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Simultaneous determination of endogenous and ^{13}C-labelled thyroid hormones in plasma by stable isotope dilution mass spectrometry

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Abstract

This study describes a capillary gas chromatography–mass spectrometry (GC–MS) method for the simultaneous determination of endogenous thyroid hormone (thyroxine, T_4) and its ^{13}C-labelled analogue ($^{13}C_6$-thyroxine) in plasma. $^{13}C_9$-thyroxine was used as analytical internal standard. A double derivatization (CH_3OH/HCl and HFBA) inducing good GC mobility was used for the GC–MS analysis of the thyroid hormones. Quantification was carried out by selected ion monitoring (SIM) of specific ions of the fragment ions (*m*/*z* 970/976/979). The detection limit of the present GC–MS–SIM method was found to be 100 pg per injection for thyroxine (S/N = 3.0). A first implementation in in vivo tests of $^{13}C_6$-T_4 like metabolic tracer was carried out under veterinary control on one cat and one rabbit. The thyroxine follow-up was done by GC–MS and based on double isotopic dilution with two different regio-selective ^{13}C-labelled molecules of the same hormone. The present paper discusses the possibilities and limitations of this methodology. The in vivo experiment demonstrated that the use of stable isotopes and mass spectrometry provide a reliable methodology for hormonal monitoring.
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Keyword: Thyroid hormones

1. Introduction

Data on human thyroid hormone metabolism were gathered until the middle of the 1970s mainly by the use of radioactive iodinated (^{125}I or ^{131}I) hormones. Due to the potential health hazards, the use of radiolabelled products in human in vivo research has become less acceptable, therefore many studies have considered tissue or cell culture. Nevertheless, the answers to many metabolic questions require an in vivo compartment analysis with tracers, to improve the understanding of thyroid diseases. For example, the common state named "low T_3 syndromes", characterised by low T_3 and, possibly, low T_4 serum, without clinical hypothyroidism, is still poorly understood as a consequence of lack of available methodology.

Mass spectrometry of plasma samples allows safe in vivo tracing and precise metabolic investigations with stable isotopes, and such technology has contributed to a better understanding of some metabolic diseases such as diabetes [1,2].

We have synthesised a thyroxine hormone with two different possibilities of regio-selective labelling with carbon 13. The biological tracer was thyroxine substituted on the inner ring with six stable isotopes ($^{13}C_6$-thyroxine or T_4^*) and the internal standard was thyroxine substituted on the inner ring and lateral chain with nine stable isotopes and ($^{13}C_9$-thyroxine or T_4IS) as shown in Fig. 1. The first stable isotope was the form used as the pharmacological agent. The plasma samples containing endogenous and exogenous (labelled) thyroxine were analysed by the double isotope dilution method.

Our aim is to develop simultaneous quantification methods of endogenous and exogenous (stable isotopically labelled) thyroxine in animal plasma by GC–MS, using $^{13}C_9$-thyroxine as the analytical internal standard. The present study shows the first results obtained using the stable isotope tracer after injection into two animals (one cat

Abbreviations: DIT, 3,5-diiodotyrosine; T_4, 3,3′,5,5′-tetraiodothyronine, endogenous thyroxine; T_4^*, exogenous thyroxine (biological tracer), $^{13}C_6$ -thyroxine; T_4IS, internal standard, $^{13}C_9$-thyroxine; T_3, 3,5,3′-triiodothyronine; *m*/*z*, fragment mass/ion charge; HFBA, heptafluorobutyric anhydride; HFB, heptafluorobutyryl; SIM, selected-ion monitoring

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and one rabbit) and discusses the possibilities and limits of this methodology.

2. Experimental

2.1. Chemicals and solvents

The labelled precursors ($^{13}C_6$-L-tyrosine and $^{13}C_9$-L-tyrosine) were obtained from ISOTEC, Miamisburg, USA: isotopic enrichment 99 at.% ^{13}C minimum, optical purity 99.5%.

All solvents used were of highest analytical grade or HPLC grade. The esterification reagent (methanolic HCl 3N) and acylation reagent (heptafluorobutyric anhydride or HFBA) were obtained from Supelco SA.

