Analytical Quality check of oil based blend in Flaxilip capsule

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ABSTRACT
Analysis of capsules containing blend in the powder form is easy, but it is bit difficult to analyse the soft gelatin capsule containing oil based blend. The purpose of this study was to develop test parameters to determine and supervise the quality of such herbal capsule formulation. Five different lots of soft gelatin Flaxilip capsule containing Linseed oil, Guggulu processed with linseed oil, Garlic oil, Fenugreek oil along with Soyabean oil as an excipient, were selected for the study. All the five lots were subjected to general capsule tests such as determination of average weight and disintegration time. Results obtained were around 1.3500g and 10minutes respectively. Specific test parameters applicable for oils like specific gravity, refractive index, acid value, peroxide value, saponification value, iodine value were applied quantitatively for quality evaluation. Standardised suitable classical methods were applied. Results in all the five lots were found to be well within inhouse limit. All the samples were subjected to heavy metals and microbiological testing. Compliance of corresponding findings with the standard pharmacopoeial guidelines assure the safe intake of the drug. For getting the better effect, the Guggulu that is Commiphora mukul used in the formulation was processed with linseed oil. Its presence was confirmed by carrying out HPTLC for E and Z guggulu sterone. Resemblance of spots at Rf ranging from 0.36 to 0.38 and 0.43 to 0.45 in Toluene : Acetone (9:1) system showed the presence of guggulu in blends of all the lots. Hence by applying all these test parameters one can ensure the quality of the soft gelatin ayurvedic capsule formulation containing oily base like in Flaxilip capsule.

Key words : Oil based, Soft gelatin capsule, Quality, Classical methods, HPTLC.

INTRODUCTION
Nowadays people are suffering from various diseases. Imbalanced intake of food and increasing stress level elevate the cholesterol level leading to various heart related diseases. Herbal remedies have been widely used in ayurveda since many centuries. More and more people prefer using herbal medicines as they are derived from natural sources and not synthetically prepared. Hence are considered to be “safe”.
Risk factors listed by the National Heart, Lung and Blood Institute are unhealthy blood cholesterol level, high blood pressure, smoking, diabetes, overweight and obesity, metabolic syndrome (a group of risk factors linked to obesity and overweight), lack of physical activity, age, family history of early heart disease. Other factors like stress, alcohol and sleep apnea also may contribute to the coronary artery diseases. Changes in the lifestyle can improve the condition. Medications used include anticoagulants, aspirin and other antiplatelet medicines, ACE inhibitors, beta blockers, calcium channel blockers, nitroglycerin, statins, and supplements high in omega -3 fatty acids, such as fish oil. Bruce Neal has explained about the heart diseases, mortality rates and even various related therapies including lipid lowering therapy.

Hyperlipidemia is an elevation of lipids (fats) in the bloodstream. These lipids include cholesterol, cholesterol esters, phospholipids and triglycerides. They are transported in the blood as part of large molecules called lipoproteins. More than half of cases of coronary artery diseases are attributable to lipid abnormalities. Various cholesterol reducing agents are available in the market, however, they are associated with myopathy and other side effects. This generates a need of natural and safe remedy for controlling the disease.
Cholesterol, a fatty substance present normally in our body, is essential for normal functioning of the body. Amongst the various types of fats high density lipoprotein (HDL), low density lipoprotein (LDL) and Triglycerides (TG) are most important. HDL is known as good cholesterol and LDL as bad cholesterol. Excessive levels of this LDL accumulate on the walls of the arteries; reduce the blood flow, leading to diseases like heart attacks, atherosclerosis, ischemaemic heart diseases etc.

Indian system of medicine has described many herbs which help in reducing the cholesterol level. Flaxilip is the soft gelatin perfectly blended capsule formulation containing Linum usitatissiumum (Linseed) oil, Commiphora mukul (Guggulu) processed with linseed oil, Allium sativum (Garlic) oil, Trigonella foenum graecum (Fenugreek) oil with the soyabean oil as an excipient or base. As all these active ingredients are...
effective medicaments, the blend composed of such ingredients can be very well used to regulate lipid metabolism, to improve cholesterol synthesis and triglyceride level as well as to enhance the HDL level. Hence Flaxilip capsule can be recommended as herbal supplement for balancing the cholesterol level. Also can be effectively used in the management of Hyperlipidemia and associated disorders. It helps in managing the cholesterol levels in the body by naturally and safely improving cholesterol synthesis and thereby regulating lipid metabolism. It reduces LDL and increases the HDL level.

