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Stabilities Studies of Formulations Containing Eucalyptus Oil

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ABSTRACT: Stability testing of herbal products is a challenging task, because the entire herb or herbal product is regarded as the active substance, regardless of whether constituents with defined therapeutic activity are known. The objective of a stability testing is to provide evidence on how the quality of the herbal products varies with the time under the influence of environmental factors such as temperature, light, oxygen, moisture, other ingredient or excipient in the dosage form, particle size of drug, microbial contamination, trace metal contamination, leaching from the container, etc. and to establish a recommended storage condition, retest period and shelf-life. The present study is reported on Eucalyptus oil which is obtained from the fresh leaves of *Eucalyptus globules.* The present research work deals with the Accelerated stability studies of three ointments containing Eucalyptus oil (i.e Mixagrip ointment, Helpex Effect ointment and Combigrip ointment) by using Gas chromatography through various stability parameters like physical, chemical and microbiological.

KEYWORDS: Eucalyptus Oil; Gas Chromatography; Accelerated Stability Studies.

INTRODUCTION

Stability of a drug can be defined as its ability in a specific container to remain within proscribed standards of physical, chemical, therapeutic and toxicological specifications to ensure the identity strength and quality and purity of a formulation. Biopharmaceutical products in storage change as they age, but they are considered to be stable as long as their characteristics remain within the manufacturer's specifications. The number of days that the product remains stable at the recommended storage conditions is referred to as the shelf life. The experimental protocols commonly used for data collection that serve as the basis for estimation of shelf life are called stability tests. Shelf life is commonly estimated using two types of stability testing: real-time stability tests and accelerated stability tests [1].

1. Real-time stability testing - a product is stored at recommended storage conditions and monitored until it fails the specification.

2. Accelerated stability testing - a product is stored at elevated stress conditions (such as temperature, humidity, and pH). Degradation at the recommended storage conditions can be predicted using known relationships between the acceleration factor and the degradation rate.

Stability studies are conducted at all phases of the drug development cycle for different purposes with the ultimate goal of having a stable product on the market. During development, stability studies are conducted to support the formulation development and safety and efficacy claims of investigational new drugs. At registration, they are conducted to ascertain the quality and shelf-life of the drug product in their intended packaging configuration. After approval, the stability studies are conducted to ensure the quality of production and to support site or other changes to the product. Stability studies of natural products are difficult as compared to pharmaceuticals because these are a complex mixture of various types of unlimited constituents. Different types of strategies such as marker compounds and metabolomic fingerprint profiling may be applied to assess the stability of natural products [2, 3].

A systematic approach should be adopted to the presentation and evaluation of stability information, which should include,

as necessary physical, chemical and microbiological test characteristics. All product characteristics likely to be affected by storage, e.g. assay value or potency, content of products of decomposition, physicochemical properties (hardness, disintegration, particulate matter, etc.), should be determined; for solid or semi-solid oral dosage forms, dissolution tests should be carried out [4].

Types of Stability

Mainly Five types of Stability are generally recognized -

Type of Stability-

1. Chemical - Each active ingredient retains its chemical integrity and labeled potency with in the specified limit.

2. Physical - The original Physical properties including appearance palatability, uniformity, dissolution and suspendability are retained.

3. Microbiological - Sterility or resistance to microbial growth is retained according to specified requirement.

4. Therapeutic- Therapeutic effect remains unchanged

5. Toxicological- No significant increase in toxicity occurs

A product may be released based on accelerated stability data, but the real-time testing must be done in parallel to confirm the shelf-life prediction. If no significant change occurs during six-month's accelerated and real time stability testing, the product will be allowed to place in the market with a provisional shelf-life of up to twenty-four months. Once the pharmaceutical product has been registered, additional stability studies are required whenever variations that may affect the stability of the active pharmaceutical substance or pharmaceutical product are made, such as major variations like the following:

a. Change in the manufacturing process.

b. Change in the composition of the pharmaceutical product.

c. Change of the immediate packaging

MATERIALS & METHODS

Materials

The apparatus and instruments used were Double beam UV spectrophotometer, High performance liquid chromatography, Gas chromatography (Agilent 6080M), Hardness tester, Dissolution apparatus, Disintegration machine, Friability machine, Infrared spectrophotometer, High performance liquid chromatography, Refractometer, Vortex mixer, Laminar hood, Microbalance, Centrifuge machine, Hot air oven, Grinding mixer, Burette, Separating funnel, Test tubes, Measuring cylinder, Beaker, Glass rod, Glass stoppered conical flask, Petridish, Ashless Filter paper.

