International Journal of Pharmaceutics 360 (2008) 77–82

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Stability indicating validated HPLC method for quantification of levothyroxine with eight degradation peaks in the presence of excipients

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ARTICLE INFO

Article history:
Received 12 December 2007
Received in revised form 20 March 2008
Accepted 12 April 2008
Available online 20 April 2008

Keywords:
Levothyroxine
Impurities
Degradation products
Validation
Excipients

ABSTRACT

A simple, sensitive, accurate, and robust stability indicating analytical method is presented for identification, separation, and quantitation of L-thyroxine and eight degradation impurities with an internal standard. The method was used in the presence of commonly used formulation excipients such as butylated hydroxyanisole, povidone, crospovidone, croscarmellose sodium, mannitol, sucrose, acacia, lactose monohydrate, confectionary sugar, microcrystalline cellulose, sodium laurel sulfate, magnesium stearate, talc, and silicon dioxide. The two active thyroid hormones: 3,3′,5,5′-tetra-iodo-L-thyronine (L-thyroxine-T4) and 3,3′,5-tri-iodo-L-thyronine (T3) and degradation products including di-iodothyronine (T2), thyronine (T0), tyrosine (Tyr), di-iodotyrosine (DIT), mono-iodotyrosine (MIT), 3,3′,5,5′-tetra-iodothyroacetic acid (T4AA) and 3,3′,5-tri-iodothyroacetic acid (T3AA) were assayed by the current method. The separation of L-thyroxine and eight metabolites along with theophylline (internal standard) was achieved using a C18 column (25 °C) with a mobile phase of trifluoroacetic acid (0.1%, v/v, pH 3)–acetonitrile in gradient elution at 0.8 ml/min at 223 nm. The sample diluent was 0.01 M methanolic NaOH. Method was validated according to FDA, USP, and ICH guidelines for inter-day accuracy, precision, and robustness after checking performance with system suitability. Tyr (4.97 min), theophylline (9.09 min), MIT (9.55 min), DIT (11.37 min), T0 (11.63 min), T2 (14.47 min), T3 (16.29 min), T4 (17.60 min), T3AA (22.71 min), and T4AA (24.83 min) separated in a single chromatographic run. Linear relationship ($r^2 > 0.99$) was observed between the peak area ratio and the concentrations for all of the compounds within the range of 2–20 μg/ml. The total time for analysis, equilibration and recovery was 40 min. The method was shown to separate well from commonly employed formulation excipients. Accuracy ranged from 95 to 105% for T4 and 90 to 110% for all other compounds. Precision was <2% for all the compounds. The method was found to be robust with minor changes in injection volume, flow rate, column temperature, and gradient ratio. Validation results indicated that the method shows satisfactory linearity, precision, accuracy, and ruggedness and also stress degradation studies indicated that the method can be used as stability indicating method for L-thyroxine in the presence of excipients.

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1. Introduction

Levothyroxine sodium pentahydrate, the sodium salt of the levo-isomer of thyroxine is an active physiological substance secreted by thyroid gland. With three ionizable moieties: carboxyl group ($pK_a = 2.4$), phenolic group ($pK_a = 6.87$) and amino group ($pK_a = 9.96$), its aqueous solubility reduces from pH 1 to 3 and increases above pH of 7 (Patel et al., 2003). Another thyroid hormone, 3,3′,5-tri-iodo-L-thyronine (T_3), is also pharmacologically active. The precursors or metabolites include di-iodothyronine (T_2), the parent compound of the iodinated series of thyroid-active hormones, thyronine (T_0), tyrosine (Tyr), di-iodotyrosine (DIT), mono-iodotyrosine (MIT) (Gika et al., 2005), as well as 3,3′,5-tri-iodo-L-thyroacetic acid, and 3,3′,5,5′-tetra-iodo-L-thyroacetic acid have no pharmacological activity.

Stability is considered one of the most important requirements of pharmaceutical product quality. Only stable preparations would promise precise delivery of the drug to the patients. Expiration dating on any drug product is based upon scientific studies at normal and or stressed conditions of certain batches and strengths of products that are developed in multiple strengths. Levothyroxine is one such example where products are available in multiple strengths.

