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Quantification of Thyromimetic Sobetirome Concentration in Biological Tissue Samples

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Abstract

Thyroid hormone is a principal regulator of essential processes in vertebrate physiology and homeostasis. Synthetic derivatives of thyroid hormone, known as thyromimetics, display desirable therapeutic properties. Thoroughly understanding how thyromimetics distribute throughout the body is crucial for their development and this requires appropriate bioanalytical techniques to quantify drug levels in different tissues. Here, we describe a detailed protocol for the quantification of the thyromimetic sobetirome using liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Keywords

Sobetirome; Thyromimetic; Thyroid hormone; LC-MS/MS; Drug quantification; Pharmacokinetics; PK-ADME

1 Introduction

The modern era of thyroid hormone analogs was predicated on a small number of biological techniques developed during the 1980s and 1990s that combined to produce extremely potent agonists. Purified thyroid hormone receptors (TRs) expressed in heterologous systems for accurate measurements of selective binding affinity, cell-based transactivation assays for assessing activity and subtype selectivity, and x-ray crystal structures of TR subtypes bound to selective ligands to illuminate their binding modes and interactions.

Quantitative analysis of the thyromimetic sobetirome by LC-MS/MS allows for the determination of tissue distribution and other pharmacokinetic properties of the drug (see Figs. 5 and 6) and is relatively straightforward. Particular care must be taken when preparing the standard curve and the following notes address these issues:

The sequence of a thyroid hormone receptor was first reported simultaneously by Weinberger [1] and Sap [2] in 1986, who determined that it was homologous with the retroviral oncogene *c-erbA*. Researchers quickly determined that other variants of the thyroid hormone receptor could be found endogenously and that they had the same binding constants for T3 when expressed in vitro [3]. The surprising result was that while T3 and T4 had roughly equal affinities for the α and β subtypes, other analogs such as tiratricol had different binding affinities for each subtype [4]. Combined with the discovery that the subtypes had varying expression levels in each tissue [5, 6], this suggested that ligand subtype selective binding and activation could be a powerful route for producing selective effects. Reports in the early 1990s demonstrated that functional thyroid hormone receptors could be expressed heterologously in *E. coli*—the α -subtype ligand binding domain (LBD) [7] and the full-length β -receptor by itself [8] or as a fusion with GST [9]. This made it practical to perform binding assays on individual TRs.

The ability to express TRs in heterologous systems also made it possible to obtain them in the quantities necessary for growing single crystals. An x-ray crystal structure of the TR α LBD bound to L-T3 was reported by Wagner in 1995 [10]. The TR β LBD bound to T3 was reported by Darimont in 1998, which allowed researchers to make structural comparisons between the two TR LBDs [11, 12]. More recently, crystal structures of a β -selective thyromimetic bound to the TR α and TR β LBDs by Bleicher in 2008 gave significant insight into the conformational changes between the receptors that are believed to be the primary basis of the ligand's β -selectivity [13].

At roughly the same time reports were published demonstrating that thyroid hormone could stimulate the expression of specific protein products. A report by Evans in 1982 detailed how thyroid hormones regulate the expression of growth hormone genes in cultured rat pituitary tumor cells—addition of thyroid hormone increased growth hormone mRNA levels fourfold [14]. Reports by Larsen in 1986 and Wight and Glass in 1987 delineated some of the promoter sequences that are recognized and bound by the thyroid hormone receptor [15–17]. They also demonstrated that the sequence was modular and could be inserted upstream of a different gene to render it responsive to thyroid hormone activation. With these molecular tools in hand, Thompson reported in 1989 that it was possible to co-transfect mammalian cells with plasmids coding for a thyroid hormone receptor and a synthetic construct containing a reporter gene driven by a thyroid hormone response element [18]. Because each receptor subtype could be overexpressed separately, this created a powerful tool for assessing compound selectivity and potency. The method was patented by The Salk Institute in 1991, which largely prevented the use of this tool in industry for many years, though it was taken up more readily by academics [19].

