METHODS

High Pressure Liquid Chormatography Determination of the Concentration and Integrity of L-Thyroxine in Free T_4 Stock Solution

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Characterization studies were designed to evaluate the concentration and integrity of the L-thyroxine (T4) molecule (3,5,3',5'-tetraiodothyronine) in the free T4 stock solution (FT4SS) (code 99544). The determination of the concentration of T4 in FT4SS is critical to ensure that the free T4 calibrators and controls are manufactured with the least number of adjustments possible. The most significant conclusions drawn from these characterization studies are the following: (1) An accurate and sensitive HPLC method has been developed to measure the T4 concentration in FT4SS. The root cause of the failure of FT4SS to pass retest/ review is the presence of an unknown T4 degradation product with significantly higher molar extinction coefficient at 230 nm than T4 itself. The L-thyroxine concentration reference comparison spectrophotometric test with the current 43 to 58 ug/ml specification range (as per scp.99544, ed. 13A) is adequate to monitor the generation of the unknown T4 degradation product. The characterized T4 degradation product is not 3,5,3'-triiodo-thyronine (T3) and it is suspected that the identity of the degradation product is reverse T3 (3,3',5'-triiodothyronine). The use of sodium l-thyroxine pentahydrate (Na- T4-5H_2O) as the equivalent of T4 (free base) is adequate provided that an excess of 15% over the desired amount of T4 is weighed.

Key words: L-thyroxine, T4, T3, HPLC, Thyroid hormones, Characterization

Thyroid hormones, thyroxine (T4) and thyronine (T3), are iodine-containing hormones secreted by the thyroid gland. They are responsible for the regulation of diverse biochemical processes essential for normal metabolic and neuronal activity. T3 is more biologically potent than T4 but T4 is normally present in human serum in approximately 50-fold excess of circulating T3 and constitute more than 90% of the circulating protein bound iodine (1-2). More than 99.9% of circulating T4 is bound to serum thyroxine binding proteins (TBP). TBPs are composed primarily of thyroxine binding globulin (TBG), thyroxine binding prealbumin (TBPA), and albumin, which binds 75%, 15%, and 10% of circulating T4, respectively (3-5). Free or unbound levels of circulating T4 (FT4) constitute less than 0.03% of total blood T4 although it is the biologically active fraction of the physiologically available hormone (6,7). It is recognized that a variety of biochemical imbalances, drug treatments, pregnancy, certain viral and bacterial infections, and other conditions affect the levels of circulating thyroid hormones (3,8). Hyperthyroidism is a disease in which, circulating blood levels of T4 are elevated while hypothyroidism is characterized by reduced T4 levels.

Clinically, FT4 measurements have long been recognized as an aid in the assessment and diagnosis of thyroid status. The Abbott Diagnostics manufacturing plant in Barceloneta, Puerto Rico, produces serum based FT4 calibrators and controls containing precisely targeted FT4 concentrations that are employed in the calibration of automatic immunoassay platforms. These calibrators and controls are manufactured using processed human serum as the solvent matrix together with commercially available sodium levothyroxine as the active ingredient in order to simulate patient serum samples. Calibrators are used to establish the concentration-instrument response relationship throughout the dynamic range of the assay. This relationship is then fixed via a nonlinear algorithm in order to quantify unknown FT4 concentrations in patient

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samples. Manufactured controls are then employed to verify that the generated calibration curve is fit to provide reliable FT4 values in patient samples.

The first step in the manufacture of FT4 calibrators and controls is to prepare a solution of concentrated T4 in order to facilitate the addition of precise quantities of T4 into the human serum solvent matrix (9). The concentrated solution of T4 is referred to as the FT4 stock solution (FT4SS) and it is the object of this investigation. FT4SS is a 0.01 N sodium hydroxide (NaOH) solution of T4 at a target concentration of 50 µg/mL. FT4SS require frozen storage at –10ºC or colder and protected from light. After the FT4SS is used to prepare calibrators and controls it is subsequently stored until again needed to manufacture new batches of these products. The stored stock solution has to be reanalyzed for T4 concentration and integrity if used two months after the preparation date (10-12) .