2.2. Stable isotopically labelled thyroxine

T_4^* and T_4IS (Fig. 1) were synthesised in our laboratory through an optimised synthesis scheme [3] based on the work of Bevilacqua et al. [4] from commercially available labelled L-tyrosine (Fig. 2). After iodination and protection of the amino and carboxylic acid groups of the $^{13}C_x$-labelled L-tyrosine, the diaryl ether bond was obtained by condensation with a diaryl iodonium salt in presence of copper (as catalyst), triethylamine and methanol. The protection were removed with a HI/glacial acetic acid mixture and the subsequent product iodinated on the 3′,5′ positions of the outer ring with a I_2/KI 3N aqueous solution.

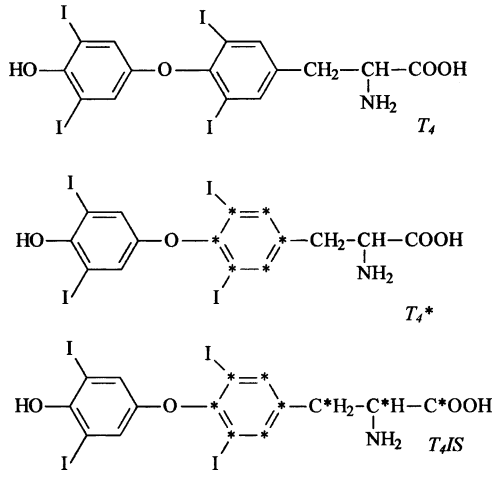

Fig. 1. T_4, T_4^* and T_4IS structure (asterisk denotes ^{13}C).

The isotopic compositions of the labelled synthesised compounds were >99 at.% for $^{13}C_6$- and $^{13}C_9$-thyroxine. The optical purity of the synthetic labelled thyroid hormones was controlled through diastereoisomer formation with L-leucine and C18 HPLC analysis: 98 ± 1% $^{13}C_x$-L-thyroxine [5]. Before use for animal tests, T_4^* was purified by C18 HPLC.

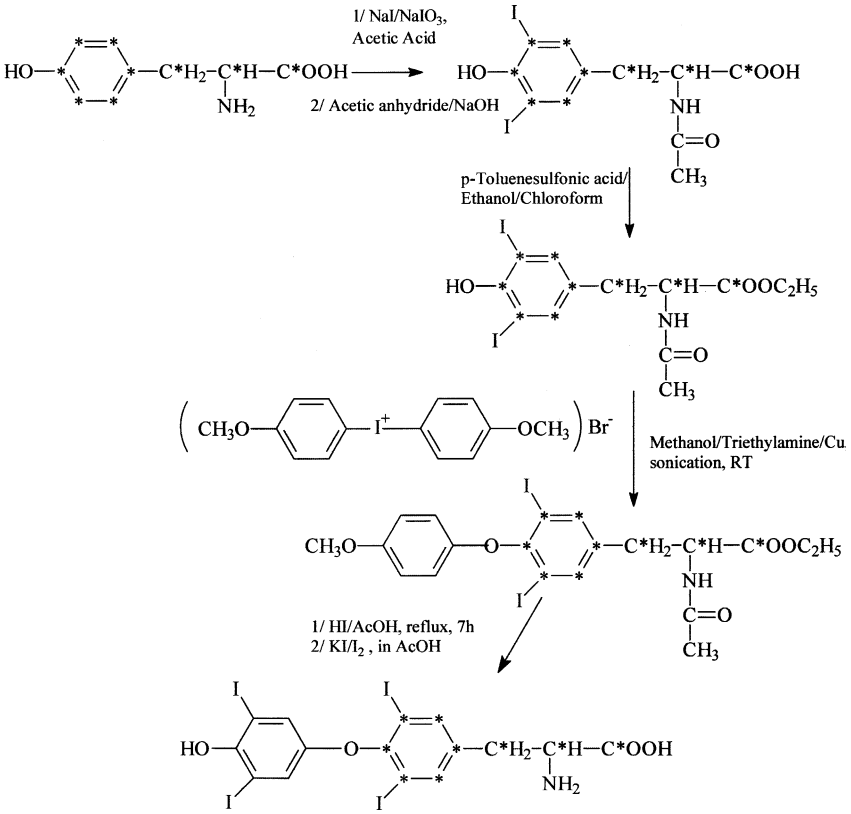

Fig. 2. Labelled thyroxine synthesis scheme (RT: room temperature, asterisk denotes ^{13}C).

