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INTRODUCTION

1 α ,25-Dihydroxyvitamin D (1 α ,25(OH)₂D), 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 α ,25(OH)₂D is of greatest clinical importance in the investigation of PTH-independent hypercalcemia¹ which is sometimes caused by over-expression of CYP27B1 (1 α -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 α ,25(OH)₂D 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 cost². However, analysis of steroids by LC-MS/MS is not always straightforward. In the case of 1 α ,25(OH)₂D, 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 α ,25(OH)₂D employing delipidation, immunoextraction with Immunodiagnostic Systems (IDS) bulk anti-1 α ,25(OH)₂D coated beads, elution with ethanol, followed by derivatization with PTAD.

MATERIALS

MATERIALS

- 1 α ,25(OH)₂VD3 and 1 α ,25(OH)₂VD2 (Cerilliant)
- 1 α ,25(OH)₂VD3-d₆ (Toronto Research Chemicals) and 1 α ,25(OH)₂VD2-d₆ (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 α ,25(OH) ₂ VD3 and 1 α ,25(OH) ₂ VD2		
	Solution Concentration (pg/mL)	Amount of solution added (μ 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

- 750 μ L of serum sample is mixed with 25 μ L of 8 ng/mL internal standards and allowed to equilibrate for 30 minutes at room temperature
- Serum is delipidated by adding 75 μ L of 5g/L Dextran Sulfate + 0.5M MgCl₂, vortex mixing, followed by centrifugation.
- 500 μ L of delipidated serum is added to a 96 well plate containing 400 μ L of IDS anti-1,25(OH)₂VD 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 α ,25(OH)₂VD3 and 1 α ,25(OH)₂VD2 with 2 aliquots of ethanol.
- Eluants are evaporated to dryness at 70 $^{\circ}$ C.

DERIVATIZATION

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

LC PARAMETERS

- Shimadzu 20LC HPLC
- Phenomenex Luna C8 50x2mm 3 μ column, maintained at 45 $^{\circ}$ 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 (μ 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 α ,25(OH) ₂ VD3	574.5
1 α ,25(OH) ₂ VD3-d ₆ IS	580.5	314.3
1 α ,25(OH) ₂ VD2	586.6	314.3
1 α ,25(OH) ₂ VD2-d ₆ 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 α ,25(OH)₂VD3 and 1 α ,25(OH)₂VD2 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

