APPLICATION NOTE

No. 21565

The advantage of pH gradient buffers is demonstrated by the ion exchange separation of charge variants of denosumab

Manish Singh, Dr. Sachin Pandey,
Thermo Fisher Scientific, APAC CoE Lab,
Ahmedabad, India

Key words

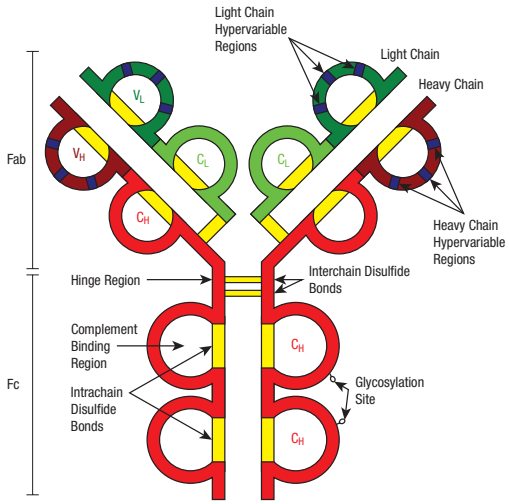
Denosumab, Monoclonal Antibody (IgG2), MabPac SCX-10, Biopharma, pH Gradient buffer, Salt Gradient, PDA detection, HPLC

Goal

- **To demonstrate the advantages of a linear pH gradient over alternative inorganic and organic buffer salt gradients for the high-resolution charge variant analysis of denosumab (IgG2) by ion exchange HPLC.**
- **To show the improved resolution of acidic charge variants by using the Thermo Scientific™ pre-mixed pH gradient buffers in conjunction with a Thermo Scientific™ MabPac™ SCX-10 column.**

Introduction

Denosumab is a novel, fully human IgG2 monoclonal antibody specific to receptor activator of nuclear factor kappa-B ligand (RANKL). It consists of two heavy and two light chains. Each light chain consists of 215 amino acids. Each heavy chain consists of 448 amino acids with four intramolecular disulphides.



The major differences between the human IgG subclasses are amino acid composition and structure of the 'hinge region', which is the part of the molecule containing disulfide bonds. This region between the Fab arms and the two carboxy-terminal domains of both heavy chains determines the flexibility of the molecule. The two heavy chains of the monoclonal antibody are connected in the hinge region by a variable number of disulfide bonds. Different IgG subclasses have different numbers of disulfide bonds:

