VALIDATED STABILITY INDICATED RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METHYL SALICYLATE, CAMPHOR AND MENTHOL IN CREAM PREPARATIONS

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Abstract

A method for the determination of three active products in pharmaceutical preparation Rheumatizen topical cream was described. The method was based on isocratic elution of Methyl Salicylate, Camphor and Menthol on reversed phase column C_8 Thermo hyperseal 250mm x 4.6mm 5µm - using a mobile phase consisting of mixture of 45% Acetonitrile: 55% (Water+0.2% Triethyl amine adjusted to pH 5 with acetic acid) at a flow rate of 1 ml/min. Quantization was achieved with Refractive index (RI) detector. Linearity of Methyl Salicylate, Camphor and Menthol was found to be from (2.4 mg/ml to 9.6 mg/ml), (0.32 mg/ml to 1.28 mg/ml) and (0.8 mg/ml to 3.2 mg/ml), with variation coefficient 0.9998, 0.997 and 0.998 respectively. The stress testing was carried out by using the solutions that had been treated with [2M NaOH, 2M HCl and 30%H_2O_2] and all of these solutions were leaved for 22 hours at room temperature in tightly closed containers without further heating to avoid the loss via evaporation of the analyets due to their volatility character.

Keywords: Methyl salicylate, Camphor,Menthol., analgesic, anti inflammatory., Reversed phase, refractive index., HPLC, drug analysis

Introduction

Both menthol (MN) and methyl salicylate (MS) are active substances in many medicines commonly used in treatment of rheumatic diseases due its analgesic and anti-inflammatory characteristics[1]. It is difficult to determine these substances in the same preparation due to their similar physical and chemical properties such as volatility and solubility. Another difficulty is a large disproportion (the ratio MS: MN is 8:1 in the medicine under examination). Thus, separation methods are recommended in analysis of these constituents. Among these methods gas chromatography (GC) was used. The gas chromatography methods were used for determining menthol and methyl salicylate in solid and liquid medicines [(2-5)], natural products [(6,7)] and biological material [(8,9)]. Methyl salicylate (MS) is a salicylic acid derivative that is irritant to the skin and is used topically in rubefacient preparations for the relief of pain in musculoskeletal, joint, and soft-tissue disorders. It is also used for minor peripheral vascular disorders such as chilblains and as an ingredient in inhalations for the symptomatic relief of upper respiratory-tract disorders[10]. Camphor (CM) acts as a rubefacient and mild analgesic and is used in liniments as a counter-

irritant in fibrositis, neuralgia, and similar conditions. It is also an ingredient of many inhaled nasal decongestant preparations but it is of doubtful efficacy. Menthol (MN) is chiefly used to relieve symptoms of bronchitis, sinusitis, and similar conditions. For this purpose it may be used as an inhalation, usually with benzoin or eucalyptus oil, as pastilles, or as an ointment with camphor and eucalyptus oil for application to the chest or nostrils (but see Adverse Effects. However, as mentioned under the section on the management of cough, the use of menthol in inhalations is unlikely to provide any additional benefit. When applied to the skin menthol dilates the blood vessels, causing a sensation of coldness followed by an analgesic effect. It relieves itching and is used in creams, lotions, or ointments in pruritus and urticaria. It has also been used to the forehead, presumably as a counter-irritant, for the relief of headache. [10, 11]

Experimental

Instrumentation

HPLC (Agilent instrument 1200 series, Germany) system equipped with vacuum degasser, mixer, autosampler, gradient quaternary pump and refractive index detector (RI). Separation and quantitation were made on reversed phase C_8 Thermo hyperseal 250mm x 4.6mm 5µm. an ultra sonic (Elma, Germany) and four digit analytical balance (AND HR 200, japan) was used.

Reagents and chemicals

Reference standards Methyl Salicylate, Camphor and Menthol were supplied from EDQM as British Pharmacopeia reference standards. Methanol HPLC grade, Triethylamine AG, Acetic acid AG and Acetonitrile were supplied from Sigma-Aldrich. Germany. The product used was Rheumatizen topical cream, the product of Medizen pharmaceutical industries.

