Supporting Information for:

Quantification of Total Vitamin D-Binding Protein and the Glycosylated Isoforms by Liquid Chromatography-Isotope Dilution Mass Spectrometry

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LERGRDYEKNKVCKEFSHLGKEDFTSLSLVLYSRKFPSGTFEQVSQLVKEVVSLTEACCAEGAD
PDCYDTRTSALSAKSCESNSPFPVHPGTAECCTKEGLERKLCMAALKHQPQEFPTYVEPTND
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LKHLSLLTTLSNRVCSQYAAYGEKKSRLSNLIKLAQKVPTADLEDVLPLAEDITNILSKCCESASE
DCMAKELPEHTVKLCDNLSTKNSKFEDCCQEKTAMDVFVCTYFMPAAQLPELPDVELPTNK
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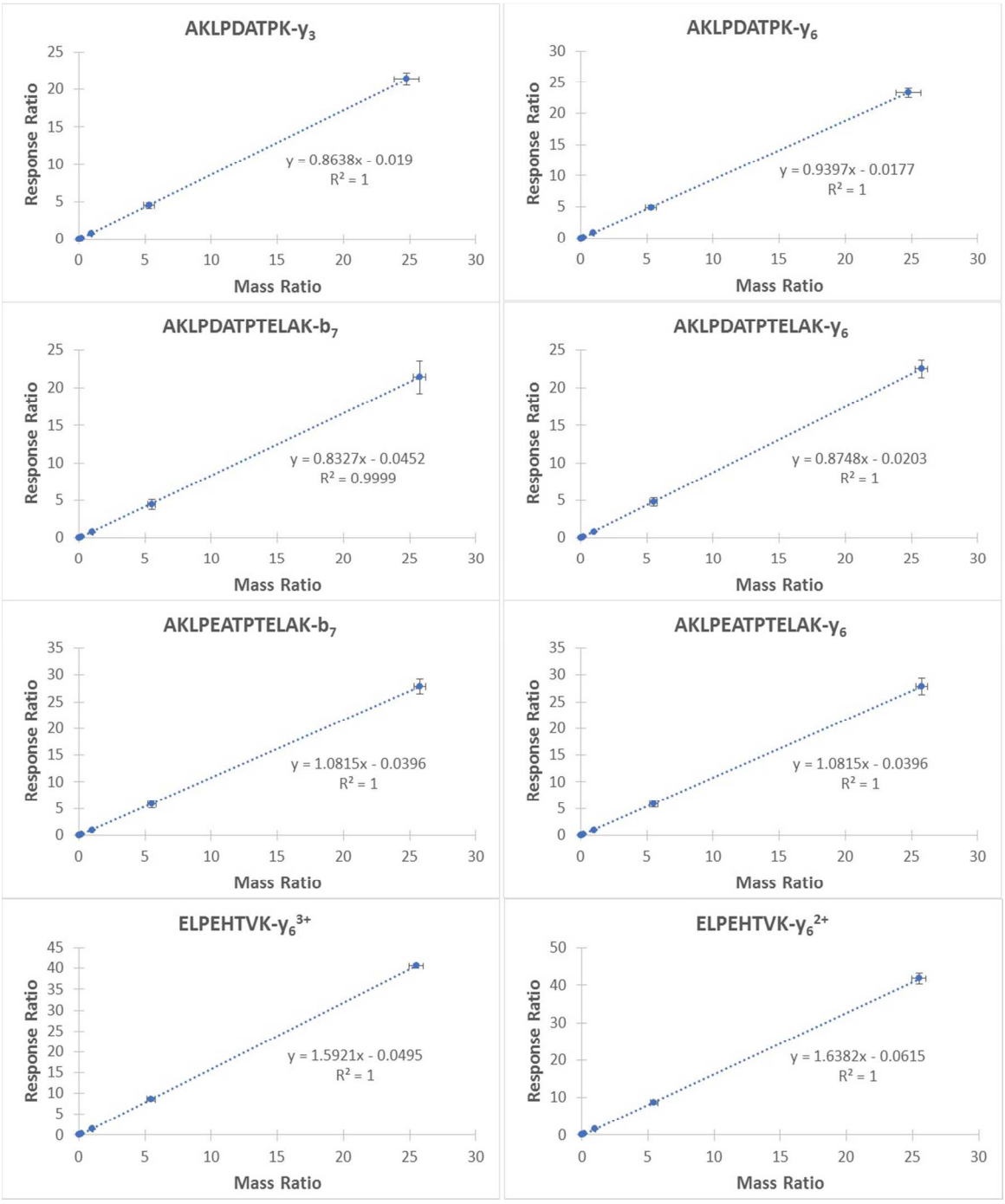
SSFIDKGQELCADYSENTFTEYKKKLAERLKAKLPDATPTELAKELAKLVNKRSDFASNCCSINS
PPLYCDSEIDAELKNILVDHHHHHH

