International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 12, Issue 9, 2020

Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE COMBINATION OF IMIQUIMOD AND SALICYLIC ACID

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Received: 19 May 2020, Revised and Accepted: 14 Jul 2020

ABSTRACT

Objective: The present study was undertaken to develop and validate an RP-HPLC method for the combination of imiquimod and salicylic acid

Methods: The method was carried out on Nucleodur C18 (250 mm × 4.6 mm I.D., 5 μm) using low-pressure gradient elution mode. The mobile phase was used as 30M potassium dihydrogen phosphate and acetonitrile (45:55) pH 6.5 adjusted using ortho-phosphoric acid. The concentration of solvents was 1-20 µg/ml and the volume of injection was 20 mcl with the flow rate of 1.0 ml/min. The absorption maxima of salicylic acid and imiquimod were found 234 nm and 226 nm, respectively.

Results: The method was validated and showed the linearity greater than 0.99% and with precision (RSD%<1). The limit of detection (LOD) and limit of quantification (LOQ) of salicylic acid was found to be 0.09756 µg/ml and 0.2956 µg/ml, respectively, and imiquimod was found to be 0.044031 µg/ml and 0.13334 µg/ml, respectively.

Conclusion: The method developed in the present study was found to be sensitive, specific, and can be applied for the simultaneous estimation of imiquimod and salicylic acid.

Keywords: Imiquimod, Salicylic acid, Reverse-phase high-performance liquid chromatography (RP-HPLC), Validation, International council for harmonization (ICH)

DOI: http://dx.doi.org/10.22159/ijpps.2020v12i9.38382. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps.

INTRODUCTION

Imiquimod is chemically 1-(2-methyl propyl)-1H-imidazo [4, 5-c] quinolin-4-amine, which is a novel synthetic compound that is a member of the imidazoquinolone family of drugs [1]. The molecular formula of imiquimod is $C_{14}H_{16}N_4$, which is a white crystalline powder and the molecular weight is 240.30g/mol. The solubility of imiquimod is freely soluble in oleic acid and lactic acid with melting point 292-294 °C and pKa value 2.7. Imiquimod is an immune response modifiers use in the treatment for external genital warts by the mechanism response modifier that acts as a toll-like receptor 7 agonists. It does not kill the viruses that cause warts directly, however, also used to treat skin problems of face and scalp that is called actinic keratoses and also for various skin cancers called superficial basal cell carcinoma. In a present-day commercially available as Aldara® 5% imiquimod cream, approved by U. S. Food and Drug Administration ("FDA") in 1997 [2-4].

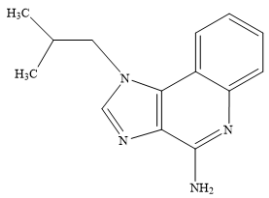


Fig. 1: Chemical structure of imiquimod

Salicylic acid (from latin salix, willow tree, from the bark of which the substance used to be obtained) is a monohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. Salicylic acid has the formula $C_6H_4(OH)$ COOH, where the OH group is ortho to the carboxyl group and is poorly soluble in water (2 g/l at 20 °C). Aspirin (acetylsalicylic acid or ASA) can be prepared by the esterification of the phenolic hydroxyl group of salicylic acid with the acetyl group from acetic anhydride or acetyl chloride. The compound is white crystalline powder and the molecular weight is 138.12 g/mol. The solubility of salicylic acid is good in ether, CCl_4, benzene, propanol, acetone, ethanol, oil of turpentine, toluene [5, 6]. It shows its anti-inflammatory action by suppressing the activity of cyclooxygenase (COX), an enzyme that is responsible for the production of pro-inflammatory mediators such as the prostaglandins [7]. Salicylic acid also has its anti-acne action [8]. Chemical structure of salicylic acid is given below:

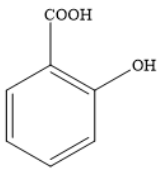


Fig. 2: Chemical structure of salicylic acid

Simultaneously steps were taken for these two drugs successfully. Therefore it was thought to develop an accurate and rapid RP-HPLC method for simultaneous estimation of the combination of imiquimod and salicylic acid. This method was validated as per the current International Conference on Harmonization (ICH) guideline [9-11].

