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# Formulation evaluation and validation of ophthalmic emulsion of docosahexaenoic acid

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## Abstract

The present study aimed at the formulation evaluation and validation of ophthalmic emulsion of docosahexaenoic acid for ophthalmic delivery of drug. Preparation of oil-in-water (o/w) type emulsion is one of the approaches to formulate drugs that are poorly water-soluble. When DHA is administered in capsule dosage form then less amount of drug reaches to the site of inflammation in the eye hence more dose is required, still the inflammation is at risk. Hence, for the direct application of DHA to the eye it can be prepared in the form of eye drops. As the fish oil is richest source of DHA it was isolated from it. The isolated compound was identified by thin layer chromatography by comparing it with standard DHA. Further analysis of isolated compound was done by gas chromatography. The oil in water emulsion was formulated containing 3% DHA oil, 1% tween 80 and 0.5% span 80. The appearance of the emulsion was milky white, the median particle size was 0.4 µm, viscosity was 30 cp, The emulsion was stable at 25ºC and 40ºC for more than 3 months. furthermore the formulated emulsion was validated for the same physical properties which are previously evaluated. The analysis of DHA in the emulsion was done by gas chromatography.

**Keywords :** Docosahexaenoic acid, fish oil, ophthalmic emulsion, gas chromatography

## 1. Introduction

The health benefits of fish oil include its ability to aid in treatment of heart diseases, high cholesterol, depression, anxiety, low immunity, cancer, diabetes, inflammation, arthritis, AIDS, Alzheimer's disease, eye disorders, macular degeneration and ulcers. It is helps in weight loss, pregnancy, fertility and skin care (particular for disorders such as psoriasis, acne).[1]

Most of the these health benefits of fish oil can be attributed to the presence of Omega 3 essential fatty acids such as Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EP A). The most common names among these for obtaining fish oil are albacore tuna, herring, mackerel, sardines, lake trout and salmon.[1]

### 1.1 DHA is present in the retina

The literature survey reveals that Saadia R *et al*., worked on topical omega-3 and omega-6 fatty acid for treatment of dry eye and concluded that topical ALA treatment led to a significant decrease in dry eye sign and anti-inflammatory changes at both cellular and molecular level.[11] Horby M *et al*., worked on effect of formula supplemented with docosahexaenoic acid and[gamma]-linolenic acid on fatty acid status and visual acuity in term infants and concluded that the addition of docosahexaenoic acid resulted in concentrations in red blood cells at similar levels as those in breast-fed infants, whereas the increase in visual acuity did not reach significance. The addition of-linolenic acid resulted in higher arachidonic acid concentrations in red blood cells.[28]

Juarez M *et al*., worked on validation of gas-liquid chromatography method for analyzing samples rich in long chain n-3 polyunsaturated fatty acids : Application to seafood and concluded that TMS-DM as methylation agent to analyze a wide spectrum of FA in different seafood matrices, as well as lack of artifacts observed on the chromatograms, provided enough evidence to suggest the proposed extraction methylation method as a suitable alternative for total FA analysis of sample rich in LC n-3 PUFA as seafood.[29]

Abu EO worked on omega-3 index determined by gas chromatography with electron impact mass spectrometry and concluded that the omega-3 index ranged from 2.3% to 6.2% among the 12 volunteers examined and there was no difference between samples taken in the fasting and postprandial states. EPA and DHA concentration ranged from 3.53 to 195.89µg/ml and 12.19 to 214.42µg/ml respectively. Thus GC-MS method has been developed for measuring the omega-3 index.[30]

Tande T *et al.* worked on validation of a method for gas chromatographic analysis of eicosapentaenoic acid and docosahexaenoic acid as an active ingredient in medicinal products and concluded that the method establishes relations between label claim of active ingredient and known reference standards. The relative standard deviation was 1% for determination of the empirical response factors of EPA ethyl esters and DHA ethyl esters relative to the internal standard,C23:0 methyl esters.[31]

**1.2 Anti-inflammatory action of n-3 PUFAs:**

Arachidonic acid is an important component of mammalian cell membranes. During the early stages of inflammation, arachidonic acid is released from the cell membrane through the activation of phospholipase A2 and serves as a substrate for the synthesis of bioactive eicosanoids (e.g., prostaglandins, leukotrienes and thromboxane) which are proinflammatory agents that increased vascular permeability, enhanced the activity of immune cells, and stimulate the release of cytokines.[2]