Chemical composition and Significance of each active ingredient is given in the ayurvedic literature. Linseed oil is a potent source of essential fatty acids which the body can not make on its own. Linseed oil contains substance called alpha linolonic acid which can lower cholesterol. This oil helps to prevent elevated blood pressure by inhibiting inflammatory reactions that causes artery hardening and poor circulation, It also protects against “Angina” and being a source of essential fatty acids can help prevent the build up of fatty deposits in the tissues. Kachcha guggul decreases LDL cholesterol and triglycerides by 15% to 20%. It increases HDL cholesterol level. Guggul sterone E and Z from kachcha gugul that is Comiphora mukul is responsible for hypolipidemic effect. It reduces absorption of dietary fat and thus can lower total and LDL cholesterol by 11 to 12 percent and triglyceride levels by up to 16%. Garlic that is Allium sativum reduces serum cholesterol. Garlic extract inhibits vascular calcification in human patients with high blood cholesterol. The lipid and glucose lowering effects of methi – Fenugreek oil that is Trigonella foenum graecum extract have been attributed to saponin. It also lowers total cholesterol and low density lipoprotein cholesterol (LDL – C). Soyabean oil can be catagorised as unsaturated fat. It is a source of Omega 3 and Omega 6 fatty acids. Soyabean oil can help to meet our daily need according to MayoClinic.com.

Linseed oil possesses an acrid taste and smell, soon becomes rancid on exposure to the air, and has the property of taking up oxygen from the air and drying to an elastic skin. Likewise all other oils can get naturally affected over the period. The unpleasant organoleptic characteristics are in part caused by the presence of free fatty acids but the major development of rancidity is brought about by atmospheric oxidation called as autooxidation. Oxidative rancidity is accelerated by exposure to heat and light, by moisture and by the presence of traces of transition metals like copper, nickel and iron, and residual natural dyes and pigments. Hence it is necessary to carry out the quantitative determination of essential quality test parameters such as specific gravity, refractive index, acid value, peroxide value, iodine value and saponification value.

In this Flaxilip capsule, Comymphora mukul that is guggul is added by processing the kachcha gugul with linseed oil. Hence the presence of gugulsterone was confirmed by carrying out well versed HPTLC.

MATERIALS AND METHODS

Raw materials
Raw materials used for this study were procured from authorized vendors in India. Flaxilip capsule – an ayurvedic capsule-formulation under investigation was prepared in the formulation laboratory of Phytomedicine department at Piramal Phytocare Ltd.

Reagents
All the chemicals and reagents used for manufacturing as well as testing purposes were of AR grade and were purchased from M/s Qualigens and M/s Merck India Ltd.

Equipments and Instruments
All the glasswares used were well calibrated and were procured from M/s Borosil. Instruments used were weighing balance (M/s Schimadzu Corporation), hot plate, heating mantles (supplied by M/s Sunbim), Abbe’s refractometer (M/s Rajdhani Scientific Instruments Co.), HPTLC (M/s Anchome, M/s Camag).

Method of analysis
General test parameters of capsule formulations such as average weight and disintegration time were carried out as per standard pharmacopoeial method.

Specific Gravity at 25°C
A thoroughly cleaned and dried pycnometer with known capacity was calibrated by filling it with boiled and cooled distilled water. The pycnometer was filled gently with the oily blend and allowed to attain the temperature to 25°C. Then the pycnometer with the blend was weighed accurately and after repeating the operation for three times, the average reading was recorded and applied to calculate the final specific gravity, using the formula...

Specific gravity at 25°C = (C – A) / (B – A)
Where, C = Weight of the pycnometer with the oily blend in g.
A = Weight of the empty pycnometer in g
B = Weight of the pycnometer with water in g
Refractive Index at 25°C

A drop of oily blend was loaded on a previously calibrated suitable refractometer at 25°C. By adjusting the light rays with the slanted mirror, reading was recorded on the refractive index scale. The distilled water was used as a blank for zero adjustment. Process was repeated thrice and the average value was recorded as the final reading of Refractive Index at 25°C.

Acid Value

About 10.00g of oily blend was weighed accurately and dissolved in 50ml of 1:1 mixture of alcohol:solvent ether, previously neutralized with 0.1M sodium hydroxide and titrated against 0.1M sodium hydroxide using phenolphthalein as an indicator. Titration reading was noted in ml as (TR) and Acid value was calculated by applying the molarity factor as.

