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Vitamin D – Acceptable Performance in Routine Labs

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13th October 2011

Sydney Convention & Exhibition Centre Darling Harbour, Sydney NSW



Outline

- LC/MS/MS 25-hydroxyvitamin D (25OHD) analysis today
- Harmonisation of calibration material
- Standardisation (National Institute of Standards and Technology NIST)
 - NIST SRM 972
- Commercial response to NIST SRM introduction
- DEQAS 2010 results
- NIST VIT D EQA pilot study 2011 results
- Overview of c3-epi 25OHD3
- Chromatographic separation of c3-epi 25OHD3

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Misconceptions about mass spectrometry

- “Mass spectrometry is a reference method”
- “Mass spectrometry is different, it will give me the right answers”
- “Mass spectrometry is accurate / precise”

- Mass spectrometry has the POTENTIAL to be all of these, but ONLY if methods are carefully developed, calibrated and validated.

- In that respect LC-MS is no different from any other analytical tool in your laboratory.

- Main difference:
 - Responsibility** - As long as clinical mass spectrometry remains almost entirely “home-brew” then **YOU** are responsible for the performance of the assays that you run. IVD guidelines will have a major impact.

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DEQAS participants (25-OHD)

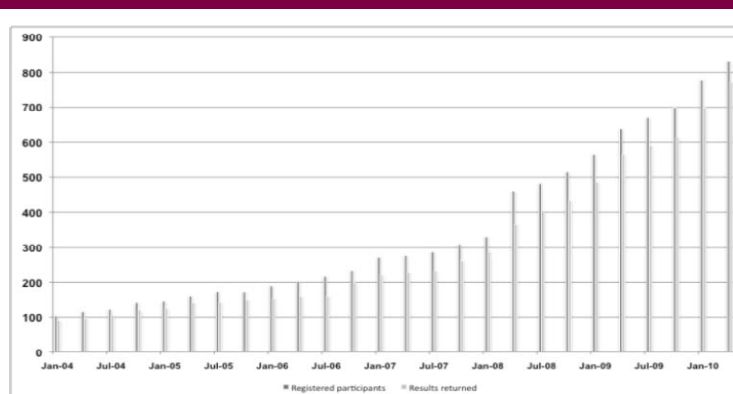


Fig.1 Number of DEQAS participants registered for the 26 distributions January 2004 to April 2010 and results submitted. (25-OHD)

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Mean inter-laboratory imprecision (CV%)

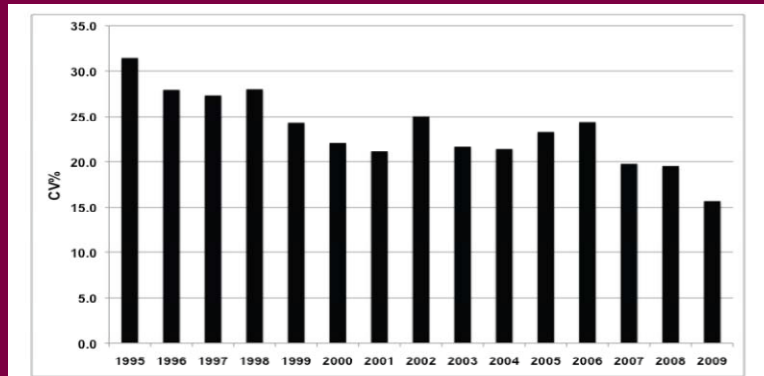
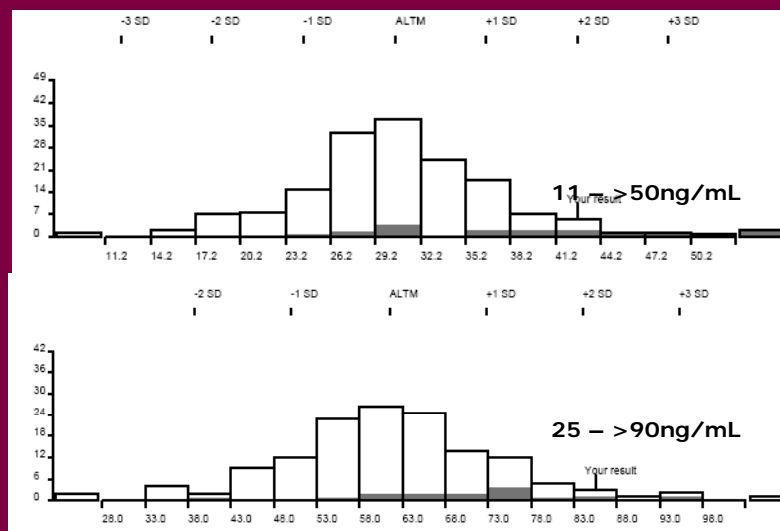


