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Determination of 3-iodothyronamine (3-T_1AM) in mouse liver using liquid chromatography-tandem mass spectrometry

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ABSTRACT

3-iodothyronamine (3-T_1AM) has been suggested as a novel chemical messenger and potent trace amine-associated receptor 1 ligand in the CNS that occurs naturally as endogenous metabolite of the thyroid hormones. Discrepancies and variations in 3-T_1AM plasma and tissue concentrations have nonetheless caused controversy regarding the existence and biological role of 3-T_1AM. These discussions are at least partially based on potential analytical artefacts caused by differential decay kinetics of 3-T_1AM and the widely used deuterated quantification standard D_4-T_1AM. Here, we report a novel LC-MS/MS method for the quantification of 3-T_1AM in biological specimens using stable isotope dilution with $^{13}C_6$-T_1AM, a new internal standard that showed pharmacodynamic properties comparable to endogenous 3-T_1AM. The method detection limit (MDL) and method quantification limit (MQL) of 3-T_1AM were 0.04 and 0.09 ng/g, respectively. The spike-recoveries of 3-T_1AM were between 85.4% and 94.3%, with a coefficient of variation of 3.7–5.8%. The intra-day and inter-day variations of 3-T_1AM were 8.45–11.2% and 3.58–5.73%, respectively. Endogenous 3-T_1AM liver values in C57BL/6J mice were 2.20 ± 0.49 pmol/g with a detection frequency of 50%. Higher liver 3-T_1AM values were found when C57BL/6J mice were treated with N-acetyl-3-iodothyronamine or O-acetyl-3-iodothyronamine. Overall, our new stable isotope dilution LC-MS/MS method improves both the sensitivity and selectivity compared with existing methods. The concomitant possibility to quantify additional thyroid hormones such as thyroxine, 3,5,3′-triiodo-$_L$-thyronine, 3,3′,5′-triiodo-$_L$-thyronine, 3,3′-diiodo-$_L$-thyronine, and 3,5-diiodo-$_L$-thyronine further adds to the value of our novel method in exploring the natural occurrence and fate of 3-T_1AM in biological tissues and fluids.

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1. Introduction

Iodothyronamines (TAMs) are suggested to be the derivatives of thyroid hormones (THs) through decarboxylation and deiodination. In 2004, Scanlan *et* al. firstly reported the existence of endogenous 3-iodothyronamine (3-T_1AM) in rat brain and its biological properties [1], which boosted the research on this compound. 3-T_1AM has been indicated to be a novel chemical messenger because of the following properties: (1) endogenous 3-T_1AM exists in human serum, rodent serum and tissues [1–3]; (2) 3-T_1AM is a multitarget ligand and can interact with trace amine-associated receptor 1 (TAAR1) [4], certain aminergic receptors, mitochondrial proteins, apolipoprotein B-100 (ApoB-100), and transient receptor potential channels [5,6]; (3) 3-T_1AM can reduce contractile performance and heart rate in the isolated perfused rat heart [7,8]; and (4) significant anapyrexia, bradycardia, and hyperglycemia were observed in mice following administration of 3-T_1AM [9–11]. The mechanisms of the biosynthesis of 3-T_1AM remain unclear, and three possible pathways have been proposed: (1) sequential deiodination and decarboxylation of TH precursors [12–14], (2) synthesis and secretion in thyroidal gland [15], and (3) synthesis by gut microbiota [7,16].

The quantification of 3-T_1AM has been achieved primarily using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and immunoassay (IA) technologies. Using monoclonal 3-T_1AM antibodies, Hoefig *et* al. established and validated a chemiluminescence immunoassay approach [3]. With this method, serum 3-T_1AM was quantified to be 66 ± 26 nM in healthy adult humans [3], 15 nM in patients after cardiac surgery [17], and 5 nM in critically ill patients [18], respectively. This method provides high sensitivity, but the selectivity might be limited by nonspecific interactions of the monoclonal antibodies [19–21]. LC-MS/MS methods, which in general provide improved selectivity, were first employed by Scanlan *et* al. to identify fragments of endogenous 3-T_1AM in rat brains extracted with 0.1 M perchloric acid and ethyl acetate [1]. Later, Saba *et* al. optimized the preanalytical procedures for 3-T_1AM in mouse blood and tissues using the Bond Elute Certify SPE cartridge which makes use of a stationary phase consisting of a mixed mode sorbent with C8 and strong cation-exchange (SCX) components. In combination with LC-MS/MS and isotope-dilution (deuterated 3-T_1AM, D_4-T_1AM), endogenous 3-T_1AM was analyzed in rodent serum and tissues such as heart, liver, kidney, skeletal muscle, stomach, lung, brain, white adipose, and ventricular tissue (**Table S1 & Fig. S1, Supporting Information**) [2,15,22–24]. Hansen *et* al. further observed 3-T_1AM in tadpole serum with their optimized LC-MS/MS approach [25] (**Table S1**).

However, significant inconsistencies and variability exist regarding endogenous 3-T_1AM. For instance, 3-T_1AM levels in human serum measured with LC-MS/MS (0.15 – 0.22 nM) were much lower than those measured by immunoassays (5 – 80 nM) [2,3,17,18,26]. Several studies (**Table S1**) reported 3-T_1AM levels below the method detection limit (< MDL) in rat and human tissues using LC-MS/MS or LC-MS methods [27,28], which might be due to the high affinity binding of 3-T_1AM to ApoB-100 [6,29] and in consequence an inefficient extraction by organic solvent. Notably, the commonly used D_4-T_1AM showed higher recovery than 3-T_1AM [29], indicating that 3-T_1AM levels might be underestimated when D_4-T_1AM is employed as an internal standard. Therefore, precise quantification of endogenous total levels of 3-T_1AM requires a method that combines an efficient disruption of 3-T_1AM protein binding with an efficient extraction procedure and a proper quantification standard.