2.3. Preparation of standard solutions

Stock solutions of T_4^* and T_4IS were prepared in the following way: 25 mg of the T_4 product were dissolved in 100 ml of 10% aqueous ammonia p.a. Dilutions of the stock solutions were carried out with pure water, to 5 parts per million (w/w) (ppm). These solutions were used for the calibration curves (see Section 2.6).

2.4. Sample preparation

2.4.1. Extraction

The protocol was based on an analytical procedure developed by Thienpont et al. [6–8]: to 1 ml of the harvest animal serum, a precise volume of the internal standard solution was added to obtain an isotope ratio close to 1:1 (T_4IS:T_4). Sixty milligrams of NaCl were dissolved under vortexing in the serum and left to equilibrate for 30 min. Two millilitres of acetone/30% HCl solution (10/0.05, v/v) were added, mixed for 5 min, and centrifuged for 20 min at 1500 × *g*, at 4 °C. After transfer of the supernatant, the pH was adjusted to pH 1 with a 30% HCl solution and was washed twice by shaking for 5 min with 0.9 ml cyclohexane. After centrifugation (1500 × *g*, 2 min, at room temperature) the cyclohexane phase was discarded. The extraction of thyroxine (T_4, T_4^* and T_4IS) was performed with 1.5 ml of ethyl acetate with shaking for 10 min. After centrifugation for 2 min (1500 × *g*, ambient temperature), the ethyl acetate phase was transferred and evaporated under dry nitrogen. The extraction was repeated with 1 ml of ethyl acetate, which was added to the first ethyl acetate extract residue, and also evaporated to dryness.

2.4.2. Derivatization

2.4.2.1. Methylation with methanolic HCl. The extraction residues or evaporated standard mixtures were redissolved in 150 μl methanolic HCl 3N. The vials were tightly closed. Derivatization was allowed to proceed for 30 min at 80 °C. After reaching room temperature, the mixture was evaporated under a stream of dry nitrogen. To suppress water traces, 100 μl cyclohexane were added and was evaporated under a stream of dry nitrogen to dryness.

2.4.2.2. Perfluoroacylation. After evaporation of the cyclohexane, 50 μl acetonitrile were added and then 50 μl HFBA. Derivatization was performed for 60 min at 60 °C in a sealed vial. The samples were then evaporated under dry nitrogen at ambient temperature, followed by the addition of 100 μl cyclohexane and evaporation under dry nitrogen to dryness. The residues were redissolved with 50 μl of anhydrous ethyl acetate. A 2.0 μl aliquot was injected for GC–MS analysis.

2.5. Gas chromatography–mass spectrometry with selected ion monitoring

The GC/MS configuration was a mass spectrometer VG Platform II Benchtop GC/MS-DS system coupled with a gas chromatograph Fisons 8000 GC (Carlo Erba) equipped with a "large volume injection" system and a FID detector. A DB-1 capillary column (J&W Scientific, 15 m × 0.25 mm (i.d.), with 0.25 μm film thickness, temperature limits: −60 to 325 °C) was used in the chromatographic step. The carrier gas was Helium, with a flow of 1.5 ml/min. The capillary column was directly coupled to the mass spectrometer.

The chromatographic program was as follows: the column was kept at 200 °C for 1 min, then carried to 280 °C with a rate of 25 °C/min. The final temperature was held for 15 min. The injector and transfer line temperatures were 280 °C. The purge valves of the injector were opened after 2 min. The top valve flow was 2 ml/min and the bottom valve flow was 10 ml/min. Mass spectrometry was performed under electron impact ionisation (70 eV). For quantitative selected ion monitoring (SIM), the mass spectrometer was set to monitor the (*m*/*z*) values 970/979 and 976/979 (after derivatization with methanolic HCl and HFBA). The voltage of the electron multiplier was set to obtain a maximum signal-to-noise ratio.

2.6. Calibration graphs

To each of the eight standards containing known amounts of thyroxine (4.06, 10.11, 15.19, 20.32, 25.4, 40.64, 80.7, 121.9 ng), thyroxine-$^{13}C_6$ (4.03, 10.08, 15.26, 20.16, 25.6, 40.33, 81.2, 122.08 ng) dissolved in pure water, 20.25 ng of thyroxine-$^{13}C_9$ were added. After evaporation of the solvent to dryness, the samples were derivatized as described in Section 2.4.2. A 2.0 μl portion of the ethyl acetate solution (50 μl) was subjected to GC–MS. The peak-area ratios were determined in triplicate. The calibration graphs were obtained by an unweighted least-square linear fitting of the peak-area ratios versus the mass ratio of thyroxine and $^{13}C_6$- to $^{13}C_9$-thyroxine on each analysis of the standard mixtures.

