Acta Poloniae Pharmaceutica – Drug Research, Vol. 60 No. 5 pp. 343–349, 2003

ISSN 0001-6837
Polish Pharmaceutical Society

VALIDATION OF A METHOD FOR SIMULTANEOUS DETERMINATION OF MENTHOL AND METHYL SALICYLATE IN PHARMACEUTICALS BY CAPILLARY GAS CHROMATOGRAPHY WITH COOL ON–COLUMN INJECTION

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Abstract: The conditions for the identification and quantitative determination of menthol and methyl salicylate in ointment Balsamum Mentholi Compositum on a hydrocarbon–ester base (vaseline–lanolin), have been established by using capillary gas chromatography with cool on–column injection and flame ionization detection (FID). The good separation of menthol (t_R = 7.2 min), methyl salicylate (t_R = 8.7 min) and thymol (t_R = 12.3 min) and camphor (t_R = 6.0 min), used as alternative internal standards, beside vehiculum constituents (peak of t_R = 15.8 min) was obtained. The method features a high sensitivity – detection limit for menthol and methyl salicylate was 0.1 ng and 5.0 ng, respectively, high accuracy, precision and recovery for active substances: 100.0% ± 2.2%, when camphor was used as an internal standard.

Keywords: menthol, methyl salicylate, capillary gas chromatography, cool on–column injection, drugs analysis, matrix constituents.

Both menthol (M) and methyl salicylate (MS) are active substance in many medicines commonly used in treatment of rheumatic diseases due to 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:M is 8:1 in the medicine under examination). Thus, separation methods are recommended in analysis of these constituents. Among these methods gas chromatography (GC) is preferred. The gas chromatography method was used for determining menthol and methyl salicylate in solid and liquid medicines (2–5), natural products (7, 8), and biological material (9, 10).

Another problem is that menthol and methyl salicylate are used mostly in ointments prepared on hydrocarbon (paraffin jelly) and ester (lanolin) bases. In previous paper (11) it has been proven that GC with cool on–column injection can be suitable for simultaneous identification and determination of active substances as well as purity evaluation in the presence of matrix constituents, since such an injection method improves the stability of injected samples, thus decreasing also the number of peaks recorded on chromatograms.

Therefore, an attempt has been made to establish conditions for identification and determination of active substances in the presence of base constituents, while considering its effect on the results obtained for an ointment containing menthol and methyl salicylate (12), by using capillary gas chromatography with cool on–column injection.

EXPERIMENTAL

Apparatus

Gas chromatograph: TRACE GC 2000 Series, CE Instruments Thermo Quest (Rodano, Italy), equipped with a FID detector (3 pg C/s, linearity 10^6), two injectors: split–splitless and cool on–column.

Injection port: cool on–column (cold on–column), secondary cooling time 0.2 min.

GC oven temperature program: from 70°C (for 1 min) up to 250°C (6 min) at a rate of 10°C/min.

FID: base body temperature 275°C.

Capillary column: WCOT Fused Silica of 25 m in length and 0.25 mm in inner diameter with the stationary phase CP–WAX 58 (FFAP)–CB of 0.2 μm in film thickness, Chrompack (Middelburg, Netherlands), catalogue no. 717;
with retention gap: 1 m, 0.50 mm ID, of uncoated fused silica tubing, PRECOL.F.S.0.05MM–MT2.MONT, CE Instruments (Rodano, Italy).

Syringe: of 10 μl in capacity and 80 mm in needle length, Hamilton (Bonaduz, Switzerland), model no. 701. Injected samples volume: 1.0 μl

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Computer: PC, Pentium 266 MHz MMX, 32 MB RAM, Adax Land-JTT Computers (Krakow, Poland).

The software: Chrom Card for TRACE ver. 1.06 for data acquisition, calculations and chromatograms registration: Microsoft Office 97 Standard, Statistica 5.1 edition '97.

(e) Paper filters: FILTRAK® No. 390, φ 9 cm (Niederschlag, Germany)

Reagents and Chemicals

Gases:

Carrier gas: helium of purity class 5.0, BOC Gazy (Siewierz, Poland), additionally passed through the filter OT3–2, R & D OXYGEN/MOISTURE TRAP, R & D SEPARATIONS.

