Development and Validation of a Gas Chromatographic Method for Determination of Menthol in Cold-Cough Syrups

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Abstract

Background
Common cold is the most common infection of the upper respiratory tract and cold-cough syrups are often prescribed. Although menthol is one of the common constituents of these syrups, quality checks on cold-cough syrups normally target the major active pharmaceutical ingredients without regard to menthol content.

Objective
To develop and validate a gas chromatography method for determination of menthol in cold-cough syrups.

Methods
A simple, rapid, robust, accurate and reliable Gas Chromatography method was developed and validated for the determination of menthol in cold-cough syrups that may also contain ambroxol, bromhexine, chlorpheniramine maleate, guaifenesin and salbutamol.

Results
Optimized chromatographic conditions were: A ZB-WAXplus 60m × 0.25mm; 0.25μm fused silica capillary column. Oven temperature program of 110 °C (2 min), ramp 10 °C/min to 190 °C (2 min). Injector port temperature maintained at 240 °C. Injection volume of 1.0 μl split in the ratio of 50:1. Carrier gas as nitrogen at 1.0mL/min which also serves as make up gas (30 mL/min) in the flame ionization detector (260 °C). Other detector gases were hydrogen (30 mL/ min) and industrial air (300 mL/min) and the diluent for samples and standards was grade chloroform.

From recovery studies, 97.56 to 102.97 % recovery was reported. Repeatability studies had a coefficient of variation of 0.55 while intermediate precision was 0.32. The method was linear over a range of 0.042 to 0.169 mg/mL with a coefficient of determination (R2) 0.9986.

Of the 21 samples analyzed, only 10 samples (47.6%) complied with assay specifications of 90.0 to 110.0 % label claim for finished products according to the United States Pharmacopeia 2016.

Conclusion and recommendation
A gas chromatographic method was developed and validated for the determination of menthol in cold-cough syrups in Kenya. This method can be used together with a validated high-performance liquid chromatography method to assay cold-cough syrups that may also contain ambroxol, bromhexine, chlorpheniramine maleate, guaifenesin and salbutamol.

This method can be useful in routine analysis such as pre-registration studies as well as post market surveillance to curb substandard and counterfeit cold-cough syrups.

Key words: Capillary column, monoterpenes, organic layer, carrier gas, total menthol, cold-cough syrup.

Introduction
Menthol is a cyclic monoterpenic alcohol obtained either naturally or synthetically from various precursors [1, 2]. The racemic mixture consists of equal parts of R and S enantiomers of cyclohexanol [5-methyl-2-(1-methylethyl)]. The most common natural isomer is the (+)-menthol and it is the one generally referred to as menthol [3-5].

Menthol is a common pharmaceutical ingredient and can be formulated as creams, ointments, balms, lozenges as well as syrups [2]. In the Kenyan market, typical multicomponent cold-cough syrup containing menthol may contain chlorpheniramine maleate, guaifenesin, salbutamol, ambroxol and bromhexine as active pharmaceutical ingredients [6].

Several High-Performance Liquid Chromatography (HPLC) methods for determination of nonvolatile active ingredients in cold-cough syrups have been published. A method for simultaneous determination of chlorpheniramine maleate, salbutamol, bromhexine, terbutaline, phenylephrine, ambroxol as well as guaifenesin has been reported [7]. Similarly, capillary electrophoresis with ultraviolet detection has been reported for determination of these active ingredients [8].

Gas chromatography (GC) methods on a packed column with a flame ionization detector as well as capillary gas chromatography with mass spectrometer detector have been published. None of these methods are applicable in...
determination of menthol. Therefore, this paper describes development and validation of a gas chromatography method for determination of menthol in cold-cough syrups that may also contain ambroxol, bromhexine, chlorpheniramine maleate, guaifenesin, and salbutamol as active ingredients.

Methods

Apparatus

Shimadzu gas chromatograph 2010 (Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20s autosampler, AOC-20i split autoinjector, a ZB-WAXplus capillary column (60 m x 0.25 mm x 0.25 m) and fitted with a flame ionization detector and mass spectrometer. Injector port and detectors held at 240°C and 260°C respectively while oven temperature programmed from 110°C (2 min) to 190°C (2 min) at 10°C/min. Injection volume (1.0µL) with a split ratio 50:1.