Fig 2. Mean inter-laboratory imprecision (CV%) of 25OHD results; distribution cycles since 1995

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LC/MS/MS Vitamin D analysis: 2007



Large spread of DEQAS results even in LC/MS/MS group

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Harmonisation of Inter-Lab Assays

Interlaboratory Variation in
25-Hydroxyvitamin D₂ and 25-
Hydroxyvitamin D₃ Is
Significantly Improved If
Common Calibration Material
Is Used

To the Editor:

Yates AM, Bowron A, Calton L, Heynes J, Field H, Rainbow S, Keevil B.

Interlaboratory variation in 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ is significantly improved if common calibration material is used.

Clin Chem 2008;54:2082-4

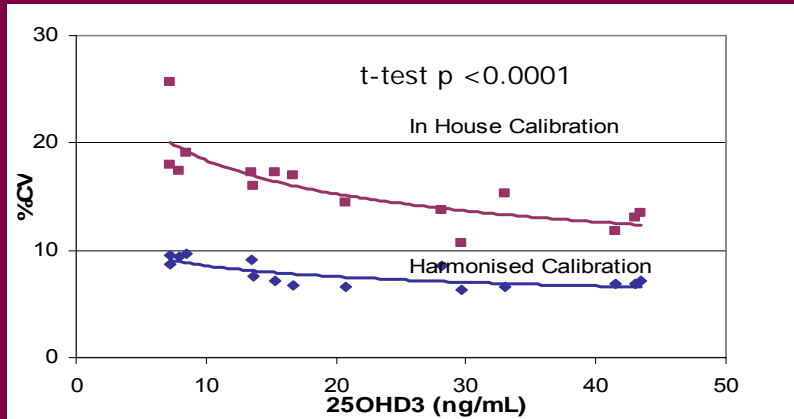
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Harmonisation of Inter-Lab Assays: Background

- This standardisation issue was highlighted at a UK Clinical User Meeting 2007
- Eight labs all running validated, routine clinical LC/MS/MS assays for 25OHD agreed to take part in an inter-laboratory study
 - Different LC and MS manufacturers
 - “Home-Brew”
 - Some using commercial standards (ChromSystems)
- Analysed 16 patient pools using their in-house calibrators and using harmonised calibrators

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Harmonisation of Inter-Lab Assays: Precision 25OHD3



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Harmonisation of Inter-Lab Assays: DEQAS

Short Report

Ann Clin Biochem 2009; 46: 79–81.

Use of a common standard improves the performance of liquid chromatography-tandem mass spectrometry methods for serum 25-hydroxyvitamin-D

Graham D Carter and Julia C Jones

Clinical Chemistry Department, Imperial College Healthcare NHS Trust, Charing Cross Hospital, London W6 8RF, UK
Corresponding author: Julia C Jones. Email: Julia.jones@imperial.ac.uk

'Use of a common standard improved agreement among laboratories using LC/MS/MS methods for 25OHD. This suggests that problems with assay standardisation contribute to inter-laboratory imprecision.'

- 25OHD2: Mean CV reduced from 21.1% to 12.6%
- 25OHD3: Mean CV reduced from 16.7% to 7.0%

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Harmonisation Study Results: Conclusions

- Variation in calibrators may explain the performance of MS in Proficiency Testing schemes
- Both inter-lab studies show the use of common calibrators should allow for the harmonisation of results between different laboratories
- What is required...
 - Harmonisation
 - SOP to prepare calibrators
 - Standardisation
 - NIST have now launched two Standard Reference Materials 972 and 2972

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Standardisation: New NIST SRM 972



- The new SRM will provide a mechanism for IVD manufacturers and clinical laboratories to identify and address variations in 25OHD measurements
- In-house calibrators and commercial calibrators and QCs can now be verified