In this study, we firstly evaluated the performance of $^{13}C_6$-T_1AM as a quantification standard for 3-T_1AM by comparing the recoveries of 3-T_1AM with $^{13}C_6$-T_1AM in fetal bovine serum (FBS). Afterwards, an LC-MS/MS approach was optimized for 3-T_1AM quantification using $^{13}C_6$-T_1AM as the quantification standard. Consequently, this method was used to examine the existence of endogenous 3-T_1AM in mouse liver. 3-T_1AM was also measured in liver samples of mice treated with N-acetyl-3-iodothyronamines (NAc-T_1AM) or O-acetyl-3-iodothyronamines (OAc-T_1AM). Other THs were also quantified together with 3-T_1AM.

2. Materials and methods

2.1. Materials and reagents

Bond Elut Certify SPE cartridges (50 mg/3 mL) were obtained from Agilent technologies (Santa Clara, CA, USA). Dulbecco's minimum essential medium (DMEM), FBS, phosphate buffered saline, and potassium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual certified stock solutions of $_L$-thyroxine (T_4), 3,3′,5-triiodo-$_L$-thyronine (T_3), 3,3′,5′-triiodo-$_L$-thyronine (rT_3), $^{13}C_6$-T_3, and $^{13}C_6$-rT_3 (100 μg/mL in MeOH/0.1 M NH_4OH) were from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_6$-T_1AM was obtained from Toronto Research Chemicals (North York, Ontario, Canada). 3,3′-diiodo-$_L$-thyronine (3,3′-T_2) and $^{13}C_6$-3,3′-T_2 were from Santa Cruz Biotechnology (Dallas, Texas, USA). 3,5-diiodo-$_L$-thyronine (3,5-T_2), and 3-T_1AM were from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_6$-T_4 was from Cambridge Isotope Laboratories (Andover, MA). NAc-T_1AM (purity ≥ 95%) was from the Department of Physiology and Pharmacology, Oregon Health and Science University. OAc-T_1AM (purity ≥ 95%) was from ASM Research Chemicals (Burgwedel, Germany) [30]. Primary individual stock solutions of these compounds (50 μg/mL for 3,3′-T_2 and $^{13}C_6$-T_4, 100 μg/mL for 3,5-T_2, 3-T_1AM, $^{13}C_6$-T_1AM, $^{13}C_6$-3,3′-T_2, NAc-T_1AM, and OAc-T_1AM) were prepared in MeOH containing 0.1 M NH_4OH. All other chemicals were of LC-MS grade or higher.

2.2. Tissue collection

C57BL/6J (12–15 weeks old females) originated from Charles River Laboratories (Sulzbach, Germany). AVM: ICR (CD-1) mice (16 weeks old females, weight: 40–45 g) were obtained from a breeding colony at the Helmholtz Center Munich (Neuherberg, Germany). All mice were housed in IVC racks (SealSafe Plus, Tecniplast, Buggugiate, Italy) under SPF conditions in IVC cages (GM 500, Tecniplast) with a 12: 12-h light: dark cycle, 20 to 24 °C, and 45% to 65% humidity. Autoclaved wood fiber (Lignocel 3/4 S, J Rettenmaier and Söhne, Rosenberg, Germany) and nesting material (Crinkle Nature, J Rettermaier and Söhne) were used as bedding and environment enrichment. Sterile-filtered tap water and an irradiated standard diet for rodents (Altromin 1314, Altromin Spezialfutter, Lage, Germany) were available without restriction. Liver samples were obtained from C57BL/6J mice. Serum, liver, kidney, skeletal muscle, stomach, intestine, white adipose tissue, lung, heart, and brain were obtained from AVM: ICR mice.

C57BL/6J male mice at the age of 8–12 weeks were housed in single cages at 22 °C with a 12-h dark-light cycle at the GTH University of Lübeck (Lübeck, Germany) or the KMW Animal Facility of the Karolinska Institute (Stockholm, Sweden). The animals were ip injected with either NAc-T_1AM or OAc-T_1AM (dissolved in 60% DMSO in PBS) at 5 mg/kg body weight once per day for five days. Afterwards, liver tissues were obtained. The samples were dissected, weighed, and snap-frozen in liquid nitrogen, and then kept at −80 °C until analysis [30].

Animal housing and handling protocols were approved by the respective local committees including the Care and Use of Laboratory animals of the Government of Upper Bavaria (Bavaria, Germany), Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung (Schleswig-Holstein, Germany), and Djurförsöksetiska Nämnd (Stockholm Norra, Sweden) according to directive 2010/63/EU.

2.3. HPLC-MS/MS

The chromatographic optimization was based on our previous technology with some modifications [31]. Briefly, TH analysis was conducted using an Agilent 6470 triple quadrupole mass spectrometry coupled with an Agilent 1290 Infinity II LC system (LC-MS/MS). The column temperature was 40 °C. The flow rate was 0.3 mL/min. The injection volume was 20 μL. Water (A) and acetonitrile (B) each containing 0.1% formic acid (v/v) were applied as the mobile phases. The optimized gradient procedure is shown in Table 1.

Data acquisition and analysis were performed using the Agilent MassHunter Workstation software [31]. We monitored four transitions for 3-T_1AM and $^{13}C_6$-T_1AM each to avoid false identification. Other THs including T_4, T_3, rT_3, 3,3′-T_2, and 3,5-T_2 were also targeted. The optimized MS/MS parameters are shown in Table 2.