Chemicals used are chloroform, alcoholic hydroxylamine, menthol, potassium hydroxide, Camphor, cineole, limonene, thymol, methyl acetate, potassium dihydrogen orthophosphate, sodium chloride, peptone, Pancreatic digest of casein, papain digest of soyabean, agar, D-glucose monohydrate, benzylpencillin sodium, chloramphenicol, potassium iodide solution, dilute hydrochloric acid, sodium hydroxide, bromothymol blue solution, acetic anhydride, toluene, 0.1M Perchloric acid, phosphoric acid, triethylamine, acetonitrile, glacial acetic acid, potassium chlorate, HPLC grade water.

Methodology

Method for Stability Studies of Formulations

The purpose of this protocol is to describe the procedure for stability studies of the finished dosage form at accelerated storage conditions.The stability studies shall be carried out to ascertain the following-

a. Stability characterstics

b. Storage conditions

Procedure

A. Selection of batches

Batches selected for the stability shall meet the following criteria-

1. Batches shall be of the same formulation to be marketed.

2. Packaging shall be same as proposed for marketing.

B. Frequency

All Pack for 3 batches shall be subjected to complete stability studies, thereafter one batch everyyear for only real time stability study.In case of any change as mentioned below atleast single batch shall be kept on complete stability studies-

- Change in suppliers for active ingredients.
- Change in the specified manufacturing process.
- Alternative site of manufacture of the product.
- Change in the primary packaging material.
- Change or replacement in the manufacturing equipment having impact on the quality of products.
- Change in the vendor of primary packaging materials.

C. Storage conditions

Table 1. Storage Conditions for Accelerated studies.

STUDY	STORAGE CONDITIONS	TIME PERIOD
ACCELERATED	40̊ C and 75%RH	6 months

D. Test requirements and sampling time points.

Following matrix illustrates the testing requirements and number of time points to be analysed for each type of storage conditions.Stability samples shall be charged within 7 days from the date of release of the batch.The initial analysis data for each batch shall be taken from certificate of analysis. If the time between release of the batch and initiation of stability studies is more than 7 days, reanalysis of the batch shall be done and data obtained shall be considered as initial for stability studies. Further stability samples shall be analyzed as per defined frequency within following time frame-

ACCELERATED CONDITION- +7 DAYS.

The following table shows the tests to be performed at different time intervals during stability study.

S.NO.	TESTS	INITIAL	2MONTHS	3MONTHS	6MONTHS
1.	Description	Y	Y	Y	Y
2.	Identification	Y	Y	Y	Y
3.	Microbiologicaltest				
a.	Total viable count	Y	Y	N	Y
b.	Total fungal count	Y	Y	N	Y
c.	Pathogens	Y	Y	N	Y
4.	Assay	Y	Y	Y	Y

Table 2. Tests for accelerated studies.

Evaluation and interpretation of Results

If a significant change occurs during 6 months testing at the accelerated storage condition, the proposed shelf life shall be based on the real time data. A significant change is considered to have occurred if a 5% change in assay from its initial value. The acceptance criteria as per the specifications are not met. Evaluation of stability data shall be done once in a year. Based on evaluation of data recommendations for change of stability study parameters, storage conditions shall be made to quality assurance and manufacturing.

Method for the Analysis of Eucalyptus Oil(Raw Material) by Gas Chromatography

For analyzing the eucalyptus oil which is used for formulation development the following parameters are to be checked in order to determine whether it is of good quality or not. After analyzing these parameters the oil can be used for making formulations that are used for various purposes.

