7.1. DRUG PROFILE

7.1.1. Methylparaben

Methyl ester of p-hydroxybenzoic acid is known as methylparaben (MET) [1-2]. It shows anti-fungal activity and thus used in a variety of cosmetics and personal-care products. In cosmetic, food and pharmaceutical industries, it is also used as preservative. In the natural form, it is associated with antimicrobial activity. MET also serves as a pheromone for a variety of insects. Thale cress [3] plant produces MET. At higher concentrations, MET exhibits toxic effects like estrogenic effect. Even at lower concentrations, it reduces the growth rate at larval and pupal stages [4]. Mostly MET is available in the liquid form and can be easily absorbed through gastrointestinal tract or skin [5]. MET, when applied directly on skin, it may cause skin aging and DNA damage [6,7]. MET easily hydrolyses into p-hydroxy benzoicacid and can be excreted through urine quickly. Though MET is practically non-toxic, some allergic reactions are reported towards ingested parabens.

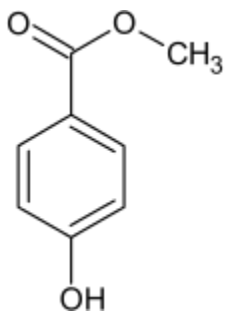


Figure.7.1. Structure of methylparaben

IUPAC name: Methyl 4 - hydroxybenzoate

Molecular formula: $C_8H_8O_3$

Molecular weight: 152.2

Melting point: 125-128^{0}C

Solubility: Soluble in water.

Administration: Oral

7.1.2. Epirubicin

Epirubicin (EPI) belongs to anthracycline medication category and is used for chemotherapy. EPI medication is preferred in patients who are suffering from breast cancer [8-17] in particular, when the patient is supposed to remove tumor through surgery. The mode of action of EPI is intercalation of DNA strands. Intercalation inhibits DNA & RNA synthesis. It also provokes DNA cleavage leading to death of cells. EPI is recommended over doxorubicin. The spatial orientation of hydroxyl groups favours its quick elimination and thus reduces toxicity. EPI is also used to treat gastric cancer, ovarian cancer, lymphomar and lung cancer. More common side effects of EPI are chest pain, sore throat, swollen glands, ulcers, fever etc.

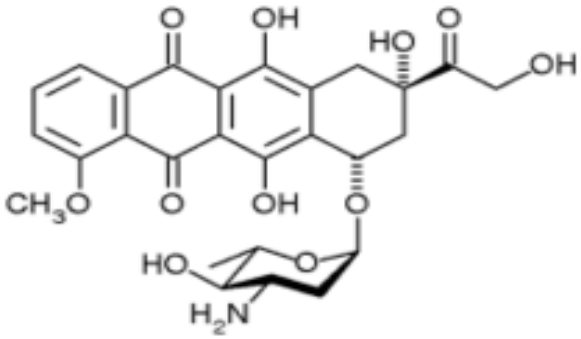


Figure.7.2. Structure of epirubicin

IUPAC name: (8R,10S)-10-((2S,4S,5R,6S)-4- amino-5-hydroxy-6- methyltetrahydro-2H-pyran-2-yl)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9, 10-tetrahydrotetracene-5,12-dione.

Molecular formula: $C_{27}H_{29}NO_{11}$

Molecular weight: 543.5

Melting point: 185^0C

Solubility: Soluble in water

Administration: Intravenous

Brand name: Ellence and Pharmorubicin PFS

7.2. LITERATURE SURVEY

Several analytical methods [18-20] have been proposed for the determination of MET and EPI individually, in the combined dosage form and in combination with other drugs. Some spectrophotometric[21] and numerous HPLC[22-47] methods were described for the estimation of MET and EPI. At the same time various analytical methods[48-52] were also available for the analysis of MET and EPI. Few stability indicating studies[53-58] were reported in addition to the biological[59-70] estimation of MET and EPI in saliva and plasma. Hence, an attempt was made for the development of sensitive HPLC method for the quantitation of MET and EPI.

Table.7.1. List of brand names of combined formulations of methylparaben and epirubicin

S.No	Brand name	Available strength		Formulation	Manufacturer
1	4 Eppedo 50	Epirubicin HCI Methylparaben	50mg, 10mg	Vial	Miracalus Pharma Pvt Ltd
2	4 Eppedo 10	Epirubicin HCI Methylparaben	10mg, 10mg	Vial	Miracalus Pharma Pvt Ltd
3	4 Eppedo	Epirubicin HCI Methylparaben	10mg, 10mg	Injection	Miracalus Pharma Pvt Ltd
4	4 Eppedo	Epirubicin HCI Methylparaben	50mg, 10mg	Injection	Miracalus Pharma Pvt Ltd

Turabi [22] et al demonstrated a linear HPLC method for the simultaneous estimation of mebendazole (MEB), MET and propylparaben (PRO) in pharmaceutical oral suspension dosage form. Inertsil ODS-3V C18 column at 247nm was employed for the successful chromatographic separation. At room temperature, mobile phase of methanol (0.05M), monobasic potassium phosphate and acetonitrile (48:32:20v/v) was used at a flow rate of 1.5ml/min. The validation approach was carried out basing on the

ICH guidelines. Retention time and linearity of the method were tabulated in **Table.7.2.** The percentage purity was found to be more than 99.0%. Thus, the proposed method was successful in terms linearity, specificity, robustness, accuracy, precision and ruggedness.

Table.7.2. Results of HPLC method of mebendazole, methylparaben and propylparaben

S.No	Parameter	Mebendazole	Methylparaben	Propylparaben
1	Retention Time	2.83min	4.14min	4.75min
2	Linearity	3.6-5. 4µg/ml	40-60µg/ml	0.4-0.6µg/ml

Shabir [25] interpreted and validated a simple HPLC method to evaluate 2-phenoxyethanol, MET, ethylparaben and propylparaben in pharmaceutical gel products. Lichrosorb system equipped with C8 (150×4.6 mm, 5 µm) column of isocratic elution was used for the detection at 258nm. A composite of acetonitrile, tetrahydrofuran and water was utilized as mobile phase at 1ml/min flow rate. Validation of the proposed method strictly followed ICH guidelines. Results of validation parameters like linearity, specificity, accuracy, precision etc were found to be within the range. The method was found to be selective and reliable. Hence, the described method can be effectively used for the routine analysis of these preservatives in the commercial formulations.

Borkar [29] et al were succeeded in developing a sensitive HPLC method for the estimation of citicoline (CIT) and MET simultaneously in oral drop formulation on Merck C8 column (250mm x 4.6mm, 5µm particle size). Mobile phase composed of monobasic potassium phosphate (0.1) and methanol (70: 30, v/v) at 294nm was used for the analysis. Good linearity was observed in the range of 80-120ppm and 8-12ppm for CIT sodium and MET simultaneously. Retention time of CIT sodium was found to be 2.06min and was 14.68min for MET. High degree of purity was obtained for both CIT and MET (>99.0%). As per the ICH guidelines the proposed method was accepted with good linearity, accuracy, precision, specificity and reproducibility.

Bhat [35] et al proposed specific HPLC method for the validation of MET in succinylcholine chloride injection. The validated method was supported with stability indicating studies. All the validation parameters were determined as mentioned in ICH guidelines. The method was confirmed to be linear in the given concentration range. Small but deliberate changes were introduced in temperature and flow rates to measure robustness. Based on system suitability conditions the developed method can be utilized for the routine analysis.

Basima and Mohammad [36] prescribed a rapid and sensitive HPLC method for the analysis of MET and PRO in topical creams containing steroids. On C18 column chromatographic separation was achieved by the use of mixture of acetonitrile and water (65:35, v/v) as mobile phase at a flow rate of 1.5ml/min. The detection was carried out by using the UV detector at 240nm. Different parameters were examined by making use of USP 30 guideline recommendations. The proposed method represented good linearity, accuracy, selectivity and robustness. Forty seven topical cream samples produced in Syria containing steroids were analyzed in the patient's terms of use and the validated method was found to be suitable.

Kurganoglu [55] et al established a different analytical technique for the determination and degradation studies of EPI. Waters spherisorb ODS1 column with a composite of acetonitrile and water (30: 70, v/v) as mobile phase was used for the chromatographic separation. The method was succeeded in terms of optimum separation when the flow rate was fixed at 0.6ml/min. Stability indicating studies were performed by the application of different stress conditions like acidic, basic, oxidation, thermal (100^0C) and UV light. Well resolved peaks were obtained for EPI and degradation products in each and every case.

Kumar [56] et al concluded a stability indicating HPLC method for the estimation of MET, ethylparaben (ETH), PRO and butylparaben (BUT) in the cosmetic products. The most important feature of the proposed method was time and the four parabens were analysed in a time period of less than 10min. The method was validated as prescribed in ICH guidelines. It was the only single HPLC assay method reported for the determination of four parabens. The method was proved to be fast and robust. Hence, the developed method can be applied in any quality control laboratories for the analysis of four parabens in bulk.

