An online solid-phase extraction–liquid chromatography–tandem mass spectrometry method to study the presence of thyronamines in plasma and tissue and their putative conversion from $^{13}$C$_6$-thyroxine

M T Ackermans, L P Klieverik, P Ringeling, E Endert, A Kalsbeek and E Fliers

Abstract

Thyronamines are exciting new players at the crossroads of thyroidology and metabolism. Here, we report the development of a method to measure 3-iodothyronamine (T$_1$AM) and thyronamine (T$_0$AM) in plasma and tissue samples. The detection limit of the method was 0.25 nmol/l in plasma and 0.30 pmol/g in tissue both for T$_1$AM and for T$_0$AM. Using this method, we were able to demonstrate T$_1$AM and T$_0$AM in plasma and liver from rats treated with synthetic thyronamines. Although we demonstrated the in vivo conversion of $^{13}$C$_6$-thyroxine ($^{13}$C$_6$-T$_4$) to $^{13}$C$_6$-3,5,3' triiodothyronine, we did not detect $^{13}$C$_6$-$^{1}$T$_1$AM in plasma or brain samples of rats treated with $^{13}$C$_6$-$^{1}$T$_4$. Surprisingly, our method did not detect any endogenous T$_1$AM or T$_0$AM in plasma from vehicle-treated rats, nor in human plasma or thyroid tissue. Although we are cautious to draw general conclusions from these negative findings and in spite of the fact that insufficient sensitivity of the method related to extractability and stability of T$_0$AM cannot be completely excluded at this point, our findings raise questions on the biosynthetic pathways and concentrations of endogenous T$_1$AM and T$_0$AM.

Introduction

Thyronamines are structural homologs of thyroid hormones (see Fig. 1). In the early 1970s, Dr M Dratman et al. (Dratman 1974) speculated about their putative biosynthesis and action. Cody et al. (1984) described the molecular structure and biochemical activity of 3,5,3'-triiodothyronamine (T$_3$AM) in 1984. It was, however, not until 2004 that Scanlan et al. (2004) showed a major physiological role for thyronamines based upon elegant experiments in rodents. Systemic administration of 3-iodothyronamine (T$_1$AM) and, to a lesser extent, of thyronamine (T$_0$AM) induces profound metabolic and cardiac effects including bradycardia, hypothermia, and hyperglycemia (Scanlan et al. 2004, Braulke et al. 2008, Zucchi et al. 2008, Dhillon et al. 2009, Klieverik et al. 2009). Both in vitro and in vivo studies have proposed potential receptors for thyronamines, i.e. the trace amine–associated receptor 1 (Hart et al. 2006, Grandy 2007, Tan et al. 2009, Panas et al. 2010) and adrenergic receptor $\alpha$2 (Regard et al. 2007). Furthermore, studies have been published addressing intra-cellular transport (Ianculescu et al. 2009) and metabolism (Piehl et al. 2008, Wood et al. 2009) of thyronamines. We recently reported that both T$_1$AM and T$_0$AM can act in the central nervous system to modulate glucose metabolism (Klieverik et al. 2009). These findings prompted us to develop an online solid-phase extraction (SPE)–liquid chromatography (LC)–tandem mass spectrometry method (XLC–MS) to study the bioavailability of thyronamines in plasma and tissues including the brain and the thyroid gland, as well as to study their hypothesized formation from thyroxine (T$_4$) using stably labeled $^{13}$C$_6$-$^{1}$T$_4$.

