

Development and validation of an inductively coupled plasma mass spectrometry method for quantification of levothyroxine in dissolution studies

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A simple, sensitive and reproducible inductively coupled plasma mass spectrometry (ICP-MS) method for the direct determination of levothyroxine (T4), based on the analysis of iodide content, in aqueous media was developed. The sample preparation consisted of addition of antimony, as the internal standard, and dilution with a 0.5% ammonia solution. The analytes were quantified at m/z 126.90 and 120.90 for iodide and antimony, respectively. The assay was linear in the concentration range of 0.1–50 ng/mL for iodide and 0.3–100 ng/mL for T4. The method was precise and accurate with lower limits of quantification (LLOQs) of 0.1 ng/mL for iodide and 0.3 ng/mL for T4. The inter-day accuracy was >94% for both analytes and the coefficient of variation (%CV) was less than 5%. The method has successfully been used for dissolution studies of T4 formulations and holds immense promise as a simple, precise and sensitive analytical technique for T4 concentration determination in *in vitro* studies. Copyright © 2008 John Wiley & Sons, Ltd.

Levothyroxine (T4), administered orally as a sodium salt, is the exclusive choice of clinicians for the treatment of hypothyroidism and various forms of thyroid neoplasia.¹ It has a narrow therapeutic index such that doses of only about 20–25% outside the therapeutic window can place patients at risk of severe adverse effects of hyper- or hypothyroidism, including harmful cardiac and/or metabolic effects.^{2,3} Careful dose titration and close clinical follow-up are therefore essential for the safe and effective use of T4, which explains the importance of bioequivalence studies in verifying switchability between available products. Despite the efforts by the US Food and Drug Administration (FDA) to address these issues, bioequivalence remains an ongoing problem. Where the current methodology for bioequivalence determination has been criticized by many experts in the field, as being insensitive in distinguishing between doses that differ by as much as 12.5%,^{4–9} the physicochemical properties of the available formulations have been somewhat ignored. The potency and also the formulation characteristics regarding drug release, i.e. dissolution, appear to be critical.^{10,11}

In vitro dissolution tests have been successfully employed as a quality control tool to ensure inter-lot manufacturing reproducibility. Furthermore, such tests can be used as a sensitive method for differentiating between formulations of the same therapeutic agent and, in many circumstances, as an alternate to more expensive bioequivalence studies for the approval of generic formulations.¹² The dissolution test for T4 as described in the United States Pharmacopeia (USP) can

be used as a one-point quality control measure, estimating the amount of drug released at the end of a certain time period (for e.g. not less than 70% of labeled amount of T4 is dissolved in 45 min according to test 1).¹³ However, the high amounts of surfactant present in the dissolution medium (0.2% sodium lauryl sulfate in 0.01 N HCl) often result in extremely rapid dissolution making it ineffective for studying dissolution profiles. Moreover, the concentration of T4 in dissolution studies is typically measured using high-performance liquid chromatography (HPLC) with ultra-violet detection (UV), as described by the USP, which suffers from the inherent lack of sensitivity. Some of the other analytical assays used for T4 determination, including immunoassays^{14–16} and HPLC-UV,^{17–19} suffer from the lack of specificity and sensitivity and/or lengthy extraction or derivatization procedures, respectively. Collectively, these drawbacks make it ineffective to discriminate between the subtle, but significant, differences in the patterns of *in vitro* dissolution of T4 products, which can further lead to differences in their absorption profile.²⁰ Hence, a sensitive analytical technique to study and compare the dissolution profiles of various T4 products is warranted.

Inductively coupled plasma mass spectrometry (ICP-MS) is a popular technique for trace element determinations because of its excellent sensitivity and elemental specificity.²¹ T4 is a good candidate for ICP-MS as it contains iodine, an element not found in other commonly used chemicals (Fig. 1). ICP-MS has already been successfully employed for the determination of iodine in other media including natural and tap water, food products and urine samples.^{22–24} It has also been used to analyze iodinated X-ray contrast agents in water.²⁵ This article describes the development of a novel ICP-MS method for the quantification of T4 based on the

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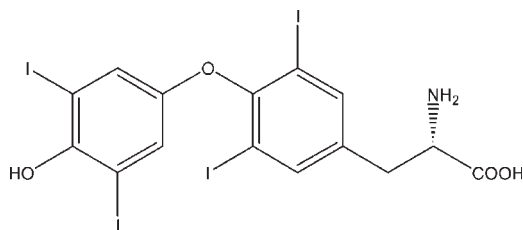


