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Simultaneous Determination of Methyl Nicotinate and Three Salicylic Acid Derivatives in Pain Relief Spray Using HPLC–DAD

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Abstract: For the first time, the high-performance liquid chromatography–diode array detector (HPLC–DAD) approach was operated for the simultaneous assessment of methyl nicotinate (MN), methyl salicylate (MS), ethyl salicylate (ES) and 2-hydroxyethyl salicylate (HES) in one pharmaceutical formulation. The limits of detection of MN, HES, MS and ES were found to be 0.0144, 0.0455, 0.0087 and 0.0061 μg/mL. The recovery percentages and relative standard deviations ranged from 93.48 to 102.12% and 0.301 to 6.341% for all active ingredients. Accordingly, the previously described data demonstrate the sensitivity, accuracy and precision of the developed method. Therefore, the investigated approach was effectively applied for the simultaneous assessment of MN, HES, MS and ES in DEEP HEAT Spray.

Keywords: methyl nicotinate; methyl salicylate; ethyl salicylate; 2-hydroxyethyl salicylate; pain relief spray

1. Introduction

Esters of salicylic acid such as methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate (Figure 1) are used as analgesic and rubefacient in many topical creams and sprays for the relief of muscle and joint pain [1–7]. Methyl nicotinate is methyl ester of nicotinic acid (Figure 1), has a vasodilator property, enhances the topical penetration of active ingredients in cream and sprays and also has an effective role for relief of pain and aches in joints, tendons and muscles [8,9].
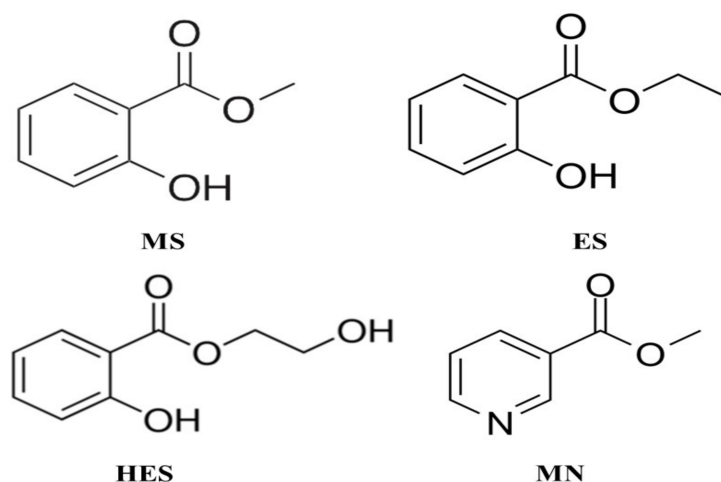

Figure 1. Chemical structures of methyl salicylate (MS), ethyl salicylate (ES), 2-hydroxyethyl salicylate (HES) and methyl nicotinate (MN).

Therefore, the presence of methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate and methyl nicotinate in one formulation enhances their efficiency for pain relief [2].

To the best of our knowledge, the literature contains a few methods for the individual determination of methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate and methyl nicotinate in different samples. High performance liquid chromatography (HPLC) was used for the individual assessment of methyl salicylate and methyl nicotinate in pharmaceutical formulations and medicinal plants [2,8,10,11], while gas chromatography mass spectrometry (GC–MS) was utilized to estimate methyl salicylate and ethyl salicylate in biological fluids [2,12].

Only one research paper published by Pauwels et al. [2] focuses on the determination of methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate in one topical formulation by using two chromatographic techniques. The two methods used by Pauwels et al. [2] included the use of a gas chromatography flame ionization detector (GC-FID), which was applied for the simultaneous assessment of methyl salicylate and ethyl salicylate, while a liquid chromatography ultraviolet detector (LC-UV) was used for the determination of 2-hydroxyethyl salicylate.

Therefore, the proposed work presents the first approach for the simultaneous assessment of methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate and methyl nicotinate in one tropical formulation based on the high-performance liquid chromatography supplied with a diode array detector (HPLC–DAD). Besides, the separation efficiency, simplicity, sensitivity, reliability and total analysis time of the investigated approach for the assessment of the four analytes will be evaluated for use in quality control protocol as well as pharmacokinetic studies.

2. Materials and Methods

2.1. Instrument

Thermo Scientific Dionex UltiMate 3000 UHPLC connected to DAD-3000 diode array detector was adjusted for the assessment of MN, HES, SA, MS and ES in solutions. The analysis data were recorded via a Chromeleon™ 7.2 Chromatography Data System. A hypersil GOLD column (250 mm length, 4 mm inner diameter, 5 μm particle size (Thermo Scientific, Waltham, MA, USA) was used for the separation of analytes.

