Acta Chimica and Pharmaceutica Indica

Research | Vol 8 Issue 1

Analytical Method for Cleaning Validation of Levothyroxine Sodium in Production Area

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Received: February 03, 2018; **Accepted:** March 07, 2018; **Published:** March 28, 2018

Abstract

The aim of this present study is to explore a rapid, selective, and sensitive HPLC method for cleaning validation for the analysis of levothyroxine sodium from production area. Chromatographic separation of levothyroxine sodium was achieved at 6.73 min by using 40: 60 volume of acetonitrile and deionized water that contain 0.05% of orthophosphoric acid mobile phase at a flow rate of 1.5 mL/min and detection UV wavelength of 225 nm. The method was validated according to USP Category I requirements. Linearity for detector response was observed in the concentration range of 0.0045–27ppm and coefficient of determination (r^2) for calibration curve was found exactly 0.9999 (r^2 > 0.99). Percent recovery was found from 83.15 to 83.80% ranging from 0.02265 to 006795mg of L-T_4. Precision and intermediate precision showed that the % RSD of both were 2.80 and 0.98 respectively which were in the range of acceptable limit (< 10) for cleaning validation. Limit of detection (LOD) and limit of quantification (LOQ) were obtained at 0.002 and 0.006 ppm respectively. The HPLC method was successfully applied for analysis of levothyroxine sodium samples from different parts of equipment's in production area.

Keywords: ***Levothyroxine sodium; Cleaning validation; HPLC; Limit of detection; Limit of quantification***

Introduction

Thyroid stimulating hormone (TSH) is secreted by the pituitary gland which is stimulated by hypothalamic TSH-releasing hormone (TRH) and inhibited by high serum levels of two hormones produced by the thyroid. This two hormones are tri-iodothyronine (T3) and tetra-iodothyronine (T4, or thyroxine), which contain three and four atoms of iodine respectively [1]. Both T3 and T4 have two enantiomers of them L-enantiomers (L-T3 and L-T4) are responsible for their biologic effects [1]. Deiodinase enzymes convert T4 to T3, which is a major source of T3 (85%) in peripheral tissues [2]. These two hormones control energy metabolism, protein synthesis, and body's sensitivity to other hormones. Cells of the fetal brain are a major target for action of T3 and T4 that play an important role in brain maturation [3-6]. Lower level of TSH hormone may causes hypothyroidism, one of the most common endocrine disorders occurring in up to 5% of the United States and the United

Citation: Islam DMD, Mohiuddin MT, Mynul Hassan MMD, et al. Analytical Method for Cleaning Validation of Levothyroxine Sodium in Production Area. Acta Chim Pharm Indica, 2018; 8(1):125
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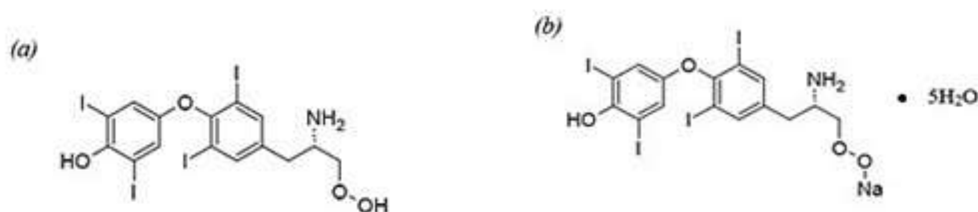
Kingdom [7-9]. In adults, the incidence of hypothyroidism is estimated to be 3.5 per 1000 per year in women and 0.6 per 1000 per year in men [10]. Most patients with the disease require life time thyroxine replacement for normalizing thyroid hormone levels in peripheral tissues. Several oral administrated FDA approved T4 tablets are now available such as, Unithroid®, Synthroid®, L-Thyroxine®, Levoxyl® and Levothyrox® [7]. In last few years some alternative L-T4 formulations have become available soft gel capsule and liquid solution are some of them. Pharmacokinetic studies revealed that the latter formulation process have more bioavailability compared to classical L-T4 tablet [11]. Softgel formulation process has some advantages like higher bioequivalent rate and lower exposure in the production environment [12]. Levothyroxine sodium (L-3, 5, 3, 5-tetraiodothyronine sodium salt) pentahydrate **(FIG. 1)** is a salt of levoisomer of thyroxine **(FIG. 1)** and serves as a replacement therapy for the inadequate secretion of T4 in the body [13]. The initial daily dose to treat hypothyroidism is approximately 1.7 µg/kg, or 100–125 mcg for a 70 kg adult [11]. Daily uptake of levothyroxine sodium should be controlled strictly otherwise it may cause hyperthyroidism and thyrotoxicosis. Hyperthyroidism refers to hyper function of the thyroid gland, whereas thyrotoxicosis refers to any state characterized by thyroid hormone excess [9].