Figure 1. Structure of levothyroxine (T4).

measurement of iodide present in T4. Both the iodide and the T4 methods were validated according to the *Validation of Analytical Procedures: Text and Methodology Q2(R1)* published by the International Conference on Harmonization (ICH).²⁶

EXPERIMENTAL

Materials

Levothyroxine sodium and ammonium hydroxide *TraceSelect*^a were purchased from Sigma Aldrich (St. Louis, MO, USA). The iodide ion standard was purchased from VHG Labs (Manchester, NH, USA) as an ammonium iodide solution in water with a final iodide concentration of 10 000 µg/mL. Antimony was used as the internal standard, which was purchased from Ultra Scientific (North Kingstown, RI, USA), supplied as a 1000 µg/mL solution in a dilute nitric acid matrix. Tuning solution for the ICP-MS instrument, namely IMS-100 (Be, S, Ce, Co, In, Pb, Mg, Ni, U at 10 µg/mL in 2% nitric acid), and multi-element ICP-MS calibration standard IMS-102 (Al, As, Ba, Be, B, Cd, Ca, Cs, Cr, Co, Cu, Ga, In, Fe, Pb, Li, Mg, Mn, Ni, K, Rb, Se, Ag, Na, Sr, Tl, U, V, Zn at 10 µg/mL in 2% nitric acid) were also purchased from Ultra Scientific. All the working solutions and samples were stored in Nalgene polypropylene plastic bottles and containers purchased from Fisher Scientific (Fairlawn, NJ, USA). Nitric acid, *TraceSelect*, was also purchased from Fisher Scientific. Purified deionized water was prepared using Milli Q50 water purification system (Millipore, Bedford, MA, USA).

Calibrators, quality control standards and sample preparation

A stock solution containing 1 mg/mL T4 was prepared in methanol and stored at 4°C until use. Aliquots of the stock, equilibrated to room temperature, were diluted with 0.5% ammonium hydroxide solution to give six calibration standards containing 0.3, 1, 5, 25, 50 and 100 ng/mL T4. Similarly, three quality control standards (QCs), representing the low, medium and high concentrations within the range of the calibrators, were prepared with final concentrations of 0.5, 10 and 75 ng/mL T4. Iodide standards with concentrations of 0.1, 0.5, 1, 5, 20 and 50 ng/mL iodide for calibrators and 0.3, 2 and 30 ng/mL iodide for QCs were also prepared from a stock solution of 10,000 µg/mL iodide. A sub-stock of antimony (Sb) with a concentration of 2 µg/mL was prepared by diluting a stock of 1000 µg/mL Sb with deionized water. A volume of 50 µL of the Sb sub-stock was added to all the calibration and QCs as an internal standard

^a*TraceSelect* is an elemental analysis grade.

(IS) to provide a final concentration of 10 ng/mL Sb. For dissolution studies, sample preparation consisted of the addition of 25 µL of IS solution (i.e. Sb sub-stock) to 1 mL of the sample and diluting it to 5 mL with 0.5% ammonium hydroxide solution.

ICP-MS instrumentation and parameters

The instrumentation included a flow injection X7 ICP-MS instrument (ThermoElectron, Waltham, MA, USA) equipped with a sample introduction system, an ICP torch comprised of three concentric quartz tubes, a quadrupole mass analyzer, a simultaneous analogue/pulse counting electron multiplier detector and an ASX-510 HS autosampler (CETAC, Omaha, NE, USA). The sample introduction system consisted of a close-coupled, variable speed, three-channel peristaltic pump, a glass concentric nebulizer and a glass single-pass conical spray chamber with a fixed impact bead. Details of the ICP-MS system and its operating conditions are given in Table 1. Prior to each analytical run, the lens voltages were adjusted and the instrument performance reports were generated using the IMS-100 tuning solution at a concentration of 1 ng/mL, in order to obtain the highest signal intensity. Also, before each run the detector was cross calibrated using IMS-102 calibration solution at a concentration of 20 ng/mL, which allowed conversion of data acquired with the analogue detector into the equivalent pulse-counting data to ensure consistency. The prepared standards and samples were injected directly onto the ICP-MS instrument with an uptake time of 60 s acquiring the data in peak-jumping mode at m/z 126.90 for iodide (obtained from the breakdown of T4) and m/z 120.90 for antimony. A washing time of 180 s was employed for all samples using 0.5% ammonium hydroxide as the washing solution. Data acquisition was performed by PlasmaLab software (ThermoElectron, Madison, WI, USA).

