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Original article

DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF METHYL SALICYLATE IN A MEDICATED CREAM FORMULATION

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Abstract

A new reversed-phase liquid chromatographic (RPLC) method for the determination of methyl salicylate in a medicated cream formulation was developed and validated. The separation was achieved using an isocratic mobile phase, on a Lichrosorb C_8 column. The eluent was monitored by photodiode array detection at 304 nm. The calibration curve showed excellent linearity (R^2 = 0.9999) over the concentration range of 25-175 µg/mL. The recovery of methyl salicylate was in the range from 99.78-100.0%. The percent relative standard deviation values for intra- and inter-day precision studies were <2.0%. The method is very simple, sensitive and robust with short runtime (<3.0 min) to enable the processing of numerous quality control samples.

Key words: *Methyl salicylate, Reversed-phase LC, Method validation, Medicated cream formulation.*

İlaç Etkin Maddesi İçeren Krem Formülasyonlarında Metilsalisilat'ın Miktar Tayini İçin Bir Sıvı Kromatografik Yöntem Geliştirilmesi ve Validasyonu

Metilsalisilat'ın bir ilaç etkin maddesi içeren krem formülasyonunda miktar tayini için yeni bir ters faz sıvı kromatografik yöntem geliştirilmiş ve valide edilmiştir. Ayrım, Lichrosorb C_8 kolonu üzerinde izokratik mobil faz kullanılarak gerçekleştirilmiştir. Elüent fotodiyot dizisi kullanılarak 304 nm de deteksiyonu ile izlenmiştir. Kalibrasyon eğrisi 25-175 µg/mL konsantrasyon aralığında çok iyibirdoğrusallık göstermiştir. (R^2=0.999). Metilsalisat'ın geri kazanımı % 99.78-100.00 arasındadır. Gün içi ve günler arası yüzde bağıl standart sapma değerleri < % 2.0 dir. Yöntem çok basit,duyarlı ve sağlamdır. Ayrıca kısa sürede tamamlanabilmesi (< 3.0 dk) nedeniyle çok sayıda kontrol numunesine uygulanabilme kapasitesine sahiptir.

Anahtar kelimeler: *Metil salisilat, Ters faz LC, Yöntem validasyonu, İlaç etkin maddesi içeren krem formülasyonu.*

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INTRODUCTION

Methyl salicylate (2-hydroxybenzoic acid methyl ester, methyl 2-hydroxybenzoate, oil of wintergreen) is a naturally occurring compound which can be found in wintergreen oil as well as in sweet Birch. Methyl salicylate (Figure 1) is a non-steroid analgesic and anti-inflammatory drug used in many medicinal formulations for over-the-counter products including muscle ache creams and ointments. Furthermore, the United States Departments of Homeland Security (DHS) and Defense are testing next generation personal protection systems to protect U.S. military personnel from chemical threats. Some of these programs focus on man-in-simulant testing, where methyl salicylate is used to test the effectiveness of chemical suits (1, 2).

Methyl salicylate has two functional groups, the alcohol OH group and the ester group ($COOCH_3$). Esters are formed by the combination of an alcohol, e.g. R-OH and a carboxylic acid, (R-COOH). In pure form, methyl salicylate is toxic, especially when taken internally. A seventeen year-old cross-country runner at the Notre Dame Academy on Staten Island, died April 3, 2007, after her body absorbed high levels of methyl salicylate through excessive use of topical muscle-pain relief products (3). For these reasons it is essential to analyse and quantify the methyl salicylate drug, which can be considered as a major part of the quality control final product release.

The literature presently describes only one analytical method for the determination of methyl salicylate in combination with camphor and menthol in ointment using gas chromatography (4). The objective of this study was therefore to develop a simple, sensitive, robust and precise reversed-phase liquid chromatography (RPLC) method for the determination of methyl salicylate in medicated cream formulation. Frequently, LC is the analytical method of choice in pharmaceutical analysis because of its specificity and sensitivity. As a best practice (5-9) the new RPLC method was validated accordance to International Conference on Harmonization (ICH) (10) and U.S. Food and Drug Administration (FDA) (11) guidelines.