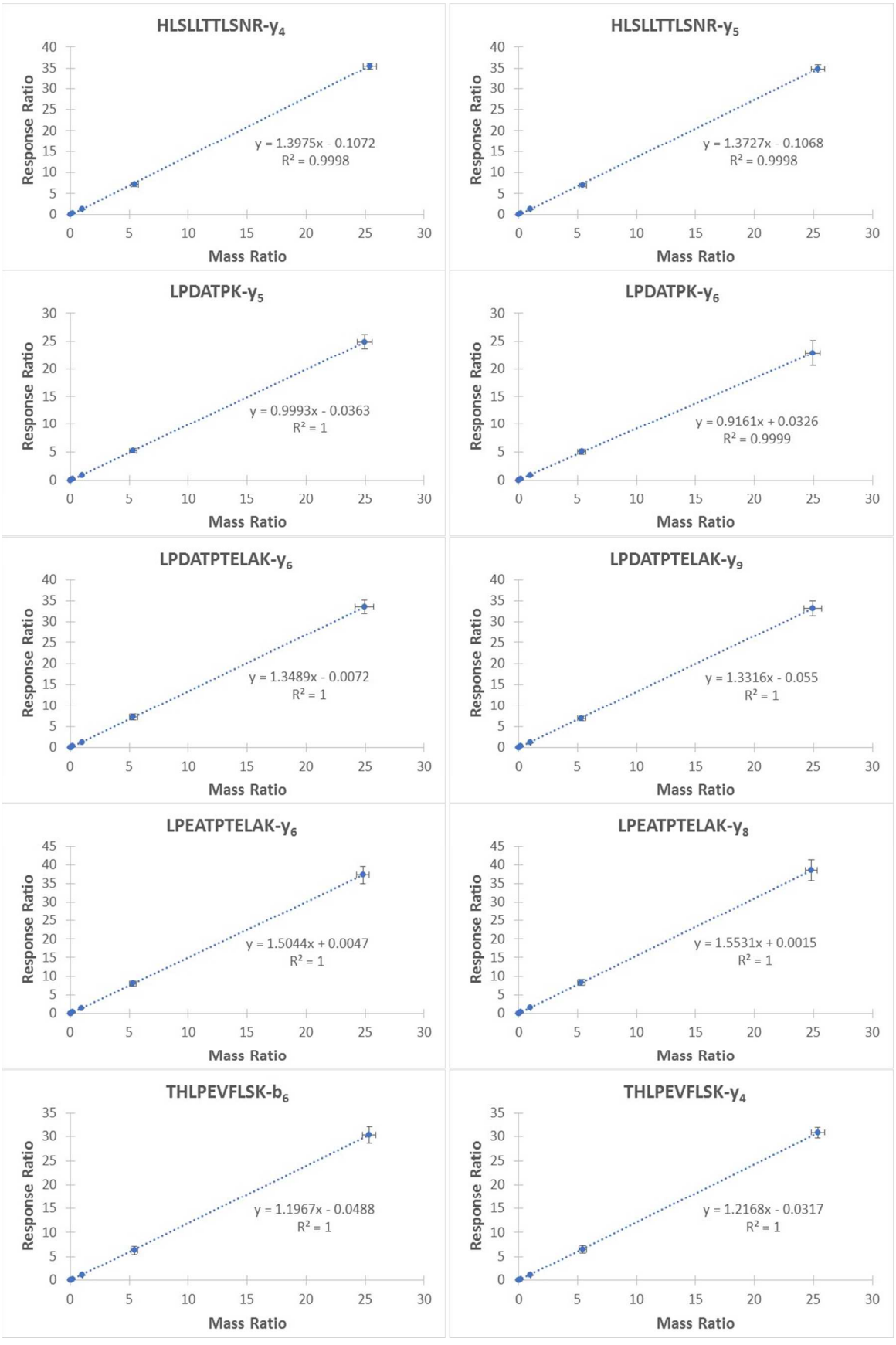
Figure S-1. Amino acid sequence of human VDBP. Peptides selected for LC-IDMS are shown in blue. The region of the sequence containing the isoform-specific tryptic peptide is underlined (GC-1f is shown). The last eight amino acids (in green) are present only in the recombinant protein.

Isoform	Peptide	Precursor m/z	Product m/z	Collision Energy (V)	Retention Time (min)
2	AKLPDATPK	314.2	345.2	1	12.3
2	AKLPDATPK	470.8	628.3	12.5	12.3
1f	AKLPDATPTELAK	452.3	697.4	2.8	19.8
1f	AKLPDATPTELAK	452.3	658.4	4.8	19.8
1s	AKLPEATPTELAK	456.9	711.4	3	19.8
1s	AKLPEATPTELAK	456.9	658.4	6	19.8
2	LPDATPK	371.2	531.3	6.4	11.4
2	LPDATPK	371.2	628.4	6.4	11.4
1f	LPDATPTELAK	578.3	658.4	17.9	20.7
1f	LPDATPTELAK	578.3	945.5	18.9	20.7
1s	LPEATPTELAK	585.3	658.4	17.3	20.7
1s	LPEATPTELAK	585.3	830.5	18.3	20.7
All	ELPEHTVK	476.8	710.4	9.8	10.4
All	ELPEHTVK	318.2	355.7	1	10.4
All	HLSLLTTLSNR	418.9	489.3	6.7	28.4
All	HLSLLTTLSNR	418.9	590.3	4.7	28.4
All	THLPEVFLSK	390.9	677.3	1.7	27.0
All	THLPEVFLSK	390.9	494.3	1.7	27.0
All	TSALSAK	339.2	489.3	1.7	7.3
All	TSALSAK	339.2	576.4	3.7	7.3
All	VLEPTLK	400.2	587.3	2.8	18.7
All	VLEPTLK	400.2	700.4	4.8	18.7
All	YTFELSR	458.2	504.3	11.6	23.6
All	YTFELSR	458.2	651.3	7.8	23.6

Table S-1. Transitions used for LC-IDMS of VDBP peptides. Peptides are either found only in one of the three isoforms (2, 1f, or 1s) or shared between the isoforms (All).

Figure S-2. Peptide calibration curves for the two transitions. Average values are shown for the instrument reponse and mass ratios of the unlabeled and labeled peptides for four sets of data analyzed on different days. Error bars show the standard deviation between the replicates. Each calibrant had CV < 15 % on both axes except for the response ratio of the lowest concentration for one transition (AKLPDATPTELAK, b7, 20.5 % CV). However, replicate runs for this transition within each day are < 8 % CV.