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0378-5173/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2008.04.018

Previous studies have shown that different dosage forms of levothyroxine are susceptible to degradation under the influence of various environmental stress factors such as humidity and temperature. Won (1992) reported that levothyroxine degrades with high temperature and extremes of pH. Levothyroxine has been a subject of FDA Advisory Committee meetings where the clinical consequences of marketing product with approved specification limits of 90–110% has been reported as a problem. There were numerous recalls of levothyroxine due to stability issues (FDA, 2006). Further, lacks of potency and stability assurances has brought in concerns from physicians regarding their therapeutic substitutions and are believed not to deliver right doses to the patients (Thyroid, 2004).

In order to understand the degradation mechanisms of levothyroxine systematically, there is a need for a reliable and simple validated stability indicating method (ICH Q1A (R2), 2003). The stability indicating method should not only identify the degradation products of levothyroxine but also quantitate them. There are numerous reported methods to assay levothyroxine (Takahashi et al., 2002; Smith et al., 1981; Rapaka et al., 1981; Garnick et al., 1984; Richheimer and Amer, 1983). However, these methods either require derivatization of levothyroxine and liothyroxine for separation on HPLC (Takahashi et al., 2002; Smith et al., 1981) or lengthy and more tedious extraction–evaporation procedures before injecting into HPLC (Rapaka et al., 1981). There are methods reported to assay content uniformity (Garnick et al., 1984) which cannot be used as stability indicating method. Richheimer and Amer (1983) reported a stability indicating assay method for levothyroxine. However, it is limited by the number of impurities. Quantification of impurities was not proposed in this method. Thin layer chromatography (TLC) has traditionally been used to identify degradation kinetics of levothyroxine (Won, 1992). However, it is not very accurate method to quantify the related compounds.

Thus none of the previously reported methods satisfied the criteria of stability indicating methods. Very recently, a novel HPLC-based assay to quantify the impurities of levothyroxine was reported in the literature (Gika et al., 2005). The method included quantification of levothyroxine and six of its degradation products. However, in an attempt to reproduce the method in our laboratory, it was found to be erroneous in the order of mobile phase gradient. The other limitation of the method was that the major acidic impurities of levothyroxine, namely, tri-iodo thyroacetic acid and tetra-iodo thyroacetic acid were not a part of impurity profile. The purpose of the current work was to modify this assay method to include these two impurities and also demonstrate that the assay was stability indicating as per FDA and ICH guidelines. Stress conditions used were high temperatures, acid and base hydrolysis, oxidation, and photolysis (Bakshi and Singh, 2002). Also some of the commonly used formulation excipients were mixed with levothyroxine and eight impurities, and the chromatography was evaluated with a good resolution of all the peaks.

2. Materials and methods

L-Thyroxine sodium (L-T_4) was obtained from KVPharmaceutical (St. Louis, MO). 3,3′,5-Tri-iodo-L-thyronine (L-T_3) 3,5-di-iodo-L-thyronine (L-T_2), 3,5-di-iodo-L-tyrosine (L-DIT), 3-iodo-L-tyrosine (L-MIT), L-thyronine (L-T_0), L-tyrosine (L-Tyr), 3,3′,5-tri-iodo-L-thyroacetic acid, and 3,3′,5,5′-tetra-iodo-L-thyroacetic acid, butylated hydroxyanisole, mannitol, sucrose, acacia, sodium laurel sulfate, magnesium stearate, Inertsil 5 μm column, 250 mm × 4.6 mm, and security guard cartridge were purchased from Sigma (St. Louis, MO). Theophylline reagents, methanol, 0.01 M NaOH, 0.1% trifluoroacetic acid (TFA), Acetonitrile, and Fisherbrand low adhesion specialty tips (21-381-83) were purchased from Fisher Sci (Suwanee, GA). Povidone (BASF, Florham Park, NJ), crospovidone (ISP technologies Inc., Wayne, NJ), lactose monohydrate (Kerry BioScience, Chicago, IL), confectionary sugar (Domino's sugar, Baltimore, MD), talc (Spectrum Chemicals, Gardena, CA), silicon dioxide (Aerosil, Evonik Degussa, Orange, CA) croscarmellose sodium, and microcrystalline cellulose (FMC Biopolymer, Philadelphia, PA),were used as received. For all studies, distilled and deionized water was used.