Materials and Methods

Plasma and tissue samples

We used heparinized plasma samples of thyronamine-treated (i.p. bolus of either 50 mg/kg T$_1$AM or 50 mg/kg T$_0$AM in 500 $\mu$l) or vehicle-treated (i.p. bolus of 500 $\mu$l saline) euthyroid, adult Wistar rats ($n=6$) that participated in the studies published previously (Klieverik et al. 2008, 2009). Moreover, plasma, hypothalamus, and neocortex samples of rats ($n=2$ per dose) treated with $^{13}$C$_6$-$^{1}$T$_4$ were analyzed to investigate whether T$_1$AM is formed from T$_4$. These rats had been equipped with a s.c. osmotic minipump delivering $^{13}$C$_6$-$^{1}$T$_4$ for a period of 10 days (Alzet 2ml2, Durect Corp., Cupertino, CA, USA; flow rate 5 $\mu$l/h; dose: vehicle, 0·44, 1·75, and 20 $\mu$g/100 g body weight per day; Klieverik et al. 2008). Furthermore, we studied...
human heparin plasma samples and serum \((n=8)\) taken from healthy volunteers by venipuncture. Finally, we studied human thyroid tissue specimens \((n=2)\) obtained from the thyroid tissue bank in the Academic Medical Center of the University of Amsterdam. As a positive control for the extraction of thyronamines from tissue, we used \((13C_6-T1AM)\) obtained from Abbott Diagnostics (Abbott Park). All other chemicals were obtained from Merck.

Recovery experiments

The recovery of the SPE was tested by comparing peak areas of identical injections of \(T1AM\), \(T0AM\), and \(d_4-T1AM\) with and without SPE \((n=10)\). In order to test the recovery of the sample pretreatment including the stability of \(T1AM\) and \(T0AM\) during the procedure, we processed human plasma and human thyroid after adding \(T1AM\) and \(T0AM\) to the sample. For the plasma samples, 10 \(\mu l\) of a mix of 1 \(\mu mol/l\) \(T1AM\) and \(T0AM\) in PBS were added to 100 \(\mu l\) plasma. Plasma was vortexed and processed as unspiked plasma. For the thyroid tissue samples, the same amount of \(T1AM\) and \(T0AM\) was added to 100 mg tissue at the same time as the internal standard (IS). The recovery was calculated as the concentration of thyronamines measured divided by the concentration added.

Sample pretreatment

All quantitative analyses were carried out using the IS method using stable isotope-labeled \(T1AM\). As IS, \(d_4-T1AM\) \((1 \mu M\) in PBS) was used. In this molecule, four hydrogen atoms are replaced by four deuterium atoms. As hydrogen and deuterium are isotopes of the same element, \(d_4-T1AM\) and \(T1AM\) act similarly both biologically and analytically. However, due to the higher atomic mass of deuterium compared with hydrogen, the molecular weight of \(d_4-T1AM\) is 4 mass units higher than that of \(T1AM\) enabling the distinction between the two molecules by mass spectrometry. No \(d_4-T1AM\) was added to the samples that were used to study the conversion from \(^{13}C_6-T4\) to \(^{13}C_6-T1AM\).

Plasma samples

Ten microliters IS were added to 100 \(\mu l\) plasma. Samples were incubated overnight \((15-17\ h)\) at 37 \(^{\circ}\)C with 20 \(\mu l\) protease K \((100\ mg/ml\) in distilled water). Thereafter, the sample was centrifuged \((3\ min\ at\ 16,000\ g)\). Five microliters of the supernatant were diluted 20 times with distilled water. This dilution was used for the measurement of protein fragments using SDS-PAGE and Coomassie Brilliant Blue coloring. To another 100 \(\mu l\) of the supernatant, 150 \(\mu l\) of 0-1% formic acid were added. The sample was placed at 4 \(^{\circ}\)C until analysis with XLC–MS.

Tissue samples

Ten microliters IS and 3 ml prechilled \(KH_2PO_4\) solution \((100\ mM\ at\ pH\ 6)\) were added to ca. 100 mg tissue. The tissue was ground until it was devoid of visible tissue fragments. Six milliliters of chilled acetic acetone \((5\ ml\ of\ 37\%\ HCl\ in\ 100\ ml\ acetone)\) were added. In order to complete denaturation, the sample was placed on ice for 10 min. After centrifugation \((10\ min\ at\ 4000\ \text{g})\), the supernatant was transferred to a clean tube and evaporated to dryness followed by the addition of 300 \(\mu l\) of 0-1% formic acid. The supernatant obtained after centrifugation \((5\ min\ at\ 4000\ \text{g})\) was placed at 4 \(^{\circ}\)C until XLC–MS analysis.

