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Development and Validation of a Gas Chromatographic Method for the Menthol Assay in Compounding Ointment

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Abstract:

The stability study of extemporal ointments is topical at the present stage of their quality control. State Pharmacopoeia of Ukraine (SPhU) established a rather shot period for their storage, which can be extended only in presence of scientifically valid information about stability of the dosage form. Such researches often require the development of modern methods for determining the quantitative content of each active ingredient. Method GC/FID was developed for the menthol assay in compounding ointment which is being prepared for stock in many pharmacies of Ukraine. It can be used for the ointment stability studies with subsequent evaluation of the possibility of extending its expiration date. In this method 30,0 m×0,25 µm column, helium as a carrier gas at a flow rate 1,26 ml/min and FID detector temperature 310 ^{0}C were used. Specificity, linearity, precision and accuracy study of the method showed possibility of its using for the ointment quality control. The recovery of menthol was found to be 92.67 %, 95.54 % and 99.54 % for the different ointment samples analysis. Validation parameters of the method allow recommending it for the assay of menthol in the studied ointment. During the analysis was determined the ointment correspondence with the SPhU requirements. The results of the menthol assay in the compounding ointment demonstrate its compliance with the SPhU requirements.

Key Words: Compounding ointment, GC/FID, menthol, quantitative determination.

INTRODUCTION

At the present stage of extemporal medicines quality control the study of their stability and the revision of the expiration dates are conducted. According to the requirements of the SPhU, the expiration date of most extemporal dosage forms is 10 days. But many compounding medicines often can have a longer shelf life. Its increasing becomes possible only in presence of scientifically confirmed information on the stability of dosage form during a certain period. In this case, there is the problem of the lack of objective methods for estimating the active ingredients quantitative content of many dosage forms. In particular, this applies to medicines that are prepared for stock. In addition to studying their stability, the preparation of such dosage forms according to the requirements of the SPhU should be regulated by a technological instruction with the steps of all active ingredients quality control.

One of the ointments that is preparing for stock in many pharmacies of Ukraine is Simanovsky ointment. Her active ingredients are menthol, phenylephrine hydrochloride and zinc oxide, which complement each other's action. The quantitative ratio of active ingredients in the ointment composition can vary slightly, but most often she is prepared according to the following prescription: phenylephrine hydrochloride 0,02; menthol 0,04; zinc oxide 0,24; wool fat 4,0; white soft paraffin 6,0.

The aim of our work was the development of simple and accurate gas chromatographic method for the menthol assay in this composition.

Menthol is the main component of mint essential oil [1-4]. It stimulates the upper airway cold receptors thereby improve nasal breathing [1, 4]. Due to its counterirritant and anesthetic properties [1], the ability to reduce skin irritation, treat sore throat, sunburn and muscle pain [3], cooling properties without lowering the temperature and weak local analgesic properties when used on the skin surface [2] menthol is often found as an active component of many anesthetics and anti-inflammatory drugs [1]. Using the method of direct UV spectrophotometry for the menthol assay in studied ointment is impossible, since there are no chromophore groups in its molecule. In addition, other ointment active ingredients can influence on the accuracy of the analysis. The volatile properties of menthol allow using the GC/FID method for its assay.

The SPhU [5] and the EurPh [6] recommend using GC for the determination of related substances in menthol. Methylene chloride is used in this procedure as a solvent. The glass column of 2 m×2 mm, the FID temperature is maintained at 200 °C, nitrogen as a carrier gas at a flow rate 30 ml/min are used for the analysis. This method is also proposed by the Indian Pharmacopoeia [7], but with using of ethanol as a solvent. The analysis is carried out using glass or stainless steel column of 4 m×2 mm, detector temperature at 240 °C and a flow rate 30 ml/min.

The Japanese Pharmacopoeia proposes to use the alkalimetric method for the menthol assay after it heating in water bath with certain amount of a mixture of dehydrated pyridine and acetic anhydride with a reflux condenser connection [8]. USP recommends GC for determining the chromatographic purity of menthol with detector temperature at 240 °C and 1.8 m×2 mm column [9].