- 2 for IgG1 and IgG4
- 4 for IgG2
- 11 for IgG3

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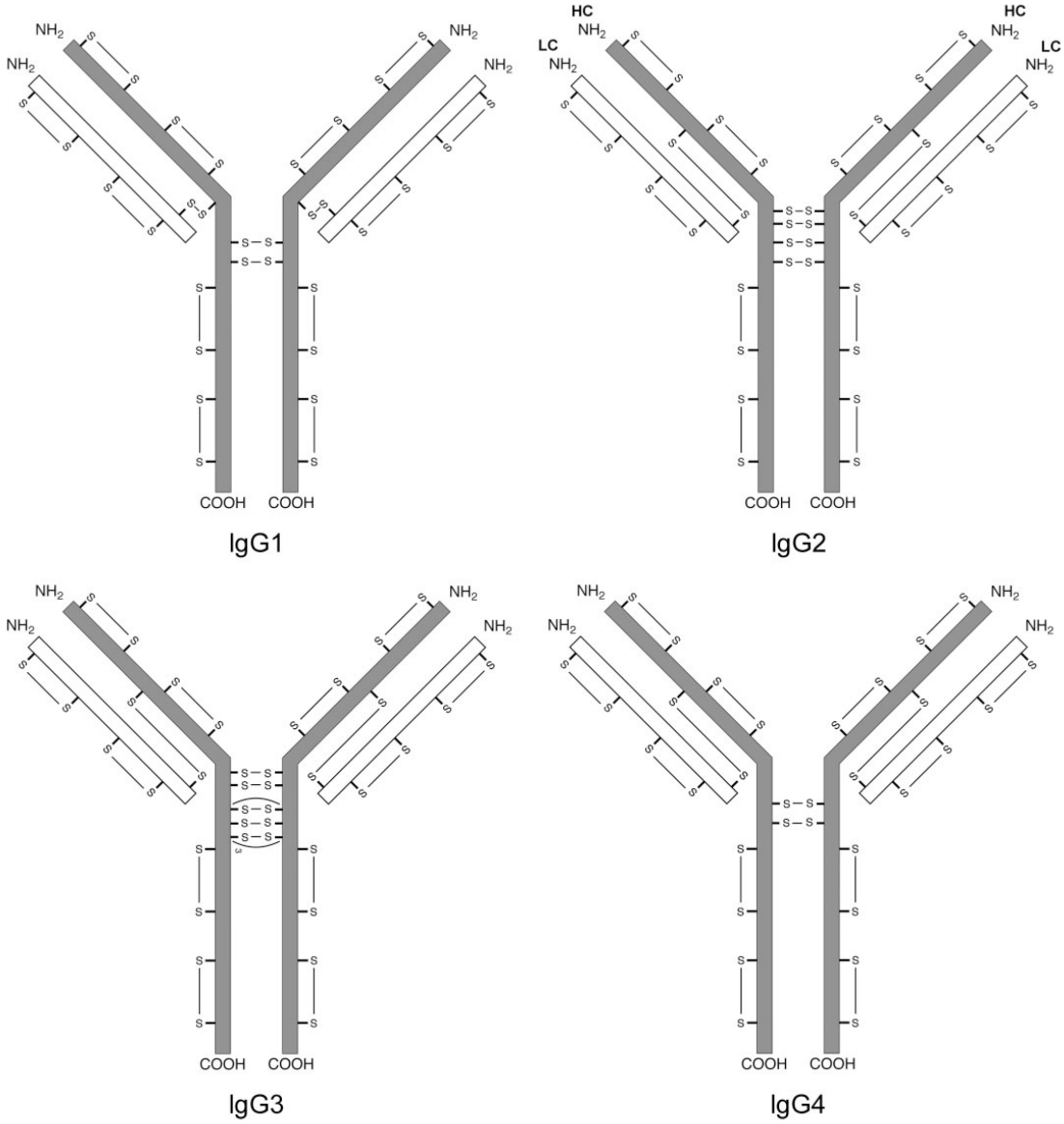

Figure 1. Schematic drawings of the human IgG subtypes indicating the originally proposed disulfide connections.

Another structural difference is the position of disulfide linkage between heavy chain and light chain. The light chain of the IgG1 is connected to the heavy chain via disulfide bond between the last cysteine residue of the light chain and the fifth cysteine residue of the heavy chain. However, for IgG2, IgG3, and IgG4 subclasses, the light chain is connected to the heavy chain via disulfide bond between the last cysteine residue of the light chain and the third cysteine residue of the heavy chain.[1]

Due to this structural property of IgG2 with respect to disulfide linkage, separation of acidic and basic variants of IgG2 becomes difficult using the conventional salt gradient method.

Charge variants of mAbs are due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Salt gradient cation-exchange chromatography has commonly been used with some success in characterizing mAb charge variants. However, significant effort is often required to tailor the salt gradient method for each individual mAb.[2] In the fast-paced drug development environment, a quick and robust platform method is desirable to accommodate the majority of the mAb analyses. Thermo Fisher Scientific recently introduced cation-exchange pH gradient buffers that meet this platform method requirement. The buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. The Thermo Scientific MabPac SCX-10 column was used for the chromatographic separations .

