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Combined RP-HPLC methodology for the determination of Clotrimazole, antioxidant and preservatives in Topical formulation

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Background: Clotrimazole is an antifungal medication that is used to treat skin infection such as athlete's foot, jock itch, ringworm and other fungal skin infection. This medication is also used to treat a skin condition known as pityriasis (*Tinea versicolor*), a fungal infection that causes a lightning or darkening of the skin of the neck, chest, arms, or legs. It is an azole antifungal that works by preventing the growth of fungus. **Aim:** To develop a combined HPLC methodology for the simultaneous determination of Clotrimazole, preservatives – Methyl Paraben (MP), Propyl Paraben (PP) and antioxidant – Butylated Hydroxyl Toluene (BHT) in topical dosage form. **Method:** A stability-indicating reverse phase-HPLC method has been developed and validated for the simultaneous determination of Clotrimazole in pharmaceutical dosage form. Clotrimazole was used as standard drug. The method was developed using a Thermo Scientific HPLC system (Ultimate 3000) with a Waters Symmetry C8 (4.6 × 150 mm I.D., 5 µm) and gradient elution consisting of buffer and acetonitrile as the mobile phase. The flow rate was adjusted to 1.0 ml/min. The column oven was set at 40 °C and the detection wavelength at 225 nm. The retention time of Clotrimazole was found to be 11.817 min. **Results:** The developed method was validated according to the ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the specified acceptance criteria. **Conclusion:** The proposed method was successfully applied to the topical dosage form for routine analysis.

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INTRODUCTION:

Clotrimazole (Fig 1) is a synthetic, imidazole derivative with broad-spectrum, antifungal activity. Its molecular weight is 344.8 g/mol with an empirical formula $C_{22}H_{17}ClN_2$. Clotrimazole is white or pale-yellow crystalline powder that is soluble in ethanol (96 %) and in methylene chloride, practically insoluble in water. Clotrimazole is commonly available without a prescription in various dosage forms, such as topical cream, ointment or vaginal suppository. It is used for

vulvovaginal candidiasis (yeast infection) or yeast infection of skin. Few HPLC methods were found for the determination of clotrimazole. Clotrimazole (CLOT) is chemically described as 1-[(2-chlorophenyl) diphenyl methyl]-1H-imidazole [1,2].

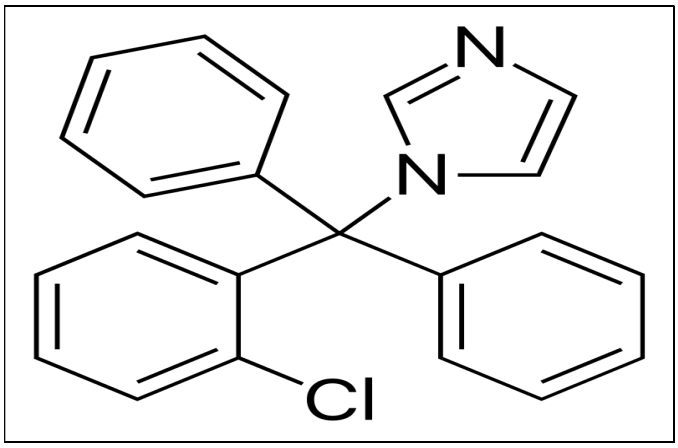

Fig 1. Chemical structure of Clotrimazole.

Methyl paraben (Fig 2) is 4-hydroxybenzoate ester resulting from the formal condensation of the carboxy group of 4-hydroxybenzoic acid with methanol. It is most frequently used as an antimicrobial preservative in cosmetics. It occurs naturally in several fruits, particularly in blueberries. It acts as a plant metabolite, an antimicrobial food preservative, a neuroprotective agent and an antifungal agent. Its molecular weight is 152.15 g/mol with the chemical formula $CH_3[C_6H_4(OH)COO]$. It is white crystalline powder, freely soluble in Alcohol and in methanol, slightly soluble in water [3,4].

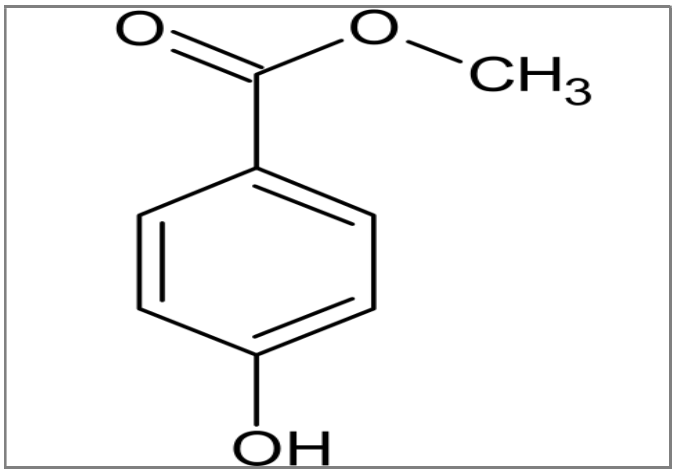

Fig 2. Chemical structure of Methyl Paraben.

Propylparaben (Fig 3), the *n*-propyl ester of p-hydroxybenzoic acid, occurs as a natural substance found in many plants and some insects, although it is manufactured synthetically for use in cosmetics, pharmaceuticals, and foods. It is a member of the class of parabens. It is a preservative typically found in many water-based cosmetics, such as creams, lotions, shampoos, and bath products. As a food additive, it has the E number - E216. Its molecular weight is 180.2 g/mol with the chemical formula $C_{10}H_{12}O_3$. It is white crystalline powder, freely soluble in water, sparingly solution in alcohol and practically insoluble in methylene chloride [3].

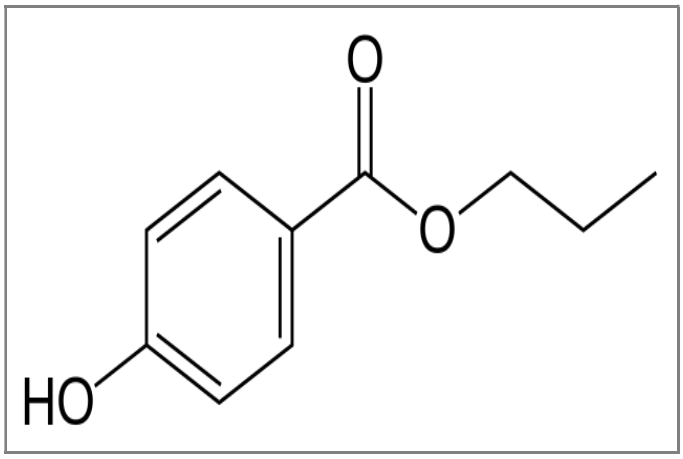

Fig 3. Chemical structure of Propyl Paraben.

