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# DEVELOPMENT AND VALIDATION OF METHOD FOR DETERMINATION OF LUTEIN BY HPLC

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## ABSTRACT

A simple selective and rapid reversed phase high performance liquid chromatographic (RP-HPLC) method for The analysis of Lutein 5% has been developed and validated. The separation was achieved from HPLC Column ( C18 250mm x 4.6 mm, 5 µm) with a mobile phase Acetonitrile, Methanol, Water and Ethyl Acetate in the ratio of 70:9·6:0.4:20. Flow rate 1.0 ml/min with UV-visible detection at 446 nm. The method is specific and it is observed that no interference with diluents. Proposed method is accurate with (99.89%-100.16%) recovery for Lutein. The proposed method was accurate, and precise for the quantification of Lutein 5%. The proposed can also be used for routine analysis in quality control. The method was validated for parameters like selectivity, sensitivity, precision, intermediate precision, accuracy, linearity, recovery & stability. This (RP-HPLC) method is suitable for determining the concentration of Lutein 5% and it can apply for routine analysis for determination of the Lutein from dosage form.

**KEYWORDS:** HPLC Column, Carotenoids, Zeaxanthin FDA, ICH, β-carotene.

## INTRODUCTION

Lutein is a highly unsaturated compound; it is inherently susceptible to the oxidative stresses associated with thermal and UV exposure. Several studies have investigated the potential for thermal and oxidative degradation of lutein in organic solvent, oil-in-water emulsion and dosed vegetable oil systems.[1,2,3,4]

Lutein (b-,3-carotene-3,30-diol) belongs to a class of oxygenated carotenoids (xanthophylls) and is present in many foods, particularly vegetables and fruits. Specifically, it is a dihydroxy derivative of a-carotene and the structure of lutein and its importance in protecting against oxidative and blue-light damage in the retina have been described previously.[5,6] Nonetheless, the mechanisms controlling the significantly higher uptake of plasma β-carotene relative to the xanthophylls lutein in the mammary gland remain unknown[7]

Analytical methods for carotenoids in foods have previously been reviewed.[8-12] Direct solvent extraction is the preferred technique for samples with low lipid content or samples that are free of xanthophyll esters. However, in samples with high fat content, such as milk and infant formulae, alkaline hydrolysis has generally been applied prior to solvent extraction[13-15] although enzymatic digestion has also been described.[16-17] Reversed-phase chromatography utilising both high performance liquid chromatography (HPLC) and ultra HPLC platforms, with either C18 or C30 column chemistries, are most commonly used for instrumental analysis.[18] However, normal phase separations have also been reported because of the superior retention of polar xanthophylls and the resolution of carotenoid isomers.[19] Due to subtle differences in the characteristic ultraviolet (UV)-visible absorbance of various carotenoids, photodiode array detection is considered to be essential for unambiguous identification. However, mass spectrometric detection methods have increasingly been used in both atmospheric pressure chemical ionization and electrospray ionization modes to identify constituent carotenoids.[20-22] Methods for the analysis of lutein in infant formulae to support label claims have recently been reported[23-24], and the analysis of lutein and zeaxanthin in vegetable oils, using solvent extraction with quantization by either reversed phase or normal phase LC, has also been described.[25-26]

Only few methods are available for determination of Lutein from pharmaceutical dosage form, so present work was undertaken with the aim to develop and validate a rapid and consistent reversed phase high performance liquid chromatographic method for determination for Lutein according to ICH guideline.[27]

## MATERIALS AND METHODS

### Reagents and chemicals

The following chemicals were used for the process: Water [HPLC grade] Acetonitrile [HPLC grade] Methanol [HPLC grade] & Ethyl Acetate all these chemicals were from Merck,

Germany and Lutein 5 % [Working Standard] were from DSM Nutritional products Ltd., Switzerland.

**Instruments**

The equipment used for the method was Analytical balance Sartorious (model: TE214S). HPLC Dionex Ultimate 3000(equipped with Auto sampler and UV-visible detector), HPLC Shimadzu Prominence (equipped with Auto sampler and PDA detector). The Column selected for the method was Cogent C18, 250mm x 4.6mm, 5µ. The flow rate was monitored at 1.0 mL/min. The wavelength selected for the method was 446 nm and the injection volume was 20µl. The temperature of the column oven was 25ºC± 2º C.