1. Appearance - Colourless or pale yellow liquid.
2. Odour - Reminiscent of 1,8-cineole
3. Relative Density- 0.906 to 0.927
4. Refractive Index- 1.458 to 1.470
5. Optical Rotation – 0˚ to +10˚
6. Solubility in Alcohol – It is soluble in 5 volumes of ethanol (70% v/v)
7. Aldehydes - To 10 ml in a ground glass-stoppered tube 25 mm in diameter and 150 mm long, add 5ml of toluene R and 4ml of alcoholic hydroxyl amine solution R. Shake vigorously and titrate immediately with 0.5 M KOH in alcohol (60%v/v) until the red changes to yellow. Continue the titration with shaking the end point is reached when the pure yellow odour of the indicator is permanent in the lower layer after shaking vigorously for 2 min and allowing separation to take place. The reaction is complete in about 15 min. Repeat the titration using a further 10ml of substance to be examined and as a reference solution for the end point, the titrated liquid from the first determination to which has been added 0.5ml KOH in a alcohol (60% v/v) is required in the second titration.
8. Assay by Gas Chromatography
Standard solution - Weigh 50 mg of standard oil and make up the volume to 25ml with chloroform.
Test solution - Weigh 50 mg of sample and make up the volume to 25ml with chloroform.
Injector temperature -250˚C
Carrier gas - Nitrogen
Run time - 30min
Injection mode - Split
Flow rate - 1.2ml/min
Procedure - Inject 2µl of standard solution and sample solution and calculate the percentage purity of sample oil.

Calculation

$$\frac{A_T \times W_S \times P \times 100}{A_S \times W_T}$$

A_S-Area of standard peak
W_S-Wt. of working standard
A_T- Area of test sample
W_T- Wt. of test sample
P- Potency (B.P 2010)

Method for Analysis of Ointments Containing Eucalyptus Oil by Gas Chromatography

The ointments which contain eucalyptus oil are analysed by following parameters to assess its stability by assessing its various parameters which are given below at different time intervals i.e At initial stage and then after 2 months, 3 months and 6 months.

Description

White soft mass filled in plastic container.

Average Fill Weight

Weigh the 20 containers of the ointment in order to determine average fill.

$$\frac{\text{Weight of total container} - \text{Weight of container}}{20}$$

Identification

The retention time of the major peak in the chromatogram of the assay preparation corresponds to that obtained from standard solution**.**

Assay

Reagent-Chloroform HPLC grade

Standard dilution-Weigh accurately 200mg of camphor W.S , 100mg of menthol W.S 20mg of thymol W.S and 100mg of methyl salicylate W.S ,20mg of eucalyptus oil W.S and 60mg of turpentine oil W.S and mix with 50ml of chloroform.

Sample solution-
Weigh 1g of ointment and mix with chloroform and dilute to 50ml with chloroform. After dilution the estimation of all ingredients is done by GC with following conditions-
Column-Capillary column DB-624(0.53mm×30m)
Oven temperature-100˚C hold 4minute,5℃/min 200℃
Injector temperature-250℃
Detector temperature -250℃
Flow rate of carrier gas-1.2ml/min
Carrier gas-nitrogen
Split flow-60ml/min
Run time-30min
Injection mode-Split

Procedure- Inject 2µl of standard and sample solution and calculate the percentage of all ingredients present in the sample by area of the respective peaks. After getting chromatogram calculate the percentage of all the ingredients present in the sample by area of the peaks.

Calculation-

$$\frac{\text{Area of sample peak} \times \text{wt. of standard} \times \text{Potency} \times 100}{\text{Area of standard peak} \times \text{sample wt.}}$$

Microbial Test of Ointments
These tests are mainly performed to check whether ointment has been contaminated with microbes or not during its storage and to check whether it is suitable for use.

A. Total Viable Aerobic Count(Bacteria & Fungi)

Sample preparation:
Transfer an accurately weighed portion of ointment to a conical flask containing 100ml buffered sodium chloride-peptone solution pH 7.0, shake to dissolve.

Procedure
Total microbial count(Bacteria and fungi)
Transfer 1.0ml of the sample preparation on to the four pre sterlised petridish,pour 15-25ml of soyabean digest agar medium in two out of four plate,and in two plate sabouraud dextrose agar with antibiotics medium.Incubate the plate of soyabean casein digest agar medium at 30-35˚ C for bacterial count and the plate of sabouraud dextrose agar with antibiotics medium at 20-25˚ C for fungal count for five days.Select plates with the highest number less than 100 colonies and calculate the number of colony forming units per gram of product [5].

Observation
Total bacterial count
Total fungi

Test for *Pseudomonas Aeruginosa*
Transfer 1ml of sample preparation and inoculate in 100 ml soyabean casein digest broth. Incubate at 32.5℃±2.5℃ for 18-24hours to obtain enriched medium. transfer 1.0 ml of enriched medium in to 100ml Mac conkey broth.Incubate at 43-45˚ C for 18-24 hrs. Streak on Mac Conkey Agar plate and incubate at 32.5 ± 2.5˚C for 18-24hrs.