The current in-house test method requires the use of two technologies to determine the concentration of T4 in FT4SS. One of the T4 concentration determinations is performed by diluting the FT4SS 1/501 into T4 stripped human serum (code 95654) followed by a TDx total T4 immunoassay of the diluted stock. TDx is an Abbott's automatic immunoassay platform that uses fluorescence polarization to measure total T4 concentration. The second T4 concentration determination method required by the in-house procedure employs spectrophotometry. A T4 absorbance reference comparison test (T4-ARCT) standard is prepared with USP levothyroxine or an equivalent T4 source. Na-T4-5H2O (code 99512) has been routinely used as the USP T4 equivalent for this particular test. The absorbance reading of the known T4 concentration of the T4-ARCT standard is then employed to calculate the T4 concentration corresponding to the stock's absorbance. In-house specifications require that the TDx Total T4 derived concentration value of the diluted stock must fall at or within 8.5 and 11.5 µg/dL and that the T4 concentration value for the FT4SS obtained from the absorbance method must fall at or within 43 and 58 ug/ml. Historically, no reanalysis of expired stock has ever passed the absorbance test. The retest passes the TDx test but always fails the absorbance tests on the high side of the specifications. With this background information, studies of an expired lot of FT4SS were pursued in an attempt to clarify the subjacent molecular substrate that would explain the observed phenomena.

The first step in these studies was to reanalyze expired FT4SS according to in-house established procedures as already described above in order to select a sample that would reproduce the typical case of a non-acceptable FT4SS lot. The second step was the development of an HPLC method that would allow the qualitative and quantitative analysis of the T4 molecule present in FT4SS and in the Na-T4-5H2O powder raw material from which the stock is manufactured and which could also be used as the USP T4 equivalent in the in-house ARCT. The developed HPLC method exhibited a high degree of specificity, accuracy and precision in the determination of the T4 concentration and in detecting the presence of a degradation product in expired FT4SS. These studies are necessary in order to assess whether the observed phenomena might negatively impact the performance of FT4 calibrators and controls in the field. In summary, the present study uses HPLC-UV to measure the concentration of the T4 molecule in FT4SS (code 99544) and in Na-T4-5H2O (code 99512) and to separate it from a degradation product that develops upon the storage of the FT4SS.

Materials and Methods

A sample of expired FT4SS was provided to the ADI's quality control laboratory (Q.C.) in order to reanalyze it according to established in-house procedures as briefly described in the introduction section above.

The HPLC method developed to determine the concentration and integrity of the T4 molecule in FT4SS (code 99544) was adapted for T4 from a T3 stock standard HPLC in-house method and is here described in detail. A Waters® High Performance Liquid Chromatograph (HPLC) was used to determine T4 concentrations. The system consists of a 600E system controller with a 700 satellite WISP autosampler coupled to a 484 UV/VIS detector and a M746 data module integration system. Materials used were reagent HPLC grade methanol, 85% phosphoric acid (PA), USP levothyroxine (T4), Na-T4-H2O, 0.1 N sodium hydroxide (NaOH) and 0.01 N NaOH.

The chromatographic conditions were: a 5 um particle size phenomenex C18, 3.9 x 300 mm column was employed using a 60% methanol; 40% distilled water isocratic mobile phase containing 1 mL 85% PA per L of solution. The flow rate employed was 1.0 mL/min. and the absorbance monitored at 230 nm.

