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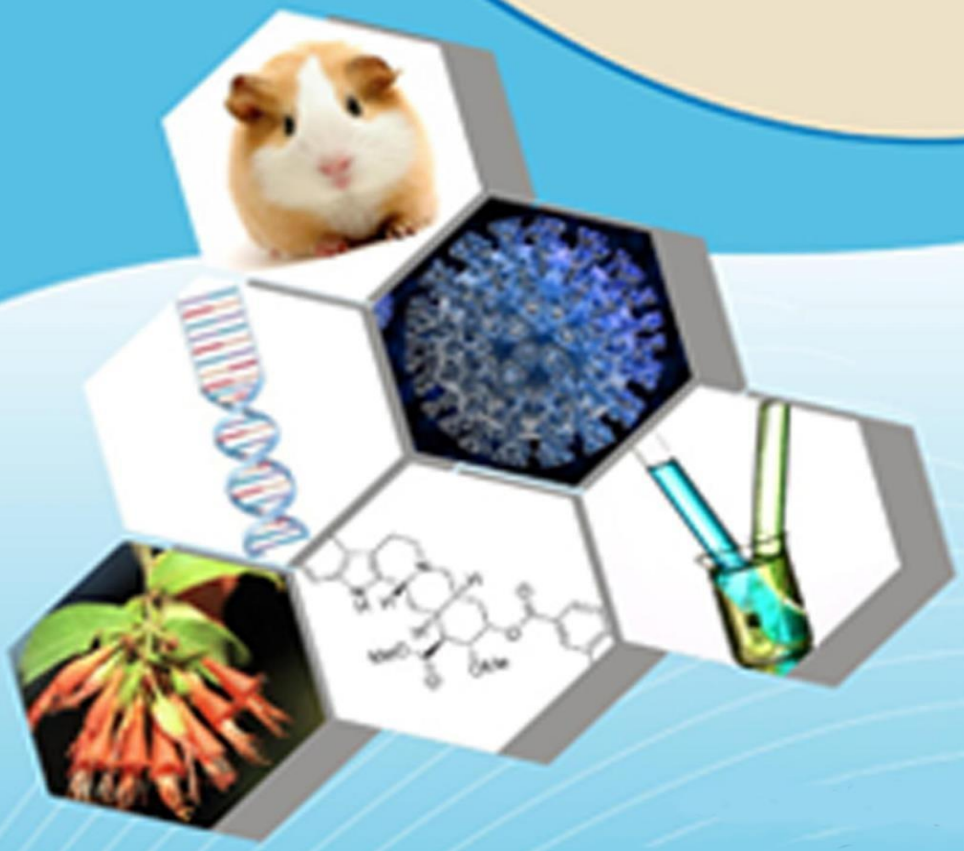

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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF MEBENDAZOLE AND PIPERAZINE BY RP-HPLC

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ABSTRACT

The method provides great sensitivity, adequate linearity and repeatability. The estimation of Mebendazole and Piperazine was done by RP-HPLC. 4.6 and the mobile phase was optimized which consists of MEOH : Phosphate buffer mixed in the ratio of 70:30 % v/ v. A Symmetry C18 (4.6 x 150mm, 5µm, Make XTerra) column used as stationary phase.

INTRODUCTION

CHROMATOGRAPHY

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

Modes of Chromatography:

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

Different modes of chromatography are as follows:

- Normal Phase Chromatography
- Reversed Phase Chromatography
- Reversed Phase – ion pair Chromatography
- Ion Chromatography
- Ion-Exchange Chromatography
- Affinity Chromatography

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- Size Exclusion Chromatography

METHOD DEVELOPMENT:(6,7,8,9)

Analytical method is a detailed description of different steps necessary to perform analytical tests which may include preparation of samples, reagents, use of apparatus, generation of calibration curve and use of formulae for calculations.

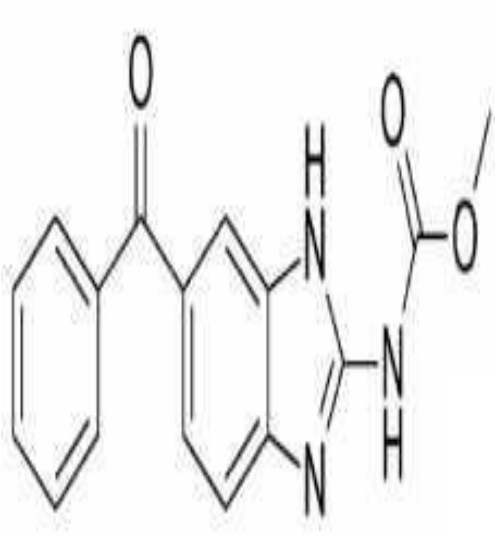
Analytical method development is required to analyze Herbal Products, New process & reactions, New molecules, Active ingredients(Macro analysis), Residues(Micro analysis), Impurity profiling etc., .

USP has published specific guidelines for method validation for compound evaluation. USP defines eight steps for validation

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantitation
- Linearity and range

DRUG PROFILE

MEBENDAZOLE



Mebendazole (MBZ) is a medication used to treat a number of parasitic worm infestations. Mebendazole is usually well tolerated.Compound that consists of a six-membered ring

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containing two nitrogen atoms at opposite positions in the ring. Piperazine exists as small alkaline deliquescent crystals with a saline taste.The piperazines are a broad class of chemical compounds, many with important pharmacological properties, which contain a core piperazine functional group.

IUPAC Name **:** methyl N-(6-benzoyl-1H-1,3-benzodiazol-2-yl)carbamate

Chemical formula **:** C16H13N3O3

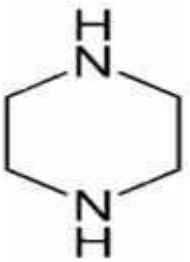
Molecular weight **:** 295.2927

Cas No **:** 31431-39-7

Category **:** Acids, Acyclic,Anthelmintics,Anti-Infective Agents

Brand Name **:** Elmin

DRUG : PIPERAZINE



Chemical Data

IUPAC Name: piperazine

Chemical formula : C4H10N2

Molecular weight **:** 86.1356

CAS No **:** 71939-50-9

Brand name **:** Cadiphylate

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LITERATURE REVIEW

1. Durgesh Rameshlal Parakh[1]*et. al., The gradient RP-HPLC method was developed on Agilent (India) C18 250 × 4.6 mm, 5 µ column using mobile phase as acetonitrile: water pH 3.0 with orthophosphoric acid (90:10 v/v) at a flow rate of 1 mL/min and detection was carried out at 234 nm using UV-Visible detector (UV 3000 M).

2. V. Rama Koteswara Rao*et.al., The mobile phase was mixture of aqueous phosphate buffer with pH 5.2: Methanol: Acetonitrile (30:20:50 v/v/v), effluent flow rate monitored at 1.0 ml/min. the stationary phase was C18 column, zodiac 5µ (4.6×250mm).

Gamal Ragab, Hanaa Saleh, et. al., Isocratic, HPLC method , using Thermo Hypersil C18gold column with mobile phase of Acetonitrile - 0.1% orthophosphoric acid (30:70,v/v) was investigated to separate the drugs from their stressed degredation products. The flow rate was 1.5 ml/min.

3.Kullai Reddy Ulavapalli1et.al., Chromatographic separation was achieved by using Inertsil ODS-3V C18,250 x 4.6 mm, 5µm column, mobile phase composed of sol-A: Potassium dihydrogenphosphate (1.0 gram in 1000 ml of HPLC Water) buffer and sol-B:Acetonitrile withgradient elution (0-5min- sol-A: 80-80; 5-7min- sol-A: 80-60; 7-10min- sol-A: 60-30; 10-15min- sol-A: 30-80 and 15-20min- sol-A: 80-80).

4.Umang Shah* ,et.al., 1% H2SO4 in methanol was selected as a common solvent for estimation of MBZ and LVM. For first order derivative method, estimation of MBZ was carried out at 307nm (ZCP of LVM) and of LVM at 232.6nm (ZCP of MBZ).

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4.AIM AND OBJECTIVE

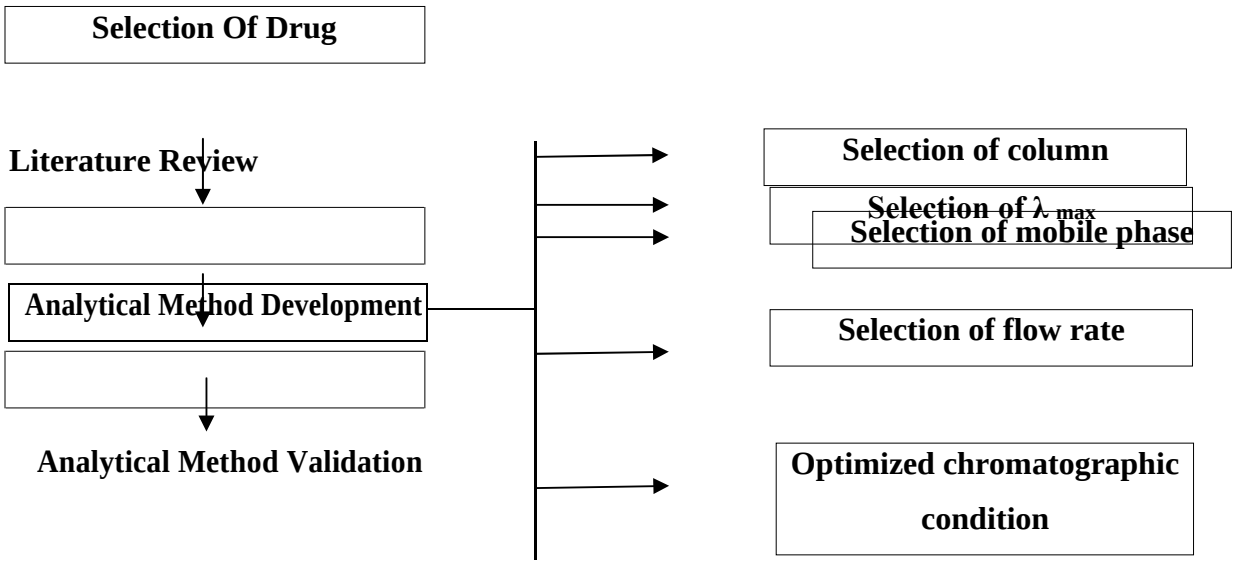
Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form.

The developed method will be validated according to ICH guidelines.

Objective of the work

- The analytical method for the simultaneous estimation of Mebendazole and Piperazine will be developed by RP-HPLC method by optimizing the chromatographic conditions.
-

5.PLAN OF WORK



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6. MATERIALS AND METHODS

MATERIALS:

T. 6.1 List of Instruments

S.No.	Instrument	Model No.	Software	Manufacturer's
1	HPLC	Waters 2695	Empower	Waters
2	UV double beam	UV 3000	UV Win 5	Lab India
3	Digital weighing	BSA224SC	-	Satorius
4	pH meter	AD102U	-	Lab India
5	Ultra	SE60US	-	-
6	Suction pump	VE115N	-	-

S.No.	Chemical	Manufacturer	Grade
1	Water	Merck	HPLC Grade
2	Methanol	Merck	HPLC Grade
3	Acetonitril	Merck	HPLC Grade
4	Potassium dihydrogen	Merck	A.R
5	Mebendazole & Piperazine	-	-

METHOD DEVELOPMENT:

Method development for simultaneous estimation of Mebendazole and Piperazine in Pharmaceutical dosage forms includes the following steps:

1. Selection of Detection wavelength:

10 mg of Mebendazole and Piperazine was dissolved in mobile phase.

