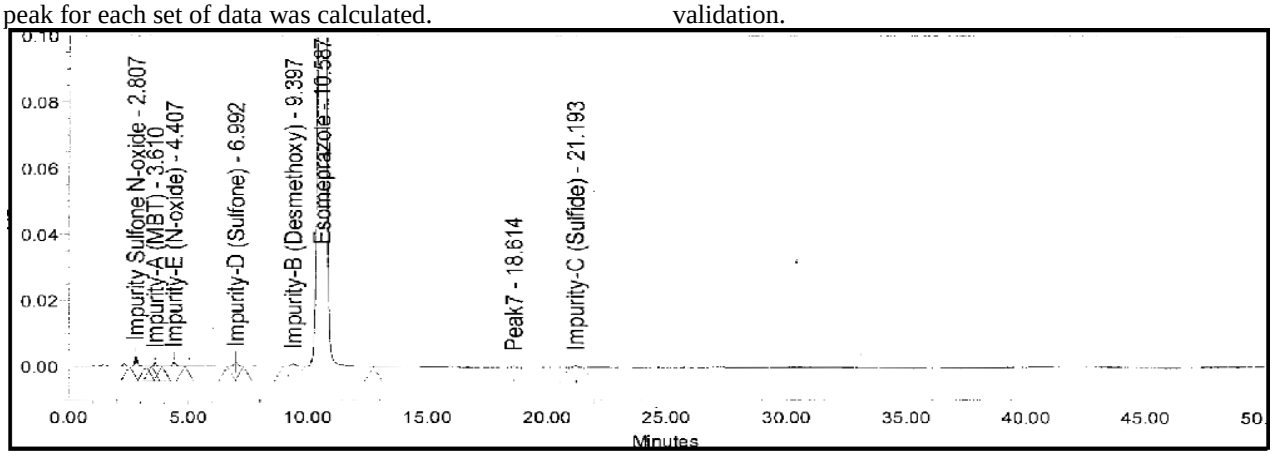

Fig.2 Chromatogram of Method Development Trial-6

METHOD VALIDATION

1. System suitability studies: The resolution between Impurity D (Sulfone) and Esomeprazole peak was calculated, which was found to be greater than 3.0 as shown in table 4.

Table :4 Observations of System suitability studies

System suitability parameter	Observations on different days during validation studies	Acceptance criteria
Resolution between Impurity D (Sulfone) and Esomeprazole peak	6.48, 6.62, 6.89, 7.04, 7.18, 7.61, 6.62, 5.43, 7.64, 7.22, 6.34, 7.65, 7.20, 8.49, 7.94	NLT 3.0

2. Stability in analytical solution: As per data given in table 5 and 6, unknown impurities at RRT 1.10 and 2.72 were gradually increasing with time, so it was concluded that Esomeprazole sample solution is not stable at 25°C and freshly prepared solution should be used.

Table: 5 Results for stability in analytical solution at 25°C-1

Interval (min.)	Impurity-A (MBT)		Impurity-B (Desmethoxy)		Impurity-C (Sulfide)		Impurity-D (Sulfone)	
	Area counts	% Diff.	Area counts	% Diff.	Area counts	% Diff.	Area counts	% Diff.
Initial	6149	-	12186	-	6751	-	16385	-
51	6205	-0.91	12104	0.67	6677	1.10	16400	-0.09
103	6290	-2.29	12066	0.98	6720	0.46	16500	-0.70
154	6263	-1.85	11951	1.93	6788	-0.55	16583	-1.21
205	6394	-3.98	12036	1.23	6847	-1.42	16633	-1.51
256	6463	-5.11	12158	0.23	6799	-0.71	16737	-2.15
378	6902	-12.25	12044	1.17	7165	-6.13	17194	-4.94
500	6953	-13.08	12041	1.19	7553	-11.88	17946	-9.53

Table: 6 Results for stability in analytical solution at 25°C-2

Interval (min.)	Impurity-E (N-Oxide)		Impurity Sulfone N-Oxide		Unknown impurity at RRT-1.10		Unknown impurity at RRT-2.72	
	Area counts	% Diff.	Area counts	% Diff.	Area counts	% Diff.	Area counts	% Diff.
Initial	12226	-	17835	-	0	-	0	-
51	12241	-0.12	17891	-0.31	4695	N/A	0	N/A
103	12187	0.32	17922	-0.49	12275	N/A	0	N/A
154	12190	0.29	17949	-0.64	20161	N/A	0	N/A
205	12225	0.01	17858	-0.13	27757	N/A	0	N/A
256	12329	-0.84	17955	-0.67	35381	N/A	0	N/A
378	12387	-1.32	17764	0.40	50576	N/A	2846	N/A
500	12431	-1.68	17187	3.63	53544	N/A	10435	N/A

3. Specificity : The purity (purity angle should be less than purity threshold) of Esomeprazole and all known related substance indicate that the peaks were homogeneous and there was no interference of Esomeprazole related substances with Esomeprazole peak and with each other.

Table: 7 Results of peak purity study

Sample	Purity Angle	Purity Threshold
Esomeprazole Magnesium (UnspikedSample)	0.64	1.73
Esomeprazole Magnesium (Spiked Sample)	0.89	1.00
Impurity A (MBT)(Spiked Sample)	0.36	0.52
Impurity B (Desmethoxy) (Spike Sample)	1.26	1.43
Impurity C (Sulfide)(Spiked Sample)	0.66	0.90
Impurity D (Sulfone)(Spiked Sample)	0.78	1.15
Impurity E (N-oxide)(Spiked Sample)	0.52	0.79
Impurity Sulfone N-oxide(Spiked Sample)	0.34	0.57

Table: 8 RRT and RT of Esomeprazole and its Related Substances

Sample	RRT	RT in spiked solution	RT in individual solution
Esomeprazole Magnesium	1.00	10.79	10.73
Impurity A (MBT)	0.35	3.76	3.76
Impurity B (Desmethoxy)	0.88	9.53	9.51
Impurity C (Sulfide)	1.97	21.28	21.27
Impurity D (Sulfone)	0.62	6.67	6.66
Impurity E (N-oxide)	0.41	4.44	4.44
Impurity Sulfone N-oxide	0.25	2.70	2.67