The chromatograms obtained for one sample are presented in Fig. 3. Individual quantification is possible with the specific *m*/*z* value of each molecule and its constant fragmentation (in *m*/*z* value and in yield). For each preceding chromatogram, the extraction of each *m*/*z* value is given.

2.7. Accuracy and reproducibility

The accuracy of the measurements were determined for endogenous and labelled thyroxine by assaying six preparations of 2.0 ml portions of human plasma spiked with 30.0 ng of thyroxine and 50.0 ng of $^{13}C_6$-thyroxine (40.5 ng of $^{13}C_9$-thyroxine as internal standard). After preparation of the sample for GC–MS–SIM as described above, the peak-area ratios (T_4/$^{13}C_9$-T_4) and ($^{13}C_6$-T_4/$^{13}C_9$-T_4) were measured.

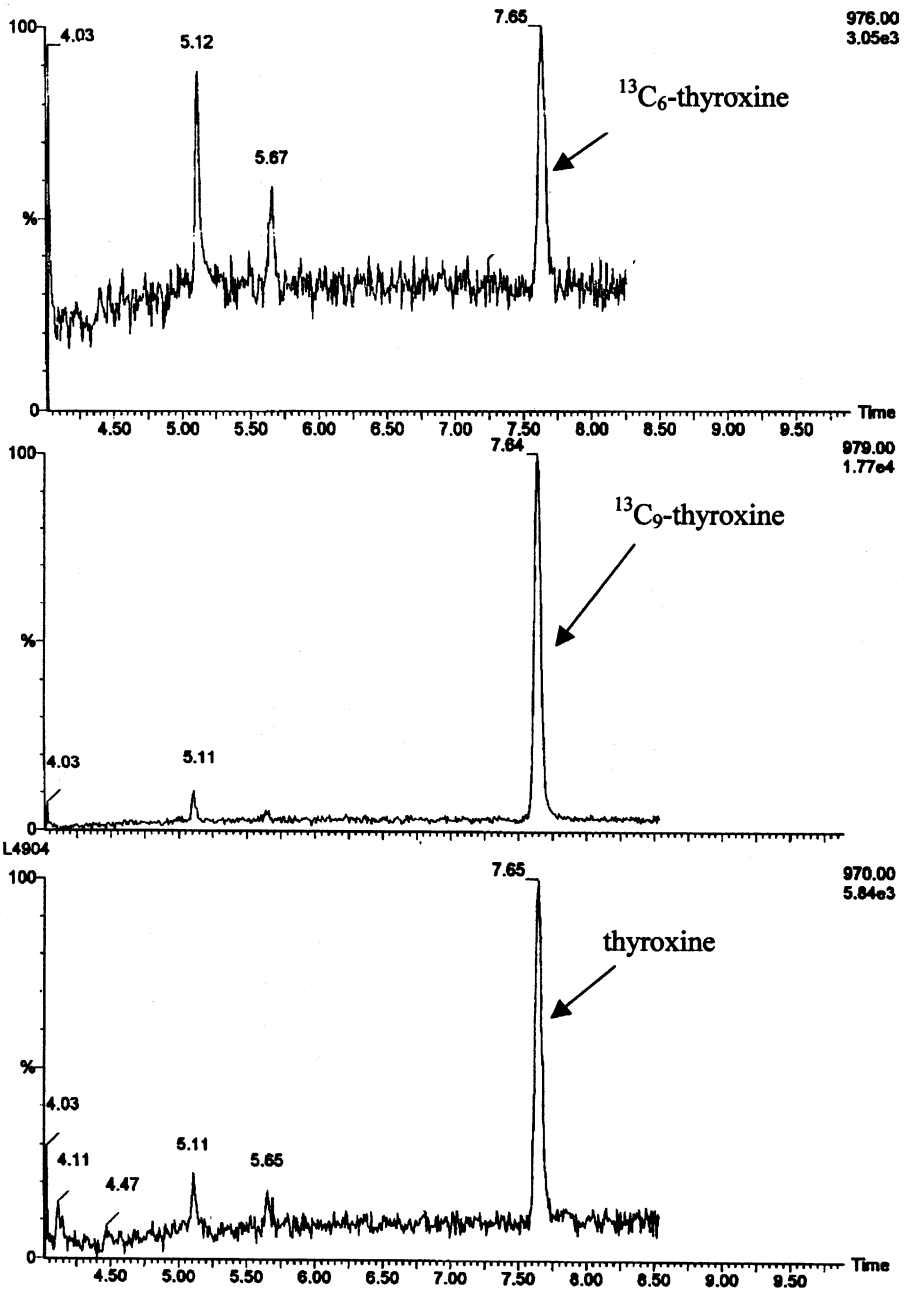

Fig. 3. Selected-ion recordings of N,O-HFB, methyl ester derivatives of $^{13}C_6$-thyroxine/$^{13}C_9$-thyroxine/thyroxine (*m/z* 976, 979, 970) after processing from 2.0 ml serum spiked with $^{13}C_6$-thyroxine (4.03 ng) and $^{13}C_9$-thyroxine (20.25 ng) as internal standard (t_r: 7.65 min).

2.8. In vivo studies: animal experiments

Two animals (one New Zealand white rabbit and one female domestic short haired cat) were administered T_4^* 100 μg once at time 0 immediately following blood collection. Blood collection (6 ml in plain tubes) was repeated at various intervals (time 0, 4 and 8 h. and 1, 2, 4, 8 and 16 days). Serum was harvested within 1 h., and stored at −40 °C until extraction and analysis.

3. Results and discussion

Stable isotope methodology has widely been accepted for investigating the pharmacokinetics and metabolism of steroids in humans. One of the major advantages of the methodology coupled with mass spectrometry is that endogenous (T_4 *m/z*: 970) and exogenous (T_4^* *m/z*: 976) compounds with the same basic structure can be easily differentiated by using a specific isotopic labelled compound (T_4IS *m/z*: 979) as analytical internal standard.