Calibration solutions

Serial dilutions of the stock solutions of \(T1AM\) and \(T0AM\) \((10\ mM\ in\ DMSO)\) at 1, 5, 10, 20, 30, 40, and 50 \(\mu mol/l\) were prepared in 0-1% formic acid. For XLC–MS, 100 \(\mu l\) of each calibration standard were pipetted in a deep well plate. To each well, 10 \(\mu l\) IS and 140 \(\mu l\) 0-1% formic acid were added. Before the deep well plate was put into the autosampler it was covered, vortexed, and centrifuged \((2\ min\ at\ 10000\ \text{g})\) to make sure that all the samples were well mixed, and that no air bubbles were left in the wells. For the linearity check, calibration was extended with concentrations of 100 and 200 \(\mu mol/l\) \(T1AM\) and \(T0AM\).

Figure 1 Structural formulae of \(T_4\), \(T_1AM\), and \(T_0AM\).
**XLC–MS procedure**

**Instrumentation** For the analysis, we used a Symbiosis Pharma System (Spark Holland, Emmen, The Netherlands) coupled to a Quattro Premier XE tandem MS system (Waters, Milford, MA, USA).

**Solid-phase extraction** SPE was achieved on an OASIS WCX cartridge (10 × 1 mm, 30 μm, Waters). The cartridge was conditioned with 1 ml acetonitrile and equilibrated with 1 ml of 10 mM ammonium acetate at pH 8:methanol (90:10). 100 μl of the sample were loaded on the cartridge using 1 ml of 10 mM ammonium acetate at pH 8:methanol (90:10), and the cartridge was washed with another 1 ml of this solution. The purified thyronamines were eluted using the high-pressure dispenser in focusing mode. During focusing, the analytes of interest are eluted from the cartridge with 200 μl H₂O:acetonitrile:acetic acid (50/50/0.6). Post-cartridge addition of aqueous solvent delivered by the LC pump reduces the percentage of organic solvent used for cartridge elution before the analytes reach the LC column. As a consequence, the analytes are trapped at the head of the column. After this focusing step, the LC gradient is started in order to separate the analytes on the LC column.

**Liquid chromatography** The LC method was adapted from Piehl et al. (2008a). Briefly, the sample was separated on a Phenomenex Synergi Polar-RP 80 Å, 4 μm particles, 50 × 2 mm (Phenomenex, Maarssenbroek, The Netherlands) using gradient elution. Flow was 0.20 ml/min. The composition of mobile phase A was H₂O:acetonitrile:acetic acid (95/5/0.6), and the composition of mobile phase B was H₂O:acetonitrile:acetic acid (5/95/0.6). Gradient program was as follows: 100% A for 2 min (high pressure dispenser focusing time), thereafter from 90% A to 10% A in 2.5 min, hold for 1 min at 10% A, and re-equilibration at 90% A for 3 min. Total runtime was 8.5 min.

**Mass spectrometry** We used ionization in the ESI+ mode with the following parameters: capillary voltage, 3-00 kV; cone voltage, 25-00 V; extractor, 3-00 V; RF Lens, 0-3 V; source temperature, 140 °C, desolvation temperature, 300 °C. Cone gas flow was 2001/h, and desolvation gas flow was 10001/h. T₀AM, T₁AM, and d₄-T₁AM were measured in the multiple reaction monitoring (MRM) mode using the following MRM transitions: T₀AM 230 > 109 and 230 > 213, T₁AM 356 > 212 and 356 > 339, and d₄-T₁AM 360 > 216 and 360 > 343. For the quantification, we used the MassLynx software (Version 4.1, Waters).