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R² = 0.9998
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y = 0.9993x - 0.0363
R² = 1
LPDATPK-y6
y = 0.9161x + 0.0326
R² = 0.9999
LPDATPTELAK-y6
y = 1.3489x - 0.0072
R² = 1
LPDATPTELAK-y9
y = 1.3316x - 0.055
R² = 1
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R² = 1
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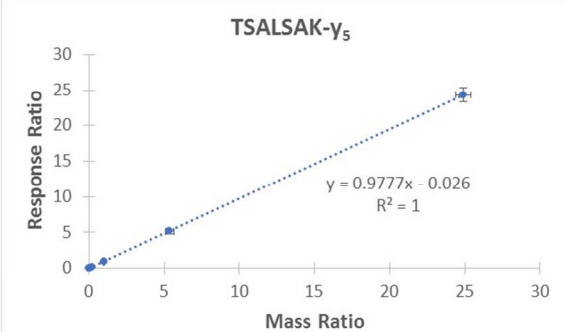
Mass Ratio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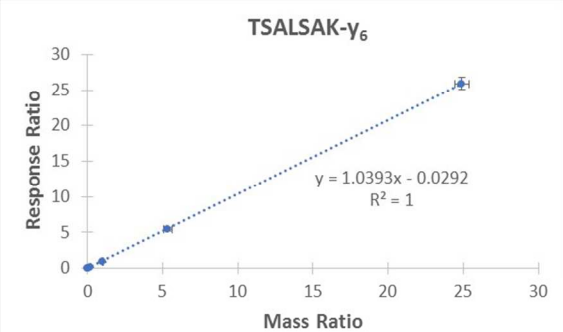
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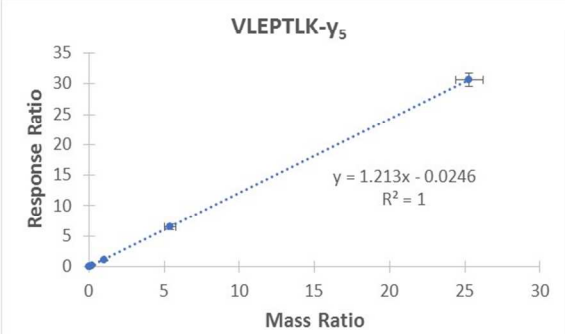
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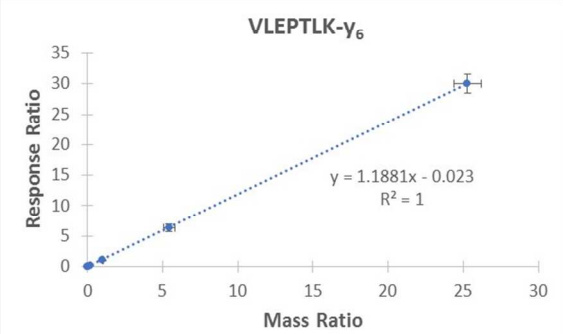
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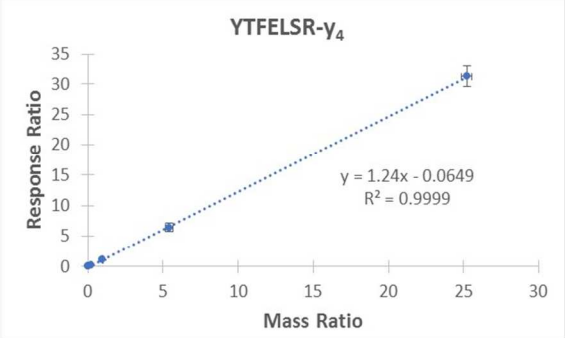
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Mass Ratio
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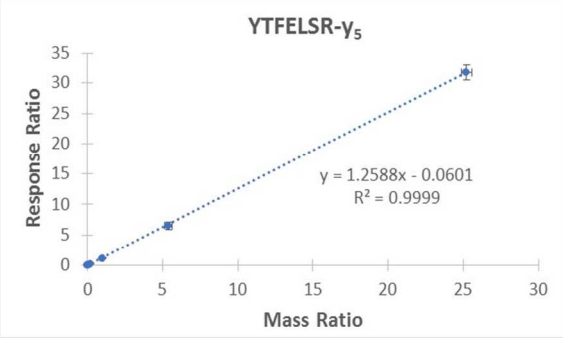

YTFELSR-y5
Response Ratio
Mass Ratio
y = 1.2588x - 0.0601
R² = 0.9999

Amino Acid	Label	Precursor m/z	Product m/z	Fragmentor (V)	Collision Energy (V)	Retention Time (min)
Alanine	None	90.1	44.1	90	10	9.2
Alanine	U-$^{13}C_3$, ^{15}N	94.1	47.1	90	10	9.2
Isoleucine	None	132.1	86.1	58	10	14.9
Isoleucine	U-$^{13}C_6$	138.1	91.1	58	10	14.9
Leucine	None	132.1	86.1	58	10	15.6
Leucine	U-$^{13}C_6$	138.1	91.1	58	10	15.6
Phenylalanine	None	166.1	120.1	78	10	18.2
Phenylalanine	U-$^{13}C_9$, ^{15}N	176.1	129.1	78	10	18.2
Proline	None	116.1	70.1	80	14	9.6
Proline	U-$^{13}C_5$, ^{15}N	122.1	75.1	80	14	9.6
Valine	None	118.1	72.1	58	10	11.6
Valine	U-$^{13}C_5$	123.1	76.1	58	10	11.6

Table S-2. Transitions used for LC-IDMS of amino acids. Unit resolution was used for the precursor and product ions and the dwell time was set to 250 ms.