Butylated hydroxytoluene (BHT) also known as dibutyl hydroxytoluene (Fig 4), is a lipophilic organic compound, chemically a derivative of phenol that is useful for its antioxidant properties. BHT is widely used to prevent free radical-mediated oxidation in fluids and other materials, and the regulation overseen by the U.S. FDA – which considers BHT as 'generally recognized safe' as a small amount to be added in food. At an earlier stage, National Cancer Institute determined that BHT was noncarcinogenic in an animal model, societal concern over its broad use have been expressed. Its molecular weight is 220.356 g/mol with empirical chemical formula $C_{15}H_{24}O$. It is white to yellow powder, slightly phenolic odor, soluble in ethanol and insoluble in water and propane-1 2-diol [5,6].

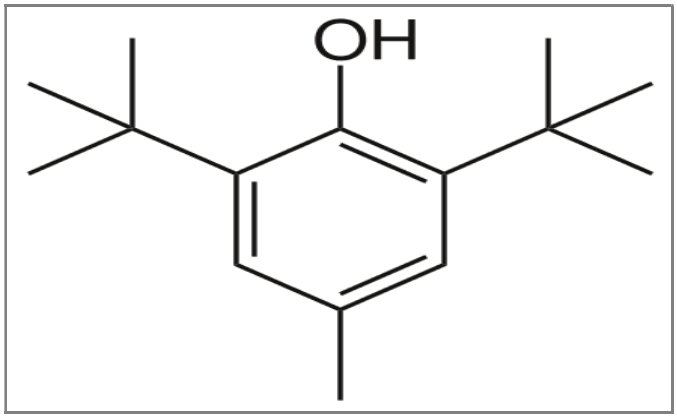

Fig 4. Chemical structure of BHT.

Few HPLC methods have been reported for the estimation of Clotrimazole. In the present work we are focused on to achieve the optimum chromatographic conditions for the simultaneous determination of Clotrimazole and preservative content (Methyl hydroxybenzoate, Propyl hydroxybenzoate), antioxidant (Butylated Hydroxytoluene) in the topical preparations. The developed method can be applied successfully to quality control and stability testing purposes. To access the reproducibility and wide applicability of the developed method, it was validated as per ICH guidelines [7,8].

MATERIALS AND METHODS:

Chemicals and reagents:

Clotrimazole, all preservatives and antioxidant's (Methyl hydroxybenzoate, propyl hydroxybenzoate, Butylated hydroxytoluene) working standards made available from Oman Pharmaceutical Products Co L.L.C. Topical formulation containing Clotrimazole cream 1 % w/w was taken from the commercial batch manufactured at Oman Pharmaceutical Products Co L.L.C. HPLC grade Acetonitrile and Methanol was procured from LobaChemie and Merck Ltd. All other chemical reagents were of analytical grade.

Buffer Solution Preparation:

Dissolved 1.0 g of Potassium dihydrogen orthophosphate and 0.5 g of tetrabutylammonium hydrogen sulphate into 1000 ml of mili q water. The mobile phase A and B were buffer (100 %) and acetonitrile (100 %) respectively (Table 1). Methanol was used as diluent.

Table 1. Gradient Program.

Time (min)	Mobile Phase A %	Mobile Phase B %
0	75	25
3	75	25
25	20	80
30	20	80
31	75	25
35	75	25

Clotrimazole standard stock solution (Solution-1):

Accurately weighed 25 mg of Clotrimazole working standard was taken in 25 ml volumetric flask. It was dissolved and diluted to volume with diluent to obtain a solution of strength 1000 µg/ml.

Methyl Hydroxybenzoate standard stock solution (Solution-2):

Accurately weighed 25 mg of Methyl hydroxybenzoate working standard was taken in 25 ml volumetric flask. It was dissolved and diluted to obtain a solution of strength 1000 µg/ml.

Propyl Hydroxybenzoate standard stock solution (Solution-3):

Accurately weighed 25 mg of Propyl hydroxybenzoate working standard was taken in 250 ml volumetric flask. It was dissolved and diluted to obtain a solution of strength 1000 µg/ml.

Butylated hydroxytoluene standard stock solution (Solution-4):

Accurately weighed 25 mg of Butylated hydroxy toluene working standard was taken in 25 ml volumetric flask. It was dissolved and diluted to obtain a solution of strength 1000 µg/ml.

Standard for Assay (Solution-5):

About 5 ml of solution-1, 10 ml solution-2, solution-3 and 5 ml of solution-4 was transferred into a 100 ml volumetric flask and diluted up to the mark with diluent. The solutions gave following strength that are Clotrimazole 50 µg/ml, Methyl hydroxybenzoate 100 µg/ml, Propyl hydroxybenzoate 10 µg/ml and Butylated hydroxytoluene 50 µg/ml.

Sample Stock preparation for related substance (Solution-6):

About 2.5 g (equivalent to 25 mg of Clotrimazole, 0.5 mg of Methyl hydroxybenzoate, 0.5 mg of Propyl hydroxybenzoate and 2.5 mg of Butylated hydroxytoluene) of sample was weighed accurately and transferred into 50 ml centrifuge tube. About 20 ml of methanol was added and the centrifuge tube was kept in a water bath for 5 min at 50 °C and the solution was cooled on ice bath for 15 min, centrifuged for 5 min at 5000 rpm. Next, the supernatant liquid was transferred carefully into a 50 ml volumetric flask. The extraction procedure was repeated again with 20 ml methanol. Finally, the combined extract was diluted with methanol to produce 50 ml. The resultant solution was filtered with 0.45 µ Nylon filter after discarding 5 ml of filtrate. Finally solutions strength were Clotrimazole 500 µg/ml, Methyl hydroxybenzoate 100 µg/ml, Propyl hydroxybenzoate 10 µg/ml and Butylated hydroxytoluene 50 µg/ml [9].

Sample Preparation for Assay (For Clotrimazole):
About 5 ml of Sample Stock solution (Solution-6) was Pipetted into 50 ml volumetric flask and diluted to volume with diluent which gave a solution of strength of Clotrimazole 50 µg/ml.

Chromatographic study:
The clotrimazole, its preservative and antioxidant content in all solutions were determined by HPLC by using the chromatographic conditions as mentioned in Table 2 [10,11].
The Chromatographic data were analysed and Specificity, Linearity and range, Robustness, precision and accuracy were determined [12,13].