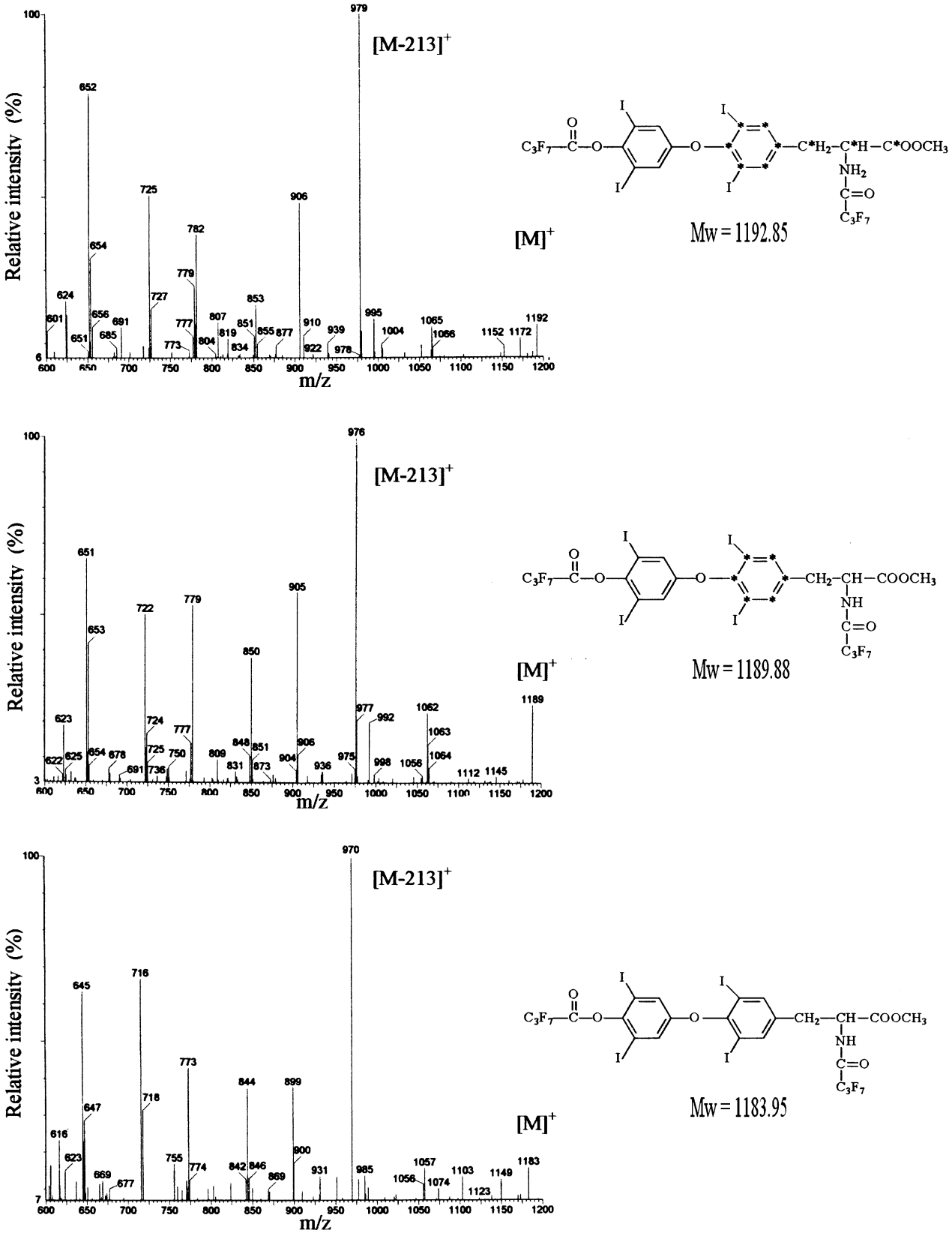

Fig. 4. Mass spectra of non-labelled and labelled T_4.

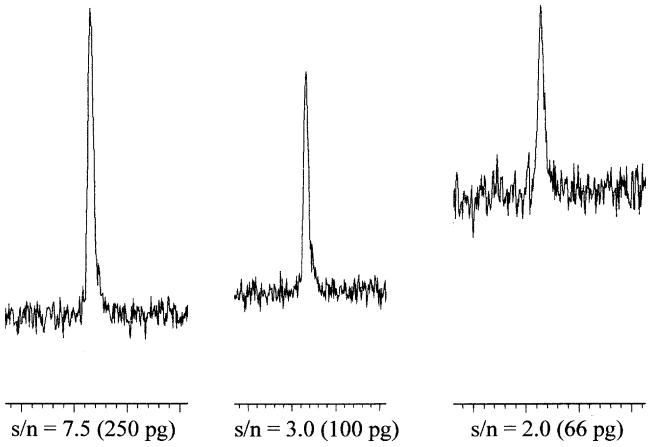

Fig. 5. Sensitivity limit for thyroxine.

We have synthesised ^{13}C-labelled compounds for this research due to the major advantages to metabolic or pharmacokinetic studies in vivo because of the stability of the label.

3.1. Sensitivity

The characteristic ions in the mass spectra (Fig. 4), the $[M\text{-}213]^+$ ions for T_4, $^{13}C_6$-T_4 and $^{13}C_9$-T_4 (*m/z* 970, 976, 979) were chosen for selected ion monitoring of the N,O-HFB, methyl ester derivatives. When a signal-to-noise ratio of 3.0 was used as a criterion for a significant response, the detection limit of the present GC–MS–SIM method was found to be 100 pg per injection of T_4 (S/N = 3.0) (Fig. 5).

3.2. Selected ion monitoring

The derivatization was applied to the simultaneous determination of T_4 and $^{13}C_6$-T_4 in serum, using $^{13}C_9$-T_4 as internal standard. Fig. 3 shows the selected ion monitoring of the O,N-HFB, methyl ester derivatives of labelled and unlabelled thyroid hormones