

Supplementary Material

Analytical Characterization for Similarity Assessment Between Biosimilar SB16 and Denosumab Reference Product Using State-of-the-art Analytical Method

Drugs in R&D

Authors:

**Eunok Jung^{1*}, Jinyoung Kim^{1*}, Misol Do^{1**}, Jihye Kim^{1**}, Hanseul Kweon¹,
Jaecil Lee¹, Jungmin Lee¹, Taekyung Yoo^{1#}, Sung-Yong Yang^{1#}**

¹ Quality Evaluation Team, Samsung Bioepis Co., Ltd, Incheon, South Korea

* First co-authors with equal contribution

* Second co-authors with equal contribution

Corresponding Author

Supplementary Table S1. List of quality attributes and analytical methods for the analytical characterization

Category	Quality attribute		Analytical method
Primary structure	Amino acid sequence		LC-ESI-MS (Peptide mapping)
	Peptide mapping		LC-ESI-MS
	N-terminal sequence		LC-ESI-MS
	C-terminal sequence		LC-ESI-MS
Post translational modification (PTM)	Oxidation		LC-ESI-MS
	Deamidation		LC-ESI-MS
Higher order structure	Secondary structure		FT-IR spectroscopy
	Secondary and Tertiary structure		CD spectrometry (far-UV and Near UV)
	Thermal stability/heat-induced protein denaturation pattern/melting temperature		DSC
	Tertiary structure/conformational dynamics		H/DX-MS
Purity and impurity	HMW and monomer		SE-HPLC
	IgG		CE-SDS (Non-reduced)
	LC+HC		CE-SDS (Reduced)
Charge heterogeneity	Acidic, main, and basic variants		CEX-HPLC and icIEF
Glycosylation	N-glycan profile		HILIC-UPLC
Biological activity	Fab-related biological activity	%Relative RANKL binding activity	RANKL binding assay by ELISA
		%Relative RANKL neutralization potency	RANKL neutralization assay by reporter gene assay
		%Relative anti-differentiation potency	Anti-differentiation assay
	Fc-related biological activity	%Relative FcRn binding activity	FcRn binding assay by SPR
		FcγRIa binding response	FcγRIa binding assay by SPR
		%Relative FcγRIIa binding activity	FcγRIIa binding assay by SPR
		%Relative FcγRIIb binding activity	FcγRIIb binding assay by SPR
		%Relative FcγRIIIa binding activity	FcγRIIIa binding assay by SPR
		%Relative C1q binding activity	C1q binding assay by ELISA
		ADCC	ADCC assay
		CDC	CDC assay

ADCC, antibody-dependent cell-mediated cytotoxicity; C1q, complement component 1q; CD, circular dichroism; CDC, complement-dependent cytotoxicity; CE-SDS, capillary electrophoresis-sodium dodecyl sulfate; DSC, differential scanning calorimetry; ELISA, enzyme-linked immunosorbent assay; FcRn, neonatal fragment crystallizable receptor; FcγRIa, Fc-gamma receptor Ia; FcγRIIa, Fc-gamma receptor IIa; FcγRIIb, Fc-gamma receptor IIb; FcγRIIIa, Fc-gamma receptor IIIa; FT-IR, fourier transform-infrared spectroscopy; H/DX-MS, hydrogen/deuterium exchange-mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HMW, high molecular weight; icIEF, imaging capillary isoelectric focusing; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; LMW, low molecular weight; RANKL, receptor activator of nuclear factor kappa-B ligand; SE-HPLC, size exclusion-high performance liquid chromatography; SPR, surface plasmon resonance

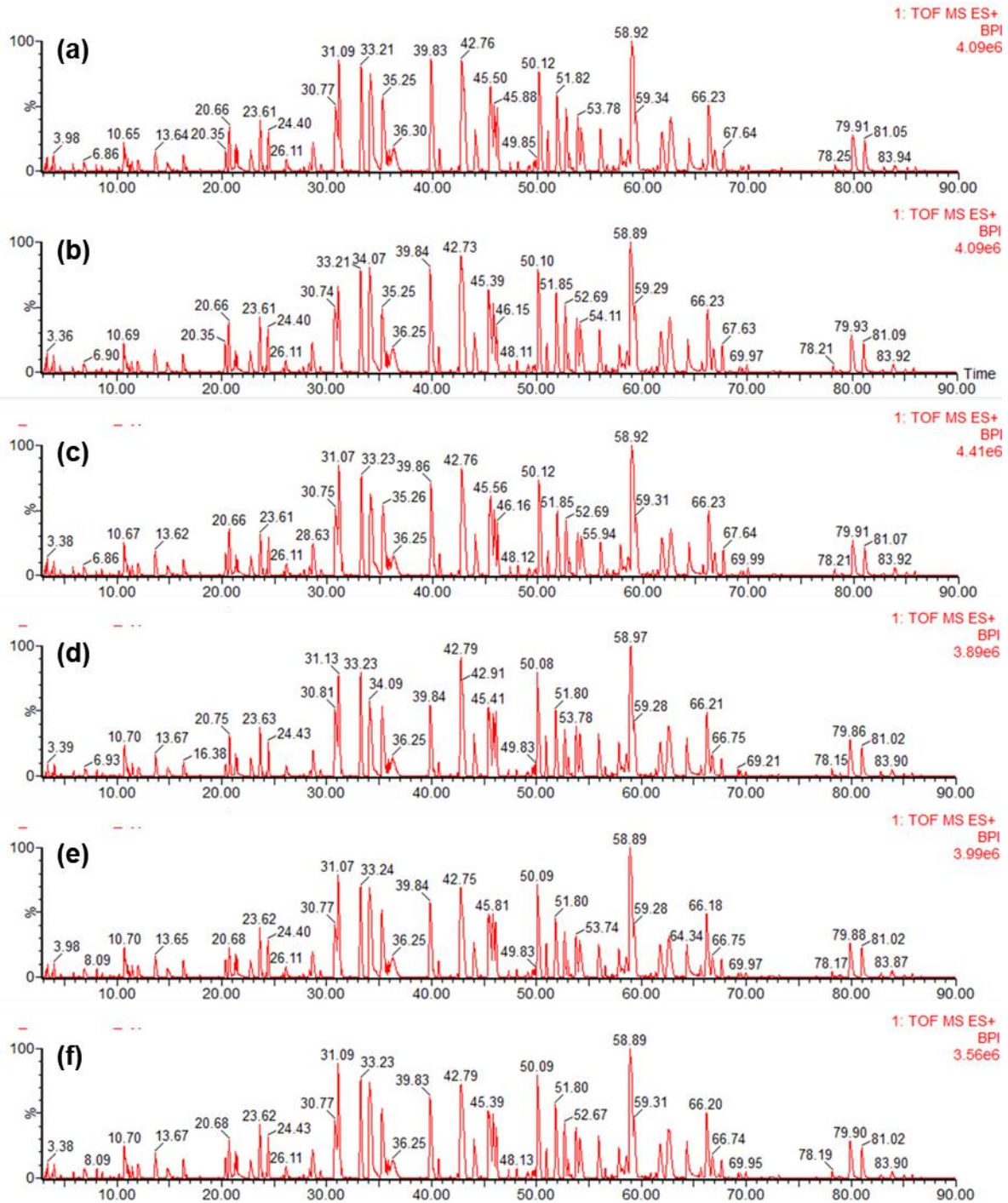
Supplementary Table S2. PTM analysis of SB16 and denosumab RP