MATERIALS AND METHODS

Salicylic acid was procured as a gift sample from SGPTC Pvt. Ltd and imiquimod drug was obtained as a gift sample from Glenmark Pharma, Mumbai India. All other chemicals like o'phosphoric acid, potassium dihydrogen phosphate, and disodium hydrogen

orthophosphate were used of AR grade and solvents like methanol, acetonitrile, and water were used of HPLC grade

Identification of standard drugs

The identification of standard drugs was carried out by melting point study, infrared spectroscopic study, and solubility study.

Rp-hplc method

Selection of detection wavelength

A known concentration of 10 µg/ml of salicylic acid and imiquimod were prepared in methanol and scanned in the UV region 200-400 nm.

Selection of mobile phase and optimization of chromatographic condition

Initially, the mobile phase was tried with methanol: water, acetonitrile: water, acetonitrile: water: methanol and then finally with phosphate buffer: acetonitrile: at various pH conditions. The following optimized parameters were used as a final method for the simultaneous estimation of salicylic acid and imiquimod.

Chromatographic condition

In the optimized parameters, the stationary phase was Nucleodur C18 (250 mm × 4.6 mm I.D., 5 μm) using low-pressure gradient elution mode. 30M potassium dihydrogen phosphate and acetonitrile (45:55) pH 6.5 were used as mobile phase and pH were adjusted using orthophosphoric acid. The concentration of solvents was 1-20 µg/ml, and the volume of injection was 20 µl with the flow rate of 1.0 ml/min. The absorption maxima of salicylic acid and imiquimod were set in 234 nm and 226 nm, respectively.

Standard stock solution preparation (100 µg/ml)

100 µg/ml solution was prepared using 10 mg of salicylic acid 1 mg of imiquimod and about 1 ml of diluent was added and sonicate to dissolve to make the standard solution.

Preparation of calibration curve

Different concentration was prepared 0.1 µg/ml to 1 µg/ml for imiquimod and 1 µg/ml to 10 µg/ml for salicylic acid from the standard stock solution, respectively.

Validation of RP-HPLC method

Linearity

Different concentrations of both the drugs were prepared for linearity i.e. salicylic acid 1-10 µg/ml and imiquimod 0.1-1 µg/ml [12, 13].

Accuracy

Recovery studies were carried out by the addition of standard drug solution at the level of 80%, 100%, and 120% to the pre-analyzed sample. Results of the recovery study were found to be within the acceptance criteria 100±10 %. In this method, the known concentration of standard drug was added to the assay sample [14, 15].

Precision

Different concentrations of both the drugs were used for intra-day and inter-day variation for six times in the same day. The concentration was 5 µg/ml of salicylic acid and 0.5 µg/ml imiquimod, and then % RSD was calculated [16, 17].

Limit of detection and limit of quantification

The limit of detection can be calculated using the following equation as per ICH guidelines [18].

$$LOD = 3.3 \times N/S$$

Where N is the standard deviation of the intercepts of the drug, and S is the slope of the corresponding calibration curve.

The limit of quantification can be calculated using the following equation as per ICH guidelines.

$$\mathrm{LOQ} = 10 \times \mathrm{N/S}$$

Where N is the standard deviation of the intercepts of the drug, and S is the slope of the corresponding calibration curve [19].

Robustness

Same samples of salicylic acid and imiquimod concentration 5µg/ml and 0.5µg/ml respectively by considered variation in the method parameters i.e. change in wavelength or change in flow rate, change in pH of the mobile phase. The change in the responses of salicylic acid and imiquimod were noted in terms of %RSD [20].

Specificity

The specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients. A volume of 20µl of individual ingredients and excipients solution was injected and the chromatogram was recorded [21].

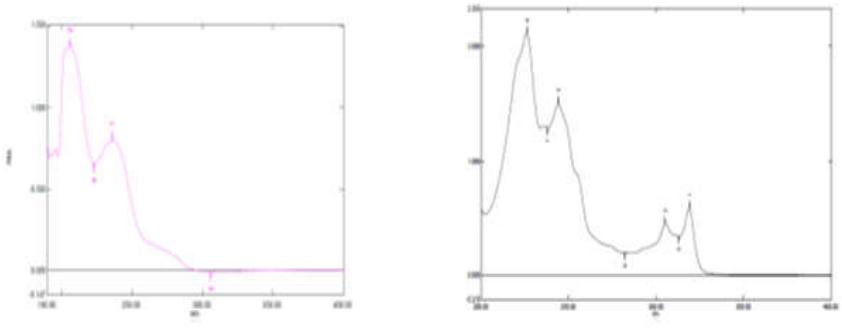
Ruggedness

The ruggedness was studied by analyzing the same samples of salicylic acid and imiquimod concentration 5µg/ml and 0.5µg/ml, respectively by changing the analyst. The change in the responses of salicylic acid and imiquimod were noted in terms of %RSD [22].