Method development

During the optimization cycle, several chromatographic conditions were attempted using reversed C_8 with two dimensions 150 mm and 250 mm length but the 250 mm was found to be more effective in resolution. Various mobile phase compositions like methanol with water were firstly used and the separation was found to be unsatisfactory due to the beak brooding. Then Acetonitrile with buffer (phosphate) in different proportions were tried in an isocratic and gradient modes. But the results was unsatisfactory since in many cases it was interfere between the

beaks of MN and CM. Triethylamine was used to enhance the resolution between MN and CM and the results was found to be good but the symmetry of the peak was not good. In order to enhance the symmetry of the peaks we replace the buffer solution with water and adjust the pH of the system with Acetic acid that acts as separation modifier. The most suitable detection technique was found to be refractive index detector with positive polarity and maintain at temperature of 25°C.

Chromatographic conditions

An HPLC method is described[12] for determination of Methyl Salicylate, Camphor and Menthol. The method is based on the separation of Methyl Salicylate, Camphor and Menthol using an Octylsilyl C_8 stationary phase, (4.6 x 250 mm), 5µm column and mobile phase composed of a filtered and degassed mixture of (Water + 0.2ml Triethylamine to pH 5.0 with acetic acid) and Acetonitrile (55:45%) V/V flowing at 1 ml / min, and Refractive Index detection with positive polarity at 25°C.

Stock standard solution

Weigh 3g of Methyl Salicylate, 0.4g of Camphor and 1g of Menthol in 50ml volumetric flask, dissolve, then complete to the mark with Methanol.

Sample preparation

Mix the content of 10 tubes of the cream (about 300g) into 1000ml beaker and mix well by the aid of a glass rod. Weigh 1 gram of the cream into 50ml volumetric flask. Add 25 ml methanol, shake well until you disperse the cream base and sonicate for 20minutes, leave at room temperature for 20 minutes to cool if you need, then complete to the mark with methanol and filter on whatmann filter paper No.1, then on micropore syringe filter 0.45µm. the expected concentration of the sample solution is 6mg/ml of MS, 0.8mg/ml of CM and 2mg/ml of MN.

Procedure

Calibration curve of MS, MN and CM

Accurately measured aliquots of stock standard solutions were transferred into 50 ml volumetric flask to make a serial dilutions of Methyl Salicylate, Camphor and Menthol from (2.4 mg/ml to 9.6 mg/ml), (0.32 mg/ml to 1.28 mg/ml) and (0.8 mg/ml to 3.2 mg/ml) respectively. The solutions were completed to volume with Methanol. A volume of 10 µL of each solution was injected in duplicates into the chromatograph. A calibration curve was obtained by plotting area under the peak (Area) against concentration (C). See fig. $2_{a\text{-}c}$

System suitability

System suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method [13]. It is achieved by 6 injections of a solution which have a concentration of 6.0 mg/ml for MS, 0.8 mg/ml of CM and 2 mg/ml of MN (represents the concentration of the assay method 100%).

Selectivity of the method

The Selectivity of the method has been confirmed by investigating the chromatograms of the solutions of standard mixture of (MS, CM and MN), the dissolved inactive materials, Cream test solution.

Method range

The concentration range for the application of the HPLC method is investigated by application of the method for the determination of the percent of Methyl Salicylate, Camphor and Menthol in samples with three different concentrations. The concentration ranged between 80% and 120% of method concentration including the test method concentration (100%)

Accuracy

The accuracy of the method for the determination of Methyl salicylate, camphor and menthol in Rheumatizen Topical Cream is performed by the addition of Methyl salicylate, camphor and menthol reference materials to 3 levels of solutions containing the inactive materials.

a) Level I: 3 set of solution of inactive materials, MS, MN and CM reference materials have final concentration about 80% of the test method concentration.

b) Level II: 3 set of solution of inactive materials, MS, MN and CM reference materials have final concentration about 100% of the test method concentration.

c) Level III: 3 set of solution of inactive materials, MS, MN and CM reference materials have final concentration about 120% of the test method concentration.