**1.3 Dry eye syndrome (DES):** It is referred to as tear film instability, a condition that typically develops from deficiencies of one or more components of the biologically complex pre-ocular tear film. This arises because the eye is no longer secreting enough tears or undergoing rapid rate of evaporation. Tears are composed of three layers: the outer oily lipid layer, the middle watery lacrimal layer, and the inner mucous or mucin layer. Each layer is produced by a different part of the eye, for example, the lacrimal gland produces the lacrimal layer. Therefore, a problem originating in any part of the eye can result in dry eyes. The recently introduced topical cyclosporine A has been shown to decrease ocular surface inflammation, stimulate tear production, and improve signs and symptoms of dry eye.[11]

**1.4 Emulsion:** Oil-in-water emulsion generally comprise of an aqueous phase having suspended therein discrete oil droplets surrounded by a layer of at least one water soluble surfactants. Emulsion stability is partly determined by particle size of oil globule of oil in water emulsion having particle size that exceeds 1µm in diameter is less stable and undergo creaming, coagulation and phase separation upon storage.[13] All ophthalmic composition comprising of an oil-in-water emulsion used directly in to the eye achieve maximum efficacy when the oil phase spread evenly and freely over the eye surface. Ophthalmic emulsion offers an advantage of being able to deliver poorly water soluble drug in solubilised form as an eye drop.

**1.5 Gas chromatography:** In gas chromatography, the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the retention time of the compound.[24] The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the two mobile phases.[25]

**1.6 Validation of the analytical method**

**Accuracy:** The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Minimum five test concentrations from 80% to 120% are normally used, for establishment of accuracy in assay of drug substance. Average recovery should be 99 to 101% of drug at each level. **Precision**

Precision may be the measure of either the degree of repeatability or reproducibility of an analytical method under normal operating conditions.

**Linearity and range:** The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The range of analytical method is the interval between the upper and lower level of analyte (including these levels) that have been demonstrated to be determined with suitable level of precision, accuracy, and linearity using method written.

**Ruggedness:** The ruggedness of an analytical method is obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

**Robustness:** The robustness of a method is evaluated by varying method parameters such as pH, ionic strength, temperature, etc., and determining the effect (if any) on the results of the method.

## 2. Materials and methods

Docosahexaenoic acid is supplied by Central India Pharmaceuticals , MIDC, Nagpur Soluble in Chloroform, n-hexane, dichloromethane. Fish oil was obtained from Janta Aqua Fisheries Udupi , Karnataka .Tween 80 was used as surfactant. UV spectrophotometer used was UV 1700 Shimadzu japan, GC used was Thermo scientific Trace GC 600, Brookfield viscometer LVT DV-1

**2.1 Isolation of DHA from Fish oil by Column Chromatography:** Isolation of DHA from Fish oil was performed as it has higher concentration of DHA. The isolation procedure was carried out by column chromatography as follows.

**Table 1 column chromatography**

| Sr no. | Solvents used for elution | Fraction no |
|---|---|---|
| 1 | Petroleum ether | 1-6 |
| 2 | Petroleum ether : n-hexane (90:10) | 7-14 |
| 3 | Petroleum ether : n-hexane (80:20) | 15-21 |
| 4 | Petroleum ether : n-hexane (70:30) | 22-29 |
| 5 | Petroleum ether : n-hexane (60:40) | 30-37 |
| 6 | Petroleum ether : n-hexane (50:50) | 38-44 |
| 7 | Petroleum ether : n-hexane (40:60) | 45-50 |
| 8 | Petroleum ether : n-hexane (30:70) | 51-58 |
| 9 | Petroleum ether : n-hexane (20:80) | 59-66 |
| 10 | Petroleum ether : n-hexane (10:90) | 67-71 |
| 11 | n-hexane | 72-76 |
| 12 | n-hexane : Chloroform (99:1) | 77-85 |
| 13 | n-hexane : Chloroform (98:2) | 86-91 |
| 14 | n-hexane : Chloroform (97:3) | 92-101 |
| 15 | n-hexane : Chloroform (96:4) | 102-111 |
| 16 | n-hexane : Chloroform (95:5) | 112-121 |
| 17 | n-hexane : Chloroform (94:6) | 122-130 |

Then concentrated fractions were subjected to thin layer chromatography by using different solvent system,detecting reagent used were 50%$H_2SO_4$ and iodine chamber.