Table 2. Chromatographic condition for analytical study.

Parameter	Specification
Instrument	HPLC
Column	Symmetry C8 (150 × 4.6) mm, 5µ
Flow Rate	1.0 ml/min
Detection wavelength	225 nm
Injection volume	10 µL
Column oven	40 °C
Run Time	35 min
Elution	Gradient

RESULTS AND DISCUSSION:
The developed method for determination of Clotrimazole, its preservative and antioxidant were validated by using the following parameters:

System suitability (Assay, Preservative and Antioxidant):
For establishing the system suitability, the procedure described in the methodology was followed before starting the analysis. System suitability data has been presented in Table 3 to 6.

Table 3. System suitability – Clotrimazole (Assay).

Injection #	Area	Tailing Factor	Plate count
1	1316.1014	1.05	82518
2	1311.0871	1.06	83635
3	1349.3046	1.03	83166
4	1308.1118	1.05	83665
5	1340.5760	1.05	83527
6	1309.0827	1.03	83214
Mean	1322.3773	1.05	83288
SD	17.91	-	-
% RSD	1.4	-	-

Table 4. System suitability – Methyl Hydroxybenzoate (Assay).

Injection #	Area	Tailing Factor	Plate count
1	810.4438	0.97	13759
2	807.9650	0.97	13835
3	829.8211	1.00	15077
4	807.8621	0.99	13808
5	826.3224	1.00	15113
6	807.3674	0.98	13975
Mean	814.9636	0.99	14261
SD	10.27	-	-
% RSD	1.3	-	-

Table 5. System suitability – Propyl Hydroxybenzoate (Assay).

Injection #	Area	Tailing Factor	Plate count
1	82.2734	1.04	60182
2	80.4338	1.05	60650
3	83.5294	1.05	62148
4	81.7100	1.05	61013
5	82.8689	1.04	63106
6	81.0528	1.05	61378
Mean	81.9781	1.05	61413
SD	1.15	-	-
% RSD	1.4	-	-

Table 6. System suitability – Butylated Hydroxytoluene (Assay).

Injection #	Area	Tailing factor	Plate count
1	725.1715	0.96	293430
2	722.7643	0.95	292798
3	743.2518	0.95	294227
4	722.1102	0.95	295364
5	739.2321	0.96	294693
6	721.1675	0.96	294622
Mean	728.9496	0.95	294189
SD	9.70	-	-
% RSD	1.3	-	-

Specificity (Assay, Preservative and Antioxidant):
There were no interfering peaks at the retention time of Clotrimazole and its preservative and antioxidant peak in the presence of excipients. Further, to demonstrate the specificity of the method, the sample had been subjected to acid, base, oxidation, thermal and photolytic degradation. This was evaluated by using a Photo Diode Array detector (PDA). The data are presented in Fig 9 to 16 for the chromatograms and Table 7 for the peak purity analysis data.

Table 7. Force Degradation study summary of Clotrimazole.

Sample Name	Final DC	PPC (Clotrimazole)	PPC (MP)	PPC (PP)	PPC (BHT)	% Dg (Clotrimazole)
AD	5 ml 0.1N HCl @100 °C for 15 min	1000	1000	1000	1000	25.2
AA	5 ml 1N NaOH @100°C for 15 min	1000	1000	1000	1000	16.4
OD	5 ml 35 % H_2O_2 @100°C for 15 min	1000	1000	1000	1000	28.2
TD	100 °C for 24 h	1000	1000	1000	1000	4.3
PD	1.2 million lux h	1000	1000	998	1000	-

AD- Acid Degradation, AA- Alkali Degradation, OD- Oxidative Degradation, TD- Thermal Degradation, PD- Photolytic Degradation, DC- Degradation Condition, PPC- Peak Purity of Clotrimazole, MP- Methyl paraben, PP – Propyl paraben, BHT – Butylated Hydroxy Toluene and Dg- Degradation.

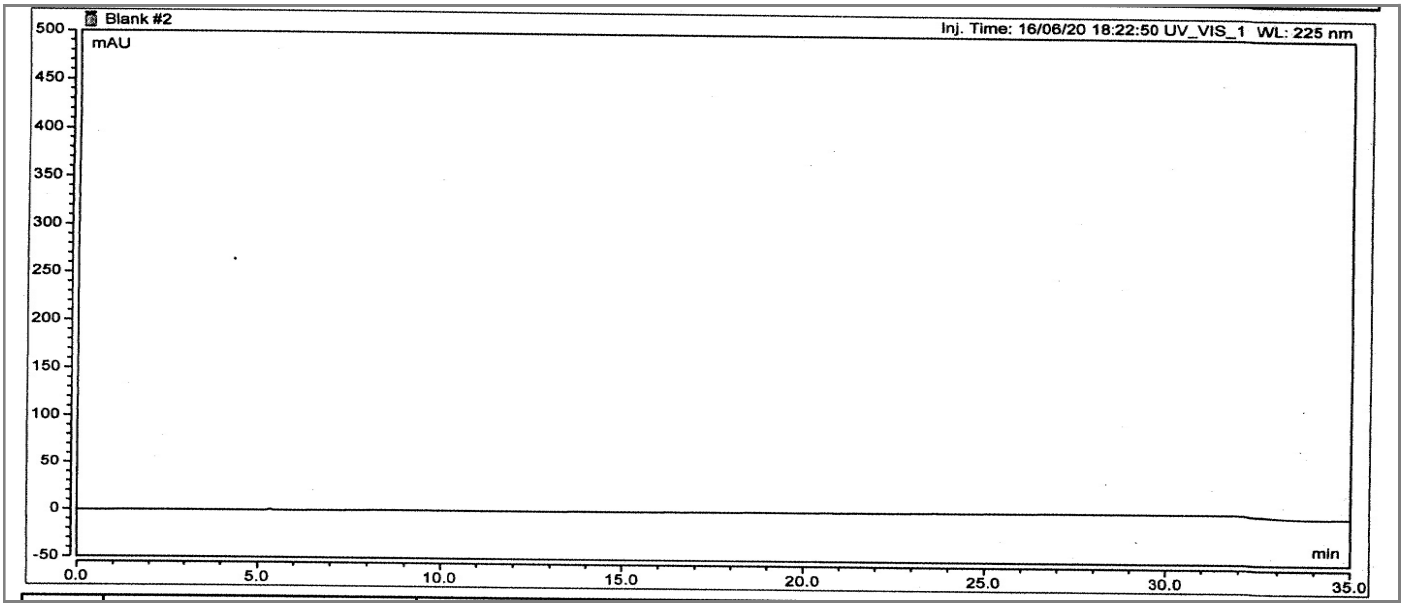

Fig 9. Reference chromatogram of Blank.