2.4. Experimental procedures

2.4.1. Recovery of 3-T_1AM in FBS and mouse liver homogenate

An aliquot of 500 μL DMEM supplemented with 10% FBS was employed as the incubation buffer. 3-T_1AM and $^{13}C_6$-T_1AM were spiked to reach concentrations at 1 ng/

Table 1
HPLC procedure optimized for 3-T_1AM analysis. Mobile phase A: H_2O containing 0.1% formic acid. Mobile phase B: ACN containing 0.1% formic acid. Flow rate: 0.3 mL/min.

	Time (min)									
	3	4	6.5	7.5	9	10	12	13	13.2	17
A (%)	95	70	62	62	60	60	0	0	95	95
B (%)	5	30	38	38	40	40	100	100	5	5

Table 2
Optimized MS/MS parameters for thyroid hormones. For each compound SRM ion-transitions are shown as *m/z* for parent ion and product ions. Four transitions were monitored for the confirmation of 3-T_1AM. Compound optimized values for retention time (t_R), fragmentor (F), collision energy (CE), collision acceleration voltage (CAV) and dwell times.

Compound	t_R (min)	Parent ion (*m/z*)	Product ions (*m/z*)	F (V)	CE (V)	CAV (V)	Dwell (msec)
Target compounds							
T_4	7.49	777.4	731.4	140	25	1	50
			633.4	140	25	1	50
T_3	6.70	651.8	605.6	120	25	1	50
			507.6	120	25	1	50
rT_3	6.96	651.8	605.6	120	25	1	50
			507.6	120	25	1	50
3,3′-T_2	6.27	525.8	479.8	90	15	2	50
			381.8	90	15	2	50
3,5-T_2	5.91	525.8	479.8	90	15	2	50
			381.8	90	15	2	50
3-T_1AM	5.79	356	339	118	24	2	50
			212	118	40	2	50
			195	118	44	2	50
			165	118	28	2	50
Quantification standards							
$^{13}C_6$-T_4	7.49	783.6	737.5	140	25	1	50
$^{13}C_6$-T_3	6.70	657.7	611.6	120	25	1	50
$^{13}C_6$-rT_3	6.96	657.7	611.6	120	25	1	50
$^{13}C_6$-3,3′-T_2	6.27	531.8	485.8	100	25	2	50
$^{13}C_6$-T_1AM	5.79	362	345	104	12	1	50
			218	118	60	2	50
			201	118	72	2	50
			171	118	28	2	50

mL (2.8 nM), 5 ng/mL (14 nM), 10 ng/mL (28 nM), 50 ng/mL (140 nM), 100 ng/mL (280 nM), 250 ng/mL (700 nM), 500 ng/mL (1400 nM), and 1000 ng/mL (2800 nM), respectively. After vortex, the mixture was incubated at 37 °C for 0, 20, 40, 60, 120, and 180 min. At each time point, samples were extracted using a liquid–liquid approach: 1 mL of methyl *tert*-butyl ether (MTBE) was added and the mixture was vortexed and centrifuged at 7000 rpm (5478g) for 10 min. The organic supernantant was decanted into a new tube. The extraction process was repeated twice more and the extracts were combined. The combined organic solution was evaporated to dryness under N_2 at 40 °C. Afterwards, the sample was reconstituted in MeOH:0.1 M HCl (1:1, v/v) and measured by LC-MS/MS.

In order to examine the influence of the FBS concentration on the recoveries of 3-T_1AM and $^{13}C_6$-T_1AM, we repeated the above experiment by using DMEM/50% FBS and 100% FBS, respectively. To investigate the influence of the pH value of the incubation solution, we adjusted the pH of DMEM/10% FBS to 10 with 1 M NaOH. Consequently, 100 ng/mL 3-T_1AM and $^{13}C_6$-T_1AM were spiked, and kept at 37 °C for 60 min. Similarly, to examine the effect of protein deproteinization, the solution was kept at 100 °C for 30 min. Thereafter, 100 ng/mL 3-T_1AM and $^{13}C_6$-T_1AM were spiked, and kept at 37 °C for 60 min. Similar processes were also applied for mouse liver homogenate. A 500-mg portion of mouse liver tissue was homogenized by ultrasonication (Bandelin Electronics, Berlin, Germany) after adding 0.5 mL 1 × phosphate buffer (154 mM NaCl, 10 mM NaH_2PO_4, pH 7.4). 3-T_1AM and $^{13}C_6$-T_1AM were then spiked and processed as described above.

2.4.2. Quantification of 3-T_1AM and other THs in mouse liver

A 200-mg portion of mouse liver was weighed and homogenized after addition of 0.5 mL 1 × phosphate buffer (154 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) at 4 °C. After spiking with the quantification standards and vortex, 1 mL 0.1 g/mL trichloroacetic acid (TCA) solution was added. The mixture was vortexed vigorously and kept on ice for 1 h. Thereafter, the homogenate was centrifuged (4000*g*, 10 min, 4 °C), and the supernatant was transferred to a new centrifuge tube. The process was repeated once by adding 0.5 mL 1 × phosphate buffer. Afterwards, 60 mg NaCl was added into the combined extracts. After vortex, the mixture was kept on ice for 1 h for equilibration. Thereafter, 3 mL acetone was added for further deproteinization. After vigorous vortex and centrifugation (2000*g*, 15 min, 4 °C), the supernatant was decanted into a new tube. This process was repeated once by adding 0.5 mL acetone. The supernatants were combined and evaporated to about 1.5 mL under N_2. The concentrated supernatant was reconstituted with 2.3 mL of 0.1 M potassium acetate buffer (pH 4.0). After vortex, the solution was further cleaned-up using Bond Elute Certify cartridges on a vacuum manifold (Supelco, Bellefonte, PA, USA). The cartridges were conditioned by sequential wetting with 2 mL methylene chloride/2-propanol (75:25, v/v), 2 mL MeOH, and 2 mL of 0.1 M potassium acetate buffer (pH 4.0). Thereafter, samples were charged, and immediately washed with 3.5 mL H_2O, 1.6 mL of 0.1 M HCl, 7 mL MeOH, and 3.5 mL methylene chloride/2-propanol (75:25, v/v). Analytes were then eluted into a vial with 2 mL methylene chloride/2-propanol/ammonium hydroxide (70:26.5:3.5, v/v/v). The elute was evaporated to dryness under N_2 at 40 °C. The residue was reconstituted with 60 μL MeOH/0.1 M HCl (1:1, v/v), vortexed rigorously, transferred to a vial, and finally injected into the HPLC system [2].