Reagents, chemicals and gases

Standard substances used in GC analysis were menthol (MEN) (Sigma-Aldrich, St Louis, USA) and Camphor (CAM) (May & Baker, Dagenham, England).

Ambroxol (AMB), guaifenesin (GUA), bromhexine (BRO), salbutamol (SAL) and chlorpheniramine maleate (CHL) were kind donations from stock of standards used at the Drug Analysis and Research Unit.

HPLC grade methanol (Sigma-Aldrich, St Louis, USA) and HPLC grade chloroform (Sigma-Aldrich, St Louis, USA). Purified water was prepared in the laboratory using Aquatron-A 4000 water still (Cole-Parmer, Staffordshire, United Kingdom) which utilizes distillation followed by filtration through a 25 µm polypropylene filter.

Nitrogen, helium (99.99 %), hydrogen of purity 99.9 % and industrial air were obtained from BOC Gases (Nairobi, Kenya).

Figure 1. Chemical structure of l-menthol

Standard preparation

Solutions used in method development and validation were prepared volumetrically. Stock standard solutions for CAM and MEN were prepared separately at a concentration of 2.06 mg/mL in chloroform. The individual working standard solutions for method development were prepared at a concentration of 0.48 mg/mL and 0.40 mg/mL for CAM and MEN respectively.

Working solutions for method validation were prepared at a concentration of 0.1 mg/mL which was regarded as 100% concentration around which various dilutions were centered.

During menthol determination, a working standard solution was prepared to contain 0.041 mg/mL MEN and 0.049 mg/mL CAM in chloroform. This mixture of working standards was labeled as solution 1.

Sample preparation

Depending on the label claim, 10-20 mL of sample syrup was measured into a 50-ml volumetric flask to which 1.2 mL of CAM stock standard solution was added. Approximately 20 mL chloroform was then added and the mixture shaken and sonicated for 5 minutes. The aqueous layer was removed and the organic layer made to volume with chloroform. The solution was then filtered through a 125 mm Whatman’s filter paper and stored in a stoppered container. This was labeled as solution 2.

Method development

Different chromatographic conditions were tested on a ZB WAXPLUS column (60 m x0.25mm x0.25µm) coated with polyethylene glycol. Carrier gas was nitrogen and detection was done using a flame ionization detector. Two solvents were tried, methanol and chloroform (both HPLC grade) and chloroform produced satisfactory results. Effects of temperature and carrier gas velocity on retention time, capacity factor and resolution were investigated [9].

Method validation

Accuracy of the method was evaluated by method of standard addition [10]. TriPLICATE determinations were made at three concentration levels corresponding to 80, 100 and 120 % concentration containing constant amount of CAM. Results of accuracy studies were then expressed as percent recovery [10, 11].

Specificity of the method was investigated by chromatographing working standard solution containing MEN and CAM each at 0.1 mg/mL and then determining resolution and asymmetry factors from the resulting chromatograms. Peak purity analysis of MEN was also conducted using GC-mass spectrometry to rule out coelution. Specificity was further evaluated by chromatographing a simulated blank syrup containing AMB, CHL, GFN, SAL and BRO and retention times of eluted peak recorded [12].

Limit of detection and limit of quantitation were established from signal to noise ratio of menthol peak resulting from serial dilutions of stock standard solutions [11].

Linearity of detector response was determined by making triplicate determinations from working concentrations corresponding to 40, 60, 80, 120 and 160 % concentration of MEN [13]. Average peak areas were recorded and plotted against concentration in order to determine the correlation coefficient.
Repeatability of the method was assessed using coefficient of variation (CV) of peak area response factors obtained from nine replicate determinations made at 80, 100 and 120 %. Intermediate precision was assessed from CV of peak area response factors obtained from triplicate determination of a 100 % standard solution over a three-day interval [11].

