Accurate Reference Standards for Accurate Quantitation of Thyroid Hormones: Impact on Clinical Reference Ranges

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Introduction

Disorders of thyroid metabolism affect millions of patients worldwide. Clinical diagnosis and treatment requires testing and monitoring of patient thyroid hormone levels. Reference ranges for thyroid hormones vary among patient sub-groups and disease states. Triiodothyronine (T3) and thyroxine (T4) are the most commonly tested thyroid hormones. Reverse triiodothyronine (rT3) is an inactive isomer of T3 formed primarily by enzymatic de-iodination at the 5-position of Thryoxine (T4).[1] Currently most thyroid hormone assays are by radioimmunoassay (RIA) which can be expensive, have limited shelf life, and lack specificity.[1] The available RIA assay for rT3 is designated for research use only in the USA.[2] There is a significant clinical diagnostic need for robust and accurate methods for determining thyroid hormone levels in general and particularly for rT3. In recent years there has been significant push to develop LC/MS/MS methods for quantitation of thyroid hormones with the potential for higher accuracy even on the low end, better specificity, and the ability to quantitate the different thyroid hormones separately.

Calibrators for LC/MS/MS methods are critical to accuracy of results and must be carefully evaluated. The importance of proper selection and certification of materials and their impact on calibrator accuracy and resulting clinical decisions is illustrated in this poster with rT3. rT3 was developed as a certified solution reference standard and evaluated at Cerilliant and LabCorp, demonstrating the impact of calibrator certification on clinical reference ranges.

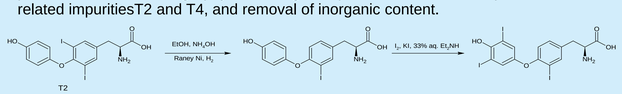
References
1. Wang, Dongli and Stapleton, Heather M., Analysis of Thyroid Hormones In Serum by Liquid Chromatography-Tandem Mass Spectrometry, Anal Bioanal Chem. 2010 July; 397(5): 1831–1839.
2. ALPCO reverse T3 RIA Product Literature

Availability of Reference Materials for Thyroid Hormones

- While the individual thyroid hormones are widely available, the level to which they are tested varies significantly. Most are offered as research grade chemicals with limited certification. Often, purity is assigned by non-specific techniques such as TLC and no information regarding impurity profile is provided. Cerilliant's testing of commercially available thyroid hormones showed that some were low purity by HPLC, and some had significant amount of residual inorganic impurities.
- The challenges with developing thyronine Certified Reference Materials (CRMs) are the low solubility of the neutral species, the zwitterionic properties of the amino-acid, and the compounds' tendency to accumulate inorganic salts and acetate salts as impurities.
- It is essential to have high purity, properly homogenized and well-characterized Reference Materials (RMs) for critical quantitative applications.

Synthesis and Purification of rT3 and T3

- Commercially available rT3 was purchased from Sigma Aldrich and tested at Cerilliant. Material was found to have low purity and deemed unsuitable for use as a RM.
- Cerilliant synthesized rT3 by selective de-iodination of T2, by hydrogenation, followed by terminal ring iodination with iodine and potassium iodide, and purification by reverse phase column chromatography. Challenges to purification included separation of related impuritiesT2 and T4, and removal of inorganic content.



- Commercially available T3 was purchased from TRC and was purified by reverse phase column chromatography.

- In both cases, control of inorganic impurity profile was achieved by extensive washing of the materials at controlled pH of 4-5. The level of inorganic impurity was determined by microash and ion chromatography.

Preparation of Certified Spiking Solutions® of Thyroid Hormones

- Cerilliant synthesized rT3 and T3 were certified along with testing of commercially available materials.
- Ampouled spiking solutions of T3 and rT3 were developed as Certified Reference Material solutions from the Cerilliant synthesized neat materials.
- A mass balance purity factor was assigned based on characterization of the neat materials.
- Spiking solutions were prepared gravimetrically, using qualified weighing techniques and balances calibrated to NIST standards.
- Spiking solutions were dispensed into amber ampoules, purged with argon, and flame sealed.
- Validated process controls were used to ensure accuracy, batch homogeneity and consistency .
- Spiking solutions were certified against an independently-prepared calibration curve.