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Standardisation: SRM 972 Certified Values

		ng/mL	nmol/L
Level 1	25OHD3	23.9 ± 0.8	59.6 ± 2.1
Level 2	25OHD2	1.71 ± 0.08	4.14 ± 0.19
	25OHD3	12.3 ± 0.6	30.8 ± 1.5
Level 3	25OHD2	26.4 ± 2.0	64.1 ± 4.8
	25OHD3	18.5 ± 1.1	46.2 ± 2.8
Level 4	25OHD2	2.40 ± 0.21	5.81 ± 0.52
	25OHD3	33.0 ± 0.8	82.3 ± 2.0
	3epi-25OHD3	37.7 ± 1.2	94.1 ± 2.9

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NIST Comparison: Commercial Material at launch

		NIST Target	UTAK	In house	Recipe	3 plus 1 ChromSys	Single ChromSys
		nmol/L	% Dev	% Dev	% Dev	% Dev	% Dev
Level 1	25OHD3	59.6 ± 2.1	+8.2	-3.7	+0.3	-17.3	+3.4
Level 2	25OHD3	30.8 ± 1.5	+7.1	-4.8	+0.8	-18.3	+2.3%
Level 3	25OHD2	64.1 ± 1.8	+15.4	+20.3	+5.3	+18.9	+20.1%
	25OHD3	46.2 ± 2.8	+13.2	+0.7	-3.6	-13.5	+8.2%

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NIST Comparison: Commercial Chromsystems Material now

		NIST Target	3 plus 1 ChromSys
		nmol/L	% Dev
Level 1	25OHD3	59.8 ± 2.0	-0.2
Level 2	25OHD3	30.8 ± 1.5	-3.6
Level 3	25OHD2	63.6 ± 4.8	-2.6
	25OHD3	46.3 ± 2.8	-2.1

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Discussion

- Inter-laboratory variation can be reduced to <10%CV through the use of harmonised calibration material
- Standardisation of the 25OHD assay has now been addressed with the launch of NIST SRM 972 and SRM 2972
- Are my reported 25OHD concentrations accurate?
 - Test your current calibration material against the NIST SRM 972 material (srminfo@nist.gov)
 - Subscribe to an EQA scheme
 - DEQAS, UK
 - NIST began a programme in 2011 which is free
 - There were a total of 41 participants in the Summer 2011 exercise. Seventeen of the datasets originated from immunoassay (IA) techniques and twenty-four from liquid chromatographic (LC) methods
 - vitdqap@nist.gov
 - RCPA, Australasia

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DEQAS results obtained using NIST traceable calibrators

L.C./MS/MS Total 25.OHD (nmol/L) for July 2010						Results for October 2010							
Summary of data; Mean results (trimmed if n= or >10)						Summary of data; Mean results (trimmed if n= or >10)							
Sample No.	376	377	378	379	380	Sample No.	381	382	383	384	385		
Standard (lot No.)						Standard (lot No.)							
CS* 0110	n=11	59.2	18.6	86.4	46.5	67.1	CS 0110	n=9	104.8	42.6	86.1	32.6	67.2
CS 4609	n=11	62.0	19.2	84.9	45.1	65.9	CS 4609	n=9	102.4	39.2	86.8	29.6	65.8
CG 0509	n=4	56.7	17.3	80.0	42.5	62.6	CG 0509	n=4	104.5	44.5	85.7	33.2	69.1
NIST	n=9	60.0	18.6	85.5	46.7	63.9	NIST	n=9	99.6	40.3	84.2	30.4	66.5
Others	n=14	59.6	18.3	83.1	45.2	65.6	Others	n=15	101.9	40.0	85.8	28.2	66.4
LC-MS/MS(All)	n=92	58.7	17.8	82.1	44.5	64.4	LC-MS/MS(All)	n=91	100.9	40.1	84.2	29.5	66.2
Summary of data; CV%						Summary of data; CV%							
Standard	n=11	8.5	10.8	9.5	8.7	10.5	Standard	n=9	11.1	7.2	14.3	11.5	8.9
CS 0110							CS 0110						
CS 4609	n=11	4.6	8.1	7.9	5.2	7.9	CS 4609	n=9	9.9	7.2	10.5	8.6	8.3
CS 0509	n=4	18.0	31.9	15.5	9.1	8.3	CS 0509	n=4	12.7	13.2	15.7	6.6	7.9
NIST	n=9	8.7	12.4	8.6	12.1	8.9	NIST	n=9	9.9	7.7	9.2	8.4	13.0
Others	n=14	7.7	14.1	6.5	8.9	6.7	Others	n=15	9.6	14.5	9.2	12.1	10.5
LC-MS/MS(All)	n=92	13.2	14.8	11.4	9.9	10.8	LC-MS/MS(All)	n=91	12.4	12.9	10.8	12.9	11.0