The chromatograms were recorded at constant flow of carrier gas of 1.0 ml/min (35 cm/sec) as a mode of the mobile phase flow in duration of all the GC oven program.

Gases fed to the detector: synthetic air: Synthetische Luft KW–Frei 20,0000% Sauerstoff (350 ml/min); hydrogen (35 ml/min) and nitrogen (make-up gas, 30 ml/min) of purity class 5.0, Linde Gaz Polska (Cracow, Poland).

Standard substances:

(–)–Menthol, puriss., 99.9%; Fluka (Buchs, Schweiz), product No. 63660, serial No. 397642/1; Methyl salicylate, 99.8%, SIGMA, M2047, 108H0121. DL–Camphor, cat. No. 841456, Merck KgaA (Darmstadt, Germany); Thymol cryst. extra pure, cat. No. 108167, Merck KgaA (Darmstadt, Germany); anhydrous lanolin and paraffin jelly according to the Polish Pharmacopoeia (FP V); Methanol gradient grade for liquid chromatography, LiChrosolv® Merck KGaA (Darmstadt, Germany).

Table 1. The validation parameters and results with statistical assessment

Method			Results		
Parameter	Internal standard	Measured for...	Mean, x	Standard deviation, σ	Relative standard deviation, RSD (%)
Retention time, t_R, (min) n = 25	T	M	7.17	0.013	0.17
		MS	8.68	0.015	0.17
		T	12.29	0.013	0.11
	C	C	6.01	0.012	0.20
		M	7.19	0.012	0.17
		MS	8.74	0.025	0.28
Relative retention time, RRT n = 25	T	M/T	0.58	0.00045	0.08
		MS/T	0.71	0.00060	0.08
	C	M/C	1.20	0.00104	0.09
		MS/C	1.45	0.00326	0.22
Peak height, H ($\mu V \cdot 10^5$) n = 12	T	M	1.06	0.042	3.99
		MS	1.76	0.142	8.06
		T	3.14	0.106	3.37
	C	C	2.94	0.315	10.71
		M	0.63	0.058	9.16
		MS	1.65	0.069	5.08
Peak area, A ($\mu V \cdot sec \cdot 10^6$) n = 12	T	M	0.50	0.017	3.38
		MS	2.53	0.079	3.13
		T	1.10	0.055	4.97
	C	C	0.88	0.037	4.25
		M	0.40	0.020	5.10
		MS	2.29	0.067	2.93
Resolution, R n = 12	T	M/MS	7.3	0.43	5.96
	C	M/MS	6.2	0.33	5.32