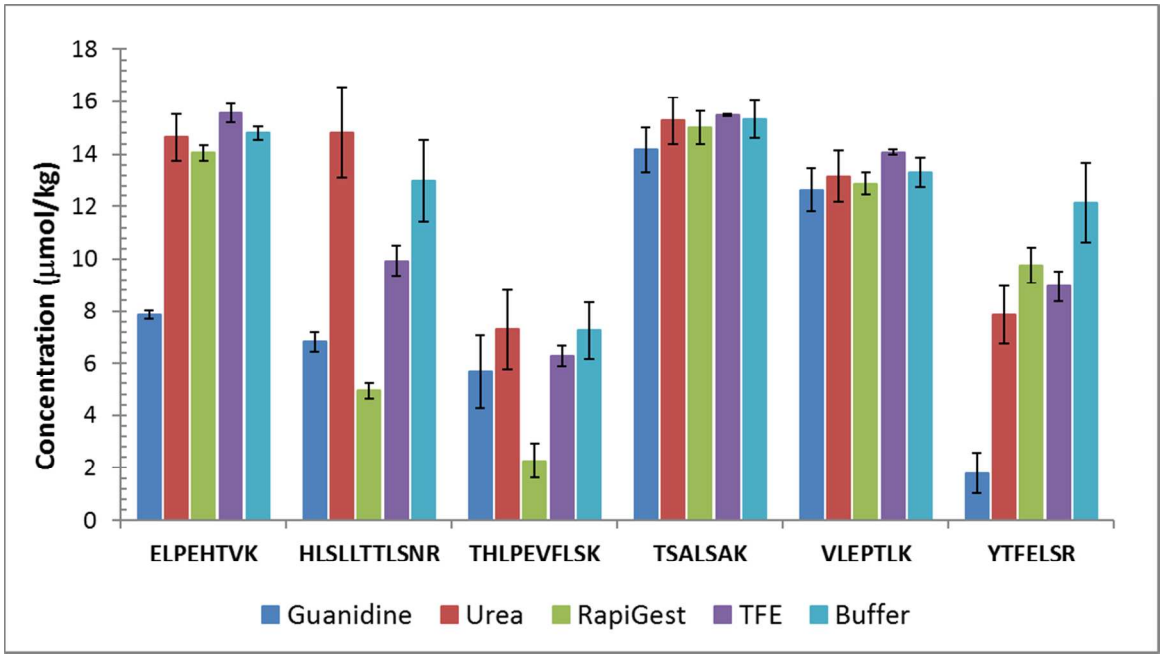


Figure S-3. Concentrations of six shared peptides for different digest protocols of pVDBP using heat denaturing. Error bars show the standard deviation between digests performed in duplicate on different days.

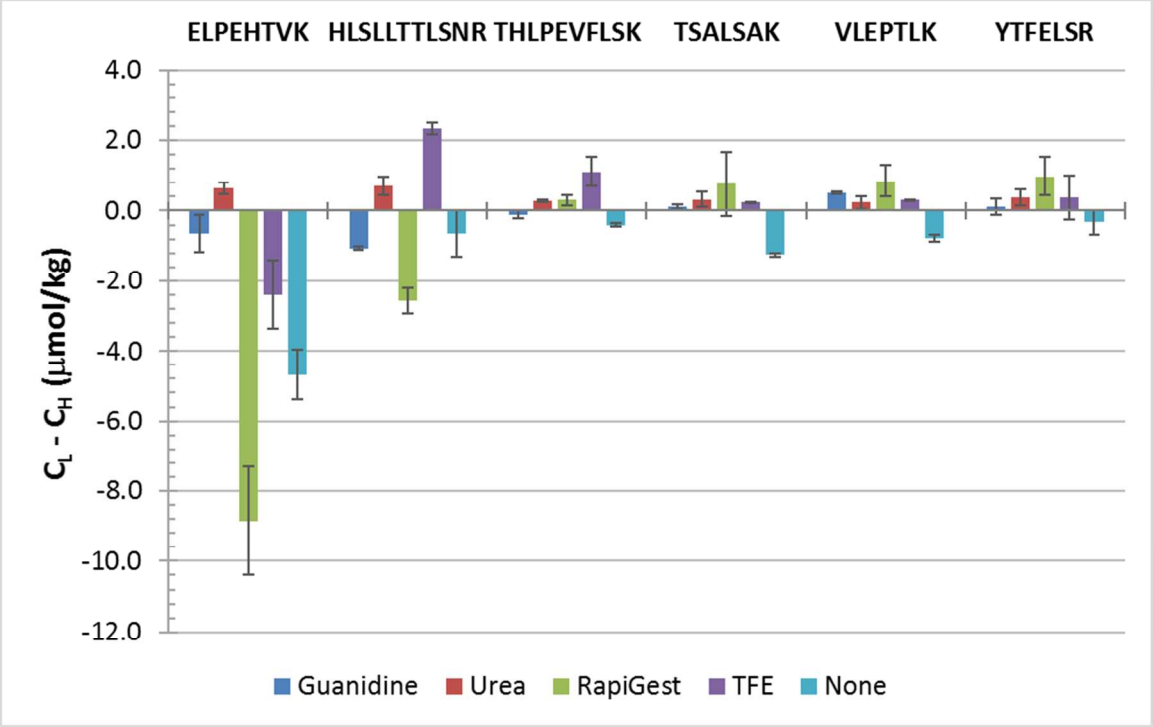


Figure S-4. Differences in peptide concentrations for pVDBP without or with heat denaturing. Samples were denatured without (C_L) or with (C_H) heating at 60 °C in the presence of guanidine, urea, RapiGest, TFE, or no denaturant (none). Urea was added following heat denaturing. Error bars show the standard deviation between experiments performed in duplicate on different days.

