DEVELOPMENT AND VALIDATION OF THE SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF MENTHOL**

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The main objective of the present study is a quantitative determination of menthol using a simple, sensitive, and reproducible method of reacting menthol with an aromatic aldehyde (salicylaldehyde) in an acidic medium. A linear response was observed between the concentration and the absorbance in the range 0.02–3 mg/5mL possessing a correlation coefficient greater than 0.999 and exhibiting good recovery values (99.54–100.40%). The wavelength of the maximum absorbance (λ_{max}) was measured at 498 nm immediately after the reaction and subsequently within 1 h. The proposed method was validated according to the ICH guidelines for parameters including linearity, precision, accuracy, range, stability, and robustness. Hence the developed method can be used in the analysis of menthol in drug formulations.

Keywords: *menthol, spectrophotometry, salicylaldehyde, ICH guidelines.*

РАЗРАБОТКА И ВАЛИДАЦИЯ СПЕКТРОФОТОМЕТРИЧЕСКОГО МЕТОДА ОПРЕДЕЛЕНИЯ МЕНТОЛА

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УДК 543.42.062:547.596.2

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(Поступила 27 марта 2019)

Проведено количественное определение ментола с использованием простого, чувствительного и воспроизводимого метода, основанного на использовании взаимодействия ментола с ароматическим альдегидом (салицилальдегидом) в кислой среде. В диапазоне 0.02—3 мг/5 мл наблюдаются линейный отклик между концентрацией и поглощением с коэффициентом корреляции >0.999 и хорошие значения восстановления (99.54—100.40%). Длина волны максимального поглощения λ_{max} = 498 нм измерена сразу после реакции и затем в течение 1 ч. Разработанный метод проверен в соответствии с руководством ICH для таких параметров, как линейность, точность, область измерений, стабильность и надежность, и может быть использован при анализе ментола в лекарственных препаратах.

Ключевые слова: *ментол, спектрофотометрия, салициловый альдегид, рекомендации ICH.*

Introduction. Menthol, or 5-methyl-2-(1-methylethyl)cyclohexanol, is a highly lipid-soluble organic compound derived from various mint plants such as corn mint oil (*Mentha Avensis)* and peppermint (*Mentha piperita)* or prepared synthetically. It is one of the cyclic monoterpene alcohols with the configuration (1R, 2S, 5R) displaying 3 asymmetric carbon atoms [1]. In nature, menthol exists as *l*-menthol, which is frequently used in various biomedical applications as it exhibits better cooling properties than the other existing

** Full text is published in JAS V. 87, No. 3 (http://springer.com/journal/10812) and in electronic version of ZhPS V. 87, No. 3 (http://www.elibrary.ru/title_about.asp?id=7318; sales@elibrary.ru).

menthol isomers [2]. Menthol is known to have various biological characteristics such as antipruritic, antiseptic, and antimicrobial activities [3].

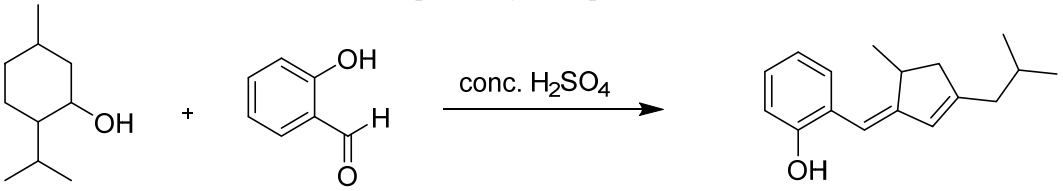
Menthol is also included in the array of daily used products such as toothpaste, perfumes, chewing gums, shampoos, liqueurs, and many medicinal products as a fragrance and flavor ingredient [2]. Menthol is a primary component in anesthetic agents and cooling formulations. It provides a cooling effect by activating the cold sensation transient receptor potential cation channel, inhibiting Ca^{++} currents of neuronal membranes when applied on skin [3].