Acid value = 5.61 X TR X Molarity Factor / Weight of the sample in g

Peroxide Value

5 to 10 g of accurately weighed oil blend sample was dissolved in 30ml mixture of 2 volumes of chloroform and 3 volumes of glacial acetic acid. 0.5ml of saturated potassium iodide solution was added and the mixture was vigorously shaken for a minute. Then 30ml distilled water was added and titrated against 0.01N sodium thiosulphate using 5 to 10 ml of starch solution as an indicator with continuous vigorous shaking, until the color was discharged. Titration reading in ml was recorded as n1. Blank was carried out under the same condition, omitting the sample & titration reading was recorded in ml as n2. Peroxide value in milliequivalents /kg was calculated as

Peroxide value = [10 (n1-n2) X Normality Factor / Weight of the sample in g]

Iodine value

Accurately weighed about 0.10g of blend was dissolved in 10ml dichloromethane in a dry iodine flask. After adding 20ml iodine monochloride solution, the mixture was allowed to stand in a dark at 15°C to 25°C for 30 minutes. Thereafter 15ml of dilute potassium iodide solution was added in the top cup. Stopper was removed carefully. The stopper as well as sides of the flask were rinsed with 100ml distilled water. After mixing vigorously the overall content was subjected to titration against 0.1M sodium thiosulphate using starch solution as an indicator. Blank was carried out in the similar manner omitting the sample. Iodine value was calculated using the formula

\[
\text{Iodine value} = 1.269 \times \frac{v}{w}
\]

Where,
\[
v = \text{the difference in ml between the titration of sample and blank}
\]
\[
w = \text{weight of the sample in g}
\]

Saponification value

About 2g of the blend was weighed accurately in a 200ml round bottom flask and boiled for about 1 hour with 25ml of ethanolic solution of potassium hydroxide by attaching reflux condenser. Then excess of alkali was titrated against 0.5M hydrochloric acid using phenolphthalein as an indicator. The operation was repeated without the substance being examined. Saponification value was calculated using the expression

\[
\text{Saponification value} = 28.05 \times \frac{v}{w}
\]

Where,
\[
v = \text{the difference in ml between the titration of sample and blank}
\]
\[
w = \text{weight of the sample in g}
\]

Identification test for GUGGUL by HPTLC

Sample preparation : 1g oily blend was extracted in chloroform by sonication. Filtered through filter paper number 1, evaporated the chloroform to dryness and reconstituted in 10ml methanol.

Reference standard:

Kachcha guggul : About 200mg kachcha guggul was extracted in chloroform, filtered through filter paper number 1, evaporated to dryness and reconstituted in 10ml methanol.

Guggul extract powder: About 100mg guggul extract powder was extracted in chloroform, filtered through filter paper number 1, evaporated to dryness and reconstituted in 10ml methanol.

Stationary phase \(\rightarrow\) TLC Silicagel 60 F 254

Solvent system \(\rightarrow\) Toluene : Acetone (9:1)

Sample volume \(\rightarrow\) 10 microlitre

Reference standard volume \(\rightarrow\) 10 microlitre
Results and Discussion

Oils and fats commence to decompose from the moment they are isolated from their natural environment. Changes occur during storage which result in the production of an unpleasant taste and odor. Such oils and fats are referred to as having become rancid. Table 1 gives the clear quantitative picture of initial and near or after expiry retesting details of essential test parameters in all five lots of Flaxilip capsule formulation.

As recorded in table 1, the description of Flaxilip soft gelatin capsule with yellow colored oily blend remain same throughout in all the lots. Average weight and disintegration time vary from about 1.300g to 1.370g and 8.00 minutes to 11 minutes respectively, even after standing till expiry date. Specific gravity lies within limit of 0.930 ± 0.01 for all five lots. The blend of flaxilip capsule is a mixture of oily extracts of more than one ingredient, hence findings cannot be compared with pharmacopoeial specification of any single oil. Therefore the inhouse limits are set to determine the acceptance level of the product.

Refraction index in lot number 3 and 4 is found to be around 1.473, whereas in rest three lots it is observed to be about 1.478. Not much variation observed at initial and retest level study. However, the acid value in all lots during retesting process is found to be slightly increased, but all the findings are well within specified limits. Free fatty acids (FFA) are the unattached fatty acids present in a fat. Some unrefined oils may contain several percent free fatty acids. The levels of free fatty acids are reduced in the refining. Dimberu and Belete estimated free fatty acid and cholesterol content in some commercial edible oils. The FFA figure is usually calculated as oleic acid in which case the acid value = 2 X FFA. From acid values for all five lots of flaxilip capsule recorded in table 2, the maximum value of FFA level observed is in lot 1 that is 1.398, which lies within acceptable limits. During storage, peroxide formation is slow at first during an induction period which may vary from a few weeks to several months according to the particular oil, the temperature etc. Dr. Matt Miller explained about oxidation of food grade oil. In flaxilip capsule. Initially the peroxide values are found to vary from 0.534 mEq/kg to 4.919 mEq/kg. Since oils filled in the capsule are not directly exposed to air, auto oxidation rate is minimum hence slight increase in the peroxide values of respective lots are observed during retesting process. But since all these values are less than 10.00 mEq/kg even after 3 years, we can accept them.