2.2. Chemicals and Materials

MN, HES, MS, ES, salicylic acid (SA), acetonitrile, formic acid and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). A Barnstead™ Smart2Pure™ water purification system was used for deionized water production.

DEEP HEAT Spray (150 mL) contains 1.6% of MN, 5% HES, 1% of MS and 5% of ES, its manufacturer and marketing authorization holder is the Mentholatum Co. Ltd., East Kilbride, Scotland, UK, and it was purchased from local community pharmacies in Saudi Arabia.

2.3. Preparation of Stock Solutions

MN, HES, MS, ES and SA (as internal standard) stock solutions were made in 50 mL of HPLC grade methanol at a concentration of 1000 μg/mL and diluted to the needed concentration using the same solvent to prepare working solutions.

2.4. Preparation of Spray Solution

In total, 2.0 mL of spray content were transferred to 50 mL glass flask and diluted to the mark by HPLC grade methanol. MN, HES, MS and ES working solutions of 14.57, 45.55, 9.11 and 45.55 μg/mL, respectively, in presence of 0.5 μg/mL of SA internal standard, were prepared after a series of dilutions for the prepared spray solution.

2.5. Chromatographic Conditions

The separation of MN, HES, SA, MS and ES was achieved through a Hypersil GOLD column (250 mm length, 4 mm inner diameter, 5 μm particles size (Thermo Scientific, Waltham, MA, USA). The composition of mobile phase was 50% methanol: 50% acetonitrile (A) and water acidified with formic acid (0.1%) (B) in the volume percent of 70:30 (*v/v*) at a flow rate of 0.5 mL/min with an isocratic elution mode. The mixture components were detected at a wavelength of 210 nm and at room temperature (25 °C). The injection volume of standard solutions and samples was 10 μL.

2.6. Validation of Assay Approach

The precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), linearity and system suitability variables were used to validate the rapid identification of MN, HES, MS and ES using the HPLC approach. The linearity of developed method was tested by using a series of concentration levels of MN, HES, MS and ES standards ranging from 0.03–100, 0.05–50, 0.03–50 and 0.03–50 μg/mL, respectively, in presence of SA as internal standard. Accuracy of the method was calculated for the five concentration levels (0.07, 0.5, 1, 5, 20 μg/mL) of MN, HES, MS and ES in triplicate by using the following formula: recovery % = determined value/added value × 100. Intra-day and inter-day precisions were evaluated for the mentioned above concentration levels of each component in triplicate by estimating the relative standard deviation (RSD%) = (σ/mean determine concentration) × 100, where σ is a standard deviation of intercept). Limit of quantification (LOQ), and limit of detection (LOD) of MN, HES, MS and ES were calculated from linear regression equations dependent on the slope and standard deviation of the intercept via applying the following formula: LOD = 3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation of intercept and S is the slope of the calibration curve. The system suitability was evaluated by calculating selectivity factor (α), resolution (R_s), capacity factor (K'), column efficiency (N), tailing factor (T) and height equivalent to theoretical plate (HETP).

