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235 Butorphanol Tartrate 10-mg/mL Nasal Spray
236 Dibucaine 1% Ointment
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238 Methotrexate 2.5-mg Capsules
239 Naproxen 25-mg/mL Oral Suspension
240 Tamoxifen 10-mg or 20-mg Capsules
241 Thiotriazine 1-mg, 2-mg, 5-mg, or 10-mg Capsules

International Journal of Pharmaceutical Compounding
Vol. 18 No. 3 | May-June 2014

www.IJPC.com
Investigation of Physical and Chemical Stability of Ointment with Herbals

ABSTRACT
The physical and chemical stability of a stock preparation ointment with active ingredients—herbal tinctures of calendula and arnica—for the treatment of hemorrhoids was studied. Evaluations for physical and chemical stability were performed initially and throughout the storage period. Physical stability of the ointment was assessed by means of visual observation in normal room light. Throughout the study period, the physical appearance of the ointment did not change. The chemical stability of the ointment was evaluated by means of a stability-indicating, thin-layer chromatography analytical technique. The shelf-life was found to be one month at 25°C ± 2°C/60% RH and two months at 5°C ± 3°C, when protected from light.

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Kateryna O. Khokhlova, PhD
Victoriya A. Georgiyants, ScD
Liliia I. Vyshnevska, ScD

INTRODUCTION
It is common in Ukraine to use herbal substances as active ingredients in the compounding of preparations. One of the more common and widely used examples is a semisolid stock preparation for the treatment of hemorrhoids.1 It is applied topically as a multicomponent ointment. The active ingredients of the multicomponent ointment are the herbal substances calendula tincture and arnica tincture. These herbs contribute to wound healing, hemostasis, anti-inflammation, antisepsis, and light anesthetic activities to the therapeutic effect of the ointment. The composition/formula of the ointment has been provided for the reader.

One of the requirements of quality assurance of a compounding preparation is the investigation of its stability. By the results of stability investigation, the beyond-use date (BUD) and the storage condition of the ointment can be determined and ensured. The purpose of this stability study was to determine the BUD and storage conditions of the ointment by use of the ointment’s physical appearance and identification tests.

The investigation of the stability of the ointment was conducted according to the requirements of existing International Conference on Harmonization’s guidance on stability,2,3 specific herbal guidelines on stability,4-6 peer-reviewed articles on stability testing of herbal medicinal products (HMPs),7 and features of BUD determination of extemporaneous formulations.10 Taking into account the nature of active ingredients of the ointment, which are a complex mixture of phytochemical constituents, the thin-layer chromatography (TLC) method was chosen for the stability investigation of identification tests. The high-performance thin-layer chromatography (HPTLC) method is a particularly suitable tool for stability tests on HMPs, including multicomponent HMPs.11 For identification tests, two groups of constituents were chosen: 1) flavonoids and 2) calendulosides. Flavonoids are a characteristic group of substances for initial herbal raw materials of ointment, such as flowers of calendula and flowers of arnica. According to the leading international pharmacopeias and specialized pharmacognosy publications, flowers of calendula and arnica are standardized by the flavonoids pattern.12-14 Calendulosides are specific for calendula flowers.15 Thus, the TLC-identification test of calendulosides is used in the monographs of the State Pharmacopeia of Ukraine (SPU) on tincture of calendula.16 Also, flavonoids and calendulosides are active groups of marker substances of the ointment.

According to a reflection paper on markers,5 since the herbal substance in its entirety is regarded as the active substance, a mere determination of the stability of the marker will not suffice. The stability of other substances present in the herbal substance, should, as thoroughly as possible, also be demonstrated. Thus, the flavonoid’s and calenduloside’s TLC fingerprints were used for stability purposes for identification tests of the ointment.

MATERIALS AND METHODS
All solvents and reagents used in the study were analytical reagent grade, and all reagents used in the study were freshly prepared.

Samples of the ointment for stock preparation (Batch 328, 329, 330) were obtained from drugstore No. 63, Kupiansk, Ukraine. Samples of the active ingredients were calendula tincture (Batch 10312; Yan, Zhytomyr Region, Ukraine) and arnica tincture (Batch 10780; DKP Pharmaceutical Factory, Zhytomyr, Ukraine).

Reference standards used were Rutin (Lot 10808; State Pharmacopeia of Ukraine, Kharkiv, Ukraine); chlorogenic acid (Lot A0285833; Acros Organics, Geel, Belgium); Hyperoside (Lot 271109; State Pharmacopeia of Ukraine); Vitezin (Lot 150910; State Pharmacopeia of Ukraine); and Calendulosides (Lot 1; State Pharmacopeia of Ukraine).