Chinmoy and Jitmanyu [58] developed a simple and precise HPLC method for the development and validation of MET, ketoconazole (KET) and mometasone furoate (MOF) in the commercial formulation. Waters X terra C18 column was utilized to achieve chromatographic separation at 250nm. Mobile phase containing triethylamine buffer and acetonitrile (40: 60, v/v) was used at 1.5 ml/min flow rate. The pH of mobile phase was maintained at 6.5 with glacial acetic acid. Linearity range reported was 0.12–15.2µg/ml for MET, 67–149.4µg/ml for KET and 0.42–7.6µg/ml for MOF. The validation approach was carried out basing on the ICH guidelines. The method was accepted in terms of linearity, LOD, LOQ, robustness and solution stability. The method was proved to be precise and accurate. The method was supported with stability indicating studies. The forced degradation chromatograms of the sample gave separate peaks for pure drugs and for degradation products. Thus, the developed stability indicating method can be safely applied for the estimation of MET, KET and MOF in pharmaceutical dosage form.

Dodde [59] et al reported an accurate HPLC method for the estimation of EPI and its metabolite epirubicinol (EPO) in saliva and plasma. Saliva and plasma samples were extracted with a mixture of chloroform and 2- propanol (6:1, v/v). Later, organic phase was evaporated under vacuum at 45^0C. Chromsep stainless steel HPLC column was

employed for the entire analysis and doxorubicin was used as an internal standard. The chromatographic detection was carried out at 474nm and 551nm. Results of some validation parameters were tabulated in **Table.7.3.** The proposed method can be comfortably used for the routine analysis of both EPI and EPO in saliva and plasma.

Table.7.3. Results of epirubicin and epirubicinol by HPLC method

S.No	**Parameter**		**Epirubicin**	**Epirubicinol**
1	Linearity (Saliva & plasma)		5 - 1000µg/ml	2 - 400µg/ml
2	%Recovery	Saliva	88.9	69
		Plasma	77.3	80
3	LOQ (Saliva & plasma)		5µg/ml	2µg/ml

7.3. EXPERIMENTAL

7.3.1. Chemicals and solvents

The drug samples and the working standard of methylparaben and epirubicin were gifted by Miracalus Pharma Pvt Ltd. The pharmaceutical formulation (4- Eppedo 10 brand: Methylparaben 10mg; Epirubicin 10mg) was procured from provincial market. Methanol, acetonitrile and water (HPLC grade) were purchased from Merck Specialties Pvt Ltd, Mumbai, India. AR grade perchloric acid and reaming buffer solutions were also purchased from Merck Specialties Private Limited, Mumbai, India.

7.3.2. Preparation of standard stock solution

Methylparaben and epirubicin in the pure form were used for the preparation of standard stock solutions separately. Accurately weighed 10mg of MET was transferred into 10ml volumetric flask. Initially, little amount of methanol was added to dissolve the drug. Complete dissolution was ensured after sonication for about 15min. Proposed volume was reached by the addition of required quantity of methanol. In the similarly way, standard stock solution of EPI was prepared. Further, these standard solutions were diluted with mobile phase to obtain different concentrations ranging from 5-30µg/ml for both the drugs MET and EPI

7.3.3. Preparation of sample solution

Composite of 20 vials [4- Eppedo 10 brand: Methylparaben 10mg, Epirubicin 10mg] were mixed in a clean dry beaker. From the formulation solution, an amount of liquid equivalent to 10mg of epirubicin was measured accurately in a 10ml calibrated volumetric flask and was dissolved in small amount of methanol and then sonicated for 5min. The solution was made up to the corresponding volume by the use of solvent. After filtration, an amount of the solution was diluted with mobile phase to a concentration of 20µg/ml of EPI. Simultaneously, as on the label claim 20µg/ml of MET was obtained.

7.4. METHOD DEVELOPMENT

7.4.1. Detection of wavelength

The active ingredients MET and EPI showed maximum overlapping at 258nm and thus the total analysis was carried out at this wavelength only.

7.4.2. Choice of stationary phase

After testing several octadecyl columns, the standard solution of MET and EPI exhibited appropriate peak response on kromasil RP-C18 column. Hence, kromasil column was used for the determination of MET and EPI.

7.4.3. Selection of mobile phase

Suitable mobile phase for the estimation of MET and EPI was selected on comparison of system suitability conditions in each and every trial. Finally, composite of methanol and sodium perchlorate (75: 25, v/v) was accepted as the apt mobile phase due to the exhibition of high resolution, column efficiency and good base line separation.

7.4.4. Flow rate

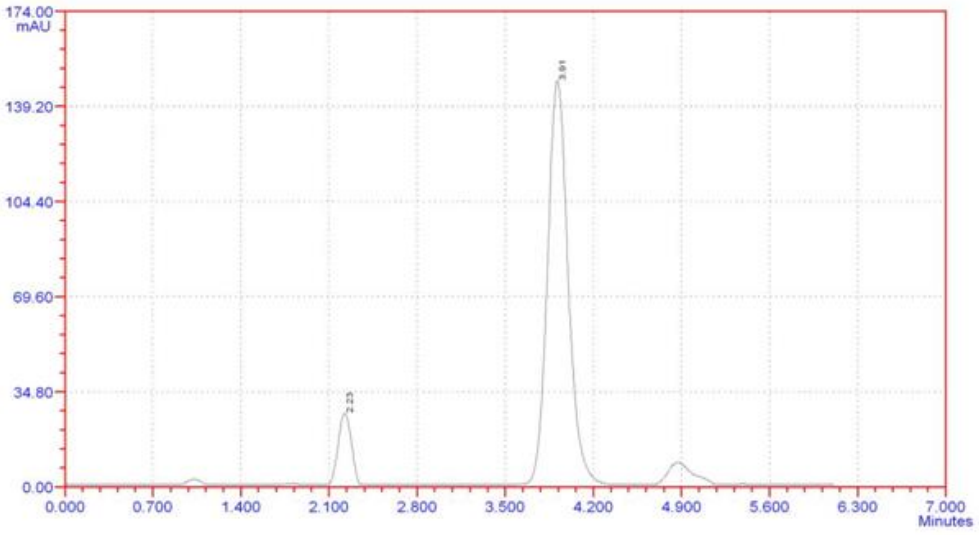
Optimum separation for the analysis of MET and EPI was attained at a flow rate of 1.0ml/min.

7.4.5. Optimized chromatographic conditions

Several systematic trials were conducted for the selection of optimum chromatographic conditions for the quantification of MET and EPI and were presented in **Table.7.4.** Corresponding trial chromatograms were given from **Figure.7.3.** to **Figure.7.11.** Optimum chromatographic conditions of MET and EPI were tabulated in **Table.7.5.** Chromatograms of blank, MET single, EPI single, standard and formulation were shown from **Figure.7.12.** to **Figure.7.16.**

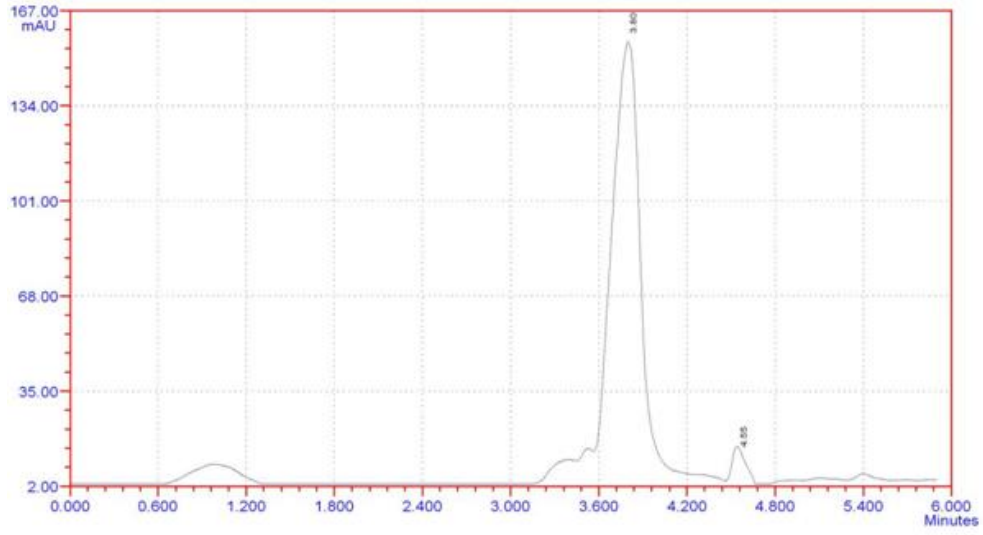
Table.7.4. Trial conditions of methylparaben and epirubicin

