

## INTRODUCTION

1 $\alpha$ ,25-Dihydroxyvitamin D (1 $\alpha$ ,25(OH)<sub>2</sub>D), the biologically active form of Vitamin D, is responsible for calcium and phosphorous homeostasis through its actions on the GI tract, kidney and bone. Routine measurement of 1 $\alpha$ ,25(OH)<sub>2</sub>D is of greatest clinical importance in the investigation of PTH-independent hypercalcemia<sup>1</sup> which is sometimes caused by over-expression of CYP27B1 (1 $\alpha$ -hydroxylase) in granulomatous and lymphoid tissue. In addition to the well-known endocrine functions, there is an increasing body of literature elucidating the paracrine and autocrine actions of 1 $\alpha$ ,25(OH)<sub>2</sub>D and interest in quantifying this compound is growing accordingly.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the 'gold standard' for clinical steroid measurement offering advantages over traditional clinical immunoassay in both specificity and cost<sup>2</sup>. However, analysis of steroids by LC-MS/MS is not always straightforward. In the case of 1 $\alpha$ ,25(OH)<sub>2</sub>D, low circulating concentration, interferences from more abundant vitamin D metabolites, and low ionization efficiency hamper analysis.

We have developed an LC-MS/MS assay for analysis of 1 $\alpha$ ,25(OH)<sub>2</sub>D employing delipidation, immunoextraction with Immunodiagnostic Systems (IDS) bulk anti-1 $\alpha$ ,25(OH)<sub>2</sub>D coated beads, elution with ethanol, followed by derivatization with PTAD.

## MATERIALS

### MATERIALS

- 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 (Cerilliant)
- 1 $\alpha$ ,25(OH)<sub>2</sub>VD3-d<sub>6</sub> (Toronto Research Chemicals) and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2-d<sub>6</sub> (Medical Isotopes) internal standards
- MS Gold VitaminD free human serum (Golden West Biologicals)
- PTAD (Sigma Aldrich), 0.5mg/mL in Acetonitrile (Sigma Aldrich)
- Dextran Sulfate and Magnesium Chloride (Sigma Aldrich)
- Ethanol (J.T. Baker)

### CALIBRATORS

- Addition of 2 standard solution levels into MS Gold serum

Table 1: Calibrator levels

Level	1 $\alpha$ ,25(OH) <sub>2</sub> VD3 and 1 $\alpha$ ,25(OH) <sub>2</sub> VD2		
	Solution Concentration (pg/mL)	Amount of solution added ( $\mu$ L)	Final Concentration in serum (pg/mL)
Blank	---	---	---
Standard 1	500	5	2.5
Standard 2	500	10	5
Standard 3	500	20	10
Standard 4	5000	5	25
Standard 5	5000	10	50
Standard 6	5000	20	100
Standard 7	5000	40	200

## METHODS

### IMMUNOEXTRACTION

- 750uL of serum sample is mixed with 25uL of 8 ng/mL internal standards and allowed to equilibrate for 30 minutes at room temperature
- Serum is delipidated by adding 75uL of 5g/L Dextran Sulfate + 0.5M MgCl<sub>2</sub>, vortex mixing, followed by centrifugation.
- 500uL of delipidated serum is added to a 96 well plate containing 400uL of IDS anti-1,25(OH)<sub>2</sub>VD coated bead slurry.
- The plate is sealed and rotated end over end at room temperature for 90 minutes.
- The beads are transferred to a filter plate, and washed 6 x with 1mL aliquots of DI water, followed by elution of the 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 with 2 aliquots of ethanol.
- Eluants are evaporated to dryness at 70°C.

### DERIVATIZATION

- 50uL of 0.5 mg/mL PTAD in ACN is added to each sample, and left at RT for 1 hour for reaction to complete
- 50uL DI water is added to quench excess PTAD, and vortex mixed.

### LC PARAMETERS

- Shimadzu 20LC HPLC
- Phenomenex Luna C8 50x2mm 3 $\mu$  column, maintained at 45°C, with 4x2mm C8 guard column
- MPA: 0.1% FA in Water
- MPB: 0.1% FA in Acetonitrile

Table 2: Gradient parameters

Time (min)	Flow ( $\mu$ L/min)	%MPA	%MPB
0	500	65	35
0.1		65	35
4		5	95
5		5	95
5.1		65	35
6.5		65	35

### MS/MS PARAMETERS

- AB Sciex API5000 triple quadrupole mass spectrometer (electrospray ionization in positive mode)