In this study, the charge variants of denosumab were analyzed using different approaches of salt gradients and pH gradient buffer. For salt gradient analysis, two different types of mobile phase were used:

- MES buffer (2-(N-morpholino) ethanesulfonic acid)
- Phosphate buffer

For pH gradient buffer analysis, linear gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 90% Thermo Scientific™ CX-1 pH gradient buffer A (pH 5.6) to 100% CX-1 pH gradient buffer B (pH 10.2). The results demonstrate the general applicability of the pH gradient method on monoclonal antibody charge variant analysis. The data obtained show that the pH gradient method delivers a higher-resolution power than the traditional salt method. The methods described here can be widely used in the development of the biosimilars of these top-selling IgG2 mAbs.

Experimental

Consumables

- CX-1 pH gradient buffer A (pH 5.6), 125 mL (P/N 083273)
- CX-1 pH gradient buffer A (pH 10.2), 125 mL (P/N 083275)
- Thermo Scientific™ MAbPac™ SCX-10 column (10 µm, 4.0 mm × 250 mm) (P/N 074625)
- Thermo Scientific™ Virtuoso™ 9 mm Wide Opening Clear Glass Screw Thread
- 1.5 mL High Recovery Vial with V-Patch (P/N 60180-VT309)
- Fisher Scientific™ Sodium phosphate dibasic anhydrous (P/N 10440481)
- Fisher Scientific Sodium phosphate monobasic anhydrous (P/N 10751135)
- Fisher Scientific MES (Fine White Crystals), Fisher BioReagents™ (P/N 10419123)
- Fisher Scientific Sodium chloride (P/N 11964051)
- Fisher Scientific HPLC grade water (P/N 10449380)
- Deionized water, 18.2 MΩ/cm resistivity

Sample handling equipment

- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Standard preparation

Denosumab (70 mg/mL concentration) was diluted to 1 mg/mL by using deionized water.

Separation conditions

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:

- SR-3000 Solvent Racks with Degasser (P/N 5035.9200)
- LPG-3400RS Separation Pump (P/N 5040.0036)
- WPS-3000TBRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)

pH Gradient:

Mobile Phase A:	CX-1 pH Gradient buffer-A Dilute CX-1 pH Gradient buffer-A diluted 10-fold using deionized water
Mobile Phase B:	CX-1 pH Gradient buffer-B Dilute CX-1 pH Gradient buffer-B diluted 10-fold using deionized water
Gradient Method:	Table 1

Salt Gradient Method 1:
Salt Gradient

Mobile Phase A:	20 mM MES Buffer pH 6.5
Mobile Phase B:	20 mM MES Buffer pH 6.5 + 300 mM sodium chloride
Gradient Method:	Table 2

Salt Gradient Method 2:
Salt Gradient

Mobile Phase A:	20 mM dibasic sodium phosphate + 20 mM monobasic sodium phosphate, pH 6.0
Mobile Phase B:	20 mM dibasic sodium phosphate + 20 mM monobasic sodium phosphate, pH 6.0, + 300 mM sodium chloride
Gradient Method:	Table 2

Table 1. Gradient conditions for pH gradient.

Time (min)	A %	B %
0	90	10
40	0	100
42	0	100
43	90	10
50	90	10

Table 2. Gradient conditions for salt gradient.

Time (min)	A %	B %
0	100	0
4	100	0
50	0	100
52	0	100
53	100	0
65	100	0

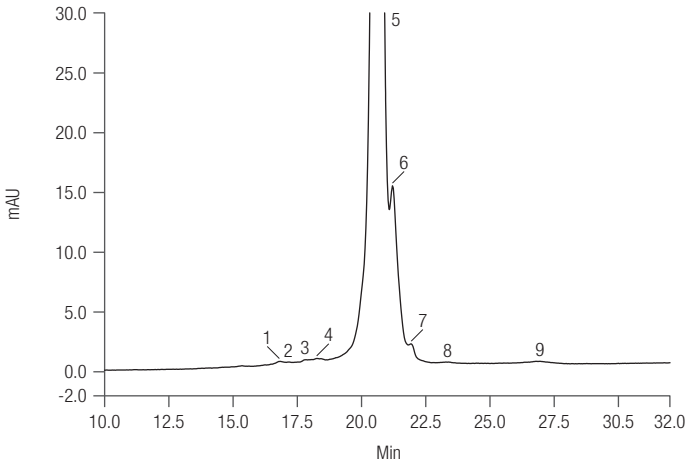

Figure 2. Denosumab charge variant analysis using salt gradient with MES buffer.