Trial	Mobile phase (v/v)	Wavelength	pH of mobile	Column	Flow rate
I	MeOH: Acetate Buffer 50:50	258nm	6.1	Kromasil RP- C18	1.0ml/min
II	MeOH: PB 50:50	258nm	5.8	Kromasil RP- C18	1.0ml/min
III	MeOH: Water: ACN 50:25:25	258nm	5.9	Kromasil RP- C18	1.0ml/min
IV	MeOH: sodium perchlorate 50:50	258nm	6.1	Kromasil RP- C18	1.0ml/min
V	MeOH: sodium perchlorate 75:25	258nm	5.5	Kromasil RP- C18	1.0ml/min
VI	MeOH: sodium perchlorate 75:25	242nm	5.5	Kromasil RP- C18	1.0ml/min
VII	ACN: sodium perchlorate 75:25	242nm	5.5	Kromasil RP- C18	1.0ml/min
VIII	MeOH: sodium perchlorate 75:25	242nm	5.4	Kromasil RP- C18	1.0ml/min
IX	MeOH: sodium perchlorate 75:25	258nm	5.4	Kromasil RP- C18	1.0ml/min



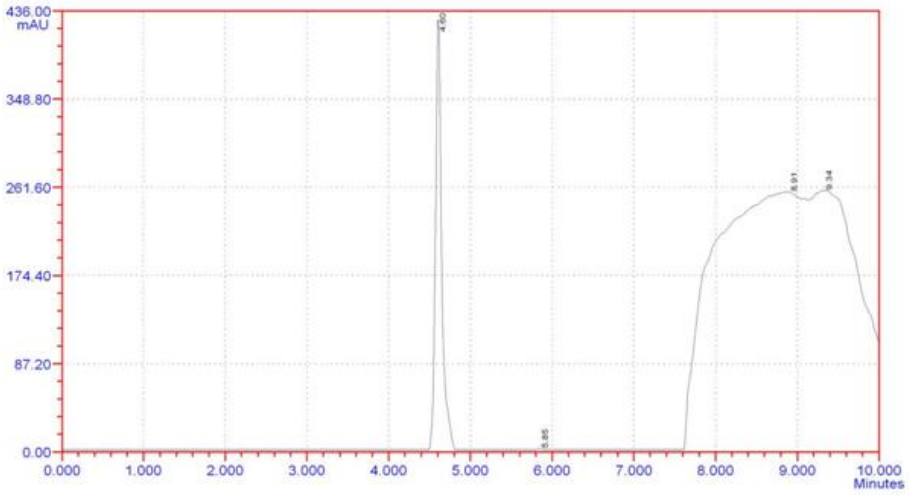
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		2.225	3143	28820.0	11.146	1.13	1174
2		3.913	14930	229738.9	88.854	0.06	1289
	Sum:		18073	258558.9	100.0000		

Figure.7.3. Trial chromatogram I



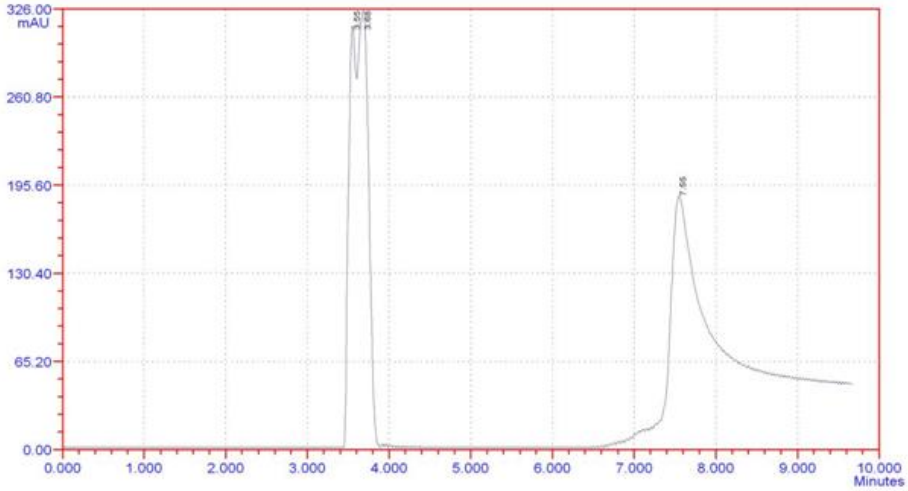
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.802	14482	179591.9	94.625	0.96	1873
2		4.552	1371	10201.5	5.375	1.18	7458
	Sum:		15853	189793.4	100.0000		

Figure.7.4. Trial chromatogram II



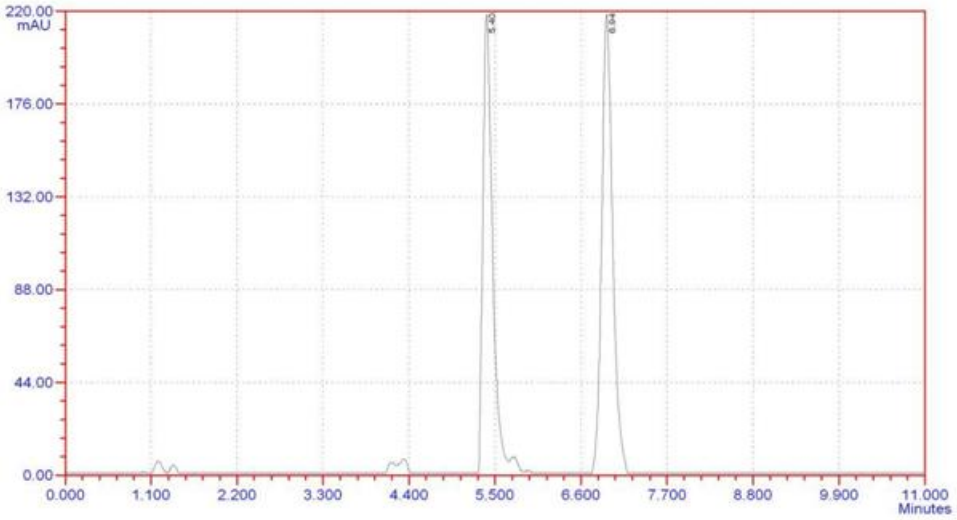
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.603	42919	233563.7	9.245	1.57	14262
2		5.855	859	6480.5	0.257	1.51	12004
3		8.907	20194	1677446.2	66.398	0.60	229
4		9.337	18186	608859.9	24.100	2.25	1550
	Sum:		82158	2526350.3	100.0000		

Figure.7.5. Trial chromatogram III



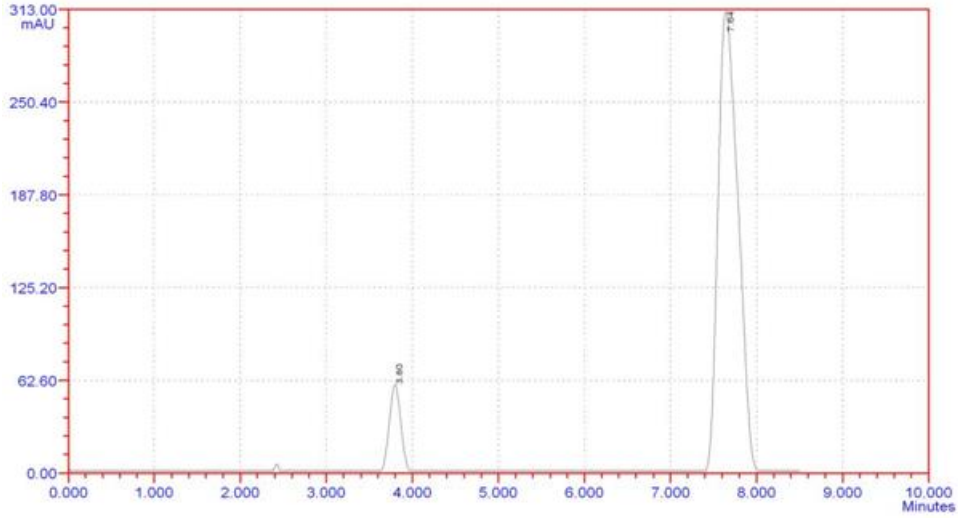
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.552	31226	197258.9	21.555	0.75	6301
2		3.683	32457	299753.4	32.755	1.70	3170
3		7.547	17190	418115.6	45.689	1.51	1919
	Sum:		80873	915128.0	100.0000		