The GC/FID method is often used to determine the quantitative content of menthol in mint essential oil. One of the authors suggested using a 30 m capillary column and detector temperature at 220 °C for this purpose [1]. In another case, GC is used with a split ratio of 1:20, silica capillary column (30 m) and detector temperature at 280 °C [10]. A GC method with 25 m×0.25 mm capillary column with a temperature program from 50 to 180 °C and a carrier gas helium was also proposed [11]. It allows separating five menthol derivatives in mint essential oil over a period of 32 minutes.

Additionally, GC/FID method was proposed for the menthol assaying in different preparations. The GC method was developed

for the assay of menthol in cough syrup with 30 m×0.32×1.0 μm column, detector temperature at 260 °C and nitrogen was a carrier gas [3]. GC determination of menthol in ointment with methyl salicylate with cool on-column injection by using capillary column (25 m) and detector temperature at 275 ^{0}C was proposed [12]. GC method was developed and validated for the simultaneous estimation of menthol, methyl salicylate and linseed oil in marketed pain relief formulation. The determination was carried out on the capillary GC with detector temperature 250 ^{0}C [13].

The GC method was proposed for the menthol analysis in the composition of Validol tablets. The temperatures of the injector and detector were at 220 ^{0}C and 250 ^{0}C respectively. The flow rate of the carrier gas (nitrogen) was 7.0 ml/min, and the split ratio was 3:1 [14].

The analysis of existing methods of menthol assay in different forms by GC/FID showed the absence of method for its quantitative determination in the studied ointment with current composition.

MATERIALS AND METHODS

Class A glassware, menthol substance (series SI/FP/1825/09-10, manufactured by Kaizen Organics PVT. LTD, India), phenylephrine hydrochloride substance (series 102252, manufactured by Unichem Laboratories Ltd, India), zinc oxide substance (series L08131014, manufactured by Zinsa, Peru) and the compounding ointment with them were used for the work.

Test Solution Preparation:

0.500 g of the ointment was weighed into a measuring beaker; 10 ml of methanol was added, covered with a polyethylene film, placed on the ultrasonic bath at 30 °C for 15 minutes. The procedure was repeated three more times with transferring the solution to the 50.0 ml volumetric flask and dilution to the mark with methanol. Ultrasonic Cleaner Set (Wise Clean WUC-A06H) was used for the sample preparation. Methanol extract was filtered through a Q-MAX RR Syringe Filters (filter diameter 25 mm, membrane 0.22 μm PTFE Hydrophobic) into the vials.

Standard Solution of Menthol Preparation:

0.020 g of the menthol substance was dissolved in the 100.0 ml volumetric flask in methanol to obtain a final concentration of 2×10^{-4} g/ml. The solution was filtered through a Q-MAX RR Syringe Filter into the vials.

Chromatographic Conditions:

The studies were carried out on the gas chromatograph GC-2010 Plus Shimadzu with a FID detector, an AOC-20i+s auto sampler and an Rxi-5MS column (30.0 m × 0.25 μm) with injection volume 1 μL, injection temperature at 260 ^{0}C, injection mode – split (split ratio 1:60), carrier gas was helium, column flow 1,26 ml/min, FID detector temperature at 310 ^{0}C. Temperature program: initial temperature 50 ^{0}C, heating up to 60 ^{0}C at a rate of 3 ^{0}C/min, heating up to 310 ^{0}C at a rate of 25 ^{0}C/min (hold time 5 minutes). Total program time – 18.33 minutes.

RESULTS AND DISCUSSION

Since menthol is easy soluble in methanol [5], its extraction during the test solution preparation was carried out with this solvent. The use of an ultrasonic bath allowed the maximum extraction of menthol from the ointment base. Since menthol has volatile properties [2], during the test solution preparation measuring beaker was coated with a polyethylene film. Based on existing research methods, a method for the menthol assay in the tested ointment with temperature program changing during the analysis from 50 °C to 310 °C was developed.

A chromatogram of the ointment methanol extract analysis was obtained (Figure 1a), the peak of menthol is well resolved. The retention time of menthol was 8.00 min.

