Development of a fast workflow to screen the charge variants of therapeutic antibodies.

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Abstract

Chemical or enzymatic modifications of therapeutic monoclonal antibodies (mAbs) having high risk towards safety and efficacy are defined as critical quality attributes (CQAs). During therapeutic mAbs process development, a variety of analytical techniques have to be used for the thorough characterization and quantitative monitoring of CQAs. This paper describes the development of a rapid analytical platform to assess and rank charge variants of mAbs. The workflow is first based on a cation exchange chromatography (CEX) comparative analysis of intact IgGs versus F(ab)'₂ and Fc sub-domains generated by IdeS digestion. This analytical procedure was validated with FDA and EMA approved mAbs. Then, functional assays and peptide mapping can be performed in a second instance. This approach can be used during the early stage of drug research and development to screen lead molecules and select optimized candidates (best clone, best formulation) which could be “easily” developed (OptimAbs).

Keywords

Cation exchange chromatography; charge variants; developability; IdeS; trastuzumab.

1. Introduction

Many post-translational modifications as well as physical and chemical degradations of mAbs have been described in the past twenty years [1]. The number and type of modifications might affect surface charge of the monoclonal antibody (mAb), either by directly changing the number of charged residues or by altering the surface charge distribution (**Fig. 1**).

Numerous variants are commonly observed when analyzing mAbs with charged-based separation techniques such as iso-electric focusing (IEF) gel electrophoresis, capillary isoelectric focusing (cIEF) gel electrophoresis, imaged capillary isoelectric focusing (icIEF), cation exchange chromatography (CEX) and anion exchange chromatography (AEX). These variants are generally referred as acidic or basic species, when compared to the main isoform. Acidic species are variants with low apparent isoelectric point (pI), and basic species are variants with high apparent pI, when antibodies are analyzed using IEF-based methods. When using chromatographic-based methods, acidic and basic species are defined based on their retention times relative to the main peak. Acidic species are, for example, the variants that elute before the main peak by CEX or after the main peak by AEX.

Charge variants include N-terminal cyclization of glutamine or glutamic acid residues at both heavy and light chains. Clipping of the C-terminal side of the heavy chain is also commonly observed: clipping of the C-terminal lysine (or when previously removed by molecular engineering), clipping of the C-terminal glycine simultaneously with amidation of the neighboring prolines. Less frequently, lysine -NH₂ moieties aldimination, acetonation and glycation also impact the mAb surface charge distribution [2]. Sialic acids described in CHO, SP2/0 or NS0 produced IgGs carry negative charges. Finally, deamidation of asparagine residues may occur, preferentially at neutral or basic pH. Deamidation takes place via formation of an intermediate succinimide, which is unstable and hydrolyzes into a mixture of two acidic isomers Asp and IsoAsp. Deamidation may also occur at acidic pH by direct hydrolysis of the side chain amide yielding only Asp. To a lesser extent, isoAsp may originate from Asp isomerization [3].

The importance of those charge modifications in terms of safety, immunogenicity and efficacy have been evaluated and ranked by Liu et al. [4]. Modifications are considered as highly critical when they are only observed in recombinant IgG, and less risky when they are common to endogenous IgG. The ranking of importance according to Liu et al. has been illustrated in **Fig. 1**. For example, N-terminal pyroglutamic residues are variants naturally occurring in IgG, not considered as critical quality attributes. On the other hand, deamidation in complementarity determining regions (CDRs) occur after inappropriate pH buffer storage may results in the loss of mAb-antigen recognition. However, in a practical case study, major

charge variants of a recombinant humanized IgG1 were isolated and compared, showing no difference in FcRn binding affinity, pharmacokinetic properties in rats and *in vitro* potency [5]. Charge related heterogeneity of mAbs is mainly characterized with IEF [6] or IEX [7]. CEX is adapted for molecules with basic isoelectric points, such as mAbs [7]. In CEX, the positive net charge of mAbs under pH conditions of the experiment would interact with negatively charged stationary phase. Elution is then performed either by pH or salt gradient (or combination of both). The separation of the variants depends on the net-surface charge, charge distribution and conformation of the protein [8]. In CEX, acidic variants such as deamidated, sialylated, glycosylated compounds elute before the main peak, while basic ones (species with C-terminal lysine, C-terminal proline amidation, uncyclized N-terminal glutamine) elute after the main peak [9].