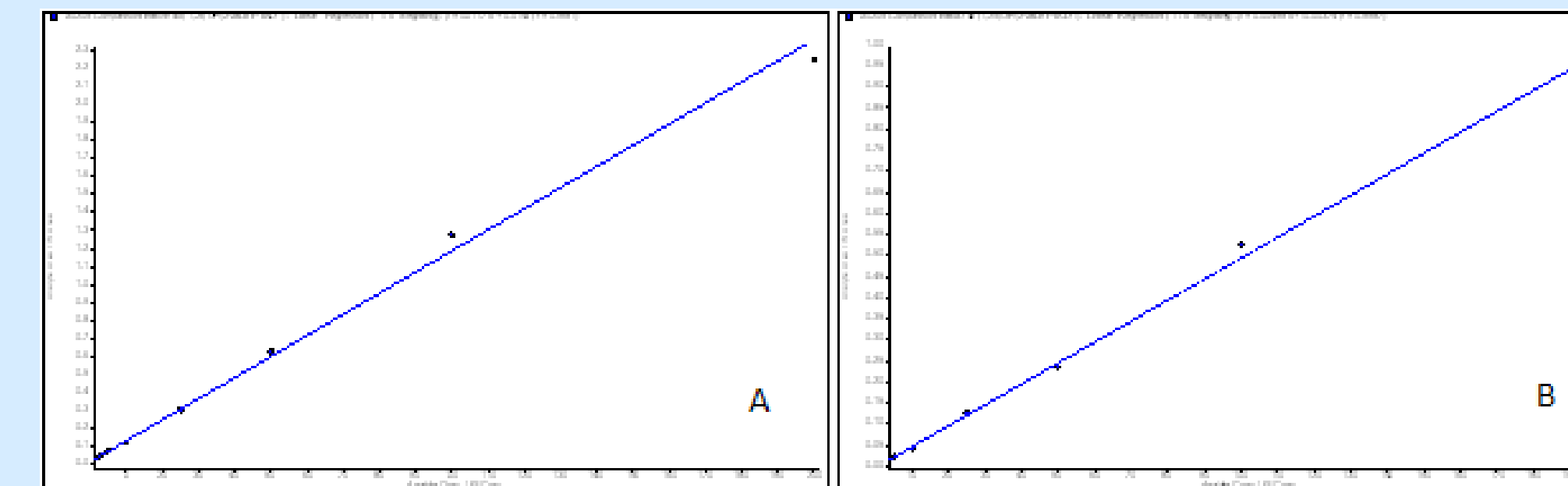


Figure 1: Calibration curves for (A) 1 α ,25(OH)₂VD3 from 2.5-200 pg/mL and (B) 1 α ,25(OH)₂VD2 from 5-200 pg/mL. Regression for both analytes is linear, 1/x.

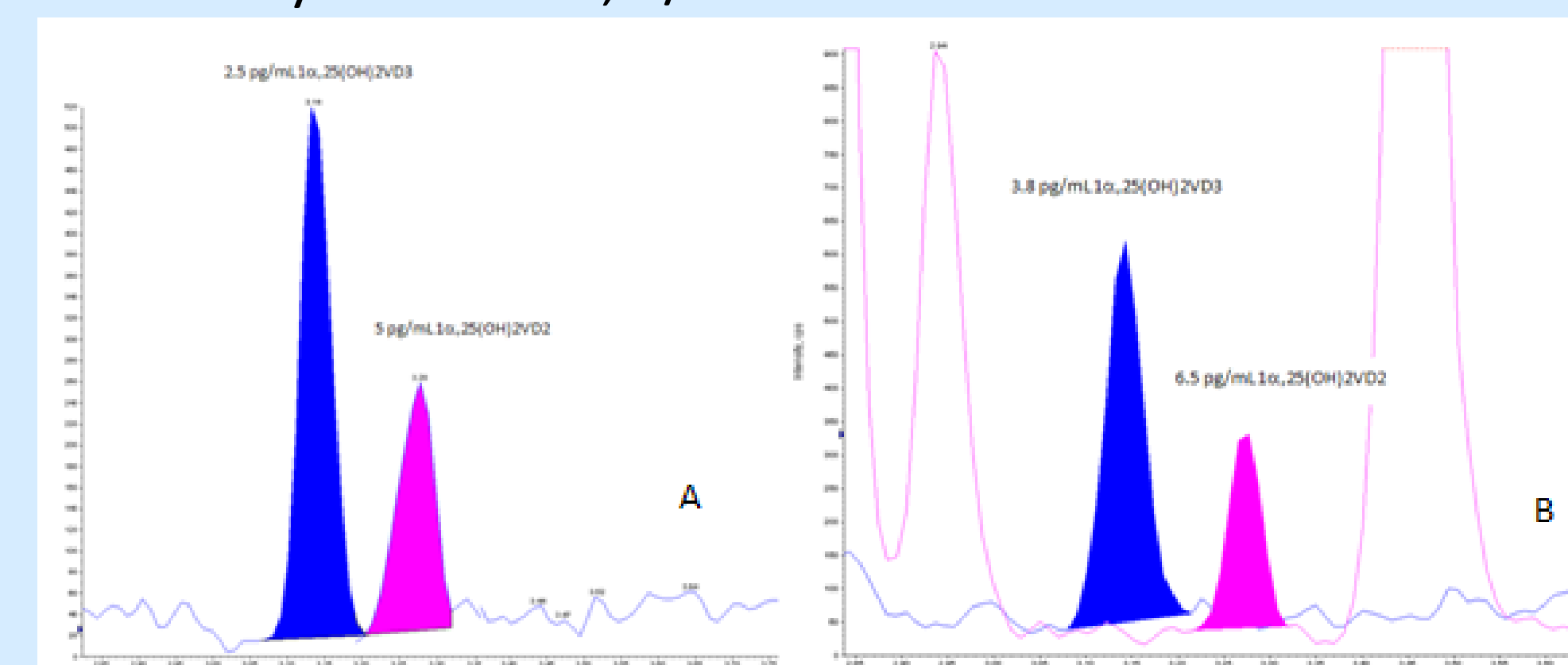


Figure 2: Representative chromatograms for (A) low level calibrator and (B) low level pooled patient sample.