after processing from pooled serum. The recordings showed no significant interfering peaks from other endogenous compounds present in serum. The recovery ratio of thyroxine from serum using solvent extraction procedure were 77.3–93.3% ($n = 9$).

3.3. Calibration graphs

Calibration graphs were prepared in the range 4.1–121.9 ng of T_4 and 4.0–121.1 ng $^{13}C_6$-T_4, with $^{13}C_9$-T_4 (20.2 ng) as internal standard for the GC–MS assay. The mixture was analysed as the N,O-HFB, methyl ester derivatives of T_4, $^{13}C_6$-T_4 and $^{13}C_9$-T_4 by monitoring the $[M\text{-}213]^+$ ion intensities at *m/z* 970 (T_4), 976 ($^{13}C_6$-T_4) and 979 ($^{13}C_9$-T_4). The peak area ratios were plotted against the mixed molar ratios of unlabelled or $^{13}C_6$-labelled thyroxine and the corresponding $^{13}C_9$-labelled compounds. A good correlation was found between the observed peak-area ratio (y) and the molar ratios (x). Unweighted least-square regression analysis gave typical regression lines $y = 1.4288x - 0.038$ ($r^2 = 0.9994$) for T_4 and $y = 1.0458 - 0.0581$ ($r^2 = 0.9988$) for $^{13}C_6$-T_4.