Table 3: MRM s for analytes and IS

Analyte	Q1 Mass (Da)	Q3 Mass (Da)
1 $\alpha$ ,25(OH) <sub>2</sub> VD3	574.5	314.3
1 $\alpha$ ,25(OH) <sub>2</sub> VD3-d <sub>6</sub> IS	580.5	314.3
1 $\alpha$ ,25(OH) <sub>2</sub> VD2	586.6	314.3
1 $\alpha$ ,25(OH) <sub>2</sub> VD2-d <sub>6</sub> IS	592.6	314.3

## EXPERIMENTAL

### PRECISION

Pooled patient samples at LOQ, low, medium and high concentrations were analyzed for within-run, between-run, and total imprecision using modified Clinical Laboratory Standards Institute (CLSI) EP-5A document (quintuplicate analysis over four days).

## EXPERIMENTAL CONT'D

### LOQ and LOD

Limit of Detection (LOD) and Limit of Quantitation (LOQ) were estimated based on signal-to-noise calculations for low pooled samples.

### RECOVERY

1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 were spiked into a patient pool at levels of 10, 20, 50, 100 and 150 pg/mL. Observed recovery was compared with expected recovery.

### INTERFERENCE TESTING

High normal levels of 25-hydroxyvitamin D metabolites and 24,25-dihydroxyvitamin D metabolites were spiked into Mass Spec Gold Serum and pooled serum, and extracted as per the procedure.

### METHOD COMPARISON

Comparison was done with a commercial DiaSorin RIA assay (ARUP Laboratories) with 48 patient samples.

## RESULTS

### CALIBRATION

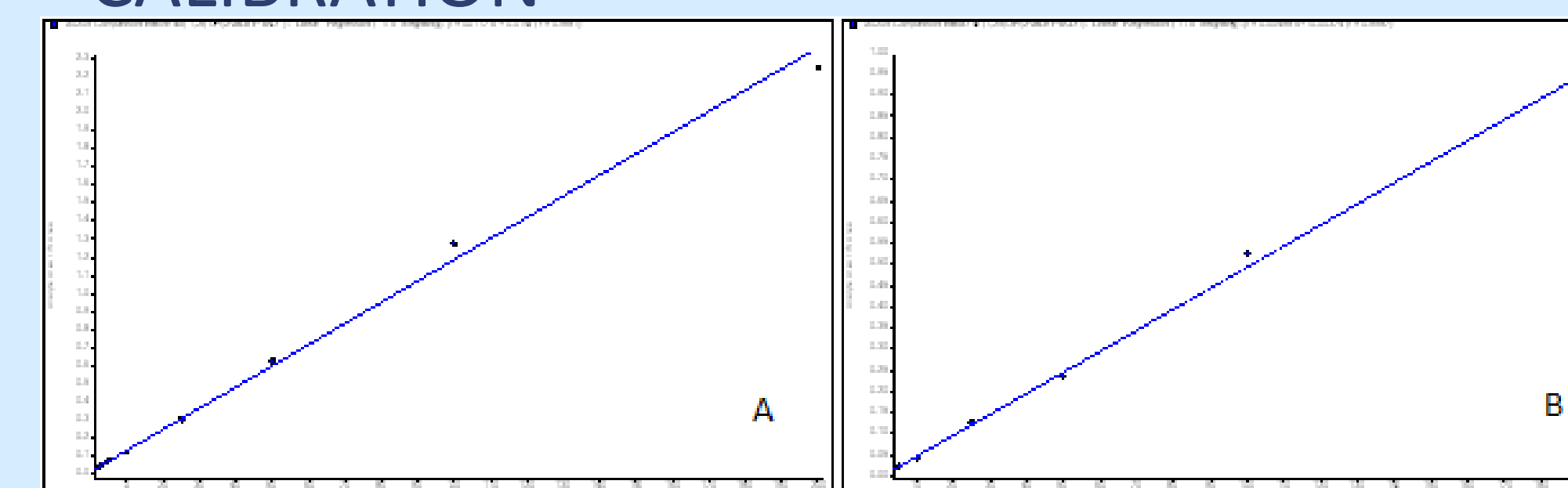


Figure 1: Calibration curves for (A) 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 from 2.5-200 pg/mL and (B) 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 from 5-200 pg/mL. Regression for both analytes is linear, 1/x.