Sample	%Oxidation		%Deamidation
	Met ₂₅₃	Met ₃₉₈	Asn _{385, 390 or 391}
SB16 PFS	2.3-2.9	0.1	0.1
EU Prolia	4.9-5.3	0.3-0.4	0.3-0.4
US Prolia	4.8-6.4	0.3-0.5	0.3-0.5
SB16 Vial	3.4-4.1	0.1-0.4	0.3-0.4
EU Xgeva	4.9-5.2	0.3	0.7-0.8
US Xgeva	5.0-5.7	0.3-0.4	0.8-0.9

Supplementary Table S3. Quantitative PTM analysis of CEX-HPLC fractions from SB16 and Prolia

Modification	Site	Sample	Fraction of CEX-HPLC					
			Whole	Acidic 1	Acidic 2	Main	Basic 1	Basic 2
pyroGlu	N-term Glu	SB16	1.8	2.2	1.8	1.5	1.4	1.4
		EU Prolia	2.4	3.5	2.1	1.7	1.7	N/A ^b
		US Prolia	2.5	3.7	1.9	1.6	1.5	N/A
Deamidation	HC Asn385, Asn390, Asn391	SB16	0.4	1.6	2.9	N/D	0.2	0.2
		EU Prolia	0.8	4.5	4.8	0.1	0.2	N/A
		US Prolia	0.9	4.2	4.4	N/D	0.2	N/A
Oxidation	Met253	SB16	2.2	3.8	3.1	1.9	3.2	3.1
		EU Prolia	5.0	7.9	6.4	4.6	8.1	N/A
		US Prolia	4.3	6.7	6.5	4.6	7.8	N/A
	Met398	SB16	0.1	0.2	0.2	0.1	0.2	0.1
		EU Prolia	0.2	0.4	0.4	0.3	0.4	N/A
		US Prolia	0.3	0.4	0.3	0.3	0.3	N/A
C-terminal α -amidation	HC C-term Gly	SB16	0.2	N/D	N/D	N/D	3.1	7.9
		EU Prolia	N/D	N/D	N/D	N/D	0.8	N/A
		US Prolia	N/D	N/D	N/D	N/D	0.8	N/A
C-terminal Lys variant	HC C-term	SB16	2.1	0.3	0.1	N/D	15.7	65.9
		EU Prolia	0.2	N/D	N/D	N/D	5.7	N/A
		US Prolia	0.2	N/D	N/D	N/D	6.2	N/A

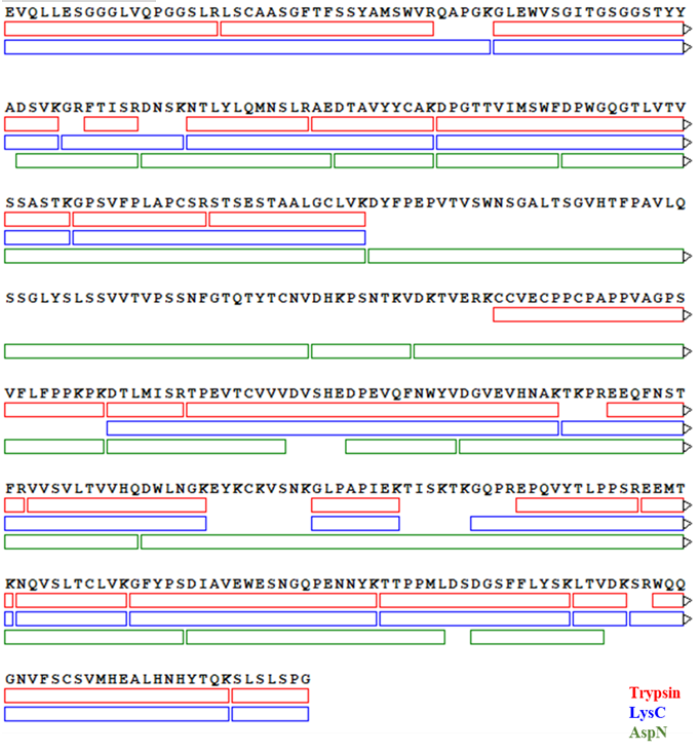
PTM, post-translational modification; CEX, cation exchange; PyroGlu, pyroglutamic acid.