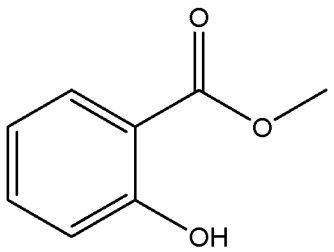

Figure 1. Chemical structure of methyl salicylate.

EXPERIMENTAL

Chemicals and reagents

Methanol (HPLC grade), acetic acid (analytical grade) and methyl salicylate (pure ≥ 99%) were obtained from Sigma-Aldrich (Gillingham, UK). Distilled water was de-ionised by using a Milli-Q system (Millipore, Bedford, MA).

LC system and conditions

The Knauer (Berlin, Germany) HPLC system equipped with a model 1000 LC pump, model 3950 autosampler, model 2600 photodiode-array (PDA) detector and a vacuum degasser was used. The data were acquired via Knauer ClarityChrom Workstation data acquisition software. The mobile phase consisted of a mixture of methanol-water (65:35, v/v) containing 1.0 % acetic acid. The flow rate was set to 1.0 mL/min. The injection volume was 20 µL and the detection wavelength was set at 304 nm. Reversed-phase LC analysis was performed isocratically at 30 ± 0.5°C using a Lichrosorb C_8 (150 mm x 4.6 mm, 5 µm) column (Jones Chromatography, Hengoed, UK).

Standard preparation

Methyl salicylate (0.1 g) was accurately weighed and added to a 100 mL volumetric flask before being dissolved in methanol. A 10 mL aliquot of stock solution was diluted to 100 mL in the mobile phase, yielding a final concentration of 100 µg/mL.

Standard solutions for the evaluation of methyl salicylate linearity were prepared over a concentration range of 25-175 µg/mL, to 25, 50, 75, 100, 125, 150 and 175%, in the mobile phase.

Sample preparation

Approximately (1.0 g) sample from final product commercially available was weighed into a 100 mL volumetric flask and twenty milliliter of methanol was added and flask was heated on a water bath up to boiling point. Then sample was cooled to room temperature and diluted in 100 mL mobile phase. The sample was filtered through 0.45 µ membrane filter and injected into RPLC.

RESULTS AND DISCUSSION

Method development

The chromatographic separation of methyl salicylate was carried out in isocratic mode using a mixture of methanol-water (65:35, v/v) containing 1.0% acetic acid as mobile phase. The column was equilibrated with the mobile phase flowing at 1.0 mL/min for about 20 min prior to injection. The column temperature was held at 30 ± 0.5°C. Twenty microliter of standard solution was injected automatically into the column. Subsequently, the liquid chromatographic behavior of methyl salicylate was monitored with a photodiode-array UV detector at 200-400 nm and signal optimized at 304 nm (Figure 2). Additionally, preliminary system suitability, precision, linearity, robustness and stability of solutions studied performed during the development of the method showed that the 20 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen. Chromatogram of the resulting solution gave excellent separation (Figure 3).

Effect of temperature

Tests were made by varying the temperature between 25°C and 50°C at 5°C steps, to study the influence of this parameter. The results showed that the variation of the temperature between 25°C and 35°C did not significantly affect any of the chromatographic parameters and only decreased the retention time of the analyte (Figure 4) so 30°C was selected as the working temperature.