* ChromSystems

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Overview of the NIST Summer 2011 exercise

- For the Summer 2011 exercise of VitDQAP, ethanolic solutions with known concentrations of 25-hydroxyvitamin D₂ (25(OH)D₂) and 25-hydroxyvitamin D₃ (25(OH)D₃) were provided as control materials for assay calibration or verification (SRM 2972)
- In addition, participants were asked to determine 25-hydroxyvitamin D in four samples of human serum (study materials).
- Report an individual concentration value for 25(OH)D₂ and 25(OH)D₃ along with a total concentration of 25-hydroxyvitamin D (25(OH)D_{Total} = 25(OH)D₂ + 25(OH)D₃)
- However, all four serum materials contain very low levels of 25(OH)D₂ (reported values < 0.40 ng/mL)
- Overall, the control solutions appeared more compatible with the LC methods and several of the immunoassay participants reported that the calibration solutions were not compatible with their method and did not provide values.

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25(OH)D2 and 25(OH)D3 in the control solution (SRM 2972)

Table 1. Summary of participant data and community results for 25(OH)D₂ (ng/mL) and 25(OH)D₃ (ng/mL) in the SRM 2972 control solutions.

25(OH)D ₂ (ng/mL)			25(OH)D ₃ (ng/mL)		
Lab	Method	SRM 2972 Value	Lab	Method	SRM 2972 Value
052	LC-UV	261.5	052	LC-UV	344.0
096	LC-MS/MS	227.3	096	LC-MS/MS	321.3
110	LC-UV	233.4	110	LC-UV	331.7
116	LC-MS/MS	241.6	116	LC-MS/MS	331.0
119	LC-MS	243.2	119	LC-MS	352.6
139	LC-UV	241.5	128	LC-MS/MS	333.5
184	LC-MS/MS	236.5	184	LC-MS/MS	338.5
185	LC-MS/MS	238.6	185	LC-MS/MS	334.6
187	LC-MS/MS	235.0	186	LC-MS/MS	320.0
188	CLIA	401.5	188	CLIA	381.5
195	LC-MS/MS	242.0	189	LC-UV	290.9
196	CLIA	210.4	196	CLIA	452.0
197	LC-MS/MS	234.3	197	LC-MS/MS	342.5
198a	LC-MS/MS	245.5	198a	LC-MS/MS	308.6
199	LC-MS/MS	233.0	199	LC-MS/MS	329.0
200	RIA	216.0	200	RIA	319.1
202	LC-MS/MS	241.0	202	LC-MS/MS	334.5
209	LC-MS/MS	240.6	209	LC-MS/MS	332.8
210a	RIA	209.4	210a	RIA	299.1
211	LC-MS/MS	236.0	211	LC-MS/MS	337.1
212	LC-MS/MS	245.8	212	LC-MS/MS	341.7
215	LC-MS/MS	226.7	215	LC-MS/MS	366.2
217	LC-MS/MS	220.8	217	LC-MS/MS	364.3
220	LC-MS/MS	232.4	220	LC-MS/MS	324.4
221a	LC-MS/MS	253.0	221a	LC-MS/MS	265.0
221b	LC-UV	177.0	221b	LC-UV	214.0
All methods	N	26	All methods	N	26
	Median	237.3		Median	333.2
	MADE	8.0		MADE	18.5
	CV%	3.4		CV%	5.6
IA methods	N	4	IA methods	N	4
	Median	213.2		Median	350.3
	MADE	4.9		MADE	61.1
	CV%	2		CV%	17
LC methods	N	22	LC methods	N	22
	Median	239.3		Median	333.2
	MADE	7.3		MADE	15.0
	CV%	3		CV%	4
LC-MS methods	N	18	LC-MS methods	N	18
	Median	238.9		Median	334.0
	MADE	6.6		MADE	13.4
	CV%	3		CV%	4
NIST Value	U_{95}	238.6	NIST Value	U_{95}	334.0
		3.8			5.2

NIST/NIH Vitamin D Metabolites Quality Assurance Program
Gaithersburg, MD 20899-8392