2.1. Preparation of calibration standards

In all cases the sample diluent used for preparing the standards and samples was the 0.01 M methanolic sodium hydroxide solution, which was prepared as described in USP monograph (USP, 2007). Two stock solutions (I and II) of each of nine components (T4, T3, T2, T0, MIT, DIT, T3AA, T4AA, and Tyr) prepared at 1000 μg/ml were prepared by dissolving them individuallly in the sample diluent. From the stock solution I, a working mix I was prepared by mixing 10 ml of each of these components and making the volume to 100 ml. In a similar way working mix II was prepared from stock II. This was used on 3 different days but final dilutions were made on each day of validation. Working mix I was used for the calibration standards, and working mix II was used for quality control samples. Six different standard solutions were prepared from the working I to yield all the nine components in a concentration range from 2 to 20 μg/ml. An internal standard, theophylline, was also added to all the above diluted calibration ranges. The standards were then transferred to an automatic injector for HPLC analysis.

2.2. Preparation of quality control (QC) standards

Three quality control standards were prepared from the working mix II to yield concentrations of 8, 10, and 12 μg/ml with 10 μg/ml as target concentration (100%). These were then transferred to an automatic injector for HPLC analysis.

2.3. Preparation of resolution mixture and system suitability standard

A combination solution containing all nine components at 10 μg/ml each and theophylline was prepared from stock solution I and was used as system suitability standard.

2.4. Chromatography

HP 1100 HPLC equipment from Agilent (Wilmington, DE) consisted of quaternary pump, an automatic injector, a diode array wavelength detector, and a column oven. Various columns and mobile phases were tested. Finally, the method was validated with a reversed phase Inertsil ODS 2 column (250 mm × 4.6 mm, 5 μm, 150 A) with a Inertsil ODS Security Guard cartridge (4.0 mm × 3.0 mm, 10 μm). It provided baseline separation with gradient conditions with 0.1% TFA (A) and acetonitrile (B) from 92 to 8% A in 25 min, at 8% A from 25 to 30 min, from 8 to 92% A from 30 to 35 min run time of 40 min for all the nine components and IS in a single chromatographic run. The flow rate was 0.8 ml/min, column temperature was 25 °C and the injection volume was 50 μl. The UV detection wavelength was set at 215, 223, 228, 232, and 240. However, all the calculations were performed at 223 nm.

2.5. Validation

Validation was carried out according to ICH and FDA guidelines for chromatographic methods (Bakshi and Singh, 2002). Specificity, selectivity, linearity, accuracy, precision, and robustness were

established for the method. System suitability and resolution was performed utilizing related compound C and ranitidine HCl as the standards.

2.6. Stress degradation studies

Stress conditions applied for degradation of levothyroxine powder include refluxing it (1 mg) at room temperature under acidic (0.1N HCl, 24 h) and alkaline (0.1N NaOH, 24 h) conditions, oxidation (3% hydrogen peroxide, 24 h), and photolysis (exposure to UV-A and UV-B rays). Also the degradation was carried out at 40 °C for a period of 14 h. All these samples were appropriately diluted with sample diluent and injected into the HPLC.

2.7. Excipient analysis

Some of the commonly used formulation excipients were selected based on the commercial product inserts. They included butylated hydroxyanisole, povidone, crospovidone, croscarmellose sodium, mannitol, sucrose, acacia, lactose monohydrate, confectionary sugar, microcrystalline cellulose, sodium laurel sulfate, magnesium stearate, talc, and silicon dioxide. Initially, all the excipients were diluted in the sample diluent, filtered, and analyzed on HPLC for detection and evaluating their retention times. Following that, L-thyroxine along with internal standard and all the eight degradation compounds were mixed with the excipient in 1:1 ratios and were analyzed on HPLC as described earlier. The detector was set at multiple wavelengths of 215, 223, 228, 232, and 240 so as to ensure non-interference of excipients with either the active pharmaceutical ingredient (API), internal standard or impurities.

3. Results and discussion

3.1. Analytical method development

Levothyroxine products have a history of stability failures which could result into sub-potency products with patients receiving less than optimal dose. The problem is aggravated while switching from one product to another although they are listed as therapeutic equivalents. To understand mechanism of levothyroxine degradation, an assay which will not only identify but quantify the impurities is essential. A reported method (Gika et al., 2005) was corrected and used with modifications to add two additional degradation products of levothyroxine, T3AA and T4AA. These two are considered to be significant degradation products for levothyroxine. Five different wavelengths were used to observe the chromatograms, but only 223 nm was used for calculation purpose. Although the peak areas were highest at 215 nm, the baseline showed very high negative drift. The wavelength of 223 also showed comparatively higher peak area for all the impurities as well as levothyroxine compared to 228, 232, and 240 nm. Therefore, that wavelength was used. However, the detection at all wavelengths was continued considering that stability samples might show some impurities which might be detected at one wavelength as opposed to other. Fig. 1 depicts the chromatogram obtained with the current method. The peaks of all the impurities and levothyroxine were well resolved. This method was further validated as given in Section 2.7. The order of elution was Tyr (4.97 min), theophylline (9.09 min), MIT (9.55 min), DIT (11.37 min), T0 (11.63 min), T2 (14.47 min), T3 (16.29 min), T4 (17.60 min), T3AA (22.71 min), and T4AA (24.83 min). The current method can be used to assay levothyroxine and its major degradation products.