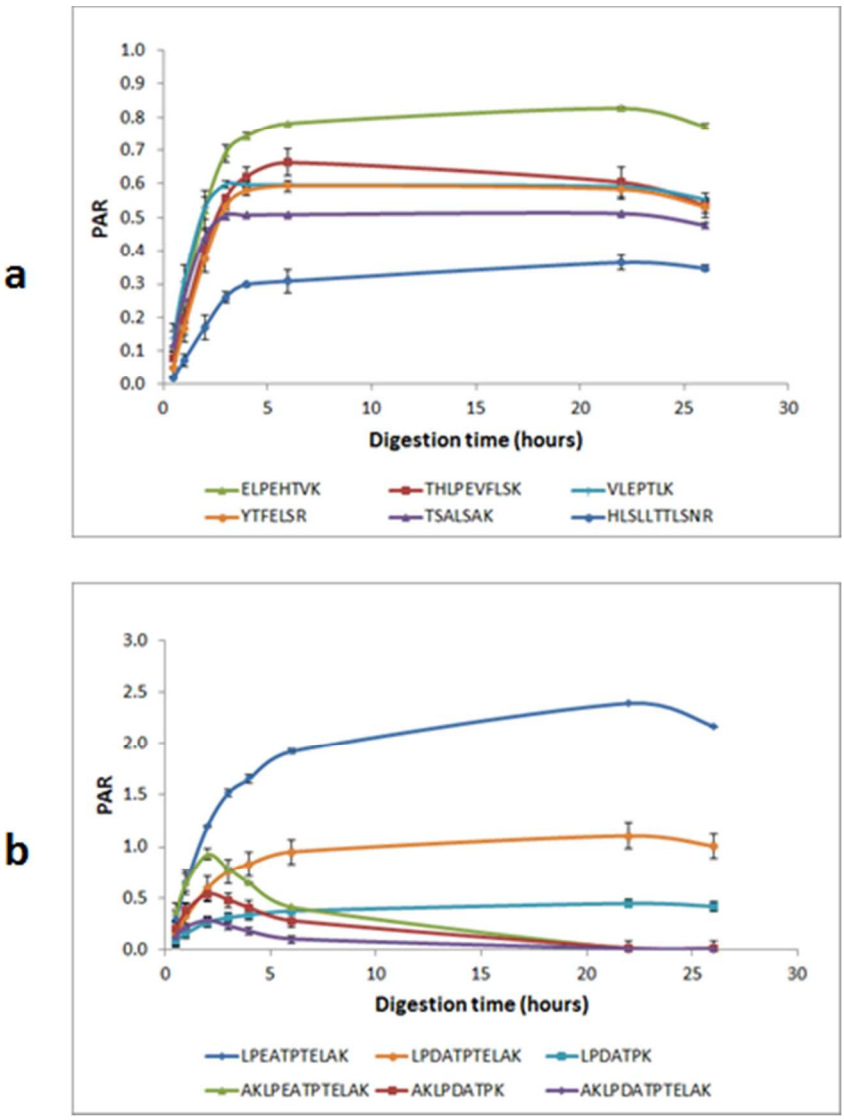


Figure S-5. Changes in VDBP peptide concentrations during tryptic digestion of pooled plasma (SRM 1950) prepared in TFE with heat denaturing. Aliquots were removed during the digestion and spiked with labeled peptides. PARs between the unlabeled and labeled peptides were plotted versus digestion time to reduce instrument variability. (a) Peptides in common between VDBP isoforms. (b) Isoform-specific peptides. Error bars show the standard deviation between replicate digestions performed on different days.

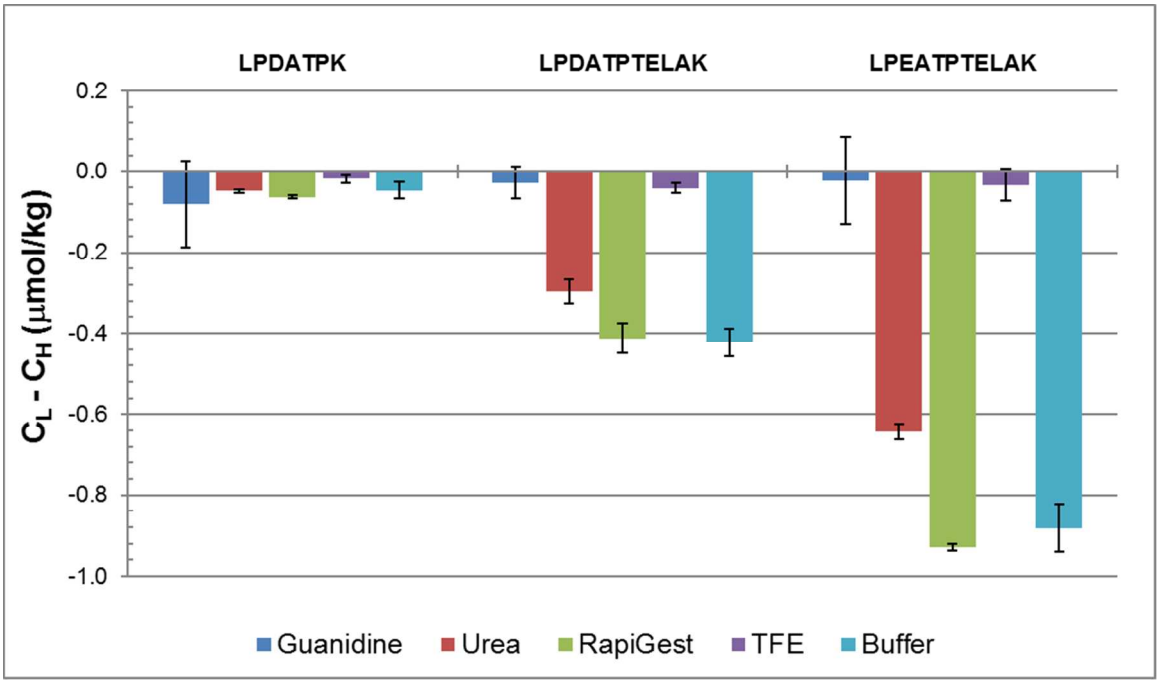


Figure S-6. Differences in isoform-specific peptide concentrations for pooled human plasma without or with heat denaturing. Samples were denatured without (C_L) or with (C_H) heating at 60 °C in the presence of guanidine, urea, RapiGest, TFE, or Tris buffer (no denaturant). Urea was added following heat denaturing. Error bars show the standard deviation between experiments performed in duplicate on different days.

