Original article

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Analytical Method Development and Validation of Purity in Nimotuzumab Products

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Abstract

Nimotuzumab, a therapeutic mAb targeting the epidermal growth factor receptor (EGFR), has gained approval for treating various indications globally including squamous cell carcinomas of the head and neck, nasopharyngeal cancer, and pancreatic cancer in numerous countries. To ensure the quality of nimotuzumab, there are several crucial elements in the biopharmaceutical manufacturing process, aiming to guarantee the safety, effectiveness and consistency of the product. Aggregation is one of the quality control topics that is very important. It can impact biological activity and induce immunogenic responses in patients. Size exclusion chromatography (SEC) stands out as the standard method for quantifying proteins and their aggregates. The study focuses on the development and validation of a specific SEC chromatographic method for measuring nimotuzumab aggregates. The validation process adheres to ICH guidelines, covering specificity, limit of detection (LOD), limit of quantitation (LOQ), precision, and intermediate precision. The developed method utilizes TSKgel® UltraSW aggregate column in tandem with a Sepax TweenTrap column, demonstrating specificity, accuracy, and precision in determining nimotuzumab purity in biopharmaceutical products. The validated method was applied to test a marketed nimotuzumab product in Thailand. In conclusion, the developed and validated SEC method proves to be a specific, accurate and precise tool for assessing nimotuzumab purities in the biopharmaceutical context.

Keywords: Aggregate, Nimotuzumab, Size exclusion chromatography

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Introduction

Monoclonal antibodies (mAbs) received initial approval over three decades ago from the United States Food and Drug Administration (US FDA) and have since demonstrated a growing market presence in the last five years [1]. The primary structure of these antibodies comprises heavy and light chains, glycosylation, disulfide bonds, and inherent heterogeneity [2]. Nimotuzumab, a therapeutic monoclonal antibody targeting the epidermal growth factor receptor (EGFR), has found application as a therapy in various countries. It has gained approval for treating multiple indications, including squamous cell carcinomas of the head and neck, glioma, nasopharyngeal cancer, and pancreatic cancer in numerous countries.

Ensuring the quality of nimotuzumab is a crucial element in the biopharmaceutical manufacturing process, aiming to guarantee the safety, effectiveness, and consistency of the end product. There are some key aspects of quality control for monoclonal antibodies such as identification, potency, impurities including aggregation. Molecular changes can occur at various stages of the manufacturing process. Aggregation can be initiated with smaller molecules, such as dimers or fragments, and progress to larger entities like sub-visible or visible particles. These alterations have the potential to reduce biological activity and may induce immunogenic responses in patients.

Size exclusion chromatography (SEC) is basically the standard method for quantitative protein and its aggregates. The sample will pass through the column and be separated by molecular weight. The highest molecular weight elutes first and the smaller which access into the pore of stationary phase will elute later like the peak of aggregate usually come before its monomer. In this study, the method of SE chromatography was developed and validated for measuring the aggregates of nimotuzumab. The validation includes specificity, limit of detection (LOD), limit of quantitation (LOQ), precision, and intermediate precision according to the ICH guideline Q2(R1).

Material and method

Material

High Performance Liquid Chromatography, HPLC (Dionex, USA, consisting of Pump, Autosampler, Column oven, UV Detector, and program Chromeleon). Analytical column (Shodex LW-803 column and TSKgel® UltraSW aggregate column). TweenTrap column (Sepax TweenTrap column). Analytical balance (Mettler, model XPR205). pH meter (Mettler, model SevenCompact S220). 0.2-µm cellulose acetate membrane filter (Grace, USA). Dibasic sodium phosphate dihydrate (Carlo Erba). Potassium dihydrogen phosphate (Carlo Erba). Sodium chloride (Carlo Erba). Dibasic sodium phosphate (Carlo Erba). Monobasic sodium phosphate (Carlo Erba). Polysorbate (Tween) 80 (Sigma Aldrich). Sodium azide (Sigma Aldrich). Isopropyl alcohol, HPLC grade (Carlo Erba). Pantetic sodium phosphate heptahydrate (Panreac). Sodium hydroxide (Carlo Erba). Nimotuzumab CIM (Code MRT(QFB) h-R3/1906, 5 mg/mL).

Method

1. Reagent preparation

1.1 Matrix solution

The excipients contained in nimotuzumab product formulation are listed in Table 1 below.

Table 1. Nimotuzumab product formulation and matrix solutions

Component	Matrix solution (1X)	Matrix solution (2X)
Nimotuzumab (mg)	-	-
Dibasic sodium phosphate (mg)	18.0	360
Monobasic sodium phosphate (mg) or Monobasic sodium phosphate monohydrate (mg)	4.5 5.175	90 103.5
Sodium chloride (mg)	86.0	1720
Polysorbate 80 (mg)	2.0	40
Sterile water for injection (mL) q.s. to	10	100

1.2 10X phosphate buffer solution (PBS)

Accurately weigh 14.4 g of disodium hydrogen phosphate dihydrate, 2.0 g of potassium hydrogen phosphate and 234.0 g of sodium chloride and dissolve with water type I,

stir the solution until homogenized. Bring the volume up to 1,000 mL by volumetric flask and mix thoroughly. The solution should be verified before use by preparing 1X PBS. The pH shall be between 7.2-7.4, if it is out of range, discard the preparation.

