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Skyline Performs as well as Vendor Software in the Quantitative Analysis of Serum 25-Hydroxy Vitamin D and Vitamin D Binding Globulin

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With the continuing expansion of mass spectrometry into the clinical laboratory for the quantification of proteins and small molecules, laboratories are being equipped with different instrument platforms from different manufacturers. This requires specific training for laboratory personnel on each data analysis software package that is unique to each instrument, which can increase method deployment time and potentially increase errors in data analysis. Skyline is a freely available, vendor-neutral software package that facilitates quantitative analysis of both protein and small molecule mass spectrometry data (1). We implemented calibration features in Skyline and utilized two methods to demonstrate the new features and compare these results to those obtained from vendor software for small molecule and protein quantification.

Because the chromatographic characteristics are slightly different for peptides and small molecules, we quantitatively analyzed serum concentrations of 25-hydroxy vitamin D [**25(OH)D**] and vitamin D binding globulin (**VDBG**), as previously described (2, 3). Briefly, 25(OH)D was immunoaffinity enriched from 154 single-donor human plasma samples and the derivatized extracted analytes and stable isotope labeled analogs were chromatographically separated using Waters Acquity UPLC coupled on-line via an ESI source operating in positive ion detection mode for analysis by multiple reaction monitoring (**MRM**) on a Waters Xevo triple quadrupole mass spectrometer. Data were collected and 25(OH)D concentrations were calculated using TargetLynx software (Waters). The same MRM transition data were imported into Skyline-daily (Ver. 3.7.1.11271) for quantification utilizing the new calibration feature of the software package (http://skyline.ms/tquant.url). Explicit retention times were set for peak picking and integration using Skyline's peak integration algorithm. Manual adjustment of integration limits was not required for these data. For both the TargetLynx and Skyline-daily software environments, concentrations were calculated using the peak area response (**PAR**) and a five-point calibration curve with 1/x weighting.

For VDBG analysis, 164 single-donor human plasma samples were tryptically digested with isotope labeled peptides spiked prior to digestion and peptides were analyzed using the same instrumentation as for 25(OH)D. Peak area data were analyzed and VDBG concentrations were calculated based on a four-point calibration curve made from human serum diluted with chicken serum. Absolute quantification of VDBG was performed utilizing the PAR of two peptides, VLEPTLK and ELPEHTVK. The concentration of the two peptides was calculated using separate calibration curves and the results averaged for absolute concentration of VDBG. As for 25(OH)D, the data were quantified in both software environments using 1/x weighting.

Concentrations of 25(OH)D and VDBG calculated using TargetLynx and Skyline-daily were compared via Deming regression using the R statistical software (Ver. 3.3.2) and Deming package. Results for Deming regression analysis of 25(OH)D are represented in the Figure, where the slope and intercept were 0.992 (95% CI: 0.977 – 1.035) and 0.230 (95% CI: −0.126 – 0.586) ng/mL, respectively, indicating that there was no statistically significant difference between the calculated concentrations from the two software packages. Similarly, for VDBG, results from Deming regression gave a slope of 1.014 (95% CI: 0.977 – 1.050) and an intercept of 6.178 (95% CI: −3.028 – 15.384) μg/mL, respectively (Figure).

One of the most important potential benefits of vendor-neutral software in the clinical laboratory is that medical scientists would only need to learn one software package for data analysis, which would be particularly important in laboratories with multiple mass spectrometers from different vendors. With additional mass spectrometric experiments being developed, including data independent analysis (4), it will be important for data analysis software packages to be capable of quantifying peptides, proteins, and small molecules. Several vendor-neutral software packages exist for quantification of peptides and proteins (e.g. Maxquant, PEAKS, etc). However, fewer vendor-neutral programs are capable of quantifying small molecules. The commercially available software package Ascent, from Indigo Bioautomation, automates quantitative analysis of chromatographic and mass spectrometry data for clinical laboratories (5), but requires a license. Skyline is freely available, open source, and supported by an academic software team and a community of users. Software updates can be strictly controlled by the end-user and historical versions of Skyline are archived and available on the Skyline web site (the version number is embedded in the Skyline document). There is also an extensive number of tutorials that facilitate rapid training and implementation of the software in the laboratory and it can be deployed on as many computers as is needed for data analysis. There are several meta-analysis review features in Skyline that allow the user to quantitatively monitor the quality of individual samples and data points, e.g. internal standard peak areas and transition ion ratios. One drawback to Skyline is that it does not have direct connectivity to laboratory information systems, which is common to all mass spectrometry vendors (with some exceptions), but current efforts to standardize mass spectrometry data outputs will be helpful.