**Method Development**

**Preparation of mobile phase**

The mobile phase was consists of Acetonitrile, Methanol, Water and Ethyl Acetate in the ratio of 70:9·6:0.4:20. (v/v). The prepared mobile phase was degassing in ultrasonic water bath for 5 minutes and it was filtered through 0.45 µ filter under vacuum filtration.

**Preparation of Diluents**

The diluents was consists of Absolute Ethanol and Mobile Phase.

**Standard preparation:** The Standard solution was prepared by accurately weighing and transferring about 200 mg Lutein 5% working standard into a 100 ml clean and dry volumetric flask. Initially about 5 ml of water was added and sonicated for 10 minutes to make dispersion. Then about 70 ml of absolute ethanol was added and sonicated for 10 minutes. The sample was kept for few minutes to cool at room temperature and the volume was made up to mark with the absolute ethanol. The content was filtered through whatman filter paper No. 1. Further from filtrate 10 ml was pipette out into 50 ml clean and dry volumetric flask and diluted up to mark with mobile phase.

**Sample preparation:** The Sample solution was prepared by accurately weighing and transferring about 200 mg Lutein 5% sample into a 100 ml clean and dry volumetric flask. Initially about 5 ml of water was added and sonicated for 10 minutes to make dispersion. Then about 70 ml of absolute ethanol was added and sonicated for 10 minutes. The sample was kept for few minutes to cool at room temperature and the volume was made up to mark with the absolute ethanol. The content was filtered through whatman filter paper No. 1.

Further from filtrate 10 ml was pipette out into 50 ml clean and dry volumetric flask and diluted up to mark with mobile phase.

**System suitability solution**

The final standard solution is used as system suitability solution and inject 20 µl of five replicate injections were injected. The chromatogram was recorded and the system suitability parameters for each of the injection were checked for % RSD of area within 1%, Tailing factor not more than 2.0.

**Method Validation**

**System suitability**

The system was deemed suitable if the following acceptance criteria were satisfied. The relative standard deviation (% RSD) of the peak area responses of Lutein from five replicate injections of standard solution is not more than 2.0%, the tailing factor is not more than 2.0.

**Specificity**

Specificity and selectivity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The standard solution was prepared and injected to the column and the retention time was checked. There were no interferences found. The method was found to be précised and specific.

**Linearity**

It is the relationship between instrument response and known concentrations of the analyte. The linearity was carried out by observing the correlation coefficient (r) of standard solution.

**System Precision**

System precision was carried out by performing six replicate injections of standard at 100% of the test concentration and calculating the % RSD of the measured area.

**Method precision**

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of sample. To demonstrate method precision, six replicate of sample against standard at 100% of test concentration was carried out and the precision of method was calculated by computing % RSD of six measurements.

**Intermediate precision (Ruggedness)**

Intermediate precision or ruggedness study of an analytical method is the degree of reproducibility of the test results obtain by the analysis of the same samples under a variety of normal test conditions. Test sample of Lutein representing single batch was analyzed by two different analysts on two different equipments on two different days. The ruggedness of the test method was calculated by measuring % RSD of six results and % RSD of results of two analysts.

**Accuracy**

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Study was carried out over a range of 80% - 120% (3 replicate each) of the test concentration. The % recovery and RSD of % recovery for each concentration was also measured.

**Range:** Data generated in linearity, precision and accuracy were considered for establishing the range of the analytical method.

**Robustness:** Robustness of the method was investigated by changing flow rate (±0.1%), changing column temperature (±5$^0$C) and ratio of components of mobile phase.

**Stability study:** The solution stability experiments were carried out under room temperature at intervals of 0h 6h 12h 18 h 24 h 30h and 48 h.