1.3 Mobile phase, 1X PBS

Transfer 100 mL of 10X PBS to 1-L volumetric flask and bring the volume up to 1,000 mL and mix thoroughly. Stir the solution with a magnetic stirrer until homogenized. Measure the pH which should be within the range 7.2-7.4 and filter through the membrane filter.

1.4 Tween 80 solution (0.2% w/v)

Prepare 0.2% w/v Tween 80 solution in water type I.

2. Standard preparation

Bring the Nimotuzumab CIM standard to stand at the ambient temperature before use about 10 minutes and prepare a series of solutions as presented in Table 2. Mix solution well before transfer to the HPLC vial. Store at 2-8˚C until use.

Table 2. Dilution of nimotuzumab CIM solution

Nimotuzumab CIM solution (%)	Concentration (mg/mL)	Nimotuzumab CIM solution used (mg/mL)	Volume of Nimotuzumab CIM solution used (µL)	Matrix solution (µL)	Total volume (µL)
100	5	Nimotuzumab CIM	-	-	-
5	0.25	5	20	380	400
2.5	0.125	0.25	100	100	200
2	0.1	0.25	80	120	200
1.25	0.0625	0.125	100	100	100
1	0.05	0.1	100	100	100
0.625	0.03125	0.0625	100	100	100
0.5	0.025	0.05	100	100	100
0.3125	0.0156	0.03125	100	100	100
0.25	0.0125	0.025	100	100	100

3. Control sample and test sample preparation

3.1 100% Control sample preparation

Use Nimotuzumab CIM solutions (5 mg/mL) for 100% control sample.

3.2 5% Control sample preparation (0.25 mg/mL)

Dilute 10 µL of nimotuzumab CIM with 190 µL of mobile phase and mix solution well before transfer to an HPLC vial. Store at 2-8˚C until use.

3.3 Test solution

Bring a sample to stand at the ambient temperature before use about 10 minutes and mix solution well before transfer to the HPLC vial. Store at 2-8˚C until use.

4. HPLC condition

Pump mode	Isocratic
Mobile phase	PBS solution
Flow rate	mL/min
Column temperature	ambient
Autosampler temperature	5ºC
Injection volume	10 µL
Detector	PDA 280 nm
Run time	40 minutes

5. System suitability

Perform the injections as follows and evaluate suitability of the system as per the criteria in Table 3.

Mobile phase	1 injection
5% control sample	3 injections
100% control sample	3 injections

Table 3. System suitability criteria

No.	Criteria	Acceptance limit
1	%RSD of area and retention time of monomer peak obtained from 3 injections of 100% control sample	≤ 2%
2	%RSD of area and retention time of monomer peak obtained from 3 injections of 5% control sample	≤ 2%
3	Detection accuracy*	4-6%

*Detection accuracy

$$\frac{\text{Average area of monomer peak obtained from 3 injections of 5\% control sample}}{\text{Average area of monomer peak obtained from 3 injections of 100\% control sample}} \times 100$$

6. Method development

6.1 Column selection

The size exclusion column (SEC) was used for purity analysis including monomer, dimer, and other higher molecular masses. There were two columns chosen for this experiment Shodex, LW-803 column (300 x 8.0 mm, 3 µm) and a TSKgel® UltraSW aggregate column (300 x 7.8 mm, 3 µm). The chromatograms from two

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columns will be compared and evaluated the peak shape (asymmetry) and the separation (resolution).

Moreover, in this experiment the TweenTrap column was used with the analysis column because there was the tween80 in the nimotuzumab formulation and its peak interferes with the interesting peaks of nimotuzumab. Sepax TweenTrap column (4.6 × 35 mm, 5 µm) was selected for eliminate the Tween 80 peak.

The chromatogram of mobile phase, matrix solution (1x) and tween80 solution was injected by using the chromatographic condition with and without TweenTrap column and evaluated the absence of Tween 80 peak. Baseline of the chromatograms of the matrix and Tween 80 solutions obtained from the injections eluted through the TweenTrap column should be as smooth as that of obtained from the mobile phase. Evaluate peak shape, %relative area and resolution of the peaks from the chromatograms obtained from the condition with or without TweenTrap column.

6.2 Injection volume

Vary the injection volume from 1-50 uL and plot a curve between injection volume and peak area. Evaluate linear regression of the curve.

7. Method validation

The developed method was validated as per the following validation parameters, specificity, limit of detection (LOD), limit of quantitation (LOQ), precision (repeatability and intermediate precision).

7.1 Specificity

Inject the mobile phase, matrix solution (1x), 100% control sample, finish product (FP) solution and Tween80 solution and evaluate the interferences from excipients, mobile phase and other reagent used in the method on the interesting peaks.