Used in the study were 50 HPTLC 20 × 10 plates, silica gel 60 F254 (Lot OB526793; Merck KGaA, Darmstadt, Germany); a TLC development chamber (Latch-Lid ChromatoTank Unit, Model 80–33; GGC, Richmond, California); a TLC syringe 50 μL (Model 1705 N SYR, Cemented NDL, 22 gauge, 2 inch, point style 3T; Hamilton Bonaduz AG, Bonaduz, Switzerland); a UV lamp (Model CM–10, Serial No. 1783400; Spectroline, Westbury, New York); a drying oven (Model 2III-0-01; Media-
labortechnik, Odessa, Ukraine); and measuring ware provided by Simax (Kavalierglass, Co. Ltd., Sázava, Czech Republic).

The reagents used in the study were diphenylborinic acid aminoethylster (Lot BCBH6834V; Sigma-Aldrich Chemie GmbH, Steinheim, Germany); polyethylene glycol (Lot 101292061; Allchem, Kharkiv, Ukraine); and anisaldehyde (Allchem, Kharkiv, Ukraine).

**Methods of Preparation**

**Thin-layer Chromatographic Identification Test**

**SAMPLE SOLUTION**

1. Transfer about 10 g of ointment, accurately weighed, to a 50-mL conical flask.
2. Add 10 mL of alcohol (70% V/V) to the ointment.
3. Heat on a boiling water-bath for 3 to 5 minutes, shaking frequently until the ointment base melts.
4. Cool the mixture on an icy bath.
5. Filter through a plug of absorbent cotton into a 50-mL volumetric flask.
6. Transfer the adsorbent cotton with the drug residue back into the 50-mL conical flask.
7. Repeat the extraction three times with 10 mL, 15 mL, and 15 mL of alcohol (70% V/V) using the method described above.
8. Rinse the 50-mL conical flask and filter with an additional quantity of alcohol (70% V/V).
9. Transfer to the same 50-mL volumetric flask.
10. Dilute to volume with alcohol (70% V/V).
11. Evaporate the solution to dry residue.
12. Dissolve the residue in 1 mL of alcohol (70% V/V).

**METHOD A: FLAVONOIDS**

**STANDARD SOLUTION 1 (OPTIONAL)**
Dissolve 2.5 mg rutin, 2.5 mg hyperoside, and 1 mg chlorogenic acid in 10 mL of methanol.

**STANDARD SOLUTION 2 (OPTIONAL)**
Mix thoroughly 2 mL of calendula tincture and 2 mL of arnica tincture.

**DERIVATIZATION REAGENT**
Natural Products (NP)/PEG Spraying Solution
NP reagent: 1 g of diphenylborinic acid aminoethylster is dissolved in 100 mL methanol.
Macrogol reagent: 5 g of PEG-400 (International Nonproprietary Name; macrogol) is dissolved in 100 mL of methanol.

**CHROMATOGRAPHIC CONDITIONS**
Stationary phase: HPTLC plates silica gel 60 F254 of appropriate size (10 × 10 cm or 20 × 10 cm)
Mobile phase: Ethyl acetate:anhydrous formic acid:glacial acetic acid:water (100:11:11:27)
Sample application: 10 mcL of sample solution and 4 mcL of standard solution are applied as 8-mm bands, min 2 mm apart, 10 mm from lower, left, and right edges of plate.

**Development:** Chromatographic chamber, saturated for 30 minutes with filter paper, developing distance 60 mm from lower edge of plate. The plate is then dried with a hair dryer (cold air) for five minutes.
**Detection:** The plate is heated at 100°C for 3 minutes, then sprayed while still hot in NP reagent, dried in a stream of cold air, then sprayed in macrogol reagent.
**Examination under UV 365 nm.**

**RESULTS**
The chromatogram obtained with the reference solution shows in the middle part, in order of increasing Rf values, a yellowish-brown fluorescent zone (rutin), a light-blue fluorescent zone (chlorogenic acid), and a yellowish-brown fluorescent zone (hyperoside).

