Short communication

Ion-trap mass spectrometry for determination of 3,5,3′-triiodo-L-thyronine and 3,5,3′,5′-tetraiodo-L-thyronine in neonatal rat cardiomyocytes

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ABSTRACT

Our short report describes a method employing electrospray ion-trap mass spectrometry (MS) connected to a reversed phase (C_8) HPLC system for monitoring of 3,5,3′-triiodo-L-thyronine (T3) and its precursor 3,5,3′,5′-tetraiodo-L-thyronine (T4) in neonatal rat cardiomyocytes. The experimental protocol allows simultaneous analysis of the free thyroid hormones in nanomolar concentration range and enables observation of their distribution in cultivation medium over time. The method is a useful tool for MS^2 identification of T3/T4 and analysis of their uptake into mammalian cells.

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1. Introduction

The heart is a high energy demand organ requiring a finely tuned metabolism in order to drive its incessant contraction/relaxation. While metabolic pathways in cardiomyocytes adapt to a short-term work burden usually by modulating metabolite traffic, long-term adaptation is governed by hormonal means. 3,5,3′-Triiodo-L-thyronine (T3) is an amino acid type hormone that affects many physiological processes in mammals, including processes coupled to regulation of cardiac functions. T3 is mainly produced by peripheral deiodination of 3,5,3′,5′-tetraiodo-L-thyronine, also known as L-thyroxine (T4), which is produced in the thyroid gland [1,2]. The chemical structures of T3 and T4 are shown in Fig. 1A.

Several analytical tools have been developed for monitoring of T3 and T4 thyroid hormones in biological material. Immunoassays, physical separation methods (equilibrium dialysis and ultrafiltration), and spectrometric detectors connected with HPLC are the most prominent methods used in research involving T3 and T4 [3,4]. It was previously shown that detection strategies based on mass spectrometry (MS) enabled analysis of selected iodinated hormones in clinical samples such as blood [4], serum [5], and human breast milk [6]. For this purpose electrospray ionization (ESI, operating in both negative and positive mode) isotope dilution MS and

MS/MS connected to GC and/or HPLC system have frequently been used [7–11]. For comparison of several detection strategies for T4 analysis see review by Holm et al. [4].

Primary cultures of neonatal rat cardiomyocytes (NRC) serve as a suitable model for evaluation of *in vitro* effects of T3 and T4 on heart metabolism and targeted protein expression. NRC could be prepared in a robust and reproducible fashion according to an optimized procedure described previously in our laboratory [12]. In case of T3 and T4 analysis in NRC, equilibrium dialysis and low-pressure chromatography with Sephadex column and measurement of ^{125}I-activity in the fractions were applied [13–15]. We aimed our effort at developing a suitable and sensitive method for T3 and T4 detection in NRC that would not require the use of radioactive or otherwise labeled hormones. As far as we are aware, MS^n procedure and modern HPLC tool for study of T3 and T4 in cardiac cell cultures has not been developed to date. Therefore we focused our interest towards the application of MS and HPLC/MS interface for T3 and T4 monitoring in NRC, also taking advantage of our experience with substance detection in difficult to handle biological materials.

2. Experimental

2.1. Chemicals

Standards of T3 and T4 as well as acetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol was obtained