2.4.3. Method evaluation

Calibration curves for all THs were obtained in neat solvents and in fortified mouse liver. Calibration standards ranging from 0.5 to 150 ng/mL, with 10 ng/mL of quantification standards were prepared in ACN: H_2O (2:8, v/v) containing 0.1% formic acid. Matrix-matched calibration curves were generated by spiking THs in the liver extract at different levels (0, 5, 10, 50, 100, 150 ng/mL), corresponding to a final concentration of 0 – 45 ng/g in liver sample.

To calculate the method detection limit (MDL) and method quantification limit (MQL), fortification of 3-T_1AM, 3,3′-T_2, and 3,5-T_2 in eight CD-1 mouse liver samples were performed at very low concentrations. MDL and MQL were calculated as 3 or 10 times the SD, respectively. Individual values were then normalized to the wet liver sample mass used for extraction to yield a final value of ng/g wet weight (ww).

The accuracy of the method was estimated by spike-recoveries at different spike levels . We assessed the precision of our method by intra-day and inter-day variations, which were calculated as the %CV of the concentrations of three or six samples spiked at 1.5, 15 and 30 ng/g, respectively. The determination of inter-day CV was conducted over two weeks and included freeze–thaw cycles.

2.5. Statistics

The concentrations were expressed as mean ± SD of at least three replicates. The differences between groups were estimated using *t*-test or analysis of variance (ANOVA). P-value < 0.05 was defined as significant. The time course of decreases of 3-T_1AM and $^{13}C_6$-T_1AM were regressed using the pseudo first order models. All the statistical analyses were performed using R (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. Recovery of 3-T_1AM in serum and tissue homogenate

The recoveries of 3-T_1AM and $^{13}C_6$-T_1AM decreased exponentially when incubated with DMEM/FBS for 5, 10, 20, 40, and 60 min (Fig. 1). Following an incubation of 10 ng/mL 3-T_1AM/$^{13}C_6$-T_1AM in DMEM/10%FBS for 60 min, only 2% of the compounds were recovered. These results are in good agreement with previous findings [29] and likely the consequence of 3-T_1AM binding to the apolipoprotein ApoB-100 [6]. The time course of 3-T_1AM/$^{13}C_6$-T_1AM decay follows a pseudo first order reaction. As shown in Table 3, the rate constants (k) of 3-T_1AM/$^{13}C_6$-T_1AM remained constant at low concentration ranges (1–10 ng/mL), and then decreased significantly at higher levels (50–500 ng/mL). An increased k-value was observed at 1000 ng/mL, probably due to the large variation of quantification at high concentrations. Similarly, the half-lives of 3-T_1AM and $^{13}C_6$-T_1AM were constant at low concentrations, but increased significantly at higher levels (Table 3). As shown in Fig. 1, 3-T_1AM and $^{13}C_6$-T1AM recoveries decreased faster in solutions with higher FBS concentrations. Specifically, the half-life of 3-T_1AM (initial concentration: 100 ng/mL) decreased from 11.1 min in DMEM/10% FBS, to 4.64 min in DMEM/50% FBS, and to 3.60 min in 100% FBS. This might again be explained by

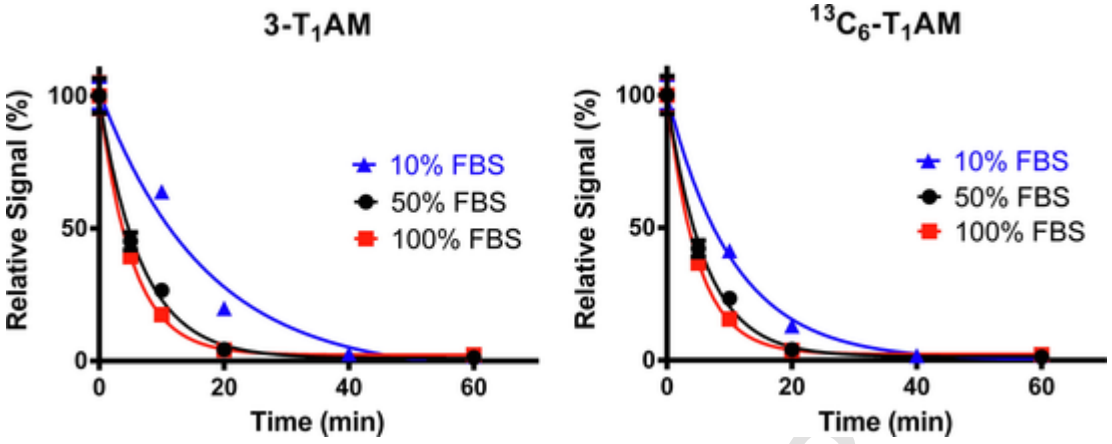

Fig. 1. Influence of FBS concentration on 3-T_1AM and $^{13}C_6$-T_1AM decay. 100 ng/mL of 3-T_1AM and $^{13}C_6$-T_1AM were spiked at time 0 in cell culture medium composed of DMEM and different levels of FBS (10%, 50%, and 100%, respectively). Data were shown as relative response and were expressed as mean ± SD of triplicates. A pseudo first order kinetic model was applied to fit the decay. DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum. 3-T_1AM, 3-iodothyronamine; $^{13}C_6$-T_1AM, isotope (^{13}C) labeled 3-T_1AM.