Figure.7.6. Trial chromatogram IV



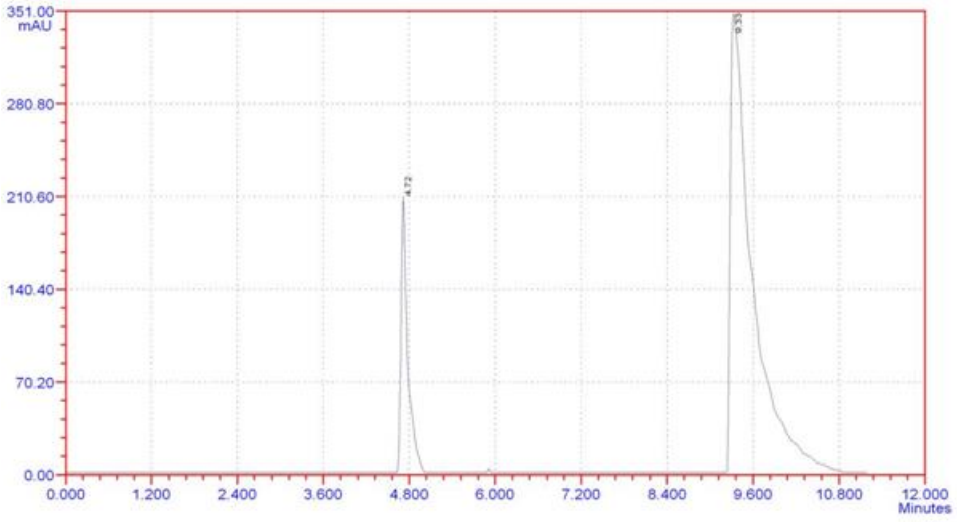
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		5.398	2251	20151.9	55.713	1.34	7247
2		6.940	1826	16018.8	44.287	1.12	12474
	Sum:		4077	36170.6	100.0000		

Figure.7.7. Trial chromatogram V



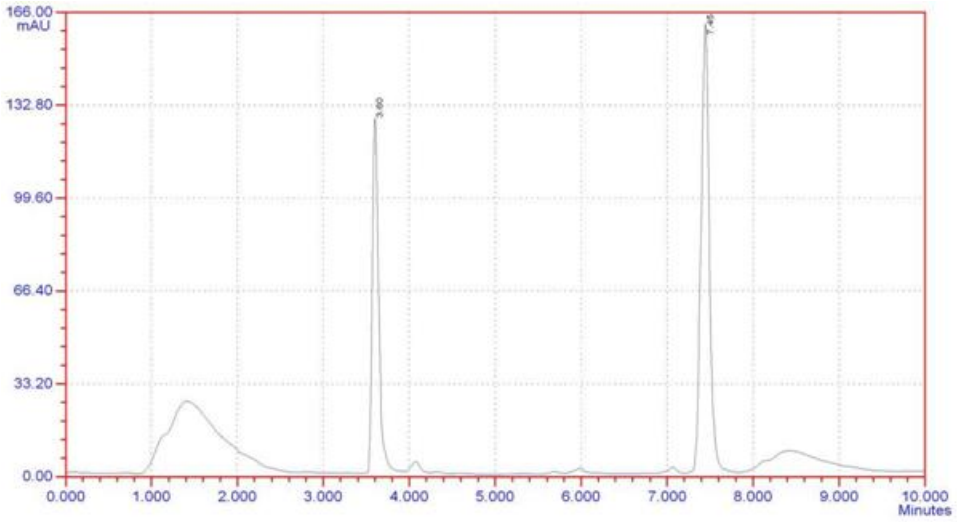
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.797	6659	80986.0	22.622	0.94	1942
2		7.642	23134	277003.2	77.378	1.18	8118
	Sum:		29793	357989.2	100.0000		

Figure.7.8. Trial chromatogram VI



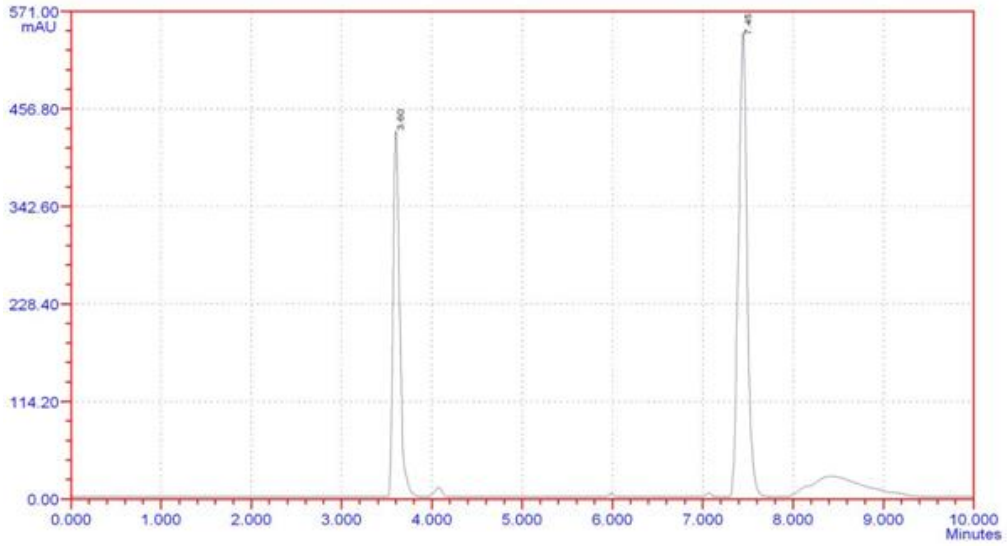
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		4.717	22001	167306.8	16.818	2.09	7668
2		9.330	35824	827494.7	83.182	8.79	3252
	Sum:		57825	994801.5	100.0000		

Figure.7.9. Trial chromatogram VII



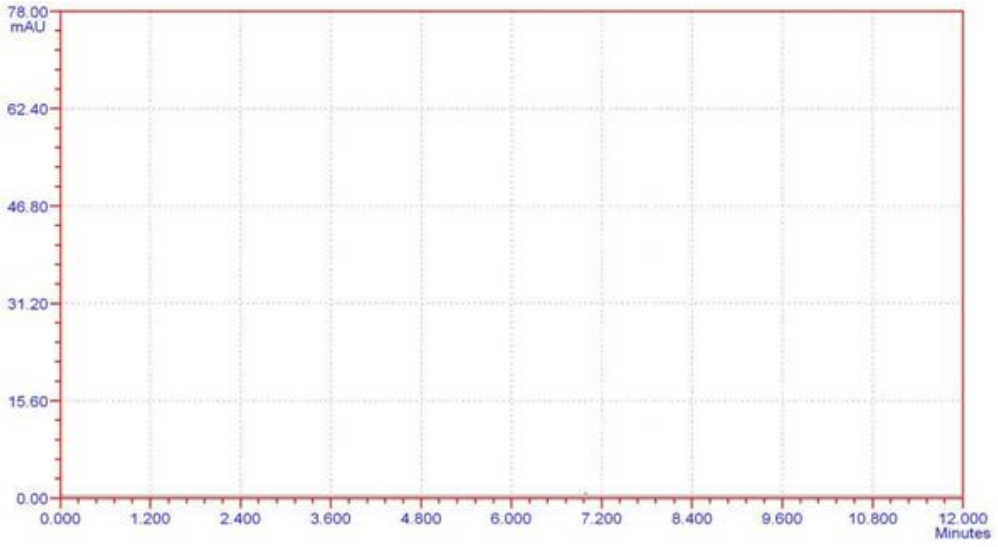
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.595	34490	133933.2	36.115	1.36	17082
2		7.448	44703	236917.4	63.885	0.93	39367
	Sum:		79193	370850.6	100.0000		

Figure.7.10. Trial chromatogram VIII



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1		3.595	43004	215177.5	36.921	2.06	10289
2		7.448	54590	367632.4	63.079	1.26	24381
	Sum:		97594	582809.9	100.0000		