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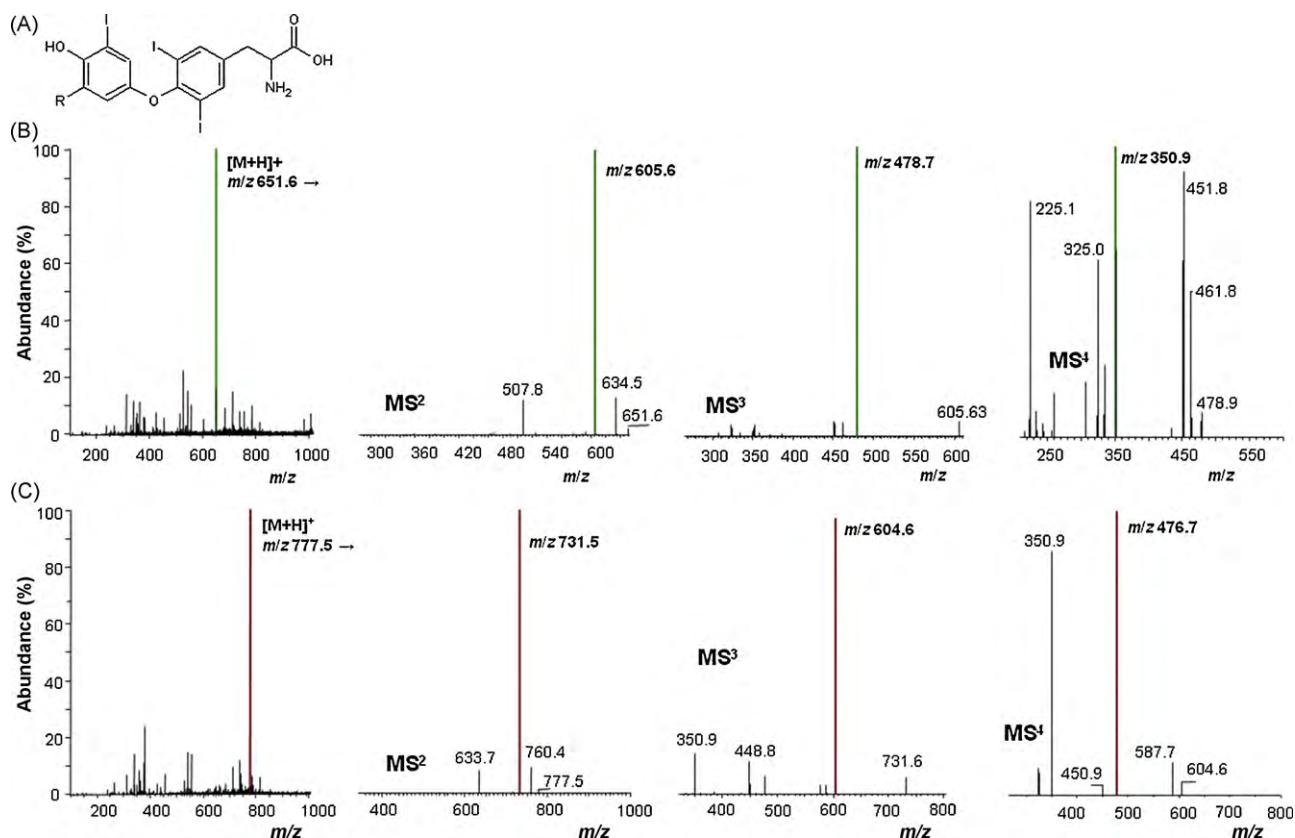E-mail address: jan.vacek@upol.cz (J. Vacek).

Fig. 1. (A) Chemical structures of T3 (R=H) and T4 (R=I). Representative mass spectra of T3 (B) and T4 (C) are shown. Experimental conditions: 1 μg ml^{-1} of T3 and T4, ESI-MS parameters: electrospray voltage (4.5 kV), transfer capillary voltage (34 V) and temperature (300 °C), flow rate (5 μl min^{-1}); for more details see Section 2.

from Merck (Darmstadt, Germany). Model standard solutions were prepared by dissolution of the thyroid hormone standards in HPLC mobile phase (see Section 2.5) in a concentration range from 1 ng ml^{-1} to 10 μg ml^{-1}. All solutions were prepared using reverse-osmosis deionized water (Ultrapur, Watrex, Prague, Czech Republic). Nitrogen and helium (99.999% for both) were obtained from Linde Gas (Prague, Czech Republic).

2.2. Neonatal rat cardiomyocytes (NRC)

Primary cultures of NRC were prepared by following the previously published and optimized procedure [12]. NRC were cultured in a medium (DMEM/Medium 199, in ratio 4/1 with penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1}) in the absence (control) or in the presence of T3 and T4 for 6 h at 37 °C in a humidified incubator. Following the treatment period the NRC (2.5 × 10^5 cells per sample) were scraped off the cultivation dish, separated by centrifugation (50 × g/2 min at room temperature, Minispin Plus, Eppendorf, Hamburg, Germany), 4-times washed in 0.5 ml of PBS, and finally added to 200 μl of HPLC mobile phase (see Section 2.5). NRC suspension was sonicated utilizing ultrasonic homogenizer UP 200S (cycle 0.5, amplitude 50, 10-times) from Hielscher Ultrasonics GmbH (Teltow, Germany), then centrifuged (14,000 × g/2 min at room temperature), and supernatants (10 μl) were analyzed by HPLC/MS.