Table 3
Dynamic parameters of the 3-T_1AM/ $^{13}C_6$-T_1AM decay in DMEM/10% FBS. 3-T_1AM and $^{13}C_6$-T_1AM were spiked at time 0 in cell culture medium composed of DMEM and 10% FBS. Data were shown as relative response and were expressed as mean ± SD. Pseudo-first order kinetic model was applied to fit the decay. DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum. 3-T_1AM, 3-iodothyronamine; $^{13}C_6$-T_1AM, isotope (^{13}C) labeled 3-T_1AM.

	3-T_1AM			$^{13}C_6$-T_1AM		
Concentration	k (1/min)	Half-life (min)	R^2	k (1/min)	Half-life (min)	R^2
1 ng/mL	0.138 ± 0.005	5.04	0.997	0.203 ± 0.013	3.41	0.993
5 ng/mL	0.135 ± 0.007	5.15	0.995	0.203 ± 0.014	3.41	0.992
10 ng/mL	0.147 ± 0.010	4.71	0.991	0.227 ± 0.018	3.05	0.989
50 ng/mL	0.093 ± 0.011	7.44	0.978	0.151 ± 0.017	4.58	0.977
100 ng/mL	0.063 ± 0.008	11.1	0.979	0.094 ± 0.006	7.34	0.995
250 ng/mL	0.033 ± 0.005	21.3	0.990	0.036 ± 0.006	19.3	0.986
500 ng/mL	0.011 ± 0.006	61.6	0.911	0.012 ± 0.007	59.4	0.907
1000 ng/mL	0.044 ± 0.004	157	0.948	0.047 ± 0004	148	0.949

the increased scavenging of 3-T_1AM and $^{13}C_6$-T_1AM by apolipoprotein constituents in the FBS.

Lorenzini *et* al. observed significant isotopic effects for D_4-T_1AM, i.e., higher recovery rates for D_4-T_1AM than 3-T_1AM, which indicates that using D_4-T_1AM as the internal standard will underestimate the level of 3-T_1AM [29]. Our results revealed that $^{13}C_6$-T_1AM is a good internal standard for 3-T_1AM analysis because 3-T_1AM and $^{13}C_6$-T_1AM showed similar decay dynamics at all concentrations. However, we were not able to directly compare $^{13}C_6$-T_1AM against D_4-T_1AM since we did not have access to D_4-T_1AM.

Next, we assessed the impact of pH and deproteinization on the recovery of 3-T_1AM and $^{13}C_6$-T_1AM from the incubation buffer (**Fig. S3**). No significant decreases in T_1AM and $^{13}C_6$-T_1AM recoveries were observed when the pH value of the solution was adjusted to 10, likely due to the deproteinization at basic condition. Similarly, the recoveries of T_1AM and $^{13}C_6$-T_1AM kept constant after deproteinization at 100 °C for 30 min (data not shown). We further investigated the decay of 3-T_1AM/$^{13}C_6$-T_1AM in liver homogenate. As shown in Fig. 2, the recoveries were almost 100% after 60 min ($p = 0.61$ for 3-T_1AM and $p = 0.98$ for $^{13}C_6$-T_1AM, Welch *t*-test, $n = 3$). This can be explained by the high concentration of binding protein (ApoB-100) in blood (76.8 – 153 mg/dL in human plasma) [32]. Whether the recoveries of 3-T_1AM and $^{13}C_6$-T_1AM by our method may also decrease in tissue homogenate after longer incubation periods of 4 h or more, as suggested before [29], remains to be determined.

3.2. Method optimization

For the calculation of instrument detection limits (IDLs) and instrument quantification limits (IQLs), the standard solution (0.5 pg/μL) was repeatedly measured 8 times. IDLs were defined as 3 times the SD, IQLs as 10 times the SD. Water (A) and ACN (B) were used as mobile phases. Initially, we examined the effects of different additives to the mobile phases. Consistent with a previous report [33], we found up to a 6-fold improved signal intensity for 3-T_1AM after the addition of 5 μM ammonium formate compared to the addition of 0.1% formic acid. Nonetheless, similar IDLs/IQLs of THs were observed in mobile phases spiked with both additives (shown in **Table S2**). Since adding 0.1% formic acid provided better peak shapes for other analytes (T_4, T_3, rT_3, 3,5-T_2, and 3,3′-T_2) [28], we selected formic acid as the additive.

Following the sample cleanup procedures described previously for 3-T_1AM analysis [1,2] (shown in **Fig. S1**), we next optimized our LC-MS/MS method for 3-T_1AM measurement using $^{13}C_6$-T_1AM as quantification standard. It is critical to disrupt the TH-protein binding for TH

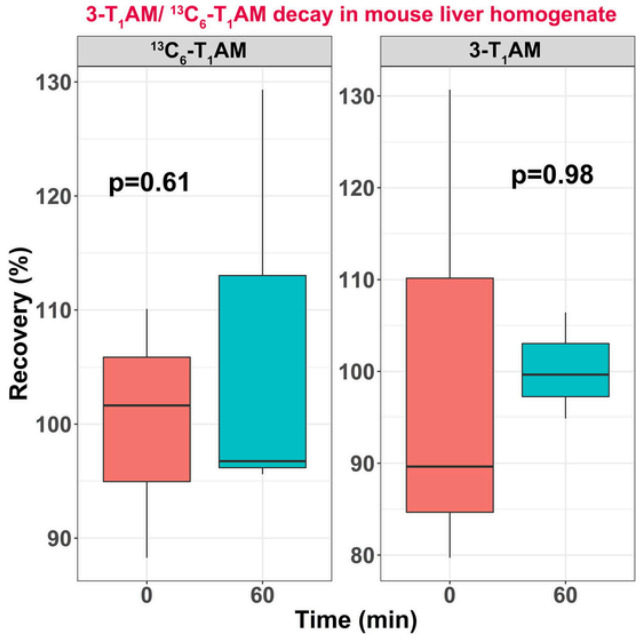

Fig. 2. Recoveries of 3-T_1AM and $^{13}C_6$-T_1AM in liver homogenate. 3-T_1AM and $^{13}C_6$-T_1AM were added at time 0 and incubated for 60 min. The percent recovery values are shown as relative signal and expressed as Box-Whisker plots.