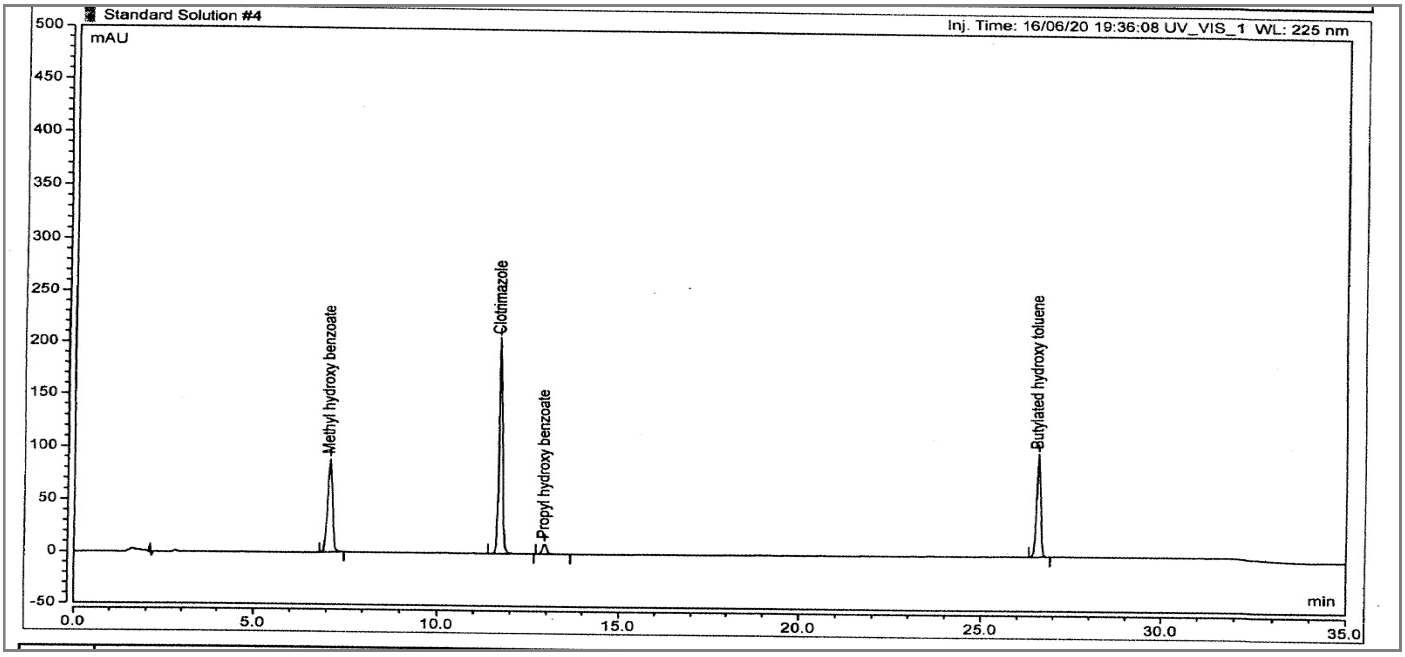

Fig 10. Reference chromatogram of Standard Solution (Clotrimazole, Preservative and Antioxidant @ 225 nm).

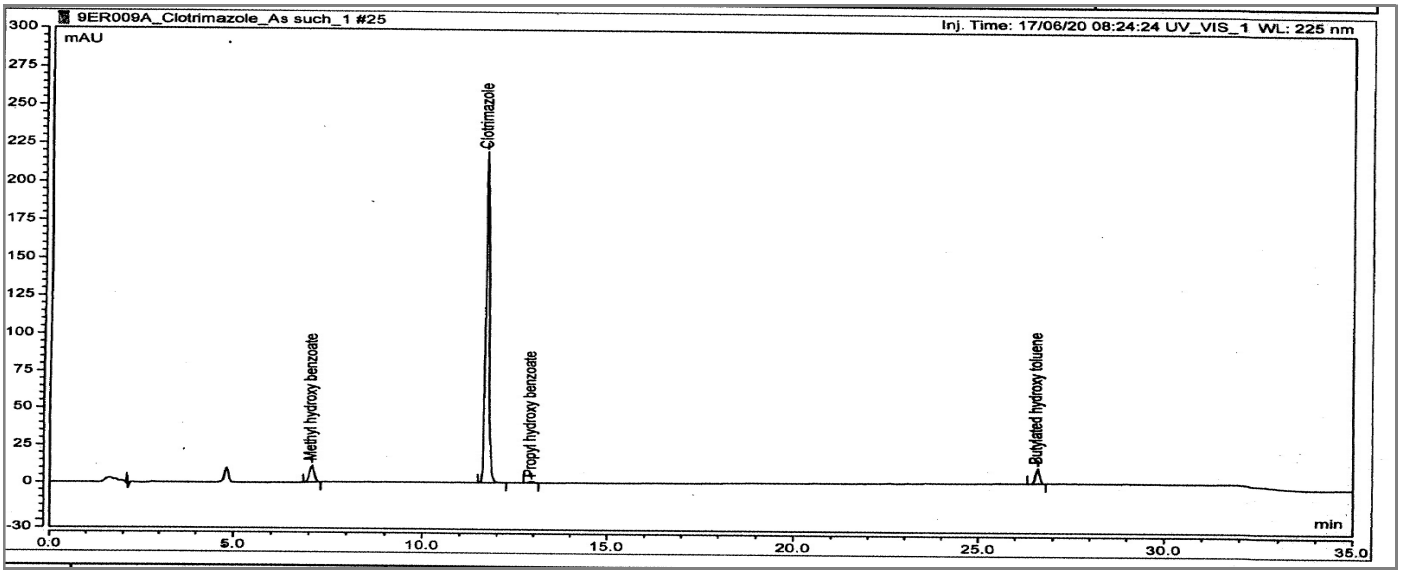

Fig 11. Reference chromatogram of as such sample (Assay – Clotrimazole, Preservative and Antioxidant @ 225 nm).