For the evaluation of system suitability was injected the extragent solution (methanol) and was recorded its chromatogram (Figure 1b). There are no peaks at the site of menthol exit. In addition, the ointment without menthol was prepared and analyzed by the recommended method. Thus the specificity of the method was determined. At the site of menthol exit also no peaks were observed (Figure 1c) which indicates that other components of the ointment will not affect the accuracy of the assay.

To prove the possibility of using the analysis method in the ointment quality control, its validation was carried out in accordance with the requirements of articles 5.3.N.2. “Validation of analytical methods and tests” and 2.2.46. “Chromatographic separation techniques” of the SPhU [15] and the EurPh [6]. During the validation, the system suitability parameters, linearity of the method, accuracy, precision, detection limit and quantification limit were determined.

To assess the possibility of using the sample preparation technique and the analysis method we calculated their total uncertainty. The forecast of the samples preparation uncertainty was made taking into account the stages of sample preparation of the standard and test solutions (Table 1). Taking into account limit of deviation in the quantitative content of components in compounding ointments ± 10 % (according to the SPhU requirements, article “Semi-solid compounding medicines”), its maximum value is: max Δ_{As}=10×0.32=3.20 %. During the uncertainty of sample preparation calculating ($\Delta_{SP,\ r}$), all stages of weighing and dilution during preparation of the standard and the test solutions were taken into account. The error of the volumetric glassware and analytical balance which were used in the preparation of solutions was taken into account during the calculations.

The obtained value (Table 1) slightly exceeds the permissible requirements ($\Delta_{SP,r} \leq 0.32 \times max\Delta_{As}=1.02$ %), which, in turn, increases the uncertainty requirements for the final analytical operation ($\Delta_{FAO,\ r}$). It characterizes the uncertainty of the equipment used. The calculated uncertainty of the final analytical operation is 0.75 %.

From the data obtained, we calculated the total uncertainty of the analysis:

$$\Delta_{As,r} = \sqrt{\Delta^2_{SP,r} + \Delta^2_{FAO,r}} = \sqrt{1.43^2 + 0.75^2} = 1.61 \text{ %}.$$

Obtained value does not exceed its maximum value (3.20 %). It indicates the possibility of obtaining correct results when using this method for the ointment analysis in other laboratories.

Table 1. Calculation of the uncertainty of sample preparation of standard and test solutions

Sample preparation operation	Uncertainty, %
Standard solution	
Weighing of menthol on an analytical balance	0.0002/0.02 × 100=1
Dilution in a 100 ml volumetric flask	0.12
The total uncertainty of the standard solution preparation	$\Delta^{st}_{SP} = \sqrt{1^2 + 0.12^2} = 1.42$
Test solution	
Weighing of the ointment on the analytical balance	0.0002/0.5 × 100=0.04
Dilution in a 50 ml volumetric flask	0.12
The total uncertainty of the test solution preparation	$\Delta_{SP} = \sqrt{0.04^2 + 0.12^2} = 0.13$
The total uncertainty of the both solutions preparation ($\Delta_{SP,r}$)	$\Delta_{SP,r} = \sqrt{1.42^2 + 0.13^2} = 1.43$

Table 2. Parameters of the system suitability for the ointment analysis by the GC method

Parameters	Results of the menthol analysis
Retention time	8.00 *(S_r=0.022 %)*
Area	6022 *(S_r=1.48 %)*
Height	3725 *(S_r=1.60 %)*
Amount	0.04 *(S_r=1.11 %)*
Plate count	669286 *(RSD=0.85 %)*
Symmetry factor	1.30 *(RSD=0.43 %)*
Capacity factor (K´)	3.66 *(RSD=0.033 %)*

Table 3. Results of the linearity study of menthol assay method

Validation characteristics	The obtained value
b	1.002
S_b	0.0006
(b-1)	0.002
a	0.0036
S_a	0.35
The criterion of *a* statistical insignificance ($a \le t(95\%, n-2) \times S_a$)	0.0036≤1.16
S_0	1.49
Requirements for the residual standard deviation	$S_0 \le \frac{\Delta_{As}(\%)}{t(95\%, n-2)} = 1.87$
r	0.9999
The critical value of the correlation coefficient (*min r*)	$R_c \ge 0{,}9985$