Cerilliant® Analytical Reference Standards a SIGMA-ALDRICH company

LabCorp Laboratory Corporation of America

Certification of Thyroid Hormone Neat Materials and Spiking Solutions

- rT3 synthesized and T3 purified by Cerilliant were certified for use as CRMs by full characterization for chromatographic purity, identity, residual solvent, residual water and residual inorganic content, determined by sulfated ash and by IC/ICP-MS. A mass balance purity factor was assigned based on chromatographic and residual impurities. Spiking solutions were prepared using mass balance purity values.
- Neat materials were also assayed by quantitative NMR against maleic acid used as an internal standard. Quantitative NMR provides an orthogonal, independent direct measurement of the mass fraction of the analyte of interest, calibrated with an internal standard, based on molar response of protons in the NMR spectrum.

Analytical Test	Method	Cerilliant rT3	Sigma rT3 * (lot# 091M1695V)	Cerilliant T3 (post purification)	TRC T3 (pre-purification lot# 11-ANR-129-1)
Chromatographic Purity by HPLC/PDA Analysis	USP<621>, SP10-0102	98.4%	91.6%	99.5%	96.2%
Chromatographic Purity by LC/MS Analysis	USP<736>, SP10-0107	98.6%	100%	99.2%	94.2%
Identity by LC/MS Analysis	USP<736>, SP10-0107	Consistent with Structure	Consistent with Structure	Consistent with Structure	Consistent with Structure
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure	Consistent with Structure	Consistent with Structure	Consistent with Structure
Residual Solvent Analysis by GC/FID Headspace	AM1087 Validated method	0.02%	0.36%	None Detected	0.07%
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	0.70%	0.87%	0.28%	0.38%
Inorganic Content by Residue on Ignition	USP<281> Sulfated ash SP10-0135	1.033%	0.585%	< 0.2%	0.255%
Inorganic Content by Ion Chromatography or ICP/MS	Outsourced	0.36% sodium (1.28% sodium acetate)	0.16% sodium 0.68% chlorine 0.37% ammonium 0.40% phosphate 0.07% sulfate 0.01% nitrate	< 100 ppm	< 100 ppm
Mass Balance Purity Factor	Purity = [(100 - (wt%OVI) – (wt% H_2O) – (wt%ROI)] * (Chrom Purity/100)	96.48% (using % sodium acetate from ICPMS instead of %ROI)	90.28%	99.22%	95.5%
Assay by Quantitative NMR	USP<761> SP10-0116, AM1370	98.15%	83.42%	99.52%	96.38%

*determined unsuitable for intended use

- The larger discrepancy between purity factor and qNMR assay for rT3 is attributed to impurities that interfere with the qNMR monitor signals for rT3.
- While qNMR is not as accurate for samples with significant impurities, this technique confirms the low purity of rT3 purchased from Sigma and indicates the material is not suitable for use as a quantitative RM.

- Analytical verification and certification of spiking solutions comprised accuracy, consistency homogeneity, and purity testing of the solution via HPLC/UV.
- Accuracy of the prepared concentration was verified by comparison to a independently-prepared calibration curve.
- Homogeneity was confirmed across each batch of reference solution prepared.
- The solution purity was evaluated for consistency with the neat material to rule out degradation and contamination during preparation.
- Cerilliant rT3 and T3 spiking solutions were certified at concentration of 100±0.6 µg/ml.

Collaborative Study between LabCorp and Cerilliant

- LabCorp evaluated the Cerilliant rT3 Certified Spiking Solution® by LC/MS/MS at two LabCorp sites.
- LabCorp testing indicated the Cerilliant spiking solution was 30 to 50% high relative to calibrators prepared in-house from powder rT3 obtained from Sigma-Aldrich.
- An investigational study was initiated at Cerilliant and LabCorp.

Cerilliant Comparison of rT3 Reference Materials

- The Cerilliant rT3 Certified Spiking Solution® made from Cerilliant neat material was compared to LabCorp stock solutions prepared from Sigma rT3 material and to a Cerilliant prepared check-standard made from Sigma rT3 material.
- Solutions were analyzed at 100 µg/mL against a Cerilliant calibration curve by both HPLC/UV and LC/MS/MS.
- Solutions were analyzed in two diluents to rule out diluent effects.