Assay validation

The validation procedure for T4 and iodide assays involved the determination of performance parameters including linearity, lower limit of quantitation (LLOQ), accuracy and precision in accordance with the ICH guideline, *Validation of*

Table 1. ICP-MS operating conditions optimized for iodide and levothyroxine

	Parameter	Conditions
Plasma	Radiofrequency power	1400 W
	Plasma gas	Argon
	Plasma gas flow rate	13 L/min
	Auxillary gas flow rate	0.90 L/min
	Nebulizer gas flow rate	0.94 L/min
Vacuum	Expansion chamber	2.0 mbar
	Quadrupole	7.7×10^{-7} mbar
Data acquisition	Monitored isotopes (m/z)	¹²⁷ I, ¹²¹ Sb
	Scanning mode	Peak jumping
	Uptake time	60 s
	Dwell time	75 ms for Sb and 100 ms for I
	Number of sweeps	100
	Acquisition time	~1 min
	Washing time	180 s

Analytical Procedures: Text and Methodology Q2(R1).²⁶ Linearity was assessed by carrying out calibration plots with six concentrations in the ranges of 0.1–50 ng/mL and 0.3–100 ng/mL for iodide and T4, respectively. From the resulting calibration curve the regression coefficient, y-intercept and slope were calculated, characterizing the calibration function. The sensitivity of the assay was expressed as LLOQ which is the lowest concentration of T4 and iodide that can be quantified with suitable accuracy and precision. The LLOQ was determined based on the standard deviation of the response and the slope expressed as:

$$\text{LLOQ} = \frac{10\sigma}{S}$$

where σ is standard deviation of the response of 10 blank samples and S is the slope of the calibration curve.

The calculated LLOQ was further validated by analysis of accuracy and precision of six samples prepared at the calculated LLOQ concentration.

Accuracy was determined as the closeness of the results for QCs to their true value. The accuracy of the ICP-MS method was also validated by comparison of the average T4 concentrations with the results obtained from split samples analyzed by an HPLC-UV assay as described in the USP 30 dissolution test 3 for T4.¹³ The correlation between the two methods was studied via linear regression and the Pearson's correlation coefficient (r) was calculated. The degree of agreement between individual results obtained from multiple analytical runs of the same sample was taken as the precision. The intra-day and inter-day precision for the LLOQ and QCs were both determined.

RESULTS

The validation results for both iodide and T4 are shown in Table 2. The LLOQs were found to be 0.1 ng/mL and 0.3 ng/mL for iodide and T4, respectively. The assay was linear in the range of 0.1–50 ng/mL for iodide and 0.3–100 ng/mL for T4 with coefficients of determination (r^2) of 0.9999 for both iodide and T4, estimated from six calibration curves run on different days. The assays were accurate and precise with an intra- and inter-day accuracy of >90% and >94%, respectively, for both iodide and T4. The inter-day imprecision of

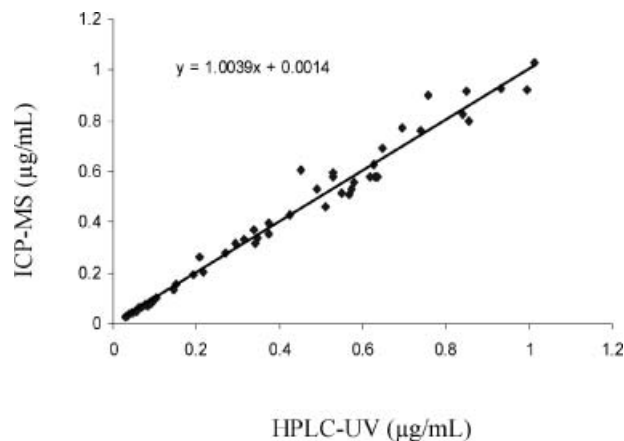


Figure 2. T4 measured by ICP-MS vs. HPLC-UV. Pearson coefficient of correlation, $r = 0.989$; $n = 53$.

iodide was less than 6% and that of T4 was less than 4%. The accuracy of the ICP-MS method was also characterized by comparing it with T4 concentrations obtained from split samples analyzed by the USP HPLC-UV method, covering a range of 0.03–1.0 µg/mL. Pearson's correlation coefficient (r) was calculated to be 0.989, which shows that the results of both methods are in excellent agreement (Fig. 2).