## RESULTS AND DISCUSSION

**Table-01: Data for System Precision**

| Standard Concentration (µg/ml) | No. of Measurement | Retention Time | Peak Area | Theoretical Plate | Tailing Factor |
|---|---|---|---|---|---|
| | 01 | 4.230 | 12026 | 9590 | 1.34 |
| | 02 | 4.230 | 12010 | 9552 | 1.34 |
| | 03 | 4.230 | 12031 | 9585 | 1.34 |
| | 04 | 4.230 | 12017 | 959 | 1.34 |
| | 05 | 4.230 | 12017 | 9559 | 1.34 |
| | 06 | 4.230 | 12034 | 9528 | 1.34 |
| Average | | 4.230 | 12022 | 9568 | 1.34 |
| Relative standard deviation | | 0.000% | 0.076 % | 0.272% | ---- |

**System suitability**

System suitability is an integral part of analytical procedures. In optimized chromatographic conditions Relative standard Deviation (%RSD) of area of Lutein 0.076% (NMT 1.0%), Average tailing factor for Lutein 1.34. (Table-01)

**Specificity**

Specificity of an analytical method is its ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures From the specificity study it is observed that the chromatogram for Lutein sample with reference standard showed positive response and Blank (Placebo) had no response, So the method was specific.

**Linearity**

The linearity of an analytical method is its ability to elicit test results directly proportional to the concentration of the analyte in samples within given range. Linearity of the method was evaluated from the correlation coefficient of calibration curves that were constructed from mean peak area of Lutein at different concentrations level (80%, 90%, 100%, 110% and 120%). Correlation coefficient of Lutein was 1.0000. (Table-02, Figure-01).

**Table-02: Data for Linearity**

| Concentration Level | Concentration (µg/ml ) | Peak Area | Correlation co-efficient |
|---|---|---|---|
| 80% | 320.8 | 9210543 | 1.0000 |
| 90% | 360.4 | 10347505 | |
| 100% | 401.8 | 11453029 | |
| 110% | 440.3 | 12641528 | |
| 120% | 480.2 | 13787103 | |

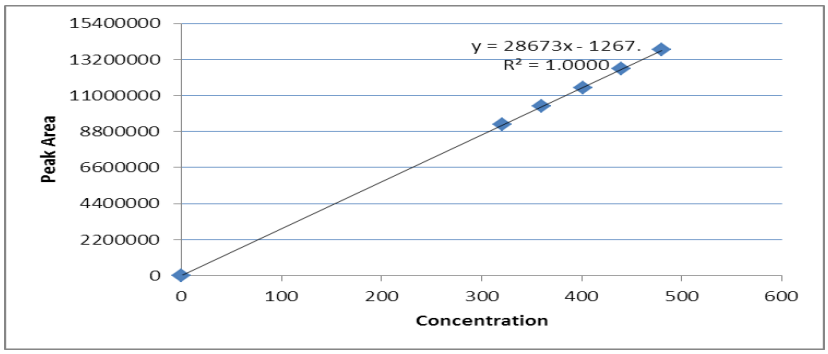


**Figure-01: Graphical Representation for Linearity of Lutein**

**System precision**

System precision was carried out by performing six replicate injections at 100% of the test concentration and calculating the %RSD, Tailing factor, resolution and Theoretical plate count. From the data it is observed that the % Relative standard deviation of area was 0.076%. Tailing factor was 1.34. Theoretical plate count was 9568. (Table-01)

**Method precision**

The result shows that the % RSD of six sample assay results was found to be 0.301%. (Table-03)

**Table-03: Data for Method Precision**

| Sample no. | Assay result in mg | % of label claim | Relative standard deviation |
|---|---|---|---|
| 01 | 5.73 | 99.83 | 0.301% |
| 02 | 5.71 | 99.48 | |
| 03 | 5.72 | 99.65 | |
| 04 | 5.75 | 100.17 | |
| 05 | 5.70 | 99.30 | |
| 06 | 5.72 | 99.65 | |

**Intermediate precision or Ruggedness**

Assay result by two different analysts at different days have been found very much close to each other and with difference of only 0.07 % and the % RSD of two analysts (12 samples) was 0.269% which was within acceptance criteria. So the method can be considered to be rugged enough. (Table-04)

**Table-04: Data for Intermediate Precision**

| Sample No. | % of label claim | |
|---|---|---|
| | Day-1 | Day-2 |
| 1 | 99.83 | 99.78 |
| 2 | 99.48 | 100.17 |
| 3 | 99.65 | 99.48 |
| 4 | 100.17 | 99.48 |
| 5 | 99.30 | 99.83 |
| 6 | 99.65 | 99.75 |
| Mean | 99.68 | 99.75 |
| % of RSD | 0.301% | 0.257% |
| % of RSD of 12 samples | 0.269% | |