Figure.7.11. Trial chromatogram IX

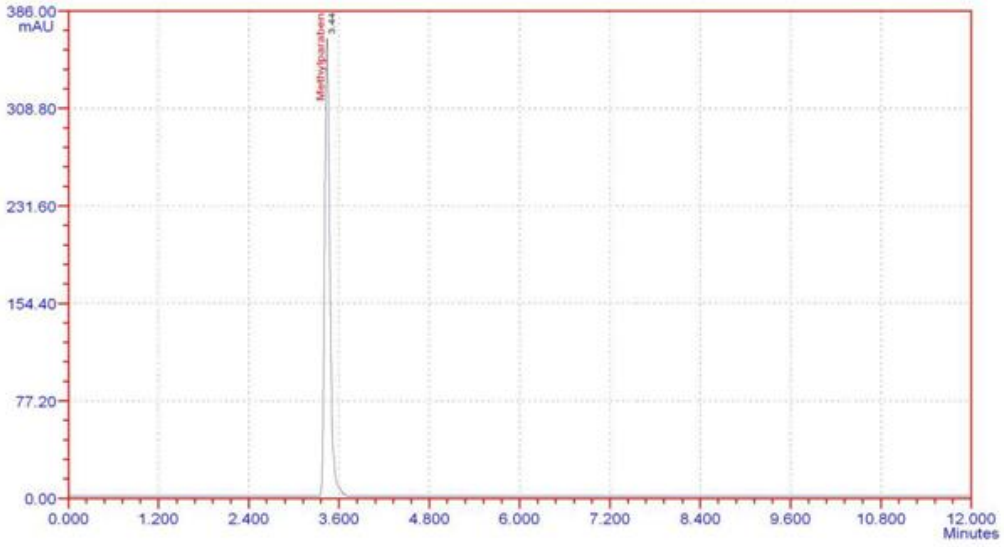


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
	Sum:		0	0.0	0.0000		

Figure.7.12. Chromatogram of blank of methylparaben and epirubicin

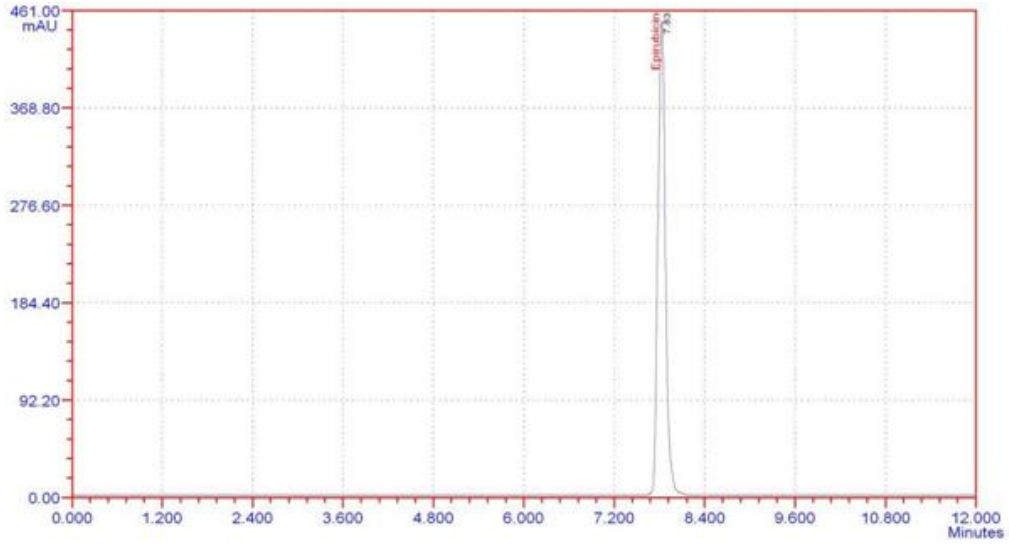
Table.7.5. Optimized chromatographic conditions of methylparaben and epirubicin

1	Pump mode	Isocratic	
2	Column	C 18 (250X4.6 mm, 5µm)	
3	Injector	Rheodyne	
4	Injector Volume	20 µl	
5	Diluent	Methanol	
6	Mobile phase	Methanol: 1% v/v sodium perchlorate 75:25 (v/v)	
7	Pump pressure	11.6±5MPa	
8	Mobile phase pH	5.3 with 1% Perchloric acid	
9	Wavelength	258nm	
10	Flow rate	1.0ml/min	
11	Run Time	12min	
12	Standard Concentration	Methylparaben	20µg/ml
		Epirubicin	20µg/ml
13	Retention Time	Methylparaben	3.42min
		Epirubicin	7.44min
14	Peak Area	Methylparaben	266834
		Epirubicin	367061
15	Theoretical Plates	Methylparaben	11689
		Epirubicin	35737
16	Tailing Factor	Methylparaben	1.12
		Epirubicin	1.12



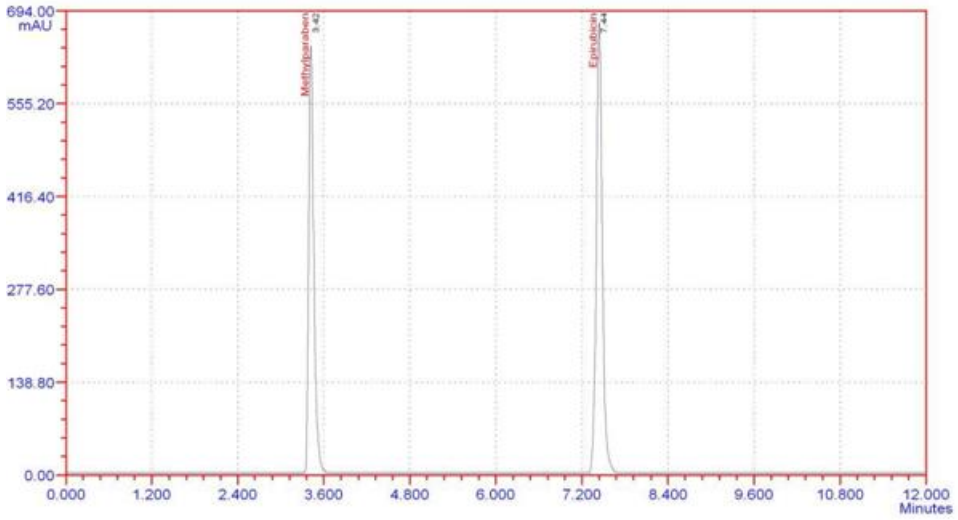
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Methylparaben	3.440	37168	264970.9	100.000	0.93	4642
	Sum:		37168	264970.9	100.0000		

Figure.7.13. Chromatogram of methylparaben single



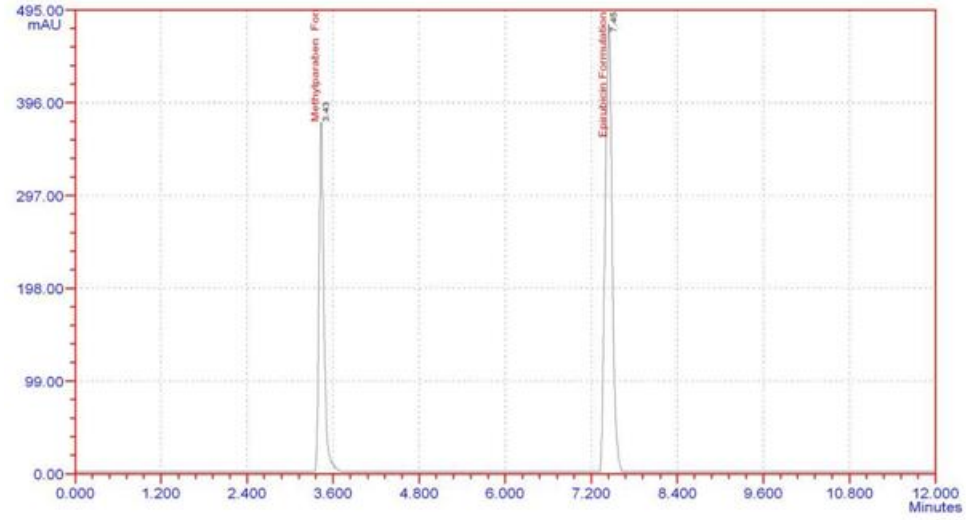
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Epirubicin	7.834	44942	362087.5	100.000	0.24	18842
	Sum:		44942	362087.5	100.0000		

Figure.7.14. Chromatogram of epirubicin single



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Methylparaben	3.417	59809	266834.5	42.094	1.12	11689	0.00
2	Epirubicin	7.442	66049	367061.1	57.906	1.12	35737	28.36
	Sum:		125858	633895.6	100.0000			

Figure.7.15. Chromatogram of methylparaben and epirubicin standard



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
	Methylparaben Formulatio	3.434	39457	265141.6	42.301	0.70	5204
2	Epirubicin Formulation	7.449	48760	361662.4	57.699	0.87	20100
	Sum:		88217	626804.0	100.0000		

Figure.7.16. Chromatogram of methylparaben and epirubicin formulation

7.5. METHOD VALIDATION

7.5.1. Specificity

Study of chromatograms of MET and EPI revealed the fact that, there was no interference of diluents and placebo in the analysis and supported the specificity of the developed method. Retention time of MET was found to be 3.42min and EPI was found to be 7.44min.

7.5.2. System suitability

Results of system suitability were expressed in **Table.7.6.**

Table.7.6. Results of system suitability of methylparaben and epirubicin

S.No	Parameter	Methylparaben	Epirubicin
1	API concentration	20µg/ml	20µg/ml
2	Retention Time	3.42min	7.44min
3	Resolution	------	28.36
4	Peak Area	266834	367061
5	Theoretical Plates	11689	35737
6	Tailing Factor	1.12	1.12

7.5.3. Linearity

Results of linearity of were specified in **Table.7.7.** Calibration curves were given in **Figure.7.17 & 7.18.** Correlation coefficient of MET was 0.999 and EPI was 0.998.