7.2 LOD and LOQ

Determine the LOD and LOQ of dimer by a signal to noise ratio of 2-3:1 and 10:1, respectively. At the LOQ level, calculate %relative peak area of the dimer comparing with the average peak area value of the nimotuzumab obtained from 100% control sample. Moreover, the six replicates of the LOD and LOQ solutions were performed and determined the accuracy and precision at the LOQ level as %recovery and %RSD. The criteria were based on AOAC Official Method for Analysis (2016), Guidelines for Standard Method Performance Requirements Appendix F, as stated in Table 5.

7.3 Precision (repeatability and intermediate precision)

Prepare six replicates of the 5% nimotuzumab CIM solution and calculate %recovery and %RSD of the %relative peak area of the dimer and monomer. Moreover, the intermediate precision was performed with the inter-day precision and in different analysts and calculate %recovery and %RSD of dimer and monomer the same as precision. The criteria were based on AOAC Official Method for Analysis (2016), Guidelines for Standard Method Performance Requirements Appendix F, as stated in Table 5.

7.4 Specification

Table 4. Validation parameters and acceptance criteria

Validation Parameter	Acceptance criteria
Specificity	Peaks of excipients, mobile phase and other reagents are not co-eluted at the RT of nimotuzumab and its dimer and related substances of higher molecular weight [4]
LOD/LOQ	LOD: Signal to noise ratio (S/N) = 2-3:1 [4] LOQ S/N = 10:1 [4]
Precision	
- Repeatability	%RSD as calculated from %relative area [2.3]
- Intermediate precision	
Inter-day study	%RSD as calculated from % relative area [2.3]
Different analysts	%RSD as calculated from % relative area [2.3]

Table 5. Expected recovery (a) and expected precision (repeatability) (b) as a function of analyte concentration [5].

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Analyte, %	Mass fraction (C)	Unit	Mean recovery, %
100	1	100%	98–102
10	10^{-1}	10%	
1	10^{-2}	1%	97–103
0.1	10^{-3}	0.1%	95–105
0.01	10^{-4}	100 ppm (mg/kg)	90–107
0.001	10^{-5}	10 ppm (mg/kg)	80–110
0.0001	10^{-6}	1 ppm (mg/kg)	
0.00001	10^{-7}	100 ppb (µg/kg)	
0.000001	10^{-8}	10 ppb (µg/kg)	60–115
0.0000001	10^{-9}	1 ppb (µg/kg)	40–120

(a)

* Table excerpted from *AOAC Peer-Verified Methods Program, Manual on Policies and Procedures* (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

Analyte, %	Mass fraction (C)	Unit	RSD_r, %
100	1	100%	1.3
10	10^{-1}	10%	1.9
1	10^{-2}	1%	2.7
0.1	10^{-3}	0.1%	3.7
0.01	10^{-4}	100 ppm (mg/kg)	5.3
0.001	10^{-5}	10 ppm (mg/kg)	7.3
0.0001	10^{-6}	1 ppm (mg/kg)	11
0.00001	10^{-7}	100 ppb (µg/kg)	15
0.000001	10^{-8}	10 ppb (µg/kg)	21
0.0000001	10^{-9}	1 ppb (µg/kg)	30

(b)

* Table excerpted from *AOAC Peer-Verified Methods Program, Manual on Policies and Procedures* (1998) AOAC INTERNATIONAL, Rockville, MD, USA.

Results and Discussion

1. Method development

1.1 Column selection

Firstly, a Shodex Protein LW-803 with µL of 5 mg/mL of nimotuzumab sample so tion studied. All four peaks of high mo ar weig (HMW), dimer, monomer and low olecular weight (LMW) were detected igure 1). L eline of the chromatogram of mob ha was smooth. There was no interfere resen d. Ho ever, the chromatogram of atrix solu shows an interference from Tw 8 mall broad peak of Tween 80 (RT about 20 mm) coeluted with main protein peaks (RT; imers about 14 and monomer about 17 min). Moreover, the 0.02% of Tween 80 solution was prepared to confirm the Tween 80 peak. Therefore, it can be concluded that peaks of the major components were interfered from Tween 80 (Figure 2).

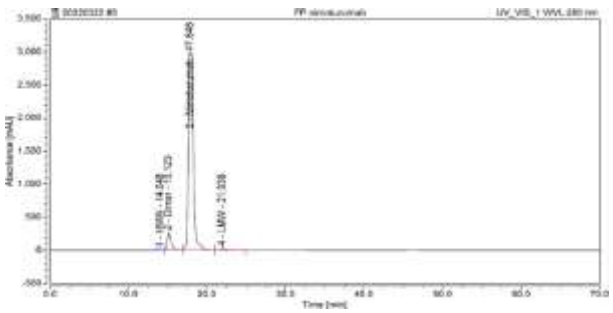


Figure 1 Chromatogram of nimotuzumab sample solution (injection volume of 50 µL)