Three solutions of USP T4 were prepared at the following concentrations, 0.1388, 0.5608 and 1.7500 mg/mL. These were used to prepare known T4 concentration standards for the construction of the HPLC calibration curve of 0.0275, 0.0555, and 0.0866 mg/ml. Each standard was injected in duplicates into the HPLC and the averaged response used to construct the calibration curve. A calibration curve of the T4 concentration versus peak area response (also referred to as "area below or under" the curve: ABC or AUC) from the working standards was prepared. The correlation coefficient, slope, and y-intercept of the linear regression analysis were calculated.

A 10 uL of undiluted and 10X diluted expired FT4SS (code 99544) were injected into the HPLC. Duplicate injections of 100 uL of 100X diluted 0.01N NaOH reference solution of Na-T4-H_2O (580.08 mg/ml) (code 99512) were also analyzed. Peak area responses (ABCs) of the sample preparations were used to calculate the T4 concentration of these samples using the generated calibration curve.

Results and Discussion

Reanalysis of Free T4 Stock Solution (Code 99544). Results of the reanalysis of expired FT4SS performed by the QC laboratory revealed that, as expected, the expired FT4SS sample passed specifications with respect to the Total T4 TDx assay at 8.79 µg/dL, where current specification are 8.5 to 11.5 µg/dL, but failed the absorbance test at 67.6 µg/ml, were current specification are 43 to 58 ug/ml. When first prepared, the same FT4SS lot passed the Total T4 TDx testing at 9.92 µg/dL and the absorbance test at 55 µg/ml. Therefore, the T4 concentration of this particular FT4SS lot appears to have decreased by 9% since it was prepared according to the TDx assay, but appears to have increased by 19% according to the absorbance reference comparison test. These are apparently conflicting T4 concentration results were further studied taking advantage of the sensitivity and resolving attributes of the HPLC technology. The results of the HPLC studies are presented and discussed below.

Each of the T4 standard solutions were injected as described in the methods section in order to determine specific retention times and T4 concentration dependent ABC responses. The peaks for duplicate injections of the standard solution containing the lowest T4 concentration are shown in figure 1. The determined average retention times (RT) and ABC responses for the three T4 standard solutions were 10.81 and 14,521 (with a 0.17% difference in ABC between replicates) for the 0.0275 mg/mL standard, 10.82 and 29,730 (with a 0.21% difference in ABC between replicates) for the 0.0555 mg/mL standard, and 10.80 and 46,703.5 (with a 0.22% difference in ABC between replicates) for the 0.0866 mg/mL standard.

Another important observation from these chromatograms is their "cleanliness and simplicity". There is a stable baseline with only two accidents: one downward deflection at approximately 3.0 min from the injection time which corresponds to the sample "front", that is, the time elapsed for injected substances that do not interact with the column to reach the detector optics and the T4 peak eluting at a RT around 10.81 minutes. This is expected since the sample contains pure T4 (USP Standard) in a 0.01N NaOH solution.