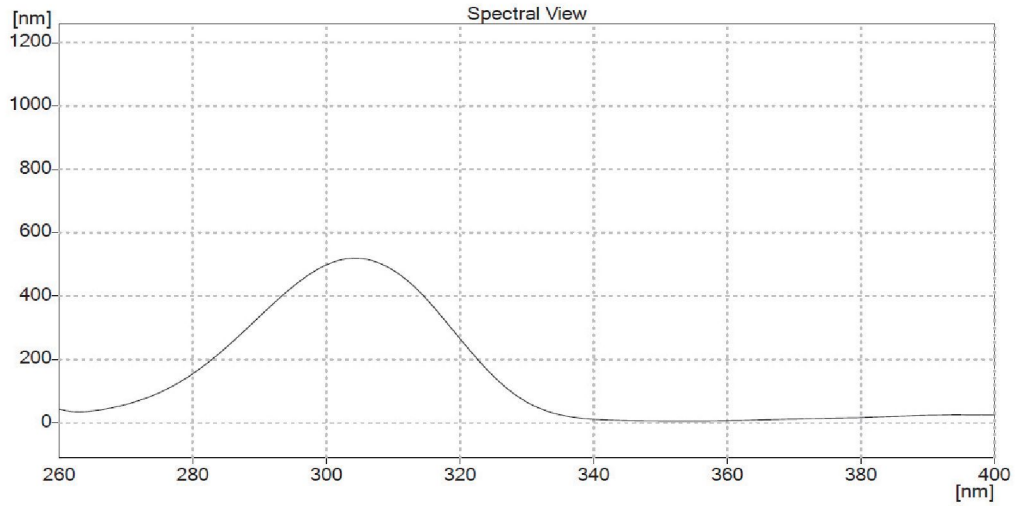

Figure 2. PDA UV spectra of the middle of the peak corresponding to the retention time of the main component of methyl salicylate.

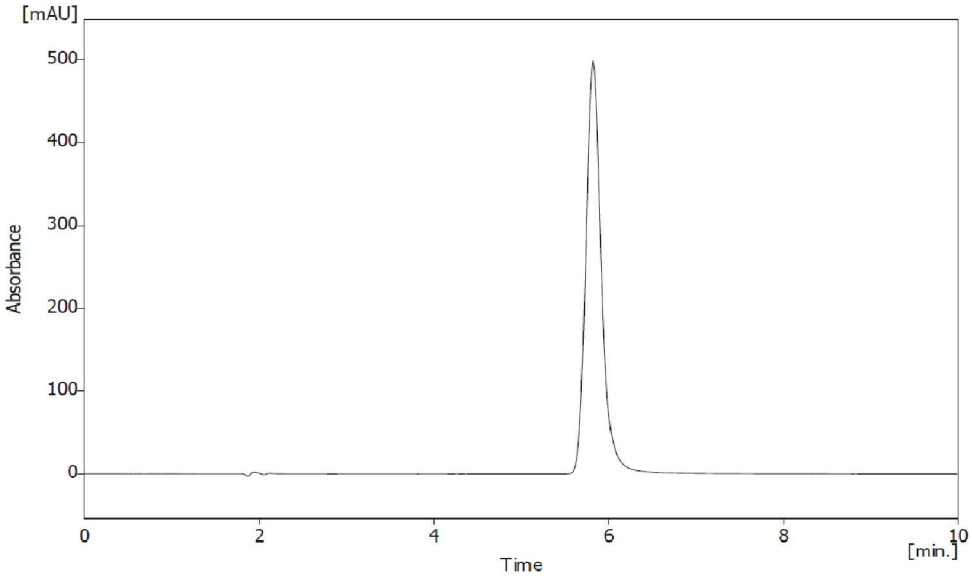

Figure 3. HPLC chromatogram of methyl salicylate.

System suitability testing

System suitability test was developed for the routine application of the assay method. Prior to each analysis, the chromatographic system must satisfy suitability test requirements (resolution and repeatability). System suitability test was performed to determine the accuracy and precision of the system from six replicate injections of solutions containing 100 μg methyl salicylate per mL. All peaks were well resolved and the precision of injections for methyl salicylate peaks were acceptable. The percent relative standard deviation (RSD) of the peaks area responses were measured, giving an average 0.16% (n = 6). The tailing factor (T), capacity factor (K), and theoretical plate number (N) were also calculated. The results of system suitability in comparison with the required limits are shown in Table 1. The proposed method met these requirements within the accepted limits (11, 12).