Flow Rate: 1.0 mL/min
Column Temperature: 40 °C
Injection Volume: 50 µL
UV Detector Wavelength: For pH gradient - 280 nm
For salt gradient - 280 nm (for MES buffer) 220 nm and 280 nm (for phosphate buffer)

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System was used for data acquisition and analysis.

Results and discussion

The CX-1 pH gradient buffer kit generates a linear pH gradient when a linear pump gradient is run from 90% CX-1 buffer A to 100% buffer B. This pH gradient method serves as a platform method for the mAb charge variant analysis, covering the pH range from 5.6 to 10.2. Denosumab was analyzed on a MabPac SCX-10 column using the full pH gradient and salt gradient method. Satisfactory separations of multiple variants were observed in denosumab.

Initially the salt gradient method with MES buffer was used for separation of charged variants. MES buffer was chosen as it falls in the Good's Buffer category because of its midrange pKa, maximum water solubility and minimum solubility in all other solvents, minimal salt effects, minimal change in pKa with temperature, chemical and enzymatic stability, minimal absorption in the visible or UV spectral range, and ability to be synthesized relatively easily.

The results obtained with MES buffer at 280 nm wavelength were good with respect to separation of basic charge variants, but acidic variants were not well separated (Figure 2 and Table 3).

Table 3. Denosumab charge variant analysis using salt gradient with MES buffer.

Peak	Peak Name	Retention Time (min)	Area (mAU*min)	Rel. Area (%)	Height (mAU)	Resolution (USP)	Plates (USP)
1	Acidic Variant-1	16.823	0.201	0.29	0.387	3.39	1745
2	Acidic Variant-2	17.167	0.117	0.17	0.322	4.86	7460
3	Acidic Variant-3	17.823	0.242	0.35	0.499	1.92	977
4	Acidic Variant-4	18.273	0.343	0.49	0.581	1.29	592
5	Denosumab	20.607	62.126	89.00	150.470	0.00	17665
6	Basic Variant-1	21.210	5.908	8.46	14.949	0.73	6873
7	Basic Variant-2	21.920	0.612	0.88	1.708	1.33	4172
8	Basic Variant-3	23.377	0.097	0.14	0.136	2.91	5283
9	Basic Variant-4	26.970	0.161	0.23	0.168	5.91	4954

To check for the better separation of acidic variants, phosphate buffer was used with the salt gradient method, but the separation obtained for acidic variant species was almost similar to that of MES buffer (Figure 3 and Table 4).

With phosphate buffer as mobile phase 2, 220 nm and 280 nm wavelengths were evaluated. As the response was high at 220 nm, we selected 220 nm as the detection wavelength for phosphate buffer. A comparison of results obtained at 220 nm and 280 nm is shown in Figure 4.

In case of salt gradient analysis separation, there was no improved resolution of acidic variants if compared with phosphate and MES buffer (Figure 5).

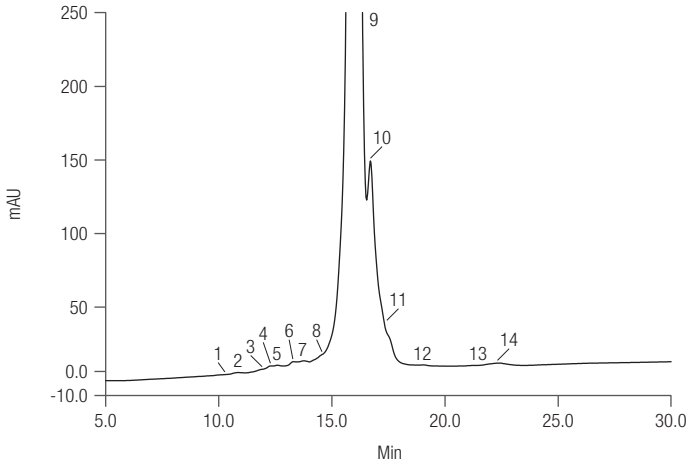

Figure 3. Denosumab charge variant analysis using salt gradient with phosphate buffer.