Whereas charge modifications of constant domains are usually common to each IgG subclass, modifications affecting variable domains require a special focus and need to be studied regardless of their potential impact on mAb binding and activity. Papain has been used so far to fragment recombinant mAbs into Fab and Fc fragments that could be chromatographically separated. Moorhouse et al. [10] developed an assay for determining the product purity of a papain-digested mAb, looking at the charge heterogeneity of each fragment. Vlasak et al. reported an extensive work based on papain digestion, followed by CEX separation and peptide mapping of isolated variants to aid identification of the degradation products of a mAb [11]. Moreover, they demonstrated a LC CDR1-isoAsp variant which was cleaved by papain in an adjacent CDR2 loop, exhibiting greater flexibility than the native form (i.e. asparagine containing form). The idea to investigate critical quality attributes of mAbs by CEX following a highly specific cleavage in the hinge area is thus attractive. It became possible with the introduction of IdeS enzyme, which rapidly generates homogenous pool of F(ab')₂ and Fc fragments without over-digestion or further degradation [12]. The benefits of IdeS for mAb and ADC characterization by LC-MS at middle level has been recently shown [13,14].

The purpose of our work is to demonstrate, using a panel of commercial and *in-house* therapeutic mAbs, the potency of IdeS digested mAbs prior to CEX separation and MS investigation for in depth and relevant charge variants identification. Assuming that Fc charge variants have limited impact on mAb activity [15,16], we therefore focused during the study on CDRs containing F(ab')₂.

Robust linear pH gradient methods have been developed by providers for analyzing antibody charge heterogeneity [17-19]. A clear advantage of those approaches over conventional salt gradient elution methods is their broad applicability for a wide range of IgG, without the need for individual optimization [7,20]. Nevertheless, in one of our recent study, pH and salt gradient modes were comparatively evaluated for a panel of 10 mAbs with pI ranking from

6.7 to 9.1, showing that both can be considered as “multi-product charge sensitive separation methods”. Whereas selectivity were quite comparable, charge variants were better resolved in the salt gradient mode and peak capacities were generally improved with this approach [21,22]. In the present study, both approaches were applied to the study of seven IdeS digested mAbs, and only the chromatograms resulting in the best variants separation were shown.

2. Materials & methods

Water was produced from a Milli-Q Water Purification SystemTM from Millipore. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich or VWR International. IdeS was purchased from Genovis (FabRICATOR Kit, Genovis). For IdeS sample digestion, mAbs were diluted in water to a final concentration of 5 mg/mL and digested with 1 unit IdeS per μ g, 30 min at 37°C. 1 M 2-(N-morpholino)ethanesulfonic acid (MES) solution (BioReagent), 1 M sodium hydroxide (NaOH) solution were purchased from Sigma-Aldrich. CEX pH gradient buffer A (pH = 5.6) and CEX pH gradient buffer B (pH = 10.2) were purchased from Thermo Fisher Scientific AG.

2.1. Cation exchange chromatography

All the experiments were performed using an HPLC system consisting in two 510 pumps, a 717 auto-injector and a 490 UV detector (Waters). Thermo MAbPac SCX-10, 4 x 250 mm, 10 μ m, strong cation exchange column was purchased from Thermo Fisher Scientific. The flow rate was set at 1 mL/min. The mAb samples (either digested or not with IdeS) were diluted in water to a final concentration of 1 mg/mL. A volume of 20 μ L was injected using low volume insert vials. A linear salt gradient from 0 to 20% B in 20 minutes was applied for analysis of adalimumab and rituximab. For the salt gradient CEX separation, the mobile phase “A” consisted of 10 mM MES in water, while the mobile phase “B” was 10 mM MES in water containing 1 M NaCl. The pH of both mobile phases was adjusted by adding 1 M NaOH solution to attain a pH of 5.7. For the pH gradient CEX separation, the mobile phase “A” was a ten times diluted CEX pH gradient buffer A (pH = 5.6), while the mobile phase “B” was a ten times diluted CEX pH gradient buffer B (pH = 10.2). A pH gradient from 0% to 100% B was applied in 30 minutes. The chromatographic signal was recorded at a wavelength of 280 nm.

2.2. Mass spectrometry

Peptide mapping was performed by LC-MS^E after trypsin digestion of collected fractions on a Synapt G2Si equipped with an Acquity UPLC H-class system (Waters, Milford, MA, USA). Digestion protocol and MS method were described in Xie et al. [23].