SPECTRUM OF PIPERAZINE:

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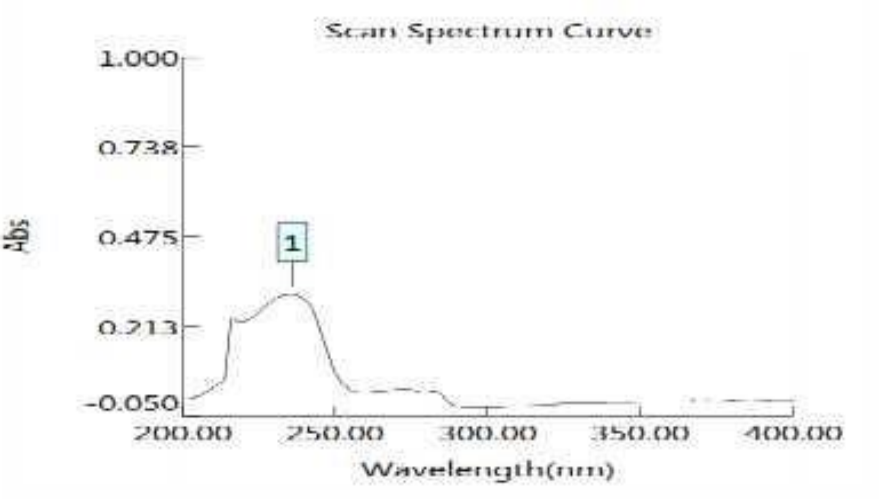
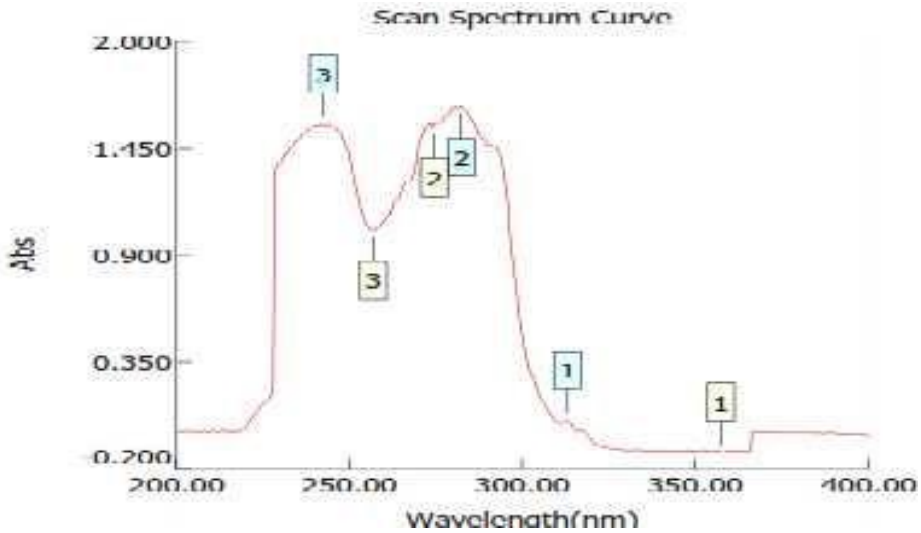


SPECTRUM OF MEBENDAZOLE :



Over line Spectrum of Mebendazole and Piperazine

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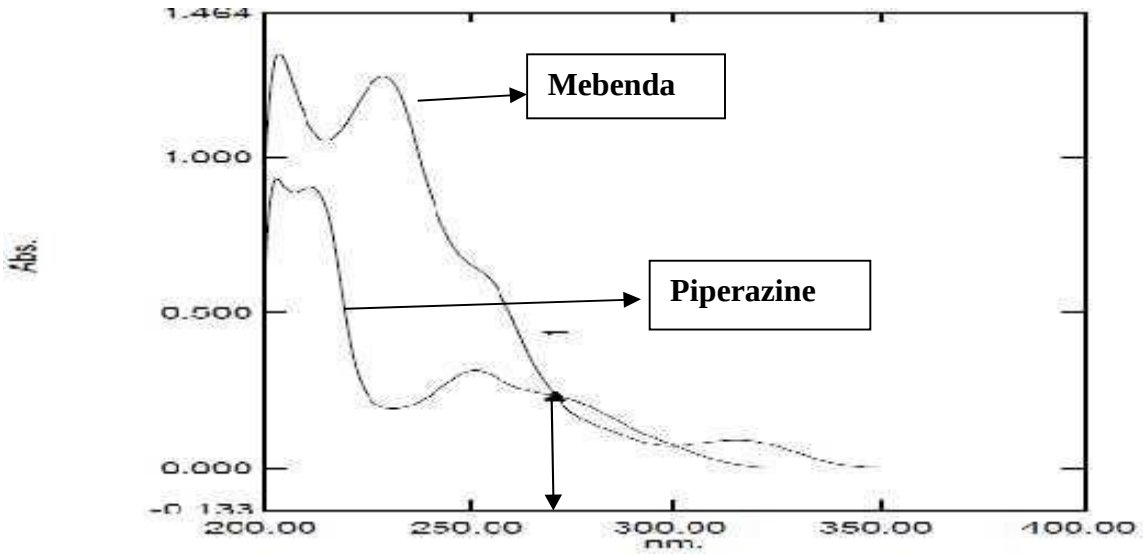

(4.6 x 250mm, 5µm, Make: Waters)]

3. Selection of mobile phase:

- Below 2: siloxane linkages are cleaved.
  Above 8: dissolution of silica.
- pH selected: 3 ±0.05
- pH controls the elution properties by controlling the ionization characteristics.

.

4. Selection of flow rate:

Flow rate selected was 1ml/min

Flow rate is selected based on

Weighed 6.8 grams of KH2PO4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

Preparation of mobile phase:

A mixture of pH 4.6 Phosphate buffer 300 mL (30%), 700 mL of MEOH (70%) are taken and degassed in ultrasonic water bath for 5 minutes.

Diluant Preparation:

Mobile phase is used as Diluant**.**

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ANALYTICAL METHOD VALIDATION

Accuracy:

Preparation of standard solution (Mebendazole and Piperazine):

Accurately weighed 10 mg of Piperazine and 10mg of Mebendazole working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks.

Preparation of Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately 5mg of Piperazine and 5mg of Mebendazole working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent.

transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent.

into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent.

Precision

A) Repeatability:

Preparation of standard stock solution:

Accurately 10 mg of Piperazine and 10mg of Mebendazole working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks

B) Intermediate Precision (Ruggedness):

Preparation of standard stock solution:

Accurately 10 mg of Piperazine and 10mg of Mebendazole working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks

Specificity

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The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak.

L.O.D:

L.O.D's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the L.O.D according to the formula.

L.O.Q:

L.O.Q's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula.

Linearity

Preparation of stock solution:

Accurately 10 tablets were weighed & crushed in mortar and pestle and weight equivalent to 10 mg of Piperazine and Mebendazole (marketed formulation) sample were transferred into a 10mL clean dry volumetric flask and about 7mL of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent.

1ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

2ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

3ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

4ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

5ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Range:

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Robustness:

b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 3 µg/ml of Mebendazole and 300 µg/ml of Piperazine in were prepared and analyzed using the varied mobile phase composition along with the actual mobile phase composition in the method.

System suitability:

5 mg of Mebendazole and 500 mg of Piperazine in working standard was accuratel y weighed and transferred into a 100ml clean dry volumetric flask and add about 20ml of diluant and sonicated to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

7. RESULTS AND DISCUSSION

Trial 1:

Mp	:	Water: Methanol (50:50%v/v)
Column	:	Thermosil C18 (4.6*150mm) 5µm
Fr	:	1.0 ml/min
Wl	:	260 nm
Ct	:	Ambient
St	:	Ambient
Iv	:	10 µl

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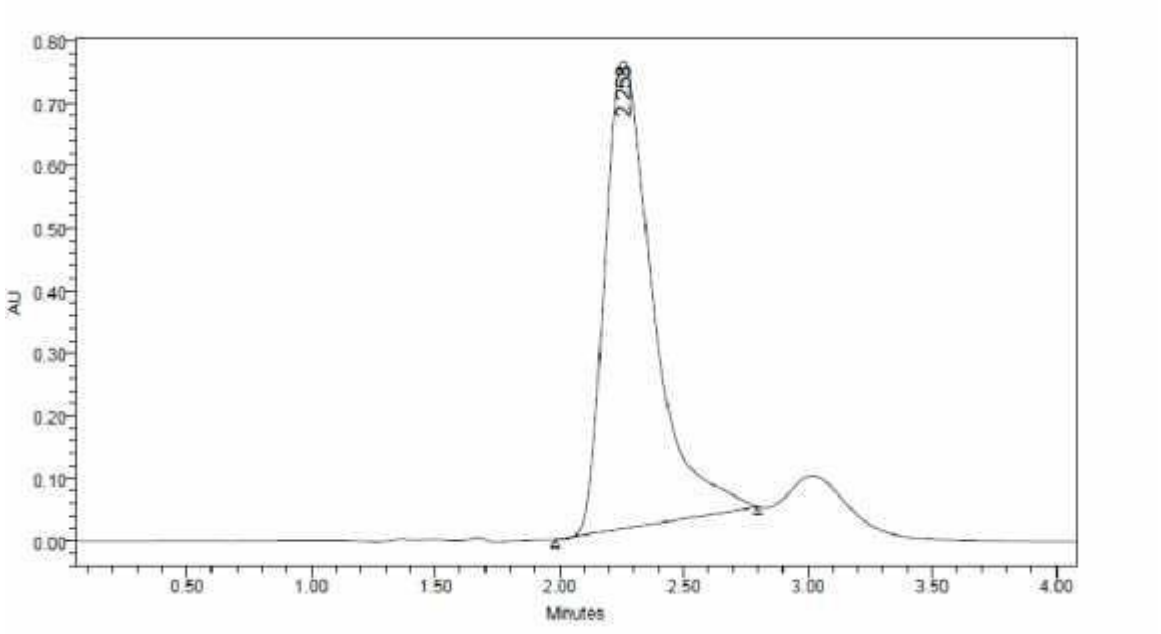

F: 7.1 Chromatogram showing trail -1

Trial 2:

Mp	:	Phosphate buffer pH 4 : Methanol (40:60%v/v)
Column	:	Termosil C18 (4.6*150mm) 5µm
Fr	:	1.0 ml/min
Wl	:	260 nm
Ct	:	Ambient
St	:	Ambient
Iv	:	10 µl

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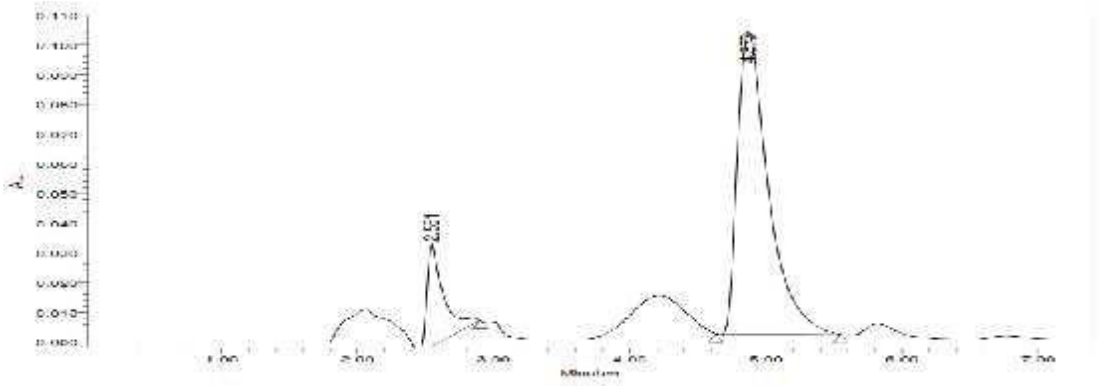