DISCUSSION

A simple, sensitive and rapid ICP-MS method, rigorously validated according to the ICH guidelines on validation of analytical procedures, for the direct determination of T4 in aqueous media has been described. T4 is an iodinated derivative of thyronine (Fig. 1), with four iodine atoms attached to its skeleton. In an ICP-MS instrument, the extremely high temperature (~10 000 K) and electron-rich atmosphere of the plasma atomizes and ionizes the T4 molecules generating I^+ ions, which are then separated based on their mass and detected. The quantification of iodide content thus determines the T4 concentration.

Ammonium hydroxide solution, 0.5%, was chosen as the solvent and diluent for all standards and samples. Memory effects are a serious problem in iodide analysis, originating

Table 2. Precision and accuracy data for iodide and levothyroxine (T4)

Analyte	Actual concentration (ng/L)	Observed concentration* (ng/L)	Inter-day precision (%CV)**	Intra-day precision (%CV)**	Inter-day accuracy (%)	Intra-day accuracy (%)
Iodide						
LLOQ	0.1	0.10 ± 0.006	5.6	3.3	94.2	92.7
QC ₁	0.3	0.28 ± 0.006	2.2	3.3	94.8	90.9
QC ₂	2.0	1.97 ± 0.028	1.4	1.5	98.4	99.7
QC ₃	30.0	30.54 ± 0.193	0.6	0.9	98.2	96.0
T4						
LLOQ	0.3	0.29 ± 0.009	2.9	3.5	99.9	97.2
QC ₁	0.5	0.49 ± 0.019	3.9	1.1	98.8	98.5
QC ₂	10.0	9.69 ± 0.184	1.9	0.8	96.9	97.6
QC ₃	75.0	74.96 ± 0.957	1.3	0.4	99.9	99.8

*Mean ± SD of six replicates.

**coefficient of variation.

from the spray chamber. The finer droplets (smaller than 10 µm) from the nebulizer are carried into the plasma whereas some of the larger droplets are deposited in the spray chamber, and then escape to waste. Formation of some species of iodine, e.g. hydrogen iodide which is also volatile in nature, is encouraged under acidic conditions and their gradual release from the spray chamber leads to memory effects. Therefore, an alkaline pH helps to minimize the memory effects.^{22,24,27} A washing time of 180 s with 0.5% ammonium hydroxide was employed, which further reduced the memory effects to negligible amounts.

Antimony (Sb) was used as an internal standard to correct for non-spectral interferences and signal instability. Indium has been previously used but it can precipitate out as In(OH)₃ at basic pH and, therefore, is not suitable with ammonia as the solvent.²⁸ Antimony, being a metalloid, works better at basic pH and has been previously used with a 1% ammonia solution.²⁹

The validation of T4 was preceded by the development and validation of the iodide assay, which was used as a model to obtain the optimum ICP-MS conditions to be applied to T4 to ensure the performance and robustness of the assay. ICP-MS is a highly sensitive technique, and the LLOQ in this case was found to be 0.3 ng/mL for T4, which is much lower than for most of the assays in use.^{17,30,31} Furthermore, the developed assays were found to be accurate and precise (Table 2). Comparison of the ICP-MS method with the USP HPLC-UV method showed a correlation coefficient of 0.989. Studying the accuracy of a method over the assay concentration range by linear regression model is a generally accepted practice.^{22,32,33} It has been shown that when using the linear regression model at least 10 samples uniformly covering the range of interest should be used and the method with smaller random error should be represented by the x-axis.^{34,35} In our data set, T4 concentrations covered the range from 0.03 to 1.0 µg/mL uniformly, and no outliers were observed (Fig. 2). The close correlation of the ICP-MS method with such a fundamentally different method demonstrates that it can be used reliably for the direct measurement of T4 concentrations.

CONCLUSIONS

Levothyroxine is a widely prescribed narrow therapeutic index drug with a questionable bioequivalence between various available formulations. Because of this, patients need to be carefully dose-titrated when being switched from one brand to another. The physicochemical properties of the drug and the formulation can significantly affect the drug release characteristics and performance of the formulation. Dissolution is one of the most important characteristics of a drug, which directly affects the drug absorption and, hence, bioavailability. Presently used analytical assays for T4 determination, including immunoassays and HPLC-UV, suffer from lack of specificity and sensitivity and/or lengthy extraction or derivatization procedures, respectively. Clearly, a sensitive and specific assay is needed to detect and quantify subtle but significant differences between various formulations. A simple, rapid and sensitive ICP-MS method was thus developed and validated according to the guidelines

published by the ICH. Currently, this method is used successfully in our laboratory to study the dissolution profiles of various available products of T4, with the objective of better characterizing their pharmacotechnical aspects.

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