Method robustness

The effect of a slight variation in the column temperature or the flow rate of the mobile phase on the retention time of the peaks corresponding to Methyl Salicylate, Camphor and Menthol was performed.

Results and discussions

Selectivity

i) The chromatogram obtained from Methyl Salicylate, Camphor and Menthol reference material shows peaks at 9.818, 11.045 & 14.575 mins respectively ± 0.025 min. see figure 1a.

ii) The chromatogram obtained from the test solution shows peaks at 9.796, 11.019 & 14.542 mins ± 0.025 min., which is nearly identical to that of Methyl Salicylate, Camphor and Menthol reference material respectively. See figure 1b

iii) The chromatogram obtained from the inactive materials, solvent or mobile phase shows no peaks at 9.818, 11.045 & 14.575 and this was considered as a proof that the inactive materials do not interfere with the determination of Methyl Salicylate, Camphor and Menthol in the product. See figures 1c and 1d.

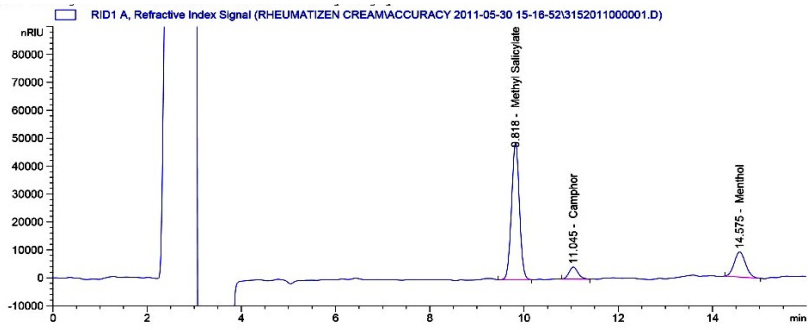


***Figure 1a: chromatogram of reference standard mixture of MS, CM and MN**

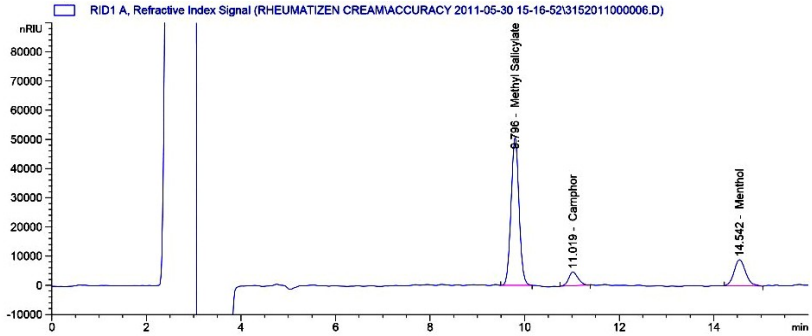


***Figure 1b: chromatogram of Rheumatezin topical cream test solution**

*Figure 1d: chromatogram represents the solvent (Methanol)

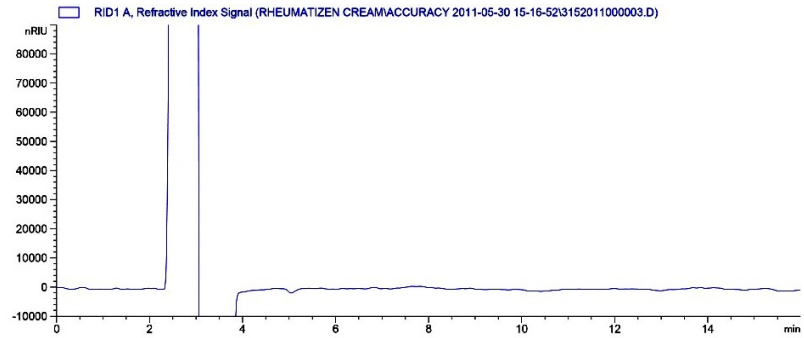


Figure 1c: chromatogram represents the solution of inactive materials *of Rheumatezin topical cream