2.3. LDH release and MTT assay

Activity of lactate dehydrogenase (LDH; EC 1.1.1.27) in the NRC medium and analysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in NRC were carried out according to the previously published procedure [16].

2.4. ESI/ion-trap MS

Quadrupole ion-trap MS detector LCQ Fleet (Thermo Scientific, Waltham, MA, USA) operating in a positive ESI mode was used for T3 and T4 analysis. ESI-MS parameters were as follows: spray voltage (4.5 kV), transfer capillary temperature (300 °C), and capillary voltage 34 V. Nitrogen was used as sheath, auxiliary, and sweep gas, and helium was used as the collision gas. The sheath, auxiliary, and sweep gas flow rates were 50, 5, and 7 (as arbitrary units), respectively. Full-scan mass spectra were acquired in the range of 50–1000 m/z ($m/z \pm 0.15$). The resolution (R) for m/z values of peaks of the thyroid hormones were measured according to the following equation: $R = m/\Delta m$, where m is the ion mass and Δm is the difference in mass between two resolvable peaks in a mass spectrum.

2.5. HPLC separation

HPLC chromatographic system Shimadzu (Shimadzu, Kyoto, Japan) equipped with SCL-10Avp controller, a vacuum degasser, a binary pump (LC-10ADvp), an autoinjector (SIL-10ADvp), a column oven (CTO-10ACvp) and a UV-detector (SPD-10Avp) was used. The system was coupled on-line to the ESI/ion-trap MS detector.

The RP-8e chromatographic column (125 mm × 4 mm, 5 μm) with pre-column system (4 mm × 4 mm, 5 μm) Purospher® STAR (Merck, Darmstadt, Germany) were applied. Injection volume was 10 μl and mobile phase consisted of methanol/2% acetic acid aque-

ous solution (63/37%, v/v). Mobile phase flow rate was 0.5 ml min^{-1}, and the temperature of the column oven was set at 30 °C.

2.6. Recovery and reproducibility

Recovery and reproducibility were evaluated with model standard solutions covering a broad concentration range, 8, 80, 800, and 8000 ng ml^{-1} of the T3 and T4 hormones. For recovery evaluation were NRC homogenates ($n = 6$) spiked with standards prior to analysis. Intra- and inter-day HPLC/MS reproducibility was tested using standard solutions diluted in HPLC mobile phase. Series of analyses ($n = 6$) were performed for 6 consecutive working days to determine the inter-day reproducibility. For other details see [17].

3. Results and discussion

The main goal of this work was to develop a method for monitoring of T3 and T4 in NRC cell cultures without using radioactive labeling of the hormones. Because the expected concentrations of hormones present were in the nanomolar range, we aimed to use an ESI/ion-trap MS based approach for selective analysis of both "native" T3 and T4. The method was optimized and applied to study the uptake and degradation of both iodinated hormones in NRC cultures.

3.1. Electrospray/ion-trap MS

In our experiments, T3 and T4 dissolved in HPLC mobile phase (1 μg ml^{-1}) were directly injected into the ESI/ion-trap MS instrument to optimize the spectrometric parameters. Tuning experiments showed that the positive ion ESI mode was more sensitive to T3 and T4 than the negative ion mode, however, the negative ionization mode can also be used in T3 and T4 ESI-MS, e.g. for standards dissolved in aqueous methanol [10]. The highest abundances of molecular ions [M+H]$^+$ of T3 (m/z 651.6) and T4 (m/z 777.5) were found at spray voltage 4.5–4.75 kV and ion transfer capillary voltage/temperature 25–35 V/300 °C. The resolution (R) for molecular ion peaks of the thyroid hormones with the optimized method were $R_{T3} = 4340$ and $R_{T4} = 5183$ (see Section 2.4). General fragmentation behaviour of both thyroid hormones in the ion-trap MS was studied at the above-mentioned conditions using normalized collision energy 25% (for T3) and 20% (for T4).