The literature reveals various methods for the determination of menthol like colorimetry [4], GC flame ionization detector [5–7], HPLC with fluorescence-labeling reagents [8], LC with refractometric detection, normal-phase HPLC with the refractive index detector for the separation of isomers [9], polarized photometric detector [10], LC with circular dichroism [11], UV spectrophotometry, and NMR spectrometry. However, all these methods have some disadvantages. They are tedious, need complicated sample preparation and expensive instrumentation, and are difficult in interpretation.

Apart from these methods, there are several research articles on the UV spectrophotometric estimation of menthol. However, menthol acts as a non-UV absorbing compound, and therefore it needs to be derivatized chemically for the substitution of a UV sensitive chromophore.

The aim of this study was to develop a simple, sensitive, rapid, and reproducible UV spectrophotometric method for the quantitative detection of menthol in pharmaceutical dosage forms, food products, and beverages.

The present study is based on the derivatization of menthol by reacting it with an aromatic aldehyde (salicylaldehyde) in the presence of concentrated sulfuric acid, resulting in a peach colored solution. Menthol gives a mixture of saturated and unsaturated hydrocarbons in reaction with concentrated sulfuric acid. In the reaction the carbocation rearrangement of the cyclohexenyl cation to the cyclopentenyl one takes place because the latter has higher stability than the former. Further the cyclopentenyl cation gives a condensation reaction with an aromatic aldehyde to form a colored reaction mixture. In our study we used salicylaldehyde. Reacting with the menthol, it produces (E)-2-((3-isobutyl-5-methylcyclopent-2-en-1-ylidene)methyl)phenol. Salicylaldehyde can also be replaced by vanillin or other aromatic aldehydes with an appropriate substituting group, and concentrated sulfuric acid can be replaced by 72% perchloric acid [12].



2-Isopropyl-5-methylcyhexan-1-ol (Mentol)

2-Hydroxybenzaldehyde (Salicylaldehyde)

(E)-2-((3-isobutyl-5-methylcyclopent-2-en-1-ylidene)methyl)phenol

The developed method has been validated as per the ICH guidelines, and its accuracy, precision, linearity, and stability were ascertained [13]. Method validation is the process of "developing a documented evidence" to ensure that the analytically developed procedure meets all requirements for applications. These studies have been executed in the pharmaceutical industry for a long time and act as an integral part of their systems and processes. The fundamental reason to validate an analytical method in pharmaceutical productions is to produce high quality and cost-effective products. This is done to test the consistency, quality, and reliability of the analytical results.

Materials and methods. Menthol was procured from Sisco Research Laboratory Pvt. Ltd. (Maharashtra, India). Salicylaldehyde was purchased from Loba Chemie Pvt. Ltd, while concentrated sulfuric acid (98% purity) of analytical grade was procured from Merck India Ltd., Mumbai, India. A single-beam UV spectrophotometer was used (Beckman Coulter, DU 730®) with a glass cuvette of 1 cm.

Spectroscopic method development. Standard stock solution of menthol (Sm) was prepared by dissolving 150 mg of menthol in 30 mL of conc. sulfuric acid. Further, a stock solution of salicylaldehyde (Ss) was prepared by adding 50 mg of salicylaldehyde in concentrated sulfuric acid (10 mL). The dilutions were made in a 5 mL volumetric flask in the range 0.02–3 mg/5 mL, and 200 μL of Ss was added in each dilution. As the reaction was exothermic, a solution of concentrated sulfuric acid and water (50:50) was added up to the mark of 5 mL while keeping them in an ice-bath.

The prepared working solution of different concentrations (0.02–3 mg/5 mL) was scanned on a UV spectrophotometer in the range of 200–700 nm against the blank (prepared in the same way excluding men-

thol) to determine the λ_{max}. After determining λ_{max}, a calibration curve was prepared for menthol in the concentration range 0.02–3 mg/5 mL. The calibration curve was plotted between the concentration at the *x*-axis and the absorbance at the *y*-axis.

Method validation. Linearity was determined by preparing several aliquots from the stock solution of menthol in the range 0.02–3 mg/mL, and the absorbance was noted down at these concentrations. Using the obtained data, a calibration curve was plotted between the concentration and the absorbance (Table 1).