Table 1. System suitability results of the proposed analytical method for methyl salicylate.

Parameter	Recommended limits	Results
Retention time (min)	-	5.82
Injection repeatability ($n = 6$)	RSD ≤ 1 % for $n \geq 5$	0.16
Capacity factor (K)	K >2	4.02
Tailing factor (T)	T ≤2	1.12
Plate number (N)	N >2000	4686

Robustness

To determine the robustness of method, the final experimental conditions were purposely altered and the results were examined. The flow rate was varied by 1 ± 0.2 mL/min. the percentage of organic modifier was varied by 65 ± 5% and column temperature was varied by 30 ± 5°C. Their effects on the retention time (TR), tailing factor (T), theoretical plate numbers (N) and repeatability of peak areas ($n = 3$) were studied. It can be seen that every employed condition, the chromatographic parameters are in accordance with established value (11). A change of mobile phase composition, flow rate and temperature had no impact on chromatographic performance. The tailing factor for methyl salicylate was found to be less than 1.3 and analyte was well separated under all the changes carried out (Table 2). Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

Stability of analytical solutions

The stability of methyl salicylate in solution containing 0.1 mg/mL of methyl salicylate was investigated. The solutions were stable during the investigated 48 h and the RSD was in between 0.08 and 0.32% for retention time (min), peak area and height. Standard solutions stored in a capped volumetric flask on a laboratory bench under normal lighting conditions for 48 h, were shown to be stable with no significant change over this period (Table 3). These results are indicated (0.5% changes in area between $T = 0$ h and $T = 48$ h). Based on these data that show quantitative recovery through 48 h, solutions of methyl salicylate can be assayed within 48 h of preparation.

Table 2. Robustness data of the developed RPLC method for methyl salicylate.

Parameter	Modification	TR (min)	T	N	RSD (%)*
Mobile phase (Methanol, ± 5%)	60%	6.49	1.22	4614	0.14
	65%	5.82	1.11	4686	0.12
	70%	5.42	1.02	4566	0.17
Flow rate (± 0.2, mL/min)	0.8	5.67	1.11	4688	0.10
	1.0	5.82	1.10	4686	0.09
	1.2	5.53	1.04	4682	0.06
Temperature (± 5°C)	25	6.14	1.14	4689	0.15
	30	5.82	1.12	4686	0.10
	35	5.47	0.98	4654	0.13

* Peak area ($n = 3$)

Table 3. Stability data for methyl salicylate

Time (h)	RT (min)*	Area (mAU s)	Height (mAU)	Recovery (%)
0	0.09	0.18	0.26	99.98
48	0.12	0.22	0.31	98.96

* RSD (%, n = 3)

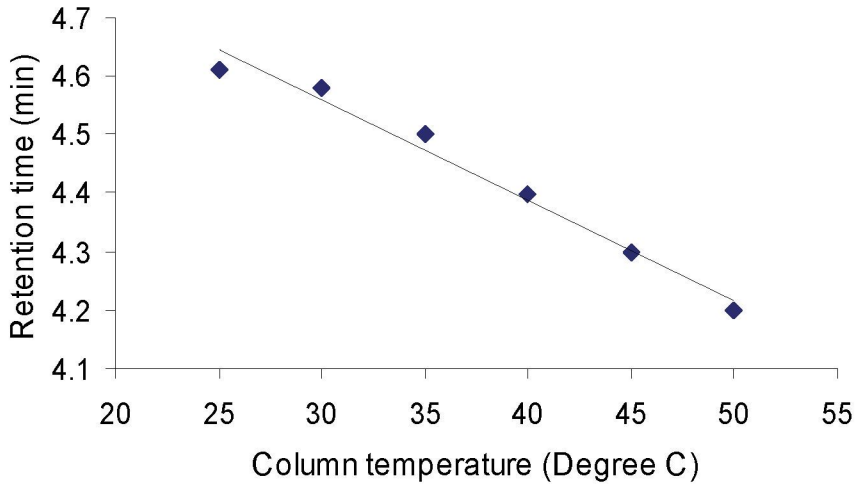

Figure 4. Effect of analytical column temperature on the retention times.