Sample	% Difference from nominal concentration	
	HPLC	LC/MS/MS
Cerilliant rT3: T-075 (100 ug/ml in Methanol/NH_3)	0.1	-1.6
Cerilliant rT3 (100 µg/ml in Ethanol)	0.7	5.1
LabCorp stock solution prepared from a different lot of Sigma material received ~4/24/12 (1 mg/ml in Ethanol, diluted to 100 µg/ml)	-14.6	-6.2
LabCorp stock solution prepared from Sigma material (100 µg/ml, Ethanol) received 061412	-9.0	-5.5
Sigma rT3 (100 µg/ml, Methanol/NH_3)	-7.5	-6.1
Sigma rT3 (100 µg/ml, Ethanol)	-10.2	-7.6

- LabCorp solutions (prepared from Sigma neat material with small weighings) were 9 to 14% low to the Cerilliant curve by HPLC. Difference by LC/MS/MS was ~5%-8%.
- The check-standard prepared from Sigma research grade material was ~7%-10% low despite adjustment for inorganic impurities and chromatographic purity by HPLC.
- The concentration difference can be attributed to the low purity and the impurity profile of the Sigma neat material.
- HPLC purity assignment assumes equivalent UV absorbance of impurities. For low purity materials such as Sigma rT3 neat material, differing UV response of the impurities and the parent analyte can become important and could be a contributor to the observed concentration difference. For high purity materials, the impact is minimal.

LabCorp Comparison of Thyroid Hormone Reference Materials in a Clinical LC/MS/MS Assay

LabCorp evaluated the Cerilliant rT3 Certified Spiking Solution® by LC/MS/MS at two sites, Burlington, NC (CET), and Esoterix, Inc (ESO). Initial results indicated the Cerilliant spiking solution was 30 to 50 % high relative to a calibrators prepared in-house from powder rT3 obtained from Sigma-Aldrich.

CET Prep Scheme for Cerilliant Material

Spike Concentration	Spike Volume	Final Concentration	Final Volume	Matrix
100 ug/mL	0.5 mL	100 ng/mL	500 mL	MeOH
100 ng/mL	0.5mL	100ng/dL	50 mL	CSS
100 ng/dL	1 mL	10 ng/dL	10 mL	CSS

CET Analysis of Cerilliant Material

	Thurs 4/19		Fri 4/20		Fri 4/28	
Replicate	10ng/mL	100ng/mL	10ng/mL	100ng/mL	10ng/mL	100ng/mL
1	15.904	159.609	14.822	153.170	15.001	148.793
2	15.262	155.929	14.105	148.417	15.220	141.454
3	15.132	157.358	14.452	151.136	NA	NA
4	16.333	158.349	14.511	151.451	NA	NA
Average	15.658	157.811	14.473	151.044	15.111	145.124
Bias (%)	56.578	57.811	44.725	51.044	51.105	45.124

Bias ~ 50%

Bias difference between LabCorp sites
- Different lots of Sigma neat material
- Different laboratory conditions (e.g. humidity),
- Different preparation schemes for stock solutions
- No clinical impact as production calibrators were prepared at one site and shared between sites.

ESO Prep Scheme for Cerilliant Material

uL	QS (mL)	Matrix	Using [ng/dL]	Final [ng/dL]
200	10	CSS	(ampoule)	200,000
500	10	CSS	200,000	10,000
50	10	CSS	200,000	1,000
1000	25	CSS	10,000	400
750	25	CSS	1,000	300
250	25	CSS	1,000	100
1250	25	CSS	1,000	50
250	25	CSS	1,000	10

Analysis of ESO Prepared Cerilliant Material by CET

Replicate	10ng/mL	50ng/mL	100ng/mL	300ng/mL	400ng/mL
1	13.729	69.906	145.534	437.324	629.449
2	13.335	70.613	145.594	491.225	619.899
Average	13.532	70.260	145.564	464.275	624.674
Bias (%)	35.320	40.519	45.564	54.758	56.169

Bias ~ 30%

When samples were shared between LabCorp facilities and analyzed by Esoterix, Inc.: Bias observed was ~30%

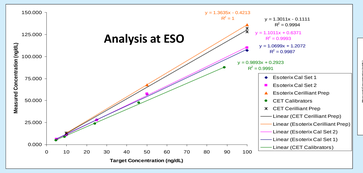
Analysis of CET Prepared Cerilliant Material by ESO

Replicate	10ng/mL	100ng/mL
1	12.700	128.000
2	13.100	132.000
Average	12.900	130.000
Bias (%)	29.000	30.000

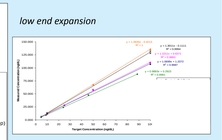
Bias ~ 30%

Analysis of ESO Prepared Cerilliant Material by ESO

Replicate	10ng/mL	50ng/mL	100ng/mL	300ng/mL	400ng/mL
1	13.3	67.6	136	419	546
Average	13.300	67.600	136.000	419.000	546.000
Bias (%)	33.000	35.200	36.000	39.667	36.500



low end expansion



Stock material from Sigma was not available for co-analysis between Cerilliant and LabCorp, therefore a new lot of material was obtained and analyzed by each company. Each company used their respective protocol to prepare stock solutions and it was shared between labs. Bias of Cerilliant material was consistent with previous data (~30%).