**Thyroid hormone measurement** Owing to the structural homology of thyronamines and thyroid hormones, the above-mentioned method, although not optimized, can also be used to detect thyroid hormones. For qualitative analyses of the stably labeled compounds, the following MRM transitions were measured: ¹³C₆-T₁AM 362 > 218 and 362 > 345, ¹³C₆-T₄ 783 > 738, and ¹³C₆-T₃ 658 > 612. As no ¹³C₆-T₃ standard was available, the retention time of T₃ was determined using unlabeled T₃ with MRM 652 > 606. Thyroid hormone status of the rats was evaluated by measuring T₄, T₃ and TSH concentrations in the plasma samples using immunoassays as reported previously (Klieverik et al. 2009).

**Analytical characterization of the method**

**Linearity and precision** We established an estimate of the linearity and precision based upon the protocols of the Committee of Clinical and Laboratory Standards Institute (CLSI) of the US using the EP Evaluator 8 software (D.G. Rhoads Associates, South Burlington, VT, USA).

**Limit of detection** To estimate the limit of detection (LOD), calibration standards were made with concentrations of 0.08, 0.17, 0.25, 0.33, 0.42, and 0.50 nmol/l. These samples were injected, and the signal to noise ratio was determined for the different peaks. The LOD was set to the lowest concentration with the signal to noise ratio > 10.

**Protein electrophoresis by SDS-PAGE**

We analyzed the protein fragments of the plasma samples using 12% SDS-PAGE with a Laemmli buffer at pH 8.3–8.5. The marker used was the Protein Plus Precision Marker. After electrophoresis, gels were stained overnight at room temperature using Coomassie Brilliant Blue G-250 (3 mg/ml) in 10% acetic acid. Gels were destained in 10% acetic acid solution in 4–8 h. To study the degradation of ApoB, we ran two human plasma samples untreated or treated with 1000 l/h. T₀AM, T₁AM, and d₄-T₁AM were measured in the multiple reaction monitoring (MRM) mode using the following MRM transitions: T₀AM 230 > 109 and 230 > 213, T₁AM 356 > 212 and 356 > 339, and d₄-T₁AM 360 > 216 and 360 > 343. For the quantification, we used the MassLynx software (Version 4.1, Waters).

**Table 1 Recovery experiments**

<table>
<thead>
<tr>
<th>(A) SPE recovery (n=10)</th>
<th>T₁AM</th>
<th>T₀AM</th>
<th>d₄-T₁AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area without SPE (cv)</td>
<td>52 540 (2%)</td>
<td>64 618 (1%)</td>
<td>65 983 (2%)</td>
</tr>
<tr>
<td>Area with SPE (cv)</td>
<td>45 268 (3%)</td>
<td>47 229 (3%)</td>
<td>58 257 (2%)</td>
</tr>
<tr>
<td>Recovery</td>
<td>87%</td>
<td>73%</td>
<td>89%</td>
</tr>
</tbody>
</table>

| (B) Sample pretreatment recovery (n=2) |
| Human plasma | 96%  |
| Human thyroid| 89%  |

109 and 212, T₁AM 356 > 212 and 356 > 339, and d₄-T₁AM 360 > 216 and 360 > 343. For the quantification, we used the MassLynx software (Version 4.1, Waters).
Figure 2  Representative chromatograms of human (A) plasma and (B) thyroid tissue, vehicle-treated rat (C) plasma and (D) liver tissue, T1AM-treated rat (E) plasma and (F) liver tissue, and T0AM-treated rat (G) plasma and (H) liver tissue. In some cases, the part of the chromatogram where T1AM or T0AM should show up is magnified by the factor indicated. Note the presence of T1AM or T0AM in samples from thyronamine-treated rats and their absence in other samples.
with the proteinase K protocol using 4% SDS-PAGE and silver staining as described by Furbee & Fless (1996). As a marker, we used the ApoA1/ApoB calibrator from Abbott Diagnostics.