F: 7.2 Chromatogram showing trail -2

Trial 3:

Mp : Phosphate buffer (0.05m) pH 4.0: Methanol (40:60%v/v)

Column : Symmetry C18 5µm (4.6*250mm) Make; waters

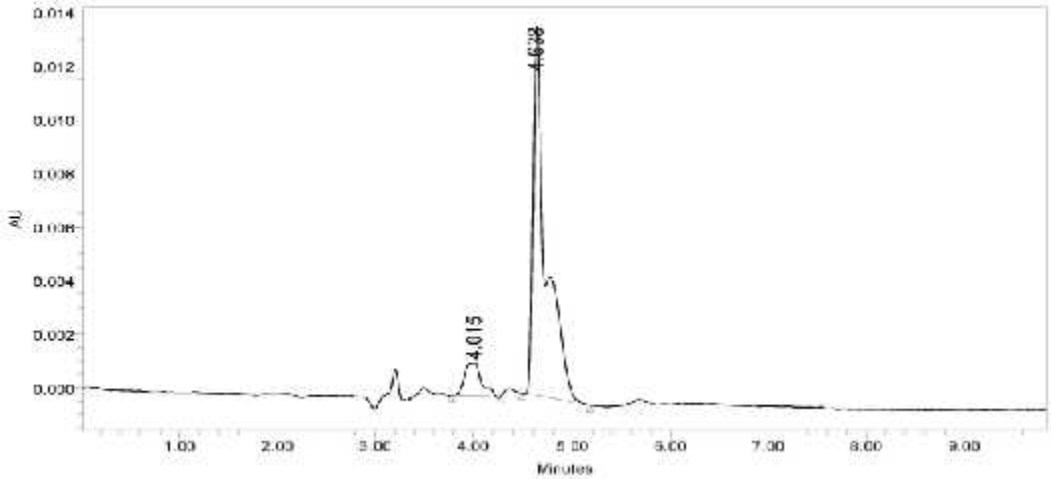
Fr : 0.8 ml/min

Wl : 260 nm

Ct : Ambient

St : Ambient

Iv : 10 µl



Chromatogram showing trail -3

Optimized chromatogram is obtained by following conditions

Column : Symmetry C18 (4.6 x 150mm, 5µm, Make: XTerra) or equivalent

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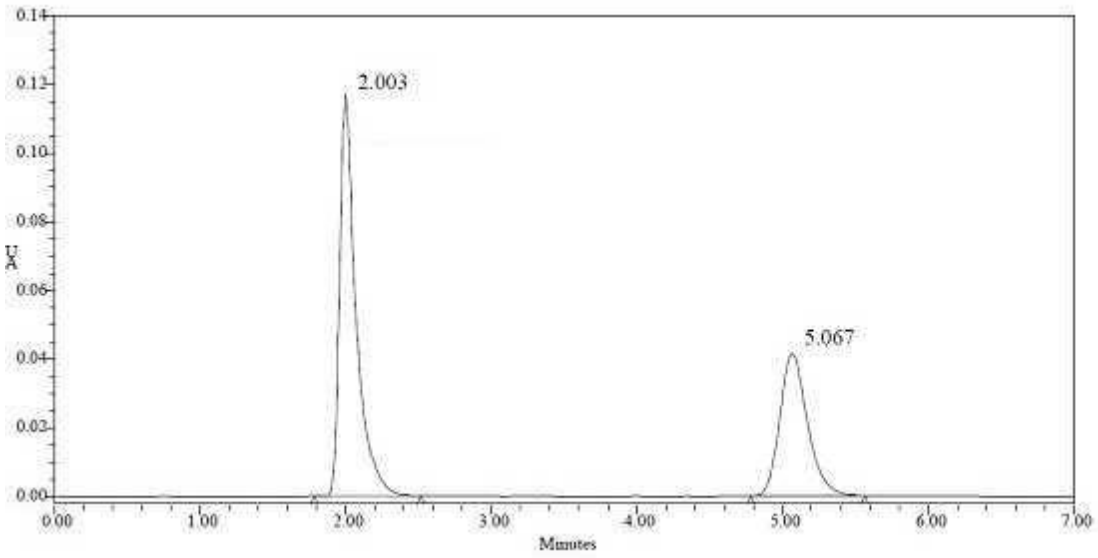
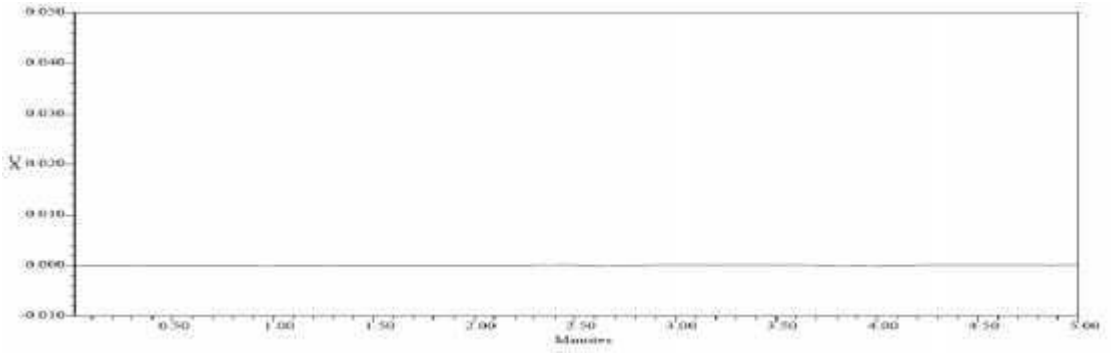
Buff pH	:	4.6
Mp	:	70% Meoh : 30% phosphate buffer ph-4.6
Fr	:	1 ml per min
Wl	:	273 nm
Temperature	:	ambient.
Rt	:	7min.



Chromatogram for Mebendazole and Piperazine

From the above chromatogram it was observed that the Mebendazole and Piperazine peaks are well separated



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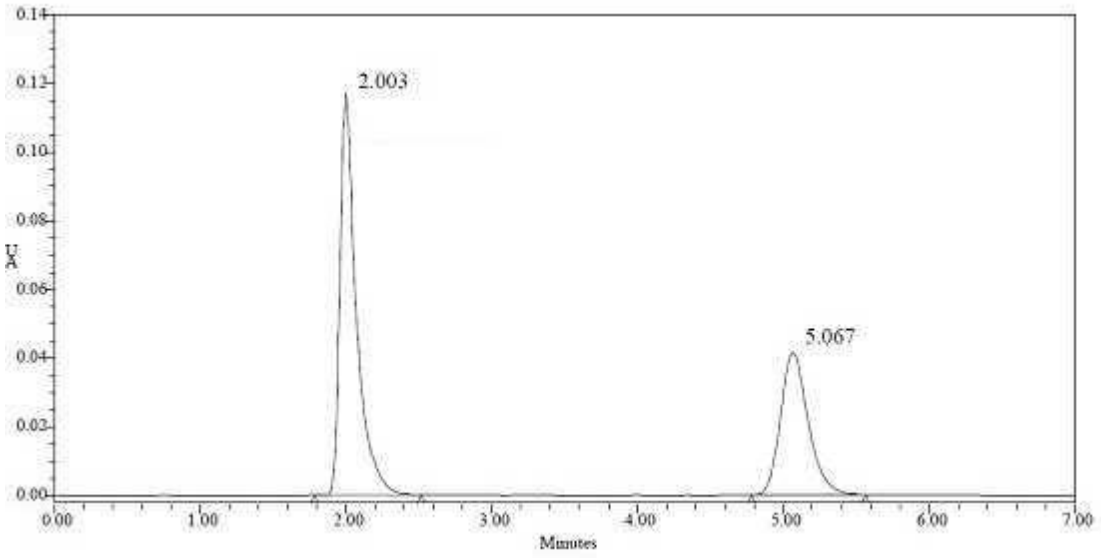
F. 7.5 Chromatogram for blank

From the above chromatogram it was observed that there are no interferences

SYSTEM SUITABILITY



F. 7.6 Chromatogram for system suitability

S. No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Mebendazole	2.003	920101	116666		1.6	2711.8
2	Piperazine	5.067	552058	41531	11.0	1.3	3428.2

Acceptance criteria:

- Resolution between two drugs should not be less than 2
- Theoretical plates should not be less than 2000
- Tailing factor should not be less than 0.9 and not more than 2.

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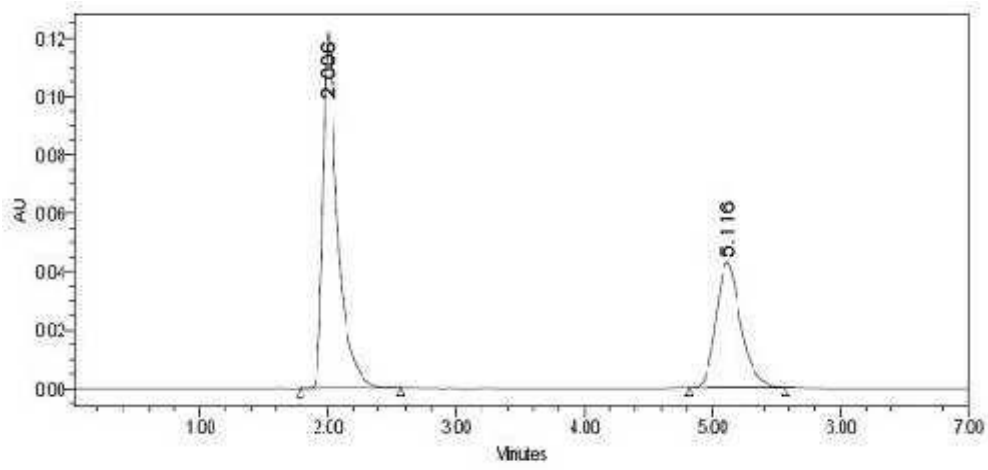
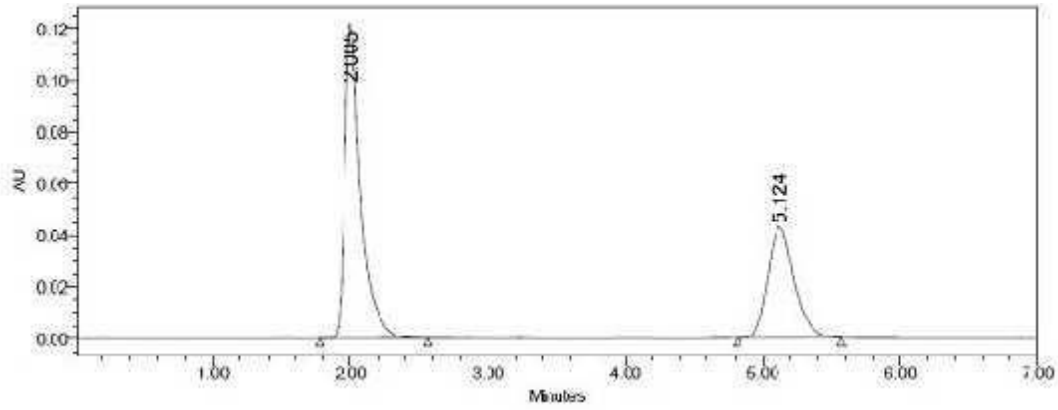
Method precision