analysis since the majority of THs in circulation or tissues are bound to proteins [34]. In particular, >90% of specially bound 3-T_1AM in human serum resides in the ApoB-100 containing low density lipoprotein fraction in a Schiff base-like manner [27], while T_4 and T_3 tend to bind with thyroxine-binding protein and transthyretin [35]. Consequently, we modified the Saba's procedure [2] to release the binding of 3-T_1AM from proteins with three different approaches: (1) TCA, which is able to precipitate the proteins and break the Schiff-base binding; (2) Urea (8 M), which is able to remove proteins from the homogenate, thereby preventing the 3-T_1AM decay observed in DMEM/FBS [25,29]; (3) Protease from streptomyces griseus (Sigma-Aldrich, USA), which is able to digest proteins [36].

Following a pretreatment with 0.1 mg/mL TCA, we observed clear peaks of three out of four transitions of both 3-T_1AM and $^{13}C_6$-T_1AM in the liver of C57BL/6J mice (Fig. 3). The signal-to-noise ratio (S/N) values of the MS/MS-fragments at *m/z* 339, *m/z* 212, *m/z* 195, and *m/z* 165 were 11.1, 143.7, 24.7, and 6, respectively. This result indicates that this approach was able to release and quantify 3-T_1AM. Fragment *m/z* 212 was employed as the quantification ion since it showed the highest response, which agrees with previous studies [2,25]. The concentration of 3-T_1AM in the liver tissue of C57BL/6J mice was quantified to be 2.20 ± 0.49 pmol/g. However, we were able to quantify 3-T_1AM in only five out of ten samples, with a detection frequency of 50%. Hansen *et* al. observed an even lower detection frequency for 3-T_1AM in tadpole serum (of 30%) [25]. The 3-T_1AM levels quantified in our study were close to those reported by Hackenmueller *et* al. with 2.4 ± 0.4 pmol/g [15]. Assadi-Porter *et* al. reported that the endogenous 3-T_1AM levels in the liver of CD-1 mice were 7.68 ± 0.85 pmol/g [24]. Previous studies also observed 3-T_1AM in rodent serum and other tissues such as heart, liver, kidney, skeletal muscle, stomach, lung, brain, white adipose, and ventricular tissue [2,15,22–24]. We applied our approach for 3-T_1AM analysis in all CD-1 mouse tissues, however, 3-T_1AM was < MDL in all samples.

3.3. Method evaluation and validation

The linearity of the calibration curve was assessed using a six-point calibration curve of the compounds in neat solvent (0 – 150 ng/mL), spiked liver (0 – 30 pg/g), as well as in liver extract (0 – 150 ng/mL). All the analytes showed good linearity in neat solvent with $R^2 > 0.999$. Mouse liver samples spiked with low levels of 3,3′-T_2, 3,5-T_2, and 3-T_1AM were measured repeatedly, and MDLs and MQLs were calculated as 3 or 10 times of the SD, respectively. As shown in Table 4, the MDLs were 0.04 – 0.07 ng/g (3-T_1AM: 0.04 ng/g), while the MQLs were 0.09 – 0.15 ng/g (3-T_1AM: 0.09 ng/g).

Spike-recoveries of the THs were calculated to assess the accuracy of our method. As shown in Table 4, the spike-recoveries of THs were 64.2% – 112% (3-T_1AM: 85.4% – 94.3%), with the coefficient of variance (CV) of triple analyses between 0.2% and 11% (3-T_1AM: 3.7% – 5.8%). These results proved the excellent accuracy of the method for quantifying T_4, T_3, rT_3, 3,3′-T_2, and 3-T_1AM. However, the recoveries of 3,5-T_2 at 1.5 ng/g and 15 ng/g spike levels were relatively low, probably because $^{13}C_6$-3,3′-T_2 was used as the quantification standard for 3,5-T_2 since isotope-labeled 3,5-T_2 ($^{13}C_6$-3,5-T_2) was not commercially available.

Last, the precision of our method was estimated based on intra-day and inter-day variations. As shown in Table 4, the intra-day variations were between 0.38% and 14.7% (3-T_1AM: 8.45% – 9.93%), while the inter-day variations ranged from 1.44% to 11.4% (3-T_1AM: 3.58% – 5.73%). These results showed that our method is of high precision.