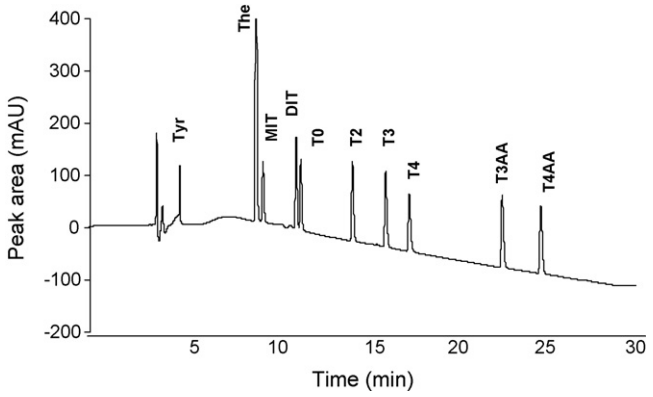

Fig. 1. Typical chromatogram of the iodothyronines and iodotyrosines separation using the HPLCs. All analytes are at 10 μg/ml. Peaks: Tyr (4.97 min), theophylline (9.09 min), MIT (9.55 min), DIT (11.37 min), T0 (11.63 min), T2 (14.47 min), T3 (16.29 min), T4 (17.60 min), T3AA (22.71 min), and T4AA (24.83 min).

3.2. Analytical method validation

Specificity was established by determining that levothyroxine, internal std and degradation products have no co-eluting peaks in preparative solvents, mobile phase (blanks), or related matrices (Fig. 1).

Selectivity was tested by running solutions containing the eight impurities and one internal standard in the same quantities and conditions as the samples to show that there was no peak at the retention times corresponding to the API.

The detection limit (LOD) was 1 μg/ml which was evaluated by measuring the baseline noise and by calculating the analyte concentration that gave S/N = 3, while the limit of quantification (LOQ) was 2 μg/ml which was established for the analyte concentration that gave S/N = 10.

Linearity was established across the analytical calibration range. At least five non-zero calibration standards and a zero calibration standard and or blanks were utilized for each calibration curve. Table 1 shows the calibration curves of levothyroxine and all eight degradation compounds on 3 different days showing a linear correlation with $R^2 > 0.99$ for all the components. Range was established by demonstrating a suitable level of accuracy, precision, and linearity.

Accuracy and precision of the analytical method was established across its analytical range (Table 2). The accuracy was measured at each quality control (QC) standard level ($n = 3$) over the analytical range as defined by the 80% of target concentration (8 μg/ml), l00% of target concentration (10 μg/ml), and 120% of target concentration (12 μg/ml), against the calibration curve. The levels were selected based on FDA and ICH guidelines (FDA, 1994; ICH Q2 (R1), 1995). Nominal values are no greater than 15% at the LLOQ and 10% at the low, intermediate and high QC levels.

Table 1
Linearity and sensitivity data

Analyte	Calibration range (μg/ml)	Slope	Intercept	R^2
Tyr	2–20	0.051 ± 0.004	0.016 ± 0.008	0.9992
MIT	2–20	0.124 ± 0.008	0.015 ± 0.002	0.9999
DIT	2–20	0.192 ± 0.014	0.009 ± 0.012	1.0000
T0	2–20	0.149 ± 0.009	0.002 ± 0.003	0.9998
T2	2–20	0.183 ± 0.013	0.008 ± 0.009	0.9999
T3	2–20	0.187 ± 0.012	0.010 ± 0.003	0.9998
T4	2–20	0.157 ± 0.011	0.014 ± 0.006	0.9999
T3AA	2–20	0.208 ± 0.014	0.007 ± 0.003	1.0000
T4AA	2–20	0.192 ± 0.014	0.012 ± 0.010	1.0000