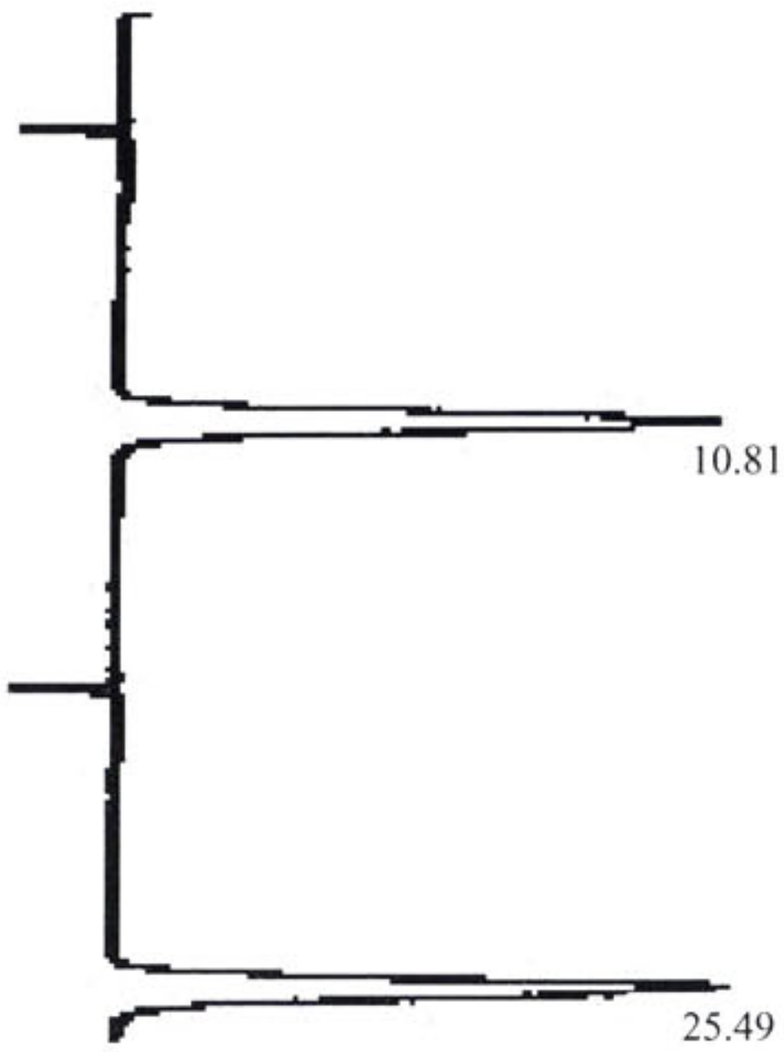

Figure 1. HPLC elution profiles of two consecutive injections (10 µl ea.) of USP T4 working standard 1. The numbers above the peaks indicate the apparent retention time of (RT) USP T4 as measured by the recorder integrator. The first peak representing USP T4 eluted with a RT of 10.81 min and a area below the curve (ABC) of 14,509. The second USP T4 peak eluted with an apparent RT of 25.49 but with a true RT similar to that of peak one (see text for clarification) and an ABC of 14,533.

The linear regression analysis of the T4 HPLC calibration curve is presented in figure 2 and in Table 1. The linearity of the calibration curve is evident exhibiting a correlation coefficient (CC) calculated at 1.0 (See Table 1). The linearity range is from 27.5 to 86.6 µg of tyroxine /mL of solution.

HPLC Qualitative and Quantitative Analyses of Expired FT4SS. HPLC chromatograms of 10 uL injections of undiluted and 10X diluted (not shown) expired FT4SS were obtained. The presence of two clearly defined peaks were observed (see Figure 3). The first peak eluted at a RT of 8.61 and the second peak eluted at a RT of 10.80 corresponding to the T4 retention time. The same elution

Table 1. U S P T_4

Working Std. No.	Volume injected	U S P T_4 mg/ml	Mean A B C
1.	10 uL	0.0275	14521.0
2.	10 uL	0.0555	29730.0
3.	10 uL	0.0866	46703.5

Linear regression analysis
Slope = 5.4 E + 05
y-Intcp = -4.4 E + 02
C C = 1.0 E + 00