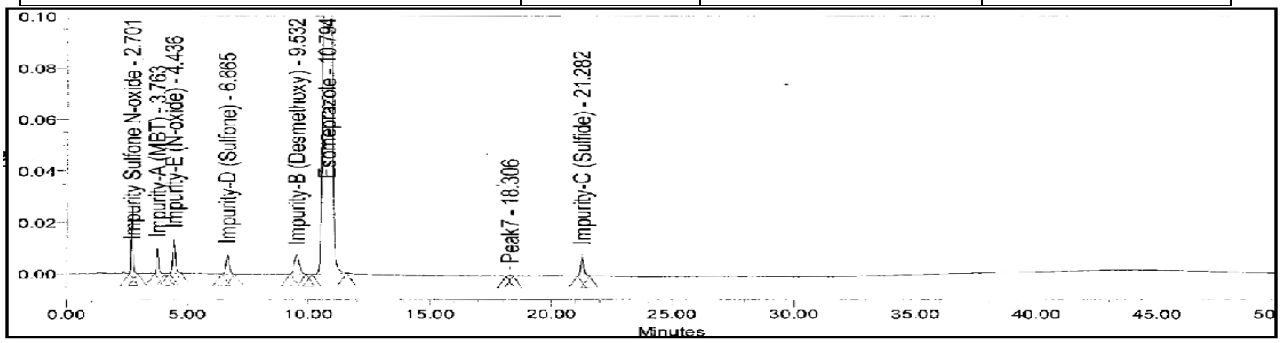

Fig.3 Chromatogram for spiked sample

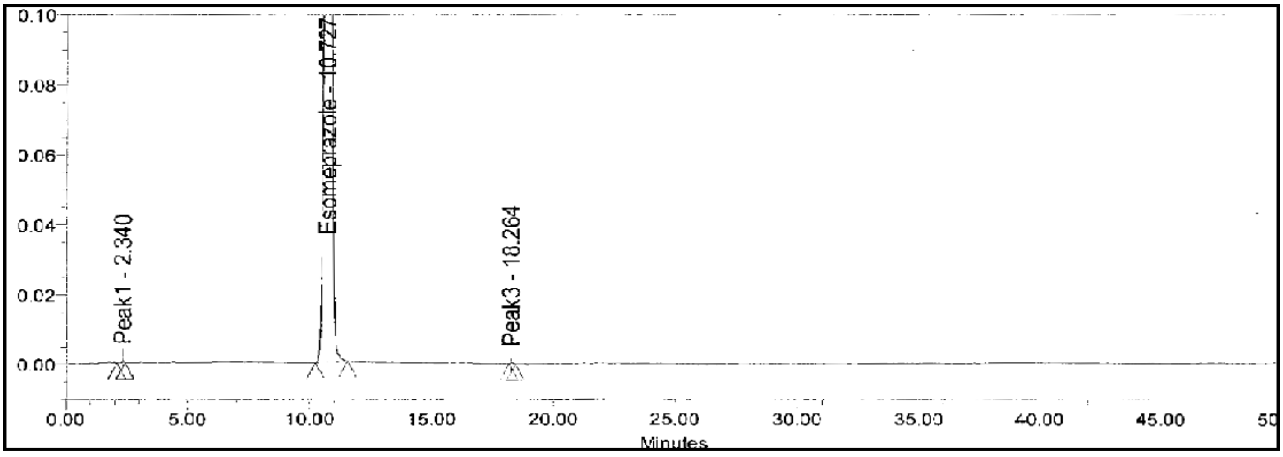

Fig.4 Chromatogram for Esomeprazole sample

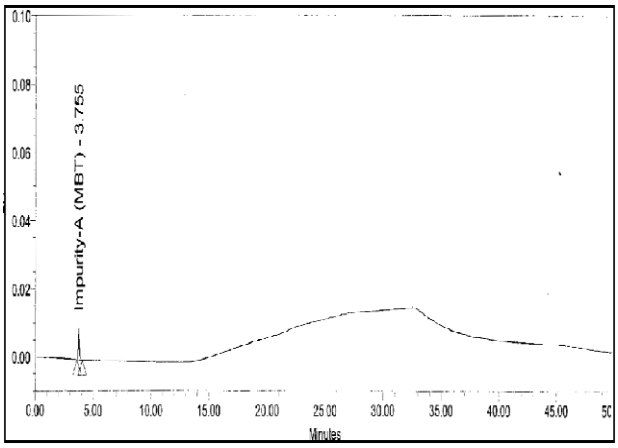

Fig. 5 Chromatogram for RT check of Impurity-A

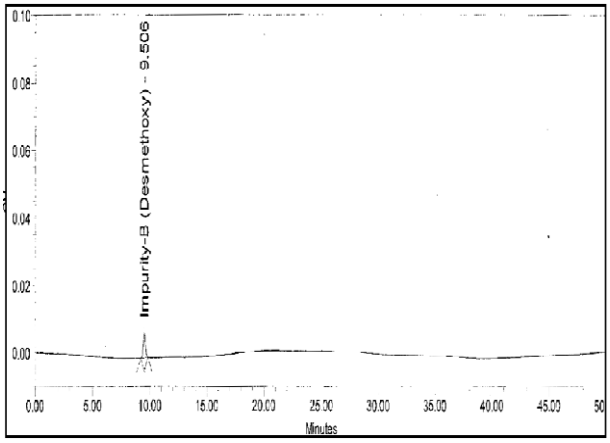

Fig. 6 Chromatogram for RT check of Impurity-B

4. Forced Degradation Studies: Esomeprazole Magnesium was observed to be susceptible to acidic, oxidative, thermal andphotolytic conditions(table 9).The study demonstrates that the method of analysis was specific, as no interference has been observed to the known impurities. Hence the method is stability indicating as shown in chromatograms of fig.7,8,9,10.11.

Table: 9 Results of forced degradation studies

Mode of degradation	Condition	Purity (% Area)	Degradation w.r.t. Control	Purity Angle	Purity Threshold
Control	No treatment	99.88	NA	0.64	1.73
Acid degradation 0.03N HCl	At Room Temp. / 2 mins	90.11	9.78	0.91	1.04
Alkali degradation 5 N NaOH	80°C / 1 hr	96.46	3.42	0.91	1.03
Peroxide degradation 1.0 % v/v H_2O_2	At Room Temp. / 1 min	77.94	21.97	0.77	1.03
Thermal degradation	105°C for 24 hrs	84.31	15.59	0.62	1.03
Photolytic degradation (exposed)	2500 Lux for 480hrs	83.23	16.67	0.57	1.03
Photolytic degradation (unexposed)	2500 Lux for 480hrs	98.38	1.50	0.77	1.02

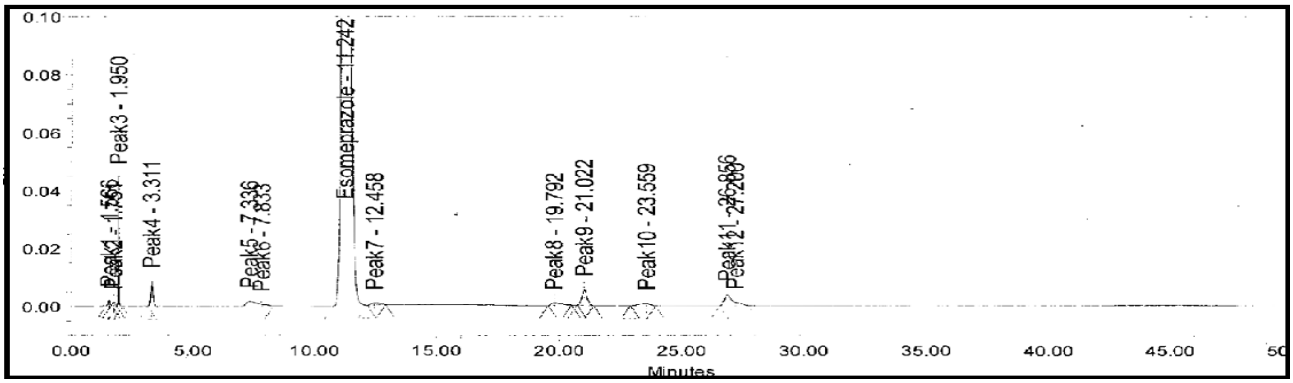

Fig.7 Chromatogram for acidic degradation studies(0.03N HCl for 2minutes)

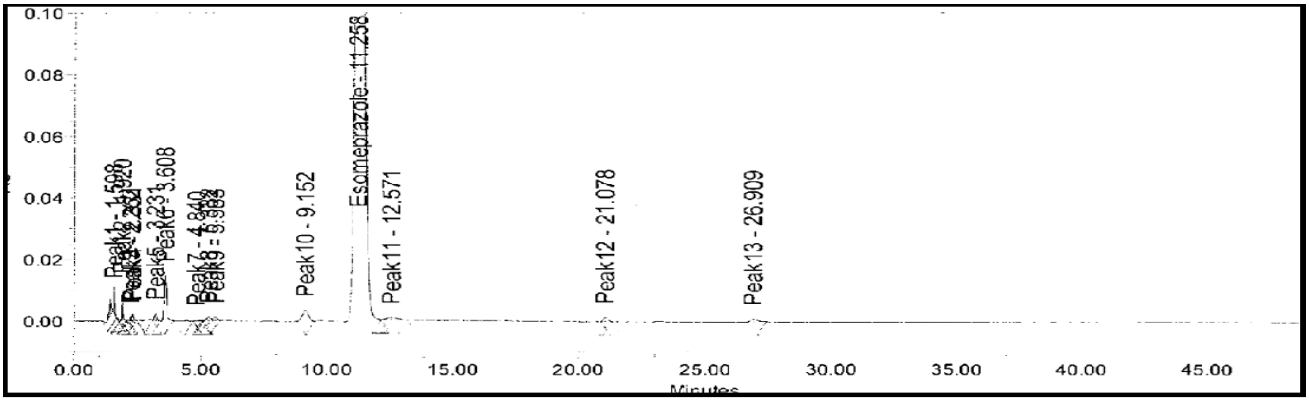

Fig.8 Chromatogram for alkaline degradation studies (5N NaOH for 1 hour at 80°C)