Table 2
Accuracy and precision data

	Intra-day ($n = 3$)			Inter-day ($n = 9$)		
	80	100	120	80	100	120
Accuracy (%)						
Tyr	96.38 ± 0.24	100.72 ± 0.56	101.62 ± 1.33	100.09 ± 3.04	100.63 ± 1.47	102.92 ± 2.21
MIT	100.52 ± 0.13	100.57 ± 0.12	100.57 ± 0.02	102.18 ± 1.34	100.62 ± 0.72	101.28 ± 0.54
DIT	96.40 ± 0.07	99.82 ± 0.11	100.53 ± 0.07	100.19 ± 2.85	100.04 ± 0.45	101.00 ± 0.46
T0	94.08 ± 0.05	97.36 ± 0.09	98.25 ± 0.10	98.19 ± 3.18	98.19 ± 1.35	99.28 ± 0.98
T2	95.04 ± 0.03	98.39 ± 0.05	99.21 ± 0.10	99.46 ± 3.32	99.43 ± 0.98	100.47 ± 0.95
T3	94.94 ± 0.34	98.20 ± 0.28	100.01 ± 0.41	99.26 ± 3.28	99.31 ± 1.27	100.59 ± 0.60
T4	96.31 ± 0.00	99.91 ± 0.10	100.71 ± 0.08	100.25 ± 2.96	100.38 ± 0.60	101.41 ± 0.56
T3AA	93.55 ± 0.04	96.86 ± 0.20	97.58 ± 0.06	97.30 ± 2.91	97.29 ± 1.07	98.29 ± 0.68
T4AA	99.59 ± 0.03	103.22 ± 0.15	103.90 ± 0.05	103.61 ± 3.02	103.55 ± 0.51	104.52 ± 0.53
			Intra-day ($n = 6$)			Inter-day ($n = 18$)
Precision (%)						
Tyr			0.94			1.48
MIT			0.12			0.70
DIT			0.08			0.43
T0			0.09			1.33
T2			0.08			0.95
T3			0.29			1.22
T4			0.10			0.58
T3AA			0.16			1.07
T4AA			0.12			0.47

The system suitability standard contains levothyroxine, internal standard, and all eight degradation products. Table 3 depicts the system suitability and resolution factors. The specifications are also given in Table 3. It was observed that all the parameters passed the USP specifications. An important system suitability parameter is resolution, a measure of how well two peaks are separated. For a reliable quantification, well-separated peaks are essential. This is a very useful parameter if potential interference peak may be of concern. The degradation impurities of levothyroxine were selected to measure the resolution parameter. It is desirable to have resolution of >1 between the two peaks. With the current method, we obtained satisfactory resolution of >1 in all cases. The tailing factor was also considered as the accuracy of quantification decreases with increase in peak tailing because of the difficulties encountered