Method validation

Linearity

Linearity was studied using seven solutions in the concentration range 25-175 µg/mL. Solutions corresponding to each concentration level were injected in triplicate and linear regression analysis of the methyl salicylate peak area (y) versus methyl salicylate concentration (x) was calculated. The correlation coefficient (r^2 = 0.9999) obtained for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of methyl salicylate (Table 4).

Table 4. Method validation results of methyl salicylate

Validation step	Parameters	Conc. (µg/mL)	Results	Acceptance criteria
Linearity	(k = 7, n = 3)	25-175	y = 1.4748x + 7788.3 (r^2 = 0.999)	r^2 = ≥ 0.999
Repeatability (Area)	RSD (%, n = 10)	100	0.14	X < 2
Intermediate precision				
Day 1, LC 1, analyst 1	RSD (%, n = 6)	100	0.19	X < 2
Day 2, LC 2, analyst 2	RSD (%, n = 6)	100	0.22	X < 2

Accuracy/recovery study

Accuracy of the method was evaluated by fortifying a methyl salicylate sample solution (200 µg/mL) with three known concentrations of reference standard (50, 100, and 150 µg/mL). Percent recoveries were calculated form differences between the peak areas obtained for fortified and unfortified solutions. Good recoveries were obtained (Table 5). No significant differences were observed between amounts of methyl salicylate added and the amounts found ($p < 0.05$).

Table 5. Recovery studies of methyl salicylate from samples with known concentration

Sample #	Percent of nominal	Amount of analyte (µg/mL)		Recovery (%, $n = 3$)	RSD (%, $n = 3$)
		Added	Found		
1	50	5.0	4.98	99.6	0.09
2	100	9.0	8.98	99.7	0.12
3	150	13.0	12.99	99.9	0.15

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day variation). Repeatability was examined by analysing six determinations of the same batch of each component at 100% of the test concentration. The RSD of the areas of methyl salicylate peak were found to be less than 0.14% (Table 4), which confirms that the method is sufficiently precise. Intermediate precision (inter-day variation) was studied by assaying five samples containing the nominal amount of methyl salicylate on different days by different analysts using different LC systems. Solutions corresponding to each concentration level were injected in duplicate. The RSD values across the systems and analysts were calculated and found to be less than 0.23% (Table 4) for each of the multiple sample preparation, which demonstrates excellent precision for the method.

Specificity

The LC-PDA/UV isoplot chromatogram (Figure 5) demonstrates a good separation of the methyl salicylate. The isoplot chromatogram data consist of UV absorption spectra from 200 to 400 nm for each point along the chromatogram. Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the methyl salicylate. This result demonstrates (Figure 6) that there was no interference from the other materials in the cream formulation and, therefore, confirms the specificity of the method.

Forced degradation studies were performed to evaluate the specificity of methyl salicylate under four stress conditions (heat, UV light, acid, base). Solutions of methyl salicylate were exposed to 60°C for 1 h, UV light using a UVL-56 lamp for 24 h, acid (1 M hydrochloric acid) for 24 h and base (1 M sodium hydroxide) for 4 h. A summary of the stress results for retention time (TR), peak area, area percent, resolution (R_s) and column efficiency (theoretical plate numbers) is shown in Table 6. Under acid (major degradation) hydrolysis condition, the methyl salicylate content decreased and additional peak was observed (Figure 7). No degradation was observed under other hydrolysis conditions (heat, UV light and base) studied (Figure 7). The additional peak detected at 1.83 min under acid condition. This was further confirmed by peak purity analysis on a PDA UV detector. The methyl salicylate analyte obtained by acid

hydrolysis was well resolved (5.82 min) from the additional peak (Figure 6), indicating the specificity of the method.