3. Results and discussion

In this study, seven therapeutic mAbs were selected, as they are representative of the most common IgGs on the market (table 1). Six of them were humanized IgG1K, one was chimeric IgG1K. Their pI ranged from 8.7 to 9.2, an alkaline range well adapted to the salt gradient mode CEX described in Fekete et al. [21,22]. Adalimumab, bevacizumab, rituximab, trastuzumab and dalotuzumab sequences are available in the IMGT database. Two *in-house* products (mAbPF1 and mAbPF2) were also included. In Fig. 2, the chromatograms resulting from either salt or pH gradient mode CEX are presented, for the intact mAb, and IdeS digested mAb. The separation of both F(ab')₂ and Fc parts was good enough to consider charge variants related to each fragment. Whatever is the separation method, the Fc fragment (which has lower pI) elute first at a retention time of around 8 to 10 minutes, while the F(ab')₂ fragment could elute at a different retention time, depending on its sequence. In the experimental conditions, based on the use of non-denaturing chromatographic elution buffer, we assume that the Fc fragment was detected as a non-covalently bound dimer. Despite the lack of hinge region after IdeS digestion, weak interactions between both Fc/2 subunits were shown to be sufficient to maintain dimerization in ammonium acetate buffer, when analyzed in off-line CZE-native MS [24]. Thus, this consideration impact the way how the data were interpreted, especially when considering possible heterodimers, due to partial C-terminal lysine clipping or partial proline amidation.

3.1. Fc charge variants

Sequence alignment of Fc fragment of the seven mAbs is presented in Fig. 3. A few differences can be noticed, especially on the amino acids at position 120 & 122 (see the numbering of figure 3), which may vary upon the mAb allotype. Adalimumab and rituximab are G1m 17,1 regarding their heavy chains, which means they have D356 & L358 instead of E356 & M358 (HC numbering) [25,26]. This allotype feature might not induce any change in the charge profile of Fc fragment. Moreover, five out of the seven mAbs still have C-terminal lysine giving raise to partial K-clipping, as commonly described [5,27]. Therefore, basic charge variants were observed and annotated as “K1” and “K2” in reference to the main peak “K0”. On the other hand, mAbPF1 and PF2 have both engineered C-termini sides without lysine at their heavy chains. Nevertheless, C-terminal proline amidation, catalyzed by peptidylglycine alpha-hydroxylating monooxygenase (PAM) in the CHO cells expressing mAbs, might explain basic variants observed in the charge profiles (-PCONH₂)[28,29].

Finally, asparagine deamidation of the Fc domain may be considered. As depicted in Fig. 3, Fc domain of IgG1 contains ten asparagine residues, which were monitored by peptide mapping [11]. These residues have large impact on protein structure, when compared to corresponding synthetic peptides [15]. Liu et al., have considered endogeneous hulgG, and showed that among the conserved sites, only Asn¹⁴⁸ (see the numbering of figure 3) was deamidated at an appreciable rate [30]. Indeed, endogeneous IgG isolated from human serum possessed 23% deamidation, naturally occurring at this site. Within the same study, it was shown that, under physiological temperature and pH conditions, *in vitro* antibody deamidation followed similar kinetics. In our panel of mAbs, the acidic fraction of the Fc part, associated with deamidation, ranges from 3% (adalimumab) to 30% for mAb PF2. It is however worth mentioning that the high deamidation rate observed on mAb PF2 might be increased by formulation storage (Tris-HCl 100 mM, pH 7.5), especially an inadequate basic pH.

3.2. Adalimumab

Adalimumab charge variants were extensively characterized by cIEF and CEX [31]. When treated by carboxypeptidase B, three peaks collapsed into lower pI (cIEF) or shorter retention time (CEX) zones, demonstrating that charge variants were due to the numbers of C-terminal heavy chain lysine. Moreover, CEX analysis of the papain digestion of the anti-TNF mAb indicated that the isoforms belong to the Fc part. In the same way, IdeS digested adalimumab (**Fig. 2**) displays one F(ab')₂ and one Fc fragment, carrying the charge heterogeneity due to C-terminal lysine (K1 & K2). Both fragments present small acidic peaks (3%). Since the product was registered in 2002, adalimumab manufacturing period includes changes in the production process and scale that required addressing consistency of its quality attribute. Representative CEX chromatograms showed that charge micro-heterogeneity of adalimumab drug substance, as measured by the proportion of lysine variants was maintained [32].

3.3. Bevacizumab

For bevacizumab, the proportion of basic variants, due to C-terminal lysine, was very low (5% for Fc-K1 & K2 after IdeS digestion, see **Fig. 2**) resulting from a near complete clipping. This difference in charge heterogeneity was due to varying degree of proteolysis by carboxypeptidases (Cps). C-terminal lysine variants were found to be sensitive to process conditions, especially components in culture medium such as lysine and arginine amounts, whereas pH and Cps levels do not impact the variant proportion [33]. The residual proportion of C-terminal lysine directly impact complement-dependent cytotoxicity (CDC). Indeed, C1q

binding and CDC requires IgG hexamerization at the cell surface, which is optimal when lysine are fully removed by Cps [34].