Table 3
System suitability parameters

		RT %R.S.D.	Peak area %R.S.D.	USP tailing	Theoretical plates ($X0^5$)	Resolution	Selectivity
Tyr	Day-1	0.05	1.38	1.00 ± 0.00	3.03 ± 0.04		
	Day-2	0.05	1.97	1.01 ± 0.01	2.54 ± 0.05		
	Day-3	0.08	1.02	1.00 ± 0.00	3.07 ± 0.05		
MIT	Day-1	0.15	0.24	1.07 ± 0.03	1.64 ± 0.02	1.82 ± 0.04	1.03 ± 0.00
	Day-2	0.23	0.07	1.10 ± 0.01	1.79 ± 0.02	1.05 ± 0.00	2.49 ± 0.05
	Day-3	0.13	0.18	1.07 ± 0.01	1.34 ± 0.05	1.03 ± 0.00	1.55 ± 0.02
DIT	Day-1	0.09	0.23	1.23 ± 0.00	2.54 ± 0.01	1.20 ± 0.00	10.3 ± 0.05
	Day-2	0.17	0.09	1.17 ± 0.00	2.61 ± 0.02	1.19 ± 0.00	10.1 ± 0.07
	Day-3	0.05	0.13	1.17 ± 0.00	2.55 ± 0.02	1.20 ± 0.00	10.3 ± 0.04
T0	Day-1	0.21	0.24	1.22 ± 0.00	2.78 ± 0.02	1.02 ± 0.00	1.54 ± 0.02
	Day-2	0.16	0.09	1.16 ± 0.00	2.84 ± 0.03	1.02 ± 0.00	1.48 ± 0.01
	Day-3	0.05	0.11	1.15 ± 0.00	2.78 ± 0.00	1.02 ± 0.00	1.53 ± 0.01
T2	Day-1	0.26	0.11	1.32 ± 0.00	3.73 ± 0.03	1.25 ± 0.00	15.9 ± 0.08
	Day-2	0.09	0.14	1.25 ± 0.00	3.72 ± 0.06	1.24 ± 0.00	15.6 ± 0.14
	Day-3	0.03	0.10	1.25 ± 0.01	3.56 ± 0.02	1.25 ± 0.00	15.6 ± 0.06
T3	Day-1	0.03	0.16	1.35 ± 0.01	4.26 ± 0.07	1.13 ± 0.00	9.52 ± 0.06
	Day-2	0.04	0.15	1.29 ± 0.00	4.16 ± 0.04	1.12 ± 0.00	9.30 ± 0.10
	Day-3	0.04	0.27	1.31 ± 0.01	3.93 ± 0.03	1.13 ± 0.00	9.18 ± 0.04
T4	Day-1	0.02	0.08	1.38 ± 0.01	4.37 ± 0.06	1.08 ± 0.00	6.43 ± 0.04
	Day-2	0.02	0.12	1.34 ± 0.00	4.18 ± 0.07	1.08 ± 0.00	6.21 ± 0.07
	Day-3	0.04	0.09	1.38 ± 0.01	3.90 ± 0.03	1.08 ± 0.00	6.14 ± 0.03
T3AA	Day-1	0.05	0.09	1.30 ± 0.01	5.76 ± 0.09	1.30 ± 0.00	23.6 ± 0.29
	Day-2	0.07	0.06	1.24 ± 0.00	5.64 ± 0.06	1.30 ± 0.00	22.8 ± 0.10
	Day-3	0.04	0.12	1.26 ± 0.00	5.40 ± 0.05	1.29 ± 0.00	22.2 ± 0.14
T4AA	Day-1	0.04	0.05	1.29 ± 0.01	6.38 ± 0.09	1.09 ± 0.00	8.97 ± 0.06
	Day-2	0.06	0.10	1.23 ± 0.08	6.18 ± 0.08	1.09 ± 0.00	8.69 ± 0.05
	Day-3	0.04	0.10	1.26 ± 0.00	5.93 ± 0.02	1.09 ± 0.00	8.58 ± 0.03
Spec		<2	<2	<2	>1	>1	>1

Table 4
Robustness with flow rate variation (nominal was 0.8 ml/min)

	Flow rate (ml/min)	RT %R.S.D.	Peak area %R.S.D.	USP tailing	Theoretical plates ($X0^5$)	Resolution	Selectivity
Tyr	0.7	0.03	0.83	0.94 ± 0.02	3.58 ± 0.02		
	0.9	0.05	1.51	0.95 ± 0.03	2.18 ± 0.06		
MIT	0.7	0.04	0.19	1.06 ± 0.01	1.87 ± 0.00	1.03 ± 0.00	1.81 ± 0.01
	0.9	0.06	0.16	1.00 ± 0.00	1.46 ± 0.01	1.04 ± 0.00	1.76 ± 0.01
DIT	0.7	0.02	0.07	1.10 ± 0.00	2.97 ± 0.01	1.02 ± 0.00	1.31 ± 0.00
	0.9	0.07	0.19	1.15 ± 0.01	2.44 ± 0.02	1.22 ± 0.00	10.69 ± 0.07
T0	0.7	0.01	0.04	1.21 ± 0.01	2.73 ± 0.02	1.18 ± 0.00	9.80 ± 0.02
	0.9	0.07	0.12	1.14 ± 0.00	2.71 ± 0.03	1.03 ± 0.00	1.70 ± 0.01
T2	0.7	0.02	0.05	1.27 ± 0.00	3.63 ± 0.05	1.23 ± 0.00	14.55 ± 0.12
	0.9	0.04	0.09	1.23 ± 0.00	3.61 ± 0.04	1.27 ± 0.00	16.56 ± 0.10
T3	0.7	0.01	0.03	1.31 ± 0.00	3.99 ± 0.05	1.12 ± 0.00	8.66 ± 0.07
	0.9	0.03	0.05	1.28 ± 0.01	4.07 ± 0.05	1.13 ± 0.00	9.79 ± 0.08
T4	0.7	0.01	0.11	1.40 ± 0.00	3.89 ± 0.04	1.08 ± 0.00	5.88 ± 0.03
	0.9	0.03	0.20	1.35 ± 0.01	4.07 ± 0.06	1.08 ± 0.00	6.48 ± 0.06
T3AA	0.7	0.01	0.06	1.29 ± 0.01	5.27 ± 0.03	1.29 ± 0.00	21.61 ± 0.10
	0.9	0.02	0.05	1.24 ± 0.01	5.67 ± 0.07	1.30 ± 0.00	23.06 ± 0.19
T4AA	0.7	0.01	0.10	1.27 ± 0.01	5.74 ± 0.05	1.09 ± 0.00	8.31 ± 0.03
	0.9	0.02	0.08	1.23 ± 0.01	6.21 ± 0.06	1.10 ± 0.00	8.96 ± 0.05
Spec		<2	<2	<2	>1	>1	>1