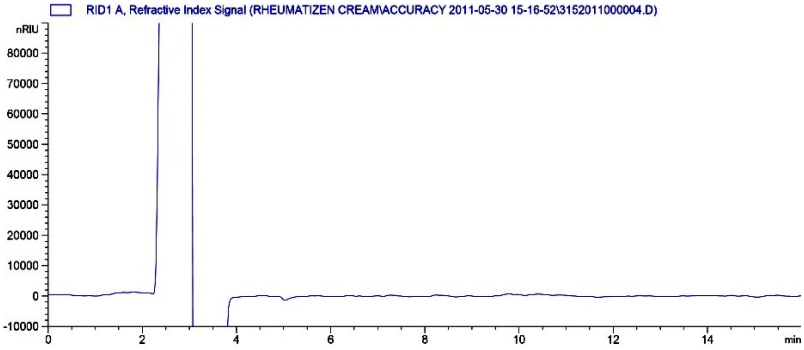


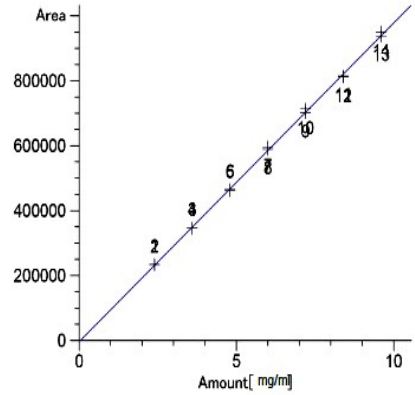
***Figure 1d: chromatogram represents the solvent (Methanol)**

Linearity of the method

In this study, seven concentrations were chosen for each analyet. Each concentration was analyzed two times. Good linearity of the calibration curve was verified by the high correlation coefficient. The Linearity of Methyl Salicylate, Camphor and Menthol was found to be from (2.4 mg/ml to 9.6 mg/ml), (0.32 mg/ml to 1.28 mg/ml) and (0.8 mg/ml to 3.2 mg/ml), with coefficient of variation of 0.9998, 0.997 and 0.998 respectively. For calibration data see table 1 and for calibration curves see figures 2a, 2b and 2c

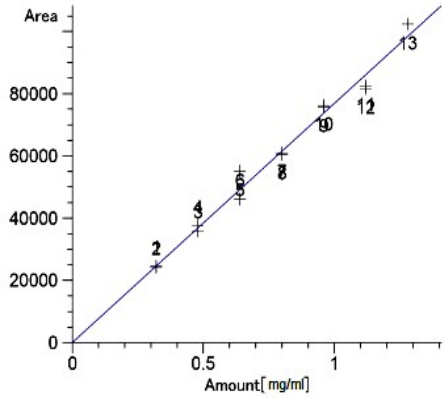
***Table 1: Linearity data of MS, CM and MN**