Test for *Staphylococcus Aureus*
Transfer 1ml of sample preparation and inoculate in 100ml soyabean casein digest broth. Incubate at 32.5℃ ± 2.5℃ for 18-24 hours to obtain enriched medium. transfer 1.0ml of enriched medium in to 100ml Mac conkey broth.Incubate at 43-45˚ C for 18-24hrs. Streak on Mac Conkey Agar plate and incubate at 32.5 ± 2.5˚C for 18-24 hrs [6].

RESULTS & DISCUSSION

Results for analysis of Eucalyptus oil (Raw material)
The eucalyptus oil sample was analyzed by various parameters to find its percentage purity so that it can be used to develop ointments which are used for various purposes. The following results are obtained-

Parameters	Results
Description	Colourless liquid
Odour	Reminiscent of 1,8-cineole
Relative Density	0.9091
Refractive index	1.461
Optical rotation	+5˚
Solubility in alcohol	Soluble in 5 volumes of Ethanol(70%v/v)
Aldehydes	Colour changes from Red to yellow
Assay(By GC)	97.92%

Table 3. Evaluation of Eucalyptus oil

Results for Stability studies of ointments containing Eucalyptus oil
The following tables shows the results of accelerated stability studies of three ointments Combigrip ointment, Helpex Effect ointment, Mixagrip ointment respectively after 2 months, 3months and 6 months through various stability parameters i.e Physical, chemical and biological.

Table 4. Evaluation of Stability of COMBIGRIP Ointment

S.NO.	TESTS	SPECIFICATION	INITIAL	2MONTHS	3MONTHS	6MONTHS
1.	**Description**	Soft mass of white colour	Complies	Complies	Complies	Complies
2.	**Average fill weight**	NLT 20gm	20.087gm	20.98gm	20.62gm	20.54gm
3.	**Identification**	The retention time of the major peak in the chromatogram of the assay preparation corresponds to that obtained fromstandard solution.	Complies	Complies	Complies	Complies
4.	**Micro biological test**					
a.	**Total viable count**	NMT 10^2 Microorganism in 1gm	11cfu/gm	12cfu/gm	-	13cfu/gm
b.	**Total fungal count**	NMT 10^1 Fungi in 1gm	Nil	Nil	-	Nil
c.	**Pathogens**	Should be absent in 1gm	Absent	Absent	-	Absent
5.	**Assay**	1-2%	1.25%	1.24%	1.24%	1.23%

Table 5. Evaluation of Stability of HELPEX Effect Ointment

S.NO.	TESTS	SPECIFICATIONS	INITIAL	2MONTHS	3MONTHS	6MONTHS
1.	**Description**	Soft mass of white colour	Complies	Complies	Complies	Complies
2.	**Average fill weight**	NLT 20gm	22gm	21.72gm	20.22gm	21.06gm
3.	**Identification**	The retention time of the major peak in the chromatogram of the assay preparation corresponds to that obtained from standard solution.	Complies	Complies	Complies	Complies
4.	**Microbiological test**					
a.	**Total viable count**	NMT 10^2 Microorganism in 1gm	14cfu/gm	11cfu/gm	-	12cfu/gm
b.	**Total fungal count**	NMT 10^1 Fungi in 1gm	Nil	Nil	-	Nil
c.	**Pathogens**	Should be absent in 1gm	Absent	Absent	-	Absent
5.	**Assay**	1-2%	1.75%	1.74%	1.73%	1.71%

Table 6. Evaluation of Stability of MIXAGRIP Ointment

S.NO.	TESTS	SPECIFICATIONS	INITIAL	2MONTHS	3MONTHS	6MONTHS
1.	**Description**	Soft mass of white colour	Complies	Complies	Complies	Complies
2.	**Average fill weight**	NLT 20gm	22.78gm	21.78gm	20.40gm	21.00gm
3.	**Identification**	The retention time of the major peak in the chromatogram of the assay preparation corresponds to that obtained from standard solution.	Complies	Complies	Complies	Complies
4.	**Micro biological test**					
a.	**Total viable count**	NMT 10^2 Microorganism in 1gm	14cfu/gm	11cfu/gm	-	15cfu/gm
b.	**Total fungal count**	NMT 10^1 Fungi in 1gm	Nil	Nil	-	Nil
c.	**Pathogens**	Should be absent in 1gm	Absent	Absent	-	Absent
5.	**Assay**	1-2%	1.21%	1.18%	1.16%	1.16%