3.4. Accuracy and reproducibility

The accuracy of the measurements were determined for thyroxine. To the plasma samples containing endogenous thyroxine were added specific known amounts of thyroxine and $^{13}C_6$-thyroxine. Table 1 shows the within-day accuracy and reproducibility in which the amounts of thyroxine and $^{13}C_6$-thyroxine determined were in good agreement with the actual amounts added. Table 2 shows the inter-day accuracy and reproducibility on the plasma samples containing only endogenous thyroxine (62.7 ± 5.8 ng/ml). The inter-assay R.S.D.s were 9.2% for the unlabelled thyroxine.

3.5. In vivo study

3.5.1. Cat study

The general profile was that of an attenuated linear propagation wave of serum T_4 concentration after the administration of 100 μg T_4^*. The first wave (Fig. 6) had a maximum positive amplitude at 8 h, with a serum T_4 concentration value double the initial value, and a maximum negative wave at 48 h, with a serum T_4 concentration half the initial value. A second attenuated wave with maximal positive amplitude at 4 days and maximal negative amplitude at 8 days is, also, recognisable from the figure. At 16 days, the value of serum T_4 approximated pre-treatment values. Serum T_4^*

Table 1
Accuracy of GC–MS–SIM determination of endogenous T_4 and $^{13}C_6$-thyroxine in human plasma using $^{13}C_9$-thyroxine as internal standard

Added (ng/ml)	Expected (ng/ml)	Found (μg/ml) Individual values[a]			Mean ± S.D. (ng/ml)	Relative error (%)	CV[b] (%)
Thyroxine							
–		53.0	51.8	55.2	53.3 ± 2.5	–	4.3
30.0	83.3	81.1	78.4	77.5	79.0 ± 1.9	−5.1	2.4
$^{13}C_6$-thyroxine							
50.0		50.1	49.1	49.8	49.7 ± 0.6	−0.6	1.0

[a] Each individual value is the mean of triplicate measurements.
[b] Coefficient of variation.

Table 2
Inter-day accuracy and reproducibility of GC–MS–SIM determination of endogenous T_4 in human plasma using $^{13}C_9$-thyroxine as internal standard (three extractions per day, 3 consecutive days)

Day	Extraction number	Mean[a] (ng/ml)	S.D. (ng/ml)	Relative error (%)	CV[b] (%)
1	1	72.0	4.0	+14.8	5.5
	2	62.5	1.4	−0.3	2.2
	3	69.5	1.9	+10.8	2.7
Results for day 1		68.0	4.9	+8.4	7.2
2	1	66.0	5.0	+5.2	7.7
	2	70.8	3.9	+12.9	5.5
	3	63.5	2.6	+1.2	4.2
Results for day 2		66.8	3.7	+6.5	5.5
3	1	59.5	4.1	−5.1	6.9
	2	60.8	2.5	+3.0	4.1
	3	55.5	1.6	−11.5	2.9
Results for day 3		58.6	2.7	−6.5	4.7
Inter-day results		62.7	5.8	–	9.2

[a] Each individual value is the mean of five time measurements.
[b] Coefficient of variation.

reached a peak at 2 h, which was 10 times greater than the initial serum T_4 concentration. It decreased subsequently with a bi-exponential slope, and was no longer detectable after a period of 8 days.

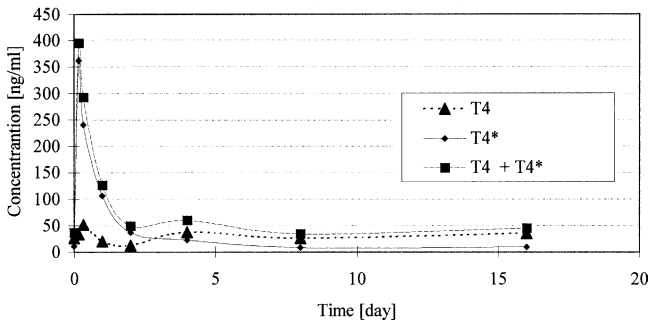

Fig. 6. Endogenous and exogenous thyroxine follow-up on one female domestic short haired cat after the administration of 100 μg T_4^* by GC–MS–SIM using $^{13}C_9$-thyroxine as internal standard.