The following m/z values for MS^n were found for T3: MS 651.6 $m/z \to$ MS^2 605.6 $m/z \to$ MS^3 478.7 $m/z \to$ MS^4 350.9 m/z, and for T4: MS 777.5 $m/z \to$ MS^2 731.5 $m/z \to$ MS^3 604.6 $m/z \to$ MS^4 476.7 m/z; MS spectra are shown in Fig. 1B and C. Using T3 and T4 [M+H]$^+$ MS precursor ions, MS^2 fragment ions [M+H−HCOOH]$^+$ 605.6 m/z and 731.5 m/z were observed (this fragmentation pattern was also shown for monoiodothyronines [18], diiodothyronines [19], and T3 or reverse-T3 [11]). Consequently, abundances of described MS^2 fragment ions were monitored for the compounds quantification in HPLC/MS experiments and analysis of real samples of NRC. In addition, MS/MS transitions were analyzed for better selectivity of T3 and T4 analysis in NRC. Specifically, the ratio between daughter ions obtained for [M+H]$^+$ of T3 (651.6 m/z) and T4 (777.5 m/z) were (605.6/634.5/507.8 m/z; 100/16/11%) and (731.5/760.4/633.7 m/z; 100/10/8%), respectively.

3.2. HPLC/MS interface

RP HPLC of T3 and T4 was carried out with column filled with particular C$_8$ stationary phase and isocratic elution based on mixture of methanol and 2% acetic acid aqueous solution (63/37%, v/v). Chromatograms acquired in TIC (total-ion current) and SIM (single-ion monitoring) modes of the compounds are shown in Fig. 2A. T4
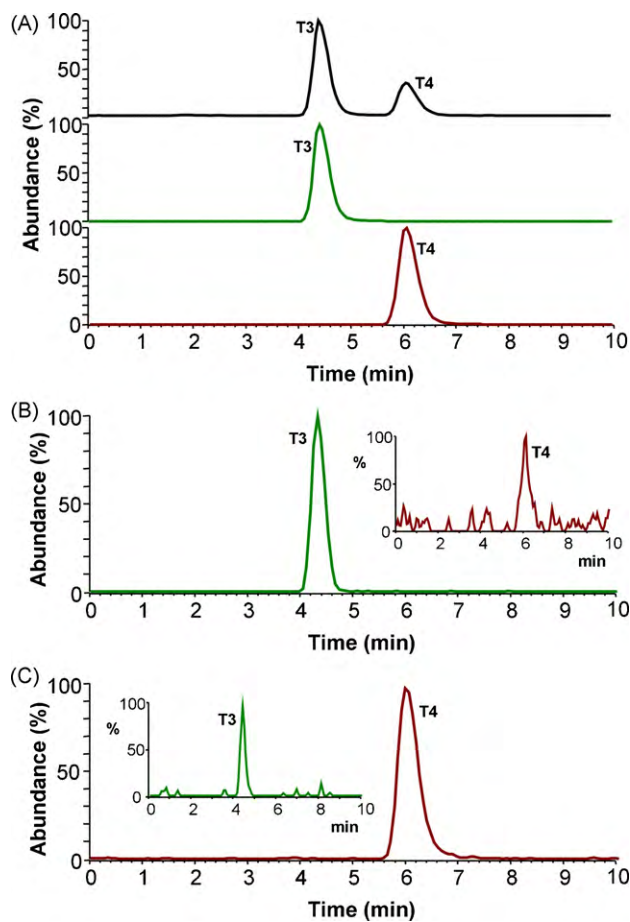

Fig. 2. Chromatographic HPLC/MS records of T3 and T4 in various samples. Panel A: model standard solutions containing 10 ng ml^{-1} of the hormone mixture (top panel) or individual hormones indicated (middle and bottom panels). Panel B: NRC culture was incubated with 0.8 μg ml^{-1} T3 for 6 h at 37 °C and the presence of T3 and T4 (inset) was detected as described in Section 2. Panel C: NRC culture was incubated with 8 μg ml^{-1} T4 for 6 h at 37 °C and the presence of T4 and T3 (inset) was detected as described in Section 2.

(t_R 6.1 min) is retained on the surface of C$_8$ stationary phase longer than T3 (t_R 4.4 min). Similar retention behaviour was observed with different RP stationary phases and several elution solvent systems [5,10,20].

The proposed HPLC/MS method provides reproducible analysis of T3 and T4 in nanomolar and micromolar concentrations. The calibrations of T3 and T4 were linear in the concentration range from 0.01 to 10 μg ml^{-1} with correlation coefficients (R^2) 0.999 for both compounds (calibration equations: T3, $y = 5009x + 138.4$; T4, $y = 2096.9x + 39.2$). T3 and T4 limits of detection (LODs, $3 \times$ S/N) were 3 ng ml^{-1} (4.6 nM) and 6 ng ml^{-1} (7.7 nM), respectively. The RP systems (usually C$_{18}$ but also more polar stationary phases [21]) were previously used by other authors with several MS analyzers and ionization sources. Not only ESI but also thermospray ionization interface have been described [22].