Table 4. Denosumab charge variant analysis using salt gradient with phosphate buffer.

No.	Peak Name	Retention Time (min)	Area (mAU*min)	Rel. Area (%)	Height (mAU)	Resolution (USP)	Plates (USP)
1	Acidic Variant-1	10.300	0.182	0.02	0.472	n.a.	n.a.
2	Acidic Variant-2	10.870	0.933	0.12	1.572	4.31	669
3	Acidic Variant-3	11.943	1.625	0.20	3.292	n.a.	n.a.
4	Acidic Variant-4	12.307	2.200	0.28	5.363	1.81	212
5	Acidic Variant-5	12.587	2.231	0.28	5.671	2.15	417
6	Acidic Variant-6	13.303	4.299	0.54	7.694	1.20	199
7	Acidic Variant-7	13.733	3.650	0.46	8.120	1.09	257
8	Acidic Variant-8	14.590	5.626	0.71	12.086	n.a.	n.a.
9	Denosumab	15.983	686.149	86.25	1446.726	0.00	8532
10	Basic Variant-1	16.710	69.930	8.79	142.298	0.84	4136
11	Basic Variant-2	17.380	11.567	1.45	27.665	n.a.	n.a.
12	Basic Variant-3	19.043	1.942	0.24	2.646	0.13	3
13	Basic Variant-4	21.497	2.583	0.32	1.095	n.a.	n.a.
14	Basic Variant-5	22.353	2.582	0.32	2.229	4.34	1590

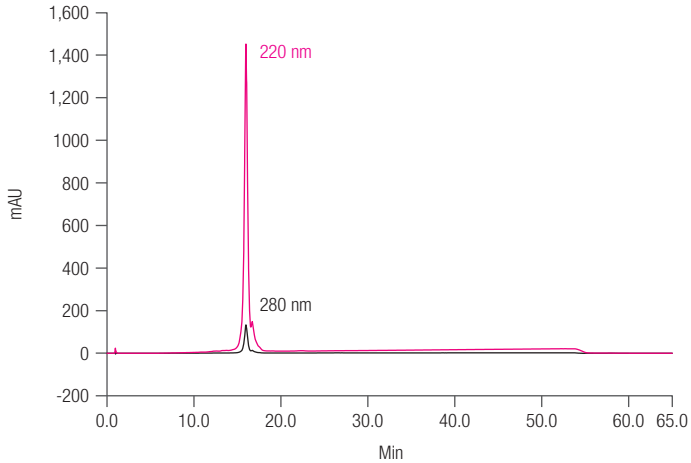

Figure 4. Sensitivity difference between 220 nm and 280 nm wavelengths when using phosphate buffer.

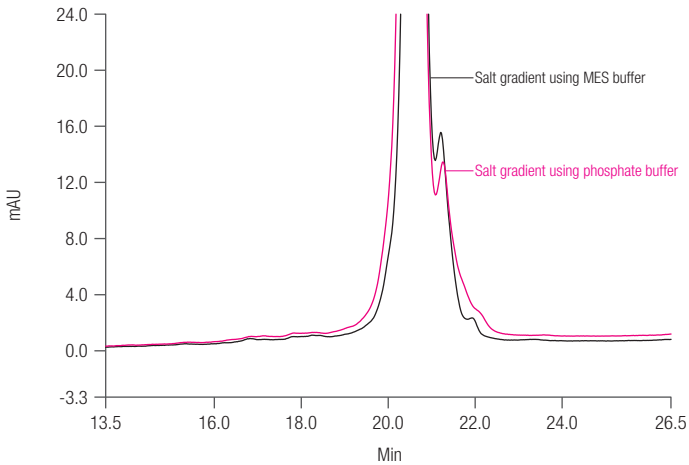

Figure 5. Comparison between profiles of MES and phosphate buffer.