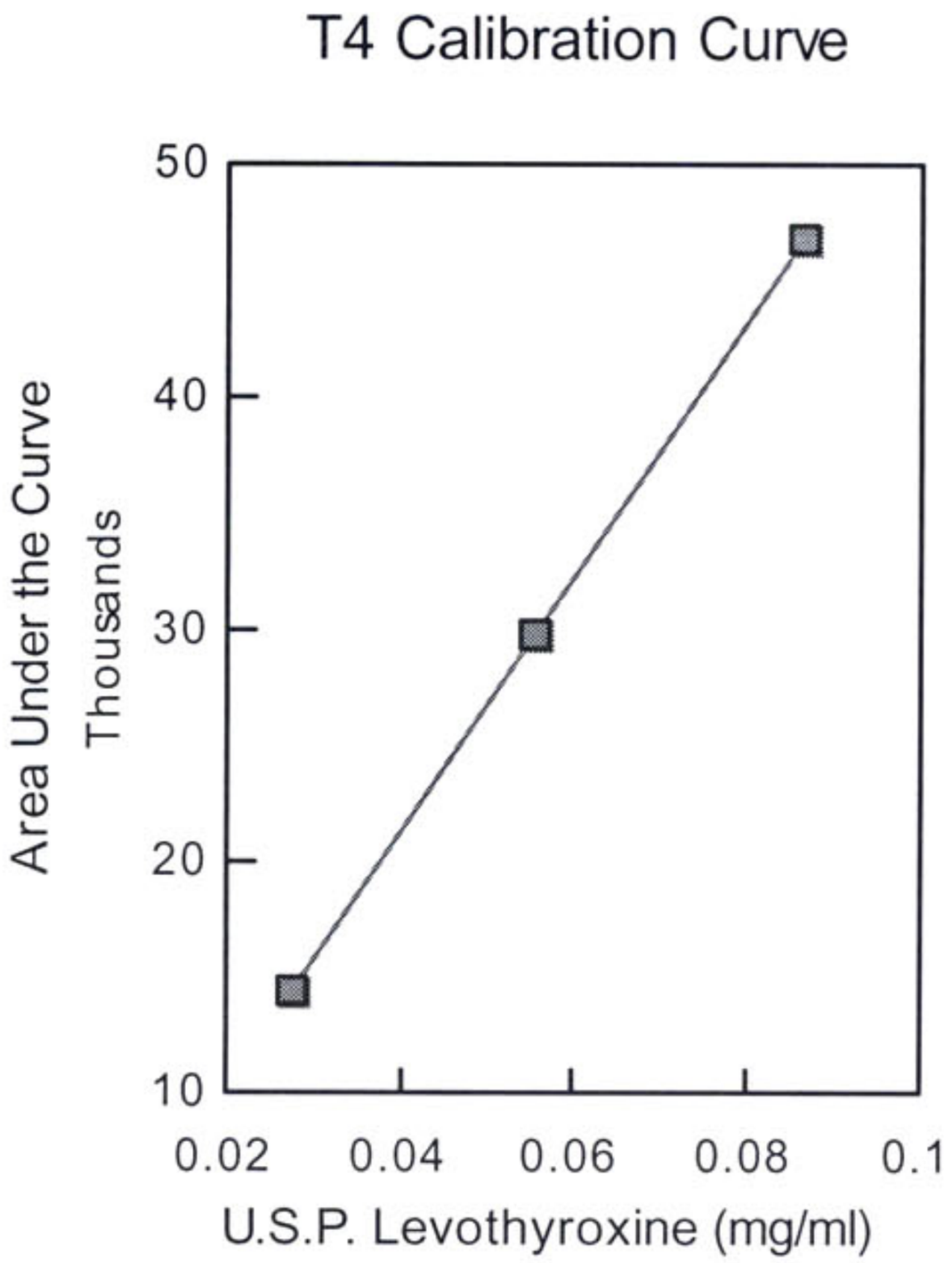

Figure 2. Plot of 10 ul injections of USP T4 working standards 1, 2, and 3 versus their average (n=2) elicited area under or below the curves. See Table 1 below for the actual values plotted and the linear regression analysis.

pattern was observed when 10X diluted FT4SS was injected precluding a concentration related artifact in the elution profile. This observation confirms the presence of a contaminant in the sample of the expired FT4SS since only a peak at RT around 10.81 corresponding to T4 would have been expected. The identity of the contaminant or degradation product eluting at a RT of 8.61 is unknown although it most probably represents a degradation product of T4 as a result of the FT4SS storage conditions.