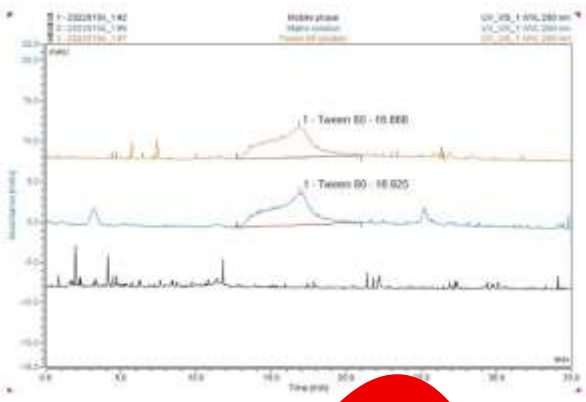


Figure 2 Overlaid chromato ms of th obile phase (black), matrix solution (blue) d 0.02% Tween 80 solution (yellow) (inje on volu of 5 µL)

Because o he i erfe e of Tween80 peak with th tere g pe ks, the TweenTrap column w ached t SE column to trap Tween 80 bef moving to the SE column. Chr atogram of atrix solution, mobile phase and Tween 80 solution from the system using Twe Trap olumn exhibits the flat and smooth eline while the other shows a broad peak of T 80 (Figure 3). The results show that weenTrap column assisted removing the Tween 80 peak since it completely trapped of Tween 80 as compared with the mobile phase (Figure 3).

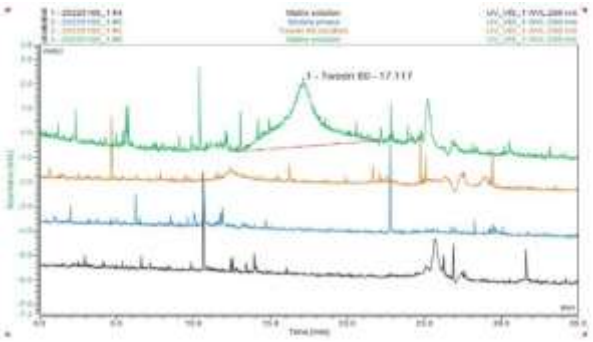


Figure 3 Overlaid chromatogram obtained from the solutions run in tandem with TweenTrap column and injection volume of 50 µL; matrix solution (black), mobile phase (blue), Tween 80 solution (yellow) and the matrix solution without TweenTrap column (green)

Two SE columns were chosen for this experiment and compared the results. They are slightly different in surface coating, hydrophilicity of the packing materials or diameter of the column. The compounds of

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interest were analyzed using the two SE columns with and without TweenTrap column. As mentioned earlier, with the injection volume of 50 µL, a small peak of Tween 80 interfered the interested compounds. However, when the injection volume was adjusted to 10 µL, Tween 80 peak became undetected and %relative area and chromatographic parameters in both SE columns with and without TweenTrap column were not different (Table 6).

Table 6. Comparison of the %relative area and chromatographic parameters obtained from two different SE columns with the injection volume of 10 µL.

Compound	% Relative area			
	TSKgel UltraSW aggregates		Protein LW-803	
	w/o TweenTrap	with TweenTrap	w/o TweenTrap	with TweenTrap
HMW	0.26	0.24	0.20	0.17
Dimer	4.44	4.39	4.46	4.39
Nimotuzumab	95.22	95.30	95.27	95.32
LMW	0.08	0.06	0.07	0.12
Dimer+nimotuzumab	99.66	99.69	99.73	99.71

Parameter	asymmetry	resolution	asymmetry	resolution	asymmetry	resolution	asymmetry	resolution
HMW	2.00	2.42	1.95	2.41	1.63	1.64	1.43	1.51
Dimer	1.81	5.01	2.32	5.05	1.62	5.06	2.08	4.91
Nimotuzumab	1.25	5.89	1.36	6.08	1.10	7.23		66
LMW	0.90	-	0.89	-	0.86	-		

Relative area and chromatographic parameters (asymmetry factor and resolution) showed that each peak (dimer and nimotuzumab) has slight tail and good resolution (Table 6). Both Shodex Protein LW-803 and TSKgel UltraSW aggregate with TweenTrap columns resulted in similar results in terms of relative area and chromatographic parameters. Both SE columns with TweenTrap columns worked well with the nimotuzumab samples. The TSKgel UltraSW aggregates with TweenTrap columns were chosen for method validation.

1.2 Injection volume

At the beginning, volume of injection of 50 µL was selected for the study since the small peak of HMW, dimer and LMW were expected to be detected. Amount injected of 250 µg nimotuzumab contained in 50 µL of the solution was loaded to the column. The results show that peak of HMW, dimer, monomer and LMW were all detected. With TweenTrap column, above chromatograms confirm the absence of Tween 80 interference at high loading (50 µL). However, peak broadening of the monomer was observed due to mass overload. This contributed to lower resolution and integration error of the peak. The appropriate volume of injection of the nimotuzumab solution was optimized.