The results presented here demonstrate that Skyline and its new calibration feature yield statistically equivalent results compared with instrument vendor software for the quantification of 25(OH)D and VDBG, making it an attractive alternative to what can be expensive proprietary software packages.

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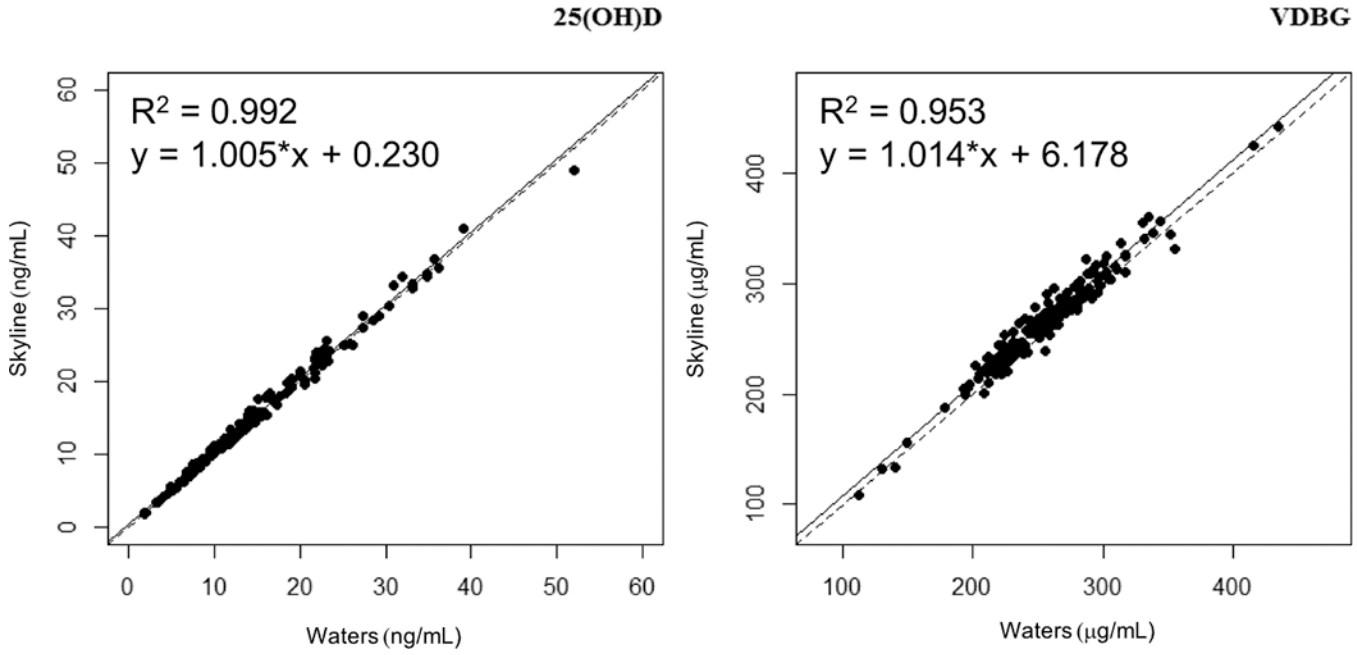

Figure.

25(OH)D concentrations from 154 serum samples and VDBG concentrations from 164 serum samples. The Deming regression equation is shown (solid line), along with the line of unity (dashed line), and the Pearson correlation coefficient (R^2).