In general, the greater the degree of unsaturation that is higher the iodine value, the greater is the liability of the oil to become rancid by oxidation. Iodine values are found to be quite stable while retesting the samples after 3 years. Lot 1 manufactured in November 2008 shows the highest iodine value around 165, whereas lots manufactured in August 2010 shows around 110 iodine value. Iodine values of all lots are well within limit.

Saponification value is inversely proportional to the mean of the molecular weights of the fatty acids in the glycerides present. Many oils have somewhat similar values with respect to their series. Those in the olive oil series fall within the range 188-196. In Flaxilip capsule, initially saponification value was varying from 152 to 186. After 3 years, the same changed as 188 to 194.

Since Khachcha guggul that is Comiphora mukul is processed with linseed oil and used in the Flaxilip formulation, the presence of Guggul in the form of E and Z guggul sterone was confirmed by HPTLC. The chloroform extract reconstituted with methanol of all individual samples were spotted on TLC plate using Linomat 5 and developed in Toluene : Acetone 9:1 system. Figure 1 shows the 3D display of all five capsule lots along with Comiphora mukul in raw form and Guggul extract powder. Figure 2 (a) and (b) show overlapping spectra of E and Z guggul sterone respectively in the corresponding range of wavelengths mentioned therein. Figure 3 depicts the photodocumentation of the plate at 254nm, 366nm and after spraying with vanillin sulphuric acid reagent, which shows clear dominating spots of Guggul sterone E and Z at Rf about 0.36 to 0.38 for Guggul E sterone and at Rf about 0.43 to 0.45 for Guggul Z sterone. The fingerprint pattern of all lots of flaxilip matches with that of Comiphora mukul, Hence presence of Comiphora mukul in the blend is confirmed. Storage stability of locally manufactured edible vegetable oils were studied and recorded by Nagassapa et al.

Table 2 and Table 3 focus on results obtained by quantitative microbiological evaluation as per standard pharmacopoeia and heavy metals observed in the product respectively. All lots are appreciably stable as pathogens are absent at initial as well as after 3 years. Strong aroma and self preservative properties of the contents used as active ingredients in the formula inhibits the growth of microorganisms.
CONCLUSION

Quality of the soft gelatin capsule containing oil based blend can be supervised by determining various values responsible for assuring the quality of oils, in turn supporting to determine the shelf life of the product. Comparing the initial analysis data with the “Retesting” data after about 3 years, help us to understand the stability of the product with respect to quality. Since oil blend used here is not open to the air and is enclosed in the capsules, which are packed in proper air tight, moisture free, HDPE containers stored at controlled temperature, initial results are not deviated much. Product Flaxilip capsule is found to be stable. Hence by applying all above quantitative as well as qualitative test parameters one can supervise and control the quality of capsule formulation containing oil based blend.

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REFERENCES

[4] Indian Pharmacopoeia, Govt. of India, Controller of Publications, New Delhi, 2007; vol.2 : 633,634.
[5] Indian Pharmacopoeia, Govt. of India, Controller of Publications, New Delhi, 2007; vol.1 : 2.4.27 : 163.
Table 1: Physicochemical parameters of Flaxilip capsule