**Results**

**Recovery experiments**

The results of the recovery experiments are given in Table 1. As can be observed in the table, the recovery of the SPE was 87, 73, and 89% for T1AM, T0AM, and d4-T1AM respectively. The recovery of the sample pretreatment was 95% for T1AM and 122% for T0AM.

**Analytical characterization of the method**

**Linearity** The accuracy tests were successful. Using the measuring range of 1–50 nmol/l, the maximum deviation for a mean recovery from 100% was 4.3% for T1AM and 5.9% for T0AM. For both the components, 7 out of 7 mean recoveries were accurate within the allowable systematic error of 6%, and 14 out of 14 results were accurate within the allowable total error of 20%. All results were linear. In the range of 5–200 nmol/l, the maximum deviation for a mean recovery was 5.4% for T1AM and 9.4% for T0AM. For both the components, 6 out of 6 recoveries were accurate within the allowable systematic error of 6%, and 12 out of 12 results were accurate within the allowable total error of 20%.

**Precision** For the calibration standard of 2.5 nmol/l, the coefficient of variation was 1.4% for T1AM and 6.1% for T0AM based upon the variation in response values (area T1AM over area d4-T1AM) of 12 measurements in one run.

**Limit of detection** In the 0.08 nmol/l sample, the signal to noise ratio was 7.03 for T1AM and 9.25 for T0AM (both <10). In the 0.17 nmol/l sample, the values were 13.72 and 17.06 respectively implying a LOD of 0.10 nmol/l referring to the concentration in the well. Considering the dilutions for the plasma and tissue samples, we inferred a LOD of 0.25 nmol/l in plasma and 0.30 pmol/g in tissue both for T1AM and for T0AM.

**Plasma and tissue samples** Figures shown are representative for the various groups, i.e. only one chromatogram of human plasma is shown, but the other seven samples were very similar. Moreover, we did not observe any difference between heparinized plasma samples and serum samples. Figure 2 shows representative chromatograms of various plasma and tissue samples. T1AM and T0AM were detected in the thyronamine-treated animals. Plasma concentrations of T1AM in T1AM-treated rats and plasma concentrations of T0AM in T0AM-treated rats were > 200 nmol/l. In the liver samples, concentrations of T1AM and T0AM were > 600 pmol/g tissue, yielding a sample higher than 200 nmol/l for the XLC–MS injection. We did not detect T1AM or T0AM above the LOD in human or vehicle-treated rat plasma, nor in the thyroid tissue samples. In Fig. 3A, we show that the proteinase K treatment of our plasma samples effectively degraded the protein using SDS-PAGE and Coomassie Brilliant Blue staining. Figure 3B shows that also larger proteins, including ApoB, are degraded in our proteinase K protocol. In Fig. 4, we show the results of the conversion study. Panel A shows the chromatograms of a standard containing 13C6-T1AM, T3, and 13C6-T4, and panel B shows the chromatograms of a blank injection of 100 µl distilled water, and panels C–E show the chromatograms of plasma, hypothalamus, and neocortex of a 13C6-T4-treated rat.
Plasma $T_4$, $T_3$, and TSH concentrations for the different doses of $T_4$ are given in Table 2. Although we detected both $^{13}C_6$-$T_4$ and $^{13}C_6$-$T_3$ in the samples of the $^{13}C_6$-$T_4$-treated rats, we did not detect $^{13}C_6$-$T_1AM$ in any of the samples. As can be observed in panel A, two peaks are clearly visible with MRM 362 $>$ 217 and 362 $>$ 345 (the $^{13}C_6$-$T_1AM$ channel). In XLC–MS, there are two ways to identify a compound: the MRM and the retention time, which is specific for a component in a chromatographic system. The experiments with $T_1AM$ and $^{13}C_6$-$T_1AM$ standards and samples from $T_1AM$-treated rats showed that in our chromatographic system the retention time of $T_1AM$ and $^{13}C_6$-$T_1AM$ is 4-8 min (Figs 2E and F, and 4A). Based upon the retention time, the peak at 5-5 min does not represent $^{13}C_6$-$T_1AM$ but another compound showing the same MRM, but with different chromatographic behavior to $^{13}C_6$-$T_1AM$. Theoretically, it could represent a $^{13}C_6$-labeled thyronamine with more than one iodine molecule, which is abolished in the ionization. However, as this peak was also present after injecting only water into the system (see Fig. 4, panel B), we concluded that it originates from one of the chemicals used for mobile phases and/or SPE solvents, thus representing an artifact. Looking closely at the MRM 362 $>$ 217 and 362 $>$ 345 in the panels of the plasma, hypothalamus, and neocortex of the $^{13}C_6$-treated rat (Fig. 4, panels C, D, and E), the only peak present is the artifact peak at retention time of 5-5 min. In summary, $T_1AM$ and $T_0AM$ were only present above the LOD in plasma of rats treated with $T_1AM$ or $T_0AM$ respectively, and we were not able to show any conversion of $^{13}C_6$-$T_4$ to $^{13}C_6$-$T_1AM$ in rat plasma, neocortex, or hypothalamus.