Injection volume of 100% control solution was varied from 1 to 50 µL. The graph between injection volume VS peak area was plotted (Figure 4). The slope of the monomer curve decreased when the volume of injection increased. Area of the monomer peak showed a trend to reach plateau at the high injection volume. At high injection volume, mass of nimotuzumab overloaded. Linear curves of both dimer and monomer peak with of 1 were obtained from the injection volume of 1 to 10 µL. Percent relative area of dimer of the injection volume of 10 µL was almost identical (4.44-4.47%). Therefore, injection volume of 10 µL was the greatest volume giving the linear relationship between injection volume and peak area and could detected all four peaks contained in the nimotuzumab solution.

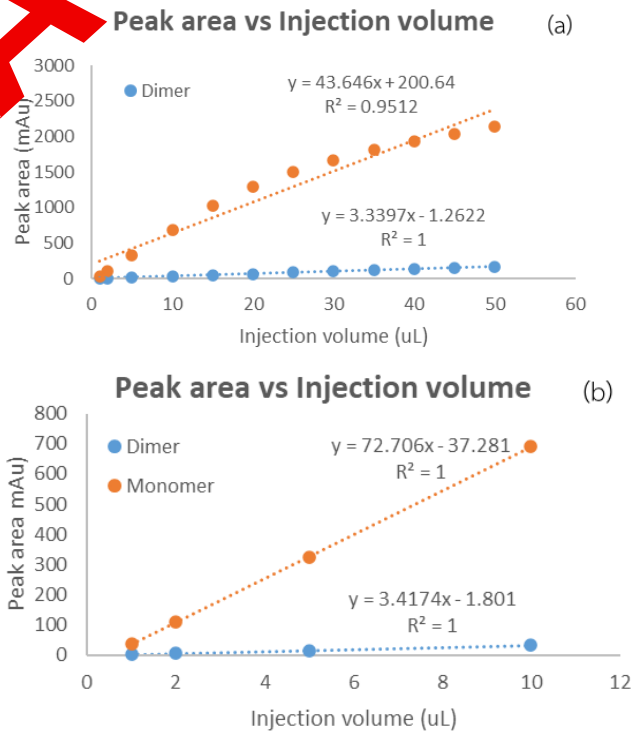


Figure 4 Graph of peak area VS injection volume; injection volume of 1-50 µL (a) and 1-10 µL (b)

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Chromatogram of nimotuzumab solution obtained from injection volume of 10 and 50 µL of 100% control sample was shown in Figure 5. Peak of monomer obtained from injection volume of 10 µL was sharp and more asymmetric than that of obtaining from the injection volume of 50 µL.

In conclusion, injection volume of 10 µL was suitable for this study. When the volume of injection was decreased from 50 to 10 µL, peak of Tween 80 was not detected. However, to ensure that Tween 80 peak was completely removed. TweenTrap column was still attached.

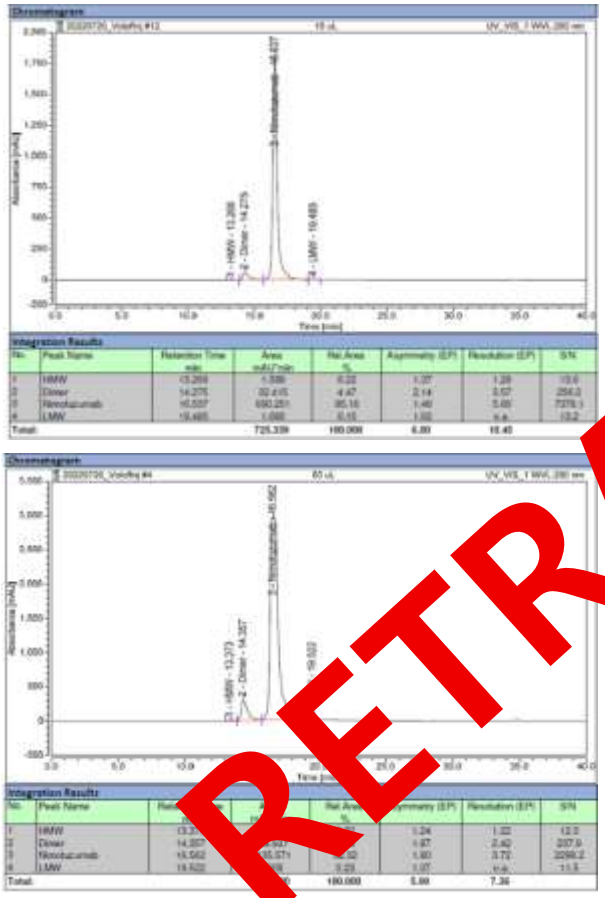


Figure 5 Chromatograms of 100% control sample obtained from the injection volume of 10 (a) and 50 µL (b)

2. Method validation

2.1 Specificity

Chromatograms showed that mobile phase, matrix solution and Tween 80 solution do not have any peaks interfering with the interested peaks (Figure 6).