The first compound to fully exploit these methods was **GC-1** (Fig. 1), reported by Chiellini in 1998 [20]. Building on the work of Yokoyama [21], it replaced the diaryl ether with a methylene bridge to produce a novel compound that demonstrated tenfold selectivity for TR β compared to TR α in both in vitro receptor binding and cell-based transactivation assays. The selectivity was demonstrated unequivocally in vivo with a *Xenopus laevis* metamorphosis assay [22] and in mice, rats, and cynomolgus monkeys it was as or more effective than L-T3 at producing decreases in serum cholesterol and triglycerides with

almost no changes in hemodynamic parameters [23, 24]. Clinical development of GC-1 for cholesterol lowering was initiated by QuatRx Pharmaceuticals in 2005. Single and multiple dose Phase 1 studies demonstrated significant decreases in serum cholesterol without affecting TSH or circulating T4 levels. While development of GC-1 as a treatment for hypercholesterolemia was discontinued, a new effort is underway to study GC-1 in the orphan genetic disease x-linked adrenoleuko-dystrophy [25].

Despite the fact that most chemical features of GC-1 had been chosen primarily for synthetic tractability rather than through rational design [26], many of them turned out to be critical for its potency and selective effects. The diaryl ether and propionic acid analogs first reported by Yokoyama [21] were much less selective than GC-1 [27]. These results suggested that GC-1 was already significantly optimized, but one site for refinement was found in the synthesis of **GC-24** (Fig. 1) [28]. Replacing the 3'-isopropyl with a benzyl group significantly reduced the binding affinity of GC-24 for TR α while leaving the affinity for TR β intact, increasing the TR subtype selectivity of the compound. The molecular basis of this selectivity was determined by mutational and crystallo-graphic analysis of the receptors [29]. While these features made GC-24 an excellent tool for probing thyromimetic function, it has not supplanted GC-1 as a clinical candidate.

This period saw many of the major pharmaceutical firms pursue thyromimetics programs, largely in an effort to develop LDL cholesterol lowering agents. This led to the exploration of novel chemotypes as research groups worked around the patented chemical space, vastly expanding the range of known active motifs. While most investigated one or two novel chemical modifications, a few went much further.

Karo Bio reported its first series of analogs in 2003 [30] based on patents filed in 1999 and 2000 [31, 32] in partnership with Bristol-Myers Squibb. This series built on earlier iodine-based thy-roalkanoic acid analogs by replacing the iodines with alkyl and halogen groups to improve metabolic stability. TR β selectivity peaked at 14-fold in the 3,5-dichloro series with the acetic acid analog (Fig. 2—**KB-141**), which also demonstrated tenfold selectivity for cholesterol lowering compared to cardiotoxicity in rats and cynomolgus monkeys [33]. An extension of the series was reported by Hangeland in 2004 that retained the 3,5-dichloro or 3,5-dibromo acetic acid core and varied the 3' position with substituted phenyl and heterocyclic groups [34]. Another attempt to modulate the selectivity of KB-141 was reported by Garg in 2007 that coupled a series of amino acids through the acetic acid group and added 5'-substituents [35]. A variation on the series (Fig. 2—**1**) was a set of compounds reported by Li in 2006 which replaced the 4'-hydroxyl group with a series of straight and branched chain amides built on a 4'-amino group [36]. No compound in this series improved on the selectivity or potency of KB-141. A new set of compounds (Fig. 2—**2**) reported by Karo Bio took the novel approach of replacing the standard biaryl core with a phenyl-naphthalene core, reported by Hangeland in 2005 [37]. Following this Karo Bio pursued a similar strategy to Bayer in synthesizing thyromimetics with fused ring heterocycles (Fig. 2—**3**). Collazo reported a series in 2006 where the inner ring was replaced by bicyclic nitrogen five- and six-membered rings with carboxylic acid appendages [38]. While a fair amount of chemical space around this basic structure was explored, the relatively simple KB-141 based on the structure of tiratricol remained the best out of these early efforts.