Reproducibility of HPLC/MS method was tested after calibration procedures in the concentration range from 8 to 8000 ng ml^{-1} of each hormone. Intra-day reproducibility of spiked T3/T4 was: 93.0/95.5% for 8 ng ml^{-1}, 103.7/101.7% for 80 ng ml^{-1}, 102.4/100.3% for 800 ng ml^{-1}, and 98.2/107.5% for 8000 ng ml^{-1}. Excellent T3/T4 reproducibility was also found in the case of inter-day measurements: 107.5/103.5% for 8 ng ml^{-1}, 96.6/92.1% for 80 ng ml^{-1}, 94.0/91.2% for 800 ng ml^{-1}, and 94.0/97.9% for 8000 ng ml^{-1}.

It is evident from current literature sources on MS of iodinated hormones that potentially interfering compounds for T3 and T4

could be their analogues or derivatives with the same or similar $[M+H]^+$ and fragmentation pattern, primarily reverse-T3, rT3 [23]. Under our experimental conditions, retention time 5.0 min and MS/MS transitions (651.6 → 634.6/507.9/605.8 m/z) with ratio (100/53/43%) for rT3 were found. These parameters were dissimilar to the parameters found for T3 or T4. We also consulted the Mass Frontier 5.01.20 software database (HighChem, Ltd.; ca. 10^5 compounds are included) for possible interfering agents of molecular weights (± 2 m/z) close to T3 or T4. The compounds naturally occurring in NRC, with the same m/z for molecular ions or their fragments as thyroid hormones were not found.

3.3. T3 and T4 in NRC

Considering the stability of both hormones, we initially applied the proposed HPLC/MS method for evaluation of T3 (0.8 μg ml^{-1}) and T4 (8 μg ml^{-1}) stability in cultivation medium during 24 h at 37 °C in the absence of NRC. The hormones were monitored in the following time intervals after their addition into cultivation medium: 0.5, 1, 2, 4, 6, 12, 24 h. The 10-fold lower concentration of T3 than T4 was applied to better reflect physiological conditions under which T3 level is several fold lower than T4 level depending on tissue or cell types [2] with roughly 20 to 1 ratio of T4 to T3 in the blood [24]. In our experiments, no changes of T4 concentration in the cultivation medium were observed during the 24 h test period. Similarly, no changes in T3 concentration in the cultivation medium were found during the first 12 h of incubation. However, a 15% decrease in T3 concentration was found in the incubation interval from 12 to 24 h suggesting somewhat longer half-life in the cultivation medium when comparing with the published data [2].

The same concentrations of T3 and T4 as noted above were added into the NRC cell cultures. Following 6 h of incubation, a cultivation interval selected as an optimum for good viability and stability of the NRC (see below), cells were washed, dissolved in HPLC mobile phase, disrupted by ultrasound, and centrifuged. The resulting supernatants contained 30.5 ng ml^{-1} of T3 and 120 ng ml^{-1} of T4 which correspond with 6.1 ng T3 and 24 ng T4 per 2.5×10^5 NRC cells ($n = 3$). The chromatograms of T3 and T4 are shown in Fig. 2B and C. In addition, residual amount of T3 was determined in NRC culture treated with the T4 and vice versa. The presence of detectable amounts of T4 even in the control culture, i.e. without any addition of hormones, could be explained as a carryover of original thyroid hormones contained in the original rat hearts. This assumption is supported by the fact that the biological half-life of T3 and T4 is 1–2 and 6–7 days, respectively [2].

Finally, the uptake of hormones into NRC cells was monitored using HPLC/MS after 6 h-long incubation, concentration range of the hormones was from 8 ng to 8 μg ml^{-1} (Fig. 3). NRC viability remained unaffected during T3/T4 treatment as verified by MTT and LDH assays (see inset in Fig. 3). The rate of uptake was lower for T3 than for T4. These results are in agreement with data presented by other authors; for example a study of T3 and T4 uptake in myoblasts and myotubes of the embryonic heart cell line H9c2(2-1) [25]. Recoveries for T3 and T4 in NRC (see Section 2.6) varied from 64.1 to 68.4% and 74.0 to 95.3%, respectively, if concentrations identical to those in uptake experiments were employed. Lower T3 recoveries, as opposed to T4, could be connected to its higher hydrophilicity and ability to react with cytosolic components in disturbed cells of homogenized samples.