RESULTS AND DISCUSSION

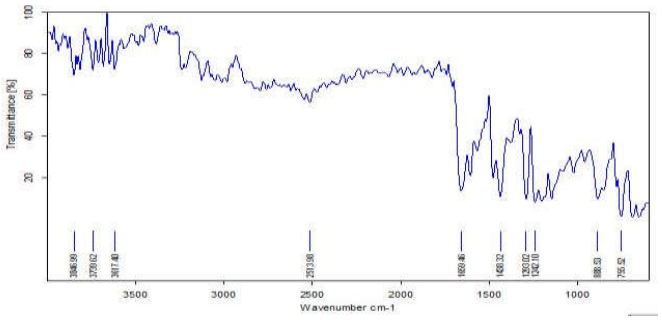
Identification of imiquimod and salicylic acid

Salicylic acid and imiquimod were observed for organoleptic properties like physical appearance, odor, and melting point. The drugs were identified with the help of UV and FTIR, and exhibited absorption maxima was 234 nm for salicylic acid and 226 nm for imiquimod when methanol was used as a solvent as mentioned in the literature (fig. 3).

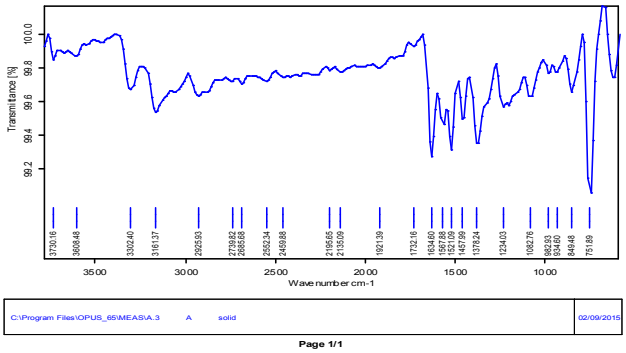


(A) (B)

Fig. 3: UV spectrum of salicylic acid (A) and imiquimod (B) in methanol



(a)



(b)

Fig. 5: FTIR spectra of (a) salicylic acid (b) imiquimod

Method validation

Linearity

The linearity of both the drugs was found within acceptance criteria. The correlation coefficient (r^2) obtained was calculated and it was found to be greater than 0.99 for salicylic acid and imiquimod is given in the below table, which is well within the acceptance criteria. Good linearity with the coefficient of correlation 0.99 indicated that the proposed method was linear within the range of 1 to 10 µg/ml, 0.1 to 1 µg/ml, respectively for salicylic acid and imiquimod.

Table 1: Linearity of salicylic acid

Conc.(µg/ml)	Area1	Area2	Area3	Mean	SD	%RSD
1	55346	55051	55866	55421	412.6439	0.744562
2	94156	94907	94550	94537.67	375.6519	0.397357
3	129106	125734	124812	126550.7	2260.491	1.786234
4	162548	165085	167974	165202.3	2714.902	1.64338
5	187989	189886	194048	190641	3099.256	1.625703
6	230434	236106	231690	232743.3	2979.099	1.279993
7	267238	268739	264993	266990	1885.274	0.706121
8	304641	308556	307314	306837	2000.613	0.652012
9	342474	341383	348660	344172.3	3924.53	1.14028
10	383247	388043	386828	386039.3	2493.371	0.645885

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, RSD =relative standard deviation

Table 2: Linearity of imiquimod

Conc.(µg/ml)	Area1	Area 2	Area 3	Mean	SD	%RSD
0.1	100795	100900	100854	100849.7	52.63396	0.052191
0.2	165045	165650	164071	164922	796.6536	0.483049
0.3	225478	220799	227558	224611.7	3461.78	1.541229
0.4	268053	266795	267947	267598.3	697.723	0.260735
0.5	330441	336627	332784	333284	3123.163	0.937088
0.6	395254	394840	397691	395928.3	1540.485	0.389082
0.7	468121	469870	463485	467158.7	3299.488	0.706288
0.8	522375	524480	525548	524134.3	1614.496	0.308031
0.9	595412	595953	595245	595536.7	370.0977	0.062145
1	655536	652236	651048	652940	2325.349	0.356135