Karo Bio's best TR β -selective compound KB2115 (Eprotirome) (Fig. 2) was patented in 2004 under Bristol-Myers Squibb [39]. An in vitro binding assay of KB-2115 demonstrated 22-fold TR β -selectivity [40], but suggestions have also been made that preferential liver uptake played a role in its selective effects in vivo [41]. A Phase I 14-day, once-daily dosing clinical trial was reported in 2008, which showed that KB-2115 could lower plasma total and LDL cholesterol with no observed drug-induced effects on cardiac parameters in overweight and hypercholesterolemic subjects [42]. A Phase II clinical trial involving once-daily dosing over 12 weeks was reported in 2010 that investigated the effects of KB2115 in addition to simvastatin or atorvastatin [43]. KB-2115 plus a statin produced significantly larger decreases in serum cholesterol and LDL compared to a statin alone without cardiotoxicity or bio-markers of bone turnover. The positive results obtained in the first two trials led to a larger Phase III trial with heterozygous familial hypercholesterolemia patients currently receiving the standard of care [44]. The study was initiated in 2011, but terminated early due to induction of chondrodysplasia in dogs during a large animal toxicology study. Though it is unclear how the drug induced cartilage damage, or whether this finding was TR mediated, further development of the drug was halted.

Metabasis Therapeutics patented a series of thyromimetics in 2004 that took a novel approach to selectivity—prodrugs designed to be metabolized in the liver into an active ligand to reduce peripheral exposure and increase selectivity [45]. The compounds were a series of phosphonic acid derivatives coupled to a standard thyromimetic core. Due to the high charge density of phosphonic acid groups reducing their passive transport through plasma membranes, an array of phosphonate ester and phosphonamide derivatives of the series were synthesized to increase the membrane permeability of the prodrug while releasing the active phosphonic acid either through simple hydrolysis or active metabolism in the liver. The in vitro binding assays reported by Boyer in 2008 found that the active forms of the series had low binding affinity and selectivity in comparison to benchmarks such as L-T3, GC-1, KB-141, and KB-2115 [46]. In vivo results were more promising—a number of compounds in the series reduced serum cholesterol by 20–48% in cholesterol-fed rats at doses of 0.2 mg/kg. The cyclic 1-(3-chlorophenyl)-1,3-propanyl prodrug (Fig. 3—**MB07811**) was selected as the best of the series due to acceptable oral bioavailability (10%) and low serum esterase activity resulting in low extra-hepatic concentrations of the active form due to specific CYP3A-mediated metabolism of the cyclic ester (Fig. 3). Full in vivo characterization was reported by Erion in 2007 [47]. MB07811 administered once daily to rats at 50 mg/kg did not evoke significant increases in heart rate, hemodynamic parameters, or heart weight. MB07811 entered into Phase 1a and 1b clinical trials in 2006 and reports in 2008 indicated that the drug was well tolerated with healthy patients in both rising single-dose and rising multiple-dose trials with significant reductions in serum cholesterol at doses ranging from 0.25 to 5 mg/kg. However, these positive results were accompanied by dose-dependent reductions in endogenous thyroid hormone levels, suggesting that the liver-selective prodrug strategy was not effective at eliminating side effects. A phase 2a trial in hypercholesterolemic patients was withdrawn before initiation in 2009.

Madrigal Pharmaceuticals, previously known as VIA Pharmaceuticals, first reported their clinical candidate thyromimetic **MGL-3196** (Fig. 4) in an abstract in 2009 [48]. The compound had initially been discovered by Roche, who licensed it to Madrigal for further development. Synthetic, in vitro, and pre-clinical in vivo data were reported by Kelly in 2014 [49]. One compound was selected for in vivo testing in diet-induced obesity (DIO) mice and demonstrated significant reductions in serum cholesterol without reducing bone mineral density, increasing heart weight, or perturbing the HPT axis. Madrigal has carried out three separate Phase 1 clinical trials with MGL-3196 in 2011, 2012, and 2015. The results of the first two studies were reported by Taub in 2013 and indicated that MGL-3196 produced the desired outcomes—reductions in serum cholesterol and lipids without changes in heart rate or TSH [50]. Madrigal was granted a new patent for use of MGL-3196 to treat resistance to thyroid hormone in 2015, but no trial results have been reported [51].