Table.7.7. Results of linearity of methylparaben and epirubicin

S.No	Methylparaben		Epirubicin	
	Concentration in µg/ml	Peak Area	Concentration in µg/ml	Peak Area
1	5	63322	5	92012
2	10	125122	10	195613
3	15	200561	15	284251
4	20	266834	20	367061
5	25	336968	25	472530
6	30	395739	30	571384

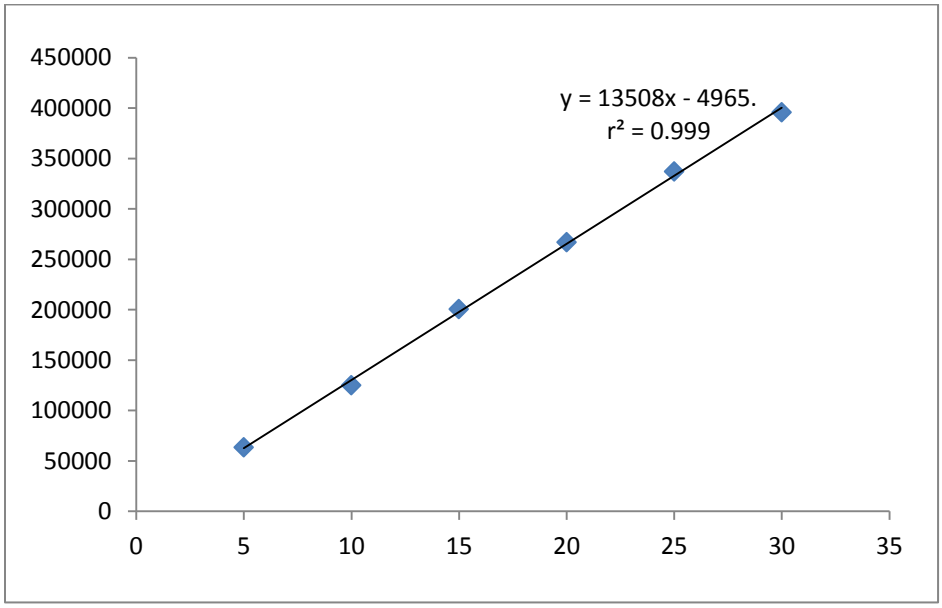


Figure.7.17. Calibration curve of methylparaben

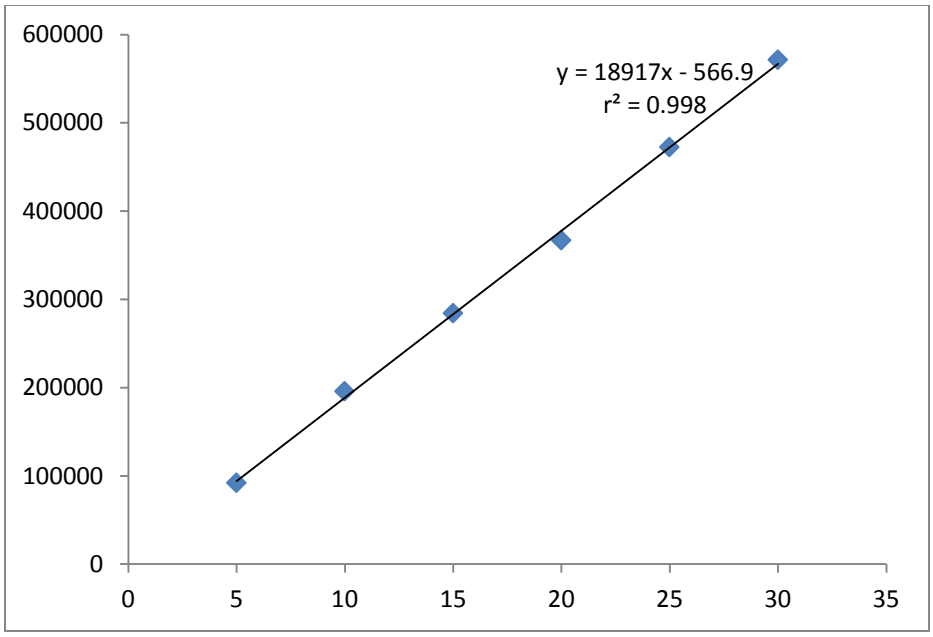


Figure.7.18. Calibration curve of epirubicin

7.5.4. Accuracy

Accuracy of the method was determined through recovery studies. Results of recovery of MET and EPI are arranged in **Table.7.8 & 7.9** respectively. Percentage recovery of MET was 98.11-100.90 and that of EPI was 98.28-101.74.

7.5.5. Precision

Precision of the method was measured through intra-day precision and inter-day precision and the corresponding results were tabulated in **Table.7.10 & 7.11.**

7.5.6. Ruggedness

Reproducibility of MET and EPI was measured by ruggedness study. Results of ruggedness were reported in **Table.7.12.**

7.5.7. Robustness

Robustness of the method was measured and the results were found to be within the limits. Results of robustness were shown in **Table.7.13**.

Table.7.8. Recovery results of methylparaben

S.No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	10	5	15	14.772	98.480
2		10	5	15	14.796	98.637
3		10	5	15	14.803	98.686
4	100%	10	10	20	19.924	99.620
5		10	10	20	20.040	100.198
6		10	10	20	20.159	100.800
7	150%	10	15	25	25.052	100.208
8		10	15	25	24.527	98.110
9		10	15	25	25.225	100.900

Table.7.9. Recovery results of epirubicin

S.No	Spiked Level	Concentration in µg/ml			Amount Found	% Recovery
		Target	Spiked	Total		
1	50%	10	5	15	15.079	100.525
2		10	5	15	14.834	98.898
3		10	5	15	14.783	98.555
4	100%	10	10	20	19.707	98.536
5		10	10	20	19.656	98.280
6		10	10	20	20.014	100.070
7	150%	10	15	25	24.761	99.044
8		10	15	25	24.997	99.989
9		10	15	25	25.436	101.746

Table.7.10. Results of intra-day precision of methylparaben and epirubicin

S. No	Methylparaben at 20µg/ml	Epirubicin at 20µg/ml
1	252458	378290
2	259025	374219
3	259617	370119
4	260282	373081
5	256733	370426
6	259602	375855
%RSD	**1.147**	**0.845**

Table.7.11. Results of inter-day precision of methylparaben and epirubicin

S. No	Methylparaben at 20µg/ml	Epirubicin at 20µg/ml
1	267346	368769
2	264058	361144
3	262198	360698
4	264624	369091
5	266447	362495
6	259632	365504
%RSD	**1.070**	**1.026**

Table.7.12. Results of ruggedness of methylparaben and epirubicin

S. No	Methylparaben at 20µg/ml	Epirubicin at 20µg/ml
1	260668	365366
2	262310	361955
3	260069	360824
4	263182	366542
5	265072	363059
6	263648	362868
%RSD	**0.717**	**0.588**

Table.7.13. Results of robustness of methylparaben and epirubicin

S.No	Condition	Change	Methylparaben		Epirubicin	
			Area	% Change	Area	% Change
1	Standard	NO Change	266834	…….	367061	……
2	MP 1	Methanol: sodium perchlorate 70:30 (v/v)	262839	1.497	364970	0.570
3	MP 2	Methanol: sodium perchlorate 80:20 (v/v)	268558	0.646	365391	0.455
4	pH 1	5.2	266300	0.200	360261	1.853
5	pH 2	5.4	266077	0.284	364819	0.611
6	WL 1	255nm	266429	0.151	361749	1.447
7	WL 2	261nm	268379	0.579	365674	0.378

7.5.8. Limit of detection and limit of quantification

LOD and LOQ values of MET and EPI were assigned in **Table.7.14.**

7.5.9. Solution stability

Stability of the standard solution of MET and EPI was determined to be 24hr. Results of solution stability were shown in **Table.7.15.**

7.5.10. Formulation

Results of MET and EPI formulation were introduced in **Table.7.16.** The percentage assay of was found to be 99.365 for MET and 98.529 for EPI.

Table.7.14. LOD and LOQ values of methylparaben and epirubicin

Drug	LOD	LOQ
Methylparaben	0.003µg/ml	0.01µg/ml
Epirubicin	0.003µg/ml	0.01µg/ml

Table.7.15. Results of solution stability of methylparaben and epirubicin

S.No	Time in Hours	Methylparaben		Epirubicin	
		Area	% Assay	Area	% Assay
1	1	262310	98.30456	371028	101.0807
2	2	263096	98.59913	360845	98.30655
3	4	265513	99.50494	365607	99.60388
4	8	264970	99.30144	366694	99.90002
5	12	263775	98.85359	360320	98.16352
6	24	261961	98.17377	361081	98.37084