n – number of analyses taken into account

M – menthol, MS – methyl salicylate, C – camphor, T – thymol

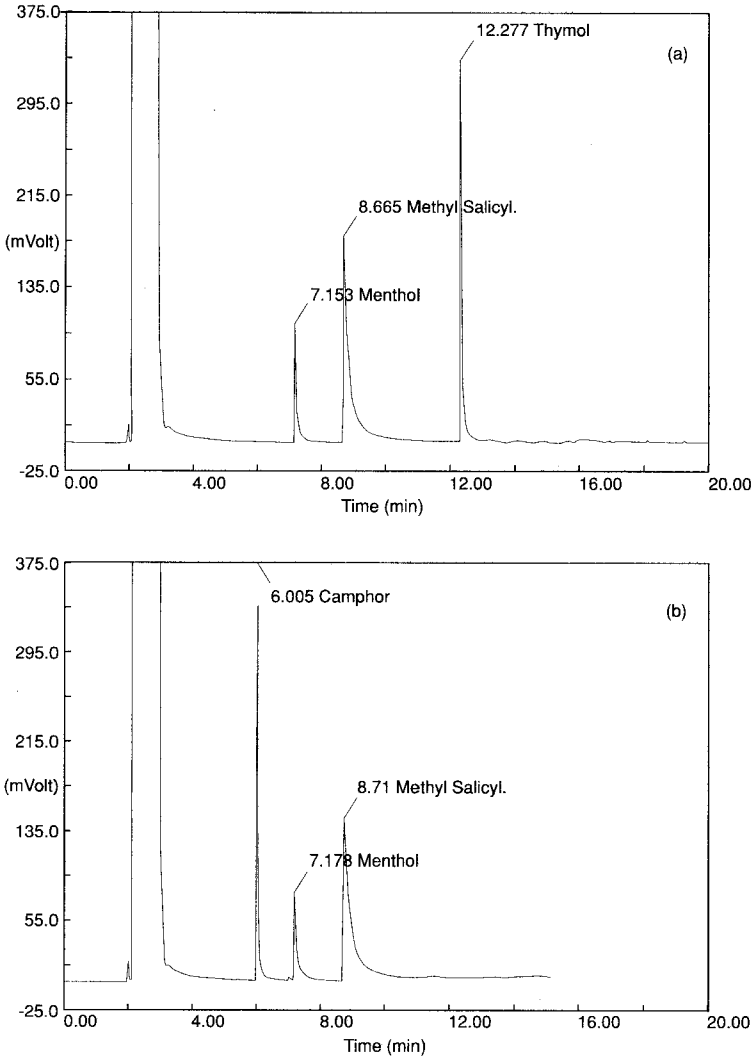

Figure 1. Chromatograms obtained for the analysed ointment extracts with internal standard: (a) thymol, (b) camphor added.

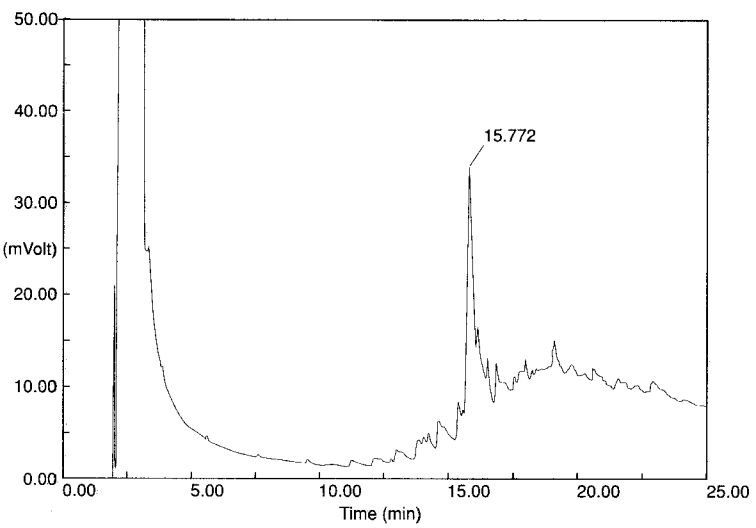

Figure 2. Chromatogram recorded for an extract containing the base constituents, i.e. lanolin and paraffin (1:1).

Preparation:
Ointment Balsamum Mentholi Compositum containing: menthol 2.5 g, methyl salicylate 20.0 g, anhydrous lanolin and paraffin jelly (1:1) and 100.0 g prepared in accordance with the Polish Pharmacopoeia (FP V). The constituents have been weighed with an accuracy of 0.1 mg.

Standard solutions
Solutions of menthol, methyl salicylate as well as camphor and thymol (internal standards) in methanol; concentration 1.00% w/v:
100 mg of each substance was weighed with the accuracy of 0.1 mg, dissolved and filled with methanol up to 10 ml;
Solutions of auxiliary constituents (lanolin and paraffin jelly) in methanol:
50 mg of each substance was weighed with the accuracy of 0.1 mg, heated in a flask of 50 ml capacity under a reflux condenser for 15 min at the methanol boiling point, cooled, filtered and filled with methanol up to 25 ml.

Preparation of solutions
The solutions were prepared similarly as those of auxiliary constituents, by weighing 525 mg of ointment with the accuracy of 0.1 mg.