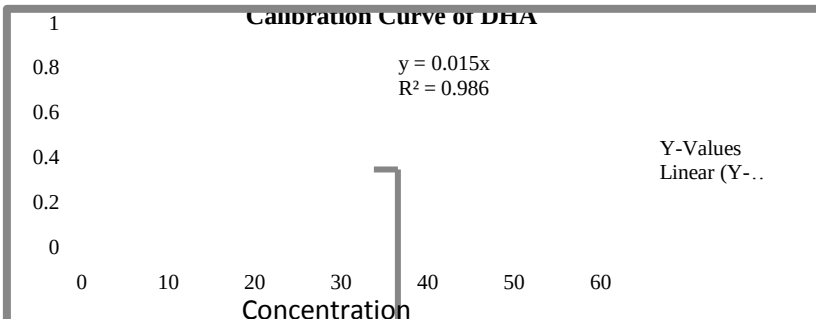
**Table 2:Thin layer Chromatography of Isolated DHA**

| Sr no. | Sample spots applied | Solvent system used | No. of spots with different locating agent | | | $R_f$ Value |
|---|---|---|---|---|---|---|
| | | | UV | Iodine | 50 % $H_2SO_4$ | |
| 1. | Isolated DHA | benzene | --- | 1 | 1 | 0.56 |

$R_f$ value of standard DHA - 0.56 . The Docosahexaenoic acid showed one spot and $R_f$ value of was found to be 0.56

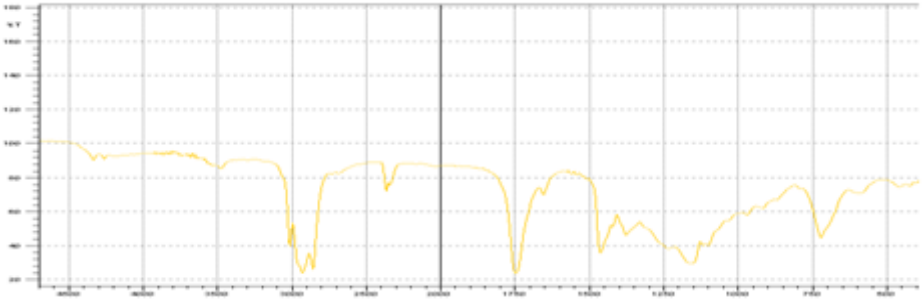
**UV Spectroscopy**

**Fig 1: Calibration curve of isolated DHA**




**FT-IR Spectra**

**Fig 2 : FT-IR spectra of DHA**



**2.2 Determination of isolated DHA by gas chromatography**

**Table 3:Chromatographic conditions:**

| | |
|---|---|
| Instrument | Thermo scientific Gas Chromatograph |
| Column | fused silica capillary column |
| Column dimension | 30 m X 0.25 mm ID X 0.25 µm film thickness |
| Carrier gas | Nitrogen |
| Detector | Flame ionization detector |
| Flow | 1-2ml /minutes |
| Injector mode | Split |
| Pressure | 73 kpa |
| Oven temperature | 110°C |
| Injector volume | 1µl |
| InjectorTemperature | 70°C |
| Detector Temperature | 190°C |
| Run Time | Upto 30 min |

**2.2.1 Preparation of Standard solution:** An accurately weighed 25 mg of standard DHA was dissolved in sufficient quantity of chloroform in 25 ml of volumetric flask later.Then, 1.0 µl of sample was injected manually into injector of gas chromatography

**Fig 3: Chromatogram of standard DHA**

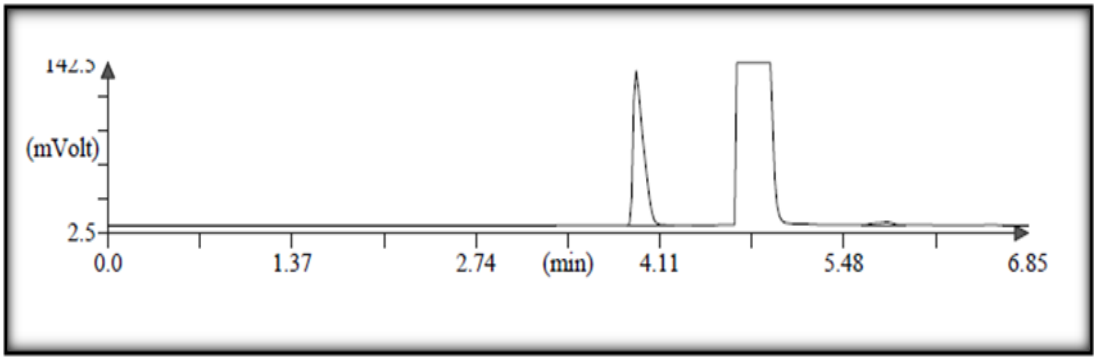



**Table 4: for chromatogram of standard DHA**

| Sr no. | Peaks | Drug sample | Ret time (min) | Area (µ*V*Sec) | Peak height |
|---|---|---|---|---|---|
| 1 | 1 | Chloroform | 3.933 | 6629819 | 127235 |
| 2 | 2 | Standard DHA | 5.011 | 200202 | 2332 |

**2.2.2 Preparation of test solution:** An accurately weighed 25 mg of isolated DHA was dissolved in sufficient quantity of chloroform in 25 ml of volumetric flask later. Then, 1.0 µl of sample was injected manually into injector of gas chromatography.