Further, for separation of acidic variants from denosumab, pH gradient buffers were evaluated with a linear pH gradient separation method. The linear gradient from pH 5.6 to pH 10.2 was generated over time by running a linear pump gradient from 90% Thermo Scientific CX-1 pH Gradient buffer A (pH 5.6) to 100% CX-1 pH Gradient buffer B (pH 10.2). The results demonstrate the general applicability of the pH gradient method on monoclonal antibody charge variant analysis. The data also show that the pH gradient method delivers higher-resolution power than the traditional salt method with MES buffer (Figure 6, Table 5, and Figure 7).

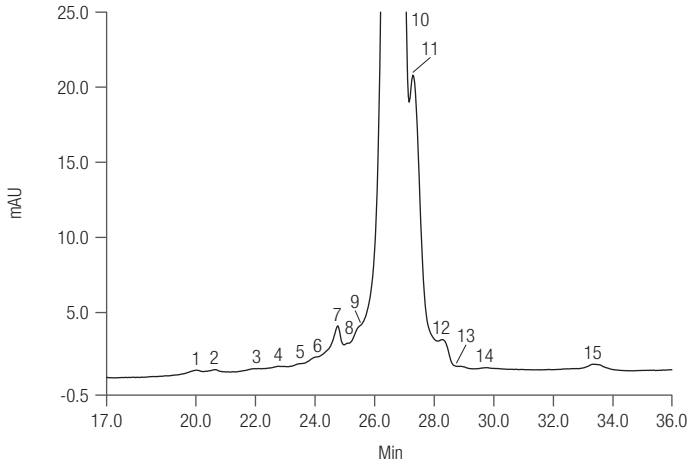

Figure 6. Denosumab charge variant analysis using pH gradient buffer.

Table 5. Denosumab charge variant analysis using pH gradient buffer.

No.	Peak Name	Retention Time (min)	Area (mAU*min)	Rel. Area (%)	Height (mAU)	Resolution (USP)	Plates (USP)
1	Acidic Variant-1	20.033	0.208	0.352	0.20	6.03	3461
2	Acidic Variant-2	20.643	0.279	0.373	0.27	5.31	3339
3	Acidic Variant-3	22.063	0.272	0.395	0.26	2.70	1177
4	Acidic Variant-4	22.793	0.396	0.522	0.39	1.91	802
5	Acidic Variant-5	23.530	0.276	0.688	0.27	n.a.	n.a.
6	Acidic Variant-6	24.107	0.509	1.114	0.50	n.a.	n.a.
7	Acidic Variant-7	24.767	1.702	3.163	1.66	2.37	16944
8	Acidic Variant-8	25.173	0.372	2.007	0.36	n.a.	n.a.
9	Acidic Variant-9	25.553	1.003	3.198	0.98	n.a.	n.a.
10	Denosumab	26.660	87.222	157.221	84.89	0.00	16291
11	Basic Variant-1	27.300	8.897	19.785	8.66	0.58	6442
12	Basic Variant-2	28.270	0.909	2.123	0.88	0.90	1716
13	Basic Variant-3	28.777	0.137	0.337	0.13	n.a.	n.a.
14	Basic Variant-4	29.730	0.219	0.210	0.21	2.31	4247
15	Basic Variant-5	33.360	0.341	0.403	0.33	6.45	11571