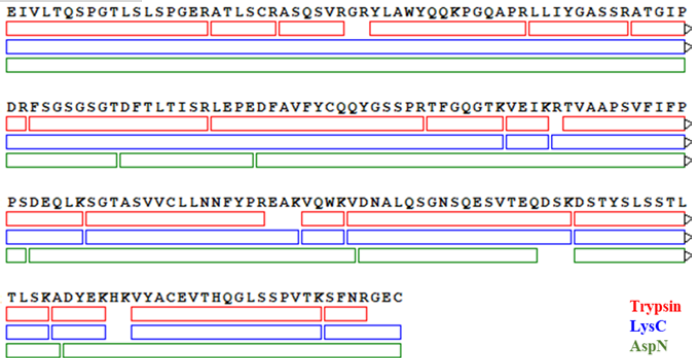
Supplementary Figure S1. Peptide mapping profiles of SB16 and denosumab RP digested with trypsin. (a) SB16 PFS, (b) EU Prolia, (c) US Prolia, (d) SB16 Vial, (e) EU Xgeva, (f) US Xgeva

(a) SB16 PFS

Heavy Chain

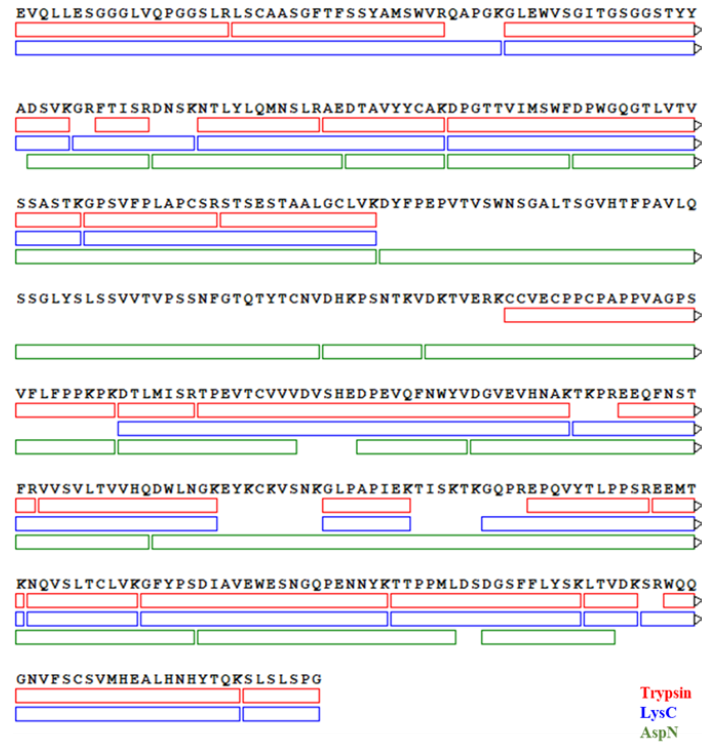


Light Chain

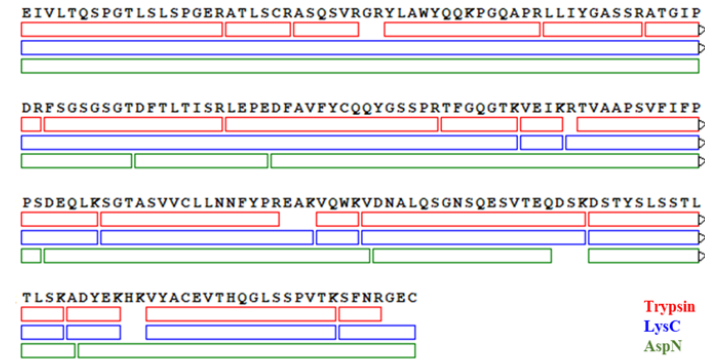


(b) EU Prolia

Heavy Chain

