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Ultrahigh-performance liquid chromatography-ultraviolet absorbance detection-high-resolution-mass spectrometry combined with automated data processing for studying the kinetics of oxidative thermal degradation of thyroxine in the solid state

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ABSTRACT

Levothyroxine as active pharmaceutical ingredient of formulations used for the treatment of hypothyroidism is distributed worldwide and taken by millions of people. An important issue in terms of compound stability is its capability to react with ambient oxygen, especially in case of long term compound storage at elevated temperature. In this study we demonstrate that ultrahigh-performance liquid chromatography coupled to UV spectrometry and high-resolution mass spectrometry (UHPLC-UV-HRMS) represent very useful approaches to investigate the influence of ambient oxygen on the degradation kinetics of levothyroxine in the solid state at enhanced degradation conditions. Moreover, the impurity pattern of oxidative degradation of levothyroxine is elucidated and classified with respect to degradation kinetics at different oxygen levels. Kinetic analysis of thyroxine bulk material at 100 °C reveals bi-phasic degradation kinetics with a distinct change in degradation phases dependent on the availability of oxygen. The results clearly show that contact of the bulk material to ambient oxygen is a key factor for fast compound degradation. Furthermore, the combination of time-resolved HRMS data and automated data processing is shown to allow insights into the kinetics and mechanism of impurity formation on individual compound basis. By comparing degradation profiles, four main classes of profiles linked to reaction pathways of thyroxine degradation were identifiable. Finally, we show the capability of automated data processing for the matching of different stressing conditions, in order to extract information about mechanistic similarities. As a result, degradation kinetics is influenced by factors like availability of oxygen, stressing time, or stressing temperature, while the degradation mechanisms appear to be conserved.

1. Introduction

Levothyroxine (T4) is a well-known and widely used drug for the treatment of hypothyroidism. The therapy is usually based on substitution of the natural hormone by a long-term treatment with synthetic levothyroxine [1]. It is a narrow therapeutic index drug, for which individual dosage levels of patients need to be established over several weeks. Consequently, products differing in content of levothyroxine due to compound instability or problems in formulation can lead to wrong medication and potentially cause severe health problems [2,3].

Levothyroxine is produced by multistep chemical synthesis [4,5]. It is distributed by the manufacturer in the crystalline form of a sodium pentahydrate, which has been reported to be stable for more than 4 years at defined conditions of 25 °C and 60% humidity [6]. Nevertheless, exposure to heat [7–9], moisture, changed humidity, or an atmosphere of dry nitrogen [10] was shown to change crystal integrity, resulting in rather instable crystalline forms or amorphous material prone to compound degradation [11] or racemization [12]. Moreover, the stability of levothyroxine is influenced by pH and light exposure [8,13]. The sensitivity of the pentahydrate to various factors demands a rigorous control of compound synthesis, packaging and storage. In this context, formulation steps such as wet granulation of the bulk material in order to reach homogeneous particle distribution are especially critical, since they can cause solid phase transformation of the pentahydrate into less stable forms [14]. Also mixing with excipients such

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as sugars or filling polymers, e.g. povidone, was shown to cause significant degradation of levothyroxine and a reduced shelf-life of the tablet [10].

A question rarely addressed is the compound sensitivity to oxygen in connection with thermal stressing. As can be found in the 2013 report of Levothyroxine tablet products of the Medicines and Healthcare products Regulatory Agency (MHRA) "a mechanistic sensitivity to oxygen predicted by solid phase degradation pathways and confirmed by thermal analytical studies with and without oxygen may be insufficiently recognized". In this context, it is known that levothyroxine shows significant and fast thermal degradation at temperatures >60 °C and that degradation products mainly derive from oxidative side chain degradation [8,15].

Nevertheless, a detailed view on the impurity profile obtained by thermal stressing with respect to the influence of ambient oxygen and the kinetics of impurity formation has not been described so far. Known methods for impurity profiling of pharmaceuticals are LC-(SPE)-NMR, GC-MS and LC-MS using dissolved samples, or solid state NMR, IR spectroscopy and powder diffractometry for direct analysis in solid phase [16–18].

Among these techniques, mass spectrometry lends itself to the detection of complex impurity patterns by offering a unique combination of specificity, selectivity, and sensitivity. Moreover, ultrahigh-performance liquid chromatography (UHPLC) has emerged as a powerful tool for rapid separations while preserving high separation efficiency [9,19]. Recently, we have shown that a combination of sequential sampling and fast UHPLC analysis interfaced to high-resolution mass spectrometry (HRMS) is suitable for comprehensively studying mechanistic aspects of thyroxine degradation [15]. Thanks to high mass resolution, the compounds separated can be safely assigned to their molecular formula, which allows a comprehensive "snapshot" of the sample constitution at the time point of sampling.