in calculating the area under the peak. A desirable USP tailing factor is <2 which is consistent with the factor obtained with the current method (Table 3).

Robustness was established by analyzing the system suitability standard ($n = 6$) at 20 and 30 °C (nominal = 25 °C), at flow rates of 90 and 110% of the nominal flow (i.e., 1 ml/min.) and injector volumes at 50 and 150% of system suitability standard injection volume. Also since gradient method was used, a slight variation in gradient was also evaluated which included 93% (A) −7% (B) and 91% (A) −9% (B). Table 4 shows the results obtained for robustness of the system. A low CV (%) indicates that the system was robust and could be used without any problem if a minor change is to occur. The determination of robustness or ruggedness is especially important for gradient elution systems which might be impacted significantly by minor variations due to gradient ratio, temperature or other factors. However the current method was found to be robust for such minor changes as demonstrated in Table 4 for flow rate variation. The specifications were met for all of the peaks under minor flow rate condition. Similar data was obtained for minor variations in injection volume, gradient, as well as column temperature (data not shown).

3.3. Stress degradation studies

Stress studies were carried out following an ICH guideline which establishes the requirements of stability indicating methods. A variety of conditions, such as pH, light, oxidation, dry heat, etc. were applied and separation of drug from the degradation products was observed in the chromatograms. Similar studies are carried out in the literature for many drugs (Bakshi et al., 2004; Ojha et al., 2003). However all the literature methods fall short in meeting the current regulatory requirements for levothyroxine assay methods (Garnick et al., 1984; Graham et al., 1974; Rapaka et al., 1981). Therefore, the current work comprised of performing forced degradation studies to establish suitability of the method as stability indicating. Another ICH guideline on stability of testing of new drug substances and products (ICH Q1A (R2), 2003) advocates the use of stability testing assay methods for highly susceptible drugs such as levothyroxine. In the current study, stress decomposition studies at temperatures in 40 °C increments above the accelerated temperature, extremes of pH and under oxidative and photolytic conditions were carried out on the drug substance, levothyroxine. The suitability of the proposed analytical method as a stability indicating method was supported by these stress degradation studies. Fig. 2 represents the stress degradation of the drug substance and the drug product in acidic, alkaline, oxidative, and photolytic (UVA and UVB) conditions, respectively. It was observed that the degraded products eluted far from the drug peak in case of oxidized sample where the degradation peaks were well separated from L-thyroxine peak. The main degradation compound formed by oxidation was observed at a retention time of 22.9 min which corresponds to T3AA. Thus it was even possible to identify the degradation compounds of L-thyroxine under the stress condition. There was no degradation observed with acidic, alkaline or with UV exposure under the conditions specified. Basic pH condition was found to enhance L-thyroxine pentahydrate stability in one of the studies (Patel et al., 2003).

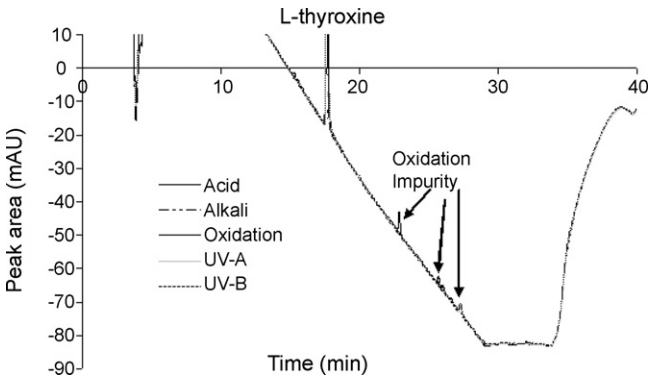

Fig. 2. Stres-degradation samples of levothyroxine with acid, alkali, oxidation, UV-A, and UV-b conditions (expanded view). The degradation compounds were seen under oxidation condition which were well separated from L-thyroxine peak. The primary degradation peak with retention time of 22.9 min corresponded to T3AA impurity. There was no degradation observed under acidic, alkaline, or photolysis conditions.