To determine robustness of the developed method, the effect of small changes on carrier gas velocity, oven temperature as well as injector port temperature on peak area and retention time of CAM and MEN was investigated. Results were summarized in a Table showing each factor and level of variation.

**Stability of working solutions**

Stability of working solutions was monitored over 72 hours under different storage and handling conditions. Solutions containing known concentration of menthol and camphor (100 %) were designated A, B, and C and handled as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Storage Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>At room temperature in a clear glass.</td>
</tr>
<tr>
<td>B</td>
<td>At room temperature in amber colored glass.</td>
</tr>
<tr>
<td>C</td>
<td>Stored in the refrigerator (2-8 °C) in a clear glass.</td>
</tr>
</tbody>
</table>

**Analysis of commercial samples**

System suitability was evaluated by injecting 1.0 μl of solution 1 into the gas chromatograph six times and calculating resolution and peak area response factor for CAM and MEN [11]. Standards (solution 1) and Samples (solution 2) were then run in triplicate and peak area response factors calculated.

**Formulae**

Peak area response factor for standard solution was calculated from the formula:

\[ RF_1 = \frac{MEN}{CAM} \]

Whereby:

- \( RF_1 \) is peak area response factor for standard solution.
- \( MEN \) is peak area due to menthol standard.
- \( CAM \) is peak area due to camphor standard.

Peak area response factor for sample solution was calculated from the formula

\[ RF_2 = \frac{MEN_t}{CAM_t} \]

Whereby:

- \( RF_2 \) is peak area response factor for sample solution.
- \( MEN_t \) is peak area due to menthol in the sample.
- \( CAM_t \) is peak area due to camphor in the sample.

Recovery was calculated from the formula

\[ \text{% Recovery} = \frac{RF_s \times 100}{RF_{std}} \]

**Results and Discussion**

**Method development**

Typical chromatograms for determination of menthol from standard preparation and from cold-cough syrup are shown in figure 2 and 3. From the chromatograms, retention time for menthol was 10.03 minutes which is considered desirable [14].

![Figure 2](image)

*Figure 2. Typical standards gas chromatogram at optimized conditions. Chloroform (CHF), camphor (CAM) and menthol (MEN). Column: ZB-WAXplus 60m x 0.25nm; 0.25μm fused silica capillary column coated with 100 % polyethylene glycol. Oven temperature 110°C (2 min), ramp 10°C/ to 190°C (2min)*

![Figure 3](image)

*Figure 3. A representative gas chromatogram for sample cold-cough syrup. Chloroform (CHF), camphor (CAM) and*
menthol (MEN). Column: ZB-WAXplus 60m x0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110°C (2 min), ramp 10°C/ to 190°C (2min)

**Method Validation**

Accuracy was assessed by means of % recoveries from standard addition method. Results at each of the three concentration levels are shown in Table 1. The method was considered accurate since % recovery values were within the recommended 98-102% [11].

**Table 1.** Percent recoveries of menthol from standard addition to a cough syrup.

<table>
<thead>
<tr>
<th>Concentration level (%)</th>
<th>Average Response factor for sample (not spiked)</th>
<th>Average Response factor for sample (spiked)</th>
<th>Average Response factor for standard</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>0.522</td>
<td>1.423</td>
<td>0.919</td>
<td>98.04</td>
</tr>
<tr>
<td>100</td>
<td>0.524</td>
<td>1.406</td>
<td>0.904</td>
<td>97.56</td>
</tr>
<tr>
<td>120</td>
<td>0.493</td>
<td>1.386</td>
<td>0.876</td>
<td>101.94</td>
</tr>
</tbody>
</table>

When validated with respect to specificity, there was no peak eluting at the same retention time as MEN or CAM from chromatograms of simulated syrup containing AMB, BRO, CHL, GUA and SAL [15]. Peak purity analysis for menthol was done using GC-MS with a purity index of 0.98.

**Figure 4.** Typical gas chromatogram of a blank syrup. Chloroform (CHF). Column: ZB-WAXplus 60m x0.25mm; 0.25µm fused silica capillary column coated with 100% polyethylene glycol. Oven temperature 110°C (2 min), ramp 10°C/min to 190°C (2 min)

Linearity was assessed at seven concentration levels, ranging from 0.042 – 0.169 mg/mL and results shown in Table 2 below.