A major lesson from these efforts to produce clinical thyromimetics is that target engagement is necessary but insufficient for success. While efforts were made to produce safe drugs through TR β selectivity and targeting to the liver, their side effects suggest that their distribution was often broader than intended. Oral administration would ensure that some fraction of the drug reached the liver and activate TRs, but it was difficult to constrain these compounds from distributing to extrahepatic tissues. Accurately determining the pharmacokinetic properties of clinical candidates is critical for understanding their effects in vivo and deciding which should advance to trials. Modern bioanalytical techniques have increased the speed and accuracy of these measurements significantly.

Sobetirome (GC-1) is currently being investigated as a therapy for a range of neurological diseases [52]. Few thyromimetics partition to the central nervous system, so it is critical to understand its distribution, so a robust and reproducible bioanalytical technique is necessary to probe its pharmacokinetic properties. Optimized extraction techniques coupled with liquid chromatography tandem-mass spectrometry (LC-MS/MS) can provide quantitative concentrations of the drug in a range of tissues. In this chapter we describe a detailed protocol for analyzing the thyromimetic sobetirome by LC-MS/MS.

2 Materials

2.1 Tissue Sample

Mouse cohorts of three or more are administered a specific dose of sobetirome (typically in $\mu\text{mol/kg}$) either via tail vein injection (i.v.), orally (p.o.), or by injection (i.p.) and the mice are euthanized at some time post-injection dictated by the particular experiment. Blood and whole brain are the most common tissues collected for analysis and are removed promptly after the animal is euthanized (*see Note 1*). Blood is immediately placed on ice (0 °C) then processed into serum and brain is directly frozen on dry ice (−80 °C). After collection tissue samples are stored at −80 °C.

¹Sobetirome levels can be measured in a variety of different tissues. Quantitation in serum and brain are described here as examples and can be extended methodologically to other tissues of interest. Concentration-time profiles provide pharmacokinetic parameters such as: area under the curve (AUC), half-life, clearance, and volume of distribution.

2.2 Internal Standard

Isotopically labeled d_6 -sobetirome [53] was used as the internal standard.

2.3 Tissue Homogenization

1. Screw cap microcentrifuge tube (2 mL, Thermo Scientific™, Waltham, MA).
2. Three GoldSpec 1/8 chrome steel balls per tube (Applied Industrial Technologies, Cleveland, OH).
3. Beadbug Microtube Homogenizer (Benchmark Scientific, Edison, NJ) or Bead Ruptor 24 (Omni International, Kennesaw, GA).

2.4 Protein Crash and Sobetirome Extraction

1. Centrifugation. Eppendorf 5415R (Eppendorf, Hauppauge, NY) and Beckman Coulter Avanti J-20 XPI with a JA-25.5 fixed angle rotor (Beckman Coulter, Fullerton, CA).
2. Thermo Scientific Savant SPD111V SpeedVac Concentrator attached to a Thermo Scientific Savant RVT4104 Refrigerated Vapor Trap connected to a Thermo Scientific VLP200 vacuum pump (Thermo Scientific, Waltham, MA).

2.5 Sample and Standard Curve Preparation for LC-MS/MS

1. Sobetirome was obtained according to the following literature procedure [54].
2. Screw thread amber vials (2 mL), glass inserts (0.2 mL), and polypropylene assembled screw threaded caps with PTFE/silicone septum (SUN-SRI, Rockwood, TN).