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 α ,25(OH) ₂ VD3				1 α ,25(OH) ₂ 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 α ,25(OH)₂VD3 and 5 pg/mL for 1 α ,25(OH)₂VD2. Estimated LOD based on S/N of 3:1 is <2.5 pg/mL for 1 α ,25(OH)₂VD3 and <5 pg/mL for 1 α ,25(OH)₂VD2.

RESULTS CONT'D

RECOVERY

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

Sample ID	Observed 1,25(OH) ₂ VD3 (pg/mL)	Expected 1,25(OH) ₂ VD3 (pg/mL)	1,25(OH) ₂ VD3 Recovery (%)	Observed 1,25(OH) ₂ VD2 (pg/mL)	Expected 1,25(OH) ₂ VD2 (pg/mL)	1,25(OH) ₂ 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

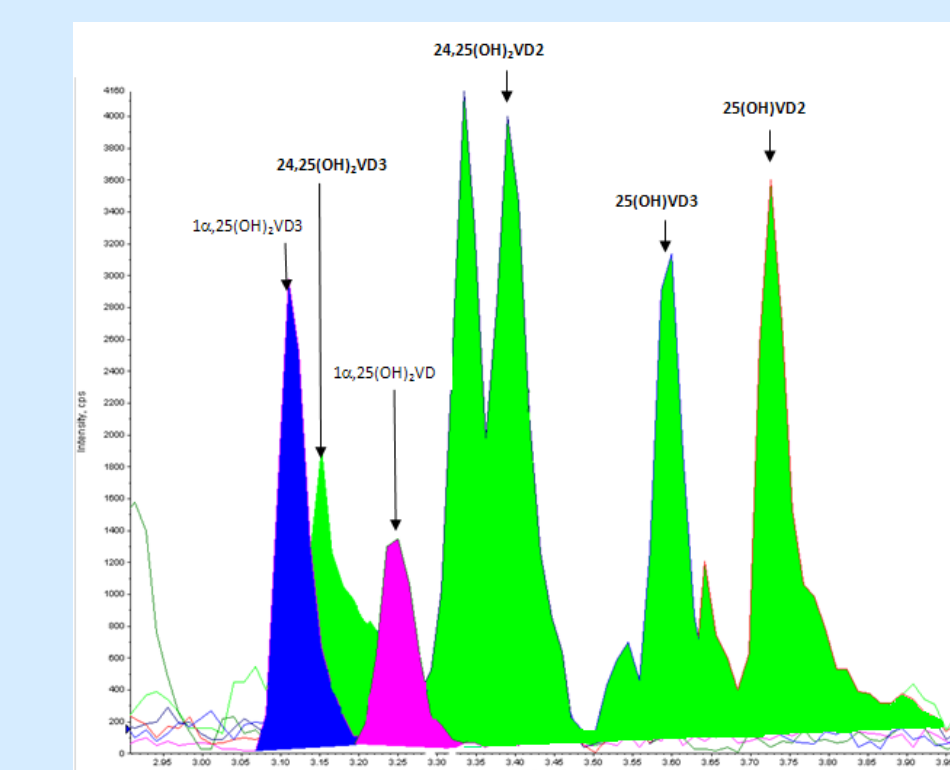


Figure 3: Overlaid chromatogram showing PTAD-derivatized MRMs for 1 α ,25(OH)₂VD, 24,25(OH)₂VD and 25(OH)VD metabolites in a pooled sample spiked with 10ng/mL 24,25(OH)₂VD and 100ng/mL 25(OH)VD metabolites. Calculated concentration of the 1 α ,25(OH)₂VD3 and 1 α ,25(OH)₂VD2 is the same for the pooled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