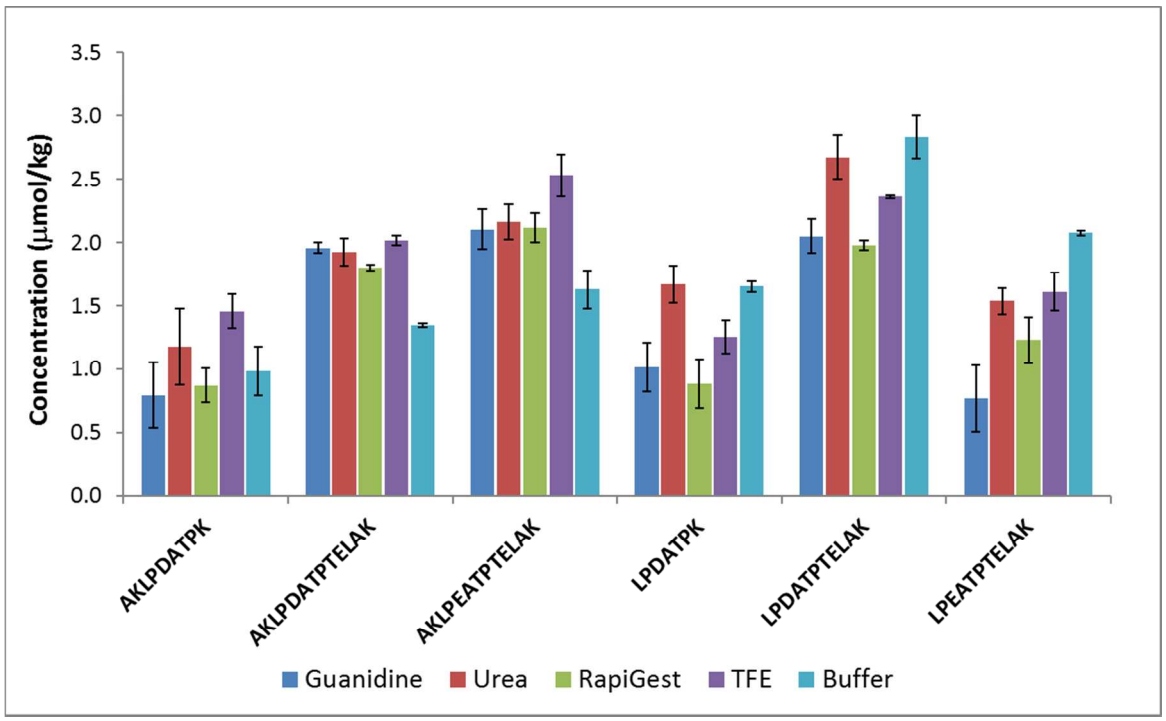


Figure S-7. Concentrations of the isoform specific peptides with and without a missed cleavage in pVDBP for different digestion conditions. Error bars show the standard deviation between digestions performed in duplicate on different days.

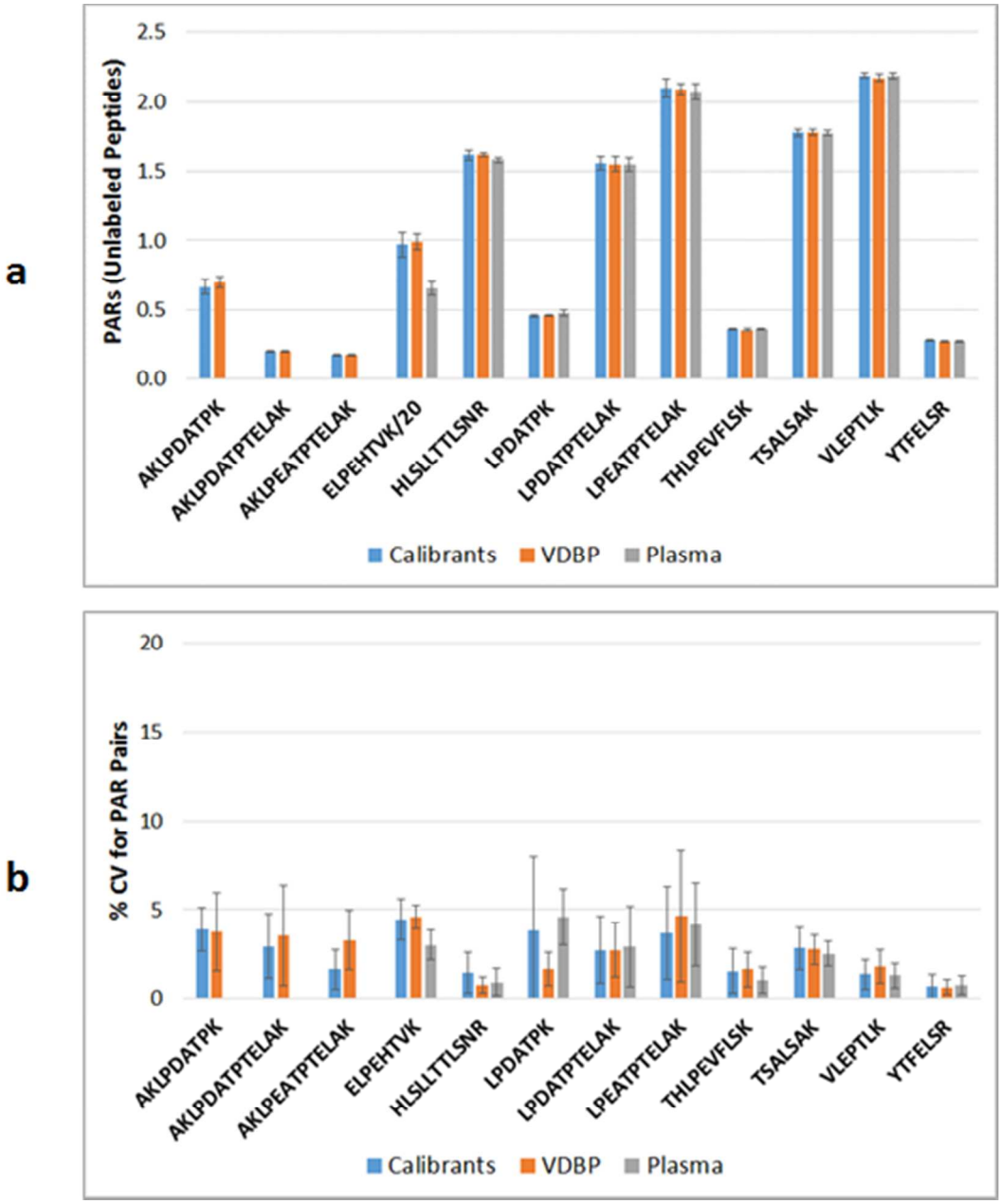


Figure S-8. (a) Average PARs of the two MRM transitions for unlabeled peptides and (b) the average % CV between PAR pairs (unlabeled and labeled peptides) are shown for calibrants, VDBP (pVDBP and rVDBP), and plasma (one pooled sample and nine individual donors). Samples were prepared using the TFE protocol with heat denaturing. Averages were calculated between 6 analyses (3 runs on two different days). Error bars show the standard deviation between the six runs. The average PAR for ELPEHTVK was divided by 20. Peptide PARs are similar for all three samples except ELPEHTVK in plasma. However, because the labeled ELPEHTVK in plasma also shows this trend (< 5 % CV), there is a matrix effect which reproducibly changes the PAR for both.

Table S-3. Intact rVDBP proteoforms and the relative amount of glycosylation measured by LC-MS.