2.6 LC-MS/MS Analysis

QTRAP 4000 hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with electrospray ionization (ESI) in negative mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps and analysis on an Applied Biosystems/SCIEX QTRAP 4000 instrument (Foster City, CA). A Hamilton PRP-C18 column: 5 μ m particle size (50 mm $\times$ 2.1 mm stainless steel) was used and kept at 40 °C, and the autosampler was kept at 30 °C. Data were acquired using SCIEX Analyst 1.6.2 software (Framingham, MA, USA) and analyzed using Multiquant 3.0.2.

3 Methods

Blood and brain tissues are collected and stored until processing as described in the above Subheading 2.1. Blood was kept on ice (0 °C) for a minimum of 30 min and then spun down at 7500 $\times g$ for 15 min. Serum (100 μ L) was collected and stored with tissues at –80 °C until samples were processed. Brain tissue was stored at –80 °C until processed.

3.1 Addition of Internal Standard and Tissue Homogenization

Serum samples were warmed to rt and 10 μ L of 2.99 μ M internal standard (d_6 -sobetirome) were added per sample. Brain samples were warmed to rt and transferred to a homogenizer tube with three GoldSpec 1/8 chrome steel balls (Applied Industrial Technologies). The resulting tube was weighed, then 1 mL of H₂O was added, followed by 10 μ L of 2.99 μ M internal standard (d_6 -sobetirome). The tube was homogenized with a Beadbug for 30 s (highest setting) or a Bead Ruptor 24 (see Subheading 4, see Note 7 for settings).

3.2 Protein Crash and Sobetirome Extraction

500 μ L of acetonitrile were added to each serum sample containing internal standard, then the mixtures were vortexed for 20 s. The samples were centrifuged at $10,000 \times g$ for 15 min at 4 °C. 90% of the upper supernatant in each sample was transferred to a glass test tube (13 $\times$ 100 mm, Fisher brand) and concentrated using a speedvac for 1.5 h at 45 °C. Brain homogenates were each transferred to a 15 mL falcon tube containing 3 mL of acetonitrile. Additional acetonitrile (1 mL) was used to wash the homogenizer tube and the solution was transferred back to the falcon tube. The samples were then centrifuged at $10,000 \times g$ for 15 min at 4 °C. Ninety percentage of the upper supernatant in each sample was transferred to a glass test tube (13 $\times$ 100 mm, Fisher brand) and concentrated using a speedvac for 4 h at 45 °C. All dried samples (brain and serum) were then dissolved in 400 μ L of 50:50 acetonitrile/H₂O and vortexed for 20 s. The resulting mixtures were each transferred to Eppendorf tubes and centrifuged at $10,000 \times g$ for 15 min. The supernatants were filtered with 0.22 μ m centrifugal filters (see Note 6).

3.3 Sample and Standard Curve Preparation for LC-MS/MS

Each extracted sample (from Subheading 3.2) was added to a screw thread amber vial (2 mL) containing a glass insert (0.2 mL) and a polypropylene assembled screw threaded cap with PTFE/silicone septum (SUN-SRi, Rockwood, TN). The standard curve was made with 100 μ L of serum from a mouse not injected with sobetirome (vehicle control, see Note 2). Processing was performed as above, but after filtering the sample was aliquoted into six vials and sobetirome was added to five out of the six vials to give final concentrations in matrix of 0.1, 1, 10, 100, and 1000 pg/ μ L (see Notes 3, 4, and 5).

⁷·Bead Ruptor 24 (Omni International, Kennesaw, GA) settings for brain homogenization are as follows: Speed: $S = 4.00$, Cycle Time: $T = 1.00$, Number of Cycles: $C = 01$, and Dwell Interval: $D = 0.00$ at 20 °C.

⁶·Final extracted brain sample solutions typically contain rather large quantities of endogenous components carried through the extraction given their solubility in acetonitrile and water combinations. Therefore, it is best not to keep these sample solutions at low temperature for any extended period of time to avoid precipitation of these endogenous components. The best results are obtained by preparing the sample solutions within hours of running them on the mass spectrometer.