NIST

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NIST pilot study method comparison

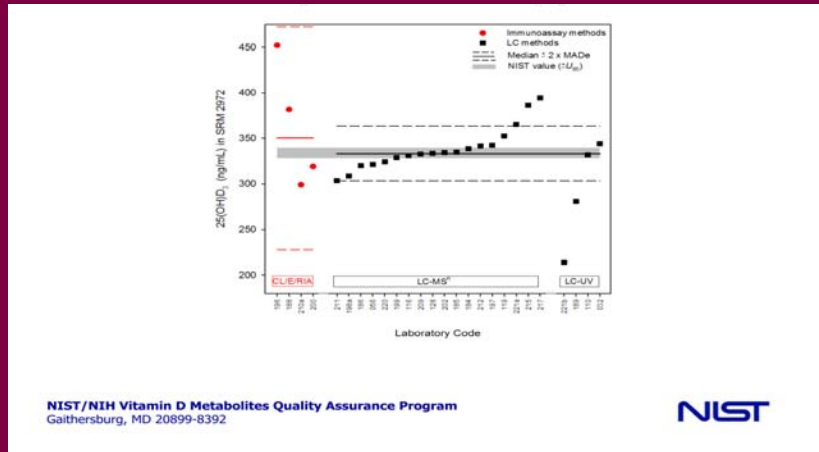
		N	7.90	14.70	45	43
All methods	Median	45	7.90	14.70	22.90	14.10
	MADE	45	1.48	2.08	3.71	1.93
	CV%	45	18.8	14.1	16.2	13.7
IA methods	N	17	17	17	17	17
	Median	17	8.00	16.00	27.08	15.90
	MADE	17	1.48	2.37	2.64	2.67
	CV%	17	18.5	14.8	10.5	16.8
LC methods	N	28	28	28	26	26
	Median	28	7.85	14.20	21.80	13.60
	MADE	28	1.41	1.56	2.00	1.11
	CV%	28	17.9	11.0	9.2	8.2
LC-MS methods	N	23	23	23	23	23
	Median	23	7.90	14.30	21.80	13.60
	MADE	23	1.11	1.33	1.93	1.04
	CV%	23	14.1	9.3	8.8	7.6
NIST Value	U_{95}	7.09	12.90	19.90	12.38	
		0.14	0.30	0.40	0.28	

NIST/NIH Vitamin D Metabolites Quality Assurance Program
Gaithersburg, MD 20899-8392

NIST

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Figure 1. 25(OH)D₂ and 25(OH)D₃ values in SRM 2972 for immunoassay and LC methods. The grey-shaded bars represent the ranges bound by the NIST certified values with $\pm U_{95}$ expanded uncertainty.

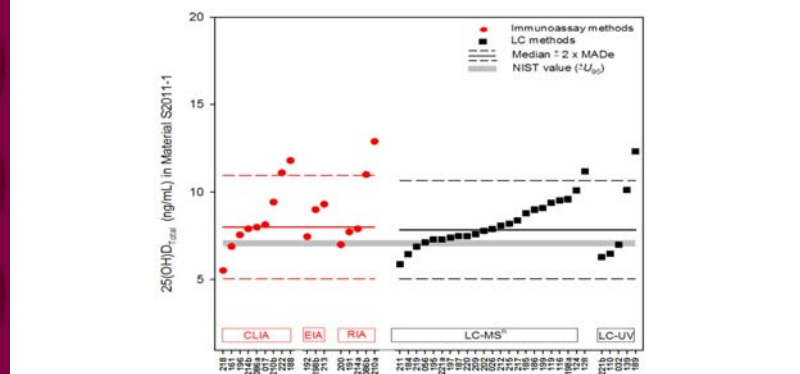


SOP on assaying ethanolic SRM 2972 not supplied

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25(OH)D in Materials S2011-1