Table 4. Recovery of menthol content in the ointment

Number of sample	Wt of sample taken	Area of menthol	Menthol concentration	Recovery of menthol, %	Mean recovery, %
1	0.5026	5972	0.03966	99.14	99.54±0.54 %
		6033	0.04006	100.15	
		5983	0.03973	99.32	
2	0.5006	5715	0.03810	95.25	95.54±0.25 %
		5741	0.03827	95.68	
		5742	0.03828	95.70	
3	0.5005	5535	0.03691	92.27	92.67±0.44%
		5588	0.03726	93.15	
		5555	0.03704	92.60	

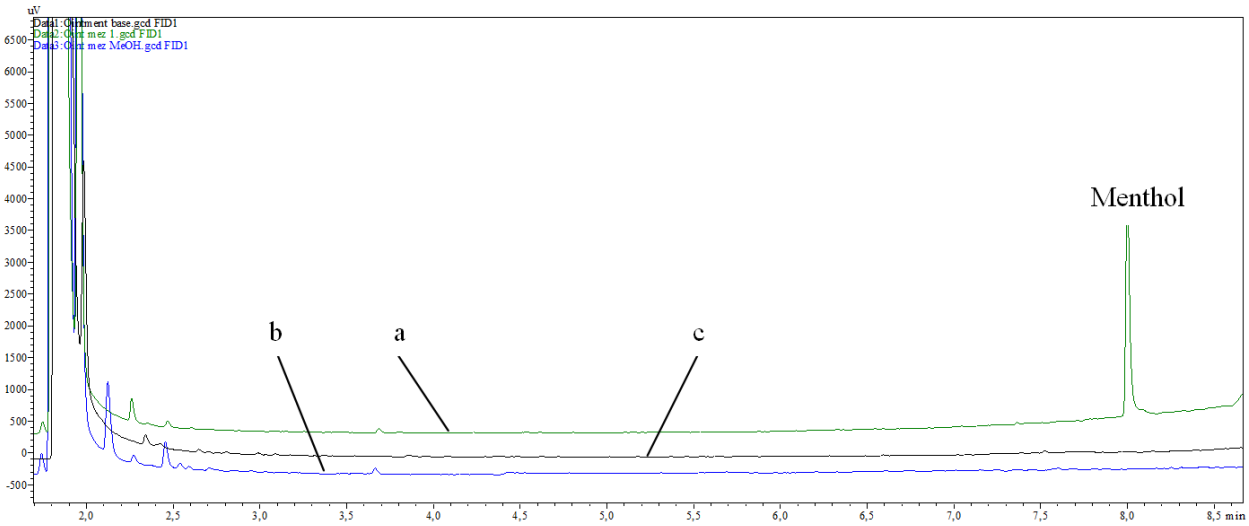

Figure 1. Chromatograms of ointment's methanol extract analysis (a); extragent solution (b) and methanol extract of ointment without menthol (c) analysis

Table 5. Method precision evaluation

Sample number	Area	Concentration
1.	5972	0.03966
2.	6033	0.04006
3.	5896	0.03915
4.	5983	0.03973
5.	6099	0.04050
6.	5985	0.03974
7.	5944	0.03947
Mean	*5987.43*	*0.03977*
RSD, %	*1.08*	*1.08*