The accurate determination of levothyroxine from the environment of production area is a major concern. There are various analytical methods cited in the literature used for the quantitative determination for levothyroxine like isotope dilution tandem mass spectroscopy [14], high performance liquid

d chromatography [13-17], inductively coupled plasma (ICP) mass spectrometry [18,19], liquid chromatography/tandem mass spectrometry (LC-MS/MS) [20] and liquid chromatography using electrochemical and MS detection [16]. This study is based on to develop a rapid, selective and sensitive HPLC method to accurately and precisely determine L-T4 from the production area at a very low concentration.

FIG. 1. **Structure of (a) thyroxine and (b) levothyroxine sodium pentahydrate.**



Materials and Methods

Materials

L-Thyroxine sodium (L-T_4) certified reference standard was purchased from the Excella GmbH & Co (Germany). Chromafil® Xtra PTFE 0.45 µm syringe filters were purchased from the Pall Corporation (Ann Arbor, MI, USA). HPLC grade acetonitrile, analytical grade phosphoric acid and Sodium Hydroxide were purchased from Fisher Scientific (Fairlawn, NJ, USA). HPLC ready deionized 18Milli-Q water was obtained, in-house, from a Milli-Q Gradient A-10 water purification system, Millipore, (Bedford, MA, USA).

Determination of the binding of PB to HSA in the absence and presence of endogenous substances

The binding of PB to HSA in the absence and presence of endogenous substances was determined using an ultrafiltration technique. Ultrafiltration was carried out using an AmiconR Ultra-0.5 mL centrifuge filter unit with an UltracelR-30 membrane (Merck Millipore Company, MA). Samples of 500 µL were centrifuged at 2,500 × g at 25 °C for 5 min. The concentration of unbound PB in the filtrates was then determined by HPLC.

Recovery calculation

The amount of levothyroxine sodium in the test samples was calculated, as quantity and percent, from the measured peak area response for the test samples (A_U) and compared to peak area response (A_s) for the standard levothyroxine solution using the following equations:

$$\text{Quantity} = \frac{\text{Au}}{\text{As}} \times \text{C} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\%\ \text{Recovery} = \frac{\text{Observed Amount}}{\text{Decleared Amount}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where C is the concentration in ppm of the USP levothyroxine working standard.

Instrumentation and chromatographic conditions

Prominence I HPLC (Shimadzu Corporation, Japan) consisted of a quaternary pump, an automatic injector, variable wavelength detector, and a column oven. Data were processed by using Lab solution 6.82-ST1 software. Separation was achieved on a Kromasil L_{10} column (4.6 mm × 250 mm, 5 µm) and ProntoSIL L_{10} column (4.6 mm × 250 mm, 5 µm) was used for intermediate precision analysis. The flow rate was 1.5 mL/min. The chromatographic conditions: Filtered and degassed 40 volume of acetonitrile and 60 volume of deionized water that contain 0.5ml of orthophosphoric acid were used as mobile phase and diluent. 10 min as equilibration time. The column temperature was controlled at 25°C and the injection volume was 100µL. The UV detection wavelength was 225 nm.