Table.7.16. Results of methylparaben and epirubicin formulation

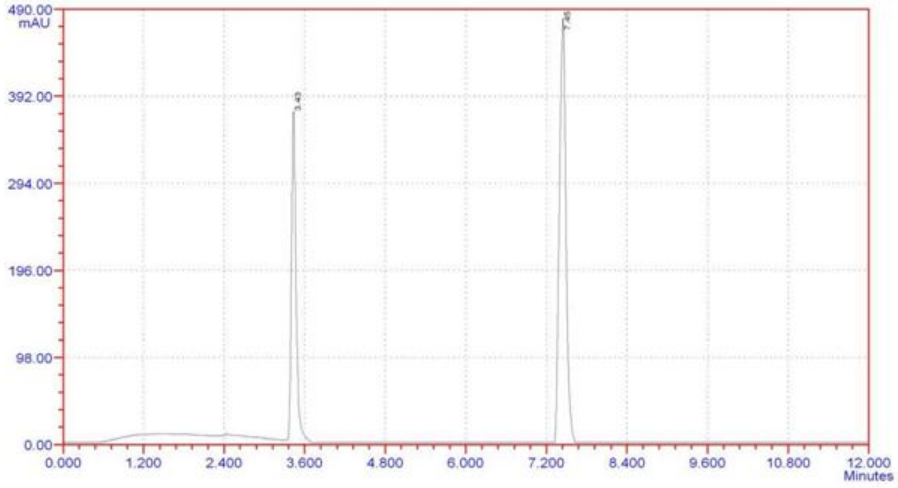
S.No	Drug	Brand	Dosage	Amount	Amount	%Assay
1	Methylparaben	4-Eppedo	10mg	20µg/ml	19.873µg/ml	99.365
2	Epirubicin		10mg	20µg/ml	19.706µg/ml	98.529

7.6. FORCED DEGRADATION STUDY

The stability of MET and EPI formulation under different stress conditions were studied and the results were presented in **Table.7.17**. Degradation chromatograms of aqueous, acidic, basic, peroxide, thermal, light and UV light were shown from **Figure.7.19** to **Figure.7.25**.

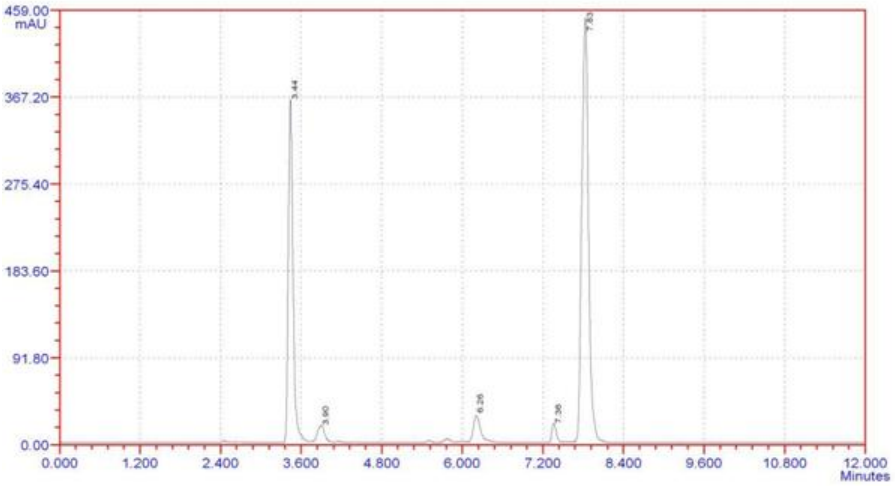
Table.7.17. Forced degradation studies of methylparaben and epirubicin

S. No	Condition	No of degradation peaks observed
1	Aqueous	0
2	Acidic	3
3	Basic	2
4	Peroxide	3
5	Thermal	1
6	Sun light	2
7	UV light	2



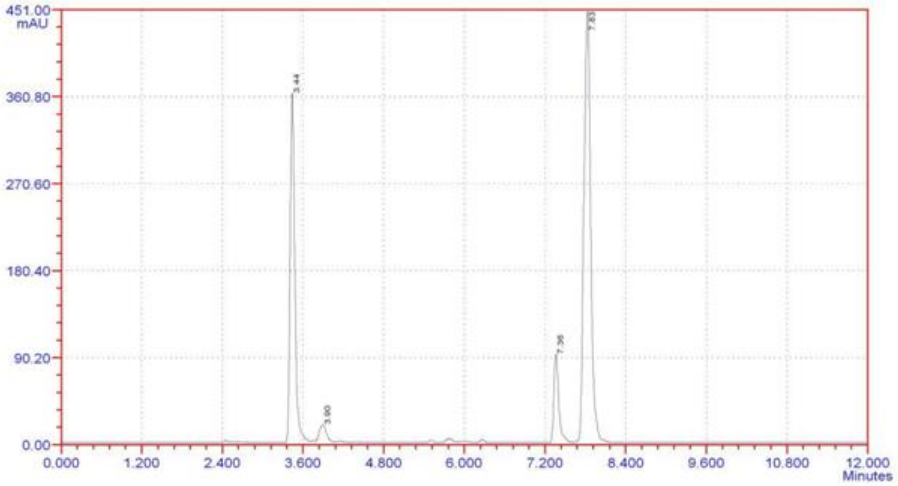
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.434	37821	256733.9	41.203	0.64	5099	0.00
2		7.449	48764	366362.2	58.797	0.66	19591	19.82
	Sum:		86585	623096.1	100.0000			

Figure.7.19. Chromatogram of aqueous degradation



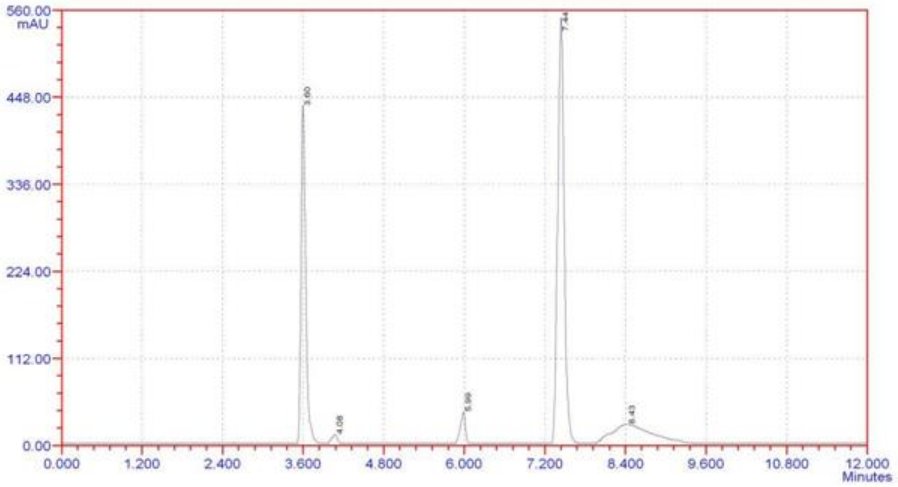
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.440	37536	250866.6	39.212	0.72	5281	0.00
2		3.899	1544	9772.2	1.527	0.82	7563	2.49
3		6.264	1210	14007.5	2.189	0.77	5835	9.32
4		7.364	9458	23625.8	3.693	0.51	173203	5.52
5		7.834	45003	341502.5	53.379	0.22	21239	3.29
	Sum:		94751	639774.7	100.0000			

Figure.7.20. Chromatogram of acid degradation



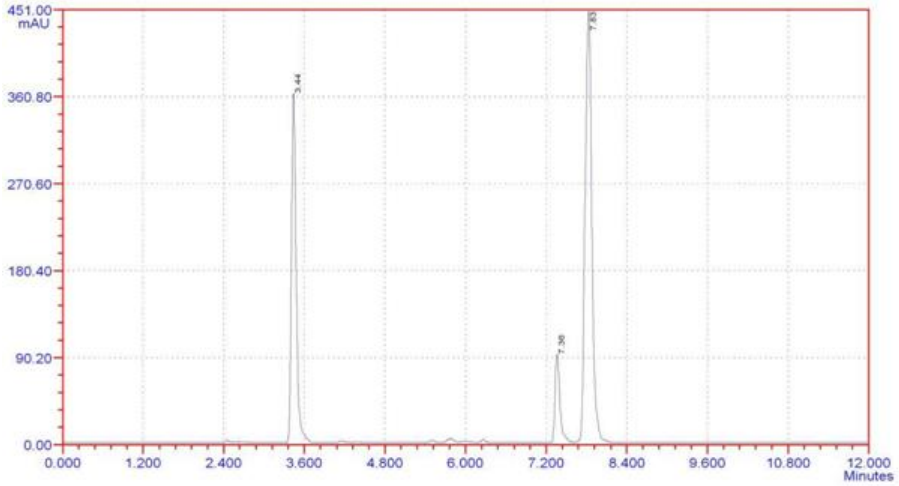
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.440	37168	241691.8	38.428	0.76	5579	0.00
2		3.899	2693	24755.8	3.936	1.22	3585	2.06
3		7.364	9250	46419.2	7.381	1.43	42916	17.21
4		7.834	44942	316075.9	50.255	0.07	24727	2.75
	Sum:		94053	628942.6	100.0000			