CET Prep Scheme for Cerilliant Material

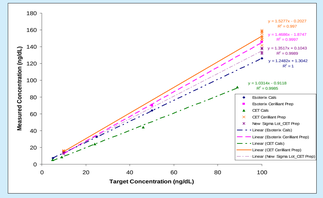
Spike Concentration	Spike Volume	Final Concentration	Final Volume	Matrix
100 ug/mL	0.5 mL	100 ng/mL	500 mL	MeOH
100 ng/mL	0.5mL	100ng/dL	50 mL	CSS
100 ng/dL	1 mL	10 ng/dL	10 mL	CSS

	CET Prep of Cerilliant Material Tues 6/12/12	
Replicate	10ng/mL	100ng/mL
1	14.156	131.744
2	12.814	137.612
3	13.266	138.328
4	14.249	133.410
Average	13.621	135.274
Bias (%)	36.213	35.274

LabCorp Prep Scheme for New lot of Sigma Material

- 4.82 mg rT3 (Lot PN121611-01) weighed out on balance.
- Ethanol volume adjusted for 90.28% purity* = 4.3514 mL (added w/ 4mL Class A volumetric pipette and Eppendorf research pipette)

*As determined by Cerilliant

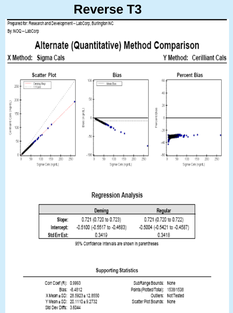


Calibrator prepared from Cerilliant Certified Spiking Solution® was determined to be more accurate and more suitable for use than the calibrator prepared from Sigma neat rT3. Calibrator was changed to use Cerilliant Certified Spiking Solution®.

Clinical Reference Ranges: Transformation of Reference Intervals

- The change in calibrator necessitated change in reference intervals for the method, requiring transformation and verification
- A study over multiple days and multiple batches was conducted to obtain a transform equation for LabCorp current reference intervals using the EP Evaluator shown below.
- The resulting slope and intercept difference (y= 0.721x - 0.51) was used to transform the existing reference intervals (based upon Sigma calibration) of adults (>16 years) from 13.5 – 34.2 ng/dL to 9.2 – 24.1 ng/dL and for children (1-15 years) from 12.2 – 32.4 ng/dL to 8.3 – 22.9 ng/dL.
- Transformed reference intervals were verified using 80 healthy adult specimens and 80 healthy children specimens.

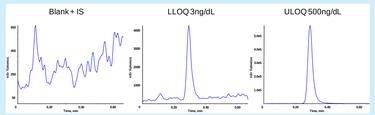
Reverse T3



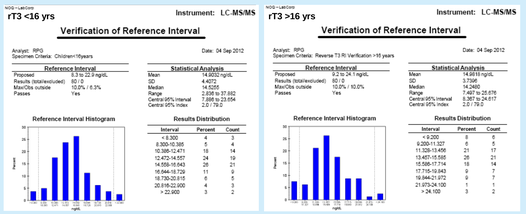
Slope (Deming) = 0.721
Intercept = - 0.51
Correlation Coefficient= 0.9993
Bias* = 29.66%

**Calculated as (bias divided by x-mean)*
*Bias %=(8.4812 / 28.5923) * 100*

Example Chromatograms



Verification of the transformed reference intervals using 80 healthy adult specimens and 80 healthy children specimens



Conclusion

- Proper characterization and certification of Reference Materials is critical for use in clinical diagnostic applications. The comparison of materials from various sources demonstrates that unless complete certification is performed, it is not possible to fully evaluate whether a material is suitable for use as a calibrator.
- Insufficient characterization and /or use of low purity research grade materials can result in incorrect therapeutic reference ranges and negatively impact clinical outcomes.
- The calibrator for the LabCorp method was changed to the Cerilliant Certified Spiking Solution® and reference ranges were re-qualified.
- Transformed reference intervals for adults (>16 years) changed from 13.5 – 34.2 ng/dL to 9.2 – 24.1 ng/dL and for children (1-15 years) from 12.2 – 32.4 ng/dL to 8.3 – 22.9 ng/dL. Transformed reference intervals were verified using specimens from 80 healthy adults and 80 healthy children.