Discussion

We have developed an analytical method to measure thyronamines in plasma and tissue requiring minimal sample pretreatment, due to the fact that the method uses online SPE. Compared with offline methods, this method has the advantages that manual sample preparation and solvent usage are minimized. In addition, an online method is more robust as human error is minimized. Using 100 $\mu$L plasma or 100 mg tissue, the detection limit of the method is 0-25 nmol/l in plasma and 0-30 pmol/g in tissue both for $T_1AM$ and for $T_0AM$. Using this method, we were able to detect $T_1AM$ and $T_0AM$ in rats treated with $T_1AM$ and $T_0AM$ respectively. The concentration of these thyronamines

Figure 4 Representative chromatograms of (A) standard 6 nmol/l, (B) blank injection of 100 $\mu$L distilled water, and (C) plasma, (D) hypothalamus, and (E) neocortex of a $^{13}C_6$-$T_4$-treated rat. As can be observed in panels C–E, a peak representing $^{13}C_6$-$T_1$ appears, indicating a $^{13}C_6$-$T_4$ to $^{13}C_6$-$T_1$ conversion. Nevertheless, we could not demonstrate the conversion of $^{13}C_6$-$T_4$ to $^{13}C_6$-$T_1AM$ as no peak appears in the $^{13}C_6$-$T_1AM$ trace. The peak in the $^{13}C_6$-$T_1AM$ chromatograms at 5-5 min in panels C–E represents an artifact, as it is also present in blank injections (see panel B and text).
is above the highest calibration standard. Braulke et al. (2008) showed that in Djungarian hamsters, serum T1AM levels were between 50 and 60 nmol/l 3 h after i.p. injection of 50 mg/kg T1AM. Besides the obvious species difference, our plasma samples were pooled samples taken between 5 and 120 min after injection, making direct comparison between their data and our data very difficult. In spite of the fact that endogenous levels of thyronamines published to date (Braulke et al. 2008, DeBarber et al. 2008, Zucchi et al. 2008) are above our LOD, to our surprise we did not detect any endogenous T1AM or T0AM.

As can be observed in Fig. 3A, the proteinase K treatment of plasma effectively degrades proteins, making protein binding as an explanation for our negative results regarding endogenous thyronamines very unlikely. ApoB 100, a 550 kDa protein that cannot be separated on a 12% gel, has been reported at various recent meetings to be the major binding protein for thyronamines. In Fig. 3B, we also show that ApoB is effectively degraded by our proteinase K treatment using a 4% SDS–PAGE and silver staining. The observation that the protein binding of thyronamines is effectively abolished by the proteinase K treatment without degrading the thyronamines follows from the recovery experiment, arguing against instability of the thyronamines during proteinase K treatment. In addition, we observed T1AM and T0AM in high concentrations in thyronamine-treated rats, supporting effective proteinase K treatment. With respect to tissue thyronamines, it could be argued that thyronamines are lost during the sample pretreatment, but again this argument can be refuted by our observations in the liver samples of the thyronamine-treated rats and by the recovery experiment in human thyroid tissue.