**Fig 4: Chromatogram of isolated DHA**

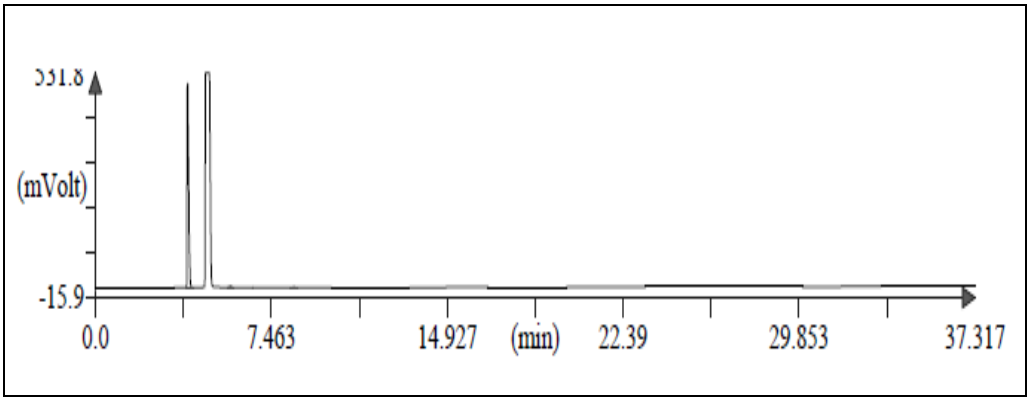


**Table 5:for chromatogram of isolated DHA**

| Sr no. | Peaks | Drug sample | Ret time (min) | Area (µ*V*Sec) | Peak height |
|---|---|---|---|---|---|
| 1 | 1 | Chloroform | 3.933 | 6629819 | 127235 |
| 2 | 2 | Standard DHA | 5.011 | 200202 | 2332 |

**2.3 Validation of the analytical method**

**Accuracy:** Accuracy can be evaluated by addition of known pure substance into test sample at different concentration (50%, 100% and 150%) and assessing the recovery of the added substance.

**Table 6: Results for the accuracy**

| Sr no. | Blank solution with spiked analyte in %(µG) | Area under curve | Recovery (%) |
|---|---|---|---|
| 1 | 50% | 346725 | 98.18 |
| | | 353354 | |
| | | 354580 | |
| 2 | 100% | 474680 | 99.69 |
| | | 483881 | |
| | | 482321 | |
| 3 | 150% | 588435 | 99.49 |
| | | 614162 | |
| | | 601935 | |
| Mean | | | 99.45 |
| % RSD | | | 0.8275 |

**Ruggedness**: The reproducibility of test results was obtained by this method for the reliability of instrument to instrument, analyte to analyte and day to day.

**1) Intraday studies:**

**Table 7: for intraday study**

| Sr No. | Day | Concentration (µg/ml). | Peak area of DHA | % RSD |
|---|---|---|---|---|
| 1 | Morning | 20 | 358315 | 1.3067 |
| | Afternoon | | 357625 | |
| | Evening | | 349951 | |
| 2 | Morning | \60 | 728931 | 0.7712 |
| | Afternoon | | 732251 | |
| | Evening | | 723983 | |
| 3. | Morning | 100 | 1198025 | 0.5340 |
| | Afternoon | | 1210005 | |
| | Evening | | 1200005 | |

**2. Interday study**

**Table 8:: Results for inter day study**

| Sr No. | Day | Concentration (µg/ml) | Peak area of DHA | % RSD |
|---|---|---|---|---|
| 1 | Day 1 | 20 | 354950 | 0.5439 |
| | Day 2 | | 355500 | |
| | Day 3 | | 358548 | |
| 2 | Day 1 | 60 | 670925 | 1.0663 |
| | Day 2 | | 680467 | |
| | Day 3 | | 685124 | |
| 3. | Day 1 | 100 | 1198025 | 1.5880 |
| | Day 2 | | 1165870 | |
| | Day 3 | | 1165468 | |

**Robustness:** The robustness of test method was carried out system suitability under normal condition and each of the altered condition maintained below.