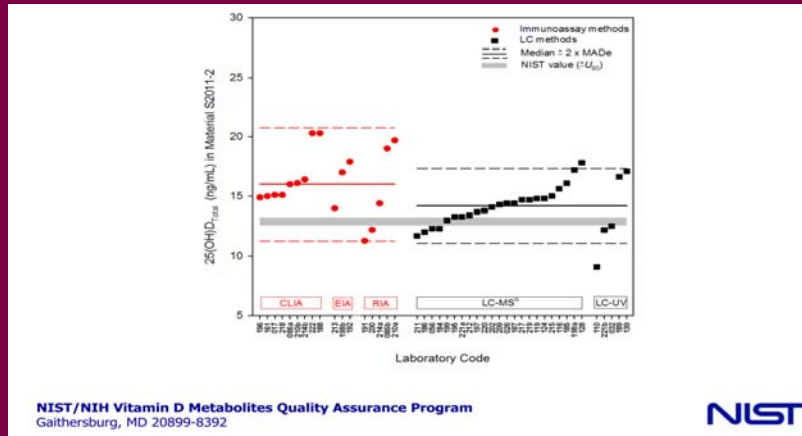
Figure 3. 25(OH)D_{Total} levels in materials S2011-1, S2011-2, S2011-3 and S2011-4 as determined by immunoassay (CLIA, EIA and RIA) and LC (LC-MSⁿ and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with \pm estimated U_{95} uncertainty.



NIST 25(OH)D_{Total} value (17.8 nmol/L)

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25(OH)D in Material S2011-2

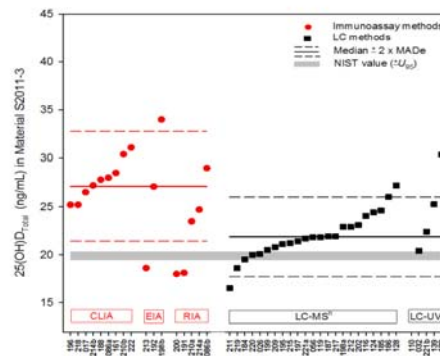


NIST 25(OH)D_{Total} value (32 nmol/L)

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25(OH)D in Material S2011-3

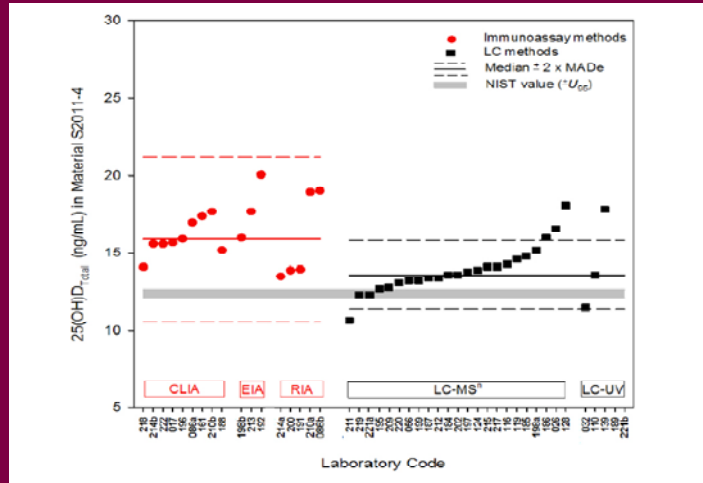
Figure 3 (cont'd). 25(OH)D_{Total} levels in materials S2011-1, S2011-2, S2011-3 and S2011-4 as determined by immunoassay (CLIA, EIA and RIA) and LC (LC-MS[®] and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with \pm estimated U_{95} uncertainty.



NIST 25(OH)D_{Total} value (50 nmol/L)

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25(OH)D in Material S2011-4



NIST 25(OH)D_{Total} value (31 nmol/L)

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Impact of C3 epimer on NIST results

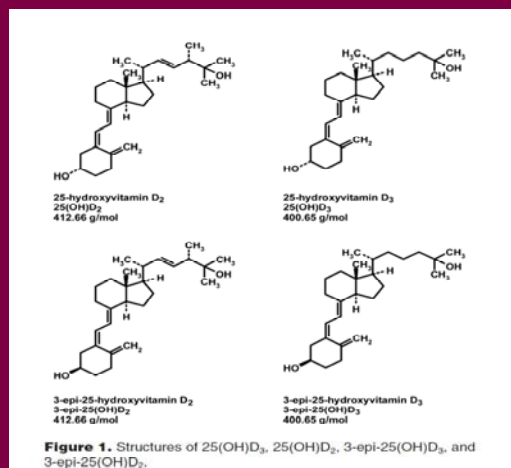
- It is notable that the NIST method separates 25(OH)D₃ and its 3-epimer, 3-epi-25(OH)D₃, which was not quantitated in the study materials.
- The 3-epi-25(OH)D₃ co-elutes with 25(OH)D₃ using typical chromatographic columns (C8, C18) and is detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV, leading to a potential bias for LC-based methods.
- One of the LC-MS/MS participants (number 56) noted using a method that separates 3-epi-25(OH)D₃ and provided values for this analyte in the study materials.
- However, the 25(OH)D₃ values reported by LC participants that use C8 and C18 columns represent the sum of 25(OH)D₃ and 3-epi-25(OH)D₃, and 25(OH)D_{Total} also includes a contribution from 3-epi-25(OH)D₃.
- 3-epi-25(OH)D₃ in all 4 samples ≤ 1 ng/mL (≤ 2.5 nmol/L)
- It is unclear how the presence of 3-epi-25(OH)D₃ affects the 25(OH)D_{Total} results from immunoassay results