The FT4SS degradation product is not T3 since T3 elutes from the column with a RT around 6.89 (chromatogram not shown) and the identified degradation product elutes with a RT of 8.61. Nonetheless, there is high probability that the contaminant is structurally very similar to both T3 and T4. Further studies may reveal the root cause of such degradation and the chemical identity of the degradation product. It will probably be necessary to find a suitable alternate solvent in the future for T4 in lieu of 0.01N NaOH. It appears that the T4 degradation product does not cross-reacts with the TDx total T4 assay since the T4 concentration of the expired material (8.79 µg/dL) does not remain stable nor appears to increase since the time of the first test to FT4SS but actually declines by 9% from 9.92 µg/dL as discussed for the retest/review study above.

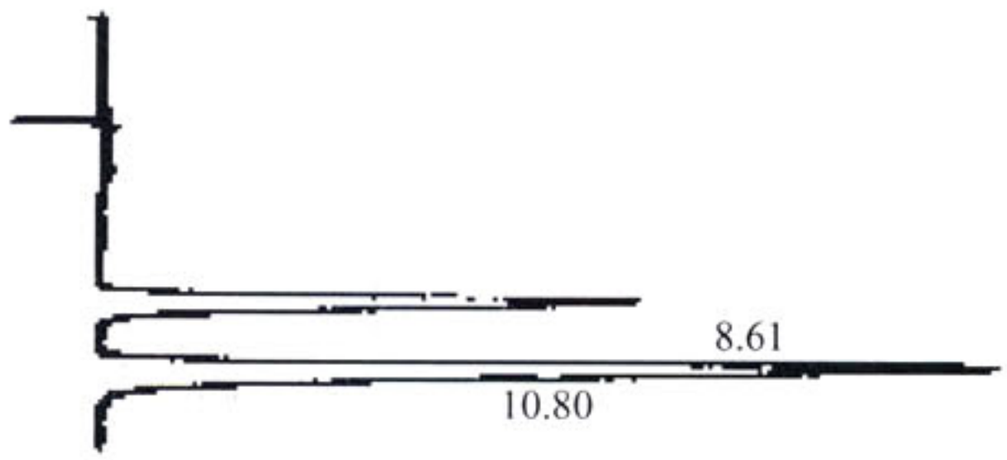

Figure 3. HPLC profile of a 10 uL injection of expired FT4SS that was subjected to QC reanalysis. At RT 10.80 min, a T4 peak was identified. At RT 8.61 min, an unidentified degradation product of T4 was evident.

The T4 concentration detected in the second peak with RT of 10.80 in the chromatogram (figure 3) as estimated using the T4 calibration curve was of 0.0463 mg/mL. This T4 concentration value passes the 3R absorbance testing requirements for the T4 concentration of the FT4SS as per scp.99544 (Specification range 43 ot 58 µg/mL). If it is assumed that the degradation product (with RT = 8.61) has a similar extinction coefficient as T4, its concentration would be 0.0193 mg/mL or 19.30 µg/mL. If these two concentrations values are added as it would happen in the case that the absorbance value of the FT4SS was measured in the spectrophotometer, the FT4SS will exhibit an "apparent" T4 concentration of 65.60 µg/mL. Comparing this value of 65.60 µg/mL with the T4 concentration value of 67.6 µg/ml obtained in the Retest/Review made by QC of this same lot of FT4SS, it is evident that the "apparently high" T4 concentration in the expired FT4SS can be explained by the presence of the T4 degradation product which apparently has a considerably higher extinction coefficient than T4 at 230 nm. Consequently, these HPLC studies clarify the root cause of the failure of expired FT4SSs to pass the absorbance test (3R) as per scp.99544 as well as the utility of the test to monitor the generation of this T4 degradation product.

HPLC Qualitative and quantitative analyses of Na-T4-5H2O. Na-T4-5H2O is used in the manufacture of the FT4SS. It is also used as the USP T4 equivalent in the absorbance reference comparison test (ARCT) to determine the T4 concentration in the FT4SS. The HPLC chromatogram presented in figure 4 below is representative of two 100 µl injections of a 100X diluted solution of the Na-T4-5H2O ARCT standard prepared to a target concentration of 580.8 mg/ml. Three peaks are observed in the chromatogram. The first peak or peaks (more like signal deflections) at an RT around 3.08 minutes exhibit

irreproducible ABCs suggesting that these peaks represent sample components not retained by the column, constituting thereby the "front" of the chromatogram.