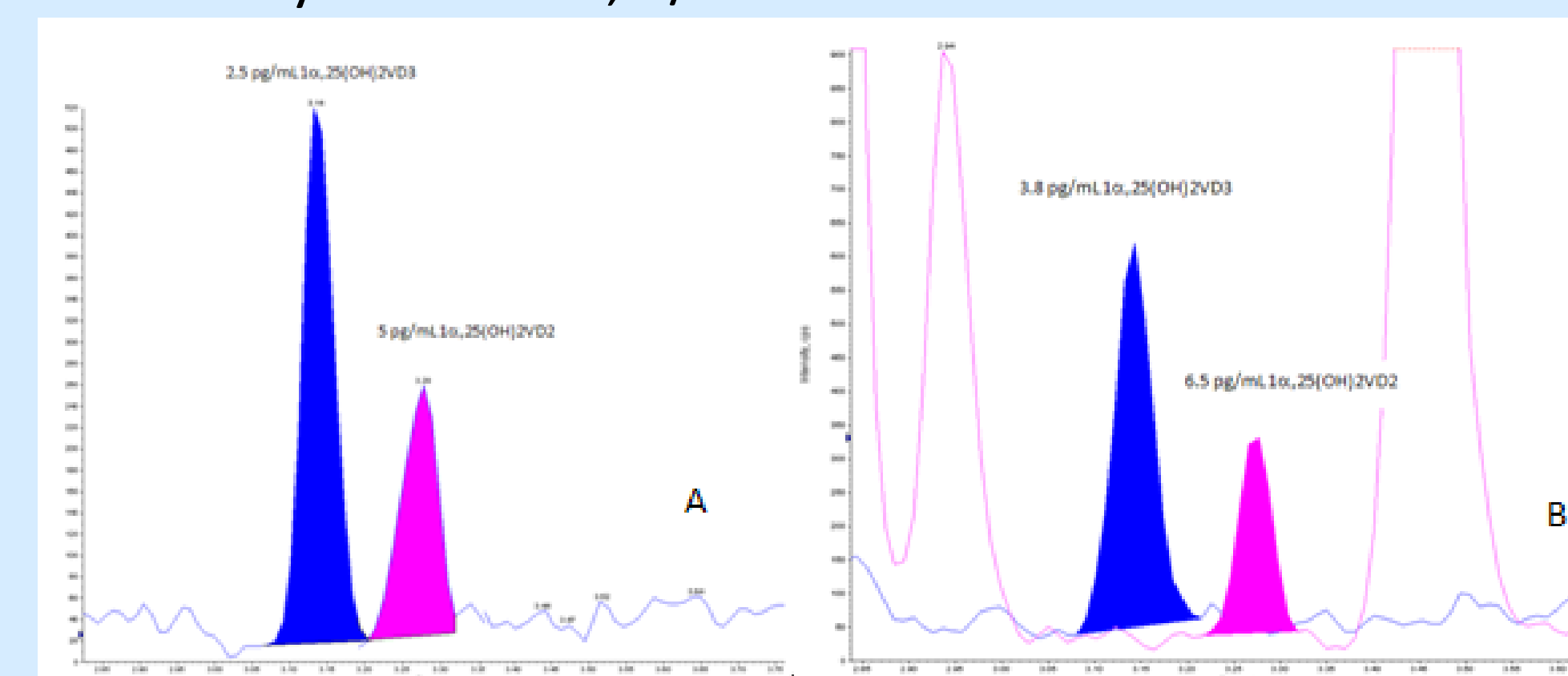


Figure 2: Representative chromatograms for (A) low level calibrator and (B) low level pooled patient sample.

### PRECISION

Table 4: Method imprecision using pooled patient samples, n=2-, except Medium n=19 (1 outlier removed) and High n=18 (2 outliers removed)

Precision Pool Level	1 $\alpha$ ,25(OH) <sub>2</sub> VD3				1 $\alpha$ ,25(OH) <sub>2</sub> VD2			
	Nominal conc (pg/mL)	WRCV (%)	BRCV (%)	TCV (%)	Nominal conc (pg/mL)	WRCV (%)	BRCV (%)	TCV (%)
LOQ	3.8	8.0	1.6	8.2	---	---	---	---
Low	7.1	7.2	3.3	7.9	6.5	10.2	9.8	14.1
Medium	33.8	8.4	7.8	11.5	16	7.5	3.1	8.1
High	84.0	5.1	4.8	7.0	54.1	6.9	3.9	7.9

### LOQ and LOD

Estimated LOQ based on S/N of 10:1 is 2.5pg/mL for 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 5 pg/mL for 1 $\alpha$ ,25(OH)<sub>2</sub>VD2. Estimated LOD based on S/N of 3:1 is <2.5 pg/mL for 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and <5 pg/mL for 1 $\alpha$ ,25(OH)<sub>2</sub>VD2.