The chromatogram obtained with the sample solution shows a yellowish-brown fluorescent zone of low intensity corresponding in position to the zone due to rutin in the chromatogram obtained with the reference solution; below and directly above it, it shows a yellowish-green fluorescent zone and a light-bluish fluorescent zone corre-

---

**Rx**

**HEMORRHOID CREAM**

*List of Ingredients (Ukraine)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calendulae Tincturiae</td>
<td>2 mL</td>
</tr>
<tr>
<td>Arnicae tincturiae</td>
<td>2 mL</td>
</tr>
<tr>
<td>Lanolin</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Vaseline</td>
<td>8.0 g</td>
</tr>
</tbody>
</table>

*List of Ingredients (Translated in English)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marigold Tincture</td>
<td>2 mL</td>
</tr>
<tr>
<td>Arnica tincture</td>
<td>2 mL</td>
</tr>
<tr>
<td>Lanolin</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Vaseline</td>
<td>8.0 g</td>
</tr>
</tbody>
</table>

*In Ukraine, all prescriptions are required to be written in Latin.*

[PHOTO PROVIDED BY THE AUTHORS: SAMPLE OF THE PACKAGED AND LABELED COMPOUNDED MARIGOLD TINCTURE OINTMENT]
**TABLE 1. Stability Tests on the Ointment Over a Period of Two Months Under Forced Degradation.**

<table>
<thead>
<tr>
<th>TEST</th>
<th>ACCEPTANCE CRITERIA</th>
<th>TIME</th>
<th>T° 5±3°C</th>
<th>T° 25±2°C</th>
<th>T° 40±2°C</th>
<th>LIGHT 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical appearance</td>
<td>Thick, viscous mass of yellow color</td>
<td>0 Days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 Days</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Month</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Months</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Identification TLC</td>
<td>According to Method A</td>
<td>0 Days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td>14 Days</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Month</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Months</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Identification TLC</td>
<td>According to Method B</td>
<td>0 Days</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calendulosides</td>
<td></td>
<td>14 Days</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Month</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Months</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* = met the requirements  
- = did not meet the requirements

**RESULTS AND DISCUSSION**

Sample application: 10 mcL of sample solution and 5 mcL of standard solution are applied as 8-mm bands, min 2 mm apart, 10 mm from lower, left, and right edges of plate.

Development: Chromatographic chamber, saturated for 30 minutes with filter paper, developing distance 60 mm from lower edge of plate.

Detection: The plate is sprayed, dried in cold air, and heated at 100°C for 2 to 5 minutes. Examination in white light.

**RESULTS**

The chromatogram obtained with the reference solution shows in the middle part two violet bands (calendulosides). The chromatogram obtained with the sample solution shows two violet zones due to calendulosides in the chromatogram obtained with the reference solution; it shows two intense violet zones in the upper part. Further zones are present.

**METHOD B: CALENDULOSIDES STANDARD SOLUTION 1**

Sonicate 5.0 mg of calendulosides with 5 mL of methanol for 10 minutes. Use supernatant after centrifugation.

**STANDARD SOLUTION 2 (OPTIONAL)**

Mix thoroughly 2 mL of calendula tincture and 2 mL of arnica tincture.

**DERIVATIZATION REAGENT**

Anisaldehyde-sulfuric Acid Spraying Solution

Carefully add 10 mL of sulfuric acid to an ice-cooled mixture of 170 mL of methanol and 20 mL of acetic acid. To this solution, add 1 mL of anisaldehyde.

**CHROMATOGRAPHIC CONDITIONS**

Stationary phase: HPTLC plates silica gel 60 F254 of appropriate size (10 × 10 cm or 20 × 10 cm).


Solutions of the ointment (three batches) were subjected to stability tests at refrigerated temperature ($t=5±3°C$) and ambient condition ($t=25±2°C$, RH 60±5%) over two months. The samples of ointment were packaged in amber-colored, glass containers (20 mL). Also, the samples of the ointment were stressed with light ($t=25±2°C$, light) and accelerated condition ($t=40±2°C$, RH 75±5%). Stability tests were conducted immediately after preparation, after 14 days, one month, and two months. Thus, the samples of the ointment were stored by storage condition: $t=5±3°C$, RH 60±5%; $t=25±2°C$, RH 75±5% in light place and $t=25±2°C$ in light place. The stability of the ointment under such conditions over two months has been investigated. The evaluation parameters were appearance and identification tests. At each test point, a sample was taken and analyzed. Physical stability of the samples of the ointment was assessed by means of visual observation in normal room light and the integrity of its container. Stability of identification tests of the samples of the ointment was analyzed according to validated TLC methods. At the end of the test period, results of all samples were compared.

**Forced Degradation Study**

Environmental factors such as temperature, light, air, and humidity can affect stability. To obtain information about the instability of the ointment during the storage period, the forced degradation of its active substances was carried out. The active substances of the ointment were stressed under forced degradation. For stress degradation, a mixture of equal quantities of calendula tincture and arnica tincture was used. It was stressed with the following: 0.1 M NaOH, 0.1M HCl, H2O2 3%, NH3 (170 g/L).