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, RSD =relative standard deviation

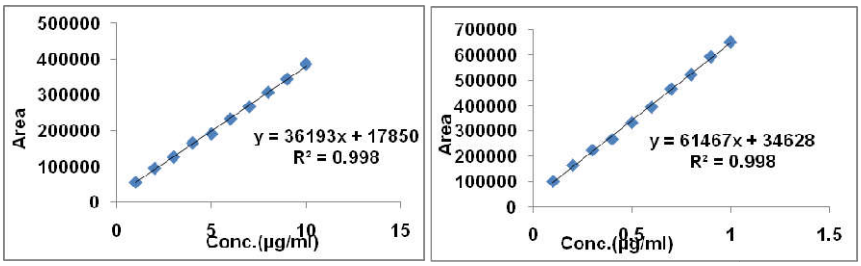


Fig. 6: Average linearity of salicylic acid (left) and imiquimod (right)

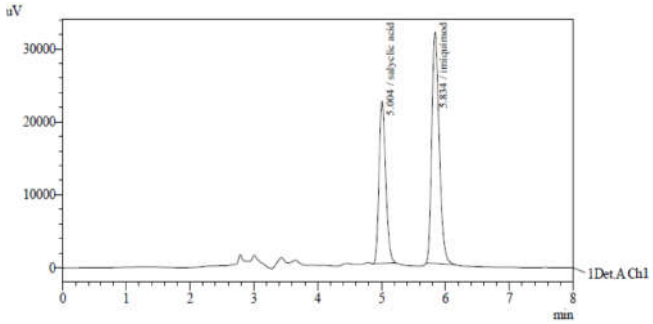


Fig. 7: Chromatogram of salicylic acid and imiquimod (4 µg/ml and 0.4 µg/ml)

Table 3: Accuracy results of salicylic acid and imiquimod

Accuracy results of salicylic acid							
Conc. level	**Area**	**Mean**	**Amount added**	**Amount recovered**	**% Recovery**	**SD**	**%RSD**
80%	167045	165052.66	4 µg/ml	4.12	103.055		
	163972		4 µg/ml	4.03	100.932	2172.9	1.3165
	164141		4 µg/ml	4.04	101.049		
100%	183748	184728.33	5 µg/ml	4.58	91.674		
	185458		5 µg/ml	4.63	92.619	882.13	0.4775
	184979		5 µg/ml	4.61	92.354		
120%	215834	217098.66	6 µg/ml	5.47	91.170		
	216921		6 µg/ml	5.50	91.671	1362.2	0.6275
	218541		6 µg/ml	5.54	92.417		
Accuracy results of imiquimod							
Conc. level	**Area**	**Mean**	**Amount added**	**Amount recovered**	**% Recovery**	**SD**	**%RSD**
80%	261795	261930.33	0.4 µg/ml	0.369	92.39	685.89	0.2619
	260825		0.4 µg/ml	0.367	91.99		
	263171		0.4 µg/ml	0.371	92.95		
100%	319439	321252.33	0.5 µg/ml	0.463	92.67	1936.4	0.6028
	321026		0.5 µg/ml	0.465	93.18		
	323292		0.5 µg/ml	0.469	93.92		
120%	375617	375861	0.6 µg/ml	0.555	92.45	1236.2	0.3289
	377201		0.6 µg/ml	0.557	92.88		
	374765		0.6 µg/ml	0.553	92.22		

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, RSD =relative standard deviation