Figure.7.21. Chromatogram of base degradation



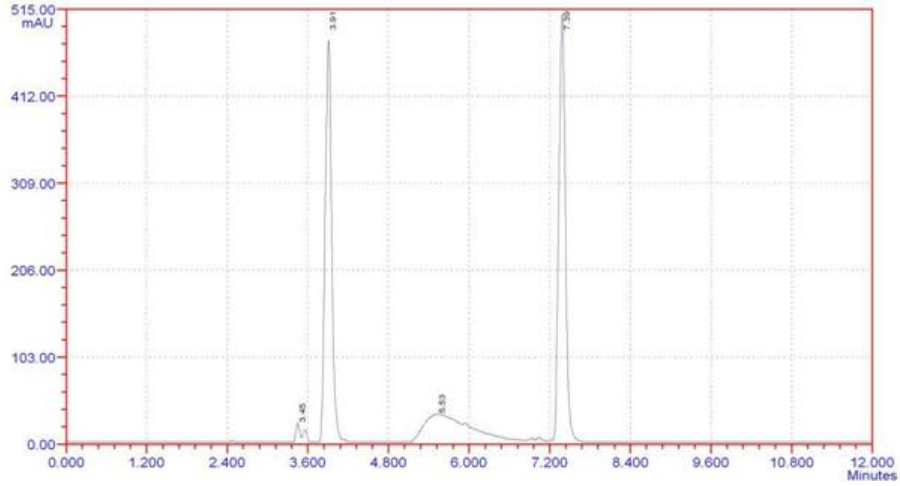
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.595	44186	247849.3	38.548	0.87	8187	0.00
2		4.075	1662	12433.4	1.934	1.09	5914	2.59
3		5.988	850	6912.0	1.075	0.94	10809	8.65
4		7.442	54998	362438.9	56.369	1.18	25415	6.97
5		8.425	864	13337.2	2.074	0.92	5937	3.15
	Sum:		102560	642970.8	100.0000			

Figure.7.22. Chromatogram of peroxide degradation



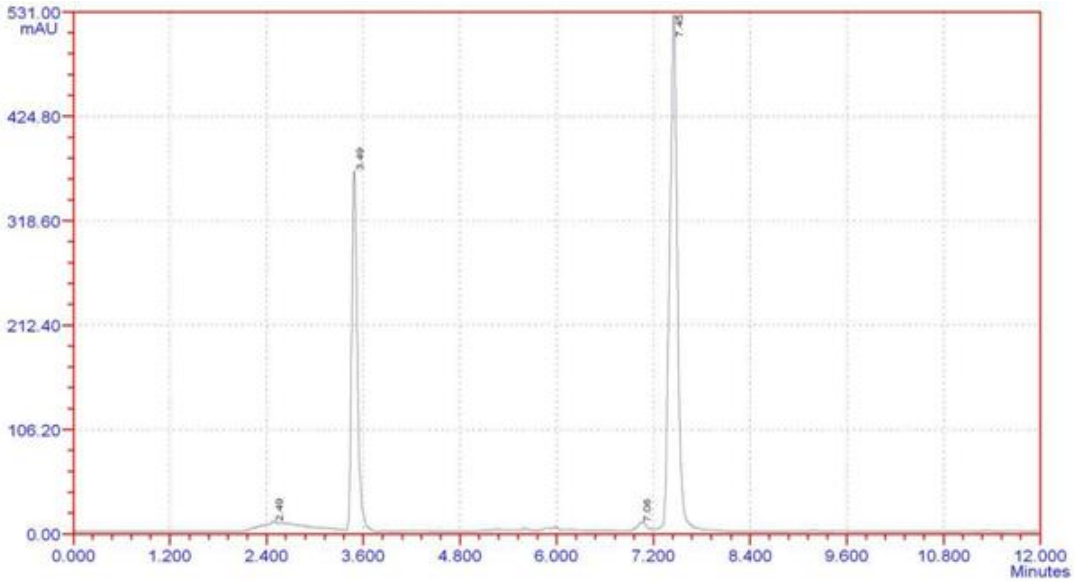
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.440	37034	255217.6	41.437	1.40	4967	0.00
2		7.364	9363	44183.3	7.174	0.93	48533	23.85
3		7.834	44867	316515.5	51.389	0.12	24576	2.82
	Sum:		91264	615916.5	100.0000			

Figure.7.23. Chromatogram of thermal degradation



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		3.448	724	6970.7	1.075	1.57	2556	0.00
2		3.910	43362	259413.4	39.999	1.05	8513	2.09
3		5.528	1088	17998.6	2.775	0.98	2226	5.07
4		7.390	51106	364170.8	56.151	0.92	21435	5.55
	Sum:		96280	648553.4	100.0000			

Figure.7.24. Chromatogram of light degradation



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		2.487	763	15727.3	2.442	0.91	290	0.00
2		3.487	37558	258231.9	40.093	1.23	5126	2.57
3		7.060	1041	8488.7	1.318	0.82	14940	16.78
4		7.452	52890	361630.7	56.147	1.12	23672	1.84
	Sum:		92252	644078.5	100.0000			

Figur.7.25. Chromatogram of UV light degradation

7.7. RESULTS AND DICUSSION

Present study was aimed to develop a precise and sensitive stability indicating HPLC method for the simultaneous quantification of MET and EPI in pharmaceutical formulation. UV spectrophotometer was utilized for the selection of wavelength. AT 258nm, the two drugs MET and EPI showed maximum absorption and hence wavelength was fixed to be 258nm throughout the analysis. Numerous trials were conducted for the choice of column, stationary phase and mobile phase. Finally, kromasil RP-C18 column was recognized as appropriate stationary phase for the complete determination. At ambient temperature, mobile phase, a mixture of methanol and sodium perchlorate was chosen as best one for the entire analysis. At a pH of 5.3 (1% perchlorate), optimum chromatographic conditions were displayed and results were furnished in **Table.7.5**.

Validation of the proposed method was initiated with the prior measurement of specificity of the method. Retention times of MET was found to be 3.42min and for EPI was 7.44min. Different properties of chromatogram were studied, in particular, tailing factor (<2.0), resolution (>2.0) and theoretical plates (> 2000) and were shown in **Table.7.6.** The chromatograms of blank **(Figure.7.12)**, MET single **(Figure.7.13)**, EPI single **(Figure.7.14)**, MET and EPI standard **(Figure.7.15)** and MET and EPI formulation **(Figure.7.16)** were studied keenly. The careful approach revealed the fact that, excipients have no effect in the analysis.

Linearity shown by MET was in the concentration range of 5-30µg/ml with the corresponding regression equation y = 13508x – 4965 (r^2 = 0.999). Linearity of EPI was also found to be in the concentration range of 5-30µg/ml with the corresponding regression equation y = 18917x –566.9 (r^2 = 0.998). Recovery studies of MET and EPI revealed the accuracy of developed method. Three spiked concentrations especially 50%, 100% and 150% were used for the recovery approach.

Results of recovery of MET and EPI were arranged in **Table.7.8 and 7.9** respectively. Precision of the method was measured by the use of adequate number of homogeneous samples. Results of intra-day **(Table.7.10)** and inter-day **(Table.7.11)** precision confirmed the preciseness of the method. The percentage RSD of MET and EPI were in the acceptable range (<2). On three different days, six replicate injections were utilized by different analyst in analyzing the ruggedness of the method. Results **(Table.7.12)** obtained were supporting the ruggedness of the method.

Robustness of the method was measured by introducing small changes in chromatographic parameters such as mobile phase, pH and wavelength. Results obtained were found to be in proper range and were furnished in **Table.7.13**. LOD and LOQ values of MET and EPI standard solution were given in **Table.7.14**. Over a period of 36hr, stability study of the standard solution of MET and EPI was examined and it was recognized **(Table.7.15.)** that, the solution was stable up to 24hr. Under the proposed chromatographic conditions, commercial formulation of MET and EPI was tested. From the results **(Table.7.16)** one can conclude that, the validation method can be safely applied for the assay of commercial formulation of MET and EPI.

Different stress conditions were applied to the standard solution of MET and EPI to measure the stability of the molecule. Results were presented in **Table.7.17**. The beauty of the degradation study lies in providing highly valuable information with respect to degradation path ways, degradation products and problems that can arise during storage. Hence, quality control and pharmaceutical industries are greatly depending on information provided through forced degradation study that plays a key role during the manufacturing, formulation development and packing of NIT and PHE in pharmaceutical formulation.

7.8. CONCLUSIONS

Interest in the present work was created by tailoring stability indicating study. As the proposed method was developed at ambient temperature, it provides the easy accessibility for the routine determination of MET and EPI. The proposed method was validated as per ICH guidelines. Samples were found to be stable up to 24hr through solution stability studies. Results of linearity, precision, recovery studies and sensitivity recommended the appropriateness of the developed method. Degradation study of MET and EPI standard solution was conducted by the application of different stress inducing conditions like aqueous, acidic, basic, oxide, thermal, light and UV light. The proposed method received great attention due to its importance in quality control. Hence the reported method can be conveniently employed for the routine analysis of MET and EPI in the pharmaceutical dosage forms.

Work area for future research includes investigation of degradation products, mechanism of action degradation products, secondary degradation products and drugs in different biological samples.

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