Alkaline degradation: Add 0.2 mL of 0.1 M NaOH to 2 mL of a mixture of tinctures; mix thoroughly.

Acidity degradation: Add 0.2 mL of 0.1 M hydrochloride to 2 mL of a mixture of tinctures; mix thoroughly.

Oxidative degradation: Add 0.2 mL of H2O2 3% to 2 mL of a mixture of tinctures; mix thoroughly.

Degradation: Add 0.2 mL of NH3 (170 g/L) to 2 mL of a mixture of tinctures; mix thoroughly.

The forced degradation solutions were kept at room temperature for a period of 24 hours and used for tests.

**RESULTS AND DISCUSSION**

The stability of the active ingredients of the ointment and samples of the ointment submitted for stability study by the identification test...
**Alcohol and Herbs Use in Compounding.** RSA. Mobile Phase: acetonitrile:glacial acetic acid:water (15:0.01:0.99). Developer: Grassia reagent, UV 366 nm.

**Storage condition 5±3°C:**

**(A) Flavonoids fingerprint.** Mobile Phase: ethyl acetate:anhydrous formic acid:glacial acetic acid:water (100:11:11:27). Developer: Natural Products reagent, UV 365 nm


**Track assignment (A):**
1. Sample of ointment (storage condition: $t = 25\pm 2^\circ C$), 10 mcL
2. Sample of ointment (storage condition: $t = 25\pm 2^\circ C$, light), 10 mcL
3. Sample of ointment (storage condition: $t = 40\pm 2^\circ C$), 10 mcL
4. Control sample of ointment, 10 mcL
5. Reference substances: rutin, chlorogenic acid, hyperoside, and vitexin (increasing $R_f$), 4 mcL
6. Sample of mixture of calendula and arnica tinctures, 5 mcL
7. Sodium hydroxide, 5 mcL
8. Hydrochloric acid, 5 mcL
9. Hydrogen peroxide, 5 mcL
10. Ammonia, 5 mcL

**Track assignment (B):**
1. Sample of ointment (storage condition: $t = 40\pm 2^\circ C$), 10 mcL
2. Sample of ointment (storage condition: $t = 25\pm 2^\circ C$, light), 10 mcL
3. Control sample of ointment, 10 mcL
4. Calendulosides A & B, 7 mcL
5. Sample of mixture of calendula and arnica tinctures, 5 mcL
6. Sodium hydroxide, 5 mcL
7. Hydrochloric acid, 5 mcL
8. Hydrogen peroxide, 5 mcL

---

**FIGURE 1. Forced degradation experiments of ointment containing tinctures of calendula and arnica.**

**FIGURE 2. Stability test on the ointment containing tinctures of calendula and arnica over a period of two months.**

Storage condition 5±3°C:

**(A) Flavonoids fingerprint.** Mobile Phase: ethyl acetate:anhydrous formic acid:glacial acetic acid:water (100:11:11:27). Developer: Natural Products reagent, UV 365 nm

were investigated by comparing the characteristic pattern of constituents, as TLC fingerprints. Fingerprints for flavonoids and calendulosides were obtained simultaneously. The results indicated that under stress conditions such as alkaline and acidity degradations, the profiles of the flavonoids and calendulosides for the active ingredients of the ointment were quite stable. Under stress conditions such as oxidative and strongly acidic degradations, the profiles of the flavonoids and calendulosides for the active ingredients of the ointment were unstable. It was observed that the characteristic zones of flavonoids disappeared or became low in intensity (Figure 1), and the characteristic zones of the shapes of calendulosides changed.

Under stress conditions such as light and accelerated temperature, the profiles of flavonoids and calendulosides of the ointment are unstable. It was observed that the characteristic zones of flavonoids and calendulosides disappeared or became low in intensity after a one-month study period.

According to the results, the physical appearance of the ointment did not change throughout the study period, except under accelerated condition ($t = 40 \pm 2^\circ C/RH 75\pm5\%$). The characteristic profile for flavonoids and calendulosides of the ointment appeared stable during one month at $t = 25\pm2^\circ C/RH 60\%$ and two months at $t = 5\pm3^\circ C$, when protected from light. In Figure 2, the fingerprints for flavonoids and calendulosides of the ointment for samples stored at refrigerated temperature ($5\pm3^\circ C$) over a period of two months are shown.

**CONCLUSION**

Based on the results of the stability test and the ointment’s physical appearance and identification tests, the ointment is stable for at least two months under the storage condition of $t = 5\pm3^\circ C$ when protected from light, and approximately one month at the storage condition of $t = 25\pm2^\circ C/RH 60\%$ when protected from light.

**REFERENCES**