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Overview C3-epimer 25OHD3

- The C3-epimer of 25OHD3 has been identified as a potential interference in the assessment of vitamin D sufficiency. Mass and fragmentation patterns are the same as 25OHD3 resulting in overestimation of 25OHD3.
- The clinical significance remains unclear.
- The C3-epimer of 25-hydroxyvitamin D₃ (25OHD3) differs only in the asymmetrical arrangement of a hydroxyl group in position C3, making chromatographic separation difficult.
- In 2006, Singh et al described a chiral chromatography method to partially separate these compounds. The study concluded that the C3-epimer was primarily detected in infants and not adults.
- More recently, NIST described a candidate reference procedure for the measurement of 25OHD3 in serum using extended reversed-phase HPLC tandem mass spectrometry that demonstrated baseline resolution of the C3-epimer from 25OHD3.

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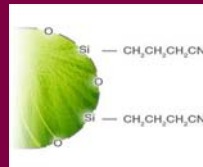
HPLC Vs UPLC

- Traditional HPLC run time is > 20 min
- Some methods employ column switching and complicated sample preparation.
- UPLC system combined with sub 2 micron particle column, run time <10 min. Baseline resolution of the C3-epimer from 25OHD3.
- Sample preparation identical to current 25 OHD3 method
- In-house calibrators assessed via NIST SRM 972 Level 4
- Poster # 80

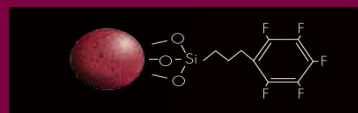
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C3-epimer chromatographic separation

- Chromatographic separation of both analytes is required before introduction into the MS
- Currently 2 stationary phases have been used successfully
 - CN

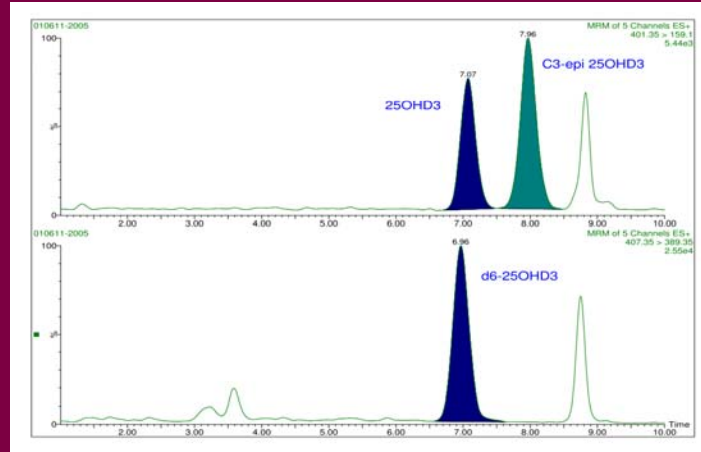


- Fluoro-Phenyl



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Chromatographic separation of 25OHD3 and c3-epi 25OHD3



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Conclusions

- Assay calibration & validation is just as important for mass spectrometry-based assays as any other assay that you use.
- There is an international effort (JCTLM) to develop resources to improve standardisation based on the principles of metrological traceability.
- Where reference standards and reference measurement services exist use them.
- Where they don't exist, it is your responsibility to use the best available means to standardise your assay.
- IVD guidelines will have a major impact on clinical mass spectrometry

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Final Thought

“There are many challenges facing the implementation of LC-MS in the clinical laboratory. But there is no doubt that this technology is making an impact on the diagnosis and treatment of patients now and will be increasingly so in the future”