4. Conclusions

We describe a procedure using ESI/ion-trap MS connected to RP HPLC for monitoring of nanomolar concentration of studied thyroid hormones (LODs 30 and 60 pg per injection for T3 and T4, respectively). Simple isocratic elution in relatively short time and sensitive MS^2 technique achievable on every HPLC system and various ordinary used MS equipments is shown. In the case of more complex biological matrices than cell cultures, highly specific MS^n determination of T3 and T4 can be employed. We applied the optimized method for analysis of T3 and T4 in NRC and expect it may also be used in other mammalian cell cultures. Our results confirm the time dependent instability of T3 in cultivation medium allowing accountability for possible T3 insensitivity phenomena in future experiments. Since the biological effects of thyroid hormones and associated pathologies continue to gain the attention of physicians and researchers alike, we anticipate our proposed HPLC/MS method may further aid the elucidation of T3 and T4 important regulation functions [13–15].

Fig. 3. T3/T4 uptake into NRC. Uptake was evaluated after incubating NRC with the indicated hormone concentration and following with determination of the hormone presence in cell homogenates. Results of MTT assay and LDH activity determination for NRC after their incubation in the presence of T3 (0.8 μg ml^{-1}) or T4 (8 μg ml^{-1}) in cultivation medium are shown in inset. Time of incubation was 6 h (37 °C) for both experiments ($n = 3$). For further details see Section 2.3.

References

[1] G.J. Kahaly, W.H. Dillmann, Thyroid hormone action in the heart, Endocr. Rev. 26 (2005) 704–728.
[2] P.G. Walfish, Triiodothyronine and thyroxine interrelationships in health and disease, Can. Med. Assoc. J. 115 (1976) 338–342.
[3] Z. Baloch, Laboratory medicine practice guidelines—laboratory support for the diagnosis and monitoring of thyroid disease, Thyroid 13 (2003) 3–126.
[4] S.S. Holm, S.H. Hansen, J. Faber, P. Staun-Olsen, Reference methods for the measurement of free thyroid hormones in blood evaluation of potential reference methods for free thyroxine, Clin. Biochem. 37 (2004) 85–93.
[5] B.F. Yue, A.L. Rockwood, T. Sandrock, S.L. La'ulu, M.M. Kushnir, A.W. Meikle, Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction-liquid chromatography/tandem mass spectrometry, Clin. Chem. 54 (2008) 642–651.
[6] B. Moller, I. Bjorkhem, O. Falk, O. Lantto, A. Larsson, Identification of thyroxine in human-breast milk by gas chromatography–mass spectrometry, J. Clin. Endocrinol. Metab. 56 (1983) 30–34.
[7] V.I. De Brabandere, P. Hou, D. Stockl, L.M. Thienpont, A.P. De Leenheer, Isotope dilution-liquid chromatography electrospray ionization-tandem mass spectrometry for the determination of serum thyroxine as a potential reference method, Rapid Commun. Mass Spectrom. 12 (1998) 1099–1103.
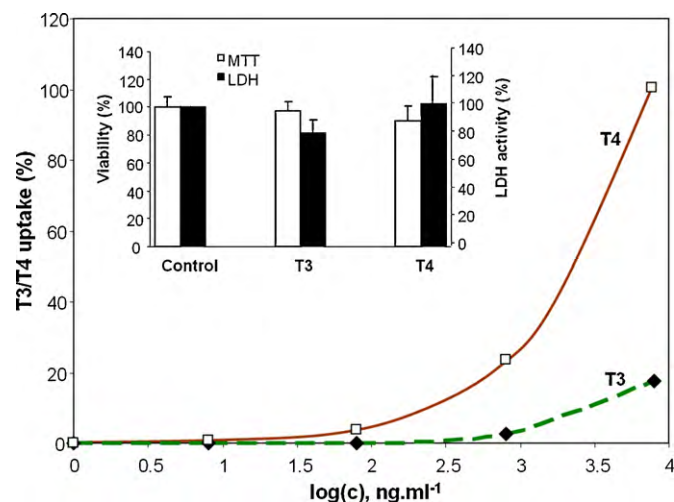
[8] L.M. Thienpont, C. Fierens, A.P. De Leenheer, L. Przywara, Isotope dilution-gas chromatography/mass spectrometry and liquid chromatography/electrospray ionization-tandem mass spectrometry for the determination of triiodo-L-thyronine in serum, Rapid Commun. Mass Spectrom. 13 (1999) 1924–1931.