Stock solution preparation (45 ppm)

45 mg of levothyroxine sodium working standard was transferred in 1000 ml amber volumetric flasks add 5 ml 1 M NaOH and 20 ml diluent and sonicate until dissolve. Allow to cool and volume up to the mark with same diluent.

Results and Discussion

The method was validated according to the United States Pharmacopeia Category I requirements. The following validation characteristics were addressed: specificity, accuracy, precision, linearity, range, LOD and LOQ.

System suitability standard

System suitability standard solution was prepared daily using stock solution, for that purpose 10 ml of stock solution was transferred in to 50 ml amber volumetric flasks and dilute up to the mark with diluent. System suitability was determined from five replicate injections of the stock solution before sample analysis. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak area, greater than 2000 theoretical plates, USP tailing factor less than 2. All critical

parameters were tested before sample run and it was found that all parameter met the acceptable criteria throughout all days which is shown in the **TABLE 1**.

TAB.1. **System Suitability Test Results (n=5).**

Parameter	Specifications	Observed results		
		Analyst 01	Analyst 02	Analyst 03
Retention Time (% RSD)	≤ 2.0	0.08	0.02	0.09
Area (% RSD)	≤ 2.0	0.09	0.19	0.67
Tailing Factor	≤ 2.0	1.51	1.44	1.52
Theoretical plates	≥ 2000	7135 ± 64	7996 ± 75	7222 ± 92
n: number of replicates per concentration levels and per series.				

Specificity

Specificity of the method was determined by comparing the system suitability standard and diluent of the sample, examined active component specifically and accurately measured without any interference of diluent peaks. The acceptance criteria were peak of active should be pure that means purity index should be higher than purity threshold and diluent should not show any interfere at the retention time (6.73 min) of Levothyroxine Sodium peak at LOD and LOQ concentration. Specificity was established by determining that peak of levothyroxine sodium standard and diluent were completely segregated **FIG. 2 and 3.**

FIG. 2. **Specificity of levothyroxine sodium from other peaks of diluent.**

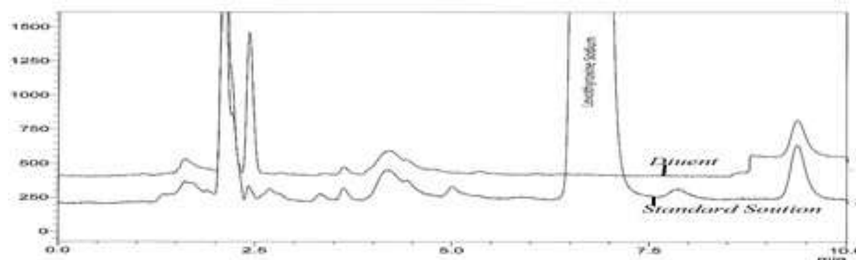
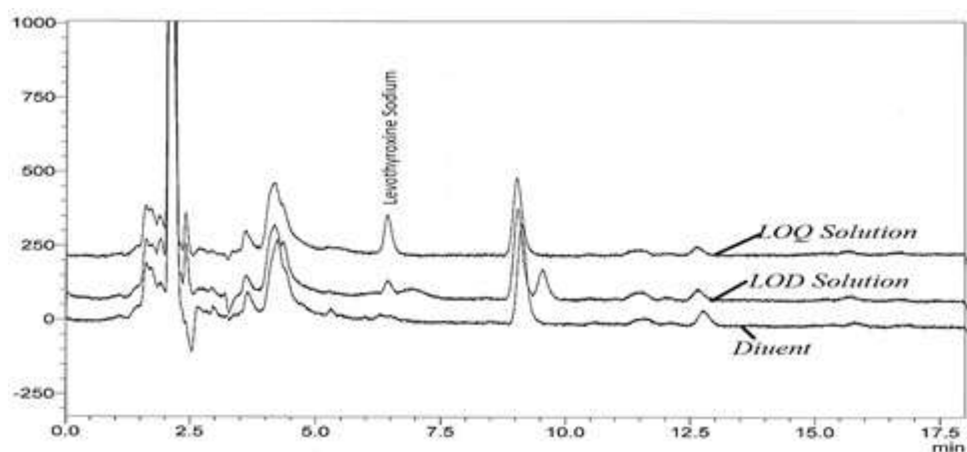