## RESULTS CONT'D

### RECOVERY

Table 5: Recovery results for patient pool spiked with 10, 20, 50, 100 and 150 pg/mL of 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 .

Sample ID	Observed 1,25(OH) <sub>2</sub> VD3 (pg/mL)	Expected 1,25(OH) <sub>2</sub> VD3 (pg/mL)	1,25(OH) <sub>2</sub> VD3 Recovery (%)	Observed 1,25(OH) <sub>2</sub> VD2 (pg/mL)	Expected 1,25(OH) <sub>2</sub> VD2 (pg/mL)	1,25(OH) <sub>2</sub> VD2 Recovery (%)
	Recovery Pool	27.2	---	---	6.12	---
Recovery Pool + 10pg	33.9	37.2	91.1%	14.9	16.12	92.4%
Recovery Pool + 20pg	46.1	47.2	97.7%	25.1	26.12	96.1%
Recovery Pool + 50pg	83.9	77.2	108.7%	64.7	56.12	115.3%
Recovery Pool + 100pg	118.0	127.2	92.8%	91.5	106.12	86.2%
Recovery Pool + 150pg	174.0	177.2	98.2%	149.0	156.12	95.4%

### INTERFERENCE TESTING

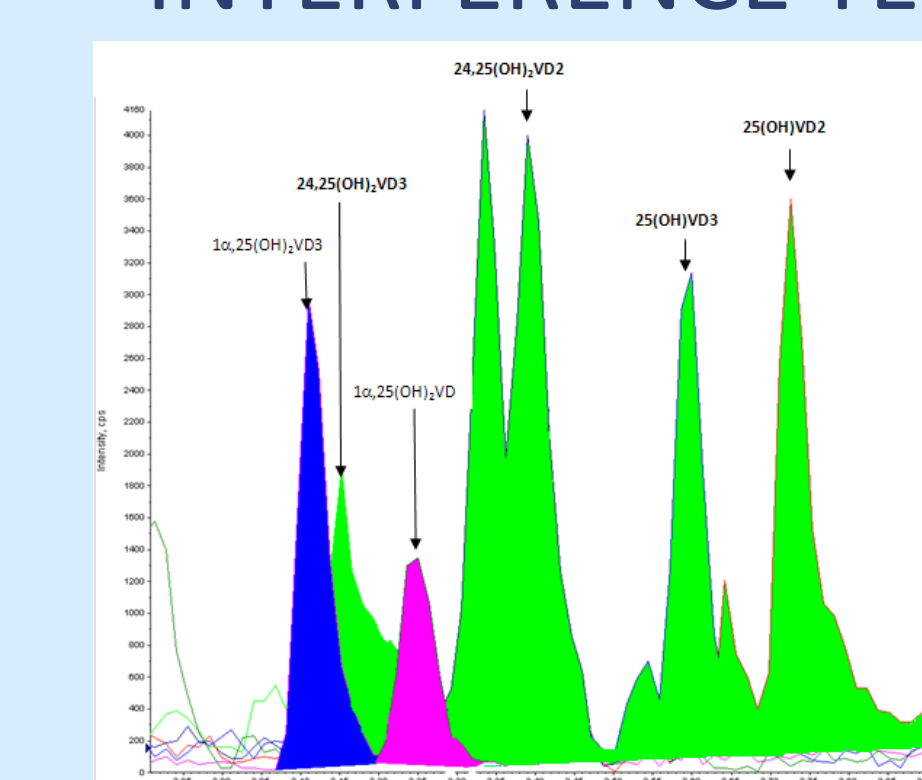


Figure 3: Overlaid chromatogram showing PTAD-derivatized MRM peaks for 1 $\alpha$ ,25(OH)<sub>2</sub>VD, 24,25(OH)<sub>2</sub>VD and 25(OH)VD metabolites in a pooled sample spiked with 10ng/mL 24,25(OH)<sub>2</sub>VD and 100ng/mL 25(OH)VD metabolites. Calculated concentration of the 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 is the same for the pooled sample with and without fortification of metabolites .

