# MSIA-SRM assay for parathyroid hormone and vitamin D binding protein: Correlation with clinical immunoassay methods and application to clinical samples.

David Taylor[1], Lewis Couchman[1], Bryan Krastins[2], Mary Lopez[2], Amol Prakash[2], David Sarracino[2], Maryann Vogelsang[2], Scott Peterman[2], Gouri Vadali[2], Sarah Robinson[3], Caje Moniz[1],

[1]King's College Hospital, London, UK, [2]ThermoFisher Scientific BRIMS Center, Cambridge, MA, USA, [3]ThermoFisher Scientific, Hemel Hempstead, UK,

## Introduction and Aims

PTH bioactivity resides in the intact molecule, PTH(1-84), however, various truncated inactive forms also circulate. Under certain conditions such as renal disease these fragments can accumulate, leading to variable cross-reactivity with the PTH immunoassays. To accurately quantify the ratio of intact hormone to its fragments is becoming increasingly significance for the diagnosis of endocrine and osteological diseases. Alongside PTH, vitamin D is also critical in the control of calcium homeostasis. Measurement of serum total vitamin D alone may be misleading because vitamin D and its metabolites circulate tightly bound to vitamin D-binding protein (VDBP), the concentration of which are known to be altered by pregnancy, liver and renal disease and, indeed, genotype.

Automated immunoenrichment prior to mass spectrometric analysis forms the basis of Mass Spectrometry Immunoassay (MSIA). Previously, we developed a multiplexed MSIA-single reaction monitoring (SRM) assay for PTH which allows quantification of four fully-tryptic monitoring peptides spanning the entire PTH sequence. Using this approach, it is possible to monitor intact PTH, as well as the degree of N-terminal fragmentation. In this study, the objective was to develop a multiplexed, MSIA-SRM-based targeted assay for VDBP and then apply the MSIA-SRM assays for PTH and VDBP to a cohort of clinical samples. Comparison of MSIA-SRM data for PTH was compared to a commercially available 'intact' PTH immunoassay.

## Methods

**Clinical Samples:** Patients were recruited following IRB-approved protocol. A total of 338 corresponding plasma and serum samples were analyzed (203 with renal impairment, 25 normal controls). Patient ages ranged from 16-88 with a mean age of 59.3 yrs. Gender distribution in the sample set was 48 % female and 51 % male.

**MSIA-SRM Assay Development:** Affinity extraction of PTH and VDBP was performed using MSIA pipette tips (absorbed with antibody) on a Versette pipetting robot (Thermo Scientific). Immune-captured material was eluted from the tips and subjected to trypsin digestion followed by liquid chromatography tandem mass spectrometry analysis. This employed a Hypersil Gold column on a TLX2 HPLC system and a TSQ Vantage triple quadrupole mass spectrometer, (both Thermo Scientific) as previously described. SRM assays were developed using Thermo Scientific Pinpoint software. Intact PTH levels were measured using a second generation methodology using an ADVIA Centaur platform (Siemens Healthcare).

## Results

**Immunoenrichment and quantification using MSIA–SRM (mass spectrometric immunoassay)**

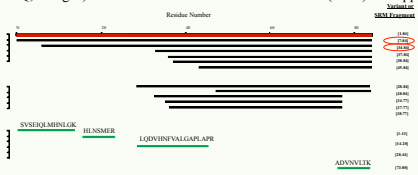
In order to develop a sensitive assay with sequence specificity for the PTH and VDBP peptides, we coupled immunoenrichment at the protein level with detection at the peptide level using SRM-MS (Figure 1).


**FIGURE 1. MSIA-SRM workflow for enrichment and quantification of low abundance proteins.**

### PTH MSIA-SRM development

**Peptide selection for PTH assay**

Following immunoenrichment, the PTH MSIA-SRM approach using four tryptic peptides spanning the length of PTH allows selective detection and quantification of specific isoforms and truncated variants of PTH (Figure 2) . Figure 3 shows calibration curves for the four tryptic peptides, all peptides behaved in a similar manner. Assay linearity was excellent, with $R^2$ values from 0.90-0.99. Assay CVs at or above the limit of quantification (LOQ, 16 ng/L) were below 10 %. The limit of detection (LOD) was approximately 5-8 ng/L.