FIG. 3. **Peak comparison of LOD and LOQ from diluent.**



Precision and intermediate precision

The precision of test method was evaluated by analyzing six swab sample solutions which were prepared and analyzed on same day by HPLC**.** 1.0 ml (45 ppm) of levothyroxine stock solution was spread out on the 10 × 10 cm surface area of a 316 grade SS plate, and dried it at ambient temperature. Dried sample was collected by using swab stick which was initially rinse into diluent. Swab stick was transferred in test tube, 5 ml diluent was added and then sample was sonicate for 10 minutes. Total six (6) replicate samples were prepared by the same procedure. The method of precision was established by injecting six replicate samples after running five standard samples. Intermediate precision was determined by applying same procedure with different analyst, HPLC and column. Critical value and results of precision and intermediate precision were summarized in **TABLE 2**. From the **TABLE 2** it was found that all parameter of cleaning validation were in permeable limit.

TABLE. 2. **Precision and intermediate precision Results (n=6).**

Parameter	Specifications	Observed results	
		Precision	Intermediate precision
Area of Sample	-	2157021 ± 2.80	1892832 ± 0.98
Tailing Factor	≤ 2.0	1.50	1.44
Theoretical plates	≥ 2000	7215 ± 79	6521 ± 56
n: number of replicates per concentration levels and per series.			

Recovery or accuracy

Accuracy Study was performed by spreading Levothyroxine Sodium solution on the 10x10 cm surface area of a 316 grade SS plate ranging from 50 to 150% (50%, 80%, 100%, 120% and 150%) which were prepared from stock solution. For this purpose 0.5, 0.8, 1, 1.2 and 1.5 ml of stock solution was spread in to the SS plate and then dried at ambient temperature. After complete dry sample were collected through swab stick which were initially rinse with diluent. Swab stick was transferred in test tube, 5 ml diluent was added and then sample was sonicate for 10 minutes. For cleaning validation recovery should be higher than 80%, and RSD of the each sample should be less than 10% [21]. Results for recovery of levothyroxine sodium

from SS plate by swab stick and there specifications were summarized in **TABLE 3**. Recovery factor was 1.20 that means this cleaning procedure was satisfactory for accurately analysis of levothyroxine sodium from production area.

Linearity and range

Standard calibration curves were prepared with sixteen calibrators over a concentration range of 0.006-27 ppm (0.006, 0.009, 0.018, 0.027, 0.054, 0.09, 0.45, 0.9, 2.25, 4.5, 7.2, 9, 10.8, 13.5, 18 and 27 ppm) for levothyroxine. The data of peak area versus active concentration were treated by linear least square regression analysis (**TABLE 4**). Coefficient of determination for cleaning validation sample should be within 0.99 to 1.0. Data from the **FIG. 4** complies that coefficient of determination value was 0.9999.

LOD and LOQ

LOD means the lowest concentration of the standard solution (six replicate samples) that can be detected but not necessarily quantitated under stated experimental conditions [22,23]. ICH and USP states that signal-to-noise ratio should be ≥3 and RSD of six replicates should be within 10 to 33 [24-27]. Six replicate sample concentration of 0.002 ppm was used to examine the LOD concentration. At that concentration S/N was found 4.11 and RSD was 12.51 **TABLE 5.**

LOQ means the lowest concentration of the standard solution (six replicate samples) that can be quantitatively determined with suitable precision and accuracy [22]. In that case signal to noise ratio should be ≥10 and RSD of six replicates should be ≤ 10 [22, 26, 27]. Six replicate sample concentration of 0.006 ppm was used to examine the LOQ concentration. At that concentration S/N was founded 11.19 and RSD was 2.37 **TABLE 5** that means at that concentration levothyroxine sodium quantitatively determined precisely.