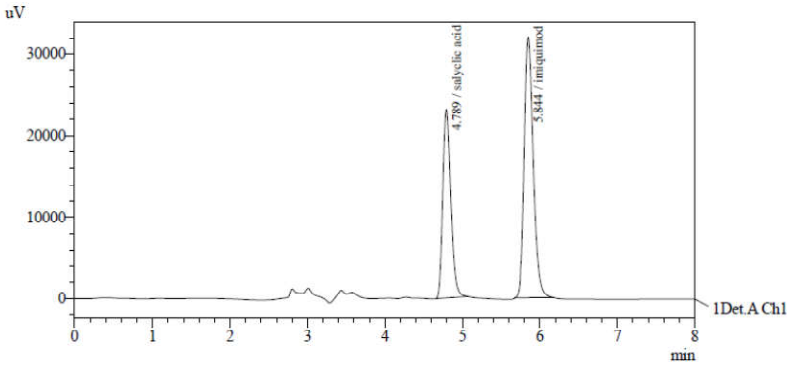


Fig. 8: Chromatogram of salicylic acid and imiquimod (80%)

Accuracy

The results indicate that the recoveries are well within the acceptance range of 80–120% (table 3). The chromatogram of salicylic acid and imiquimod drugs at level 80% was shown in fig. 8.

Precision

The %RSD for the area of six replicate injections was found to be within the specified limits i.e. for salicylic acid, it was 0.742393% and imiquimod it was observed 0.995204%. Whereas the % RSD for intra and inter-day precision of salicylic acid and imiquimod were observed below 2% [18-20]. The low values of % RSD indicate that the method is precise. % RSD for interday precision and intraday precision was given below that was found to be within the specified limits.

Limit of detection and limit of quantitation

The LOD was found to be 0.09756 µg/ml and 0.044031 µg/ml for salicylic acid and imiquimod respectively and LOQ was found to be 0.2956 µg/ml and 0.13334 µg/ml salicylic acid and imiquimod respectively which showed that sensitivity of the method was high.

Robustness

The Percentage of RSD should not be more than 2. The %RSD obtained for change of flow rate, change in wavelength, and pH variation in the mobile phase was found to be below 2, which was within the acceptance criteria. Hence the method was robust.

Ruggedness

The results were found within a specified limit, % RSD was less than 2. Data are tabulated in table 9 and fig. 10.

Specificity

Specificity of salicylic acid and imiquimod are shown in fig. 11 and table 10. All the data were within limits. %RSD of salicylic acid in specificity was observed 0.186425% and for imiquimod, it was 0.384456%.

Table 4: Repeatability of salicylic acid and imiquimod

S. No.	Salicylic acid		Imiquimod	
	Conc.(µg/ml)	Area	Conc.(µg/ml)	Area
1	5	180729	0.5	312844
2	5	180281	0.5	318685
3	5	181842	0.5	311867
4	5	182989	0.5	310441
5	5	183886	0.5	316627
6	5	182048	0.5	312784
Mean		181962.5	Mean	313874.7
Standard Deviation		1350.877	Standard Deviation	3123.692
%RSD		0.742393	%RSD	0.995204

*Mean for six independent analysis, mean±SD, n=6, Conc. =concentration, RSD =relative standard deviation

Table 5: Intraday and intraday precision of salicylic acid and imiquimod

Intraday precision				
S. No.	**Salicylic acid**		**Imiquimod**	
	Conc.(µg/ml)	**Area**	**Conc.(µg/ml)**	**Area**
1	5	194588	0.5	371303
2	5	194165	0.5	366835
3	5	195142	0.5	369995
4	5	194057	0.5	370772
5	5	195544	0.5	368306
6	5	194999	0.5	369934
Mean		194749.2	Mean	313874.7
Standard Deviation		582.2053	Standard Deviation	3123.692
%RSD		0.298951	%RSD	0.995204
Interday precision				
S. No.	Salicylic acid		Imiquimod	
	Conc.(µg/ml)	Area		Conc.(µg/ml)
1	5	192990	1	5
2	5	193563	2	5
3	5	193229	3	5
4	5	193825	4	5
5	5	193199	5	5
6	5	193807	6	5
Mean		193435.5	Mean	369890.8
Standard Deviation		347.2819	Standard Deviation	2046.481
%RSD		0.179534	%RSD	0.553266

*Mean for six independent analysis, mean±SD, n=6, RSD =relative standard deviation

Table 6: Limit of detection and quantitation

API	parameter	Linearity 1	Linearity 2	Linearity 3	Intercept SD	Slope mean	LOD	LOQ
Salicylic acid	Intercept	18267	19774	17719	1064.14112	35992.3333	0.09756	0.2956
	Slope	35900	35722	36355			µg/ml	µg/ml
Imiquimod	Intercept	34160	34149	35575	820.144499	61466.3333	0.044031	0.13334
	Slope	61543	61575	61281			µg/ml	µg/ml