Sample solutions for examinations
For calibration:
Standard solutions, respectively: 50.0 µl of menthol solution, 400 µl of methyl salicylate and 100 µl of internal standard (thymol or camphor) were taken and filled with methanol up to 10 ml.
For analyte determining:
800 µl of preparation solution were taken and 100 µl of internal standard solution (thymol or camphor) were added and filled with methanol up to 10 ml.
For recovery determining:
400 µl of preparation solution were taken, standard solutions of 20.0, 25.0 or 30.0 µl menthol solution, 160, 200 or 240 µl of methyl salicylate and 100 µl of thymol or 75.0 µl of camphor solutions as an internal standard were added and filled with methanol up to 10 ml.

RESULTS

Effect of parameter variations on the results of measurements (13,14).
The effect was investigated when establishing the conditions for chromatographic analysis.
The following procedure has been taken into account: i.e. sample preparation, analysis duration, effect of temperature and the mobile phase flow or pressure.

For this purpose, standard solutions and preparation solutions were injected into the column separately and mixed with internal standards. The experiments were carried out at fixed carrier gas flow rate or pressure, in isothermal or programmed mode of chromatograph oven at temperatures ranging from 40°C to 270°C. The temperature rise rate was changed from 5 to 20°C/min, while changing the initial and final isotherm durations and the secondary cooling time of cool on–column injector was 0.05–0.30 min. The volumes of 0.5 to 1.5 µl were injected. Each measurement cycle was preceded by checking the system tightness, column evaluation coefficient and baseline stability. The retention time, area and height of peaks used for establishing the measurement conditions were recorded.
The estimated values of recorded parameters are listed in Table 1. Examples of chromatograms are presented in Figure 1.

Selectivity and specifity of the method
The effect of base constituents on the results of menthol and methyl salicylate determination was investigated. To do it, the preparation extracts (Figure 1) and base constituents (Figure 2) were analysed and chromatograms were recorded. No peaks of retention times typical of menthol, methyl salicylate and camphor or thymol, used as internal standards, were found in Figure 2, whereas an additional peak of approximate retention time of 15.8 min was visible.

Accuracy and precision
The accuracy of the method was estimated by the values of relative error (in %) for the results of analyses performed for samples containing the same amounts of analytes (Table 3) and for recovery (in %) on model mixtures composed of the specified amount of preparation to which 80%, 100% or 120% of constituents under investigation were added (Table 2).
The precision was defined by indicating the absolute standard deviation (σ) and relative standard deviation (%RSD), (Table 3), assuming that the result of individual determination $Y = \bar{x} \pm 2\,\sigma$ (confidence interval μ = 95%).

Linearity
The chromatograms of two–component standard solutions were recorded and the changes of peak areas (A) for individual constituents were analysed (in the range 60–150% of examined analytes concentration), within the concentrations of 3.0 to 7.5 mg/100 ml for menthol and 24.0–60.0 mg/100 ml for methyl salicylate.

Table 2. Recovery of menthol and methyl salicylate

Internal Standard	Thymol				Camphor			
ANALYTE	METHYL SALICYLATE		MENTHOL		METHYL SALICYLATE		MENTHOL	
Calculation basis	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area
Added (%)	80 100 120				80 100 120			
Expected (mg)	0.45 0.50 0.55		3.60 4.00 4.40		0.45 0.50 0.55		3.60 4.00 4.40	
Found, mean (mg; n = 3)	0.37±0.02 0.44±0.02 0.49±0.03	0.43±0.002 0.48±0.003 0.53±0.005	3.06±0.19 3.71±0.16 4.89±0.29	3.48±0.02 3.80±0.01 4.58±0.03	0.44±0.006 0.51±0.001 0.57±0.042	0.45±0.004 0.49±0.002 0.53±0.001	3.57±0.20 3.76±0.09 4.94±0.06	3.36±0.015 3.87±0.004 4.87±0.012
Relative Std Deviation (%)	6.13 4.97 6.70	0.47 0.70 1.00	6.14 4.41 5.92	0.63 0.29 0.74	1.45 0.21 0.72	0.87 0.48 0.88	6.28 2.34 3.21	0.46 1.10 0.26
Recovery (%)	80.0 88.0 89.1	95.6 96.0 96.4	85.0 92.5 110.9	96.7 95.0 104.1	98.4 103.7 104.5	100.3 97.8 96.2	99.1 93.9 112.3	93.4 96.8 106.1
Recovery, mean (%)	87.7	96.0	96.1	98.6	102.2	98.1	101.8	98.8