FIG. 4. **Linearity for levothyroxine sodium.**

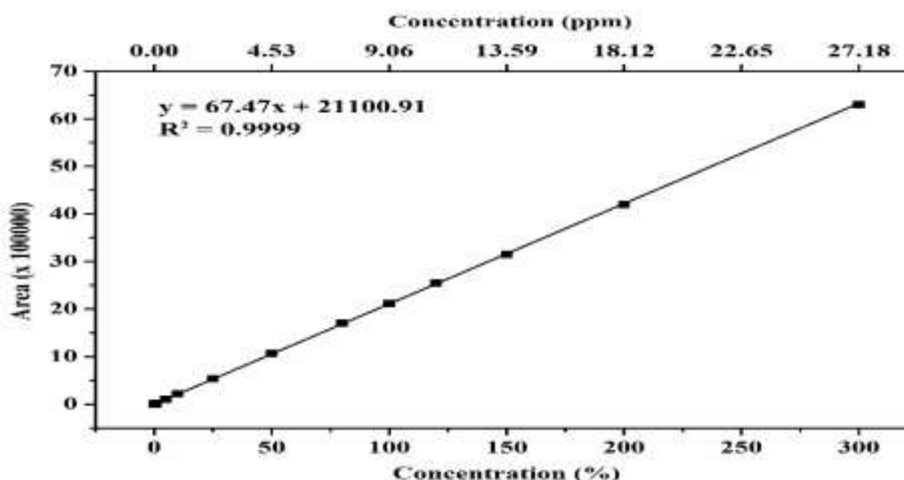


TABLE 3. **Accuracy Results (n=6).**

Parameter	Specifications	50%	80%	100%	120%	150%
Area of Sample	-	927047 ± 0.19	1503889 ± 0.23	1852183 ±1.69	2155581±0.43	2750230 ± 0.20
Declared amount (mg)	-	0.0225	0.036	0.045	0.054	0.0675
Recovery (mg)	-	0.01887 ± 0.19	0.0306 ± 0.23	0.03769 ± 1.69	0.04385 ± 0.43	0.05731 ± 0.20
Recovery (%)	≥ 80	83.85	85	83.76	81.2	82.9
n: number of replicates per concentration levels and per series.						

TABLE 4. **LOD and LOQ results (n=6).**

Parameter	LOD		LOQ	
	Critical value	Observed value	Critical value	Observed value
Concentration	-	0.002 ppm	-	0.006 ppm
Signal to noise ratio	Close to 3	4.11	Close to 10	11.19
% RSD	10.0-33.0	12.51	≤10.0	2.37
n: number of replicates per concentration levels and per series.				

Determination of levothyroxine sodium in production area

The validated method was successfully applied for the determination of levothyroxine sodium from different parts of the production environment. In production area after cleaning with IPA levothyroxine sodium was found in very low concentration which is shown in the **TABLE 5**. Maximum levothyroxine sodium was found in internal surface of the granulation and it was 2.69 ppm, which was within limit of cleaning process validation (less than 9 ppm).

TABLE 5. **Swab samples from different parts of production area.**

Sample ID	Concentration (ppm)
Inside the bin of Blister	0.13
Feeding disk of Blister	0.05
Blister hopper	1.27
Bin lid of Granulator	0.38
Internal surface of Granulator	2.69
Surface gate of Compressor	0.14
Inner O Matic of Compressor	BDL
Inner chute of Compressor	0.25

Window flap	0.09
Metal detector	BDL
Surface disk of deduster	0.04
BDL: Below detection limit.	

Conclusion

A simple and efficient HPLC method was developed and validated for levothyroxine sodium. The method addressed each of the analytical validation characteristics such as accuracy, precision, specificity, linearity, LOD and LOQ met the USP acceptance criteria. The usefulness of this method is demonstrated by successful application for the analysis of levothyroxine sodium in different parts of production area.

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