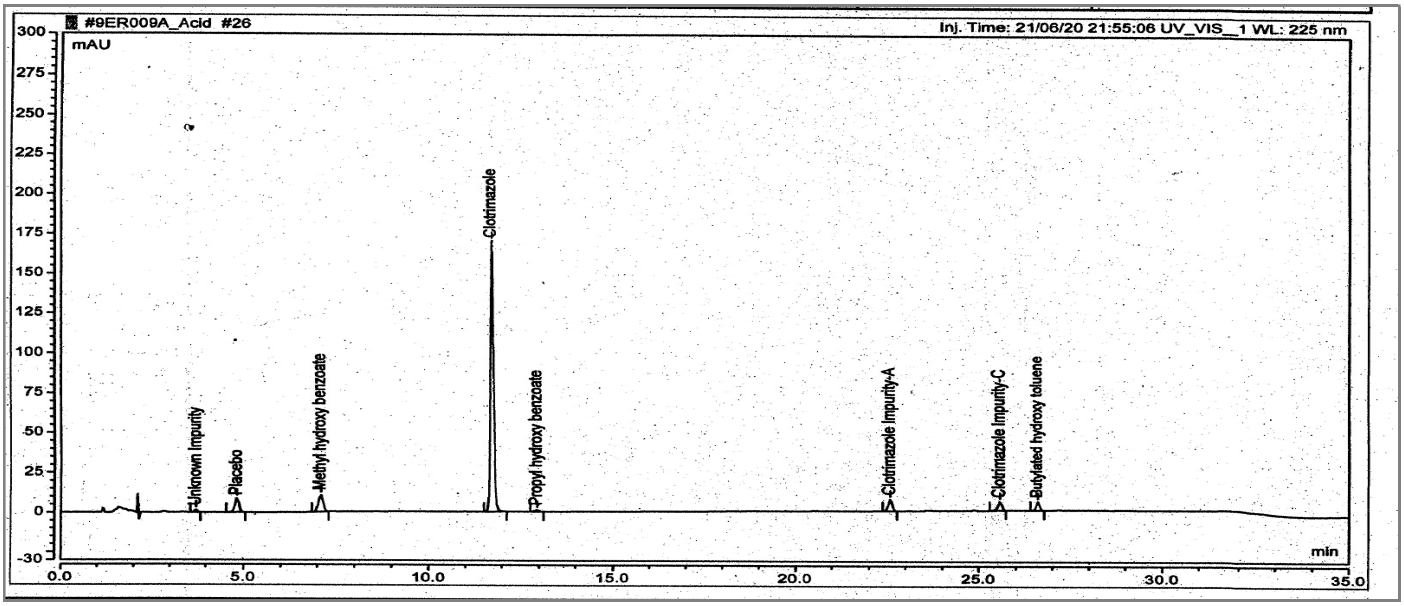

Fig 12. Reference chromatogram of Acid degradation (Assay - Clotrimazole).

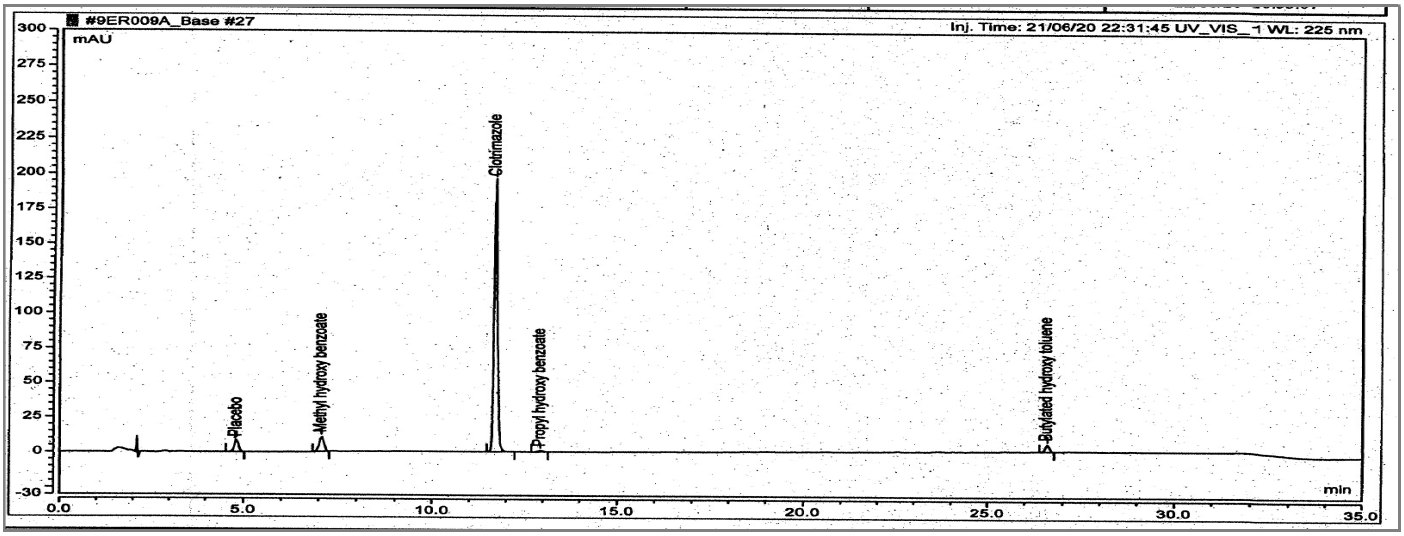

Fig 13. Reference chromatogram of Base degradation (Assay - Clotrimazole).