**Accuracy**

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The result shows that Average % recovery at different accuracy level is 99.54% -100.29% and % RSD was 0.240%. For individual % recovery meets the acceptance criteria.(Table-05, Figure-02)

**Table-05: Data for Accuracy**

| Concentration Level | Sample No. | Amount added in (µg/ml) | Amount Recovered in (µg/ml) | % Recovery |
|---|---|---|---|---|
| 80% | Sample-1 | 325.6 | 324.1 | 99.54 |
| | Sample-2 | 324.4 | 325.2 | 100.25 |
| | Sample-3 | 325.6 | 326.1 | 100.15 |
| 100% | Sample-1 | 403.2 | 403.3 | 100.02 |
| | Sample-2 | 403.2 | 402.5 | 99.80 |
| | Sample-3 | 404.8 | 404.2 | 99.85 |
| 120% | Sample-1 | 480.4 | 481.0 | 100.12 |
| | Sample-2 | 480.2 | 480.5 | 100.06 |
| | Sample-3 | 480.2 | 481.6 | 100.29 |

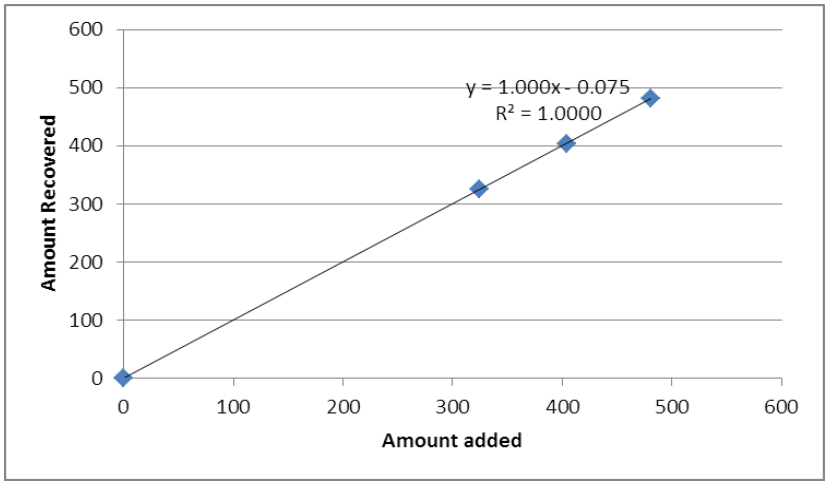


**Figure-02: Graphical Representation for Accuracy of Lutein**

**Range:** The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of the analyte within the extremes of the specified range of the analytical procedure. Based on the linearity, precision and accuracy results, the Range of the method was determined as 80% to 120% of the target concentration.

**Robustness**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The robustness of this method was determined by analyzing the same batch of sample by deliberately changing the method parameters like machine, ratio of mobile phase and column temperature. From the results presented on table it is clear that the system suitability criteria meet with the acceptance limit. Hence the method is robust. (Table-06)

**Table-06: Data for Robustness**

| Sl. No. | Changing Parameters | Assay results (%) |
|---|---|---|
| 01. | Flow rate actual | 99.68 |
| | Flow rate change to 0.8 ml per minute | 100.17 |
| | Flow rate change to 1.2 ml per minute | 100.13 |
| 02. | Mobile phase ratio actual | 99.68 |
| | Mobile phase ratio change to Acetonitrile, Methanol, Water and Ethyl Acetate in the ratio of 68:9.6:0.4:22. | 99.33 |
| | Mobile phase ratio change to Acetonitrile, Methanol, Water and Ethyl Acetate in the ratio of 72:9.6:0.4:18. | 99.50 |
| 03. | Column oven temperature actual | 100.05 |
| | Column oven temperature change to 25°C | 100.33 |
| | Column oven temperature change to 35°C | 100.33 |

**Stability study**

From the stability study data, it was observed that the test sample solution is found to be stable up to 48 h at ambient condition.

**CONCLUTION**

The method adopted for estimation of Lutein by RP-HPLC is precise, linear, accurate, rugged and robust enough. The sample solution is found to be stable up to 48 h at ambient condition. Hence this method can be considered validated for its intended purpose to establish the quality of the drug substance during routine analysis with consistent and reproducible results.

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