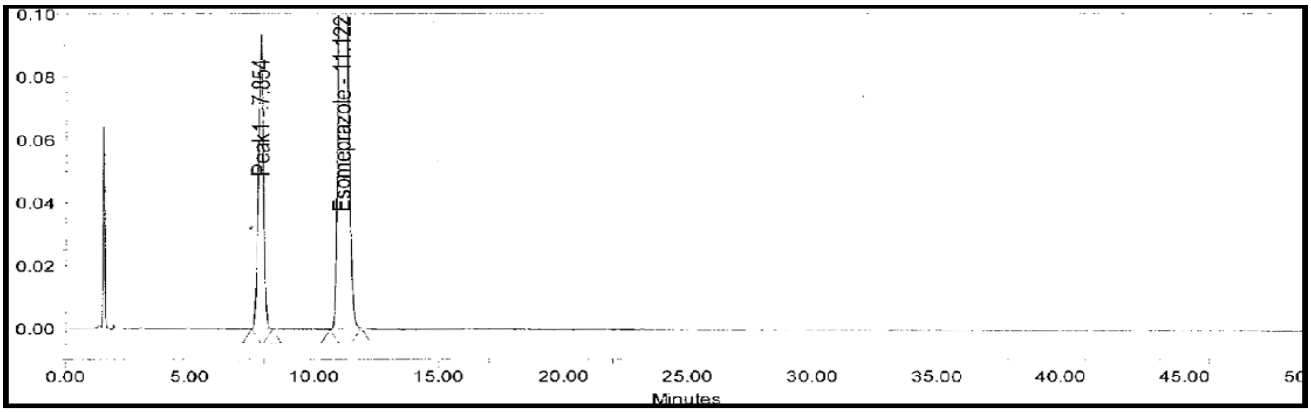

Fig.9 Chromatogram for forced degradation studies (1% v/v H_2O_2 for 1 minute)

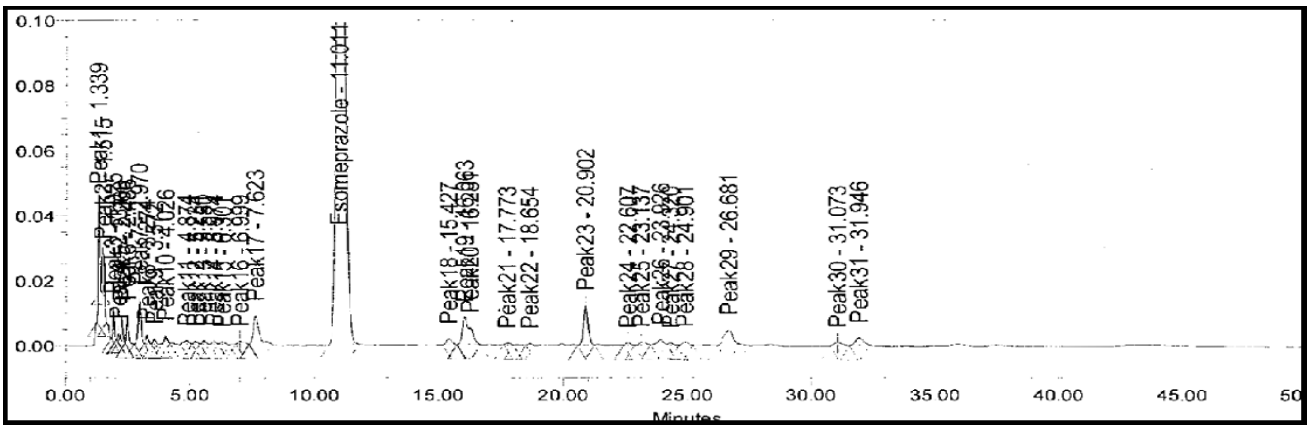

Fig.10 Chromatogram for thermal degradation studies (at 105 ºC for 24 hour)

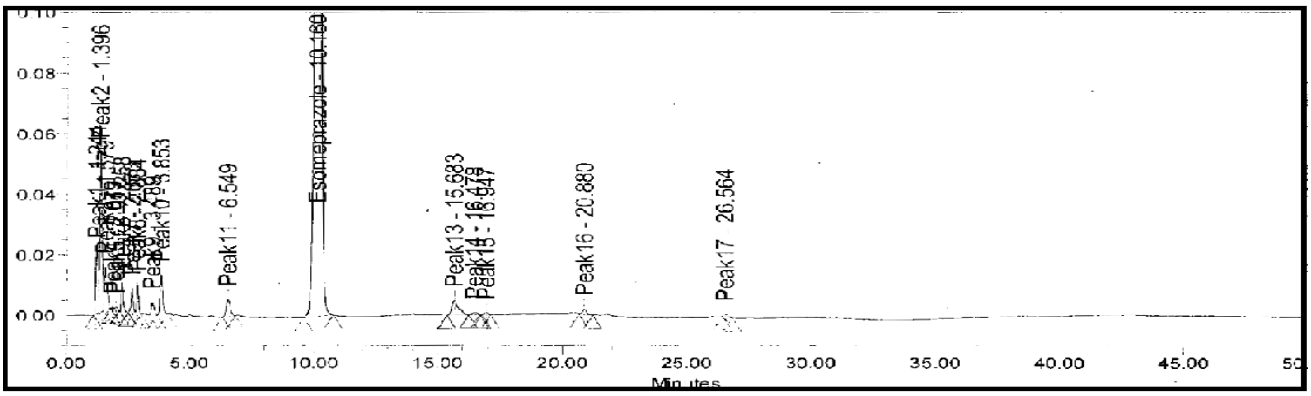

Fig.11Chromatogram for photolytic degradation studies (exposed to 2500 lux)

5.Limit of Detection and Quantitation- The Limit of Detection and Quantitation for all known related substances and Esomeprazole was determined. Table no.10,11,12,13,14,15 indicated that %RSD for LOQ and LOD were within the limit.

Table: 10 LOD and LOQ for Esomeprazole

Esomeprazole	**LOD**	**LOQ**
Conc. (µg/mL)	0.014	0.041
% w/w	0.010	0.029
Injection	**Area counts**	
1	517	1994
2	791	2051
3	826	2074
4	735	2020
5	701	2100
6	727	2101
Mean	**716**	**2057**
SD	**107.7**	**43.5**
RSD (%)	**15.04**	**2.11**

Table: 11 LOD and LOQ for Impurity A(MBT)

Impurity A (MBT)	**LOD**	**LOQ**
Conc. (µg/mL)	0.015	0.045
% w/w	0.011	0.032
Injection	**Area counts**	
1	476	1735
2	644	1791
3	556	1830
4	578	1693
5	476	1807
6	553	1815
Mean	**547**	**1779**
SD	**64.1**	**53.2**
RSD (%)	**11.72**	**2.99**

Table: 12 LOD and LOQ for Impurity B (Desmethoxy)

Impurity B (Desmethoxy)	**LOD**	**LOQ**
Conc. (µg/mL)	0.015	0.044
% w/w	0.011	0.031
Injection	**Area counts**	
1	1008	3566
2	1287	3587
3	1185	3464
4	1273	3429
5	995	3472
6	1020	3409
Mean	**1128**	**3488**
SD	**136.6**	**72.7**
RSD (%)	**12.11**	**2.08**

Table: 13 LOD and LOQ for ImpurityC (Sulfide)

Impurity C (Sulfide)	**LOD**	**LOQ**
Conc. (µg/mL)	0.015	0.044
% w/w	0.011	0.031
Injection	**Area counts**	
1	510	2088
2	833	2021
3	751	2103
4	665	2124
5	706	2092
6	702	2019
Mean	**695**	**2075**
SD	**107.2**	**44**
RSD (%)	**15.42**	**2.12**

Table: 14 LOD and LOQ for Impurity D (Sulfone)

ImpuritySulfoneN-oxide	LOD	LOQ
Conc. (µg/mL)	0.014	0.041
% w/w	0.010	0.029
Injection	Area counts	
1	1065	3696
2	1216	3603
3	1256	3601
4	1165	3605
5	976	3600
6	1015	3615
Mean	**1116**	**3620**
SD	**113.5**	**37.6**
RSD (%)	**10.17**	**1.04**

Table: 15 LOD and LOQ for Impurity E (N-oxide)

Impurity D (Sulfone)	LOD	LOQ
Conc. (µg/mL)	0.014	0.043
% w/w	0.010	0.031
Injection	Area counts	
1	705	2343
2	828	2357
3	682	2352
4	837	2352
5	609	2426
6	750	2361
Mean	**735**	**2365**
SD	**88.2**	**30.4**
RSD (%)	**12.00**	**1.29**

