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# Analytical validation of stability-indicating reverse phase HPLC method for simultaneous estimation of Levothyroxine and Liothyronine

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**Abstract:** A stability-indicating reverse phase high performance liquid chromatography method was developed and validated for Levothyroxine and Liothyronine. The wavelength selected for quantitation was 285 nm. The method has been validated for linearity, accuracy, precision, robustness, limit of detection and limit of quantitation. Linearity was observed in the concentration range of 10-100 µg/ml for both drugs. For RP-HPLC, the separation was achieved by C-18 column (Phenomenax, 250 x 4.6 mm i.d.) coupled using in mobile phase Acetonitrile: Water (25:75) as mobile phase with flow rate 1 ml/min. The retention time of Levothyroxine and Liothyronine were found to be 2.08 min and 5.02 min, respectively. During force degradation, drug product was exposed to hydrolysis (acid and base hydrolysis), $H_2O_2$, thermal degradation and photo degradation. The percent degradation was found to be 10 to 20% for both Levothyroxine and Liothyronine in the given condition. The method specifically estimates both the drugs in presence of all the degradants generated during forced degradation study. The developed methods were simple, specific and economic, which can be used for simultaneous estimation of both drugs in any combination of dosage forms.

**Key words:** Levothyroxine and Liothyronine, RP-HPLC method, forced degradation and validation

## Introduction:

Stability of pharmaceuticals is defined as the ability to retain the quality, purity, Identity, and safety throughout the shelf life of products. It is of main concern because drug substance and products lose its

Potency and quality after the time passes, due to change in environmental conditions such as temperature, light, and humidity. It is essential that quality of drugs should maintain throw out the life cycle of drug products for their safe and effective use. The need for constant monitoring of the drug substance and product for their quality and purity has the origin of the development of various stability testing methods. Stability testing now becomes a regulatory requirement for filing NDA and ANDA to USFDA and various regulatory agencies [1]. Stability indicating methods had originated from advancement in various analytical instrument technologies. Stability indicating methods of drug substance and products have the ability for separation, identification, qualification, and quantification of all impurities associated with drug substance and drug product at any storage Conditions to give the exact concentration of drug substance or analyte at any time point over the shelf life of products and beyond. These are helpful to understand the degradation pathways as well as obtaining knowledge about impurities developed during processing which should not be present in drug products or have a specific limit, if present. [2]. However, no method is reported for simultaneous estimation of these two drugs by reverse phase HPLC. The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drug substances and products" requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. An ideal stability-indicating method is one that resolves the drug and its degradation products efficiently [3-5]. Consequently, the implementation of an analytical methodology to determine Levothyroxine and Liothyronine simultaneously, in presence of its degradation products is rather a challenge for pharmaceutical analyst. Therefore, it was thought necessary to study the stability of both drugs under acidic, alkaline, oxidative, UV and photolytic conditions. This paper reports validated stability-indicating HPLC method for simultaneous estimation of Levothyroxine and Liothyronine in presence of their degradation products. The proposed method is simple, accurate, reproducible, stability-indicating and suitable for routine determination of both drugs in combined dosage form. The method was validated in compliance with ICH guidelines [6].

## Materials And Methods

Levothyroxine and Liothyronine of API were supplied as gift samples by Intas Pharm. Pvt. Ltd. India. Water and Acetonitrile (HPLC grade) were obtained from Rankem, Ranbaxy Fine Chemical Limited, New Delhi, India.

### Preparation of mobile phase:

The samples were injected through a Hamilton, Bonodaz AG microliter syringe. Chromatographic separation was performed on C-18 column (Phenomenax, 250 x 4.6mm i.d.) coupled with a guard column of the same material. The mobile phase was composed of Acetonitrile: Water (25:75 v/v) and pH of mobile

phase was adjusted to 6.2 with phosphate buffer solution sonicated for 10 min and filtered through 0.45 µm membrane filter and used as mobile phase. The flow rate was maintained at 1.0 mL.min-1.

**Preparation of stock solutions:** Stock solutions were prepared by weighing 10 mg Levothyroxine and Liothyronine separately. The weighed quantity of drugs were transferred to two separate 100 ml volumetric flasks. Volumes were made up to the mark with mobile phase to obtain a solution containing 100 µg/ml of Levothyroxine and Liothyronine. The HPLC analysis was performed on reversed-phase high-performance liquid chromatographic system with isocratic elution mode using a mobile phase of Acetonitrile: Water (25:75 v/v) on a on C-18 column (Phenomenax, 250 x 4.6mm i.d.) coupled with a guard column of the same material. with 1 ml/min flow rate at 300 nm using UV detector.

**Calibration curves for Levothyroxine and Liothyronine:** Levothyroxine and Liothyronine in a ratio of 1:3. Appropriate aliquots of Levothyroxine and Liothyronine stock solutions were taken in different 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 10-100 µg/ml for both drugs, respectively. The solutions were injected using a 20 µl fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting average peak areas versus concentrations and regression equations were computed for both the drugs (Table 1) [7].