²·Careful handling and processing of the matrix sample (vehicle control, not administered sobetirome) should be taken as to not contaminate with sobetirome.

³·The standard curve concentrations: 0, 0.1, 1, 10, 100, and 1000 pg/ μ L are reproducibly useful for analysis of tissue sobetirome concentrations after dosing mice (approximate average weight = 26 g) at a 1 mg/kg or 3.05 μ mol/kg dose. The standard curve must be adjusted accordingly if analyzing tissues from dosing much lower or higher than approximately 1 mg/kg.

⁴·Common complications arise from poor accuracy at the extreme low and the extreme high ends of the standard curve. If this problem occurs, it is helpful to add additional points to the standard curve around these low and high ends.

⁵·Resulting calculated concentrations in pg/ μ L from the standard curve are normalized by tissue weight and converted to ng/g or ng/mL.

3.4 LC-MS/MS Analysis

All samples were run on a QTRAP 4000 hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with electrospray ionization (ESI) in negative mode with a Hamilton PRP-C18 column: 5 μ m particle size (50 mm $\times$ 2.1 mm stainless steel, *see* Note 8). Data were acquired using SCIEX Analyst 1.6.2 software (Framingham, MA, USA) and analyzed using Multiquant 3.0.2.

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⁸. Sobetirome and *d*₆-sobetirome internal standard were analyzed using a QTRAP 4000 hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems) with electro-spray ionization (ESI) in negative mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps and analysis on an Applied Biosystems/SCIEX QTRAP 4000 instrument (Foster City, CA). The instrument was operated with the following settings: source voltage –4500 kV, GS1 50, GS2 60, CUR 15, TEM 650, and CAD MEDIUM. The scheduled multiple- reaction- monitoring (MRM) transitions are based on the precursor ion *m/z* and their corresponding diagnostic product ions. Compounds were infused individually and instrument parameters optimized for each MRM transition (Table 1). The gradient mobile phase was delivered at a flow rate of 0.5 ml/min, and consisted of two solvents, A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in 90% acetonitrile, 10% water. A Hamilton PRP-C18 column: 5 μ m particle size (50 mm $\times$ 2.1 mm stainless steel) was used and kept at 40 °C, and the autosampler was kept at 30 °C. Gradient was as follows, initial concentration of B was 10%, held for 0.5 min, followed by an increase to 98% B over 4.5 min, held for 0.9 min, dropping back to 10% B over 0.1 min, and held at 10% B for 2 min for a total run time of 8 min. Data were acquired using SCIEX Analyst 1.6.2 software (Framingham, MA, USA) and analyzed using Multiquant 3.0.2.

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GC-1 R = iPr

GC-24 R = benzyl

Fig. 1.
The GC series developed by the Scanlan group

Fig. 2.
Karo Bio thyromimetics

Fig. 3.
Metabolic activation of thyromimetic prodrug MB07811 developed by Metabasis
Therapeutics

Fig. 4.
Thyromimetic developed by Madrigal Pharmaceuticals

Fig. 5. Brain (left) and serum (right) concentration-time profile curves for sobetirome dosed at 3.05 $\mu\text{mol/kg}$ by i.p. injection

Fig. 6.
Concentration-time profile from intravenously dosed sobetirome (3.05 $\mu\text{mol/kg}$)

Table 1Multiple reaction monitoring (MRM) information for sobetirome (GC-1) and deuterated sobetirome (d₆-GC-1)

Compound	Retention time (min)	Q1 Mass	Q3 Mass	DP	EP	CE	CXP
<i>GC-1</i>	2.88	327.3	269.3	−80	−10	−28	−21
GC-1	2.88	327.3	269.0	−80	−10	−26	−26
GC-1	2.88	327.3	135.0	−80	−10	−48	−11
<i>d₆ GC-1</i>	2.86	333.0	275.2	−80	−10	−34	−5
<i>d₆GC-1</i>	2.86	333.0	141.1	+90	−10	−48	−7