A second small peak with a shoulder was registered at an RT of 3.58 minutes in both chromatograms. The fact that this shouldered peak eluted just after the "front" and exhibit reproducible ABCs in both chromatograms

Figure 4. HPLC elution profile (RT in minutes) of a 100 µL injection of 100X diluted Na-T4-5H2O reference standard solution (580.8 mg/ml) prepared to be used in the T4 ARCT.

suggests real contaminants of this T4 preparation; these contaminants will additively contribute to the total absorbance of the T4 ARCT standard solution. In other words, not all of the absorbance signal generated by the T4 ARCT standard solution prepared with Na-T4-$5H_2O$ is accounted for by the presence of T4. This is in contrast with the reference material prepared with USP T4 as shown in the chromatogram presented in figure 1. The peak eluting at 10.94 minutes is T4 (Figure 4).

Using the HPLC standard curve developed in figure 2 and table 1 above and the average ABCs around 10.94 minutes (average of 27,037 and 27,079 equals 27,058), a T4 concentration of 505.5 µg/ml was calculated for the undiluted T4 ARCT standard prepared with Na-T4-$5H_2O$. The T4 ARCT standard in question was prepared by weighing Na-T4-$5H_2O$ using an analytical balance (0.0001 g) and dissolving it in 0.01N NaOH. The actual T4 concentration should have been 580.8 mg/ml. Based on the observed T4 concentration of 505.5 mg/ml, there is a 14.9% difference between the observed and the expected T4 concentration in the T4 ARCT standard solution. This difference can be readily accounted for by the fact that the weight contributions of sodium and water in Na-T4-$5H_2O$ were not taken into consideration. The total atomic weight of sodium (23.0) plus that of five water molecules (18.0 per water molecule) equals 113.0. Since T4 has a molecular weight of 776.9, the weight contribution of sodium and water in the Na-T4-$5H_2O$ molecule is 14.5%, clearly accounting for most of the difference between the expected and the HPLC observed T4 concentration in the T4 ARCT standard solution. It is demonstrated then that the differences in molecular composition between the Na-T4-$5H_2O$ and the USP T4 free base result in a lower than expected T4 concentration in the T4 ARCT standard solution when the weighted amount of Na-T4-$5H_2O$ is not corrected for the sodium and water content. This weight correction in Na-T4-$5H_2O$ is essential for the weight equivalency between Na-T4-$5H_2O$ and the USP T4 standard.

So far, the discussion applies only to the situation where the T4 ARCT standard and the FT4SS are prepared using Na-T4-$5H_2O$ as the USP T4 equivalent. In practice for manufacturing purposes, this is the most common situation due to the cost differential between Na-T4-$5H_2O$ and the USP T4 standard. Other combinations are still acceptable from the point of view of manufacturing regulations for the manufacture and analysis of the FT4SS. The ideal and most expensive combination would be to use the USP T4 standard to prepare both the FT4SS and the T4 ARCT standard solution. This situation is straight forward since the amount of T4 weighted and diluted is the only contributor to the absorbance observed in both the FT4SS and T4 ARCT standard solution. The situation would be different in the case were the FT4SS is prepared using Na-T4-$5H_2O$ and the T4 ARCT standard solution prepared using USP T4. In this case, the true amount of T4 in the FT4SS would be 85% of that obtained in the T4 ARCT because the absorbance measured from the FT4SS would contain a 15% weight contribution from Na and water. Finally, it remains to be discussed the case where the FT4SS is prepared using USP T4 and the T4 ARCT standard solution prepared using Na-T4-$5H_2O$. In this case, the estimated amount of T4 calculated to be present in the FT4SS would be 85% of the actual amount since the true T4 absorbance in the T4 ARCT standard solution would be overestimated by 15%. Once absorbance differences between the USP T4 standard solutions and the Na-T4-$5H_2O$ solutions are adjusted based on the evidence presented here, adequate equivalency between these two sources of T4 would have been established.

