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Levothyroxine sodium revisited: A wholistic structural elucidation approach of new impurities via HPLC-HRMS/MS, on-line H/D exchange, NMR spectroscopy and chemical synthesis

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ABSTRACT

The structural elucidation of unknown pharmaceutical impurities plays an important role in the quality control of newly developed and well-established active pharmaceutical ingredients (APIs). The United States Pharmacopeia (USP) monograph for the API Levothyroxine Sodium, a synthetic thyroid hormone, features two high pressure liquid chromatography (HPLC) methods using UV-VIS absorption detection to determine organic impurities in the drug substance. The impurity profile of the first USP method ("*Procedure 1*") has already been extensively studied, however for the second method ("*Procedure 2*"), which exhibits a significantly different impurity profile, no wholistic structural elucidation of impurities has been performed yet. Applying minor modifications to the chromatographic parameters of USP "*Procedure 2*" and using various comprehensive structural elucidation methods such as high resolution tandem mass spectrometry with on-line hydrogen-deuterium (H/D) exchange or two-dimensional nuclear magnetic resonance spectroscopy (NMR) we gained new insights about the complex impurity profile of the synthetic thyroid hormone. This resulted in the characterization of 24 compounds previously unknown to literature and the introduction of two new classes of Levothyroxine Sodium impurities. Five novel compounds were unambiguously identified *via* isolation or synthesis of reference substances and subsequent NMR spectroscopic investigation. Additionally, Collision-Induced Dissociation (CID)-type fragmentation of identified major impurities as well as neutral loss fragmentation patterns of many characterized impurities were discussed.

1. Introduction

The active pharmaceutical ingredient (API) levothyroxine sodium is the sodium salt of the L-isomer of thyroxine, an active physiological compound found in the thyroid gland of mammals. This thyroid hormone is essential for cell differentiation, cellular metabolism, development processes and maintenance of well balanced neurological and physiological functions [1]. Preparations of levothyroxine sodium (LT_4Na) are indicated in replacement or supplemental therapy for patients with hypothyroidism, a common disorder of the endocrine system in which the thyroid gland does not provide sufficient amounts of the thyroid hormone [2–4]. The presence of organic impurities in an API can be critical and may have a significant impact on the quality, efficacy, and safety of the finished drug product [5]. Therefore, the determination of impurities in drug substances is crucial to guarantee the safety of the patient.

The official United States Pharmacopeia (USP) monograph for the determination of related substances in LT_4Na delineates two different high-performance liquid chromatographic methods with detection *via* UV–Vis absorption (HPLC-UV) (so-called "*Procedure1*" and "*Procedure 2*") [6]. The choice of the analytical method used is based on the synthetic route of the drug substance itself. Although impurity profiles have been extensively studied [7–10], the identification of unknown impurities in LT_4Na is still a challenging task, and just recently, detailed investigations of one impurity profile of LT_4Na prepared according to "*Procedure1* 'have been published [11–13]. While one mass spectrometric study was conducted for "*Procedure2*', no wholistic impurity profile has been established yet [4].

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Therefore, this study aims at the comprehensive investigation of previously unknown impurities in LT_4Na focussing on the mostly uninvestigated USP '*Procedure2*' by using a multitude of structural elucidation techniques. An efficient workflow for the determination of organic impurities should enable high resolution (tandem) mass spectrometric (HRMS and HRMS/MS) investigations both in positive and negative ionization modes [14–16]. Thereafter hydrogen/deuterium (H/D) exchange experiments, as well as nuclear magnetic resonance (NMR) spectroscopy including heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) shall corroborate the mass spectrometric analyses of the unkown compounds [17–19]. Finally, the synthesis and isolation of selected levothyroxine derivatives will facilitate unambiguous structural identification [20,21].

2. Experimental

2.1. Chemicals and materials

Three aged LT_4Na batches, LT_4-*N*-methylamide, liothyronine (LT_3) and *O*-methyl diiodothyronine were provided by Sandoz GmbH (Kundl, Austria). Unknown impurities **21**, **27,** and **59** (Table 2) were isolated from levothyroxine batches *via* semi-preparative HPLC. Unknown impurities **26** and **41** (Table 2) were produced *via* chemical synthesis (Section 2.4 and 2.5 for synthesis of impurities **26** and **41**). The three unknown impurities **26**, **41** and **59** were further purified *via* semi-preparative HPLC. 2-hydroxy T_4-acetic acid (Apin Chemicals Ltd., UK), T_4-formic acid (Organic Consultants, USA), and T_4-formaldehyde (Molcan Corporation, USA) were purchased from commercial sources. Acetonitrile (ACN) LC–MS grade was purchased either from Fluka (Chromasolv®, Buchs, Switzerland) or VWR (Hipersolv®, Radnor, PA, USA). LC–MS grade methanol (MeOH) and sulfamic acid were obtained from Fluka (Chromasolv®). Deionized water (18.2 MΩ·cm^{-1}) was prepared using the Milli-Q system from Millipore (Billerica, MA, USA). Formic acid (FA) (98%) as an eluent additive for LC–MS was obtained from Fluka (Buchs, Switzerland). NaOH solution (1 M) was purchased from Merck (Darmstadt, Germany), NaOH pellets were acquired from Amresco LLC (Solon, OH, USA). The semi-preparative HPLC for isolating impurity **59** was performed using acetonitrile and HPLC grade water which were obtained from Fluka (Buchs, Switzerland). For the online H/D exchange experiments and NMR investigations D_2O, MeOH-d_4, ACN-d_3, Dimethyl sulfoxide-d_6 (DMSO-d_6) and formic acid-d_2 were purchased from Sigma-Aldrich (Steinheim, Germany). For the synthesis of impurities **26** and **41** 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxybenzotriazole hydrate (HOBt), methylamine (2.0 M) in tetrahydrofuran and pure tetrahydrofuran LC/MS grade were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Preparation of sample and standard solutions