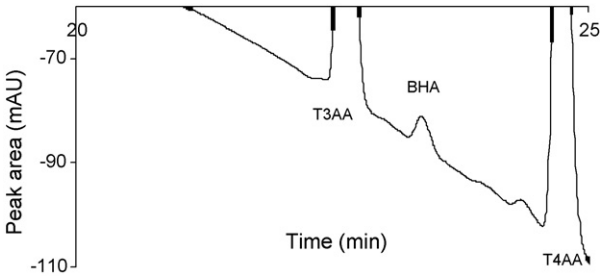

Fig. 3. Expanded view of chromatograph of L-thyroxine degradation compounds (T3AA and T4AA) with formulation excipient, BHA. The retention times are T3AA (22.71 min), BHA (23.36 min) and T4AA (24.83 min).

3.4. Excipient analysis

The current method was used to assay various excipients which are commonly used in L-thyroxine formulations. The information was obtained from package insert or label of currently marketed levothyroxine drug products which included synthroid, unithroid, levothroid, levoxyl, levo-T, levothyroxine sodium from Mylan and Genpharm. When assayed individually, only butylated hydroxyanisole was found to elute with retention time of 23.36 min. The excipients povidone, crospovidone, croscarmellose sodium, microcrystalline cellulose, magnesium stearate, talc, and silicon dioxide were insoluble and therefore did not elute at any wavelength. The soluble excipients such as mannitol, sucrose, acacia, lactose monohydrate, confectionary sugar, and sodium laurel sulfate did not elute in the chromatographic run. They do not have chromophore needed for UV detection and thus were not detected by UV. Thus, none of the other excipients eluted. L-Thyroxine along with all the impuritites and internal standard were mixed with BHA and were run by current method on HPLC. The chromatogram is shown in Fig. 3. It was observed that BHA was well separated from T3AA and T4AA which eluted at 22.71 and 24.83 min, respectively. Thus there was no intereference or co-elution from BHA. This method can thus be applied to analyze L-thyroxine even in the presence of formulation excipients and therefore can be used to analyze drug from the formulated products. This can also be used to study the effect of excipients on L-thyroxine stability. In one of the studies, effect of formulation excipients on L-thyroxine stability was studied (Patel et al., 2003). However, it failed to show whether the analytical method used was adequate to separate L-thyroxine from the excipients. In order to evaluate stability or potency of a formulation, it is imperative to have an adequate stability indicating method as well as the method which can well separate excipients from the active on interest.

4. Conclusions

In conclusion, the current study indicated that this method can be used as a stability indicating assay as well as for potency assay of levothyroxine from drug products. This method offers the separation of levothyroxine from eight impurities, namely3,3′,5-tri-iodo-L-thyroacetic acid (T3AA), 3,3′,5,5′-tetra-iodo-L-thyroacetic acid (T4AA), 3,3′,5-tri-iodo-L-thyronine (L-T_3) 3,5-di-iodo-L-thyronine (L-T_2), 3,5-di-iodo-L-tyrosine (L-DIT), 3-iodo-L-tyrosine (L-MIT), L-thyronine (L-T_0), and L-tyrosine (L-Tyr). The method employed is under gradient condition in 40 min of total run. The method has been validated and it has been shown that it is reliable, linear, and precise both in upper and lower concentration range as well as robust with minor variations in chromatographic parameters. Therefore, it can be applied for quantification of the active compound and all of the eight degradation compounds. Excipient analysis study indicated that the method was found to separate L-thyroxine from the commonly used formulation excipient, butylated hydroxyanisole. The other excipients did not interfere with the analysis as they did not elute in the chromatographic run.

Acknowledgements

Authors wish to express their sincere thanks to Everett Jefferson and Anthony Ciavarella from DPQR for their invaluable help.

Disclaimer. The opinions expressed in this work are only of authors, and do not necessarily reflect the policy and statements of the FDA.

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