METHOD COMPARISON

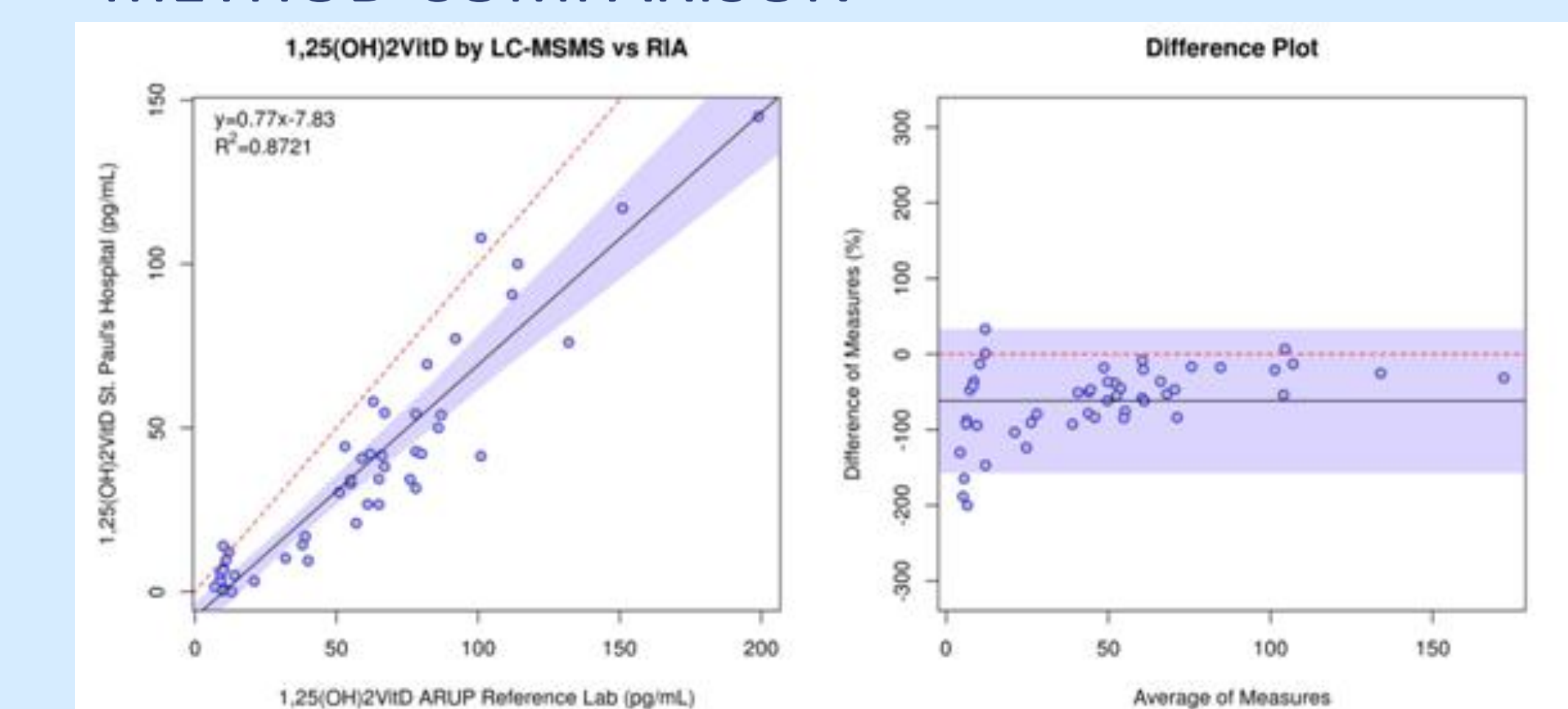


Figure 4: Method Comparison between SPH LC-MS/MS method and ARUP RIA method. Passing-Bablok regression -7.83+0.77x; r²=0.8721.

DISCUSSION

A number of other methods of 1 α ,25(OH)₂D analysis have been developed. Approaches are generally labour intensive and sample preparations have involved a combination of: protein precipitation, immunopurification, derivatization, and Li⁺ adduct formation^{3,4,5}. The present method is no exception to this but affords quantitation down to 2.5 pg/mL for 1 α ,25(OH)₂VD3 and 5 pg/mL 1 α ,25(OH)₂VD2 with total precision of 7.0-11.5% for 1 α ,25(OH)₂VD3 and 8.1-14% for 1 α ,25(OH)₂VD2 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[®]). Investigation of suitability for routine clinical use is ongoing.

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