The preparation of sample solutions for the HPLC-UV determination of organic impurities was done according to the USP monograph ("*Procedure 2*") of LT_4Na (*method I*). For solution A, 9.7 g sulfamic acid were dissolved in 2000 mL water, 1.5 g NaOH pellets were added and the pH was adjusted to pH 2.0 with 1 M NaOH solution. Solvent 1 was prepared by mixing MeOH and solution A in a 9:1 (v:v) ratio, solvent 2 was prepared by mixing ACN/solution A 3:7 (v:v) with solvent 1 at the ratio of 1:1 (v:v). To get the sample solution, 20 mg of LT_4Na were dissolved with 20 mL of solvent 1 and a portion of this solution was further diluted with solvent 2 to yield a final concentration of 200 μg/mL.

For HPLC-HRMS/MS investigations (*method II*) the LT_4Na sample solutions were prepared by dissolving the samples in pure MeOH to obtain a final concentration of 4000 μg/mL. The injection volume of the LT_4Na sample was 25 μL. The online H/D exchange experiments were conducted with a 500 μg/mL MeOH-d_4/D_2O 1:1 (v:v) sample solution and an injection volume of 100 μL.

2.3. Instrumentation

2.3.1. High performance liquid chromatography (HPLC) – Method I

The HPLC-UV investigations, purification of synthesis products and isolation of impurities were performed on an HPLC–MS system (Model LCMS 2020, Shimadzu Corporation Kyoto, Japan) equipped with a photodiode array detector (Model SPD 20A). LabSolutions version 5.60 (Shimadzu) was used for process monitoring, data acquisition and system control. All analytical chromatographic separations were carried out with a YMC C18 Pro Column (150 mm x 4 mm, 3 μm) (YMC, Kyoto, Japan). The UV detection wavelength was 225 nm. Solvent A and pure ACN were used as mobile phases A and B, respectively. The flow rate was set to 1.0 mL/min, injection volume was 25 μL and oven temperature was 25 °C. The linear gradient elution for *method I* was performed as follows: T_{min}/solvent A:solvent B; T_0/70:30; T_{10}/70:30; T_{40}/20:80; T_{50}/20:80; T_{53}/70:30; T_{75}/70:30.

For the purification procedure of impurities **26** and **41** and isolation of impurities **21** and **27,** 0.10% (v/v) formic acid in H_2O was used as mobile phase A and ACN as mobile phase B. The flow rate was set to 1.0 mL/min, injection volume was 100 μL, and oven temperature was 25 °C. The linear gradient program was set as follows: T_{min}/solvent A:solvent B; T_0/50:50; T_5/50:50; T_6/10:90; T_8/10:90; $T_{8.5}$/5:95; $T_{14.5}$/5:95; $T_{15.5}$/50:50; $T_{20.5}$/50:50.

2.3.2. High performance liquid chromatography-mass spectrometry (HPLC–MS) – Method II and H/D exchange

The HPLC-HRMS/MS investigations were performed on an HPLC system (Model Dionex UHPLC Ultimate 3000 Thermo Fisher Scientific, Germering, Germany) coupled with a linear ion trap-Orbitrap mass spectrometer (Model LTQ-Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The UHPLC system was controlled by Chromeleon version 7.2, the mass spectrometer was operated by XCalibur version 2.1 (all from Thermo Fisher Scientific). Chromatographic separation was carried out using the YMC C18 Pro column, UV detection was measured at 225 nm. For *method II* 0.10% (v/v) formic acid in H_2O was used as mobile phase A and 0.10% (v/v) formic acid in ACN as mobile phase B. The flow rate was set to 1.0 mL/min, injection volume was 25 μL and oven temperature was 25 °C. The linear gradient program for method I was set as follows: T_{min}/solvent A:solvent B; T_0/70:30; T_{10}/70:30; T_{44}/30:70; T_{50}/11:89; T_{55}/11:89; T_{58}/70:30; T_{70}/70:30. The LTQ-Orbitrap XL mass spectrometer was tuned and calibrated in negative and positive ionization mode following the procedures described by the manufacturer. The MS fine-tuning was done by mixing 10 μL/min of LT_4 Na (10 μg/mL) *via* a T-piece with the HPLC column effluent of 1000 μL/min ACN:H_2O (70:30 v/v) containing 0.1% formic acid. The optimized mass spectrometric parameters in negative and positive mode are collected in Table 1.

Online H/D exchange experiments were conducted with an HPLC system (Model 1200, Agilent Technologies,Waldbronn, Germany) coupled to an triple quadrupole ESI mass spectrometrer (Model 6460C, Agilent Technologies) with a Jetstream interface. The chromatographic parameters were identical to HPLC-HRMS/MS investigations, *method 2*, except for the use of a deuterated mobile phase A (0.10% v/v formic acid-d_2 in D_2O) and pure ACN as mobile phase B. The mass spectrometric parameters were as follows: gas temperature, 300 °C; gas flow, 5 L/min; nebulizer pressure, 45 psi; sheath gas temperature, 300 °C; sheath gas