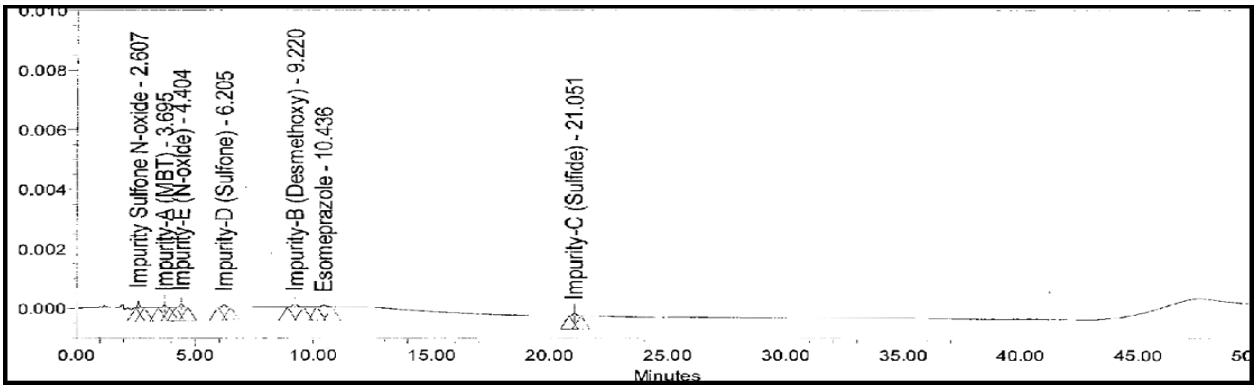

Fig.12 Chromatogram for DL-QL Determination (at 0.01% level)

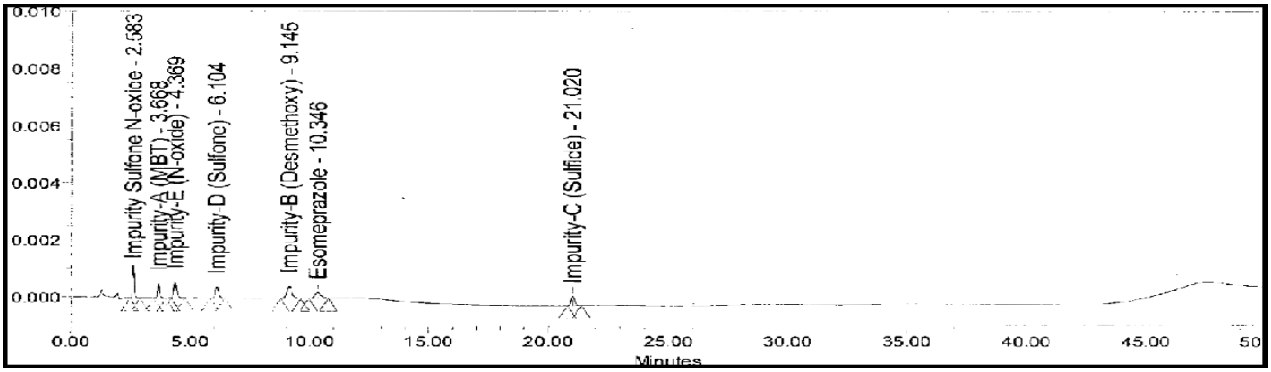

Fig.13 Chromatogram for DL-QL Determination (at 0.05% level)

6.Linearity or Range: The linearity of response for Esomeprazole and all related substances were determined in the range from LOQ to 120% of specification levels. Correlation coefficient found to be more than 0.990 as shown in table 16. Hence the method was found to be linear.

Table: 16Linearity of Esomeprazole

Esomeprazole		
***Conc. Level**	**Conc. (µg/mL)**	**Area counts**
LOQ	0.040	2185
80%	0.211	11386
90%	0.238	12893
100%	0.264	14152
110%	0.290	15689
120%	0.317	16930
Slope		**53526**
Intercept		**73**
Correlation coefficient		**0.9999**
Response Factor		**1.00**

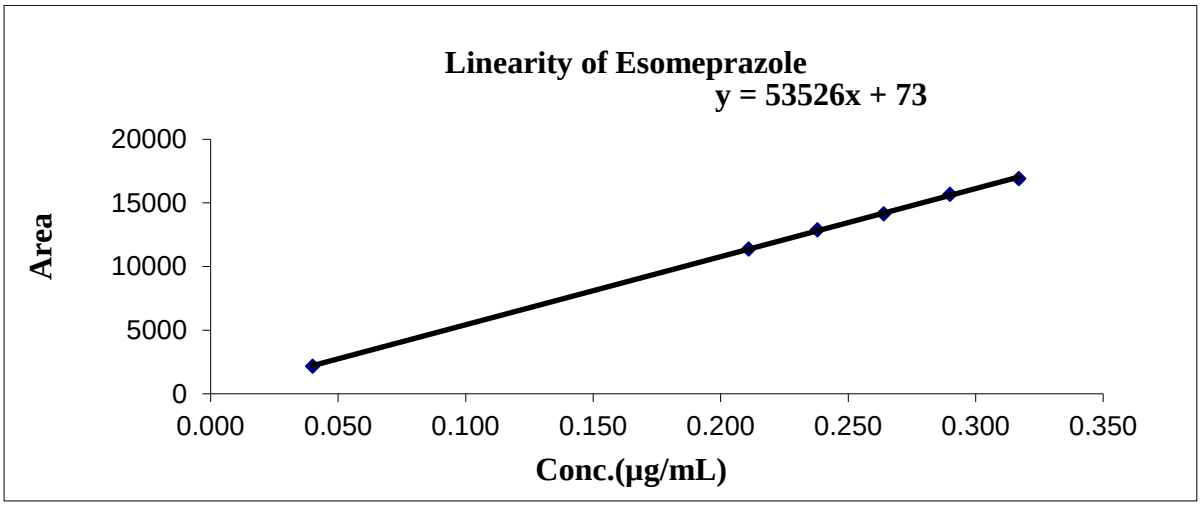

Fig 14 Linearity of Esomeprazole

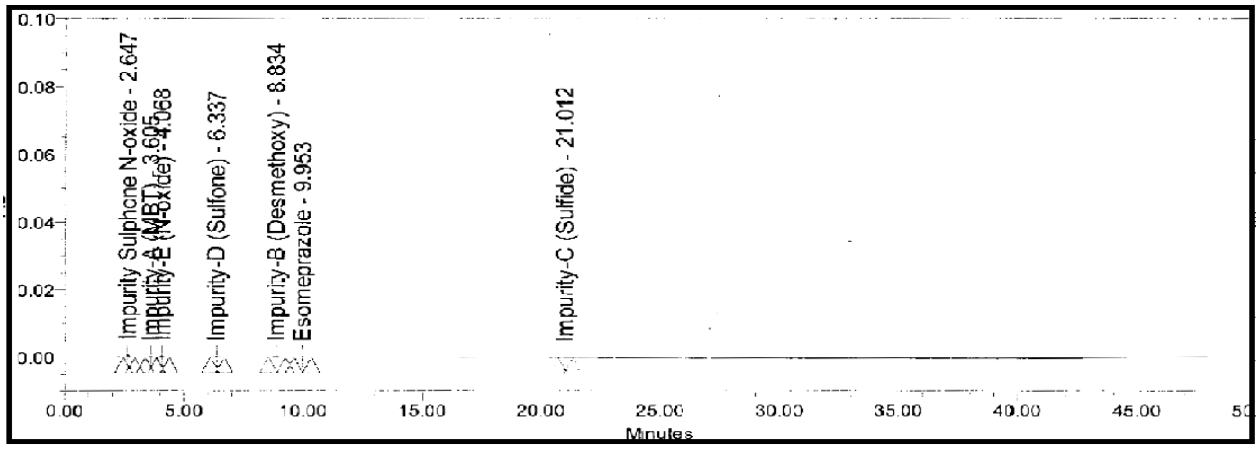

Fig.15 Chromatogram for linearity (at QL level)

7.Accuracy: % Recovery of results found to be in the range of 80-120 and individual and cumulative (overall) % RSD of % Recovery was well within the limit i.e. not more than 10.0.