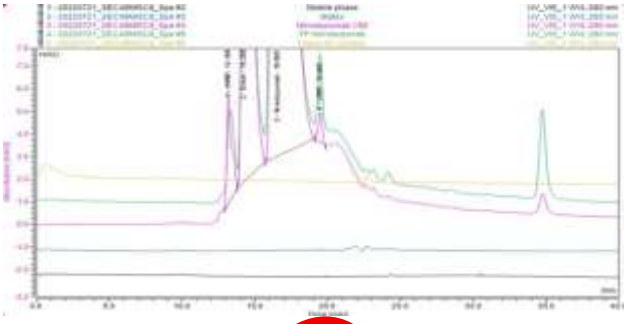


Figure 6 Chromatograms of mobile phase, matrix (1x), 100% control sample, FP solution and Tween 80 solution obtained from the injection volume of 10 µL.

Moreover, peaks of HMW, dimer, nimotuzumab and LWM were well separated. Resolution between the HMW-dimer, dimer-nimotuzumab and nimotuzumab-LMW were 2.29, 3.37 and 5.60, respectively (Figure 5a). Therefore, this method was specific for determination of nimotuzumab and its impurities.

2.2 LOD and LOQ

A series of nimotuzumab CIM solutions in matrix of concentration of 0.125-2.5% (6.25-125 µg/mL) was injected to the HPLC for determining the LOD and LOQ of dimer. LOD and LOQ of the dimer were measured based on signal to noise ratio at about 3:1 and 10:1, respectively.

For the LOD determination, peak of dimer was not observed from 0.125% (6.25 µg/mL) and 0.3125% (12.5 µg/mL) nimotuzumab CIM solutions, while peak of dimer was detected with signal to noise ratio of 6.2:1 in 0.5% (25 µg/mL) nimotuzumab CIM solution (Figure 7). To confirm, six replicates of 0.5% nimotuzumab CIM solutions were prepared and injected. The signal to noise ratio of six replicates was in a range of 7.3-9.9. Therefore, the solution at LOD level was 0.5% (25 µg/mL) nimotuzumab CIM.

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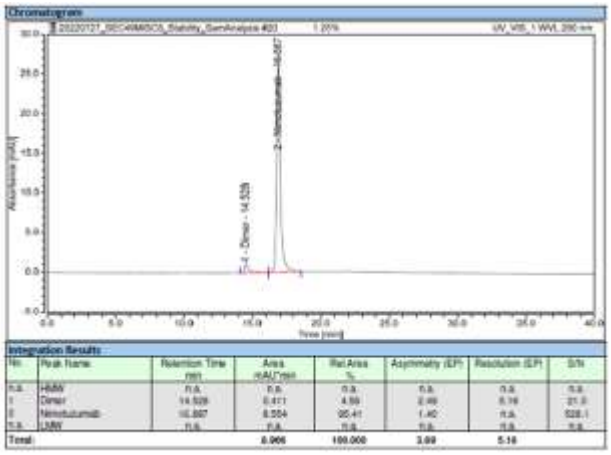
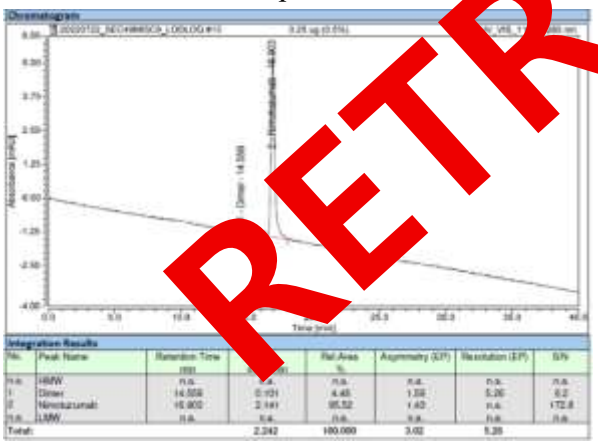


Figure 7 Chromatogram of 0.5% nimotuzumab CIM obtained from the injection volume of 10 µL.

For the LOQ determination, signal to noise ratio of dimer peak was 21.0:1 from 1.25% (62.5 µg/mL) nimotuzumab CIM solution (Figure 8). To confirm, six replicates of th solution were prepared and injected. The signa to noise ratio of six replicates was in a e of



16.7:1-28.3:1.

Figure 8 Chromatogram of 1.25% nimotuzumab CIM obtained from the injection volume of 10 µL.

The expected precision and accuracy were based on AOAC Official Method for Analysis (2016), Guidelines for Standard Method Performance Requirements Appendix F, as stated in Table 5. Firstly, the %analyte of the dimer at LOQ level should be calculated as following explanation.

The concentration of sample solution was 5 mg/mL nimotuzumab (100% nimotuzumab CIM solution) or 0.5% g/mL and the limit of dimer w s _ %. Therefore, the %dimer at limit w 0.5 x /100) = 0.035% analyte. Howe r, T LOQ evel was 1.25% nimotuzume CIM sol . The %analyte of dimer sh uld .00043 5%. As per Table 5a and l t the l el of analyte 0.0001%, the recovery hould be 80-110% and %RSD epeatabili should be 11%.

he results from the replicates LOQ s as in Table 7. % Recovery of dimer of 6 eplicate samples was between 81-93% which met the criteria of 80-110% (Table 7). The %RSD of 6 replicates was 4.68% (Table 7), which was smaller than the expected precision of 11%. Therefore, the solutions at LOD and LOQ level were 0.5 and 1.25% of nimotuzumab CIM or 25 and 62.5 and µg/mL, respectively.