RetTime [min]	Lvl Sig		Amount [mg/ml]	Area	Amt/Area	Ref Grp	Name
9.804	1	1	2.40000	2.33865e5	1.02623e-5		Methyl Salicylate
		2	2.40000	2.33014e5	1.02998e-5		
		3	3.60000	3.44694e5	1.04441e-5		
		4	3.60000	3.45684e5	1.04141e-5		
		5	4.80000	4.61739e5	1.03955e-5		
		6	4.80000	4.64890e5	1.03250e-5		
		7	6.00000	5.96070e5	1.00659e-5		
		8	6.00000	5.88575e5	1.01941e-5		
		9	7.20000	7.01506e5	1.02636e-5		
		10	7.20000	7.12875e5	1.01000e-5		
		11	8.40000	8.14820e5	1.03090e-5		
		12	8.40000	8.11610e5	1.03498e-5		
		13	9.60000	9.37794e5	1.02368e-5		
		14	9.60000	9.50121e5	1.01040e-5		
11.003	1	1	3.20000e-1	2.45878e4	1.30146e-5		Camphor
		2	3.20000e-1	2.41794e4	1.32344e-5		
		3	4.80000e-1	3.58565e4	1.33867e-5		
		4	4.80000e-1	3.74568e4	1.28148e-5		
		5	6.40000e-1	5.51700e4	1.16005e-5		
		6	6.40000e-1	4.61215e4	1.38764e-5		
		7	8.00000e-1	6.10482e4	1.31044e-5		
		8	8.00000e-1	6.05229e4	1.32181e-5		
		9	9.60000e-1	7.57338e4	1.26760e-5		
		10	9.60000e-1	7.62612e4	1.25883e-5		
		11	1.12000	8.25571e4	1.35664e-5		
		12	1.12000	8.17100e4	1.37070e-5		
		13	1.28000	1.02338e5	1.25075e-5		
14.524	1	1	8.00000e-1	6.08275e4	1.31519e-5		Menthol
		2	8.00000e-1	6.40159e4	1.24969e-5		
		3	1.20000	9.12752e4	1.31471e-5		
		4	1.20000	8.52709e4	1.40728e-5		
		5	1.60000	1.18785e5	1.34697e-5		
		6	1.60000	1.19480e5	1.33914e-5		
		7	2.00000	1.49634e5	1.33659e-5		
		8	2.00000	1.68608e5	1.18618e-5		
		9	2.40000	1.82250e5	1.31687e-5		
		10	2.40000	1.87155e5	1.28236e-5		
		11	2.80000	2.13159e5	1.31357e-5		
		12	2.80000	2.11182e5	1.32587e-5		
		13	3.20000	2.50096e5	1.27951e-5		
		14	3.20000	2.43803e5	1.31254e-5		



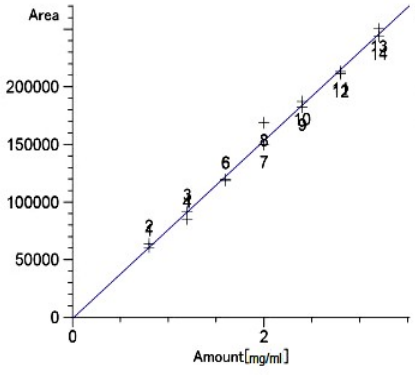
Methyl Salicylate at exp. RT: 9.804
RID1 A, Refractive Index Signal
Correlation: 0.99974
Residual Std. Dev.: 6650.93956
Formula: y = mx + b
m: 98270.84887
b: -3832.89118
x: Amount
y: Area

***Figure 2a: Calibration curve of MS**



Camphor at exp. RT: 11.003
RID1 A, Refractive Index Signal
Correlation: 0.99490
Residual Std. Dev.: 2956.93373
Formula: y = mx + b
m: 76899.45974
b: 50.06780
x: Amount
y: Area

***Figure 2b: Calibration curve of CM**



Menthol at exp. RT: 14.524
RID1 A, Refractive Index Signal
Correlation: 0.99753
Residual Std. Dev.: 5367.60910
Formula: y = mx + b
m: 77176.76536
b: -1027.23493
x: Amount
y: Area

***Figure 2b: Calibration curve of MN**

System suitability of the method

For the results of system suitability see tables 2a, 2b, 2c and 2d. The resolution between MS and CM was found to be 3.7 and between CM and MN 8.78.

***table 2a:System suitability data for MS**

Injection	Peak Area	Retention time
1	548564	9.778
2	556320	9.785
3	562797	9.785
4	569527	9.791
5	566650	9.790
6	563592	9.809
Average	561241	9.79
SD	7626	0.011
RSD	1.358	0.108

table 2b:System suitability* data for CM

injection	Peak Area	Retention time
1	54099	11.01
2	57511	11.02
3	61649	11.01
4	57072	11.02
5	63021	11.02
6	55933	11.04
Average	58214	11.02
SD	3430	0.009
RSD	5.893	0.079

table 2c:System suitability* data for MN

injection	Peak Area	Retention time
1	131650	14.54
2	149308	14.54
3	144681	14.54
4	142007	14.54
5	131733	14.55
6	142410	14.55
Average	140298	14.55
SD	7154	0.005
RSD	5.099	0.034