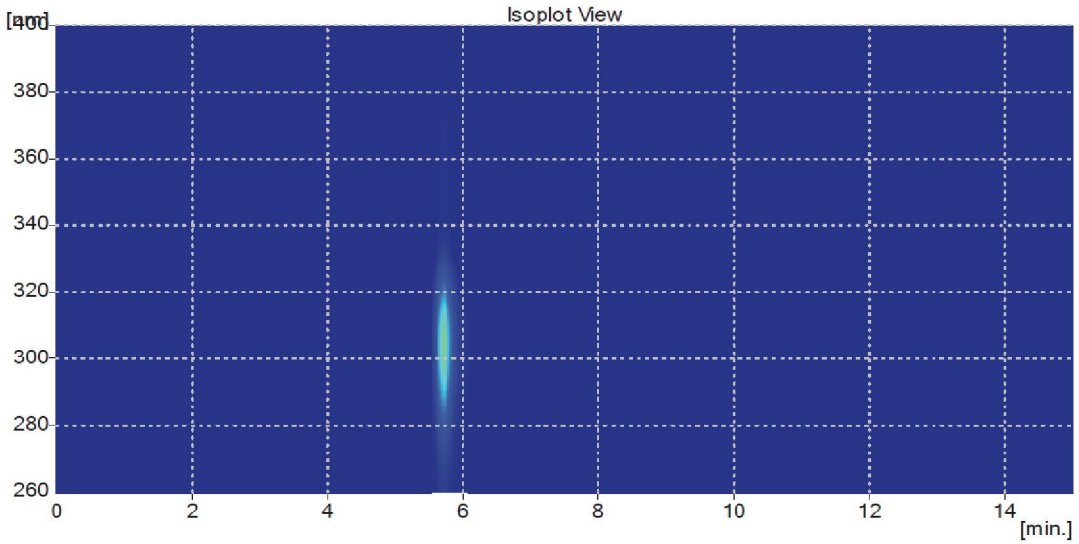

Figure 5. LC PDA/UV isoplot chromatogram of methyl salicylate.

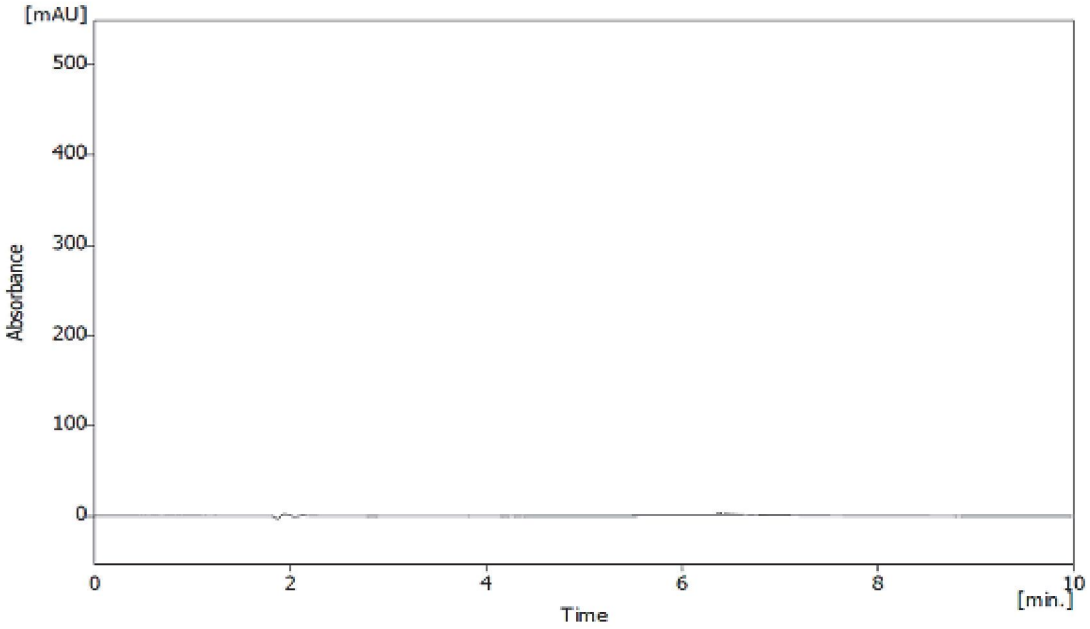

Figure 6. LC chromatogram of placebo.