Table: 17 Accuracy study for Impurity A (MBT)

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.044	0.031	0.047	106.45	**102.15**	**3.724**	**3.65**
		0.031	0.045	100.00			
		0.031	0.045	100.00			
100%	0.147	0.105	0.121	101.90	**102.55**	**0.566**	**0.55**
		0.104	0.121	102.88			
		0.104	0.121	102.88			
120%	0.177	0.125	0.138	99.20	**101.65**	**2.465**	**2.42**
		0.124	0.140	101.61			
		0.121	0.140	104.13			
Overall					**102.12**	**2.285**	**2.24**

Table: 18 Accuracy study for Impurity B (Desmethoxy)

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.042	0.030	0.035	103.33	**101.11**	**1.923**	**1.90**
		0.030	0.034	100.00			
		0.030	0.034	100.00			
100%	0.139	0.099	0.103	100.00	**100.34**	**0.589**	**0.59**
		0.098	0.102	100.00			
		0.098	0.103	101.02			
120%	0.167	0.118	0.118	96.61	**98.02**	**1.767**	**1.80**
		0.117	0.118	97.44			
		0.114	0.118	100.00			
Overall					**99.82**	**1.933**	**1.94**

Table: 19 Accuracy study for Impurity C (Sulfide)

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.043	0.031	0.033	106.45	**106.45**	**0.000**	**0.00**
		0.031	0.033	106.45			
		0.031	0.033	106.45			
100%	0.144	0.103	0.102	99.03	**99.68**	**0.560**	**0.56**
		0.102	0.102	100.00			
		0.102	0.102	100.00			
120%	0.173	0.123	0.118	95.93	**98.09**	**2.047**	**2.09**
		0.121	0.119	98.35			
		0.119	0.119	100.00			
Overall					**101.41**	**3.988**	**3.93**

Table: 20 Accuracy study for Impurity D (Sulfone)

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.041	0.029	0.043	106.90	**110.34**	**3.445**	**3.12**
		0.029	0.045	113.79			
		0.029	0.044	110.34			
100%	0.273	0.194	0.213	103.61	**102.94**	**0.782**	**0.76**
		0.192	0.208	102.08			
		0.192	0.210	103.13			
120%	0.327	0.232	0.236	96.55	**97.53**	**1.063**	**1.09**
		0.229	0.235	97.38			
		0.224	0.233	98.66			
Overall					**103.60**	**5.868**	**5.66**

Table: 21 Accuracy study for Impurity E (N-oxide)

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.041	0.029	0.043	91.28	**96.83**	**4.949**	**5.11**
		0.029	0.046	98.41			
		0.029	0.046	100.79			
100%	0.138	0.098	0.125	110.09	**109.66**	**1.132**	**1.03**
		0.097	0.124	110.52			
		0.097	0.122	108.38			
120%	0.166	0.117	0.138	103.42	**103.42**	**0.300**	**0.29**
		0.116	0.137	103.12			
		0.114	0.135	103.72			
Overall					**103.30**	**6.113**	**5.92**

Table: 22 Accuracy study for Impurity Sulfone N-oxide

Recovery Level	Conc. (µg/mL)	Amount Added (%w/w)	Amount Found (%) w.r.t RF	% Recovery	Mean	SD	% RSD
LOQ	0.041	0.029	0.029	100.00	**100.00**	**0.000**	**0.00**
		0.029	0.029	100.00			
		0.029	0.029	100.00			
100%	0.204	0.145	0.146	100.69	**100.92**	**0.404**	**0.40**
		0.144	0.145	100.69			
		0.144	0.146	101.39			
120%	0.245	0.173	0.170	98.27	**99.63**	**1.471**	**1.48**
		0.171	0.170	99.42			
		0.168	0.170	101.19			
Overall					**100.18**	**0.957**	**0.96**

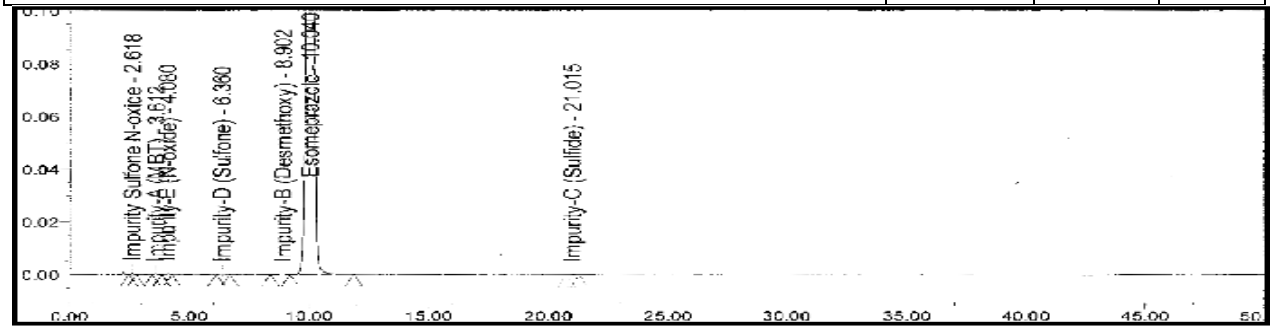

Fig.16 Accuracy at LOQ/ Test-1

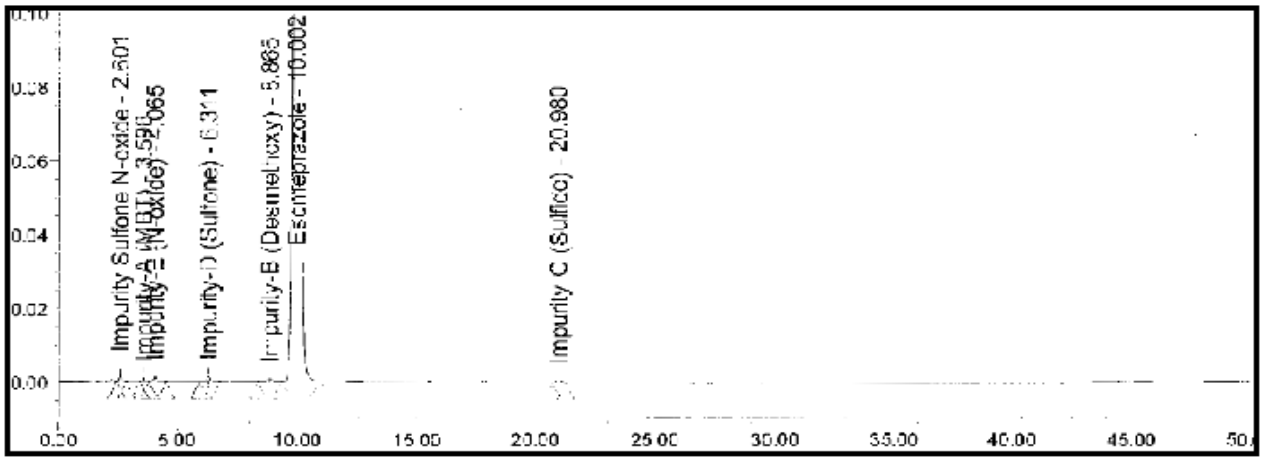

Fig.17 Accuracy at specificity 100%/ Test-1

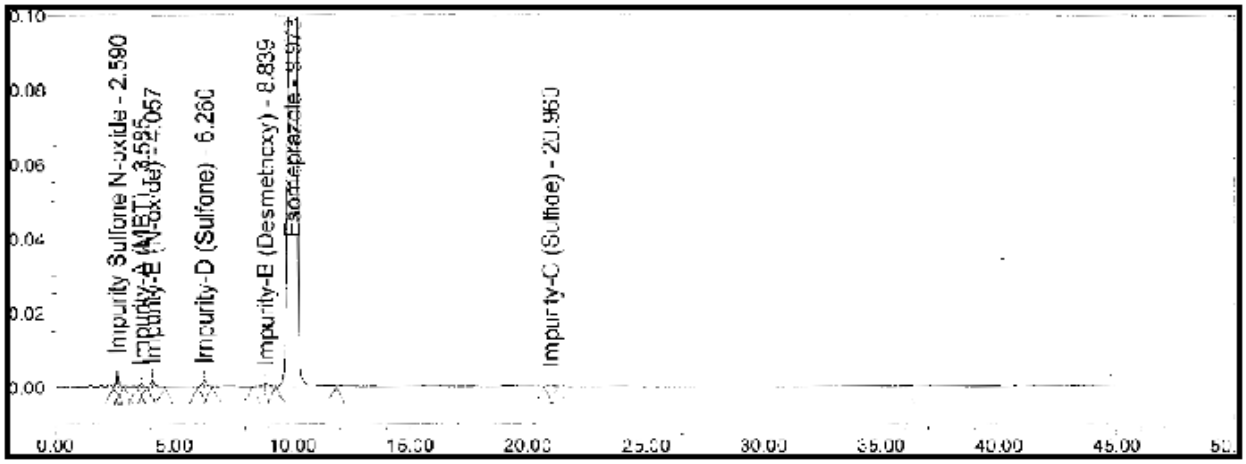

Fig.18 Accuracy at specificity 120%/ Test-1

8.Precision: R should be ≥ 3.0,RSD of resolutions, obtained from six replicate injections of reference solution (a) should not be more than 2.0% for system precision, which was found to be well within the limit. %RSD should not be more than 10.0 of individual and total impurity for method and intermediate precision. From table 23,24,25,26, it was observed that method met the precision criteria.