Chromatogram for sample injection -1



Chromatogram for sample injection-2

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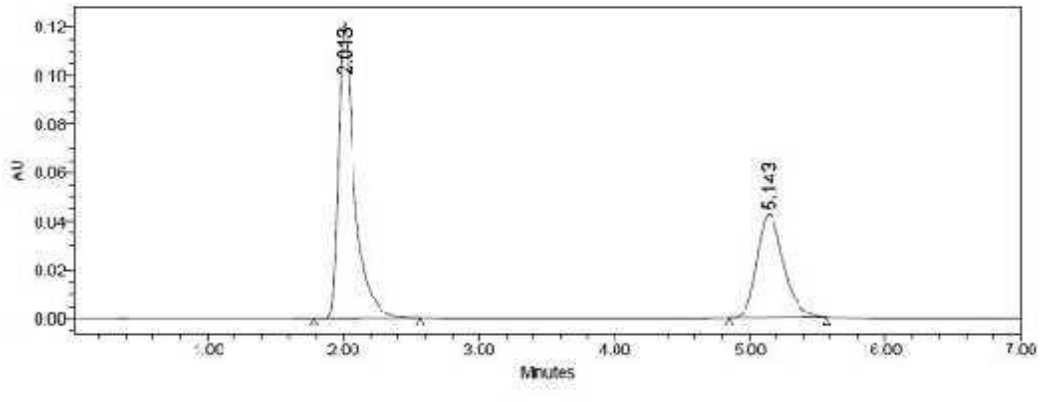
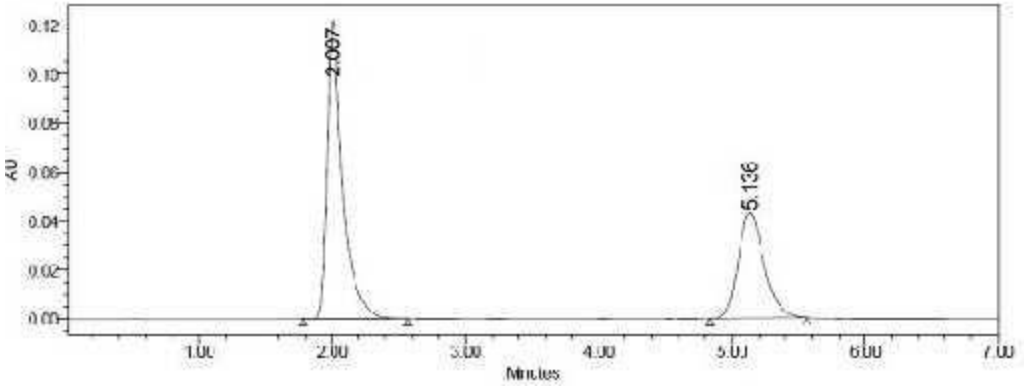
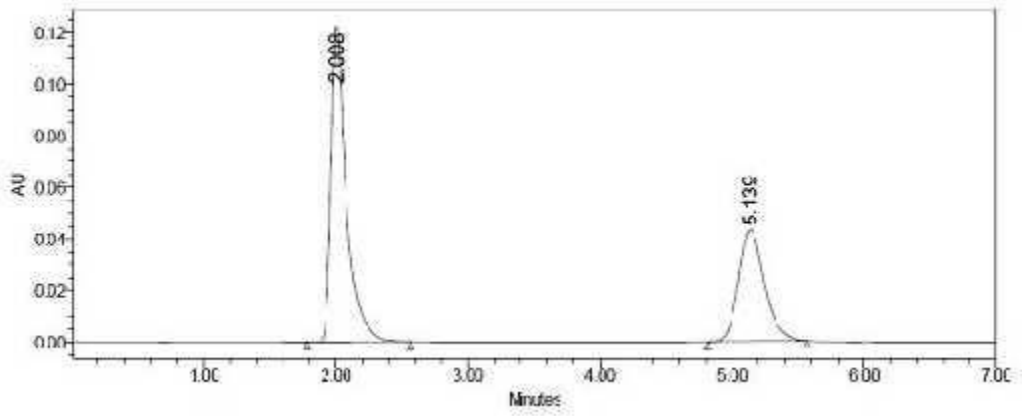


Chromatogram for sample injection-3



Chromatogram for sample injection-4



Chromatogram for sample injection-5

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S. No	Sample area	Standard area	Percentage purity
1	983375	971536	101.04
2	985049	973007	101.03
3	982956	975717	100.54
4	985219	978909	100.44
5	994145	981422	101.09
Average			**100.84**
%RSD			**0.304**

T. 7.3 Results of method precision for Piperazine

S. No	Sample area	Standard area	Percentage purity
1	592403	577531	101.36
2	592352	580381	101.85
3	592357	577723	102.32
4	592323	582190	101.44

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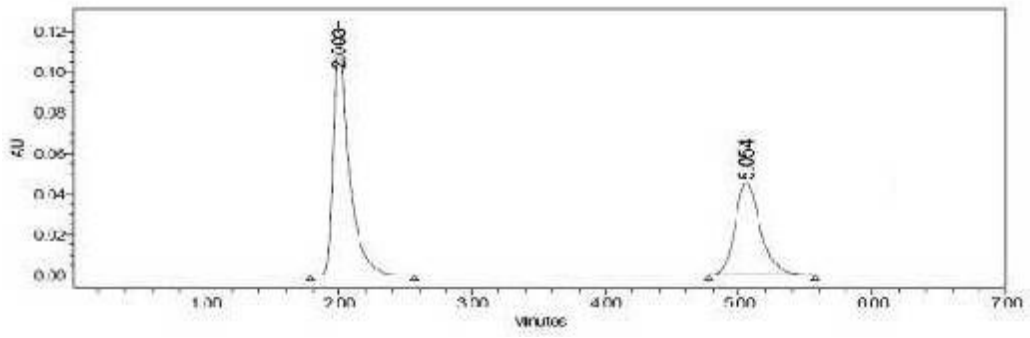
5	596525	583378	101.09
Average			**101.24**
%RSD			**0.46**

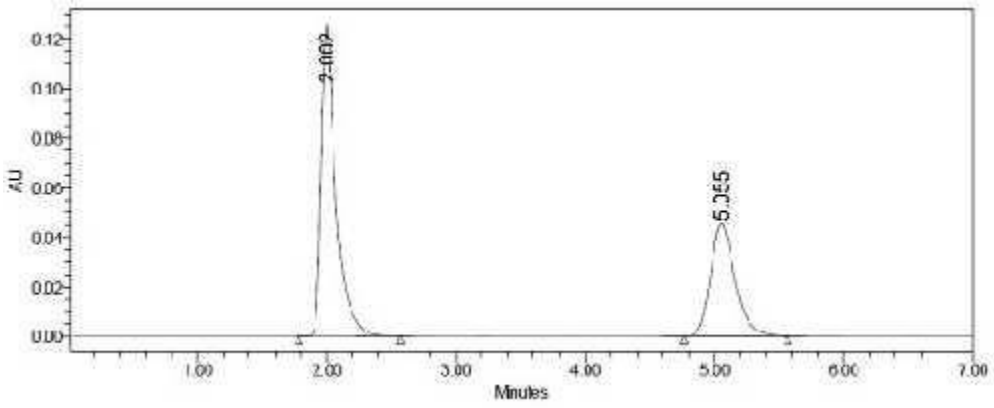
Acceptance criteria:

%RSD for sample should be NMT 2

- The %RSD for the standard solution is below 2, which is within the limits hence the method is precise.



F. 7.12 Chromatogram for sample injection-1



F.7.13 Chromatogram for sample injection-2

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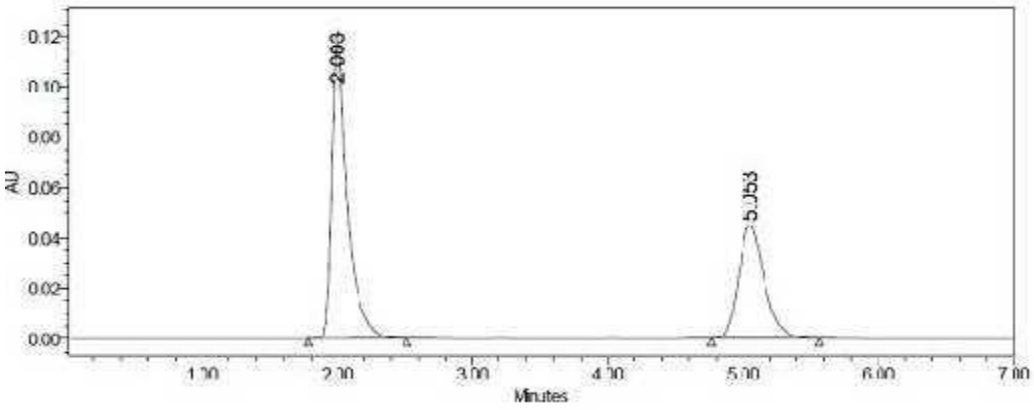
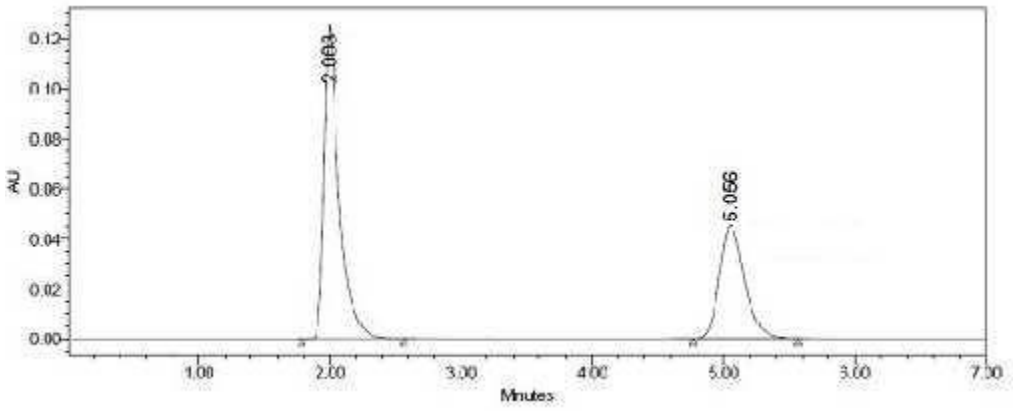
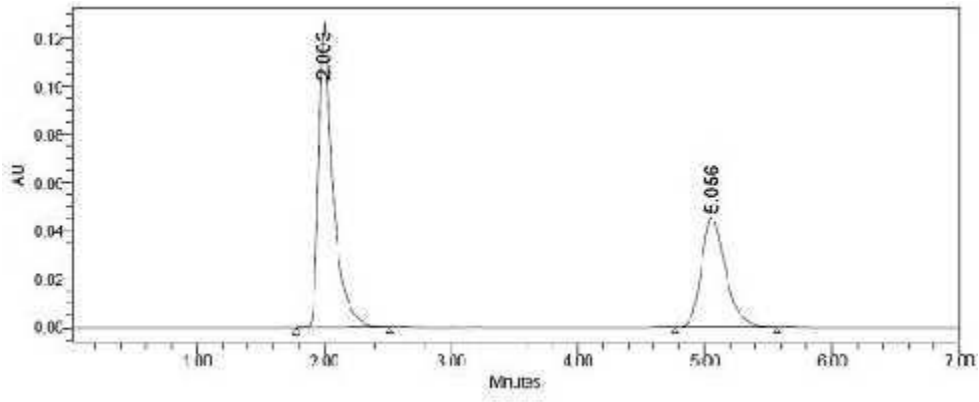