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Test parameter</th>
<th>Specification</th>
<th>Lot 1</th>
<th>Lot 2</th>
<th>Lot 3</th>
<th>Lot 4</th>
<th>Lot 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Description</td>
<td>Soft gelatin capsule filled with yellow oil</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>Average weight (g)</td>
<td>About 1.300 0</td>
<td>1.323 4</td>
<td>1.308 9</td>
<td>1.362 5</td>
<td>1.364 9</td>
<td>1.342 6</td>
</tr>
<tr>
<td>4</td>
<td>Disintegration time (min)</td>
<td>Not more than 60</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>Specific gravity of the blend at 25°C</td>
<td>0.930 ± 0.01</td>
<td>0.926 2</td>
<td>0.926 2</td>
<td>0.930 0</td>
<td>0.931 1</td>
<td>0.920 8</td>
</tr>
<tr>
<td>6</td>
<td>Refractive Index of the blend at 25°C</td>
<td>About 1.480</td>
<td>1.480</td>
<td>1.478</td>
<td>1.479</td>
<td>1.476</td>
<td>1.473</td>
</tr>
<tr>
<td>7</td>
<td>Acid value of the blend</td>
<td>Not more than 8.00</td>
<td>2.239</td>
<td>2.797</td>
<td>1.960</td>
<td>2.618</td>
<td>1.677</td>
</tr>
<tr>
<td>8</td>
<td>Peroxide value of the blend (mEq/kg)</td>
<td>Not more than 10.00</td>
<td>0.534</td>
<td>1.370</td>
<td>1.900</td>
<td>2.405</td>
<td>3.313</td>
</tr>
<tr>
<td>9</td>
<td>Iodine value of the blend</td>
<td>110.0 0 to 200.0 0</td>
<td>163.4 5</td>
<td>165.4 8</td>
<td>135.2 2</td>
<td>130.4 4</td>
<td>114.7 2</td>
</tr>
<tr>
<td>10</td>
<td>Saponification value of the blend</td>
<td>150.0 0 to 200.0 0</td>
<td>186.0 4</td>
<td>194.0 8</td>
<td>181.2 4</td>
<td>190.5 8</td>
<td>178.4 4</td>
</tr>
</tbody>
</table>

C – Complies  P – present
Table 2: Microbiological testing as per USP / BP

<table>
<thead>
<tr>
<th>S. No</th>
<th>Test parameter</th>
<th>Specifications</th>
<th>Lot 1</th>
<th>Lot 2</th>
<th>Lot 3</th>
<th>Lot 4</th>
<th>Lot 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>Total aerobic microbial count (cfu/g)</td>
<td>NMT 1000</td>
<td>30</td>
<td>&lt;10</td>
<td>20</td>
<td>&lt;10</td>
<td>10</td>
</tr>
<tr>
<td>ii</td>
<td>Total combined yeast/molds count (cfu/g)</td>
<td>NMT 1000</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>iii</td>
<td>Bile – Tolerant Gram Negative Bacteria (cfu/g)</td>
<td>NMT 100</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>iv</td>
<td><em>Escherichia coli</em> in 10g</td>
<td></td>
<td>Shoulde be abs.</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
</tr>
<tr>
<td>v</td>
<td><em>Salmonella spp.</em> in 10g</td>
<td></td>
<td>Shoulde be abs.</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
</tr>
<tr>
<td>vi</td>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>Shoulde be abs.</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
</tr>
<tr>
<td>vii</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td>Shoulde be abs.</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
</tr>
<tr>
<td>viii</td>
<td><em>Clostridia spp.</em></td>
<td></td>
<td>Shoulde be abs.</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
<td>Abs</td>
</tr>
</tbody>
</table>

cfu/g: colony forming unit per gram  
NMT: Not More Than  
Abs: Absent
Table 3: Content of Heavy metals in Flaxilip capsule (as per department of AYUSH)

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Test parameters</th>
<th>specifications</th>
<th>Lot 1</th>
<th>Lot 2</th>
<th>Lot 3</th>
<th>Lot 4</th>
<th>Lot 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arsenic as (As)</td>
<td>NMT 3.00</td>
<td>&lt;0.10</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td>2</td>
<td>Lead as (Pb)</td>
<td>NMT 10.00</td>
<td>&lt;0.10</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td>3</td>
<td>Mercury as (Hg)</td>
<td>NMT 1.00</td>
<td>&lt;0.10</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>4</td>
<td>Cadmium as (Cd)</td>
<td>NMT 0.30</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.20</td>
<td>&lt;0.20</td>
<td>&lt;0.30</td>
</tr>
</tbody>
</table>

ppm: part per million  
NMT: Not More Than

Fig. 1: 3D Display of all tracks -> 5 lots of Flaxilip with Kachcha guggul and Gugul extract

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Fig. 2: Overlapping spectra of  
(a) E Gugulu sterone (Rf 0.36-0.38) and  
(b) Z Gugulu sterone (Rf 0.43-0.45)  
5 lots of Flaxilip with Kachcha guggul and Gugul extract

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Fig. 3: Flaxilip Lot 1 to 5, kachcha guggul and Gugul extract powder 5 lots of Flaxilip with Kachcha guggul and Gugul extract
<table>
<thead>
<tr>
<th>Under 254 nm</th>
<th>Under 366nm</th>
<th>After spraying with VSR</th>
</tr>
</thead>
</table>

[Images of gel electrophoresis under different wavelengths and after spraying with VSR]