Introducing Liquid Chromatography - Mass Spectrometry into the Clinical Laboratory: Using Vitamin D Testing as a Practical Example

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ABSTRACT

Objective: Five years ago our vitamin D (VitD) testing volume and the associated reference laboratory cost increased exponentially. We were unable to identify an automated VitD immunoassay for our existing chemistry instruments and were not satisfied with the performance of other immunoassay platforms. Although this hospital laboratory had no previous experience with mass spectrometry, we decided to open an LCMSMS laboratory section for VitD testing and, depending on the success of our experiment, expand testing in the future.

Methods: We developed our in-house LCMSMS assay for measurement of serum 25(OH) vitamin-D2 and -D3 (OHD2, OHD3, respectively). Our method was calibrated to the NIST SRM 2972 standard by value assignment to the albumin based calibrators. Sample preparation involves addition of hexa-deuterated internal standard (IS) and protein crash, followed by reversed phase separation on a 2.1x50 mm C18 column. MS analysis is performed by APCI in the positive mode. Two MRM transitions are monitored for the analytes and one for the IS.

Results: Assay development, validation and technologist training took approximately 8 months and we had to train a second MT shortly after going live to assure continuity of testing. Injection to injection time is 6.5 minutes, allowing testing of 120-130 patient samples a day and we perform testing 4-5 days per week. Sample preparation takes place during the day shift and the LCMSMS is run overnight unattended. Each batch contains a 6-point calibration curve and 3 levels of QC material. Each run is reviewed by a technologist according to set criteria before reporting results. Calibrator lots are prepared every three months and assay drift is monitored by NIST reference material and CAP proficiency subscription. No calibration drift has been detected during >4 years of continuous operation. Assay AMR are 6-200 and 4-200 ng/mL for OHD2 and OHD3, respectively. Assay CVs are 4% - 10% throughout the AMR. Total VitD is reported as the sum of OHD2 and OHD3. The assay has no interference from lipemia or hemolysis. When compared to CDC target, our in-house VitD assay remains within acceptable limits, regardless if OHD2 is present or absent in the proficiency samples while the commercially available VitD immunoassays all exhibit variable degree of bias depending on the VitD composition of the samples. Although the LCMSMS method has high start-up cost, return on investment (ROI) is extremely low (<<1 year) when compared to reference lab costs. ROI would be even faster in a commercial laboratory setting due to high dollar value of CMS reimbursement for VitD testing.

Conclusion: Introducing LCMSMS method in a hospital laboratory requires significant initial monetary investment and, due to the lack of familiarity with LCMSMS techniques by medical technologists, initial training and development of standard operating procedures are time consuming. However, the very low cost of ongoing testing provides rapid ROI. Additionally, LCMSMS provides a stable analytic platform that contribute to improved patient care. Because of our positive experience with LCMSMS we have installed additional systems and are expanding our mass spectrometry based testing.

INTRODUCTION

NKF clinical practice guidelines for bone metabolism in chronic kidney disease (Am J Kidney Dis. 2003 Oct;42(4 Suppl 3):S1-201)

- Phosphorous, total Ca, PTH and **25-OH vitamin D** should be measured
- Target concentrations and frequency of measurement are dependent on CKD stage (eGFR)
- 30%+ of CKD patients are vitamin D deficient

Follow up studies in subjects w/o kidney disease suggested vitamin D deficiency is common in US population

Vitamin D testing trends at our institution:

Calendar Year	Vitamin D Orders/Yr	Increase (x fold)	Annual Sendout Cost ($)
2005	250		$7,500.00
2006	1,320	5.3	$39,600.00
2007	3,600	14.4	*$68,400.00
2008	4,755	19	$90,300.00

Vitamin D sendout cost became our largest single reference lab expense; testing had to be performed in house.

Commercial vitamin D immunoassays in 2007:

- Diasorin RIA
- Various EIAs
- BioRad HPLC (not FDA approved in US)
- Major manufacturers had assays “in development”

The results from different assays didn’t match each other.

No assay was available for our chemistry or immunoassay platform.

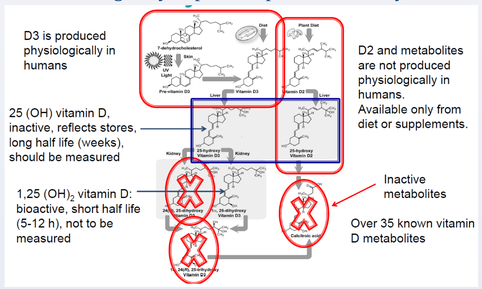
Didn’t want to purchase a new, dedicated analyzer just for vitamin D testing.

Can we develop our own assay, using LCMSMS?

LCMSMS METHOD DEVELOPMENT

Analytical challenge of vitamin D testing:

The many isoforms of vitamin D require special analytical techniques to measure the biologically important species accurately



Performance goals for our vitamin D method:

Selectivity must measure 25-OH vitamin D2 and 25-OH vitamin D3 without interference from other vitamin D species

Sensitivity shall be in the ng/ml (ppm) range

Imprecision must be <10% CV at deficient (low) vitamin D concentrations

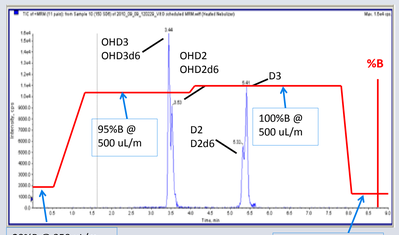
Cost must be low: Liquid-liquid extraction (protein crash) sample prep