TABLE 1. Linearity Studies for Different Concentrations of Menthol

Sr. No.	Concentration, mg/5 mL	Absorbance
1	0.02	0.107
2	0.08	0.135
3	0.5	0.335
4	0.75	0.482
5	1	0.616
6	2	1.105
7	3	1.573

The accuracy of the method was determined with the help of recovery studies at three levels (50, 100, and 150%) by the standard addition method; 0.04 mg/5 mL solution was taken as a reference solution, and its 50, 100, and 150% was added to it to produce its recovery data. The absorbance was recorded at 498 nm (Table 2).

This study was performed at two levels: intraday and interday. In the intraday variation, solutions with a concentration of 1, 2, and 3 mg/5 mL were prepared in triplicate and analyzed for the same concentrations, and the absorbance was determined for three consecutive days (Table 3).

TABLE 2. Recovery Studies for Three Different Concentrations of Menthol

Sr. No.	Reference concentration, mg/5 mL	Amount added, %	Recovery, %
1	0.04	50	100.4040
2	0.04	100	99.5454
3	0.04	150	100.2424

TABLE 3. Intraday Precision Studies for Three Different Concentrations of Menthol

Sr. No.	Concentration, mg/5 mL	Measured absorbance	RSD, %
1	1	0.6136±0.0025	0.4100
2	2	1.1040±0.0026	0.2396
3	3	1.5736±0.0040	0.2568

Stock solutions of menthol and salicylaldehyde were left to stand at room temperature for 24 h, and samples were prepared from them to determine the absorbance and check their stability and correlation coefficient [14].

The limit of detection determines the lowest amount of analyte detected in the sample but not necessarily quantitated. The limit of quantification is the lowest amount determined quantitatively with suitable accuracy and precision. These limits were calculated using the slope of the calibration curve and the standard deviation of the response involving the formula as per the ICH guidelines:

$$LOD = 3.3\sigma/S,$$

$$LOQ = 10\sigma/S,$$

where σ is the standard deviation and S is the slope of the linear graph [15].

Robustness measures the ability of the analytical procedure to remain unaffected at different temperature conditions. The analysis was carried out at 18°C, and the samples were scanned using UV spectrophotometer.

Results and discussion. Several methods suggest derivatization of menthol with aromatic aldehydes including salicylaldehyde, vanillin, dimethylaminobenzaldehyde (DMAB), etc. followed by its estimation by

UV spectrophotometric analysis, which depends on factors such as acidic conditions, temperature, and time. Guerin and Ekkert have reported that higher alcohols and phenols undergo dehydration with concentrated sulfuric acid and further react with aromatic aldehydes to give a colored compound. Based on this observation, Dangwal reported the derivatization of menthol with vanillin and DMAB wherein the reaction mixture is heated for complete derivatization [16]. Another study was conducted by Anastasia–Sandu et al., in which an ethanolic solution of menthol was reacted with (1%) salicylaldehyde in a highly acidic medium without heating to give a red orange colored product [17].

All the above methods were tried by us in the laboratory, but these methods did not meet the requirements of the ICH guidelines for necessary analytical validation. Hence, another methodology given by Auterhoff and Bertram was tried, in which samples were prepared by keeping them in an ice-bath [12]. Low temperature was maintained, given the exothermic nature of the reaction. Subsequently, the samples were scanned in the wavelength range 200–700 nm. A calibration curve was obtained within the range 0.02–3 mg/5 mL, and the maximum absorbance (λ_{max}) was displayed at 498 nm (Fig. 1). This method followed the Beer–Lambert law and hence was found suitable for future validation purposes.

A linear response was seen between the concentration of samples (x-axis) and the absorbance at λ_{max} (y-axis). The regression value or correlation coefficient (R^2) was 0.999 in the concentration range 0.02–3 mg/5 mL (Fig. 2).

To optimize the accuracy and reliability of the proposed method via the standard addition method, recovery studies were established across three different concentrations, i.e., 50, 100, and 150% [13]. The results were found to be in agreement with the true value and possessed a high degree of concordance from 99.54–100.40% (Table 2). Hence, the developed method is acceptable and accurate.