**Table 9: Change in carrier gas pressure**

| Sr n | Parameter | Concentration (µg | Peak are | Observation |
|---|---|---|---|---|
| 1 | Carrier gas pressure 73 Kpa | 20 | 352950 | 75.5 Kpa |
| | | | 352900 | |
| | | | 352948 | |
| 2 | % RSD | | | 0.5487 |

**Table 10 :Change in initial oven temperature**

| Sr no. | Parameter | Concentrati (µg/ml) | Peak area | Observation |
|---|---|---|---|---|
| 1 | Initial oven temperature 110ºC | 20 | 352950 | 140º C |
| | | | 353600 | |
| | | | 356650 | |
| 2 | % RSD | | | 0.50574 |

**2.4 Formulation of the emulsion[32]:** Oil-in-water emulsion was prepared containing DHA oil as an oil phase and varying concentrations of Tween 80 and span 80 as an emulsifying agent.Preparation of emulsion was performed in two steps. Initially water was placed in glass beaker; Tween 80 was added to the water and mixed. Sodium borate and boric acid as buffering agent and ascorbic acid,were then dissolved in solution. sodium chloride was added to the solution. In the next step, oil phase was mixed with oil soluble surfactant span80. It was then added to previously prepared solution and then emulsified by homogenizer at 4000 rpm for 1 hr. This mixture was then adjusted to pH6.8 to 7.4 by adding NaOH. This was then sonicated to get emulsion without any undissolved matter. Water was then added to this and was then sonicated for 2 minute to get uniform emulsion.

**Table 11: for optimized formula**

| Sr no. | Ingredients | % w/v |
|---|---|---|
| 1 | DHA oil | 3 |
| 2 | Tween 80 | 1 |
| 3 | Span 80 | 0.5 |
| 4 | Boric acid | 0.6 |
| 5 | Sodium borate | 0.09 |
| 6 | Sodium chloride | 0.4 |
| 7 | Ascorbic acid | 0.2 |
| 8 | EDTA | 0.05 |
| 9 | Water | Up to 100 |

**Evaluation**: Evaluation of the prepared emulsion was done by considering the physical parameters of the emulsion. Ph was measured by digital pH meter. Viscosity was calculated by Brookfield viscometer. Refractive index was calculated by Abbes refractometer. Globule size was measured by motic plus particle size analyzer

**Table 12: Validation of the physical parameters of the emulsion**

| Test sample | pH | Globule size | R.I. | Viscosity | Drug content |
|---|---|---|---|---|---|
| E3a | 7.10 | 0.42µm | 1.3120 | 30.20 cp | 98.40% |
| E3b | 7.11 | 0.41µm | 1.3210 | 30.04 cp | 98.18% |
| E3c | 7.12 | 0.41 µm | 1.3102 | 30.41 cp | 98.10% |
| E3d | 7.11 | 0.41µm | 1.3121 | 30.21 cp | 98.25% |
| S.D | ± 0.0132 | ± 0.005 | ± 0.00488 | ± 0.1515 | ± 0.1273 |
| %RSD | ± 0.1855 | ± 1.2121 | ± 0.372 | ± 0.5016 | ± 0.1297 |

## 3. Discussion

The present study was undertaken in order to carry out formulation of ophthalmic emulsion containing docosahexaenoic acid, its evaluation and validation. Docosahexaenoic acid (DHA) was isolated from fish oil by column chromatography by using different solvents in increasing order of polarity.(Table1) Fractions 51-58 and 72-85 showed single spot on thin layer chromatography in benzene mobile phase having $R_f$ value 0.56 when compared with standard DHA which matched with the $R_f$ value of standard DHA.(Fig 2, Table 2) Further isolated oil was then subjected to UV and IR spectra (Fig 1) and from the standard calibration curve of isolated DHA Fig 8 and Table 5) in n-hexane it was concluded that drug obeys beers lamberts law. From the gas chromatography analysis it was found that retention time of isolated oil and standard DHA was at 5.73 and 5.01 respectively. (Figure 3, 4) The percentage purity of isolated compound was found out to be 95.21%. This developed method was then validated to know its accuracy, precision, ruggedness, robustness. The optimized formulation was further validated for its physical parameters.

## 4. Conclusion

From the experimental study it was concluded that DHA a constituent of fish oil which is known to be beneficial for dry eye and hence it can be used to formulate the ophthalmic emulsion. The proposed method for GC was very sensitive, quick, precise and suitable for routine determination of Docosahexaenoic acid.

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