Proteoform	Observed Mass (Da)	Mass Error (ppm[a])	Intensity
GC-1f[b]	52224.2	-13.8	2.248E+07
+ GalNAc	52427.9	-4.6	4.403E+06
+ GalNAc-Gal	52589.6	-12.6	8.406E+05
+ GalNAc-Gal-$NeuNAc_2$	53173.1	4.8	2.361E+07
+ $GalNAc_2$-Gal_2-$NeuNAc_2$	53540.5	43.6	1.545E+06
Total intensity			5.288E+07
Glycosylated proteoform intensity			3.039E+07
% Glycosylation			57.5

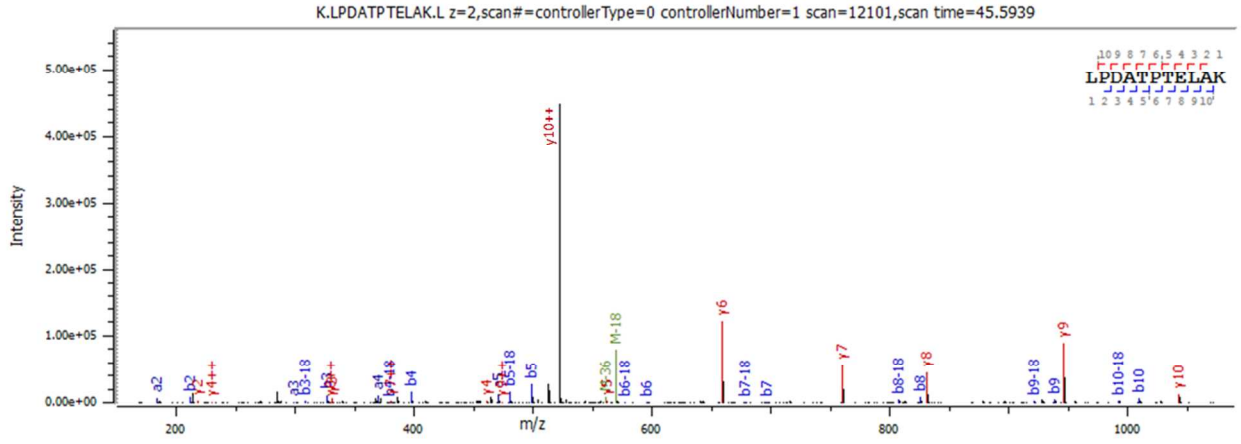
[a]ppm is equivalent to the SI units of mg/kg.
[b]The recombinant protein contains a polyhistidine tag at the C-terminus (Fig. S1).

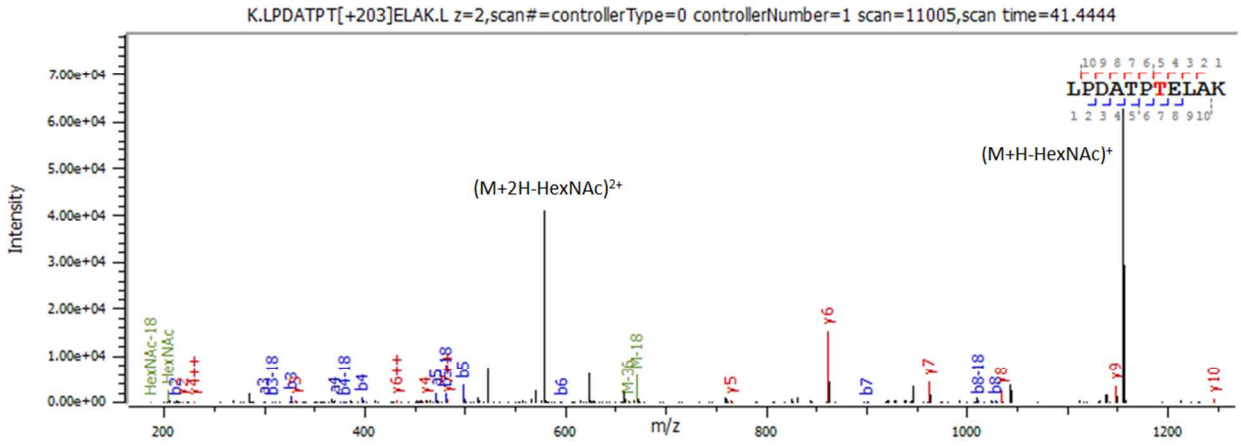
Table S-4: Isoform-specific tryptic peptides with and without glycosylation identified from bottom-up analysis of rVDBP. The MS/MS spectra were searched with Byonic against the UniProtKB human and the 78 mammalian O-glycan databases. The site of modification is noted with an asterisk (*).

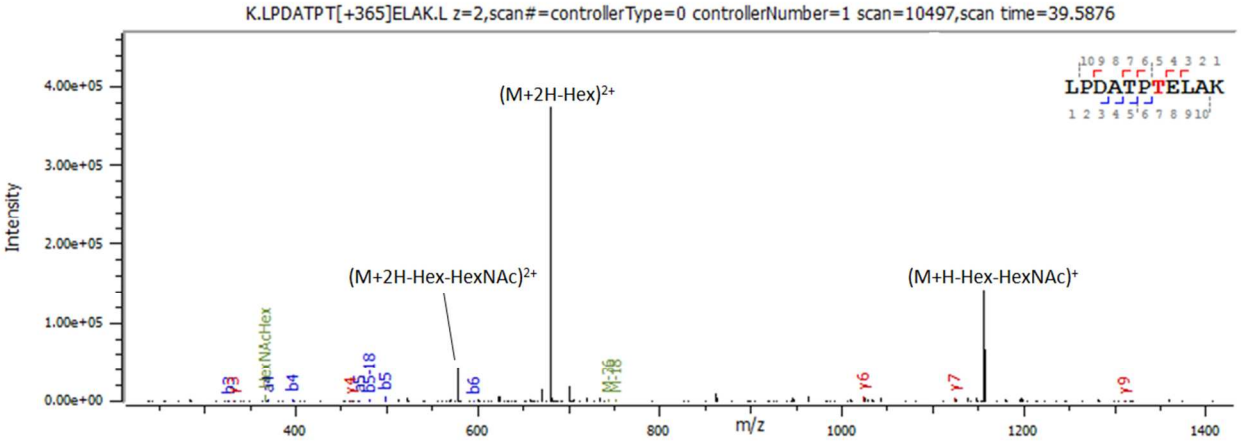
Peptide	Modification	Δ mass (Da)	MH+	Error (ppm)	z	PEP
LPDATPTELAK	none		1155.6264	0.75	2	1.24E-15
LPDATPT*ELAK	HexNAc	203.1	1358.7093	3.23	2	1.46E-13
LPDATPT*ELAK	HexNAc-Hex	365.1	1520.7599	1.43	2	1.60E-12
LPDATPT*ELAK	HexNAc-Hex-NeuAc	656.2	1811.8540	0.44	2	1.15E-13
LPDATPT*ELAK	HexNAc-Hex-$NeuAc_2$	947.3	2102.9486	0.02	2	2.90E-12
LPDAT*PT*ELAK[a]	$HexNAc_2$-Hex_2-$NeuAc_2$	1312.5	1234.5405	-2.84	2	NA