Conclusions

According to the evidence presented, several conclusions can be drawn concerning the present study. An accurate and sensitive HPLC method has been developed to measure the T4 concentration of the free T4 stock solution (FT4SS). Because of the separation power of HPLC, the methodology implemented in the current study allowed for the discovery of a heretofore unknown degradation product of T4 present in expired FT4SS. This degradation product of T4 was shown to be the root cause of the failure of the FT4SS to pass retest/review specifications as per scp.99544 because the T4 degradation

product exhibited significantly higher molar extinction coefficient at 230 nm than T4 itself. The higher extinction coefficient of the T4 degradation product caused an increase in the total absorbance in degraded FT4SS such that the upper specification limit for the absorbance reference comparison test was surpassed. Since the l-thyroxine absorbance reference comparison test with the current 43 to 58 µg/ml specification range as per scp.99544 was able to detect the generation of the T4 degradation product, this test is found to be adequate to monitor the molecular integrity of the FT4SS. Furthermore, the characterized T4 degradation product is not 3,5,3'-triiodothyronine (T3) since its HPLC elution profile was different from that of T3 itself. It is suspected that the identity of the degradation product could be *reverse T3* (3,3',5'-triiodothyronine) because this compound could be generated from T4 by a single loss of an iodine atom. Finally, the use of sodium l-thyroxine pentahydrate (Na-T4-5H_2O) as the equivalent of T4 (free base) is adequate provided that an excess of 15% over the desired amount of T4 is weighed in order to correct for the extra sodium and water content in this T4 salt.

Future efforts should be directed at elucidating the molecular structure of the T4 degradation product described here. The use of tandem HPLC-mass spectroscopy could provide direct evidence of the molecular weight of the degradation product which would in turn indicate how similar or different it is as compared with the molecular weight of T4 itself. Also, it is important to establish if this degradation product interacts with the anti-T4 polyclonal antibody used in the Abbott's TDx free T4 immunoassay. Significant cross-reactivity of the degradation product with the anti-T4 polyclonal antibody may interfere with the performance of the immunoassay as T4 degrades through out the lifetime of the free T4 calibrators and controls.

Resumen

Se diseñó un estudio para evaluar las concentraciones y la integridad de la molécula de L-tiroxina (T4) 3,5,3',5'-tetraiodotironina en una solución matriz (FT4SS) (code 99544). La determinación de la concentración de T4 en FT4SS es sumamente crítica para asegurar que los calibradores que están libres de residuos de T4 y los controles que sean manufacturados con el menor número de ajustes químicos posibles. Los resultados mas significativos de este estudio de caracterización son los siguientes: 1) se desarrolló un método certero y sensitivo para determinar los niveles de T4 en FT4SS. 2) se determinó la razón por la cual FT4SS fracasaba las pruebas de rigor de re-examen /revisión a causa del producto de degradación de T4 con coeficiente de extinción significativamente mas alto a 230 nm, que resultó mas alto que el del mismo T4. 3) La comparación de la concentración de l-tiroxina de referencia usando espectrofotometría en el rango de 43 a 58 ug/ml (scp. 99544, ed. 13A) es la adecuada para monitorear la degradación del producto desconocido de T4; 4) el producto de degradación característico del T4 no es 3,5,3'-triiodotironina (T3). El uso de pentahidrato de sodio de l-tiroxina (Na-T4-5H_2O) como el equivalente de T4 (base libre) es adecuado dado que un exceso de 15% sobre la cantidad deseada de T4 sea pesada.

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