*mean±SD, n=3, LOD =limit of detection, LOQ =limit of quantitation

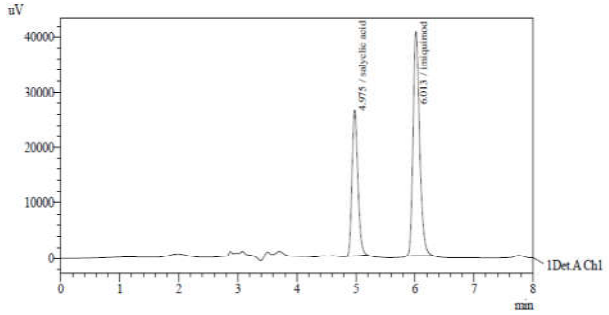


Fig. 9: Chromatogram of repeatability of salicylic acid and imiquimod

Table 7: Robustness of salicylic acid and imiquimod at different flow rates and column temperature

Change in flow rates						
Conc.(µg/ml)	**0.690 ml/min**		**0.700 ml/min**		**0.710 ml/min**	
	Area		**Area**		**Area**	
	Salicylic acid	**Imiquimod**	**Salicylic acid**	**Imiquimod**	**Salicylic acid**	**Imiquimod**
5, 0.5	185355	336750	185716	331298	179209	322635
5, 0.5	186835	331907	185978	327514	180008	319692
5, 0.5	184881	331433	185234	332858	181071	323943
Mean	185690.333	333363.33	185642.7	330556.7	180096	322090
SD	1019.24743	2942.4993	377.3822	2748.048	934.114	2177.273
%RSD	0.54889634	0.8826704	0.203284	0.831339	0.518676	0.675983
Change in column temperature						
Conc.(µg/ml)	35 °C		35 °C		35 °C	
	Area		Area		Area	
	Salicylic acid	Imiquimod	Salicylic acid	Imiquimod	Salicylic acid	Imiquimod
5, 0.5	183450	326827	189534	366900	190840	366438
5, 0.5	182884	325182	194165	366835	193563	366114
5, 0.5	187570	326333	193229	369038	195142	369995
Mean	184634.667	326114	192309.3	367591	193181.66	367515.7
SD	2557.77742	844.0835	2448.649	1253.56	2176.2036	2153.268
%RSD	1.38531808	0.258831	1.273286	0.34102	1.1265063	0.585898

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, RSD =relative standard deviation

Table 8: Robustness of salicylic acid and imiquimod at different mobile phase ratio and different wavelength

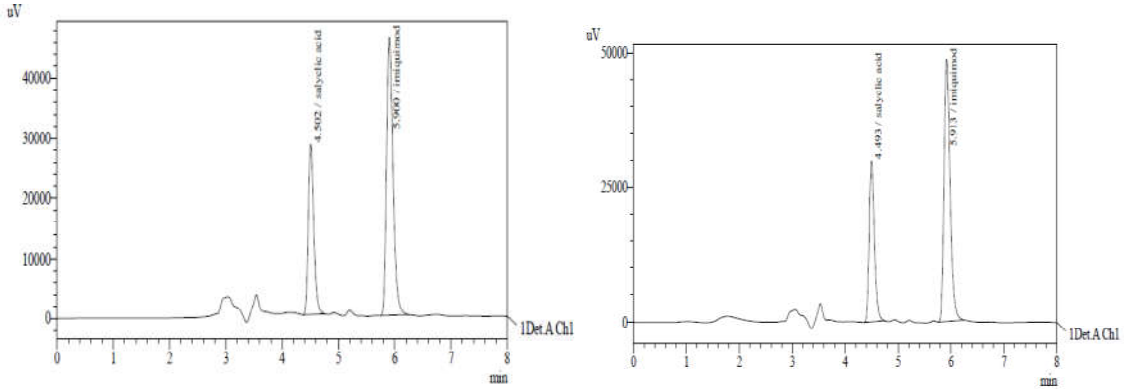
Conc.(µg/ml)	**Buffer: ACN 49.9:50.1**		**Buffer: ACN 50.1:49.9**		**Buffer: ACN 50:50**	
	Area		**Area**		**Area**	
	Salicylic acid	**Imiquimod**	**Salicylic acid**	**Imiquimod**	**Salicylic acid**	**Imiquimod**
5, 0.5	192990	371471	193807	370423	189534	366900
5, 0.5	194999	369934	194057	370772	194165	366835
5, 0.5	195544	368306	189264	368613	193229	369038
Mean	194511	369903.7	192376	369936	192309.3	367591
SD	1345.11598	1582.718	2697.968	1158.964	2448.648	1253.56
%RSD	0.69153723	0.427873	1.402445	0.313288	1.273286	0.34102
Change in wavelength						
Conc.(µg/ml)	222 nm		227 nm		232 nm	
	Area		Area		Area	
	Salicylic acid	Imiquimod	Salicylic acid	Imiquimod	Salicylic acid	Imiquimod
5, 0.5	167812	287540	185799	320120	147045	394888
5, 0.5	168691	286264	184977	319351	147211	388641
5, 0.5	167785	287674	183279	321442	146878	392983
Mean	168096	287159.3	184685	320304.3	147044.66	391764.5
SD	515.46192	778.2707	1285.126	1057.617	166.50025	4417.296
%RSD	0.3066473	0.271024	0.695847	0.330191	0.11323107	1.127539