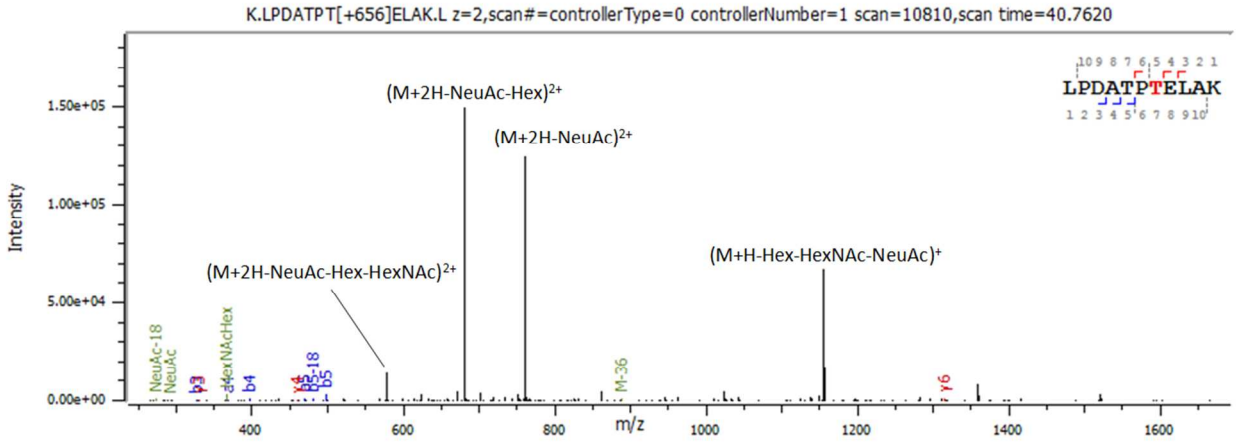
[a]Peptide was identified manually. Although both T residues appear to be modified, the structures of the glycans could not be determined.

MS/MS spectra of the rVDBP isoform-specific peptide without or with glycosylation:





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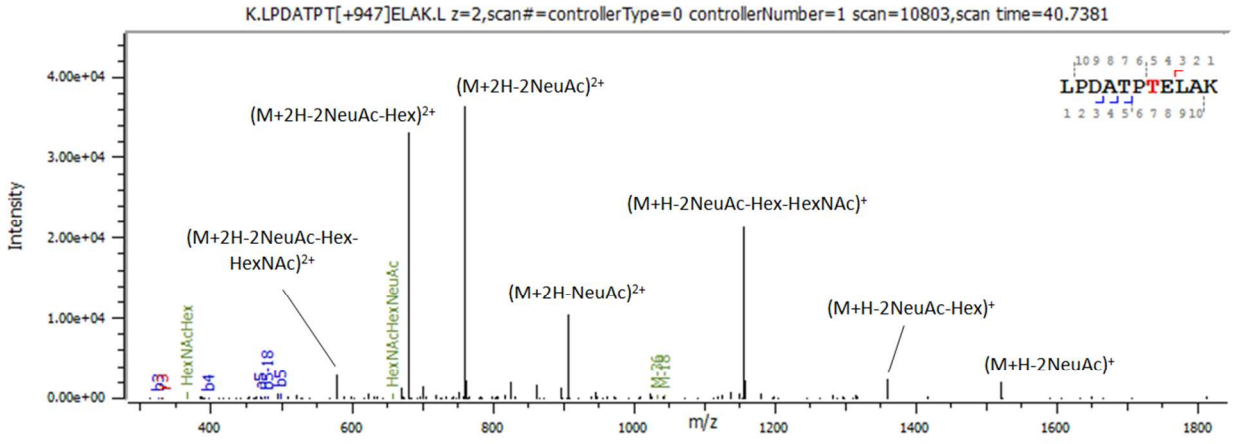
Intensity
m/z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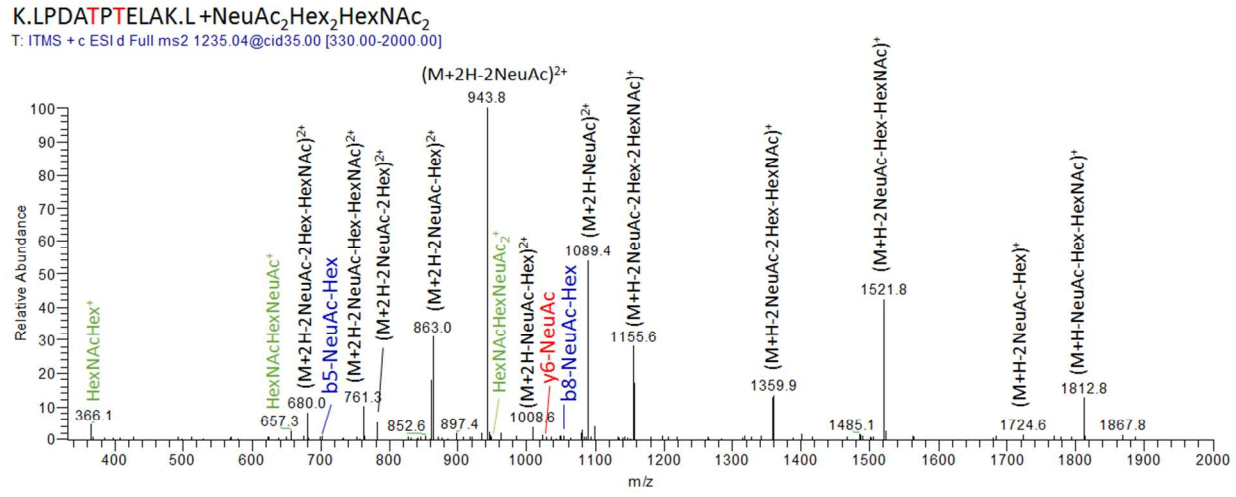
Intensity
m/z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Relative Abundance
m/z