Table: 23 System Precision

Injection No.	**1**	**2**	**3**	**4**	**5**	**6**
Resolution	7.72	7.71	7.74	7.72	7.74	7.73
Mean	**7.73**					
S.D	**0.01**					
%RSD	**0.16**					

Table: 24 Method Precision

Sample No.	Area Percent w.r.t. Response Factor						
	Imp. A	Imp. B	Imp. C	Imp. D	Imp. E	Imp. Sulfone N-oxide	Total impurity
1	0.10	0.09	0.10	0.21	0.11	0.14	0.75
2	0.10	0.09	0.09	0.21	0.11	0.14	0.74
3	0.11	0.10	0.10	0.22	0.11	0.14	0.78
4	0.10	0.10	0.10	0.21	0.11	0.14	0.76
5	0.11	0.09	0.10	0.21	0.11	0.14	0.76
6	0.10	0.09	0.10	0.21	0.11	0.14	0.75
Mean	**0.10**	**0.09**	**0.10**	**0.21**	**0.11**	**0.14**	**0.76**
SD	**0.005**	**0.005**	**0.004**	**0.004**	**0.000**	**0.000**	**0.014**
%RSD	**5.00**	**5.56**	**4.00**	**1.90**	**0.00**	**0.00**	**1.84**

Table: 25Intermediate Precision-1

Sample No.	Area Percent w.r.t. Response Factor							
	Impurity-A		Impurity-B		Impurity-C		Impurity-D	
	MP	IP	MP	IP	MP	IP	MP	IP
1	0.10	0.11	0.09	0.09	0.10	0.10	0.21	0.21
2	0.10	0.11	0.09	0.09	0.09	0.10	0.21	0.21
3	0.11	0.11	0.10	0.10	0.10	0.10	0.22	0.21
4	0.10	0.11	0.10	0.09	0.10	0.10	0.21	0.21
5	0.11	0.11	0.09	0.09	0.10	0.10	0.21	0.21
6	0.10	0.11	0.09	0.09	0.10	0.10	0.21	0.20
Mean	**0.10**	**0.11**	**0.09**	**0.09**	**0.10**	**0.10**	**0.21**	**0.21**
SD	**0.005**	**0.000**	**0.005**	**0.004**	**0.004**	**0.000**	**0.004**	**0.004**
% RSD	**5.00**	**0.00**	**5.56**	**4.44**	**4.00**	**0.00**	**1.90**	**1.90**
Overall Mean	**0.11**		**0.09**		**0.10**		**0.21**	
Overall SD	**0.005**		**0.005**		**0.003**		**0.004**	
Overall %RSD	**4.55**		**5.56**		**3.00**		**1.90**	

Table: 26Intermediate Precision-2

Sample No.	Area Percent w.r.t. Response Factor					
	Impurity-E		Impurity Sulfone N-oxide		Total Impurity (%)	
	MP	IP	MP	IP	MP	IP
1	0.11	0.10	0.14	0.13	0.75	0.74
2	0.11	0.10	0.14	0.13	0.74	0.74
3	0.11	0.11	0.14	0.13	0.78	0.76
4	0.11	0.11	0.14	0.14	0.76	0.76
5	0.11	0.11	0.14	0.13	0.76	0.75
6	0.11	0.10	0.14	0.13	0.75	0.73
Mean	**0.11**	**0.11**	**0.14**	**0.13**	**0.76**	**0.75**
SD	**0.000**	**0.005**	**0.000**	**0.004**	**0.014**	**0.012**
% RSD	**0.00**	**4.55**	**0.00**	**3.08**	**1.84**	**1.60**
Overall Mean	**0.11**		**0.14**		**0.75**	
Overall SD	**0.005**		**0.005**		**0.013**	
Overall %RSD	**4.55**		**3.57**		**1.73**	

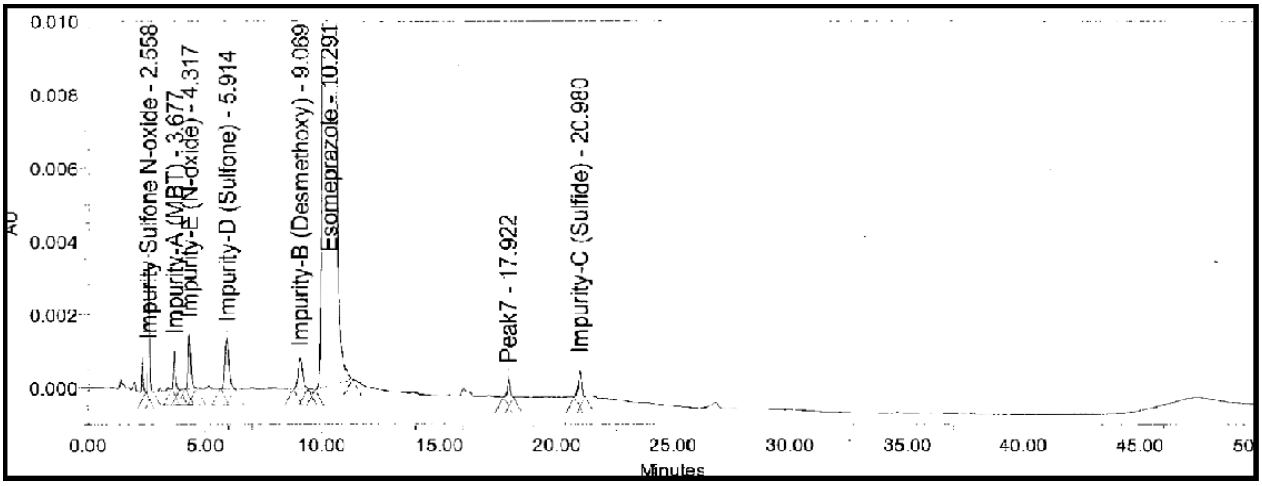

Fig.19 Chromatogram for IP-1

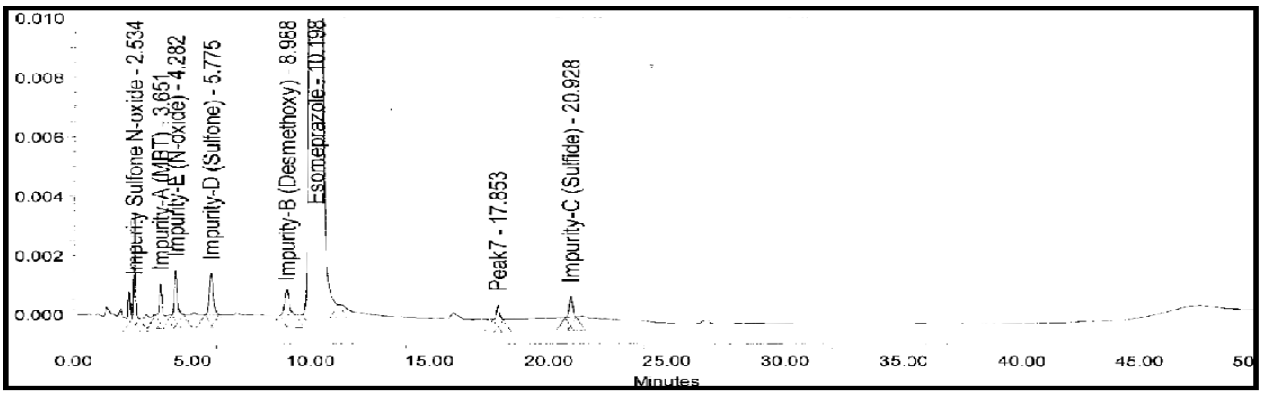

Fig.20 Chromatogram for IP-2

9.Robustness: System suitability [Resolution between Impurity-D (Sulfone) and Esomeprazole peak] was checked at each variable condition and data found to be within the acceptance criteria. Sample solution spiked with all known related substances was analyzed under each condition and relative retention time of each related substance calculated at each variable condition(table 27).