The results were analysed by using the linear regression method. The obtained 7–points curves are characterized by a 95% confidence interval and:

for menthol: $A = 1237 \cdot 10^6 \cdot c - 972 \cdot 10^3$, correlation $r = 0.97667$,

for methyl salicylate: $A = 8177 \cdot 10^5 \cdot c - 284 \cdot 10^4$, correlation $r = 0.97737$,

when: $[A]$ = 0.1·μV·sec, $[c]$ = %w/v

Limits of detection and quantitation

The chromatograms of one–component standard solutions were recorded and the ratio of detector signal (peak height), for a sample containing the specified amount of analyte, to the baseline noise level was analysed.

The limit of detection was assumed to be the amount of analyte for which the signal to baseline noise ratio was 3 or more, while for the limit of quantitation – was not less than 6.

The limits of detection were 0.1 ng for menthol and 5.0 ng for methyl salicylate. The limits of quantitation were 0.25 ng and 10.0 ng, correspondingly.

The results presented above enabled the basic conditions for measurements and quantitative determination method to be set.

Quantitative analyses

Chromatographic analysis conditions:

The 1.0 μl of samples were injected with a syringe into the capillary column through the cool on–column injector at fixed secondary cooling time: 0.20 min; The samples were injected by using the air plug technique (15) with control of the injecting volume before and after injection.

Carrier gas flow rate fixed at 1.0 ml/min (35 cm/sec).

Chromatograph oven temperature program: isotherm 70°C (duration: 1 min), temperature rise at 10°/min to 250°C (6 minutes).

Detector: maximum sensitivity, base body temperature 275°C; hydrogen 35 ml/min, air 350 ml/min, nitrogen (make–up) 30 ml/min.

Internal standard method. Internal standards: thymol or camphor.

Calibration method: multilevel, based on averaged RF – detector response factors, calculated for integrated peak areas, A (RF_A) or peak heights, H (RF_H) of menthol and methyl salicylate. (The appropriate standard sample solutions containing analysed constituents as well as thymol or camphor as an internal standard were injected into the column.)

Table 3. Quantification of menthol and methyl salicylate in ointment under examination. Results.

Internal Standard	Thymol				Camphor			
ANALYTE	MENTHOL		METHYL SALICYLATE		MENTHOL		METHYL SALICYLATE	
Calculation basis	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area
Multilevel Calibration								
Averaged RF, n = 6	0.720	0.938	0.154	0.604	0.482	1.071	0.134	0.646
Minimum	0.617	0.866	0.131	0.571	0.457	1.031	0.123	0.618
Maximum	0.829	1.026	0.169	0.630	0.508	1.115	0.144	0.671
Standard Deviation	0.0666	0.0661	0.0169	0.0259	0.0180	0.0333	0.0081	0.0179
Confidence level 95%	0.0533	0.0529	0.0135	0.0207	0.0144	0.0266	0.0065	0.0143
Relative Std Deviation (%)	9.26	7.04	10.95	4.29	3.74	3.11	6.02	2.77
Content (mg)								
ANALYTE	MENTHOL		METHYL SALICYLATE		MENTHOL		METHYL SALICYLATE	
Calculation basis	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area	Peak Height	Peak Area
Expected	0.50	0.50	4.00	4.00	0.50	0.50	4.00	4.00
Found, mean (n = 6)	0.459	0.459	3.526	3.633	0.490	0.499	3.725	3.807
Minimum	0.430	0.456	3.344	3.614	0.477	0.486	3.611	3.688
Maximum	0.486	0.461	3.774	3.657	0.497	0.518	3.862	3.883
Standard Deviation	0.0163	0.0015	0.1340	0.0154	0.0068	0.0121	0.0963	0.0966
Confidence level 95%	0.0130	0.0012	0.1072	0.0123	0.0054	0.0110	0.0770	0.0773
Relative Std Deviation (%)	3.55	0.34	3.80	0.51	1.38	2.43	2.59	2.53
Rel. Error (%)	8.2	8.2	11.85	9.18	0.50	0.60	6.88	4.83

$$RF_A = (A_i \cdot c_{IS}) / (A_{IS} \cdot c_i)$$

A – peak area, H – peak height, c – concentration, $_i$ – analyte, $_{IS}$ – internal standard