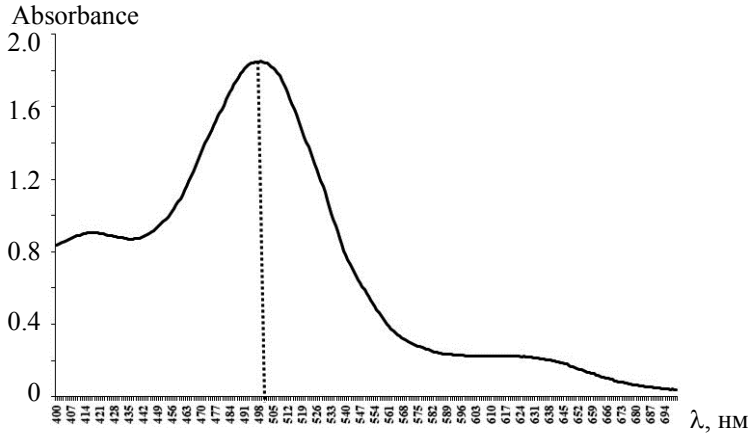


Fig. 1. Absorption spectrum of menthol.

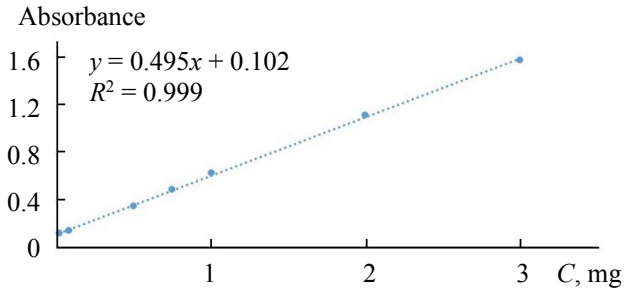


Fig. 2. Calibration curve of menthol.

The precision of the schemed method was determined in two ways, i.e., intraday and inter-day variation [14]. The intraday studies were performed for three different concentrations in a triplicate fashion thrice a day. The absorbance was reported in the format of average ± standard deviation. The precision was re-

solved by calculation of the relative standard deviation (RSD in %). All the three RSDs were found to be <1%, whereas the average RSD was equal to 0.3021% (Table 3). These results were only valid up to 1 hour after the reaction started, as the regression value of the curve between the concentration and the absorbance decreased thereafter. Thus, the samples were not stable after this time period, and the interday studies were also inapplicable. The most probable reason for these results could be the mixture of saturated and unsaturated hydrocarbons formed due to the reaction between menthol and concentrated sulpfuric acid, out of which the unsaturated part reacts with salicylaldehyde to give a peach color. Consequently, the analysis needs to be performed within 1 hour of the reaction to give appropriate results.

The stock solution for menthol, which was treated as a standard when maintained at room temperature for 24 h, was found to decompose, as a low correlation coefficient was observed. A possible reason for this could be the evolution of an oily layer on the solution of menthol and the concentrated sulfuric acid surface, which was classified as a mixture of saturated hydrocarbons.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by the standard deviation method using the slope of the calibration curve. The LOD and LOQ values determined according to the ICH guidelines were about 0.0416 and 0.126 mg/5 mL, respectively, implying the sensitivity of the developed method.

The robustness of the method was examined when the temperature conditions for the stock solutions were changed from 25 to 18°C. The samples made from these solutions did not obey the Beer-Lambert law, which suggests the instability of the sample.

Conclusions. The proposed method was validated successfully according to the parameters of the ICH guidelines. This UV spectrophotometric study proposes an accurate, reproducible, cost-effective, simple, and sensitive method for the analysis of menthol in drug formulations. The method was found to follow the Beer–Lambert law within the concentration range 0.02–3 mg/5 mL with an excellent regression value of 0.999. The wavelength of the maximum absorbance (λ_{max}) was detected at 498 nm with a UV spectrophotometer. The recovery values were found to be 99.54–100.40%. The study was able to establish the decomposition and instability of the samples after 1 h of the reaction. Thus, this method is suitable and convenient for the determination of menthol in various menthol-containing products.

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