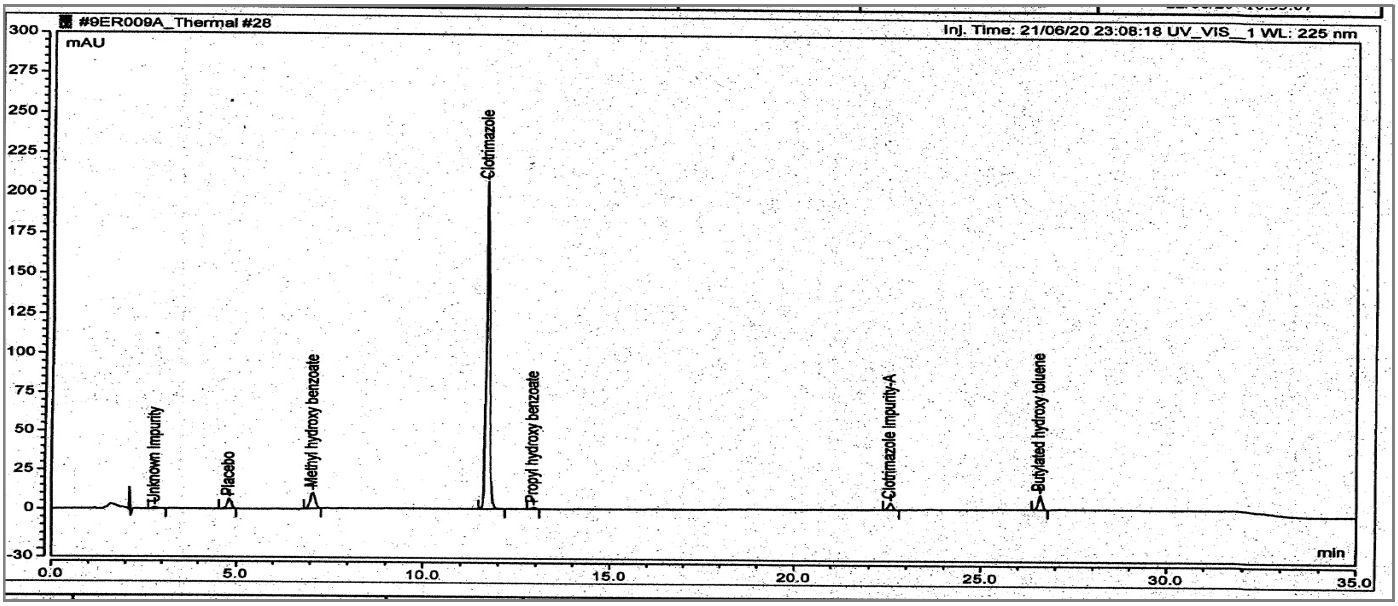

Fig 14. Reference chromatogram of Thermal degradation (Assay - Clotrimazole).

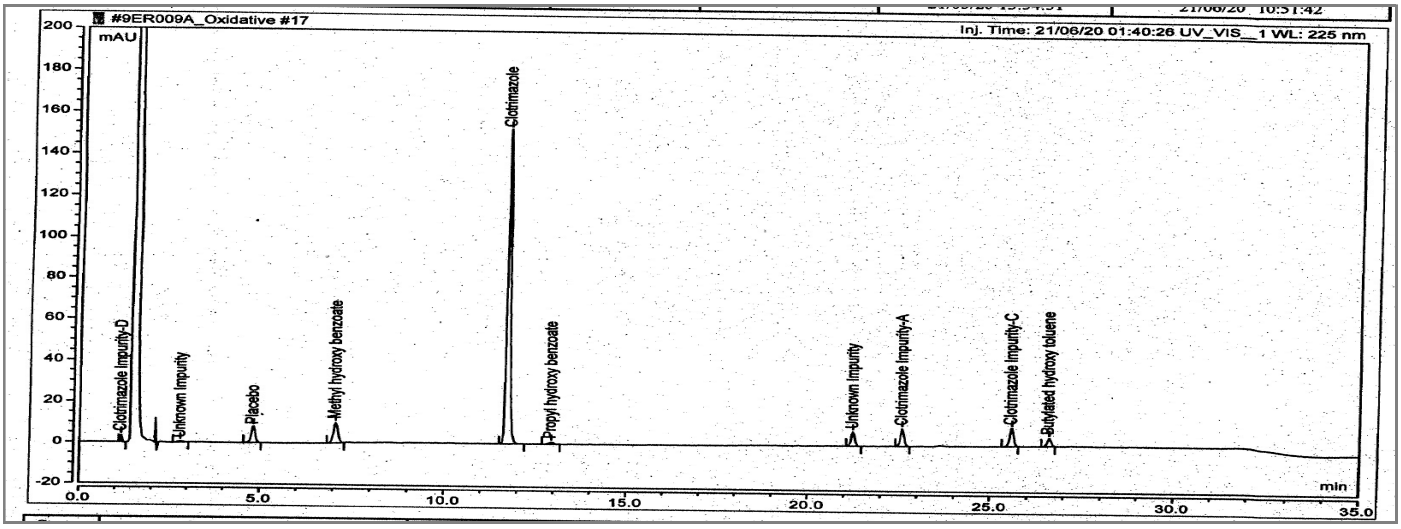

Fig 15. Reference chromatogram of Oxidative degradation (Assay– Clotrimazole).

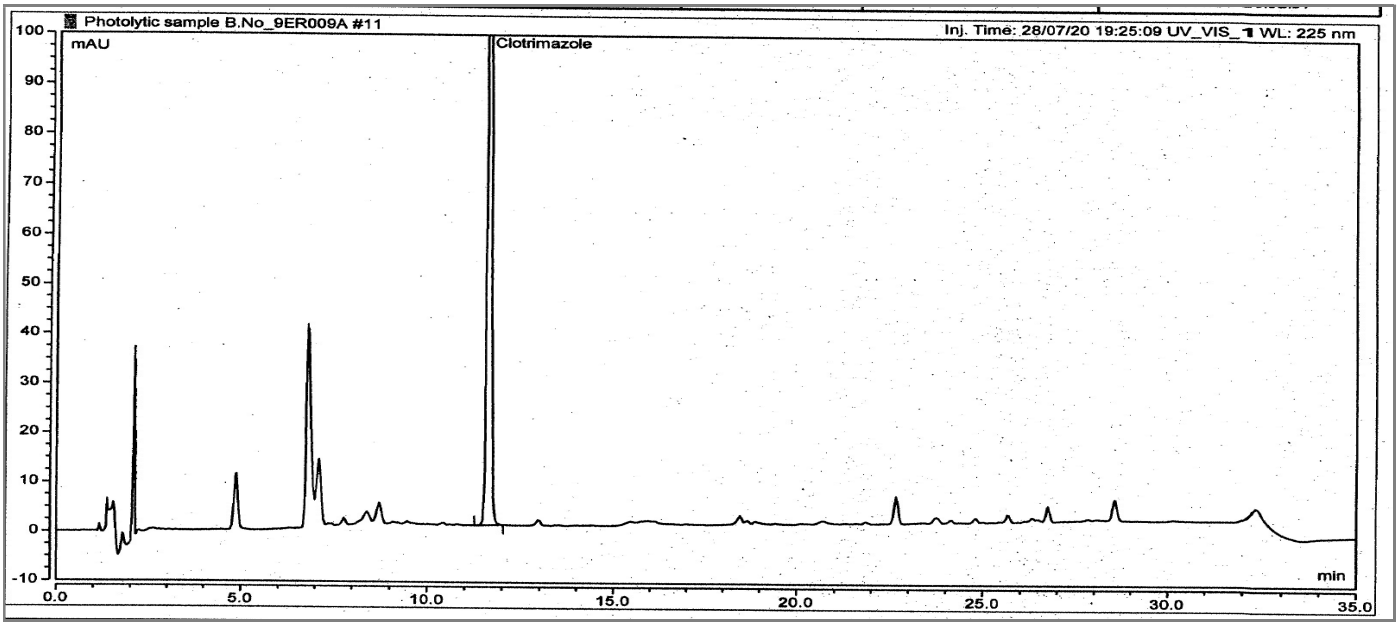

Fig 16. Reference chromatogram of Photolytic degradation (Assay – Clotrimazole).