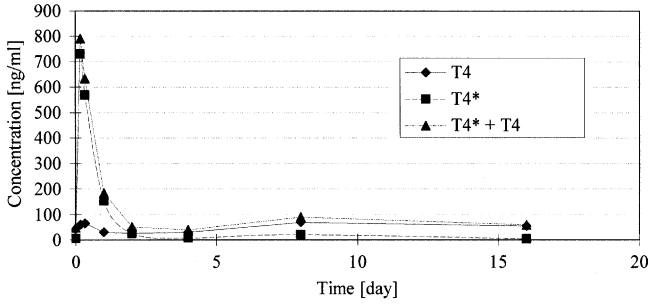

Fig. 7. Endogenous and exogenous thyroxine follow-up on one New Zealand white rabbit after the administration of 100 μg T_4^* by GC–MS–SIM using $^{13}C_9$-thyroxine as internal standard.

3.5.2. Rabbit study

The results general profile (Fig. 7) was that of an attenuated propagation wave as in the case of the cat, with a longer period: after 16 days, the curve is still at half of the second period. The T_4^* decreased subsequently with a bi-exponential slope.

4. Discussion

The data presented here show that the use of stable isotopes provides a valuable tool to monitor thyroid hormone metabolism. Within a few hours after administering T_4^*, both animals became hyperthyroxinemic, with serum total thyroxine (i.e. $T_4 + T_4^*$) concentration reaching values at least ten times greater than the initial serum T_4 value (before administration of the stable isotope). In these two animals, there was an abrupt decrease of endogenous serum T_4, as reflected by the decrease of its serum concentration. While it is not possible to determine whether this is due to a decrease of thyroid synthesis or an increase of thyroid hormone catabolism, hyperthyroid states are known to induce a marked increase of deiodinase activity in peripheral tissues, mainly in the liver.

Excluding possible effects related to the individual tested animals metabolic state, we propose, then, the following scheme compatible with damped oscillations of endogenous T_4:

(1) the first peak is likely to be associated with a displacement of endogenous T_4 from the extra cellular compartment to the blood compartment, this displacement being involved by the large amount of exogenous T_4^* administered as stable isotope;
(2) after the exogenous T_4^* administration, an increase in deiodinase activity, due to hyperthyroxinemia, involves, first, an increase of deiodinase 1 activity, and a drop of endogenous serum T_4 when the animal is hyperthyroid [9,10];
(3) in a second step, intratissular mechanisms exert a feedback mechanism on deiodinase activity, the activity of which decreases, resulting in increasing concentrations of endogenous serum T_4.

We have no hypothesis, at the moment, for this second-step feed-back mechanism. Points 2 and 3 repeated, until normal physiological serum T_4 are attained. Damped oscillations of serum thyroid hormone levels have been previously described in neonates [11,12].

During the following days, as exogenous serum T_4^* is rapidly cleared and as T_4 catabolism is increased, there is a transient state of hypothyroidism (low serum total thyroxine concentration), followed by a second wave of hyperthyroxinemia/hypothyroxinemia cycle.

This profile is typically the one described in ondulatory mechanics, and it reflects a quite efficient mechanism of homeostasis of serum T_4 around a biological haemostat –the

physiological serum T_4 concentration. This aspect of wave response for dosing T_4^* was not analysable with radioactive tracers, as classical immunoassays do not distinguish between radioactive and cold hormones. The stable isotope methodology offers an interesting solution to this problem.

5. Conclusion

The present method provides a sensitive and reliable technique for the simultaneous determination of thyroxine and their stable isotopically labelled counterparts in plasma with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies of thyroxine.

This first study is limited in the number of animal experiments and the possibilities of exploitation of this results in the field of the metabolic study. More animals and dose-ranging experiments would be required to allow a more conclusive evaluation of the data.

Our major aim was to demonstrate the potential of the technology based on the double isotopic dilution with two specific labelled thyroxine for thyroid hormone metabolic study in humans and animals.

Some improvements in the analytical equipment (use of LC–MS/MS) and protocols (extraction procedure: solid phase extraction method) will allow the T_3 and T_4 serum concentrations to be followed.

Acknowledgements

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