Ion suppression elimination: Deuterated IS will be used

Injection-to-injection time must be <10 min to be able to handle current and projected workload (~120 samples/day plus calibrators and QC)

Chromatography - final assay conditions:

Phenomenex C18 column, 50 mm x 2.1 mm x 3 um
Binary buffer system (Buffer A: H2O, Buffer B: Acetonitrile)
Column temperature: 55 C
Run time: 6.5 min



MSMS conditions:

APCI positive mode
sMRM acquisition
Each run is reviewed manually before reporting results

Compound	MRM 1	MRM 2
OHD2	395/269	395/159
OHD3	383/365	383/257
D2	397/379	397/271
D3	385/259	385/367
OHD2-d6 (IS)	401/209	--
OHD3-d6 (IS)	389/371	--
D2-d6 (IS)	403/385	--

IS, Calibrators and QCs:

Vitamin D2, D3, 25OH-D2, 25OH-D3 are from Sigma / Fluka
Liquid calibrators are prepared in 3% BSA at 6 levels, from 5 – 150 ng/mL

Assigned concentrations of each batch of calibrators are directly derived from NIST SRM 2972 standard material and checked against NIST SRM 972 serum based calibrators before use

Hexa-Deuterated IS (25OH-D2d6, 25OH-D3d6 and D2d6) from Medical Isotopes are prepared in 80% ACN : 20% MeOH

Commercial QCs are obtained from UTAK Laboratories at 3 concentrations

RESULTS

Lead time from installation to live production:

Assay development time, method validation, writing the SOP and training of one technologist took 8 months

Vitamin D LCMSMS assay performance summary:

Assay working range – AMR (established by S/N ratio calculation):

- OH-D2: 6-200 ng/ml, LLD < 4 ng/ml
- OH-D3: 4-200 ng/ml, LLD < 1 ng/ml

Assay imprecision (CV):

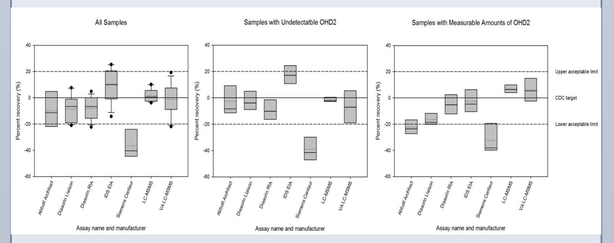
	~10 ng/mL	~30 ng/mL	~70 ng/mL
OH-D2	8.9%	7.1%	7.6%
OH-D3	9.0%	6.3%	5.5%

Carry over: not detected up to 5,000 ng/ml

No interference from hemolysis or lipemia

Sample requirements: 1 mL serum (red or gold top) preferred, EDTA and heparin samples acceptable

Comparison of the performance various vitamin D assays on the CAP ABVD proficiency events:



Commercial immunoassays show different degree of bias depending on the presence or absence of 25-OH vitamin D2 in the sample.

Our LC-MSMS methods remains within CDC recommended limits regardless of the 25-OH vitamin D2 content of the sample.

No calibration drift detected through 19 lots of calibrators.

Financial analysis of in-house, LCMSMS vitamin D testing:

Test prices	Reference lab to preferred client	$18.00
	Reference lab to small volume client	$38.00
	Request-A-test, direct to consumer lab (https://requestatest.com/vitamin-d-25-hydroxy-testing)	$59.00
	Hospital lab billing to patient	$148.69
Reimbursement	CMS (CPT 82306)	$52.00
	BC/BS	$77.32

Realized savings by in-house testing

Calendar year	Number of reportable results	Annual savings
2010	12,900	$249,000.00
2011	16,500	$260,000.00
2012	19,000	$298,000.00

Annual savings was calculated by using the lowest contracted price from a reference lab, multiplied by the number of tests for the calendar year, then the actual cost of testing by LCMSMS was subtracted from the product.

Return on Investment (ROI): less than one year.

Receiving CMS reimbursement can make vitamin D testing by LCMSMS revenue (profit) generating.

CHALLENGES, UNIQUE TO LCMSMS

High acquisition cost
- $250,000 - $600,000 depending on configuration

No FDA approved methods are available
- Method development and validation must be accomplished by the testing lab
- Method validation requires extra effort due to FDA regulations of LDTs

Expertise is not readily available in clinical laboratories
- Few pathologists / laboratory directors are trained in LC-MSMS
- MTs are not taught chromatography and mass spectrometry in school

LCMSMS workflow is foreign to clinical laboratories
- Scaling up validated methods requires special expertise
- Preventive maintenance schedule, other logistics issues must be developed in house

LESSONS LEARNED DURING OPERATION

Training and personnel
- At least two fully trained technologists are needed for continuous operation

Documentation
- Maintenance, column, reagent logs and acceptance testing records must be developed before go live

Maintenance
- Full service maintenance contract is mandatory for all instruments and components (compressor, gas generator, HPLC, MS)

Ancillary instrumentation is needed to optimize work flow
- Repeater pipettes, autosampler trays, labeler, bar code reader, robotics for sample preparation

Scheduling non-productive time (hardware maintenance, software upgrade) can be difficult with high workload
- Second instrument would be ideal but not necessary

SUMMARY AND CONCLUSIONS

LCMSMS has unique characteristics that makes it well suited for measurement of clinically important compounds.

LCMSMS has distinct advantages over immunoassays when the analyte of interest has numerous homologues.

The extra burden associated with developing, validating and performing LCMSMS based LDTs is manageable but special expertise is needed.

LCMSMS can be employed profitably in the clinical lab despite its high acquisition cost.

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