3.4. Application

NAc-T_1AM and OAc-T_1AM are the phase II metabolites of 3-T_1AM, and they do not show metabolic or thermogenic properties [30,37]. In principle, 3-T_1AM can be produced following treatment of NAc-T_1AM and OAc-T_1AM through hydrolysis. Our optimized method was further validated by analyzing 3-T_1AM and other THs in the liver tissues of mice dosed with NAc-T_1AM and OAc-T_1AM. As shown in Fig. 3, all of the four transitions of both 3-T_1AM and $^{13}C_6$-T_1AM were clearly observed. The concentration of 3-T_1AM was quantified to be 10.3 ± 3.5 ng/g and 25.8 ± 1.3 ng/g in four C57BL/6J mouse liver samples after treatment with NAc-T_1AM (5 days, i.p., 5 mg/kg) or OAc-T_1AM (5 days, i.p., 5 mg/kg), respectively (Table 5). We also examined the stability of NAc-T_1AM and OAc-T_1AM during sample preparation. The results revealed that 3-T_1AM can be generated from OAc-T_1AM (transformation rate: 40%) during sample cleanup, while NAc-T_1AM kept stable. Therefore, 3-T_1AM observed in the liver of NAc-T_1AM-treated mice was likely degradation product of NAc-T_1AM in the mouse circulation. Considering this transformation, it remains to be determined whether 3-T_1AM found in the liver of OAc-T_1AM-treated mice is generated via degradation in the mouse circulation or sample processing. A previous study also observed 3-T_1AM (13.1 ± 0.10 pmol/g) in the brain of mice injected i.c.v with 3-T_1AM (1.32 μg/kg) [23]. However, we do not have access to 3-T_1AM-treated mouse tissues in this study.

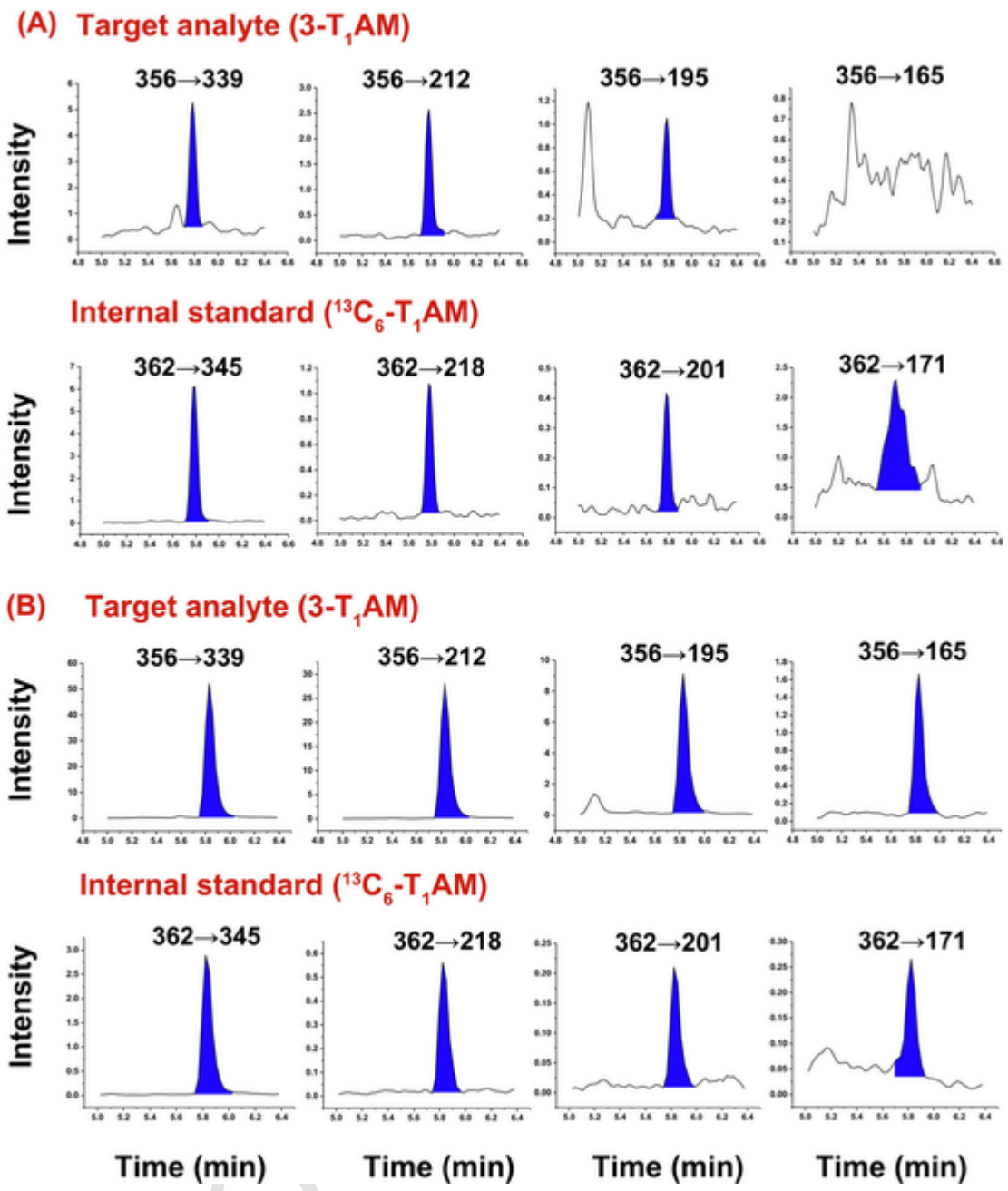

Fig. 3. Representative chromatograms of 3-T_1AM and $^{13}C_6$-T_1AM observed in (A) liver of C57BL/6J mouse; (B) liver of C57BL/6J mouse treated i.p. with 5 mg/kg NAc-T_1AM for 5 days. Four transitions were monitored for 3-T_1AM (356 → 339, 356 → 212, 356 → 195, 356 → 165) and $^{13}C_6$-T_1AM (362 → 345, 362 → 218, 365 → 201, and 365 → 171), respectively. NAc-T_1AM, N-acetyl-3-iodothyronamine.

Using our optimized approach, we were able to quantify additional THs in mouse liver (Table 5). For instance, T_4 and T_3 were found in all the treated and control groups. Besides, in the liver tissue of wildtype mice treated with T_3 orally for 14 days at 0.5 mg/L, 3,5-T_2 was quantified with mean ± SD concentrations of 2.49 ± 4.41 ng/g. These results prove that our method can be used for analyzing several THs simultaneously.