***table 2d:System suitability data for chromatographic system**

compound	Capacity factor	Symmetry	NTP
MS	2.93	0.99	16677
CM	3.42	0.83	14671
MN	4.83	0.88	17752

Method range

The results obtained indicate that the HPLC method is applicable for the determination of Methyl Salicylate, Camphor and Menthol concentrations as low as 2.4 mg/ml (80% of the method concentration) and as high as 9.6 mg/ml, (120 % of method concentration) for Methyl Salicylate and as low as 320µg/ml (80% of the method concentration) and as high as 1280µg/ml (120% of the method concentration)for Camphor and as low as 0.8 mg/ml (80% of the method concentration) and as high as 3.2 mg/ml (120% of the method concentration) for Menthol. For the results see tables 3a, 3b and 3c.

Overall conclusion:

*table 3a: Range data for MS

Concentration Range	MS Concentration found mg/ml	% Yield
80% 100% 120%	29.78 29.88 31.04	99.268 % 99.604% 103.460%
Mean % Standard deviation Relative standard deviation (RSD %)		100.777 2.330 2.312

*table 3b: Range data for CM

Concentration Range	Camphor Concentration found mg/ml	% Yield
80% 100% 120%	4.167 4.096 4.170	104.18% 102.41% 104.24%
Mean % Standard deviation Relative standard deviation (RSD %)		103.61 1.042 1.005

***table 3c: Range data for MN**

Concentration Range	Menthol Concentration found mg/ml	% Yield
80% *100% 120%	10.105 9.531 9.966	101.05% 95.317% 99.659%
Mean % Standard deviation Relative standard deviation (RSD %)		98.676 2.991 3.032

Accuracy

For MS The mean % recovery for samples of set I - III (presenting concentration ranged from 80 %-120 % of the method concentration) is 101.02%, the Standard deviation for samples of set I - III is 2.742 and Relative standard deviation (RSD %) is 2.714. This proves that the method is accurate for the determination of Methyl Salicylate in Rheumatizen Topical Cream. See table 4a for the accuracy data for MS

Table 4a: Accuracy data of MS

Set No.	mg of Methyl Salicylate added	mg of Methyl Salicylate found	% Recovery of Methyl Salicylate
I	240	236.74	98.64%
II	300	301.2	100.4%
III	360	374.4	104.0%

For CM The mean % recovery for samples of set I - III (presenting concentration ranged from 80 %-120 % of the method concentration) is 98.183%, the Standard deviation for samples of set I - III is 3.725 and Relative standard deviation (RSD %) is 3.794. This proves that the method is accurate for the determination of Camphor in Rheumatizen Topical Cream. See table 4b for the accuracy data for CM

*Table 4b: Accuracy data of CM

Set No.	mg of Camphor added	mg of Camphor found	% Recovery of Camphor
I	32	30.17	94.286%
II	40	39.42	98.555%
III	48	48.82	101.71%

For MN The mean % recovery for samples of set I - III (presenting concentration ranged from 80 %-120 % of the method concentration) is 95.197%, the Standard deviation for samples of set I - III is 2.967 and Relative standard deviation (RSD %) is 3.117%. This proves that the method is accurate for the determination of Menthol in Rheumatizen Topical Cream. See table 4c for the accuracy data for MN

*Table 4c: Accuracy data of CM

Set No.	mg of Menthol added	mg of Menthol found	% Recovery of Menthol
I	80	74.45	93.064%
II	100	93.94	93.941%
III	120	118.30	98.585%

Repeatability

It is performed by applying the proposed method for determination of MS, CM and MN in Cream 6 times. The repeatability data are listed in table 5.

***Table 5: reputability data of the method.**

NO	% MS	% CM	%MN
1	103.38	106.19	109.98
2	100.27	95.691	109.49
3	101.03	98.312	112.96
4	106.45	98.964	110.93
5	99.272	96.492	109.57
6	100.96	101.94	109.07
Mean % Standard Deviation Relative Standard Deviation	101.89 2.609 2.561	99.598 3.896 3.912	110.34 1.434 1.300

Inter-analyst precision

It is performed by analyzing the same samples of Rhumatizen topical cream by three different analysts in short time intervals. The data are collected in table 6.