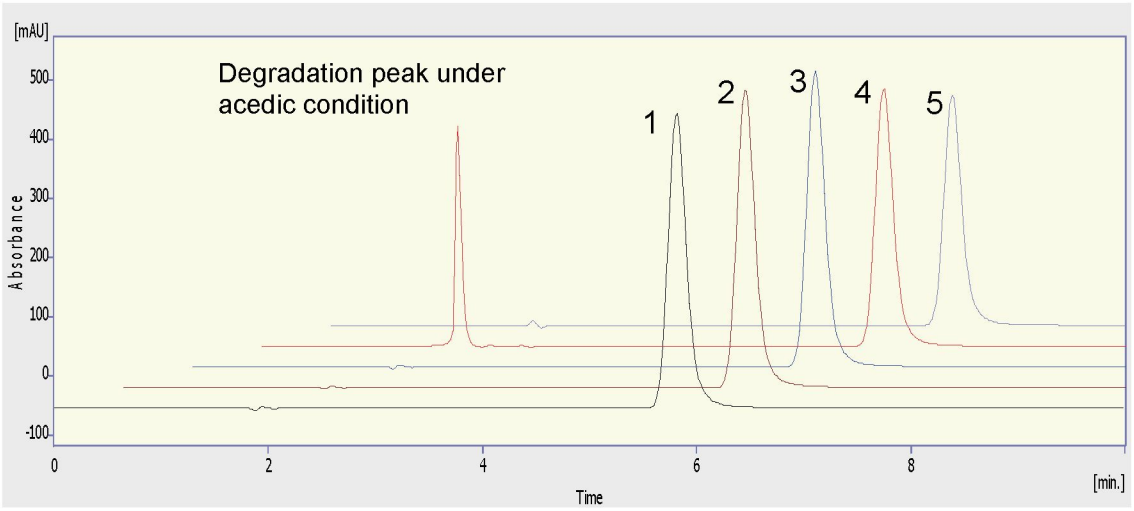

Figure 7. HPLC chromatograms of methyl salicylate obtained under stress conditions (1) fresh reference standard; (2) heat at 60°C; (3) UV light; (4) acid; (5) base.

Table 6. Force degradation studies data for methyl salicylate

Stress conditions	Sample treatment	RT (min)	Area (mAU.s)	Assay (%)	R_s	Efficiency (th.pl)
Reference	Fresh solution	5.82	6234.93	99.72	-	4686
Heat	60 °C for 1 h	5.80	6364.56	98.94	-	4659
Light	UV Light for 24 h	5.82	6319.64	98.87	-	4686
Acid	1M HCl for 24 h	5.82	5354.81	75.6	5.322	4686
		1.83*	1637.44	24.4	-	4190
Base	1M NaOH for 4 h	5.80	4895.87	98.95		5545

* Degradation peak

Limits of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of methyl salicylate was determined based on standard deviation (σ) of response and slope (s) (10). Methyl salicylate solutions were prepared in the range 0.02-25 µg/mL and injected in triplicate. Average peak area of analyte was plotted against concentration. LOD and LOQ were calculated by using the following equations: LOD = (3.3 σ)/s, LOQ = (10 σ)/s.

The LOD was determined to be 2.4 µg/mL and LOQ was found to be 14 µg/mL for methyl salicylate with CV less than 2% for six replicate injections.

CONCLUSION

A new reversed-phase liquid chromatographic method with UV spectrophotometric detection was developed for the determination of methyl salicylate in medicated cream formulation. The method was validated and the results obtained were accurate and precise with RSD < 1% in all cases and no significant interfering peaks were detected. The method is specific, simple, selective, robust and reliable for routine use in quality control analysis of methyl salicylate raw materials, bulk samples and final medicated cream product release.

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