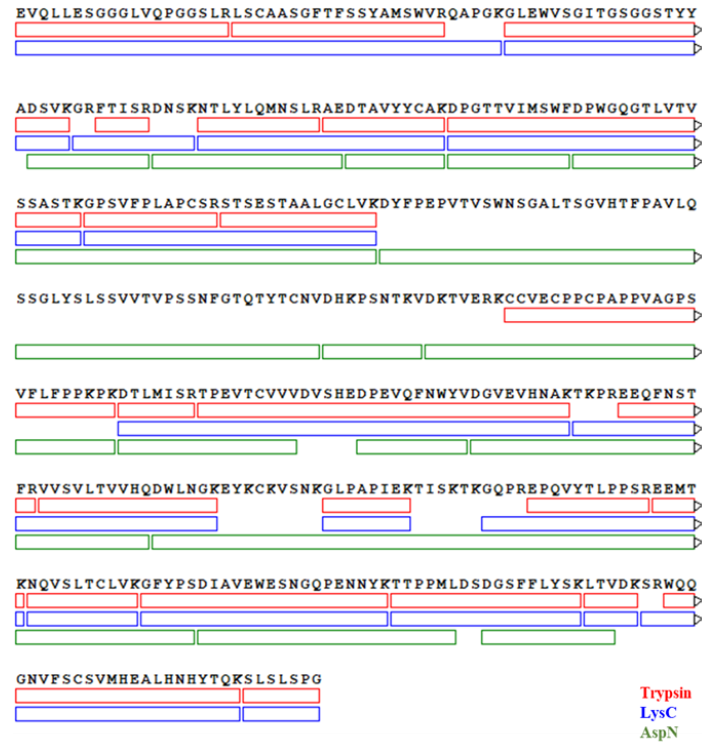


Light Chain



(c) US Prolia

Heavy Chain

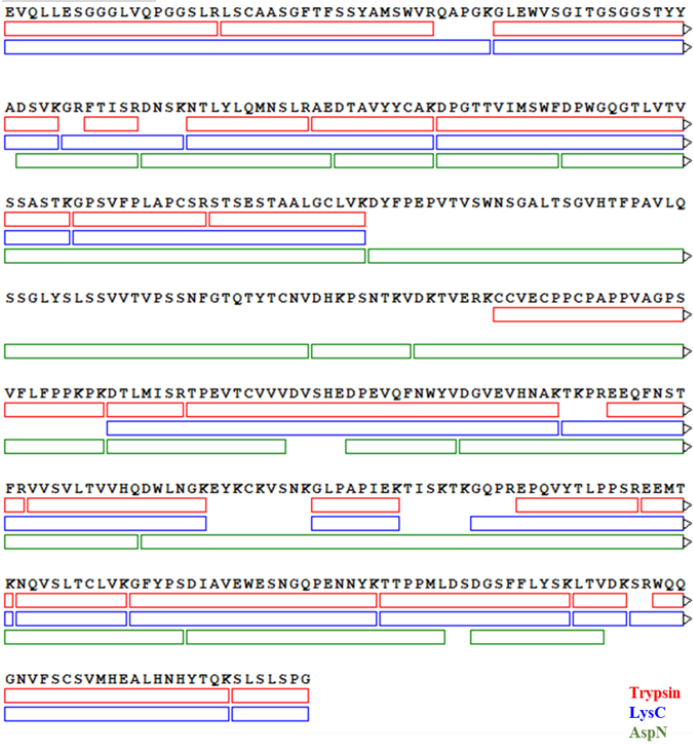


Light Chain

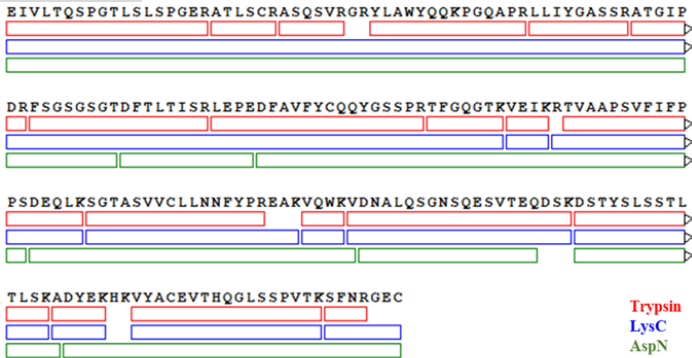


(d) SB16 Vial

Heavy Chain

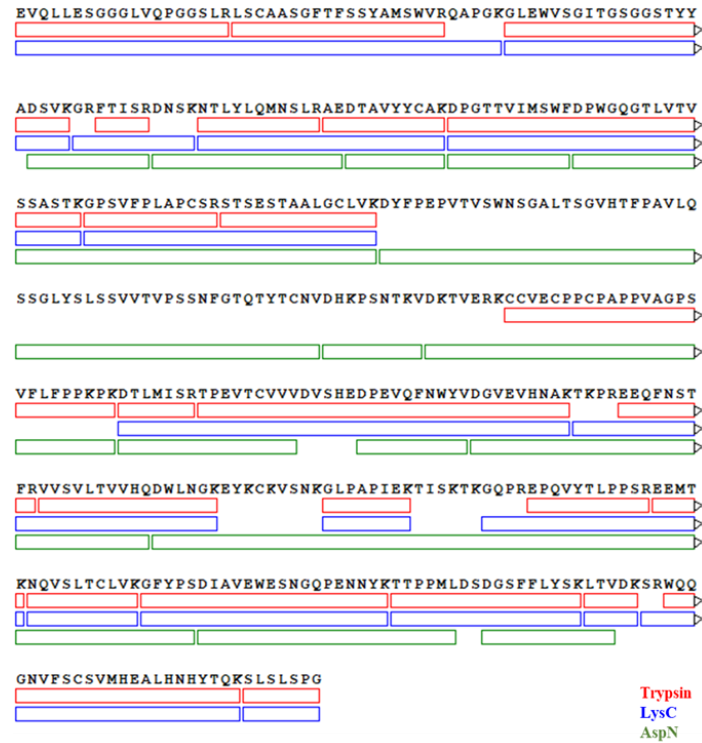


Light Chain

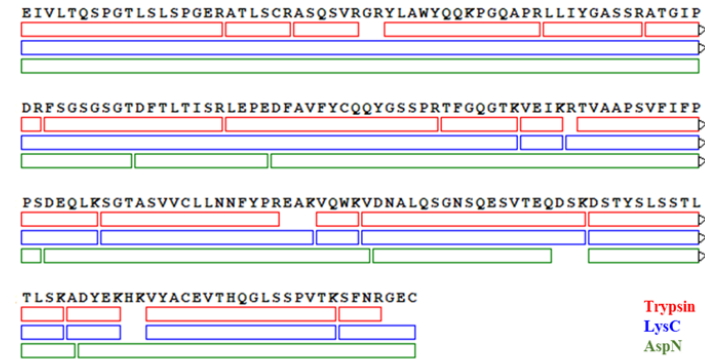


(e) EU Xgeva

Heavy Chain

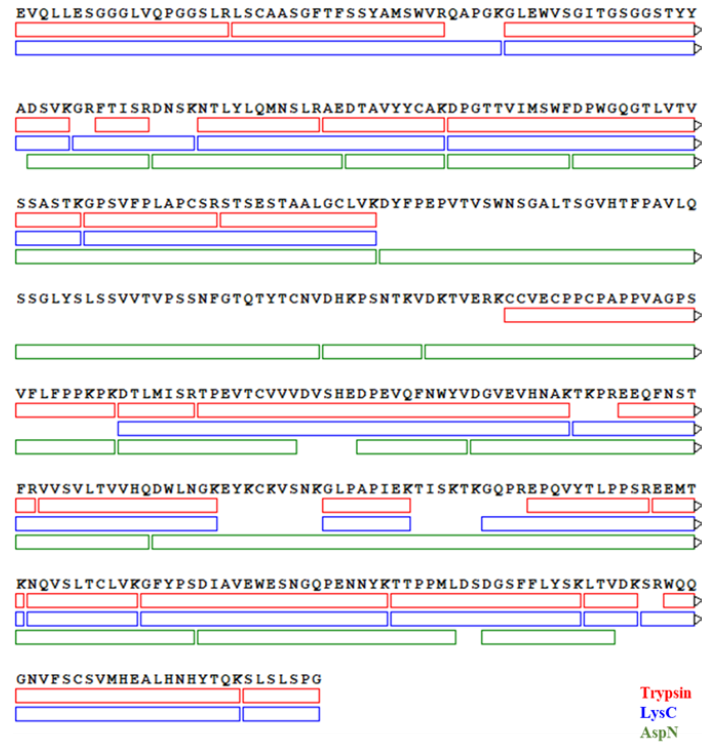