F. 7.14 Chromatogram for sample injection-3



F. 7.15 Chromatogram for sample injection-4



F. 7.16 Chromatogram for sample injection-5

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S. No	Sample area	Standard area	Percentage purity
1	979556	984395	99.30
2	982467	984039	99.64
3	979717	983976	99.36
4	978909	984278	99.28
5	981432	973915	100.57
Average			**99.63**
%RSD			**0.54**

T. 7.5 Results of Intermediate precision for Piperazine

S. No	Sample area	Standard area	Percentage purity
1	583416	593403	99.12
2	583657	594352	99.01
3	584731	593357	99.52
4	583594	592673	99.61

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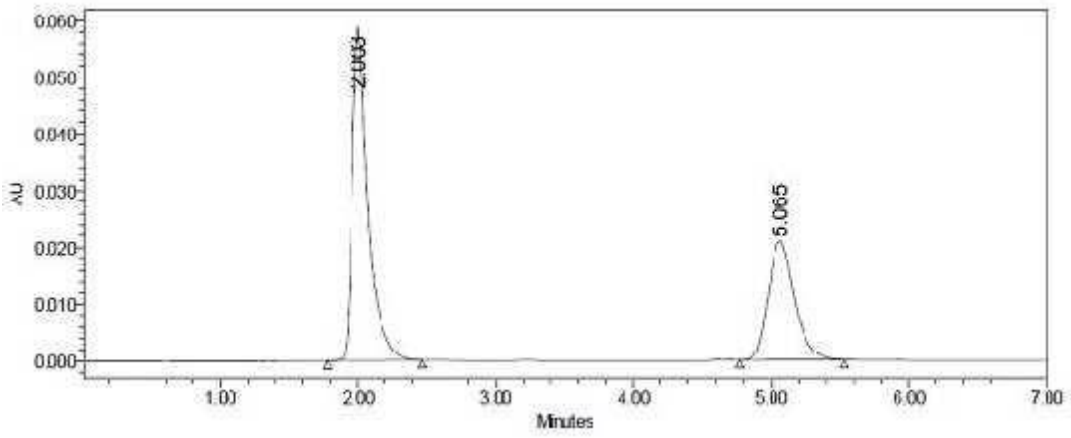
5	597649	593671	99.12
Average			**99.27**
%RSD			**0.27**

Acceptance criteria:

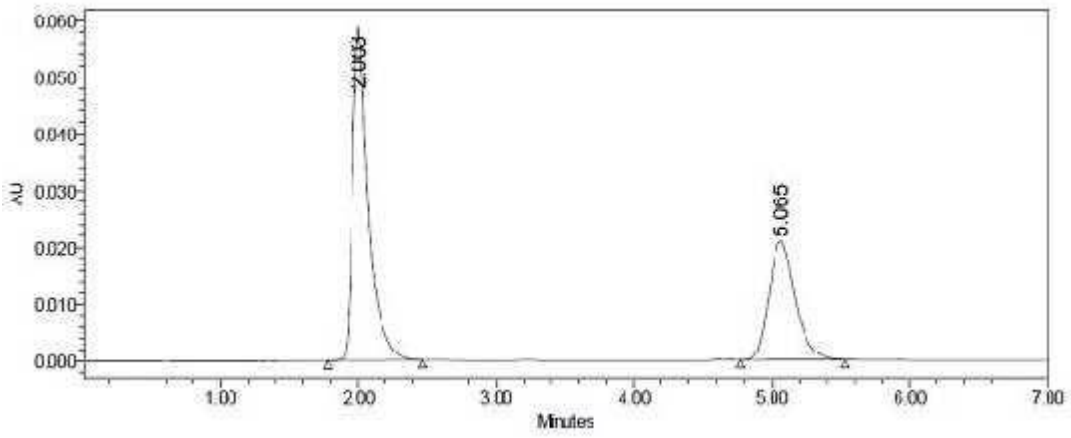
%RSD of five different sample solutions should not be more than 2

- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY



Chromatogram for sample concentration-50%



Chromatogram for sample concentration-50%

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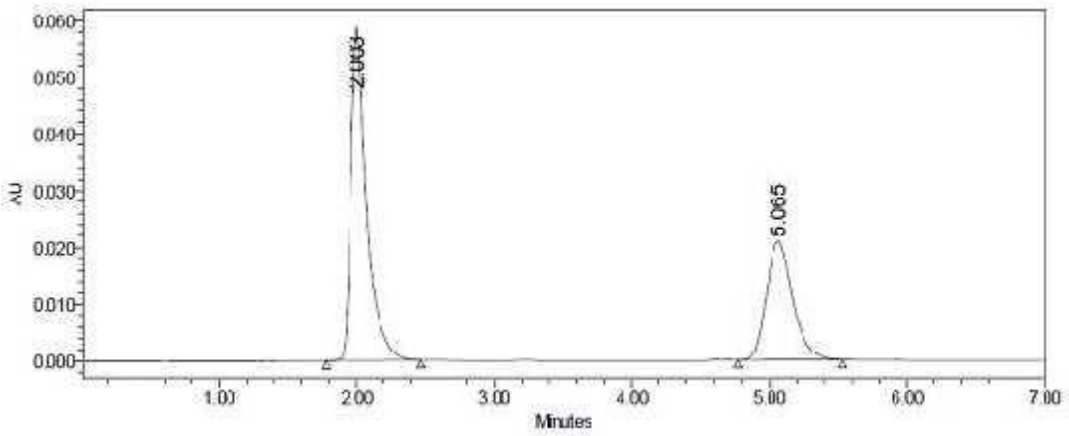
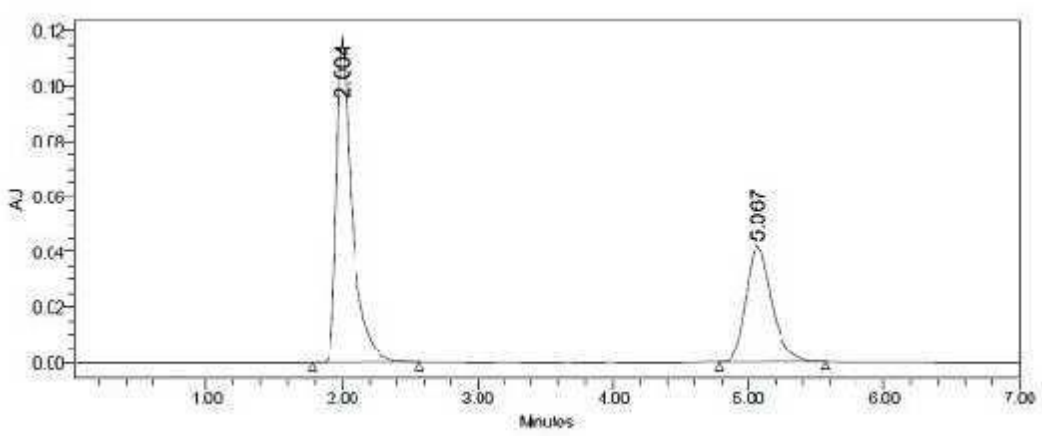


Chromatogram for sample concentration-50%



(Chromatogram for sample concentration-100%

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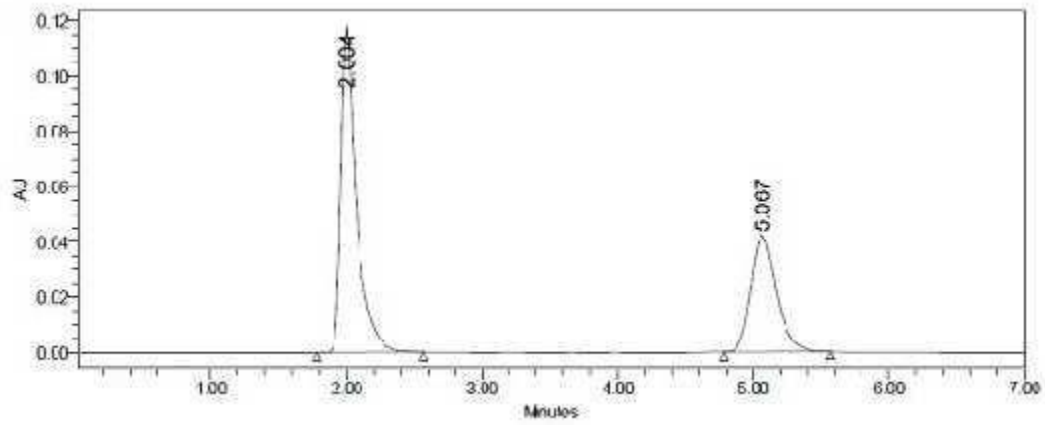
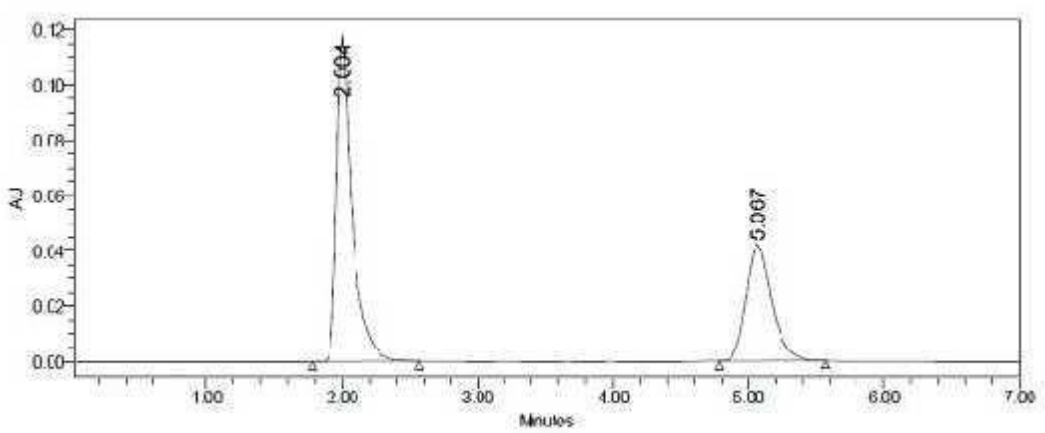