MSIA antibody binding site = aa 67-79

**FIGURE 2. PTH variant map.**

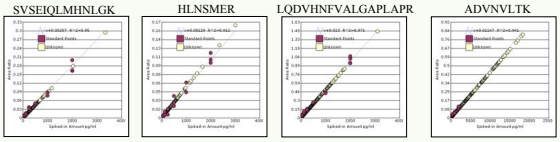



**FIGURE 3. PTH peptide SRM calibration curves.**

**Correlation with immunoassay**

Figure 4A demonstrates that good correlation exists between MSIA-SRM and immunoassay only when SRM results were averaged across all tryptic peptides. However, when the SRM values from the N-terminal, aa 1-13 tryptic peptide only were considered the correlation was poorer at 0.67. As expected, these results suggest that the immunoassay measures some inactive PTH fragments.

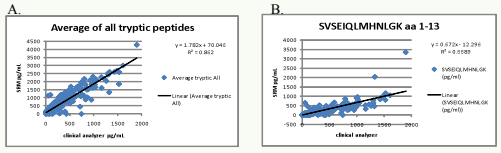



**FIGURE 4. Correlation graphs of SRM and clinical immunoassay data.**

**PTH heterogeneity in clinical samples**

As shown in Table 1, several PTH variants reported in the literature were detected in our samples. These include N-terminally truncated variants as well as phosphorylated PTH. Surprisingly, we were unable to detect the tryptic peptide corresponding to amino acids 7-13. This is consistent with an absence of PTH(7-84) in our clinical samples, despite this being proposed in the literature as a common PTH variant.

| Surrogate Peptide sequence | aa | Type | Observed in Clinical samples |
|---|---|---|---|
| SVSEIQLMHNLGK | 1-13 | tryptic, N terminal active site | yes |
| HLNSMER | 14-20 | tryptic, Internal | yes |
| LQDVHNFVALGAPLAPR | 28-44 | tryptic, Internal | yes |
| ADVNVLTK | 73-80 | tryptic, Internal | yes |
| | | | |
| FVALGAPLAPR | 34-44 | variant, previously observed or reported in literature | yes |
| VALGAPLAPR | 35-44 | variant, previously observed or reported in literature | yes |
| LMHNLGK | 7-13 | variant, previously observed or reported in literature | no |
| HLNS(phospho)MER | 14-20 phospho on S 17 | variant, previously observed or reported in literature | yes |

**TABLE 1. PTH variants detected in the clinical samples.**

**PTH variants in renal disease**

Using an SRM corresponding to amino acids 34-44, those with end stage renal failure (ESRF) could be clearly separated from those with CKD stages 2-5, as well as healthy controls (Figure 5A). However, when an SRM corresponding to amino acids 35-44 was used, no difference between ESRF, CKD stages 2-5 and controls was observed (Figure 5B). Finally, when samples were stratified according to CKD stage and immunoassay PTH result compared to that of the MSIA-SRM intact PTH (N-terminal tryptic peptide), gross overestimate of PTH by immunoassay is demonstrated in those with ESRF (CKD stage 1) (Figure 6).