Table: 27 System Suitability of Robustness Condition

Set No.	Robustness Parameter	Resolution
I	Control (Specificity)	7.64
II	Flow rate (–10%)	6.62
III	Flow rate (+10%)	6.49
IV	Column Oven Temp. (-5°C)	7.07
V	Column Oven Temp. (+5°C)	7.27
VI	pH Change (– 0.2 pH units)	5.87
VII	pH Change (+ 0.2 pH units)	8.09
VIII	Mobile Phase composition change (–2 %)	8.74
IX	Mobile Phase composition change (+2 %)	6.28
X	Wavelength (–5 nm)	6.13
XI	Wavelength (+5 nm)	6.39

Table: 28Results for Robustness

Robustness Parameter	Relative Retention Time					
	Imp. A (MBT)	Imp. B(Des-methoxy)	Imp. C (Sulfide)	Imp. D (Sulfone)	Imp. E (N-oxide)	Imp.Sulfone N-oxide
Control (Specificity)	0.35	0.88	1.97	0.62	0.41	0.25
Flow rate (–10%)	0.35	0.88	1.85	0.63	0.42	0.26
Flow rate (+10%)	0.35	0.88	2.09	0.63	0.42	0.26
Column Oven Temp. (-5°C)	0.35	0.88	1.98	0.62	0.42	0.26
Column Oven Temp. (+5°C)	0.35	0.89	2.04	0.59	0.43	0.25
pH Change (– 0.2 pH units)	0.34	0.89	2.01	0.75	0.42	0.28
pH Change (+ 0.2 pH units)	0.36	0.89	2.11	0.61	0.42	0.26
Mobile Phase composition change (–2 %)	0.30	0.88	1.67	0.56	0.39	0.22
Mobile Phase composition change (+2 %)	0.42	0.89	2.43	0.60	0.46	0.28
Wavelength (–5 nm)	0.35	0.88	1.99	0.62	0.42	0.26
Wavelength (+5 nm)	0.35	0.88	1.99	0.62	0.42	0.26

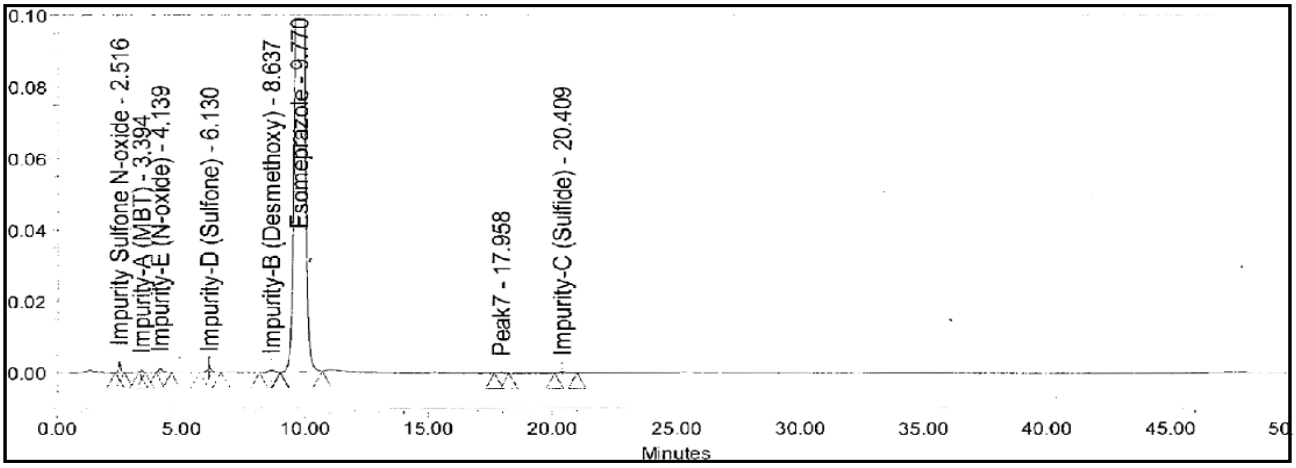

Fig.21 Chromatograms for robustness studies (flow rate increase)

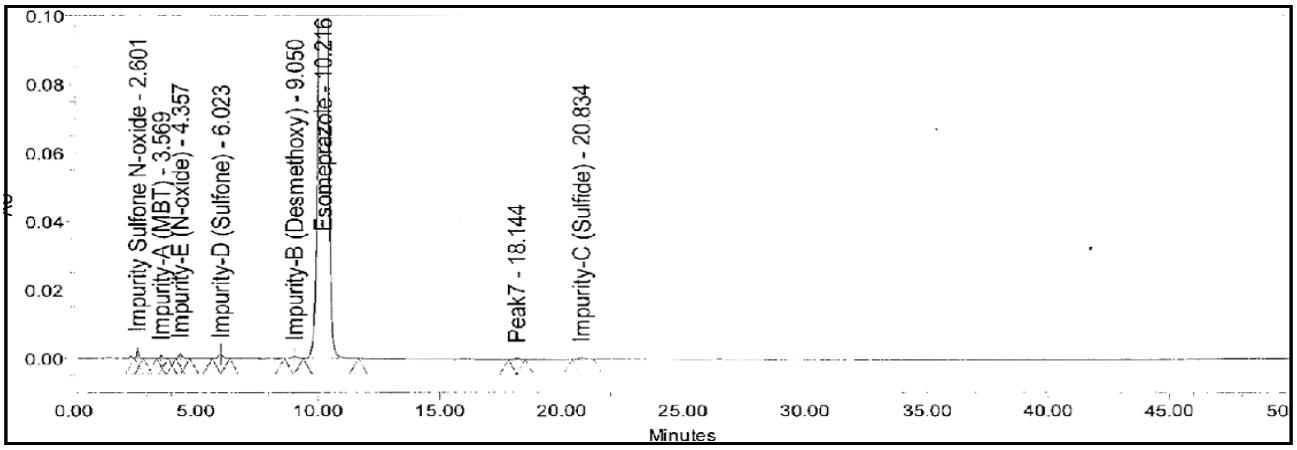

Fig.22 Chromatograms showing robustness studies(Temperature decrease)

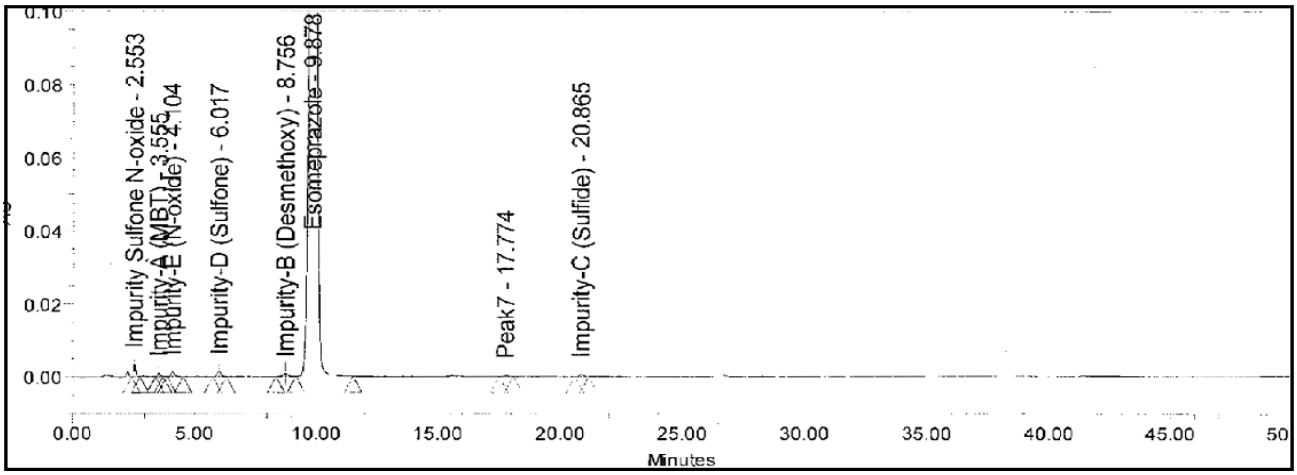

Fig.23 Chromatograms for robustness studies (pH decrease)