(Chromatogram for sample concentration-100%



Chromatogram for sample concentration-100%

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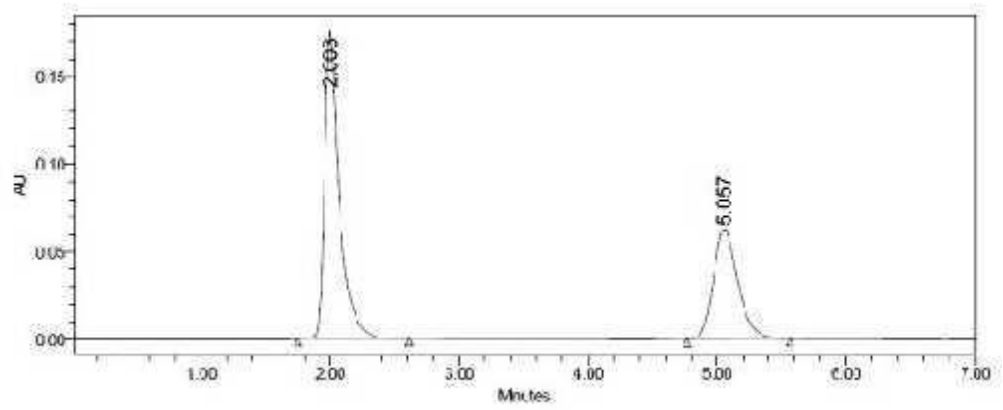

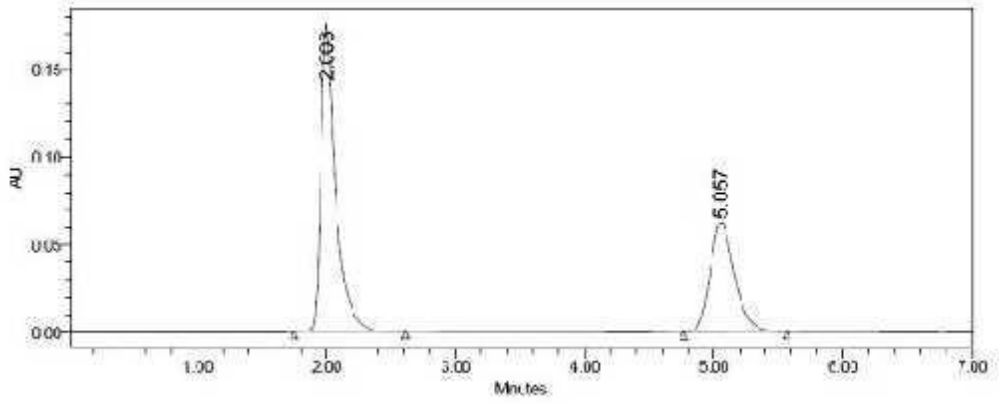

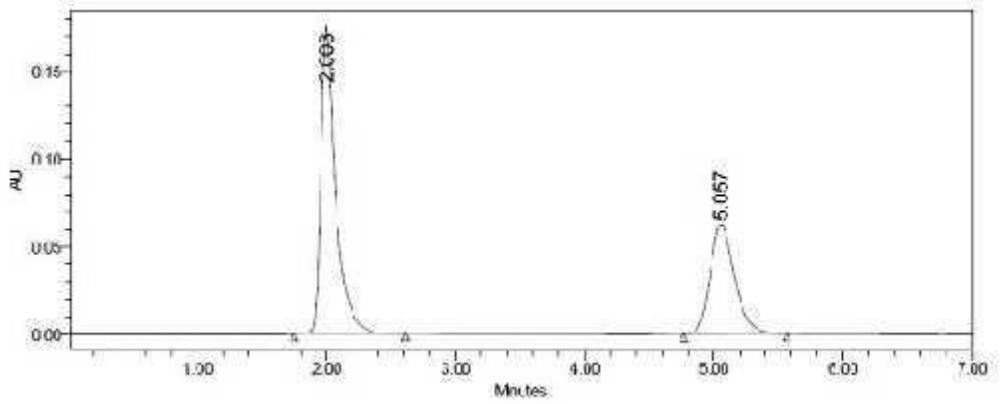

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Sample concentration	**Sample set no**	**Sample area**		**Assay**		**% Recovery**	
		Mebendazole	**piperazine**	**Mebendazole**	**piperazine**	**Mebendazole**	**piperazine**
50%	**1**	460064	276931	24.9	25.0	99.8	100
	2	460124	276694	24.6	24.9	99.6	99.6
	3	460216	276891	24.8	24.9	99.8	99.6
	Average Recovery					**99.7%**	**99.7%**
100%	**1**	923429	554156	49.9	50.0	99.8	100
	2	923654	554897	49.8	49.9	99.6	99.8
	3	923742	556371	49.8	49.9	99.6	99.8
	Average recovery					**99.6%**	**99.8%**
150%	**1**	1387901	828113	74.8	75.0	99.8	100
	2	1385360	828794	74.9	74.9	99.8	99.8
	3	1386984	828349	74.6	74.8	99.6	99.8
	Average recovery					**99.7%**	**99.8%**

Acceptance criteria:

The percentage recovery at each level should be between (97-103%).

- The results obtained for recovery at 50%, 100%, 150% are within the limits.

LINEARITY

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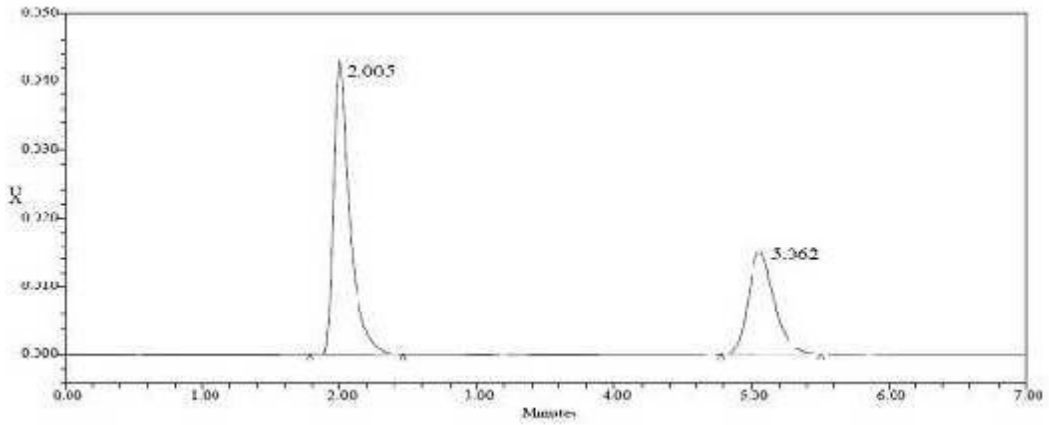
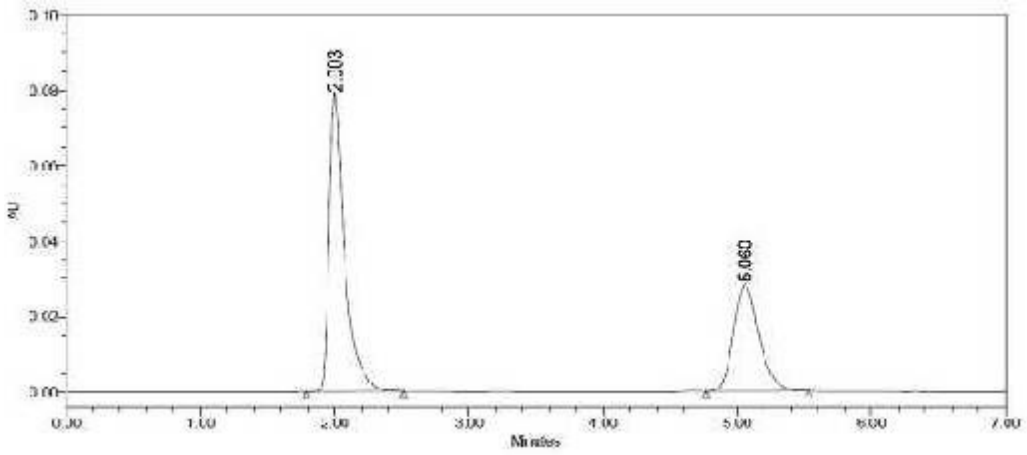
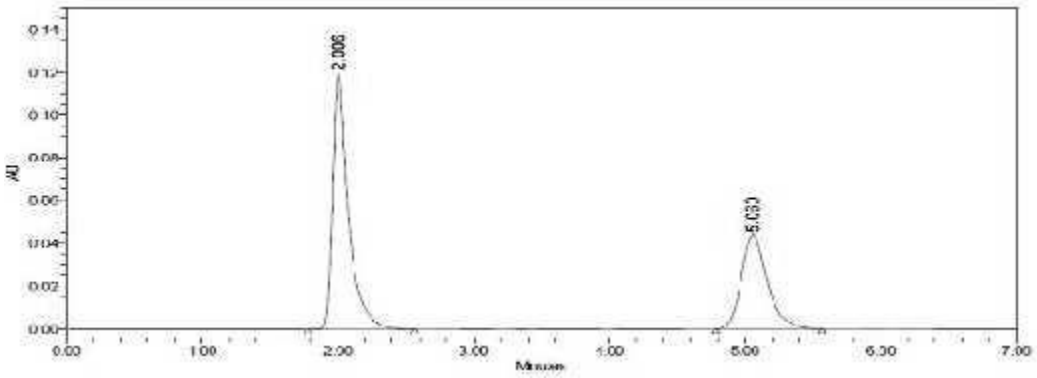


Chromatogram for linearity concentration- 25%



Chromatogram for linearity concentration-50%



Chromatogram for linearity concentration-75%

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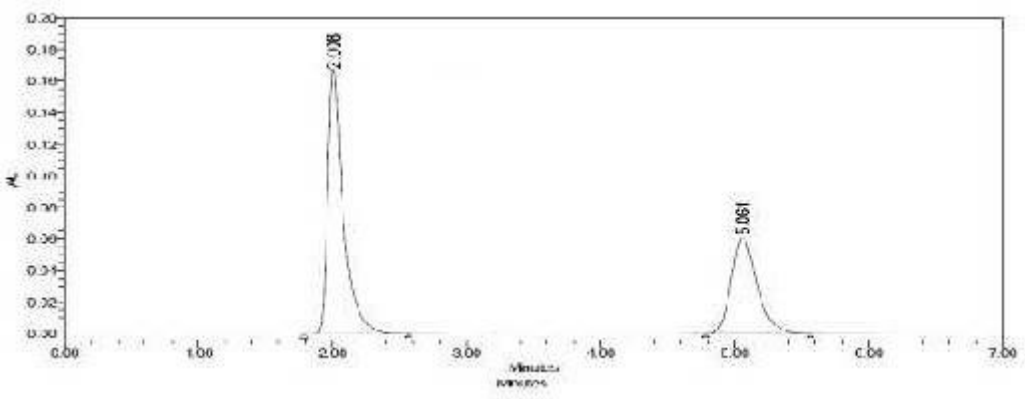
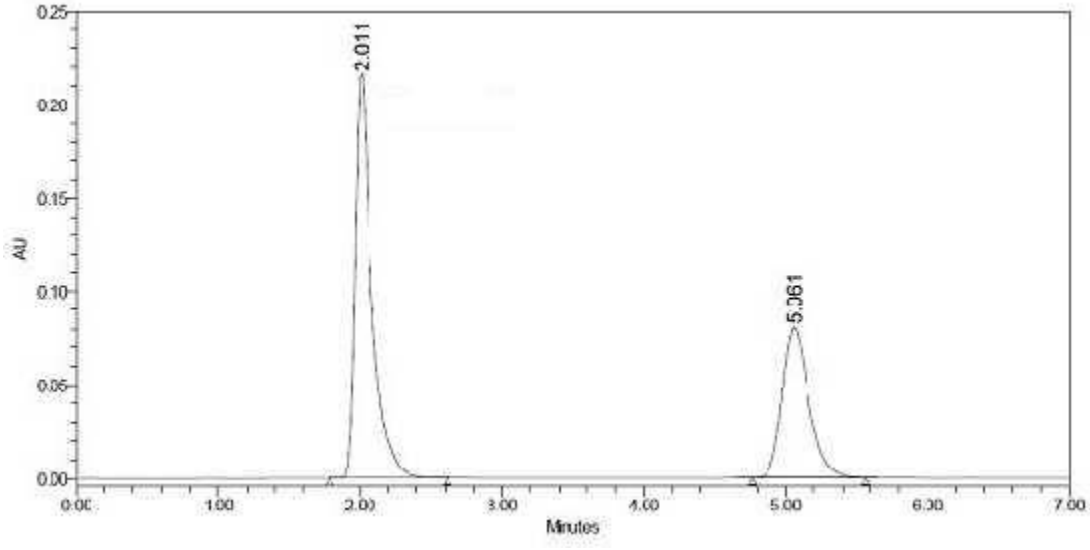