Light Chain



(f) US Xgeva

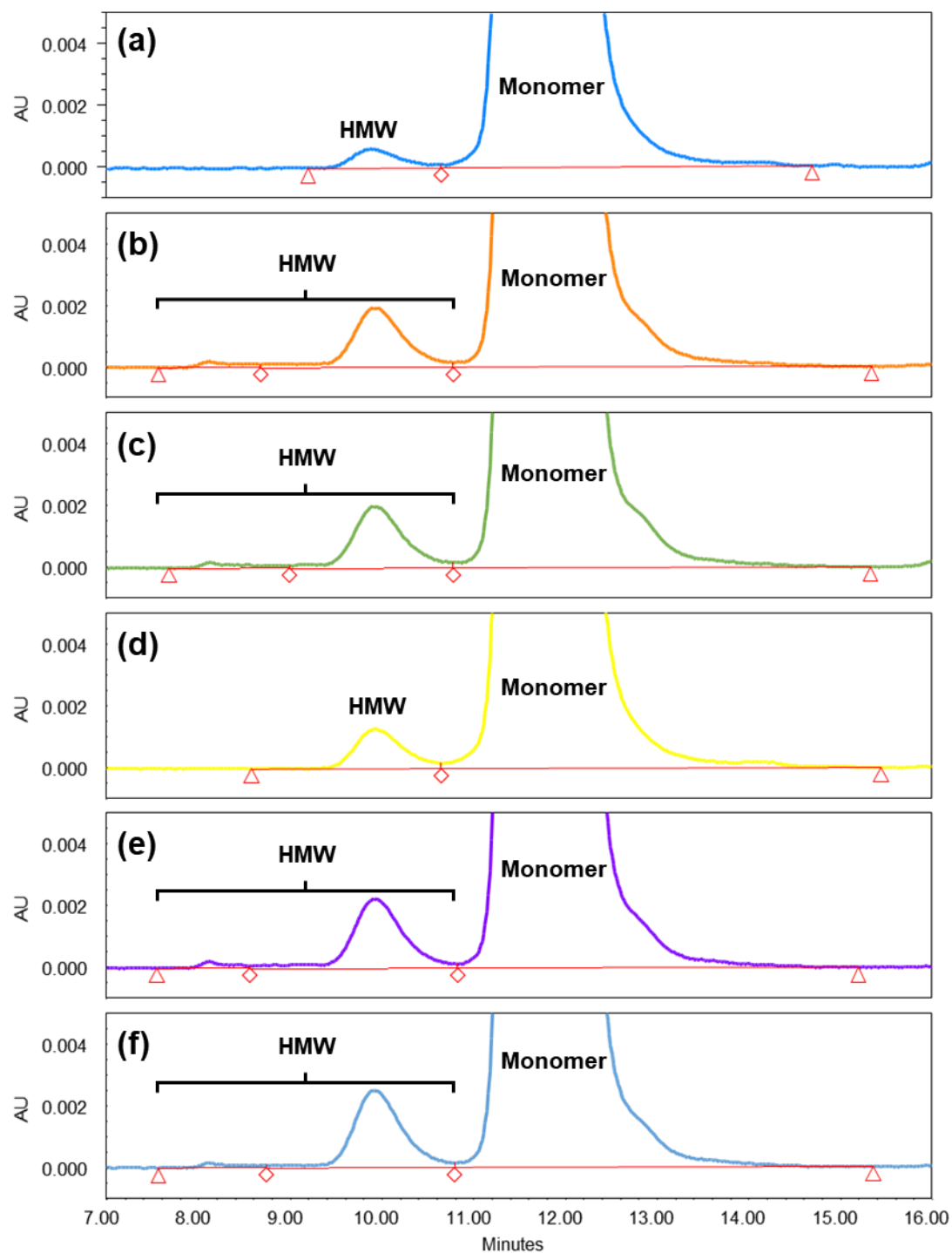
Heavy Chain



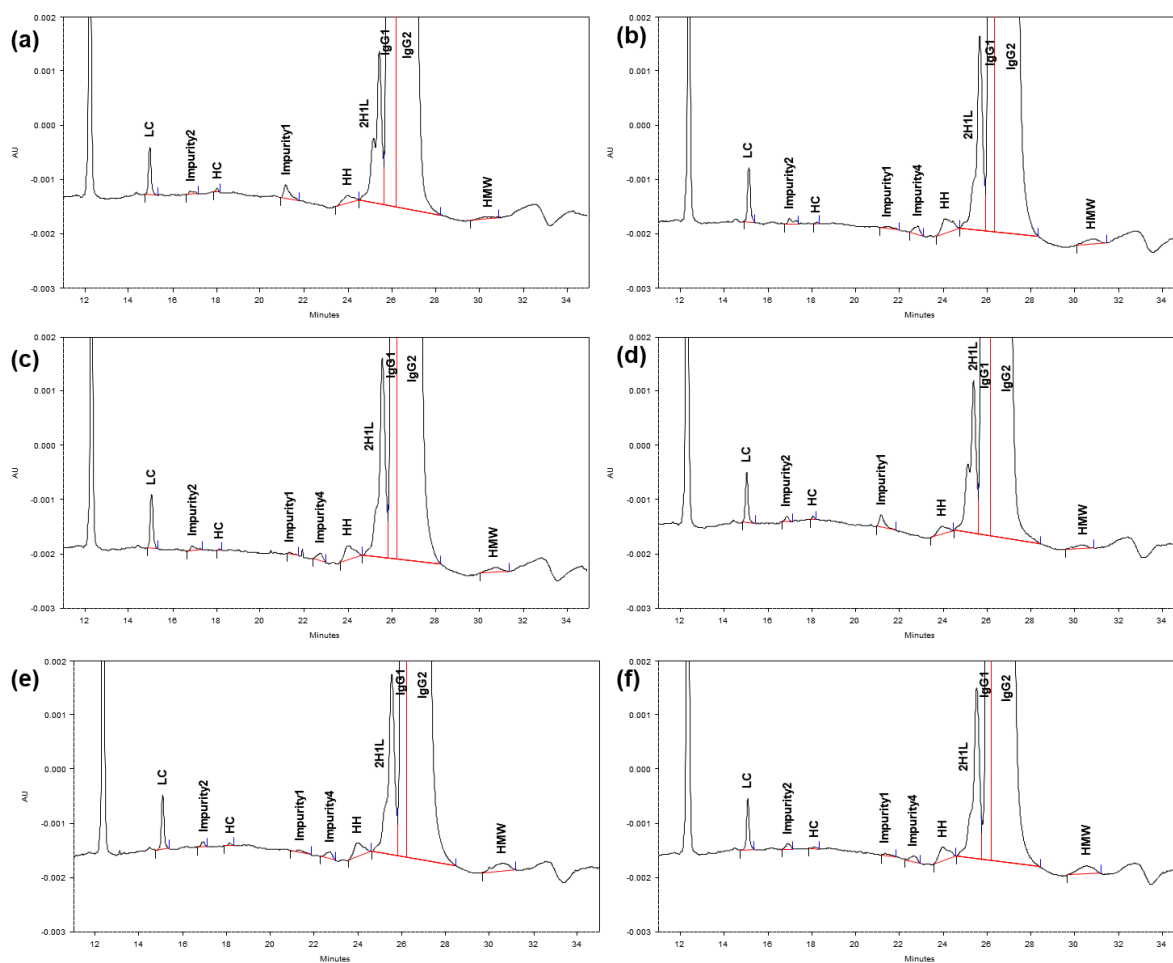
Light Chain



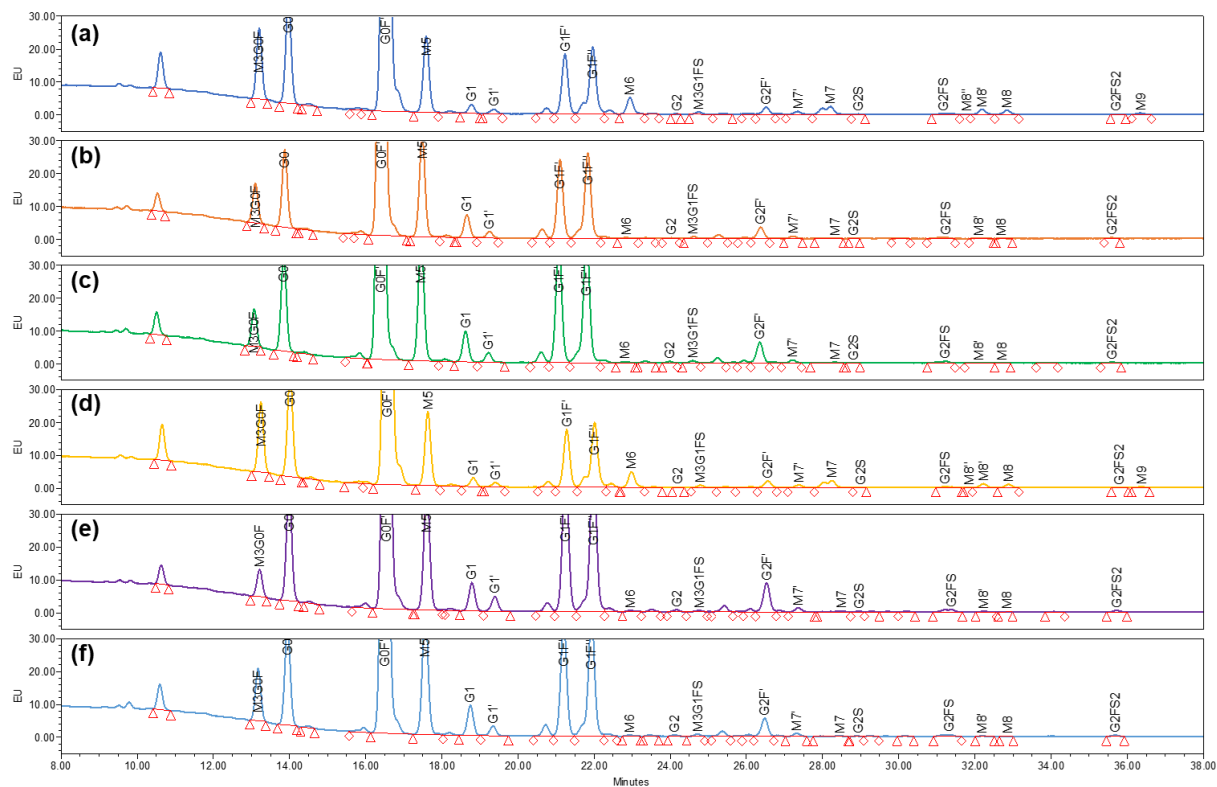
Supplementary Figure S2. Peptide coverage map of SB16 and denosumab RP. Peptide mapping was performed using three different endopeptidases (trypsin, Lys-C, and Asp-N) to ensure comprehensive sequence coverage. The combined use of multiple enzymes provided overlapping peptide fragments that confirmed 100% amino acid sequence coverage for both SB16 and denosumab RP. (a) SB16 PFS, (b) EU Prolia, (c) US Prolia, (d) SB16 Vial, (e) EU Xgeva, (f) US Xgeva. The colored bars represent peptides detected by each enzyme: trypsin (red), Lys-C (blue), and Asp-N (green).