Table 7 %Recovery and %RSD of 6 replicates of LOQ solution

Injection #	Area (dimer) Nimotuzumab CIM solution		%Recovery
	1.25%	100%	
1	0.381	34.936	0.87
2	0.355	34.830	0.81
3	0.389	34.837	0.89
4	0.400	-	0.92
5	0.407	-	0.93
6	0.390	-	0.89
Average	0.387	34.868	0.89
%RSD	4.68	0.17	4.68

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2.3 Precision (repeatability and intermediate precision)

Since the detection accuracy was proved with 5% control sample, 5% nimotuzumab CIM solution was prepared for the study of accuracy and precision of dimer and nimotuzumab. The expected precision and accuracy were based on AOAC Official Method for Analysis (2016), Guidelines for Standard Method Performance Requirements Appendix F, as stated in Table 5. Firstly, the %analyte of the dimer of 5% control sample should be calculated as following explanation.

The concentration of sample solution was 5 mg/mL nimotuzumab (100% nimotuzumab CIM solution) or 0.5% g/mL and the limit of dimer was ≤ 7%. Therefore, the %dimer at limit was 0.5 x (7/100) = 0.035% analyte. However, in 5% control sample the %analyte of dimer should be 0.035% analyte.

As per Table 5a and b, at the level of analyte 0.001%, the recovery should be 80-110% and %RSD (repeatability) should be 7.3%. For the analyte (monomer) of 5%, the recovery should be 97-103% and %RSD (repeatability) should be 2.7%.

Repeatability

%RSD of each day were 2.26, 1.25, and 1.36% for dimer and 1.75, 0.83, and 1.42% for nimotuzumab (Table 8).

Intermediate precision

%RSD of inter-day and different analysts of %relative area were 2.36% and 2.24% for dimer and 1.81% and 1.63%, respectively, for nimotuzumab.

Mean recovery of inter-day and different analysts of %relative area were 101.1% and 101.0%, respectively, for dimer and 101.7% and 102.0%, respectively, for nimotuzumab.

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Table 8a. Precision (repeatability and intermediate precision); dimer

Injection #	Analyst 1									Inter-day Precision	Analyst 2			Different analysts
	Day 1			Day 2			Day 3				Day 1			
	5%	100%	%relative	5%	100%	%relative	5%	100%	%relative	%relative	5%	100%	%relative	%relative
1	1.658	758.261	0.2170	1.766	763.225	0.2323	1.754	758.294	0.2321	-	1.734	766.001	0.2257	-
2	1.720	766.926	0.2251	1.781	758.721	0.2343	1.745	755.387	0.2309	-	1.759	770.425	0.2290	-
3	1.743	766.736	0.2282	1.813	758.781	0.2385	1.764	753.389	0.2334	-	1.759	768.175	0.2290	-
4	1.746	-	0.2286	1.821	-	0.2395	1.758	-	0.2326	-	1.764	-	0.2296	-
5	1.756	-	0.2299	1.801	-	0.2369	1.811	-	0.2397	-	1.741	-	0.2266	-
6	1.766	-	0.2312	1.773	-	0.2332	1.782	-	0.2358	-	1.737	-	0.2261	-
Average	**1.732**	**763.974**	**0.2266**	**1.793**	**760.242**	**0.2358**	**1.769**	**755.690**	**0.2341**	**0.23217**	**1.749**	**768.200**	**0.2277**	**0.2311**
SD	**0.0392**	**4.9488**	**0.0051**	**0.0225**	**2.5832**	**0.0030**	**0.0240**	**2.4665**	**0.0032**	**0.00547**	**0.0131**	**2.2121**	**0.0017**	**0.0052**
RSD	**0.02**	**0.01**	**2.26**	**0.01**	**0.00**	**1.25**	**0.01**	**0.00**	**1.36**	**2.36**	**0.01**	**0.00**	**0.75**	**2.24**

Table 8b. Precision (repeatability and intermediate precision); nimotuzumab

Injection #	Analyst 1									Inter-day Precision	Analyst 2			Different analysts
	Day 1			Day 2			Day 3				Day 1			
	5%	100%	%relative	5%	100%	5%	100%	%relative	5%	%relative	5%		%relative	5%
1	36.890	760.761	4.59175	38.918	765.660	4.85501	38.275	760.739	4.80596	-	38.587	68.501	76788	-
2	38.234	769.469	4.75110	39.078	761.168	4.87400	38.255	757.821	4.80357	-	38.875	772.968	30176	-
3	38.601	769.290	4.79451	39.680	761.233	4.94537	38.676	755.834	4.85387	-	29	702	81987	-
4	38.457	-	4.77748	39.536	-	4.92831	38.519	-	4.83512	-	8.901		4.80482	-
5	38.591	-	4.79333	39.330	-	4.90389	39.551	-	4.95824	-		-	4.78659	-
6	38.825	-	4.82100	38.837	-	4.84539	39.474	-	4.94906	-			4.77106	-
Average	**38.266**	**766.507**	**4.75486**	**39.230**	**762.687**	**4.89199**	**38.792**	**758.131**	**4.86764**		**38.79**	**770.724**	**4.79200**	**4.82662**
SD	**0.70160**	**4.97670**	**0.08313**	**0.34089**	**2.57490**	**0.04043**	**0.58049**	**2.46718**	**0.0692**	**0.087**	**0.17365**	**2.23358**	**0.02042**	**0.07875**
RSD	**0.02**	**0.01**	**1.75**	**0.01**	**0.00**	**0.83**	**0.01**	**0.00**		**1.81**	**0.00**	**0.00**	**0.43**	**1.63**