Chromatogram for linearity concentration-100%



Chromatogram for linearity concentration-125%

Concentration (µg/ml)	Peak area of Mebendazole	Peak area of Piperazine
25	296800	179891
50	653819	387781
75	983775	599708
100	1342535	799619
125	1694286	1019614

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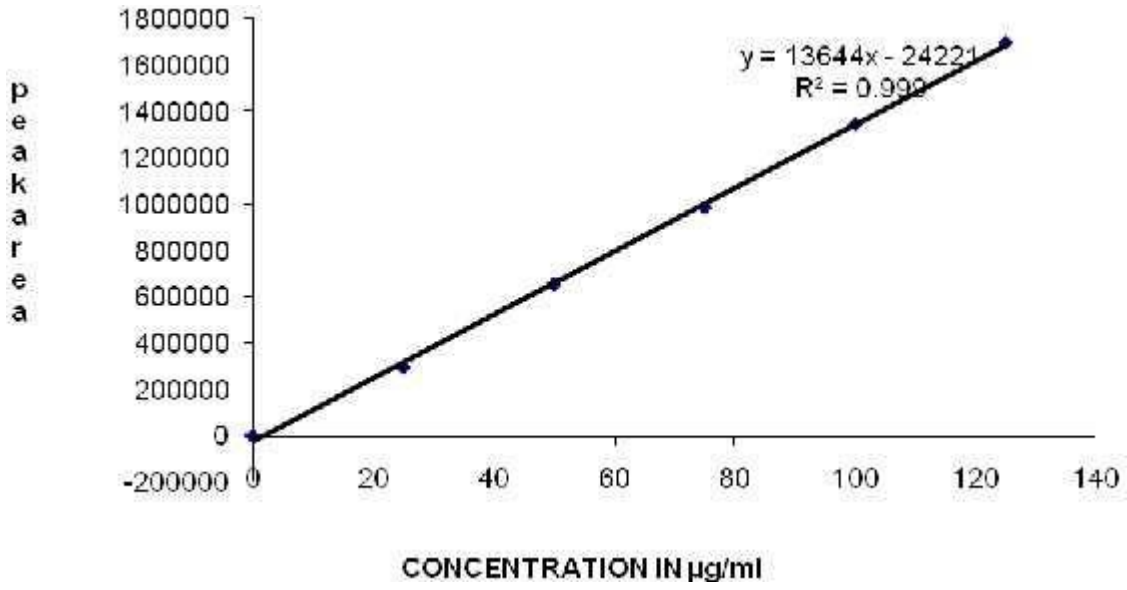
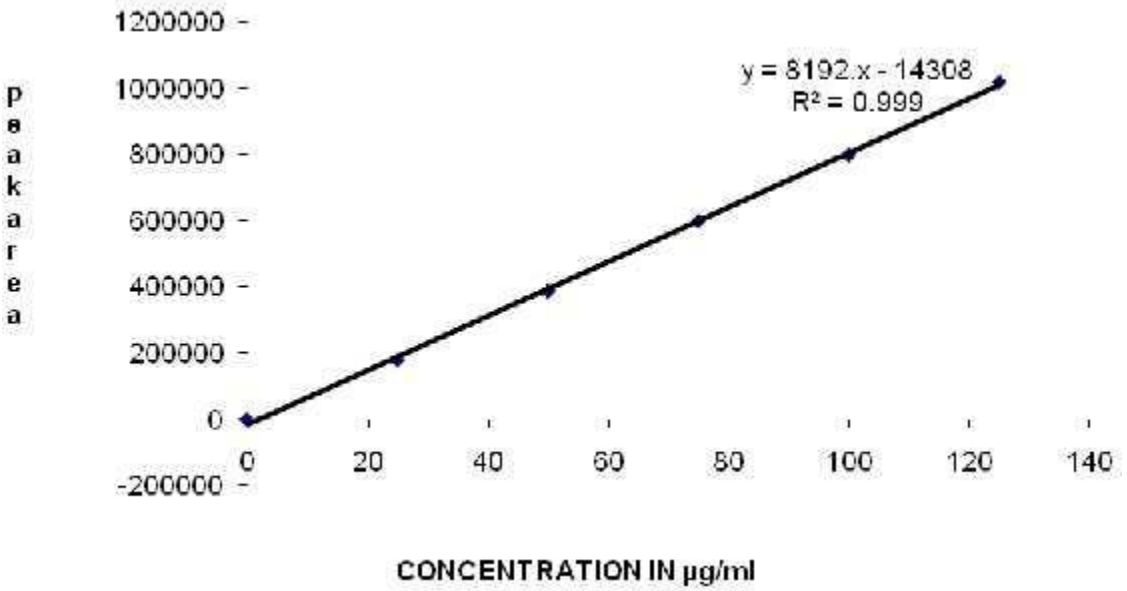


F. 7.31 Calibration graph for Mebendazole at 273 nm



F.7.32 Calibration graph for Piperazine at 273 nm

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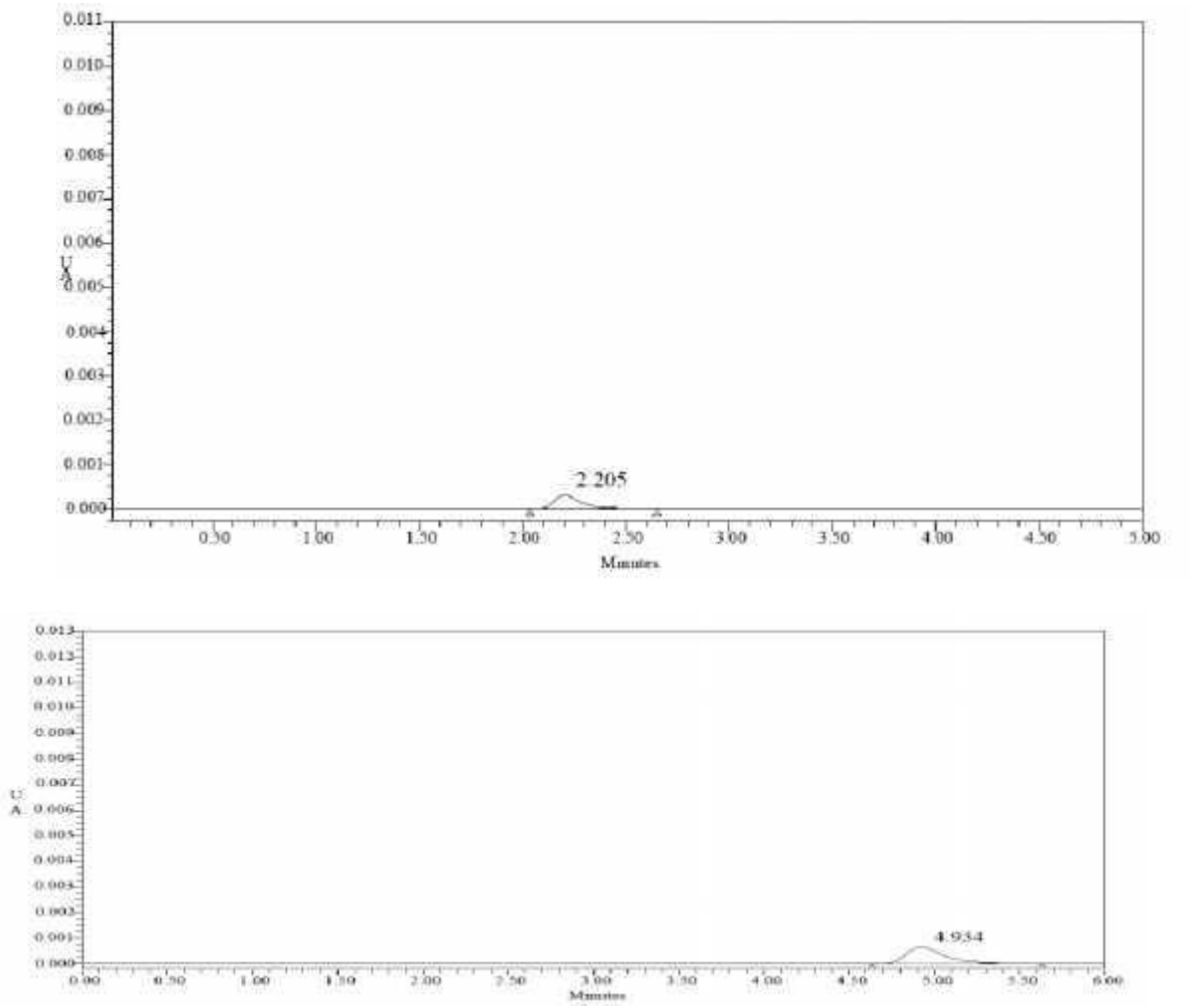
Parameters	Mebendazole	Piperazine
Slope (m)	13644	8192
Intercept (c)	24221	14308
Correlation coefficient (R^2)	0.999	0.999

Acceptance criteria:

Correlation coefficient (R^2) should not be less than 0.999

- The correlation coefficient obtained was 0.999 which is in the acceptance limit.