**Table 1: Linear Regression Data for Calibration Curve**

| Parameters (units) | Levothyroxine | Liothyronine |
|---|---|---|
| Linearity range (µg/ml) | 10-100 µg/ml | 10-100 µg/ml |
| r2 | 0.9974 | 0.9989 |
| Slope | 32869 | 28281 |
| Intercept | 9856 | 9148 |

**Forced degradation studies:** Forced degradation studies of both the drugs were carried out under conditions of hydrolysis, dry heat, oxidation and sun light. Both API were weighed, powdered, a quantity of powder equivalent to 50 mg of Levothyroxine was transferred to 50 ml volumetric flask and dissolved with mobile phase up to mark. The solution was filtered through 0.2 µm Nylon membrane filter paper and diluted with mobile phase (100 µg/ml). The sample solution was prepared to give final concentrations of 5 and 15 µg/ml for Levothyroxine and Liothyronine, respectively. This sample stock solution (100 µg/ml) was used for forced degradation studies. Forced degradation in alkaline condition was performed by taking 0.1 ml of sample stock solution of Levothyroxine and Liothyronine in separate round bottom flasks. Then 0.1 ml of 0.1 N NaOH was added and this mixture was placed for 1 h at room temperature [8]. Forced degradation in acidic condition was performed by keeping the 0.1 ml of sample stock solution in contact

with 0.1 ml of 0.1N HCl for up to 2 h at room temperature. Degradation with hydrogen peroxide was performed by taking 0.1 ml of sample stock solution and adding 0.1 ml of 3% (w/v) hydrogen peroxide in the flask. This mixture was kept for up to 1 h at room temperature. For dry heat degradation, 5 µg/ml Levothyroxine and 15 µg/ml Liothyronine solution was put in oven at 75° for 1 h. The photo stability was also studied by exposing 5 µg/ml Levothyroxine and 15 µg/ml Liothyronine solution was put into direct sunlight at 1 h. For HPLC analysis, all the degraded sample solutions were diluted with mobile phase to obtain final concentration of 5 µg/ml Levothyroxine and 15 µg/ml Liothyronine solution [9]. Similarly mixture of both drugs in a concentration of 5 µg/ml Levothyroxine and 15 µg/ml Liothyronine solution was prepared prior to analysis by HPLC. Besides, solution containing 5 µg/ml Levothyroxine and 15 µg/ml Liothyronine solution was also prepared without being performing the degradation of both the drugs. Then 20 µl portions of the above solutions were injected into HPLC system and analyzed under the chromatographic condition described earlier [10-11].

**Results And Discussion**

The mobile phase consisting of Acetonitrile: Water (25:75) as mobile phase, at 1 ml/min flow rate was optimized which gave two sharp, well-resolved peaks with minimum tailing factor for Levothyroxine and Liothyronine. The retention times for Levothyroxine and Liothyronine were 2.08 min and 5.02 min, respectively. UV overlain spectra of both drugs showed that both drugs absorbed appreciably at 285 nm, so this wavelength was selected as the detection wavelength. The calibration curve for 2.08 min and 5.02 min, respectively was found to be linear over the range of 10-100 µg/ml respectively. The data of regression analysis of the calibration curves. The proposed method was successfully applied to the determination of both drugs in their combined dosage form. The degradation study indicated that the drug degrades as shown by the decreased areas in the peaks when compared with peak areas of the same concentration of the non-degraded drug, without giving any additional degradation peaks. Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peaks of both the drugs under non degradation condition.

For forced degradation with 0.1 N HCl at 2 h, 0.1 NaOH at 1 h, 3% v/v H2O2, 75° at 1 h and photo degradation at 1h were done. The % degradation was found to be 10 to 20% for both drugs in their tablet dosage form in the given condition using developed HPLC method. In the proposed study, a stability-indicating HPLC method was developed for the simultaneous estimation of both drugs and validated as per ICH guidelines. Statistical analysis proved that method was accurate, precise, and repeatable. The developed method was found to be simple, sensitive and selective for analysis of both drugs in combination without any interference from the excipients. The method specifically estimates both

the drugs in presence of all the degradants generated during forced degradation study. Assay results for combined dosage form using proposed method showed 99.02±0.14% of Levothyroxine and 97.33±0.257% of Liothyronine. The results indicated the suitability of the method to study stability of Levothyroxine and Liothyronine under various forced degradation conditions acid, base, dry heat, oxidation and photolytic degradation. It can be concluded that the method separates the drugs from their degradation products; it may be employed for analysis of stability for their tablet dosage form.

**Table 2: Summary of Degradation Studies for Levothyroxine and Liothyronine Dosage Form**

| Degradation condition | Time | Percent degradation (%) | |
|---|---|---|---|
| | (h) | Levothyroxine | Liothyronine |
| Acid (0.1N HCL) at room temperature | 2h | 5.13 | 20.52 |
| Alkali, (0.1N NaOH) at room temperature | 1h | 0.76 | 12.28 |
| Oxidation, (3% $H_2O_2$) at room temperature | 1h | 10.68 | 19.9 |
| Dry heat (70° ) | 1h | 7.52 | 13.3 |
| Direct sunlight | 0.5h | 0.48 | 16.805 |

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