3. Result

3.1. Development and Optimization Processes

Several chromatographic experiments were tested to obtain the HPLC chromatograms with the best separation and resolution of MN, HES, MS and ES peaks in a short time of analysis, e.g., mobile phase composition, elution mode, rate of flow, kind of column, temperature of column and recognition wavelength. Three distinct columns, including Thermo Scientific ACCLAIM™ 120 C8 (4.6 × 150 mm, 5 μm), Hypersil GOLD (4 × 250 mm, 5 μm) and ACCLAIM™ 120 C18 (4.6 × 150 mm, 5 μm), were tested at different temperatures. Finally, the Hypersil GOLD (4 × 250 mm, 5 μm) column and 25 °C were found to be the best column and temperature for the separation and determination of the MN, HES, MS and ES mixtures. Furthermore, the mobile phase compositions were examined using a variety of solvents (methanol and acetonitrile), acids (formic acid, acetic acid and phosphoric acid) and buffers as well as elution mode and flow rate. The suitable separation and resolution were achieved by the isocratic elution mode using 50% methanol and 50% acetonitrile (A) and water acidified with formic acid (0.1%) (B) in the volume percent of 70:30 (*v/v*) at a flow rate of 0.5 mL/min with an isocratic elution mode. On the other hand, the UV detector was adjusted at 210 nm in order to get the desired sensitivity for MN, HES, MS and ES. Under these conditions, separation of MN, HES, MS and ES was carried out in 11 min and retention times were 5.57, 6.03. 8.09, and 10.04 min, respectively, as shown in Figures 2a and S1. The good separation of MN, HES and SA was evidenced by focusing the time range on the three components only as displayed in Figure 2b.
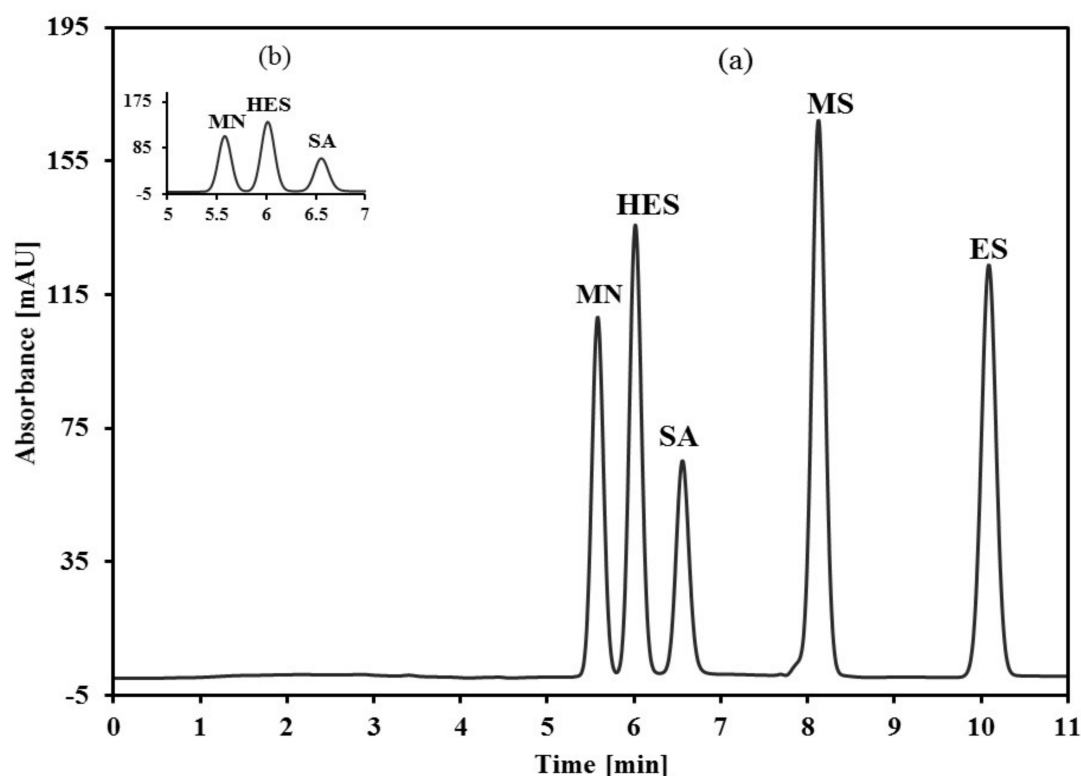
Figure 2. Chromatogram for MN, HES, SA, MS and ES at optimum conditions. (**a**) complete chromatogram; (**b**) the enlarged view of the scale range from 5 to 7 min.

3.2. Method Validation

The ICH recommendations [13] were applied for validation for the efficiency of the HPLC approach.

3.2.1. Linearity and Calibration Curve

The linear range and calibration curve equation of MN, HES, MS and ES were described in Table 1. Correlation coefficient values exceeded 0.99, as listed in Table 1.

Table 1. The results of regression equations, LOD and LOQ.

Analyte	Regression Equation	R	Linear Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
MN	Y = 0.968 x − 0.713	0.999	0.03–100	0.0144	0.0478
HES	Y = 2.485 x + 0.315	0.996	0.05–50	0.0455	0.1516
MS	Y = 3.151 x + 0.083	0.998	0.03–50	0.0087	0.0289
ES	Y = 2.926 x + 0.579	0.998	0.03–50	0.0061	0.0204

3.2.2. LOD and LOQ Assessment

The sensitivity of the investigated HPLC method toward MN, HES, MS and ES was confirmed by the calculation of LOD and LOQ. According to the formulas mentioned above, the LOD and LOQ of MN, HES, MS and ES were depicted in Table 1. The LOD and LOQ values of each component refer to the great sensitivity of the investigated approach when compared with the reported methods [2,8,10–12].

3.2.3. Accuracy and Precision

Accuracy and precision of the suggested HPLC approach for the assessment of MN, HES, MS and ES were calculated according to the formulas mentioned above by testing five concentration levels of each component (0.07, 0.5, 1, 5 and 20 µg/mL) for three replicates as

depicted in Table 2. The recovery (%) and relative standard deviation (RSD%) were found in the range from 93.48 to 102.12% and 0.301 to 6.341%, respectively. The obtained results of intra- and inter-day assays were within the accepted limits.

Table 2. Accuracy and precision of the proposed approach for the assessment of MN, HES, MS and ES.