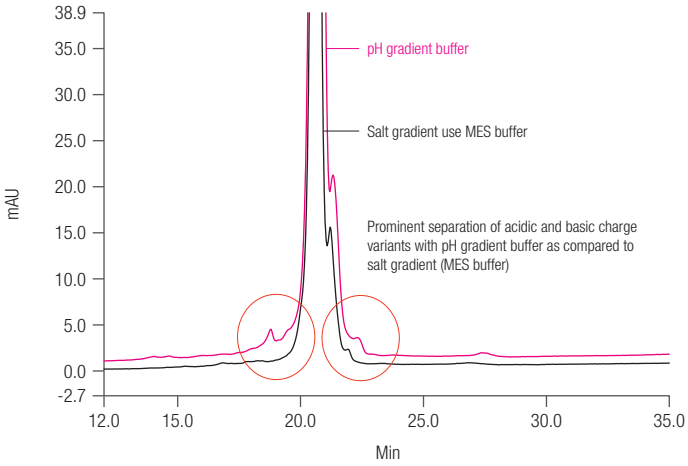

Figure 7. Comparison between profiles of MES and pH gradient buffer.

A comparison of all the results from the salt gradient (MES and phosphate) and pH gradient buffers was performed in order to evaluate the best method for separation of charge variants from denosumab (Figure 8 and Table 6).

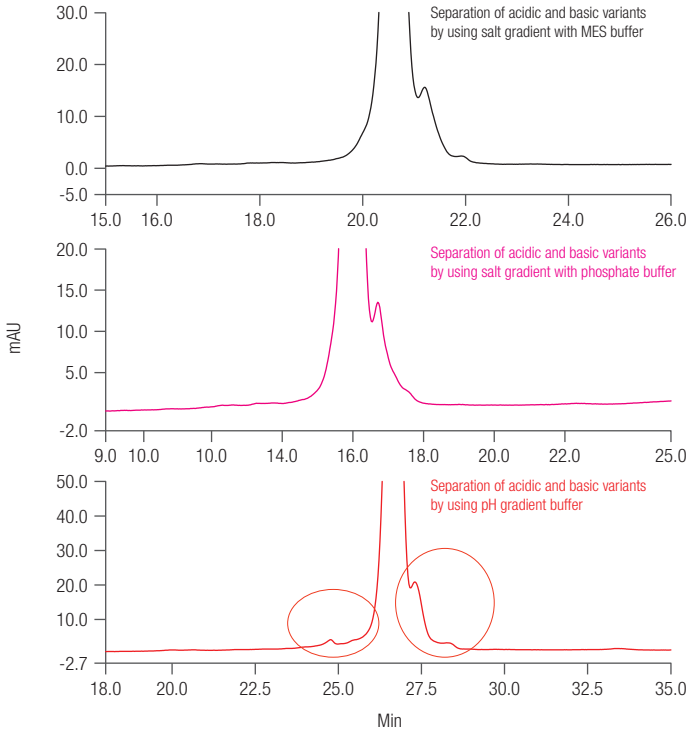

Figure 8. Comparison between profiles of MES, phosphate, and pH gradient buffers.

Table 6. Comparison of % charge variant in denosumab by pH and salt gradient (MES & phosphate).

Sample Name	% Acidic Variants	% Denosumab	% Basic Variants
Denosumab Standard (by pH gradient method)	4.89	84.89	10.21
Denosumab Standard (by salt gradient method using MES buffer)	1.3	89.00	9.71
Denosumab Standard (by salt gradient method using phosphate buffer)	2.61	86.25	11.12

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Conclusions

- In this study various approaches of salt and pH gradients were evaluated for separation of charge variants of denosumab.
- MES is the salt buffer of choice when compared to alternative inorganic salt buffers such as phosphate.
- The pH buffer gave enhanced separation of charge variants and should be chosen as an alternative to salt gradients when enhanced separation is required.
- The pH buffer has additional advantages, as the salt concentration can be kept low, yielding fewer buffer interferences (e.g., online or offline two-dimensional LC [2D-LC]). Also, pH-gradient IEC is promising for high-throughput and fast screening of antibodies.

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