### METHOD COMPARISON

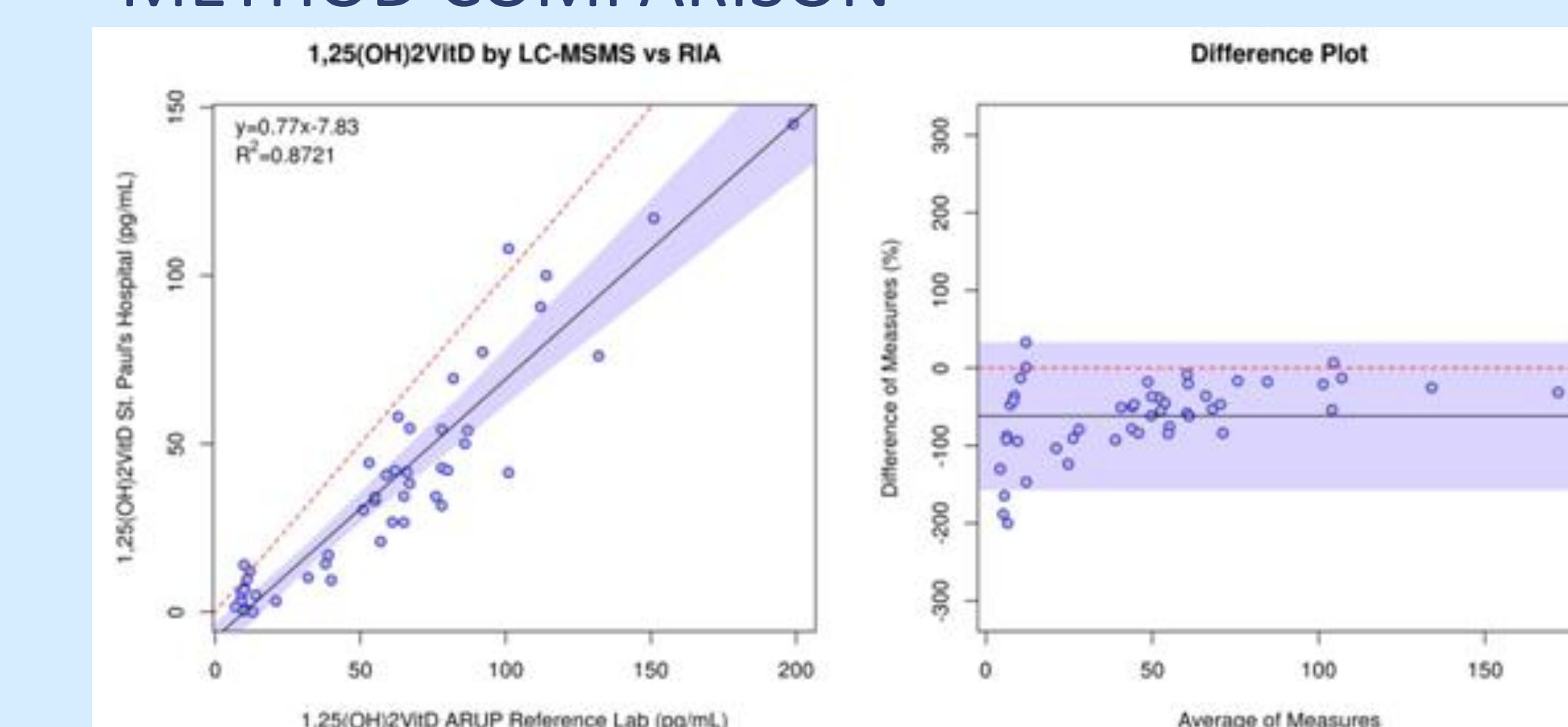


Figure 4: Method Comparison between SPH LC-MS/MS method and ARUP RIA method. Passing-Bablok regression -0.77x+7.83; r<sup>2</sup>=0.8721.

## DISCUSSION

A number of other methods of 1 $\alpha$ ,25(OH)<sub>2</sub>D analysis have been developed. Approaches are generally labour intensive and sample preparations have involved a combination of: protein precipitation, immunopurification, derivatization, and Li<sup>+</sup> adduct formation<sup>3,4,5</sup>. The present method is no exception to this but affords quantitation down to 2.5 pg/mL for 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 5 pg/mL 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 with total precision of 7.0-11.5% for 1 $\alpha$ ,25(OH)<sub>2</sub>VD3 and 8.1-14% for 1 $\alpha$ ,25(OH)<sub>2</sub>VD2 concentrations typical of patient care settings. The method differs from previously published approaches as it uses delipidation instead of generic protein crash and, like the method of Strathmann *et al*, has the benefit of employing the IDS immunopurification gel which is the less expensive of the two available commercial immunopurification products (IDS gel and the ImmunoDiagnostik Immunotube<sup>®</sup>). Investigation of suitability for routine clinical use is ongoing.

## REFERENCES

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