*Conc. =concentration, mean±SD, n=3, ACN =acetonitrile, SD =standard deviation, RSD =relative standard deviation

Table 9: Ruggedness study by analyst 1 and analyst 2

Conc.(µg/ml)	**Analyst 1**		**Analyst 2**	
	Area		**Area**	
	Salicylic acid	**Imiquimod**	**Salicylic acid**	**Imiquimod**
5, 0.5	193825	371153	203497	386815
5, 0.5	194588	371303	198780	384112
5, 0.5	193199	371146	196176	380535
Mean	193870.6667	371200.6667	199484.3333	383820.6667
SD	695.6251385	88.69235223	3710.973502	3150.120051
%RSD	0.358808865	0.023893371	1.860283181	0.820727054

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, %RSD =relative standard deviation



(A) **(B)**

Fig. 10: Chromatogram of salicylic acid and imiquimod by analyst 1 (A) and analyst 2 (B)

Table 10: Specificity data of salicylic acid and imiquimod

Conc.(µg/ml)	Specificity	
	Area	
	Salicylic acid	Imiquimod
5, 0.5	189068	336047
5, 0.5	188871	335741
5, 0.5	189556	333678
Mean	189165	335155.3
SD	352.6514	1288.524
%RSD	0.186425	0.384456

*Conc. =concentration, mean±SD, n=3, SD =standard deviation, %RSD =relative standard deviation

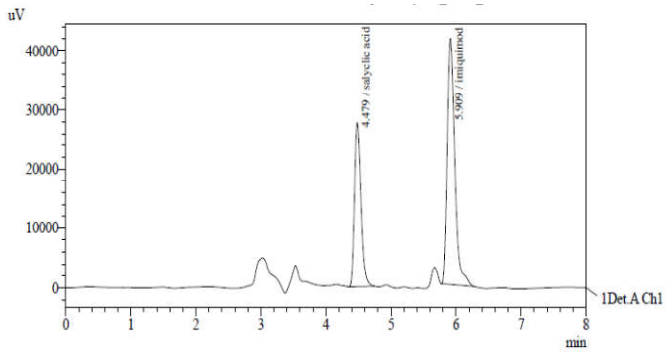


Fig. 11: Chromatogram of salicylic acid and imiquimod (specificity)

CONCLUSION

The RP-HPLC method was carried out on Nucleodur C18 (250 mm × 4.6 mm I.D., 5 μm) using low-pressure gradient elution mode. The limit of detection (LOD) and limit of quantification (LOQ) of salicylic acid was found to be 0.09756 µg/ml and 0.2956 µg/ml, respectively, and imiquimod was found to be 0.044031 µg/ml and 0.13334 µg/ml, respectively. The developed and validate method for imiquimod and salicylic acid was found to be sensitive, specific, and accurate and can be effectively applied for the simultaneous estimation of imiquimod and salicylic acid.

ACKNOWLEDGMENT

The Author is highly grateful to Dr. R. K Abhilashi, Chairman Abhilashi Group of Institutions, Mandi, India, for providing necessary facilities to carry out the research.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the author has contributed equally.

CONFLICT OF INTERESTS

Declared none

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