Pietsch et al. (2007) showed that thyronamines are substrates of human liver sulphotransferases. Therefore, another reason for the absence of endogenous T1AM or T0AM in our method could be that they are present as sulfoconjugates. In that case, their molecular weight would be higher, and ionization would be altered so they would not appear in the very specific MRM transitions of the unconjugated thyronamine. However, given the fact that we observe T1AM and T0AM in thyronamine-treated animals in such high concentrations (> 200 nmol/l plasma or > 600 pmol/g tissue), we do not expect the thyronamines to be mainly present as sulfoconjugates.

The biosynthetic pathway of conversion thyroid hormone to thyronamines would require both deiodination and decarboxylation. Piehl et al. (2008b) showed that thyronamines are substrates for the human deiodinases. The decarboxylating enzyme, however, still remains to be identified. Pyridoxal-5-phosphate-dependent aromatic L-amino acid decarboxylase (AADC) is a promising candidate, although Hoefig et al. (2009) recently reported that recombinant human AADC does not efficiently catalyze the decarboxylation of rT3. De novo synthesis of thyronamines would require oxidative coupling of two molecules of tyrosine and aromatic ring iodination, processes that are also involved in the biosynthesis of thyroid hormone. Although there are hints that several organs/tissues are capable of generating some thyroid hormone (Taurog & Evans 1967, Obregon et al. 1981, Meischl et al. 2008), the thyroid is considered the primary source of generating thyroid hormone. Therefore, if de novo synthesis was substantial, we would expect to find thyronamines in thyroid tissue. However, we did not find any endogenous T1AM or T0AM in thyroid tissue. In vivo conversion of thyroid hormones has been postulated as a biosynthetic route for thyronamines (Zucchi et al. 2008, Scanlan 2009). To investigate this route, we treated rats with 13C6-T4 for 10 days in different doses. This did not result in the appearance of detectable 13C6-T1AM, arguing against the biosynthesis of T0AM from thyroid hormones under euthyroid or hyperthyroid conditions. In support of in vivo deiodination of stably labeled T4, we were able to detect 13C6-T3 in plasma and brain tissue indicating that the exogenous 13C6-T4 was metabolized.

In conclusion, we have developed a simple and sensitive method to determine T1AM and T0AM in plasma and tissue samples. Using this method, we could identify T1AM and T0AM in plasma and liver of thyronamine-treated animals. Unexpectedly, we did not detect any endogenous T1AM or T0AM in plasma or thyroid tissue samples, nor could we demonstrate the in vivo conversion of 13C6-T4 to 13C6-T1AM. Although insufficient extraction from plasma or tissue, instability of the thyronamines, or insufficient sensitivity of the method cannot be completely excluded at present, these findings raise questions about the biosynthetic pathways and concentrations of endogenous T1AM and T0AM.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Table 2** Mean (n=2) concentrations of TSH, thyroxine (T4), and 3,5,3’-triiodothyronine (T3) in plasma of rats treated with 13C6-T4

<table>
<thead>
<tr>
<th>Dose 13C6-T4 (µg/100 g body weight per day)</th>
<th>TSH (mU/l)</th>
<th>T4 (nmol/l)</th>
<th>T3 (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.97</td>
<td>82</td>
<td>0.98</td>
</tr>
<tr>
<td>0.44</td>
<td>0.79</td>
<td>94</td>
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<td>1.75</td>
<td>0.59</td>
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<td>1.14</td>
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<tr>
<td>20</td>
<td>&lt;0.20</td>
<td>201</td>
<td>3.34</td>
</tr>
</tbody>
</table>

*“n=1.”*
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