Table 9a. Recovery; dimer

Injection #	Analyst 1									Inter-day Precision	Analyst 1			Different analysts
	Day 1			Day 2			Day 3				Day 1			
	5%	100%	%recovery	5%	100%	%relative	5%	100%	%relative	%relative	5%	100%	%relative	%relative
1	1.658	34.846	94.6	1.766	35.012	101.2	1.7	34.9	100.9	-	1.734	35.113	98.5	-
2	1.720	35.168	98.1	1.781	34.813	102.1	1.745	34.77	100.3	-	1.759	35.329	99.9	-
3	1.743	35.198	99.4	1.813	34.842	103.9	1.764	34.658	101.4	-	1.759	35.205	99.9	-
4	1.746	-	99.6	1.821	-	10	1.7		101.1	-	1.764	-	100.2	-
5	1.756	-	100.1	1.801	-		1.811		104.1	-	1.741	-	98.9	-
6	1.766	-	100.7	1.773	-	101.6	1.782		102.5	-	1.737	-	98.6	-
Average	**1.732**	**35.071**	**99**	**1.793**	**34.**	**103**	**1.8**	**34.8**	**102**	**101.1**	**1.749**	**35.216**	**99**	**101**
SD	**0.04**	**0.20**	**2.23**	**0.02**		**1.29**	**.02**	**0.13**	**1.38**	**2.36**	**0.01**	**0.11**	**0.74**	**2.20**
RSD	**0.02**	**0.01**	**2.26**	**0.01**	**0.**	**1.25**	**0.01**	**0.00**	**1.36**	**2.34**	**0.01**	**0.00**	**0.75**	**2.19**

Table 9b. Recovery; nimotuzuma

Injection #										Inter-day Precision	Analyst 1			Different analysts
	Day 1			Day 2			Day 3				Day 1			
	5%	100%	%recovery	5%	200%	%relative	5%	100%	%relative	%relative	5%	100%	%relative	%relative
1	36.890	758.261	96.6	38.918	763.225	102.4	38.275	758.294	101.3	-	38.587	766.001	100.5	-
2	38.234	766.926	100.1	39.078	758.721	102.8	38.255	755.387	100.1	-	38.875	770.425	101.2	-
3	38.601	766.736	101.1	39.680	758.781	104.4	38.676	753.389	101.2	-	39.029	768.175	101.6	-
4	38.457	-	100.7	39.536	-	104.0	38.519	-	100.8	-	38.901		101.3	-
5	38.591	-	101.0	39.330	-	103.5	39.551	-	103.5	-	38.746		100.9	-
6	38.825	-	101.6	38.837	-	102.2	39.474	-	103.3	-	38.614		100.5	-
Average	**38.266**	**763.974**	**100**	**39.230**	**760.242**	**103**	**38.8**	**755.7**	**102**	**101.7**	**38.792**	**768.200**	**101**	**102**
SD	**0.70**	**4.95**	**1.84**	**0.34**	**2.58**	**0.90**	**0.58**	**2.47**	**1.38**	**1.85**	**0.17**	**2.21**	**0.45**	**1.63**
RSD	**0.02**	**0.01**	**1.83**	**0.01**	**0.00**	**0.87**	**0.01**	**0.00**	**1.36**	**1.82**	**0.00**	**0.00**	**0.45**	**1.61**

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Conclusion

A selective and sensitive SE chromatographic method was developed for the determination of purity of nimotuzumab in biopharmaceutical products. Chromatographic separation was accomplished on a Tosoh® UltraSW aggregate column (7.8 x 300 mm, 3 µm) run in tandem with the Sepax TweenTrap column (4.6 × 35 mm, 5 µm) using PBS as the mobile phase at flow rate of 0.5 mL/min and monitored at 280 nm. The method was validated to achieve the requirements. Validation parameters included specificity, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability) and intermediate precision (inter-day precision and different analysts). The data shows that all parameters met the acceptan criteria. The validated method was used to test th marketed nimotuzumab product and the chromatogram obtained was in agree w the reference standard. In addition, one-w k st ility of the 10X PBS and mobile ase ( ) was evaluated. The 10X PBS and ile phas were stable for at least one week at 2-8 and ambient temperature, respecti y. In sum ary, the developed and validate thod was specific, accurate, and p for he termination of purities of nim uzum b prod t in Thailand.

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