In the present work, we use this approach to investigate the influence of ambient oxygen on the degradation kinetics of thyroxine bulk material by UV detection and give a mechanistic estimation of oxygen consumption. Moreover, we use time-depended HRMS data to provide a classification of degradation profiles with respect to degradation kinetics and chemical structures, and show their dependence on the availability of oxygen during stressing as well as on the mechanism of degradation. Due to the high information content of HRMS data, automated data processing is mandatory for data treatment. Finally, we show that bioinformatic tools can also be used to align differently stressed samples in order to gain valuable information about mechanistic aspects of thyroxine degradation.

2. Material and methods

2.1. Chemicals and samples

Acetonitrile (Optigrade for LC-MS) was obtained from Promochem (Wesel, Germany). Methanol (LC-MS CHROMASOLV) was purchased from Fluka (Buchs, Switzerland). Deionized water (18.2 MΩ cm) was prepared using the Milli-Q system from Millipore (Billerica, MA, USA). Formic acid (98–100% purissimum) was obtained from Sigma–Aldrich (Steinheim, Germany) and sodium hydroxide (pro analysi) was from Merck (Darmstadt, Germany). Thyroxine sodium pentahydrate bulk material for in-house stressing as well as moderately stressed thyroxine was kindly provided by Peptido GmbH (Bexbach, Germany).

2.2. Sample preparation

In-house thermal stressing was performed by placing 5 g of untreated thyroxine bulk material into an open petri dish and a closed glass bottle, respectively, and stressing them at 100 °C for 1044 h using the column oven of a gas chromatograph (Agilent, Model HP 5890, Series II, Waldbronn, Germany). Working solutions for UHPLC-(UV)-HRMS analysis were prepared by dissolving 2.0 mg of collected samples in 1.0 mL water/methanol (50:50, v/v) containing 400 mg/L sodium hydroxide. The injection volume was 6.0 μl.

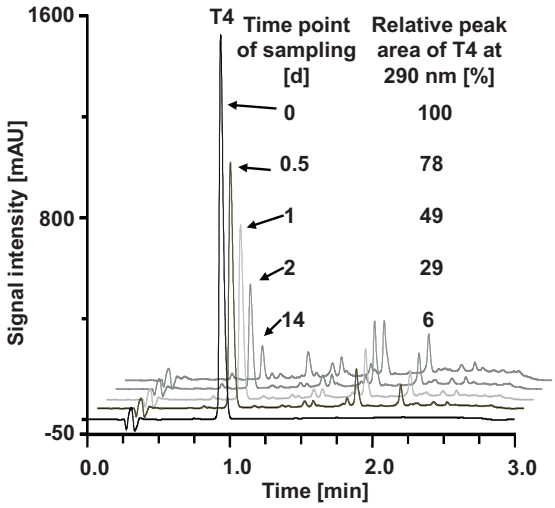

Fig. 1. UV chromatograms describing the thermal degradation of thyroxine at 100 °C. Column, Hypersil GOLD, 100 × 2.1 mm i.d., 1.9 μm particle size; sample, 6.0 μL of 2.0 mg/mL thyroxine samples after different stressing times at 100 °C; mobile phase, (A) water + 0.10% formic acid, (B) acetonitrile + 0.10% formic acid; gradient: 30–80% B in 2.1 min, 0.30 min at 80% B; flow rate: 1000 μL/min; column pressure, 620 bar; column temperature, 60 °C; detection, UV at 290 nm.

2.3. Instrumentation

Chromatographic separations were performed in 100 × 2.1 mm i.d. Thermo Hypersil GOLD columns packed with 1.9 μm particles (Thermo Fisher Scientific, Reinach Switzerland). UHPLC-UV-HRMS analysis was performed using an Accela™ UHPLC System, equipped with a pump capable of handling column backpressures up to 1000 bar and a 20 Hz photodiode array detector, which was controlled by LC-Devices version 2.01 from Thermo Fisher Scientific (Bremen, Germany). The Accela™ system was directly coupled to a linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific) equipped with an Ion Max electrospray ionization (ESI) source and controlled by XCalibur™ version 2.0.7. Chromatographic conditions are given in the legend of Fig. 1.

2.4. Automated data processing

By manual screening of a moderately stressed sample treated for six months at 40 °C and thereafter 16 h at 60 °C, which serves as a model for long term sample storage and represents the reference standard, a list of m/z values and approximate RT positions for all signals related to thyroid hormones was extracted. In order to focus on the more relevant impurities with a distinct signal-to-noise ratio, the list was filtered by setting a threshold of 0.01% peak height relative to the sum of peak heights of all impurity masses found. The resulting "mass list" (see Table S1) has 176 entries and was provided to the OpenMS tool "EICExtractor" [20]. The EICExtractor tool enables quantification of LC-MS signals within parameter-guided mass and retention time deviations over multiple samples and provides an aligned output table containing intensities extracted from the designated positions. The tool supports "master compounds", each of which describes a group of chemically related m/z values