***Table 6: Inter-analyst precision data of the method.**

Analyst	Mean % Methyl Salicylate	Mean % Camphor	Mean % Menthol
A	98.688	99.303	100.78
B	99.829	100.23	100.84
C	102.08	102.51	101.86

Inter-day precision

It is performed by analysis of a sample of Rheumatizen topical cream each day for a period of 3 days. The results are presented in table 7.

***Table 7: Inter-day precision data of the method.**

Day	**% Methyl Salicylate**	**% Camphor**	**% Menthol**
1	106.16	105.5	107.94
2	101.1	95.38	105.3
3	100.1	103.63	105
Mean % recovery Standard Deviation Relative Standard Deviation	3.256 3.178		

Robustness

Effect of slight variation in flow rates on the retention time are listed below in table 8, 9 and 10. And the effect of slight variation in the column temperatures are listed below in table 11, 12 and 13.

* Table 8: Effect of slight variation in flow rate on the retention time of MS.

Flow rate	**Retention time**
0.9 ml/min	9.796
1 ml/min	9.48
1.1 ml/min	8.898

* Table 9: Effect of slight variation in flow rate on the retention time of CM.

Flow rate	**Retention time**
0.9 ml/min	11.038
1 ml/min	10.673
1.1 ml/min	10.027

* Table 10: Effect of slight variation in flow rate on the retention time of MN.

Flow rate	Retention time
0.9 ml/min	14.619
1 ml/min	13.987
1.1 ml/min	13.28

. *table 11:Effect of slight variation in column temperature on retention time of MS

Column temperature	Retention time	Peak Area
20° C	9.188	750635
30° C	9.979	748302

*table 12:Effect of slight variation in column temperature on retention time of CM

Column temperature	Retention time	Peak Area
20° C	10.42	76093
30° C	11.062	75056

*table 13:Effect of slight variation in column temperature on retention time of MN

Column temperature	Retention time	Peak Area
20° C	13.744	189838
30° C	14.301	189695

Stress testing

The stress testing was performed by studying the effect of 2M NaOH, 2M HCl and 30% H_2O_2 on the reference materials of MS, CM and MN. The solution in each case was prepared by transferring 5ml of stock standard solution into three 50 ml volumetric flask s. in the first flask add 20 ml of 2M NaOH and in the second flask add 20 ml of 2M HCl and on the third flask add 20 ml of H_2O_2. Close the flasks well (to avoid loss due evaporation) and leave it at room temperature for about 22 hours. After the specified time neutralize the solutions of both NaOH and HCl by using the pH meter and adjusting with NaOH or HCl to the neutral pH (7.5). In the case of H_2O_2 remove the dissolved oxygen by a stream of nitrogen gas. Complete each flask to the volume with Methanol.

Effect of 2M NaOH on the HLPC chromatogram of Actives :

The result of stress testing with 2M NaOH show that under the pre described conditions only MS has affected by only decrease in the peak response and neither MN nor CM had been

affected. This indicates good stability of MN and CM but poor stability of MS under the basic conditions. The decrease in the % of MS was found to be about 71.35% see figure 3a.