[9] S.S.C. Tai, D.M. Bunk, E.V. White, M.J. Welch, Development and evaluation of a reference measurement procedure for the determination of total 3,3′,5-triiodothyronine in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry, Anal. Chem. 76 (2004) 5092–5096.

[10] J. Gu, O.P. Soldin, S.J. Soldin, Simultaneous quantification of free triiodothyronine and free thyroxine by isotope dilution tandem mass spectrometry, Clin. Biochem. 40 (2007) 1386–1391.

[11] Y.T. Zhang, A.H. Conrad, G.W. Conrad, Detection and quantification of 3,5,3′-triiodothyronine and 3,3,5′-triiodothyronine by electrospray ionization tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 16 (2005) 1781–1786.

[12] S. Chlopcikova, J. Psotova, P. Miketova, Neonatal rat cardiomyocytes—a model for the study of morphological, biochemical and electrophysiological characteristics of the heart, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. 145 (2001) 49–55.

[13] M.E. Everts, F.A. Verhoeven, K. Bezstarosti, E. Moerings, C. Hennemann, T.J. Visser, J.M.J. Lamers, Uptake of thyroid hormones in neonatal rat cardiac myocytes, Endocrinology 137 (1996) 4235–4242.

[14] H.H.A.G.M. van der Putten, B.J.L.J. Joosten, P.H.M. Klaren, M.E. Everts, Characterization of uptake and compartmentalization of 3,5,3′-tri-iodothyronine in cultured neonatal rat cardiomyocytes, J. Endocrinol. 171 (2001) 183–192.

[15] F.A. Verhoeven, H.H.A.G.M. van der Putten, G. Hennemann, J.M.J. Lamers, T.J. Visser, M.E. Everts, Uptake of triiodothyronine and triiodothyroacetic acid in neonatal rat cardiomyocytes: effects of metabolites and analogs, J. Endocrinol. 173 (2002) 247–255.

[16] M. Maines, Current Protocols in Toxicology, John Wiley & Sons, Inc., USA, 1998.

[17] D.T. Burns, K. Danzer, A. Townshend, Use of the terms "recovery" and "apparent recovery" in analytical procedures—(IUPAC recommendations 2002), Pure Appl. Chem. 74 (2002) 2201–2205.

[18] Y.T. Zhang, A.H. Conrad, R. Thoma, G.W. Conrad, Differentiation of monoiodothyronines using electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 481–486.

[19] Y.T. Zhang, A.H. Conrad, R. Thoma, G.W. Conrad, Differentiation of diiodothyronines using electrospray ionization tandem mass spectrometry, J. Mass Spectrom. 41 (2006) 162–168.

[20] N. Soukhova, O.P. Soldin, S.J. Soldin, Isotope dilution tandem mass spectrometric method for T4/T3, Clin. Chim. Acta 343 (2004) 185–190.

[21] I. Gostomski, R. Braun, C.G. Huber, Detection of low-abundance impurities in synthetic thyroid hormones by stationary phase optimized liquid chromatography-mass spectrometry, Anal. Bioanal. Chem. 391 (2008) 279–288.

[22] M. Andre, R. Domanig, E. Riemer, H. Moser, A. Groeppelin, Identification of the thermal degradation products of G-triiodothyronine sodium (liothyronine sodium) by reversed-phase high-performance liquid chromatography with photodiode-array UV and mass spectrometric detection, J. Chromatogr. A 725 (1996) 287–294.

[23] S. Piehl, T. Heberer, G. Balizs, T.S. Scanlan, J. Kohrle, Development of a validated liquid chromatography/tandem mass spectrometry method for the distinction of thyronine and thyronamine constitutional isomers and for the identification of new deiodinase substrates, Rapid Commun. Mass Spectrom. 22 (2008) 3286–3296.

[24] A. Bielecka-Dabrowa, D.P. Mikhailidis, J. Rysz, M. Banach, The mechanisms of atrial fibrillation in hyperthyroidism, Thyroid Res. 2: 4 (2009).

[25] H.H.A.G.M. van der Putten, B.J.L.J. Joosten, P.H.M. Klaren, M.E. Everts, Uptake of tri-iodothyronine and thyroxine in myoblasts and myotubes of the embryonic heart cell line H9c2(2-1), J. Endocrinol. 175 (2002) 587–596.