- **7.3.5 LIMIT OF DETECTION FOR MEBENDAZOLE AND PIPERAZINE**



F.7.34 Chromatogram of Piperazine showing L.O.D

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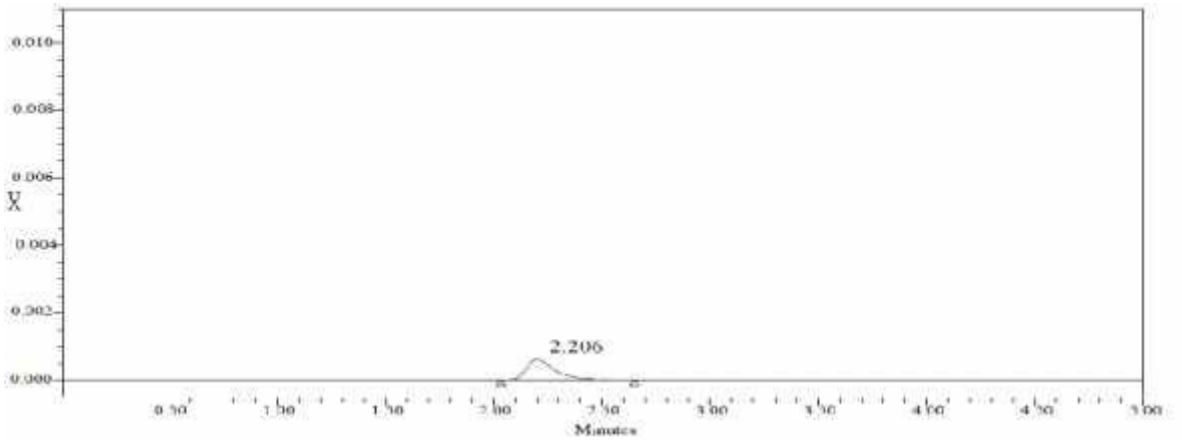
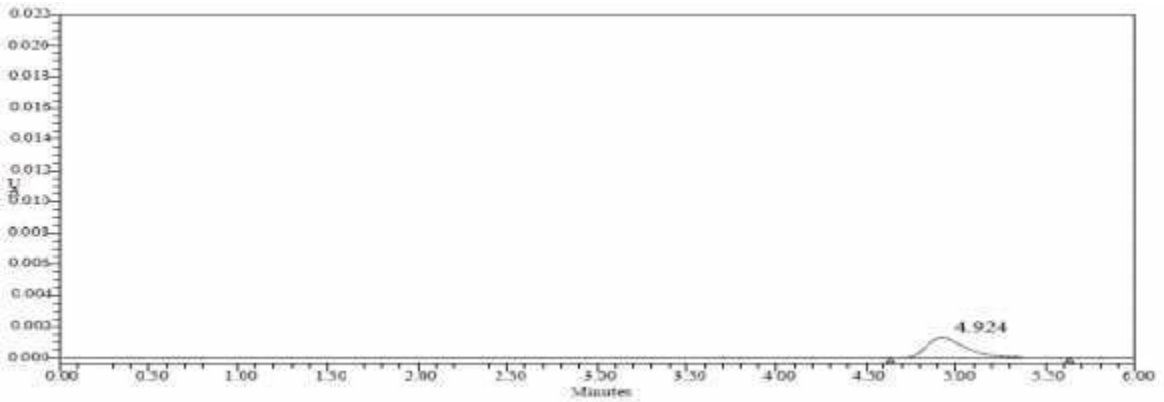
T. 7.9 Results of L.O.D

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Mebendazole	56	176	3.14
Piperazine	56	154	2.75

7.3.6 LIMIT OF QUANTITATION FOR MEBENDAZOLE AND PIPERAZINE



Chromatogram of Mebendazole showing L.O.Q



Chromatogram of Piperazine showing L.O.Q

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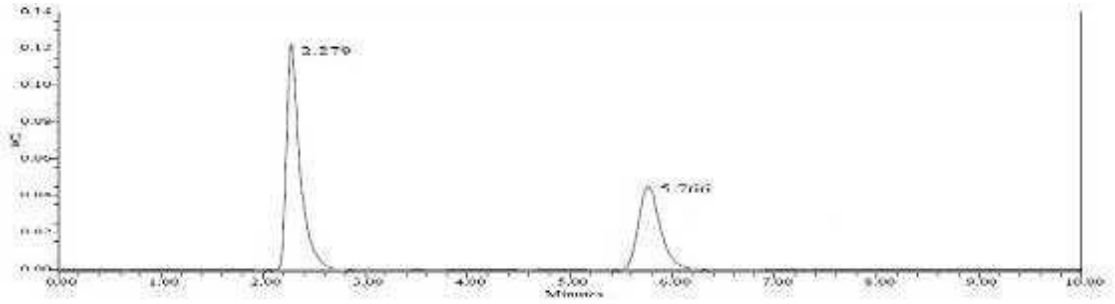
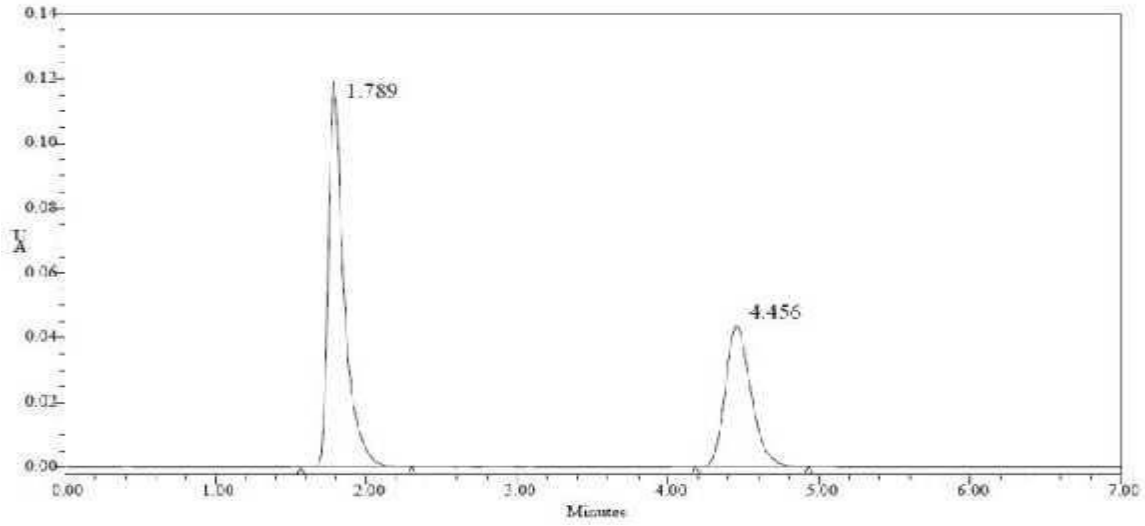
T. 7.10 results of L.O.Q

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Mebendazole	56	563	10.05
Piperazine	56	558	9.96

ROBUSTNES
S Variation
in flow



F. 7.37 Chromatogram showing less flow of 0.7ml/min



F.7.38 Chromatogram showing more flow of 0.9ml/min

Variation of mobile phase composition

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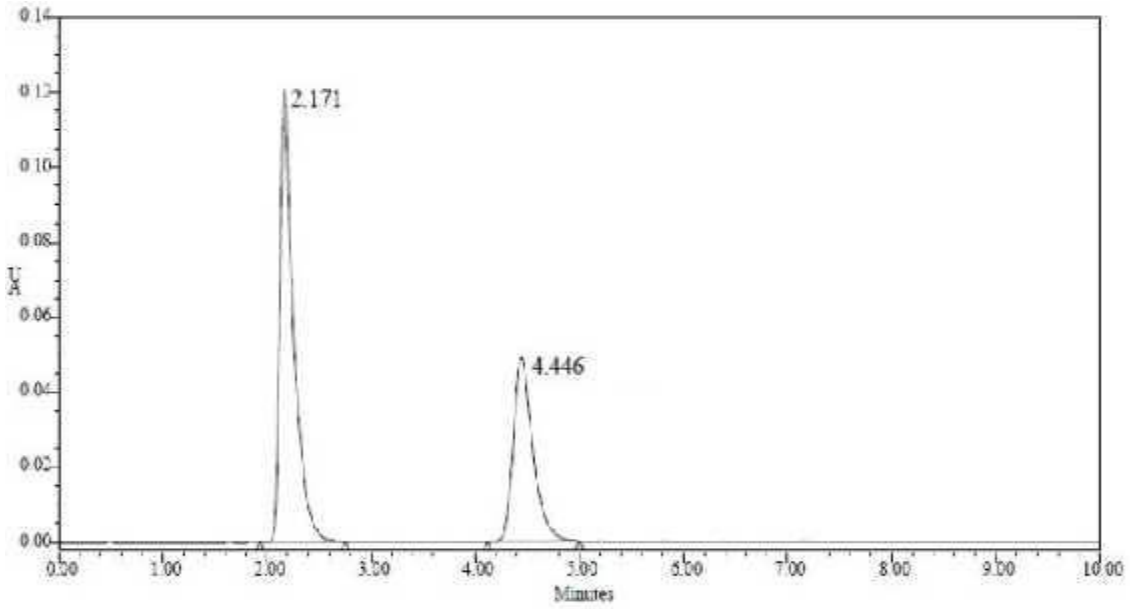

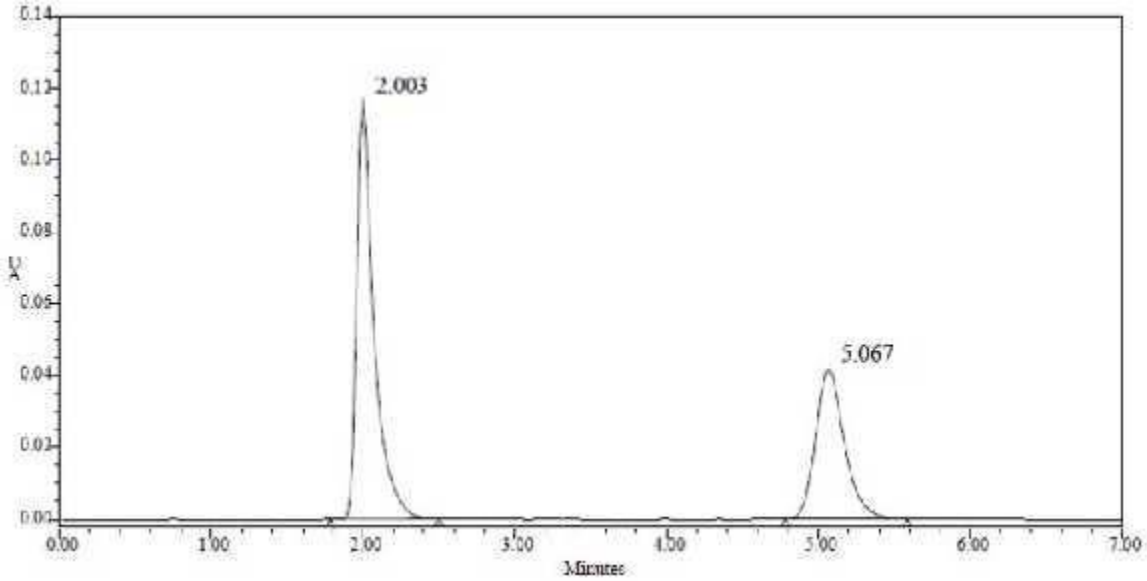

S. No	peak area for Less flow (0.7 ml/min)		peak area for More flow (0.9 ml/min)	
	Mebendazole	**Piperazine**	**Mebendazole**	**Piperazine**
1	983465	575351	971563	592641
2	985134	580381	973021	592352

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3	983467	587724	975674	595471
4	985217	583190	978974	594416
5	994245	584468	984542	583453
Mean	**986306**	**582223**	**976755**	**591667**
%RSD	**0.45**	**0.80**	**0.53**	**0.80**

S. No	**peak area for Less organic(70%)**		**Peak area for More organic (90%)**	
	Mebendazole	**Piperazine**	**Mebendazole**	**Piperazine**
1	984565	574371	981565	593761
2	986134	585481	983527	592462
3	984268	587627	985489	594491
4	986216	585362	987954	596316
5	995247	585448	994672	587353
Mean	**987286**	**583658**	**986641**	**592877**
%RSD	**0.45**	**0.90**	**0.51**	**0.57**

Acceptance criteria:

Percentage RSD should not be more than 2.

- The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 2, which is within the acceptance criteria.

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8. SUMMARY AND CONCLUSION

The estimation of Mebendazole and Piperazine was done by RP-HPLC. Mobile phase was optimized which consists of MEOH : Phosphate buffer mixed in the ratio of 70:30 % v/ v. A Symmetry C18 (4.6 x 150mm, 5µm, Make XTerra) column used as stationary phase. The detection was carried out using UV detector at 273 nm.

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