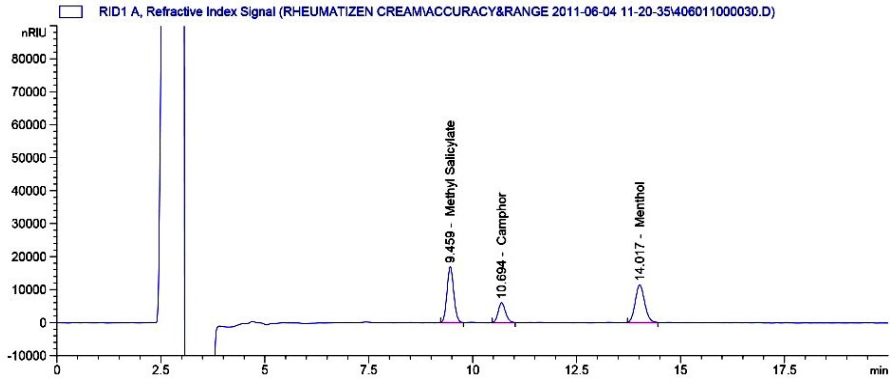
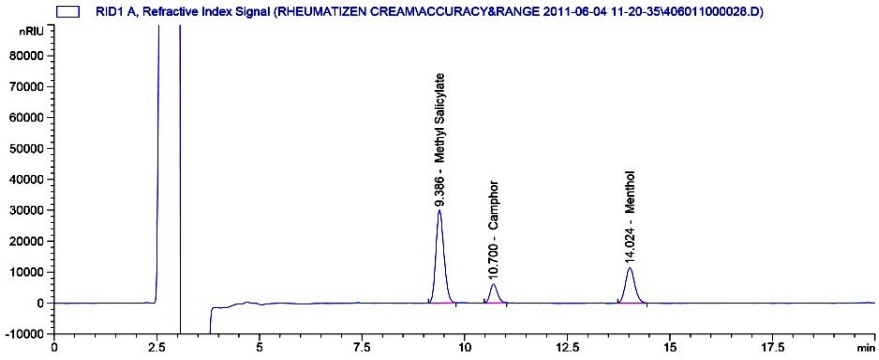


***figure 3a: effect of 2M NaOH on the MS, CM and MN.**

Effect of 2M HCl on the HLPC chromatogram of Actives:

The result of stress testing with 2M HCl show that under the pre described conditions only MS has affected by only decrease in the peak response and neither MN nor CM had been affected. This indicates good stability of MN and CM but moderate stability of MS under the acidic conditions. The decrease in the % of MS was found to be about 39.2% see figure 3b.



Effect of 30% H_2O_2 on the HLPC chromatogram of Actives:

The result of stress testing with 30% H_2O_2 show that under the pre described conditions only MS has affected by only decrease in the peak response and neither MN nor CM had been affected. This indicates good stability of MN, CM and MS under the oxidation conditions. The decrease in the % of MS was found to be about 4.75% see figure 3c.

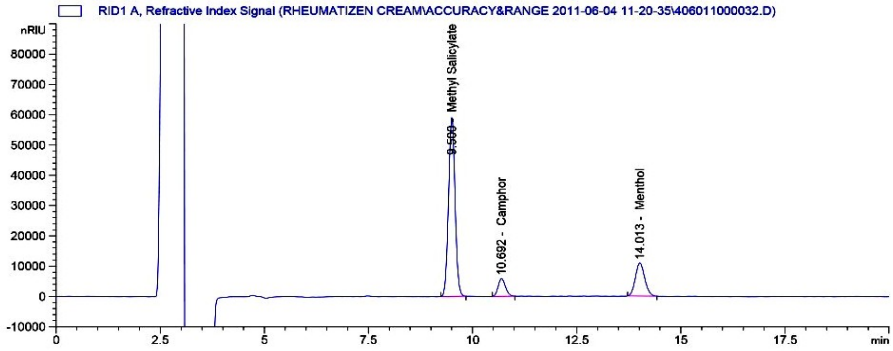


***figure 3c: effect of 30% H_2O_2 on the MS, CM and MN.**

Overall conclusion:

A fast and easy HPLC method was used to identify and quantify the three contents (Methyl salicylate, Camphor and Menthol) of the pharmaceutical preparation (Rheumatizen topical cream). The validated HPLC method for the determination is linear over the concentration range. The method is specific, simple, accurate, robust and precise. It is useful in performing the stability studies for the product. The stress testing shows that MS has poor stability in alkaline pH due to the effect of the base on the ester linkage in the molecule. MS has moderate stability in the acidic medium and good stability against oxidation. For CM and MN they have good stability in Acidic, basic mediums and also against oxidation.

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