**Table 2.** Various concentrations of menthol with respective average peak areas

<table>
<thead>
<tr>
<th>Concentration level (%)</th>
<th>Actual concentration (mg/mL)</th>
<th>Average peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>0.169</td>
<td>36111</td>
</tr>
<tr>
<td>140</td>
<td>0.148</td>
<td>32213</td>
</tr>
<tr>
<td>120</td>
<td>0.127</td>
<td>26942</td>
</tr>
<tr>
<td>100</td>
<td>0.106</td>
<td>21852</td>
</tr>
<tr>
<td>80</td>
<td>0.084</td>
<td>17952</td>
</tr>
<tr>
<td>60</td>
<td>0.063</td>
<td>13227</td>
</tr>
<tr>
<td>40</td>
<td>0.042</td>
<td>8558</td>
</tr>
</tbody>
</table>

This data was further subjected to linear regression analysis with exact concentration of menthol (x-axis) being plotted versus peak area (y-axis) (Figure 5). Values for correlation coefficient, y-intercept, slope of the regression line and residual sum of squares are summarized in Table 3. The results illustrate a correlation between peak area and concentration within the range 0.042-0.169 mg/mL. Correlation coefficient was found to be 0.9986 for menthol which meets the validation acceptance criteria [11, 16].

**Figure 5.** Linearity curve for Menthol

**Table 3.** Linear regression analysis for menthol

<table>
<thead>
<tr>
<th>Drug</th>
<th>Slope of regression curve</th>
<th>y-intercept</th>
<th>Correlation coefficient (R²)</th>
<th>Residual sum of squares</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menthol</td>
<td>218,187</td>
<td>-626.43</td>
<td>0.9986</td>
<td>835131.8</td>
</tr>
</tbody>
</table>

**Table 4.** Precision results for menthol

<table>
<thead>
<tr>
<th>Concentration level (%)</th>
<th>Coefficient of Variation of peak area Response Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repeatability (n=3)</td>
</tr>
<tr>
<td>120</td>
<td>0.34</td>
</tr>
<tr>
<td>100</td>
<td>0.32</td>
</tr>
<tr>
<td>80</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Table 5.** Effect of temperature and carrier gas velocity on peak parameters.

<table>
<thead>
<tr>
<th>Parameter varied</th>
<th>Compound</th>
<th>CV of retention time</th>
<th>CV of response factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven temperature (°C)</td>
<td>CAM</td>
<td>0.39</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>MEN</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEN/CAM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Injector port temperature (°C)</td>
<td>CAM</td>
<td>0.0082</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEN</td>
<td>0.0049</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEN/CAM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carrier gas velocity (mL/min) (0.98, 1.0, 1.02)</td>
<td>CAM</td>
<td>0.73</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEN</td>
<td>0.65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MEN/CAM</td>
<td>-</td>
<td>0.37</td>
</tr>
</tbody>
</table>

**Results for sample analysis**

The system was suitable for analysis since resolution between CAM and MEN was >1.5 while CV of peak area response factors was <2.0 [3, 11].
From analyses of 21 samples, menthol content ranged from 26.3 to 107.8% label claim. It was however noted that the lowest recorded content belonged to a sample whose menthol was incorporated as a flavor. 10 samples, all locally manufactured, complied with USP 2016 specifications (90.0-110.0%) for finished products [2].

**Conclusion and Recommendation**

A gas chromatographic method was developed for the determination of menthol in cold-cough syrups in Kenya. This method was used together with a validated HPLC method to assay cold-cough syrups that may also contain AMB, BRO, CHL, GUA, and SAL.

Evaluation of menthol content in cold-cough syrups using the developed and validated method is crucial so as to ensure that only quality products are in the market for optimal therapeutic outcomes. The validated method can be useful in routine analysis such as conducting pre-registration analysis as well as post-market surveillance to curb substandard and counterfeit cold-cough syrups containing menthol.

**References**