Linearity and range (Assay, Preservative and Antioxidant):

Standard solutions containing Clotrimazole and its preservative (Methyl hydroxybenzoate, Propyl Hydroxybenzoate) with antioxidant (Butylated hydroxytoluene) were prepared. Linearity was determined by six different concentrations (50, 80, 90, 100, 110, 120 and 150 % respectively of the target concentration for Assay). The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate coefficient of correlation, slope and intercept. In general, a value of correlation coefficient (r) > 0.999 is considered as the evidence of an acceptable fit for the data to the regression line.

The results obtained are presented in the Table 8 to 11, which demonstrates that the current method was linear for the three analytes in the range specified above with a correlation coefficient better than 0.999. The plots have been represented in Fig 5 to 8.

Table 8. Linearity of Clotrimazole (Assay).

Level	Conc. (µg/ml)	Area
1	25.160	686.4328
2	35.224	950.6871
3	40.256	1079.0310
4	45.288	1229.2264
5	50.320	1334.1624
6	61.390	1609.0696
7	75.480	2029.9623
Correlation coefficient (r)		0.9993
Regression coefficient (r^2)		0.9986
Slope		26.3666
Intercept		19.3405

Table 9. Linearity of Methyl hydroxy benzoate (Assay).

Level	Conc. (µg/ml)	Area
1	50.080	391.0930
2	70.112	544.6187
3	80.128	636.6697
4	90.144	706.0294
5	100.160	770.0501
6	122.195	928.3115
7	150.240	1173.2866
Correlation coefficient (r)		0.9991
Regression coefficient (r^2)		0.9982
Slope		7.6786
Intercept		8.3816

Table 10. Linearity of Propyl hydroxy benzoate.

Level	Conc. (µg/ml)	Area
1	5.000	39.1353
2	7.000	54.6154
3	8.000	62.2455
4	9.000	71.3101
5	10.000	77.4693
6	12.200	93.8913
7	15.000	118.8291
Correlation coefficient (r)		0.9993
Regression coefficient (r^2)		0.9986
Slope		7.8816
Intercept		-0.6092

Table 11. Linearity of Butylated hydroxy toluene.

Level	Conc. (µg/ml)	Area
1	25.240	381.0181
2	35.336	528.6868
3	40.384	600.7052
4	45.432	684.3215
5	50.480	743.6569
6	61.586	898.0238
7	75.720	1133.7753
Correlation coefficient (r)		0.9993
Regression coefficient (r^2)		0.9986
Slope		14.7312
Intercept		6.7654

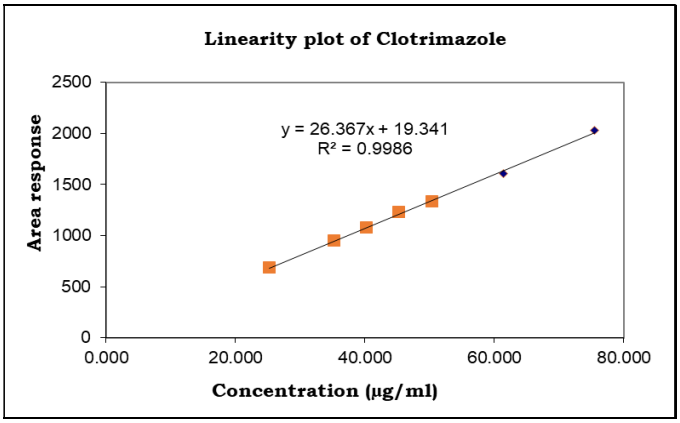

Fig 5. Linearity Plot of Clotrimazole – Assay.

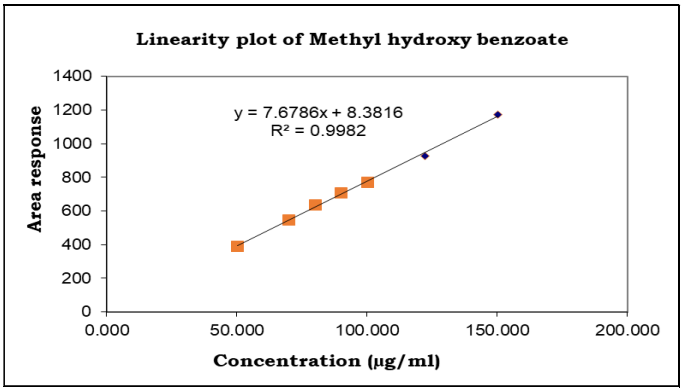

Fig 6. Linearity Plot of Methyl hydroxy benzoate – Assay.

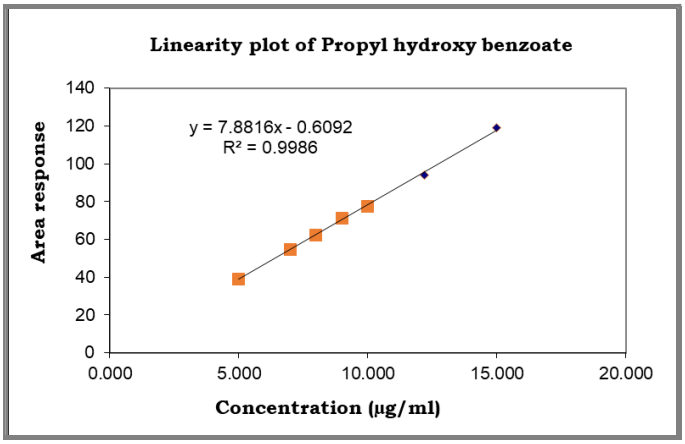

Fig 7. Linearity Plot of Propyl hydroxy benzoate.