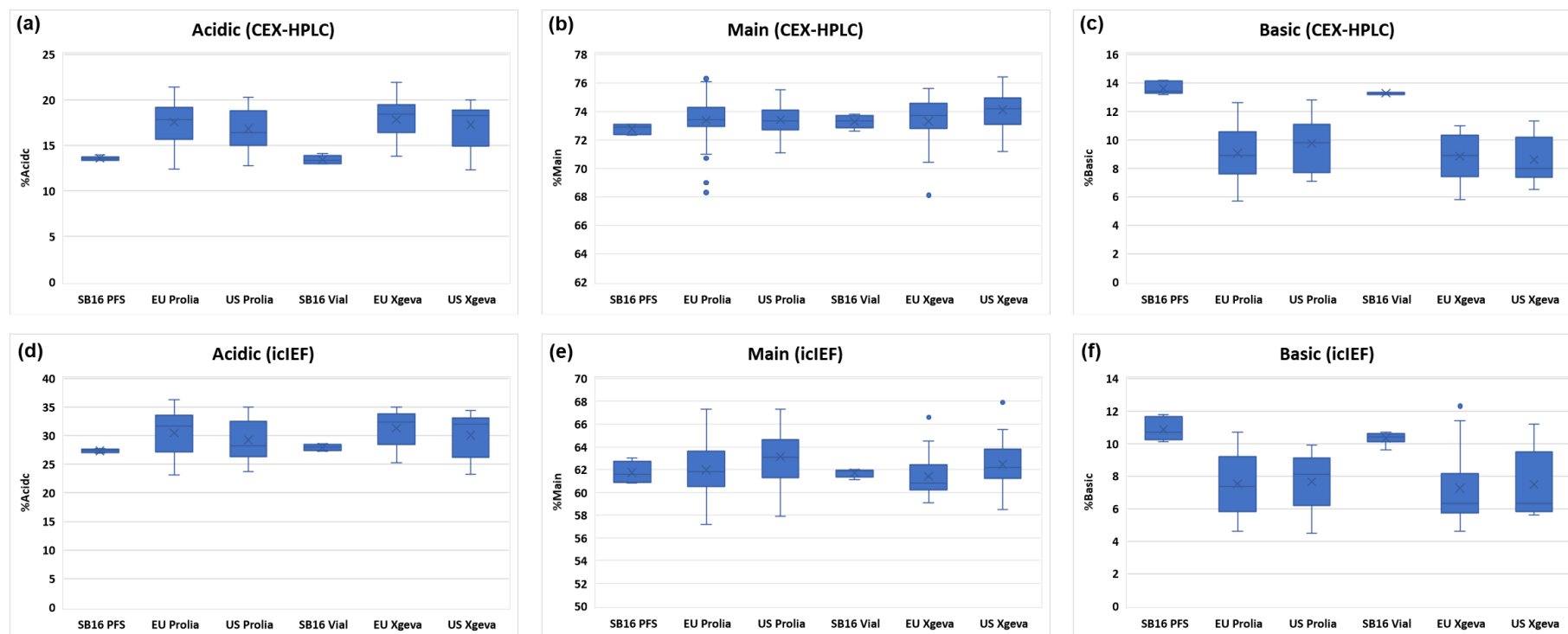
Supplementary Figure S3. Zoom view for SE-HPLC chromatograms of SB16 and denosumab reference products to compare HMW impurities at baseline level. (a) SB16 PFS, (b) EU Prolia, (c) US Prolia, (d) SB16 Vial, (e) EU Xgeva, (f) US Xgeva. *HMW*, high molecular weight; *AU*, absorbance units; *SE-HPLC*, size exclusion-high performance liquid chromatography.