Then, the chromatograms of analysed preparation sample solutions with internal standard added were recorded. The concentrations of analytes were computed from the following equation:

$$c_i = (A_i \cdot v_{IS}) / (A_{IS} \cdot v_S \cdot RF_A)$$

where v_{IS}/v_S is the ratio of internal standard volume to the total sample volume, v_S – sample.

The results of determination are shown in Table 3.

DISCUSSION

The chromatograms of analysed preparation sample solutions (Figure 1) show the peaks of active substances – menthol (t_R approx. 7.2 min) and methyl salicylate (t_R approx. 8.7 min), and internal standards used – camphor (t_R approx. 6.0 min) or thymol (t_R approx. 12.3 min). The retention times of menthol and methyl salicylate and internal standards differ suffi-

ciently for identification and quantitative analysis purposes.

The relative retention time (RRT) of menthol and methyl salicylate with respect to camphor or thymol are within ±0.5% (relative standard deviation, $\%RSD_{RRT} < 0.5$) (Table 1).

The chromatogram of the auxiliary medicine constituents (Figure 2) indicates, however, that their presence may have an effect on the results of determination, in particular quantitative, for components of retention times exceeding 10 min. Above this limit, as follows from Figure 3, a rising tendency of baseline is clearly visible. The baseline becomes more irregular and only one peak of retention time approx. 15.8 min is well developed.

This problem might refer mainly to thymol with a retention time of 12.3 min and the results obtained for the preparation extracts by using thymol as an internal standard. The solutions of ointment extracts subjected to quantitative analysis had concentrations of base constituents several times lower than the extract presented in Figure 2. Thus, an additional peak mentioned above is not present on their chromatograms (Figure 1). This, however, did not eliminate completely the effect of detector signal rise and baseline irregularities above 10 min on chromatograms recorded for the analysed extracts. This could have an effect on the results of quantitative determination.

In the next step, the method for quantitative determination of menthol and methyl salicylate was validated. It has been found that the method features a high detection and quantitation of analytes. The limit of detection was 0.1 ng for menthol and and 5.0 ng for methyl salicylate, respectively. The limit of quantitation was 0.25 ng and 10.0 ng, correspondingly. The high recovery of active substances was also reached (when using camphor as an internal standard and for calculations based on peak areas): 98.8% and 98.1% for menthol and methyl salicylate, respectively (Table 2). The linearity was maintained in a wide concentration range: from 3.0 to 7.5 mg/100 ml for menthol and from 24.0 to 60.0 mg/ml for methyl salicylate.

There is a small discrepancy between the results of quantitative determination for constituents of the analysed ointment listed in Tables 2 and 3 and obtained by using two different internal standards. The results obtained by using camphor as an internal standard feature a higher accuracy than those of thymol. This confirms the above suggestion that the presence of matrix constituents may affect the results of analysis for components of retention time exceeding 10 min, thus also thymol (retention time of 12.3 minutes) by shifting the baseline.

The calculations in the internal standard method were based both on peak areas and heights, while taking the advantage of chromatograph software. The comparable results were obtained, thus indicating that these computing methods can be used alternatively.

CONCLUSIONS

Based on the results presented above, one can conclude that the determination of active substances in the examined medicine was less affected the auxiliary constituents when using camphor as an internal standard instead of thymol. This has been confirmed by statistical analysis of quantitative determinations in the terms of accuracy of these two methods expressed by the mean relative error and recovery.

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Received: 22.04.2002