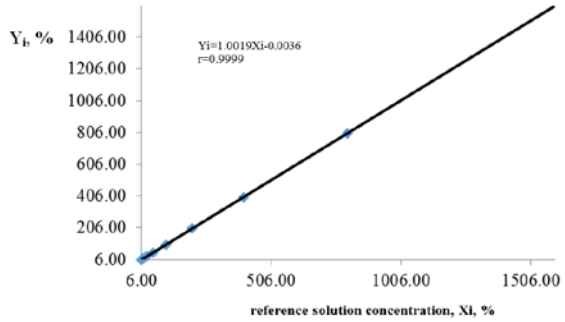

Figure 2. Calibration curve for the menthol assay method

To prove the system suitability for the ointment analysis the following parameters were determined by using software: plate count, symmetry factor, capacity factor (K´). Received parameters were evaluated for compliance with the requirements of the article 2.2.46. "Chromatographic separation techniques" [6, 15]. The reproducibility of the response as an estimated percentage relative standard deviation (S_r, %) was calculated for the parameters of retention time, area, height and amount. It was evaluated by four consecutive series of injections of ointment methanol extract. Its maximum value among all investigated parameters is 1.60 % (for the height parameter) and it doesn't exceed the permissible value (1.92 %) for deviation ± 10 %. The symmetry factor is within the permissible limits (from 0.8 to 1.5). These results testify the suitability of the system for the ointment analysis.

The linearity of the method was evaluated by constructing a calibration curve at nine concentration levels (Figure 2) ranging from $7{,}812\times10^{-7}$ to 2×10^{-4} g/ml of menthol. It was built using the internal standard peak area versus the concentration of the analyte. During the analysis, the parameters characterizing the linear dependence were determined. Parameter of slope (b) slightly differs from one, requirements for statistical insignificance of intercept (a) are fulfilled (Table. 3). The value of the residual standard deviation (S_0) does not exceed the allowable criterion and the correlation coefficient (r^2) not less than its critical value. The obtained results indicate linearity of the method on the entire range of concentrations investigated (Table. 3).

During the process of the linearity study, the limit of detection and the limit of quantification of the method were determined. LOD value was 2.51×10^{-7} g/ml and LOQ value was 7.58×10^{-7} g/ml. These data shows that the proposed method is sensitive for the determination of the menthol in studied ointment. We used a concentration of menthol 3.88×10^{-5} g/ml for the ointment analysis.

The accuracy and precision of the method were estimated simultaneously with linearity studies. Systematic error of the method (δ) was determined for the accuracy estimation. It value (δ=0.87 %) exceeds its statistical insignificance (δ>0.073 %), but at the same time fulfilling the requirements for its practical insignificance (δ<1.02 %). The recovery of the procedure was evaluated by analyzing three ointments prepared at different times and in different pharmacies. The results of the studies (Table 4), including low RSD values indicate that the method is accurate.

To study the precision of the method, the Δ_Z value was determined. Its value should not exceed max $\Delta_{As.}$ The value obtained indicates compliance with requirements (Δ_Z=0.38≤3.20 %). Method precision evaluation was performed also by repeatability estimation. It was done by seven independent determinations of the test sample solution and RSD (%) calculation.

The RSD value of menthol concentration measured during the assessment of method precision was 1.08 %. It value low enough and corresponds to the requirements of the SPhU and the EurPh (for seven parallel injections it must be not more than 3.08 %).

The obtained data testify the possibility of using the method for determining the quantitative content of menthol in the tested ointment. Advantages of this method are simple and fast sample preparation with direct further determination of menthol in the test solution. The developed method can be used to analyze the chemical stability of ointment. The data of the study will allow to evaluate the possibility of increasing the shelf life of the ointment.

During the analysis, an assessment was made of the compliance of the ointment with the requirements of the SPhU by the indicator of quantitative content of menthol. According to the article "Semi-solid compounding medicines" requirements (SPhU) the permissible deviation in the quantitative content of extemporal ointments active ingredients should not exceed ± 10 %. The average value of the quantitative content of menthol (Table 5) in the ointment samples is 0.03977 g (according to the prescription 0.04 g), which is 99.43 % from the prescribed amount. Thus the ointment meets the requirements of the SPhU.

CONCLUSIONS

1. For the stability study of the compounding ointment with menthol the GC/FID method was developed for its assay. The obtained results of the stability studies of this ointment will make it possible to evaluate the possibility of increasing its shelf life.
2. The validation parameters of the method: linearity, specificity, precision and accuracy testify the possibility of its using for determining the quantitative content of menthol in the studied compounding ointment.

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