The following graphs shows the percentage of Eucalyptus oil in Combigrip ointment, Helpex effect ointment and Mixagrip ointment at different time points-

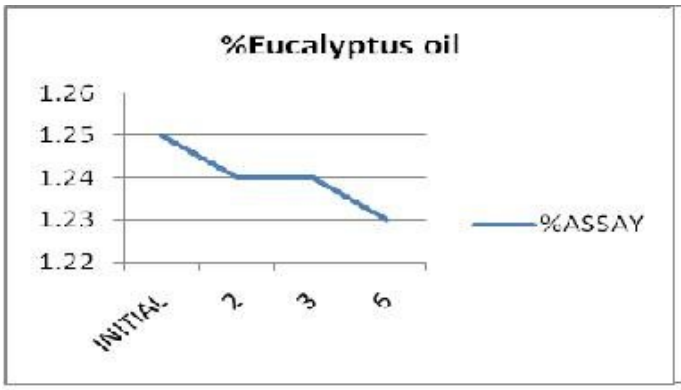

Fig 1. Combigrip ointment

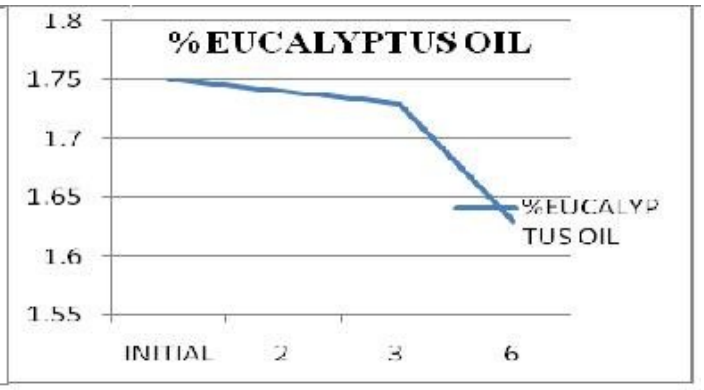

Fig 2. Helpex effect ointment

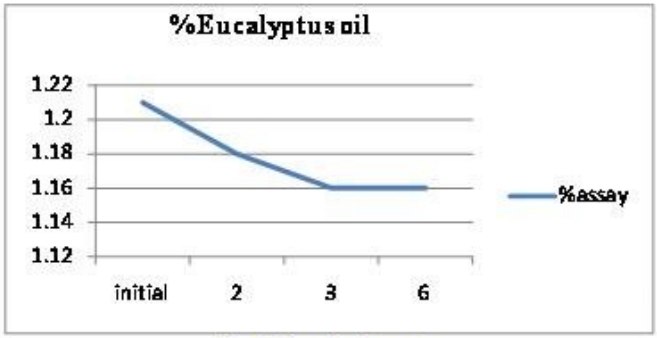

Fig 3. Mixagrip ointment

CONCLUSION

Stability testing is the primary tool used to assess expiration dating and storage conditions for pharmaceutical products. Many protocols have been used for stability testing, but most in the industry are now standardizing on the recommendations of the International Conference on Harmonization (ICH). In this accelerated stability studies are conducted of three different ointments containing Eucalyptus oil i.e Mixagrip ointment, Helpex effect ointment and Combigrip ointments. The studies are conducted by keeping them at accelerated condition and by conducting testing at different time points i.e initial analysis, 2 months, 3 months and finally 6 months.The preparations are analysed for various parameters physical, chemical and biological. After the testing it was concluded that the ointments are stable as the results were within the specifications of the protocol and there is no degration. So the preparations are stable as there is no significant error. This kind of testing help us to define shelf life of the preparation and recommend appropriate storage conditions for the preparations. Stability studies are a critical part of the drug development process and are essential for drug product marketing approval. Hence these studies are very important to ensure quality of production. So these should always be conducted before marketing. Stability testing is interwoven through the entire fabric of the drug product lifecycle. A detailed knowledge of the stability requirements and the impact on other areas (e.g., container closure, process changes) is needed to properly design and evaluate stability studies in order to ensure minimal delays and minimize costs in developing a new drug product.

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