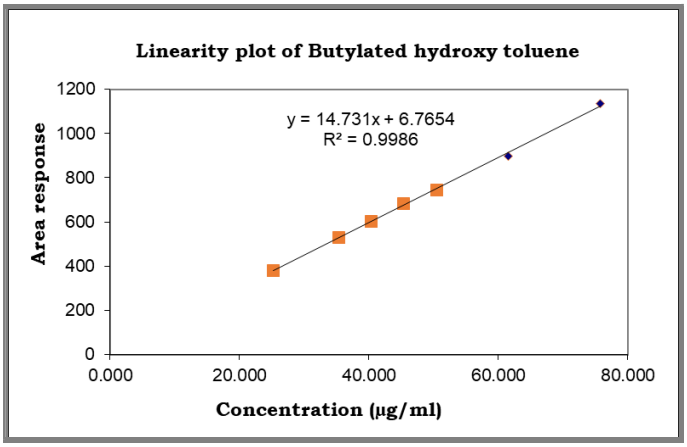

Fig 8. Linearity Plot of Butylated hydroxy toluene.

Table 12. Percentage Assay result of Method Precision and Intermediate Precision.

Sample ID #	Opizole Cream (B. No. 9ER09A)			
	Clotrimazole		Methyl hydroxy benzoate	
	MP	IP	MP	IP
1	102.1	99.9	103.5	103.8
2	100.8	99.0	103.0	103.1
3	100.3	100.3	103.1	104.3
4	99.7	100.3	102.3	105.1
5	99.4	100.6	102.0	104.5
6	100.5	100.8	103.3	102.7
Mean	100.5	100.2	102.9	103.9
SD	1.0	0.6	0.6	0.9
% RSD	1.0	0.6	0.6	0.9
% Diff	0.3		1.0	

Diff – Difference, MP – Method Precision, IP-Intermediate Precision.

Precision (Assay, Preservative and Antioxidant):

For Assay, precision was determined by preparing the standard and sample as per the methodology. The sample was prepared in six replicates and injected into the chromatograph. The % Assay value of each preparation was calculated and finally the % RSD of the six replicate preparations was deduced. The data obtained for six replicate standard injections and the six sample preparations have been presented in Table 12 and 13.

Table 13. Percentage Assay result of Method Precision and Intermediate Precision.

Sample ID #	Opizole Cream (B. No. 9ER09A)			
	Propyl hydroxy benzoate		Butylated Hydroxy Toluene	
	MP	IP	MP	IP
1	103.0	99.6	97.4	96.0
2	103.1	100.0	97.0	95.5
3	102.0	101.4	97.2	96.5
4	101.5	100.4	96.4	97.3
5	101.5	101.2	96.2	96.7
6	100.7	98.7	97.4	95.0
Mean	102.0	100.2	96.9	96.2
SD	0.9	1.0	0.5	0.8
% RSD	0.9	1.0	0.5	0.9
% Diff	1.8		0.7	

Diff – Difference, MP – Method Precision, IP-Intermediate Precision.

Ruggedness (Assay, Preservative and Antioxidant):

Ruggedness of method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot and using a different HPLC system. The sample was prepared in six replicates and injected into the chromatograph.

The % Assay value of each preparation was calculated and finally the % RSD of the six replicate preparations was deduced. The data obtained for six replicate standard injections and the six sample preparations have been presented in Table 12 and 13.

Accuracy (Assay, Preservative and Antioxidant):

For Assay, the accuracy of the proposed method had been demonstrated by the recovery study performed by the standard addition method at levels 50, 100 and 150 % of the target concentration. The data obtained had been presented in Table 14 to 17.

CONCLUSION:

This intended study concludes that the proposed method is economical, simple, sensitive and reliable. Also, it is found to be accurate, precise, specific, stability indicating and rugged.

Hence, it can be employed for the routine estimation of Clotrimazole, anti-oxidant (BHT) and preservatives (methyl paraben and propyl paraben) in Clotrimazole Cream topical dosage form.

Table 14. Accuracy of Clotrimazole (Assay).

Level	Mean Area	Added value (µg/ml)	Found value (µg /ml)	% Recovery	% Mean Recovery	% RSD
50 %	682.5243	24.990	25.400	101.6	101.8	0.2
	685.2150	24.990	25.501	102.0		
	682.9736	24.990	25.417	101.7		
100 %	1340.0056	49.980	49.869	99.8	99.8	0.0
	1339.7396	49.980	49.859	99.8		
	1339.5014	49.980	49.850	99.7		
150 %	2039.2697	74.969	75.892	101.2	101.1	0.2
	2036.8379	74.969	75.802	101.1		
	2032.3419	74.969	75.634	100.9		

Table 15. Accuracy of Methyl hydroxy benzoate.

Level	Mean Area	Added value (µg/ml)	Found value (µg /ml)	% Recovery	% Mean Recovery	% RSD
50 %	401.2186	43.770	44.568	101.8	101.8	0.2
	400.7054	43.770	44.511	101.7		
	400.2757	43.770	44.463	101.6		
100 %	779.2771	87.540	86.563	98.9	99.8	0.0
	780.7101	87.540	86.722	99.1		
	780.2644	87.540	86.672	99.0		
150 %	1190.4486	131.309	132.236	100.7	101.1	0.2
	1183.1541	131.309	131.426	100.1		
	1182.6397	131.309	131.368	100.0		

Table 16. Accuracy of Butylated hydroxy toluene.

Level	Mean Area	Added value (µg/ml)	Found value (µg /ml)	% Recovery	% Mean Recovery	% RSD
50 %	377.9837	25.144	25.372	100.9	100.8	0.1
	377.5184	25.144	25.341	100.8		
	377.2996	25.144	25.326	100.7		
100 %	747.9469	50.289	50.206	99.8	99.8	0.1
	748.4907	50.289	50.242	99.9		
	747.1494	50.289	50.152	99.7		
150 %	1137.5327	75.433	76.356	101.2	100.7	0.4
	1130.3239	75.433	75.872	100.6		
	1127.8239	75.433	75.705	100.4		

Table 17. Accuracy of Propyl hydroxy benzoate.

Level	Mean Area	Added value (µg/ml)	Found value (µg /ml)	% Recovery	% Mean Recovery	% RSD
50 %	38.9314	5.029	5.045	100.3	100.0	0.3
	38.7139	5.029	5.017	99.8		
	38.7706	5.029	5.025	99.9		
100 %	76.9963	10.058	9.978	99.2	99.2	0.1
	76.9486	10.058	9.972	99.2		
	76.9060	10.058	9.967	99.1		
150 %	117.6035	15.087	15.241	101.0	100.5	0.4
	116.7594	15.087	15.132	100.3		
	116.7719	15.087	15.133	100.3		

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