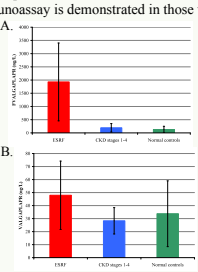



**FIGURE 5. SRMs corresponding to PTH amino acids (A) 34-44 and (B) 35-44 amongst patients with ESRF (n=203) CKD stages 2-5 (110) and normal controls.**

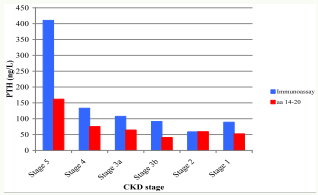



**FIGURE 6. Comparison of immunoassay data with MSIA-SRM data for amino acids 1-13 (MSIA-SRM intact PTH) for patients with chronic kidney disease (CKD).**

### Vitamin D binding protein (VDBP) MSIA-SRM development

**VDBP peptide selection and assay characteristics**

Figure 7A illustrates the amino acid sequence for VDBP. Highlighted in red are the tryptic peptide sequences selected for the SRM assay. Figure 7B shows calibration curves for 4 of the peptides, all of which demonstrated similar behavior. Linearity was excellent ($R^2$ 0.95-0.99) with CVs at or above the LOQ < 11 %.

**Correlation of VDBP SRM abundance values with Clinical groups**

Preliminary work comparing a group of patients with low bone density against controls suggests that low bone density is associated with higher levels of VDBP (mean 234 mg/L) than in patients with CKD (mean 214 mg/L). Whether such differences alter interpretation of serum total VitD requires further investigation.

A.

>sp|P02774|VTDB_HUMAN Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1

MKRVLVLLLAVAFGHALERGRDYEKNKVCKEFSHLGKEDFTSLSLVLYR
KFPSGTFEQVSQLVKEVVSLTEACCAEGADPDCYDTRTSALSAKSCESNSP
FPVHPGTAECCTKEGLERKLCMAALKHQPQEFPTYVEPTNDEICEAFRKD
PKEYANQFMWEYSTNYGQAPLSLLVSYTKSYLSMVGSCCTSASPTVCFLK
ERLQLKHLSLLTTLSNRVCSQYAAYGEKKSRLSNLIKLAQKVPTADLEDVL
PLAEDITNILSKCCESASEDCMAKELPEHTVKLCDNLSTKNSKFEDCCQEK
TAMDVFVCTYFMPAAQLPELPDVELPTNKDVCDPGNTKVMDKYTFEL
SRRTHLPEVFLSKVLEPTLKSLGECCDVEDSTTCFNAKGPLLKKELSSF
IDKGQELCADYSENTFTEYKKKLAERLKAKLPDATPKELAKLVNKRSDFAS
NCCSINSPPLYCDSEIDAELKNIL

B.

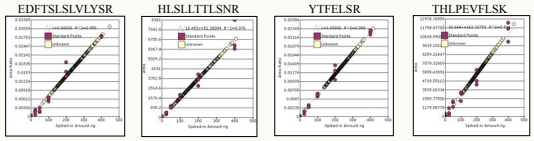



**FIGURE 7. Vitamin D binding protein (VDBP). A. Sequence and surrogate peptides for SRM, B. calibration curves for 4 of the 6 peptides.**

## Conclusions

We have developed multiplexed, high-throughput, quantitative and precise SRM assays for both PTH and VDBP. The MSIA-SRM approach allows rapid and automated enrichment to achieve high sensitivity (ng/L) and selectivity. Simultaneous monitoring of intact and variant PTH species allows the precise quantification of active and inactive forms. Correlation of the PTH MSIA-SRM assay using only the N-terminal peptide (aa 1-13) with the traditional immunoassay confirms that commercial immunoassays overestimate the amount of active PTH. Further, no evidence for PTH(7-84) was found in the data set. Further work will validate the utility of VDBP measurement in the routine assessment of vitamin D status.

**Contact:** David Taylor
Dept. of Clinical Biochemistry
King's College Hospital
London
SE5 9RS
davidtaylor8@nhs.net

**Reference:**
Lopez MF, Rezai T, Sarracino DA, Prakash A, Krastins B, Athanas M, Singh RJ, Barnidge DR, Oran P, Borges C, Nelson RW. Selected Reaction Monitoring-Mass Spectrometric Immunoassay Responsive to Parathyroid Hormone and Related Variants. Clin Chem. 2010 56:281-90