Analytes	Conc. Added (µg/mL)	Intra-Day			Inter-Day		
		Conc. Found (µg/mL) ± SD	Recovery (%)	RSD (%)	Conc. Found (µg/mL) ± SD	Recovery (%)	RSD (%)
MN	0.07	0.068 ± 0.002	97.25	2.672	0.067 ± 0.003	95.38	3.979
	0.5	0.510 ± 0.008	101.91	1.653	0.496 ± 0.011	99.22	2.115
	1	1.007 ± 0.059	100.68	5.886	1.006 ± 0.035	100.62	3.519
	5	5.099 ± 0.194	101.97	3.810	5.018 ± 0.205	100.36	4.082
	20	19.853 ± 0.073	99.263	0.369	19.85 ± 0.061	99.23	0.301
HES	0.07	0.068 ± 0.001	97.29	1.380	0.069 ± 0.004	97.95	6.341
	0.5	0.508 ± 0.013	101.68	2.532	0.503 ± 0.023	100.59	4.571
	1	0.982 ± 0.049	98.180	5.028	1.012 ± 0.033	101.21	3.218
	5	4.80 ± 0.091	96.08	1.890	4.719 ± 0.211	94.38	4.467
	20	20.06 ± 0.180	100.28	0.897	19.614 ± 0.709	98.07	3.616
MS	0.07	0.070 ± 0.001	100.50	1.421	0.069 ± 0.002	98.73	2.794
	0.5	0.503 ± 0.007	100.61	1.445	0.473 ± 0.008	94.58	1.782
	1	1.021 ± 0.011	102.12	1.121	0.943 ± 0.011	94.27	1.129
	5	4.915 ± 0.034	98.29	0.683	4.674 ± 0.041	93.48	0.861
	20	19.982 ± 0.086	99.91	0.429	20.082 ± 0.092	100.41	0.461
ES	0.07	0.068 ± 0.002	97.02	2.949	0.067 ± 0.002	96.23	2.786
	0.5	0.510 ± 0.008	102.05	1.637	0.492 ± 0.005	98.47	0.971
	1	0.993 ± 0.018	99.33	1.838	1.008 ± 0.012	100.76	1.191
	5	7.828 ± 0.076	96.56	1.580	4.871 ± 0.046	97.39	0.951
	20	19.786 ± 0.154	98.93	0.776	19.871 ± 0.348	99.36	1.749

3.2.4. System Suitability Testing (SST)

Several SST parameters were measured including selectivity factor (α), resolution (Rs), capacity factor (K′), column efficiency (N), tailing factor (T) and height equivalent to theoretical plate (HETP) to check and ensure ongoing HPLC system performance for the simultaneous determination of MN, HES, MS and ES. As depicted in Table 3, the values of Rs, α, T, K′, N, HETP ranged from 3.195 to 15.96, 1.16 to 1.41, 0.941 to 1.03, 1.05 to 2.50, 8543 to 17918 and 0.00008 to 0. 002, respectively. These values were found to be within the recommended limits [7], suggesting the accessibility and efficiency of the investigated HPLC approach for the determination of the four analytes.

Table 3. System suitability testing parameters of the proposed HPLC method.

Parameters	Obtained Value				Reference Value [13]
	MN	HES	MS	ES	
Resolution (R_s)	3.195	15.96	12.125	9.60	<1.5
Selectivity factor (α)	1.16	1.17	1.41	1.37	< 1
Tailing factor(T)	0.941	0.947	1.00	1.03	>1.5–2 or >2
Capacity factor(K′)	1.05	1.30	1.82	2.50	1–10 acceptable
Column efficiency (n)	8543	13877	17918	17479	Increase with efficiency of the separation
HETP [b]	0.002	0.001	0.0008	0.00008	The smaller the value the higher the column efficiency

HETP [b] = height equivalent to theoretical plate, (cm/plate).

3.3. Application of the Method

The investigated HPLC–DAD approach was successfully operated for the simultaneous assessment of MN, HES, MS and ES in DEEP HEAT Spray. The values of the recovery percentage of MN, HES, MS and ES, ranged from 92.04% to 101.14% with the standard deviation not exceeding 0.56% as depicted in Table 4, support this point.

Table 4. Analysis of MN, HES, MS and ES in Deep Heat Spray by the proposed HPLC method.

Component	Taken (μg/mL)	Recovery %
MN	14.57	97.88 ± 0.01
HES	45.55	92.04 ± 0.56
MS	9.11	101.14 ± 0.13
ES	45.55	94.39 ± 0.40

4. Conclusions

For the first time, an unsophisticated, dependable, accurate and precise HPLC approach was established for the simultaneous determination of methyl nicotinate, methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate in one formulation. In addition, the investigated method has the advantage of eluting the four analytes in a short analytical run time. The recovery percentages and relative standard deviations ranged from 93.48 to 102.12% and 0.301 to 6.341% for all analytes. As a result, the proposed quantitative approach can be used successfully for quality control laboratories and routine analysis of the methyl nicotinate, methyl salicylate, ethyl salicylate and 2-hydroxyethyl salicylate.

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