4. Conclusion

In summary, we observed the same decay dynamics of 3-T_1AM and $^{13}C_6$-T_1AM in FBS, which demonstrates the utility of $^{13}C_6$-T_1AM as a proper internal standard for 3-T_1AM analysis. By comparing different approaches to release and quantify endogenous 3-T_1AM, we revealed that a combination of TCA treatment, solid/liquid extraction, liquid/liquid extraction, and Bond Elute Certify SPE procedure provided the best performance on sample cleanup. We were able to measure endogenous 3-T_1AM in the liver tissue of C57BL/6J mice with a detection frequency of 50%. Clear peaks of three out of four transitions were observed.

Good evaluation parameters such as MDLs/MQLs, accuracy, and precision were obtained, which proved the high sensitivity and selectivity of the optimized approach. We were able to measure 3-T_1AM together with other THs in

Table 4
Method validation parameters of the optimized method for analyzing THs in mouse liver. MDL, method detection limit; MQL, method quantification limit.

	T_4	T_3	rT_3	3,3′-T_2	3,5-T_2	3-T_1AM
MDL (ng/g)	0.04	0.04	0.05	0.05	0.07	0.04
MQL (ng/g)	0.09	0.09	0.12	0.12	0.15	0.09
Spike-recovery (%), n = 3						
1.5 (ng/g)	107 ± 4	112 ± 1	106 ± 2	107 ± 2	64.2 ± 0.2	85.4 ± 5.8
15 (ng/g)	102 ± 2	109 ± 4	99.4 ± 1.5	105 ± 7	66.0 ± 4.3	89.2 ± 3.7
30 (ng/g)	101 ± 5	111 ± 5	103 ± 5	109 ± 11	96.3 ± 8.0	94.3 ± 4.1
Intra-day variation (%), n = 3						
1.5 (ng/g)	0.38	5.34	13.2	7.63	14.7	8.45
15 (ng/g)	2.41	1.36	1.02	5.42	13.2	11.2
30 (ng/g)	1.72	4.76	4.49	3.58	9.79	9.93
Inter-day variation (%), n = 6						
1.5 (ng/g)	4.03	3.90	8.91	10.0	11.4	5.73
15 (ng/g)	2.12	6.25	9.24	4.28	6.47	5.25
30 (ng/g)	1.44	5.39	5.04	3.47	2.91	3.58

Table 5
THs measured in liver tissues of C57BL/6J mice. Data are shown as mean ± SD.

Group	THs (ng/g mouse liver)					
	T_4	T_3	rT_3	3,3′-T_2	3,5-T_2	3-T_1AM
Wildtype [a]	23.4 ± 3.3	4.47 ± 0.62	< MDL	< MDL	< MDL	< MDL
Wildtype + T_3 [b]	0.51 ± 0.18	29.3 ± 5.15	< MDL	< MDL	2.49 ± 4.41	< MDL
TRa1 [c]	32.6 ± 4.0	8.56 ± 1.16	< MDL	< MDL	< MDL	< MDL
TRa1 + T_3 [d]	0.41 ± 0.13	30.5 ± 6.3	< MDL	< MDL	< MDL	< MDL
NAc-T_1AM control [e]	25.3 ± 2.2	3.58 ± 0.84	< MDL	< MDL	< MDL	< MDL
NAc-T_1AM [f]	28.6 ± 6.8	3.65 ± 0.93	< MDL	< MDL	< MDL	10.3 ± 3.5
OAc-T_1AM control [g]	26.1 ± 4.9	4.25 ± 0.47	< MDL	< MDL	< MDL	< MDL
OAc-T_1AM [h]	31.3 ± 1.0	4.04 ± 0.43	< MDL	< MDL	< MDL	25.8 ± 1.3

Abbreviations: N-acetyl-3-iodothyronamine; O-acetyl-3-iodothyronamine.
[a] Wildtype (3 replicates).
[b] Wildtype treated with T_3 oral 0.5 mg/L for 14 days (6 replicates).
[c] Mice with mutant TRa1 (6 replicates).
[d] Mice with mutant TRa1 treated with T_3 oral 0.5 mg/L for 14 days (4 replicates).
[e] Untreated wildtype control mice for NAc-T_1AM (5 replicates).
[f] Wildtype mice treated i.p. with 5 mg/kg NAc-T_1AM for 5 days (4 replicates).
[g] Wildtype control mice for OAc-T_1AM (4 replicates).
[h] Wildtype mice treated i.p. with 5 mg/kg OAc-T_1AM for 5 days (5 replicates).

the liver of mice treated with NAc-T_1AM and OAc-T_1AM, thus showing that our method is a useful and versatile tool for the measurement of endogenous 3-T_1AM in mouse tissues.

Authors' contributions

Z.-M. L. was involved in the study design, measurement performance, data analysis and manuscript preparation. M. M., S. G, J. M., S. C. S., and P. T. P. were involved in sample collection and manuscript review. M. D. A. and K.-W. S. were involved in the study design, data interpretation, and manuscript review. All authors have read and approved the final version of this manuscript.

CRediT authorship contribution statement

Zhong-Min Li: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Manuel Miller:** Resources, Writing - review & editing. **Sogol Gachkar:** Resources. **Jens Mittag:** Resources, Writing - review & editing. **Sonja C. Schriever:** Resources, Writing - review & editing. **Paul T. Pfluger:** Resources, Writing - review & editing. **Karl-Werner Schramm:** Supervision, Conceptualization, Project administration, Writing - review & editing. **Meri De Angelis:** Supervision, Conceptualization, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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