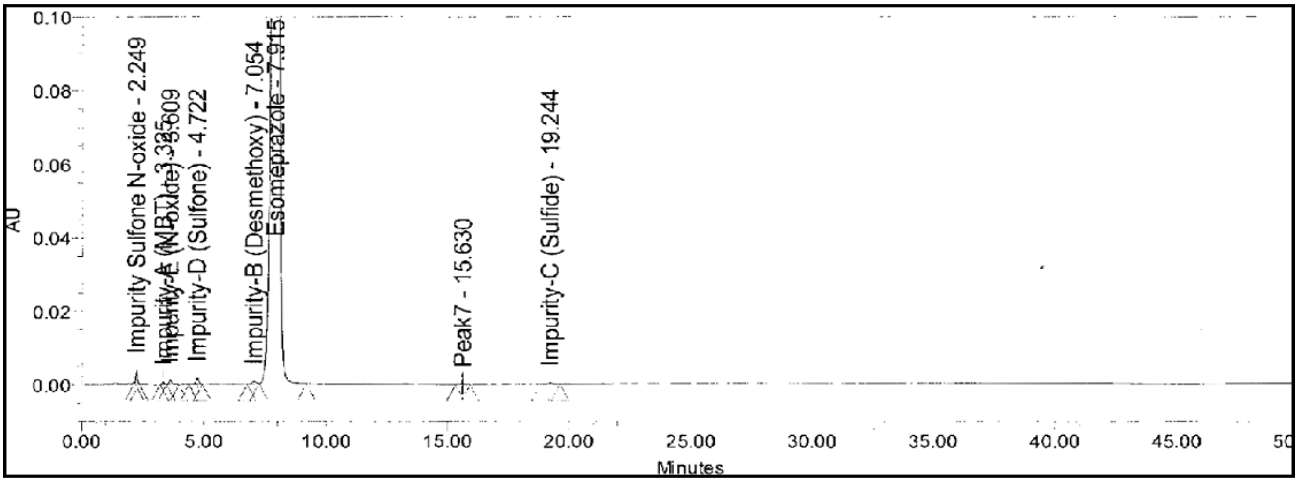

Fig.24Chromatograms for robustness studies (Mobile phase composition increase)

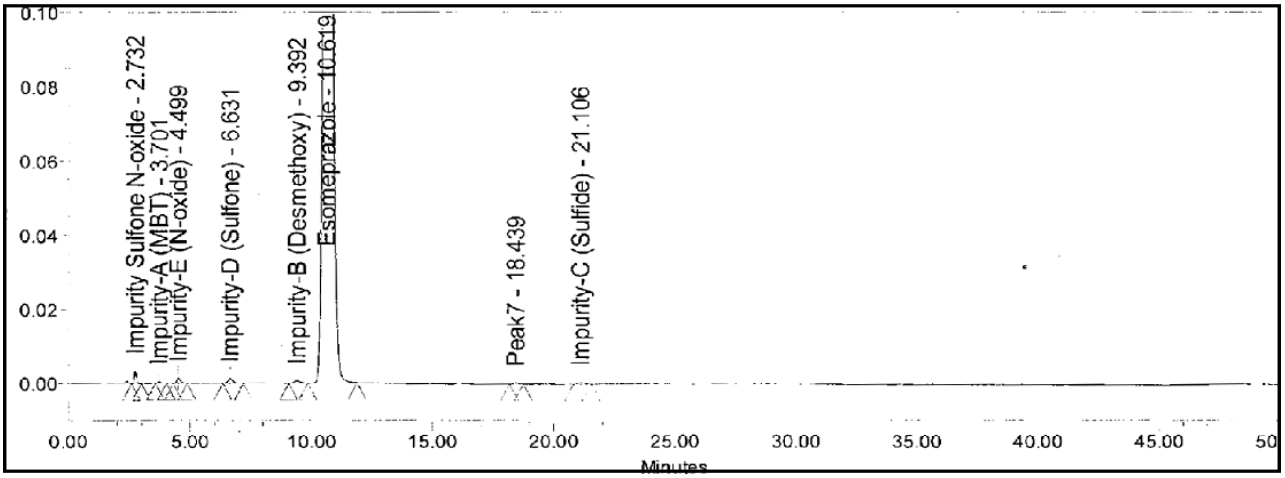

Fig.25Chromatograms for robustness studies (wavelength decrease)

Validation summary results for estimation of related substances of Esomeprazole Magnesium by HPLC(table: 29)

Validation parameter	Acceptance criteria	Results		
System suitability	Resolution between impurity D (sulfone) and esomeprazole peak should be more than 3.0	**Pass**		
Stability in analytical solution	% Difference in peak area for all impurities should not be more than 10.0% for spiked sample solution.	The esomeprazole solution was not stable at 25 ºC, use freshly prepared solution for study.		
Specificity	Peak purity should pass.	Peak purity angle was less than peak purity threshold for all impurities and esomeprazole.		
Forced degradation studies	Peak purity should pass.	Peak purity angle was less than peak purity threshold for all impurities and esomeprazole.		
Limit of Detection (LOD)	% RSD not more than 33.0	**Name**	**µg/ml**	**%RSD**
		Esomeprazole Magnesium	0.014	15.04
		Impurity A	0.015	11.27
		Impurity B	0.015	12.11
		Impurity C	0.015	15.42
		Impurity D	0.014	12.00
		Impurity E	0.014	13.59
		Impurity Sulfone N-oxide	0.014	10.17
Limit of Quantitation (LOQ)	% RSD not more than 10.0	**Name**	**µg/ml**	**%RSD**
		Esomeprazole Magnesium	0.041	2.11
		Impurity A	0.045	2.99
		Impurity B	0.044	2.08
		Impurity C	0.044	2.12
		Impurity D	0.043	1.29
		Impurity E	0.041	1.16
		Impurity Sulfone N-oxide	0.041	1.04
Linearity and range	Correlation coefficient should not be less than 0.990	**Name**	**Correlation coefficient**	
		Esomeprazole Magnesium	0.9999	
		Impurity A	1.0000	
		Impurity B	0.9997	
		Impurity C	0.9999	
		Impurity D	1.0000	
		Impurity E	0.9998	
		Impurity Sulfone N-oxide	1.0000	
Accuracy	%Recovery should not be less than 80 and not more than 120. Individual (at each recovery level) and cumulative(overall) % RSD of % recovery shall not be more than 10.0.	**Name**	**Overall %Recovery**	
		Impurity A	102.12	
		Impurity B	99.82	
		Impurity C	101.41	
		Impurity D	103.60	
		Impurity E	103.30	
		Impurity Sulfone N-oxide	100.18	
System precision	Resolution between esomeprazole and impurity D should be more than 3. % RSD should be less than 2.0.	Resolution was found greater than 3.0 % RSD was 0.16		
Method precision	%RSD should not be more than 10.0 for all known impurities.	%RSD of individual impurities was found less than 10.0		
Intermediate precision	%RSD should not be more than 10.0 for all known impurities.	%RSD of individual impurities was found less than 10.0		
Robustness	System suitability criteria should pass.	In each variable condition the resolution was more than 3 and RRT compared with ideal condition		

CONCLUSION:

The RP-HPLC method for the determination of related substances in esomeprazole has been developed and was specific, sensitive, precise, accurate, rapid and robust. The method allows quantification of six potential related substances (Impurity A(MBT), Impurity B (Desmethoxy), Impurity C (Sulfide), Impurity D (Sulfone), Impurity E (N-oxide), Impurity Sulfone N-oxide) of esomeprazole. The mobile phase A, B consists of 0.01M Disodium hydrogenphosphate:acetonitrile (80:20, v/v) and (30:70, v/v) respectively, pH-7.6 with orthophosporic acid, which were run as per the optimized gradient program. ZorbaxSB C-8, (150cm×4.6 mm, 5µm) was used as the stationary phase. 1.0mL per minute was employed as the flow rate and injection volume was kept as 40µL. Detection wavelength was kept as 280 nm. The method was validated as per ICH Q2 (R1). The developed method can be conveniently used by quality control department to determine the related substances in regular esomeprazole production samples.

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