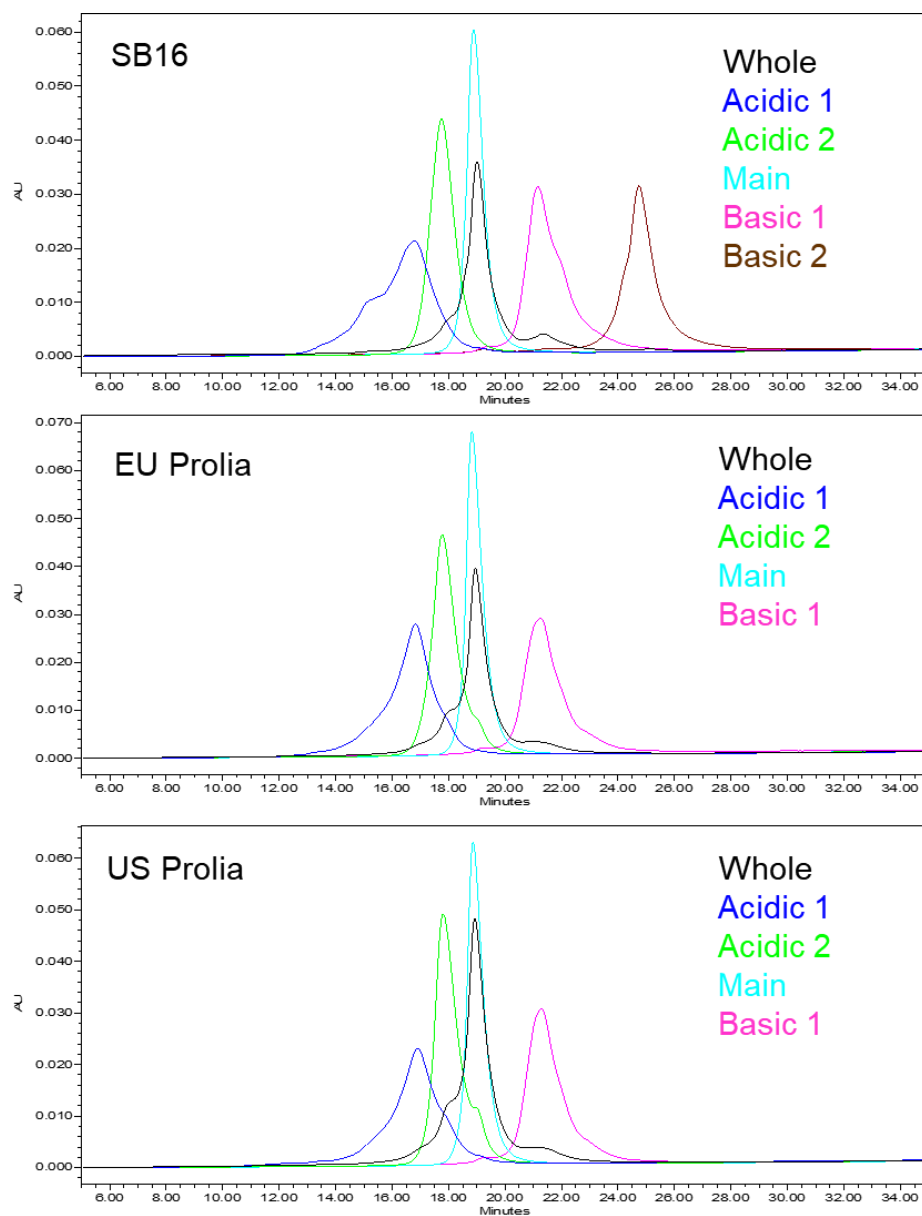
Supplementary Figure S4. Zoom view for CE-SDS (non-reduced) electropherograms of SB16 and denosumab reference products to compare 2H1L impurities at baseline level. (a) SB16 PFS, (b) EU Prolia, (c) US Prolia, (d) SB16 Vial, (e) EU Xgeva, (f) US Xgeva. *CE-SDS*, capillary electrophoresis-sodium dodecyl sulfate; *2H1L*, two heavy chains and one light chain species; *IgG*, immunoglobulin G; *AU*, absorbance units.



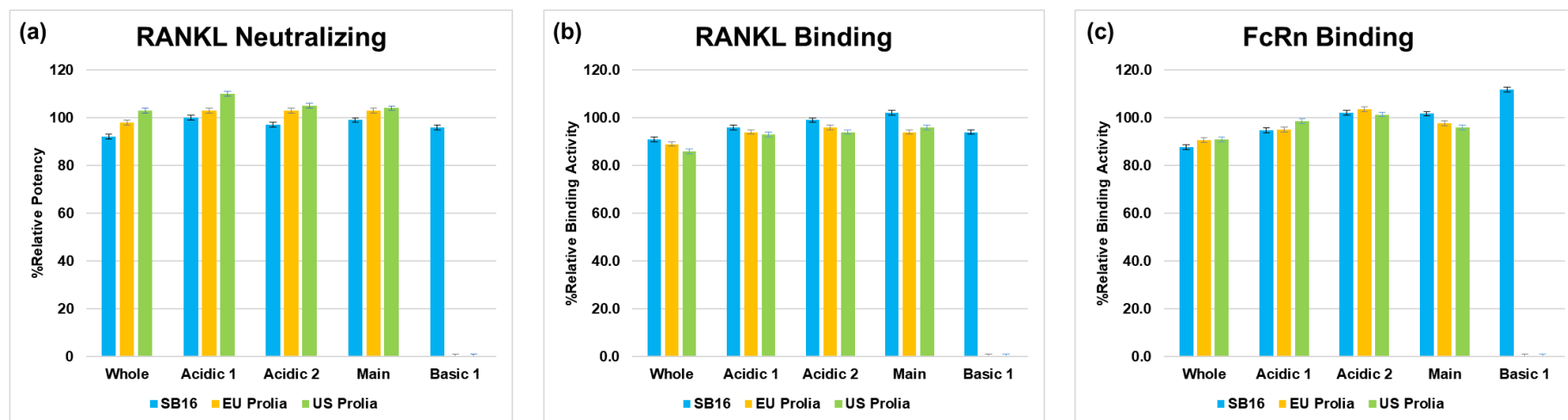
Supplementary Figure S5. Zoom view for N-glycan profile of SB16 and denosumab reference products at baseline level. (a) SB16 PFS, (b) EU Prolia, (c) US Prolia, (d) SB16 Vial, (e) EU Xgeva, (f) US Xgeva.



Supplementary Figure S6. Median and range of charge variants (%Acidic, %Main, %Basic) for SB16 and denosumab RP lots analyzed by CEX-HPLC and icIEF (a-c) CEX-HPLC results: (a) %Acidic, (b) %Main, (c) %Basic. (d-f) icIEF results: (d) %Acidic, (e) %Main, (f) %Basic. Each graph shows the median (center line) and range (whiskers) icIEF, imaging capillary isoelectric focusing; CEX, cation exchange; RP, reference product; EU, European Union; US, United States; PFS, pre-filled syringe.



Supplementary Figure S7. CEX-HPLC fractionation and overlay analysis of enriched charge variant peaks from SB16 and Prolia.



Supplementary Figure S8. Biological activity comparison across CEX-HPLC fractions of SB16 and denosumab RP. (a) RANKL neutralization potency, (b) RANKL binding activity, (c) FcRn binding activity. *CEX*, cation exchange; *RANKL*, receptor activator of nuclear factor kappa-B ligand; *FcRn*, neonatal fragment crystallizable receptor.