3.4. Rituximab

In the case of rituximab, IdeS digestion allows simplification of the mAb basic charge profile. The Fc fragment carries 10% of the modifications, due to residual C-terminal lysine, while the F(ab')₂ fragment carries charge heterogeneity due to the N-termini of LC and HC (12%). Cyclization occurs through the rearrangement of the parent reactive Gln residues. Since pyroglutamate (pE) loss a primary amine during the conversion, rituximab becomes more acidic. Incomplete cyclization results in heterogeneity which is observed as multiple peaks. Basic variants of rituximab were shown to significantly decrease along the life of this biopharmaceutical product. Indeed, for batches with expiry dates in 2010 or later (here 2014), the relative amounts of basic variants were reduced from ~30-50% to ~10%, indicating an alteration of the product related to a change in manufacturing process [35]. Both N-terminal cyclisation and C-terminal lysine clipping are phenomena naturally occurring in IgGs, which do not impact safety, PK and PD, and therefore are not considered as critical quality attributes (CQA). Such variations illustrate the range of micro-variations that can be accepted by health authorities [36].

3.5. Dalotuzumab

The CEX charge variants profile of dalotuzumab has been previously elucidated. As shown in **Fig. 2**, the chromatogram of intact mAb still gives comparable qualitative results as the one published. The two basic variants observed on intact mAb and Fc fragment were interpreted as C-terminal lysine variants on heavy chains. CEX and IEF analysis combined with carboxypeptidase B confirmed this hypothesis [37]. Within an extensive work, including papain digestion, CEX fractionation plus Edman degradation and Isoquant, Vlasak *et al.* characterized the acidic variants. They were mostly due to deamidation of Asn33 located in the first LC complementary determining region (CDR1) leading to Asp and isoAsp variants [11]. This Asn, followed by a glycine residue, was thus clearly identified as a deamidation hot spot. Although the two variants Asp and isoAsp have identical net charge, they were chromatographically separated, suggesting some level of structural differences [11]. It is known that isoAsp residues from Asn deamidation tend to gradually isomerize to Asp. According to F(ab')₂ fragment from IdeS digestion (and not Fab, as reported in Vlasak *et al.*), the acidic profile does reflect heterogeneity from Asp and isoAsp occurring on possibly one or two arms. Without any further investigation, it is therefore neither possible to discriminate between Asp and isoAsp, nor to determinate the proportion of each of them in the acidic F(ab')₂ fraction.

3.6. Trastuzumab

Trastuzumab charge variants have been extensively analyzed and a number of modifications in the CDRs were reported as critical quality attributes (CQAs) following functional evaluation [1,38]. Indeed, trastuzumab sequence contains three hot spots in the CDRs: -Asn³⁰-Thr- (LC) ; -Asn⁵⁵-Gly- (HC) ; -Asp¹⁰²-Gly-(HC). Seven forms of rhuMAb HER2 could be resolved by CEX using a combination of analytical techniques [39]. Four acidic variants contain, either at one or two arms, deamidation of Asn³⁰ or Asn⁵⁵ plus, eventually, Asp¹⁰² isomerization. Two basic variants are due to single isomerization or succinimide from Asp¹⁰². In a clear manner, those seven peaks are well resolved in the F(ab')₂ from CEX profile on **Fig. 2** and could be identified by homology and consistency with published data. Potency assays measured the ability of the antibody to prevent the proliferation of a cell line derived from a human adenocarcinoma that over-expresses her2/neu gene [39]. The form with one deamidated light chain was shown to have a slightly lower potency, whereas the form with isoAsp¹⁰² in one heavy chain CDR was greatly affected. Those results were consistent with previous studies demonstrating that trastuzumab antiproliferative activity requires a bivalent antibody [40].

3.7. mAbPF1

This *in-house* recombinant antibody reveals quite homogeneous profiles. Nevertheless, basic charge variants from untreated mAb were collected, and then analyzed by LC-MS after IdeS digestion and reduction (data not shown). Light chain and Fd fragments displayed masses in agreement with the ones calculated from sequences. However, Fc/2 displayed a loss of -58 Da when compared to the expected one, which fits with a C-terminal glycine deletion and C-terminal alpha-amidated proline, and is consistent with the shift in retention time observed by CEX. C-terminal amidation has been reported for the first time by Johnson et al. [41] and further described as resulting from effect of copper concentration in cell culture media of IgG1 mAbs [28]. This modification affecting the Fc fragment of mAbPF1 is clearly illustrated in **Fig. 2**, within two basic variants (12%) either related to one or both arms of the mAb. Moreover, N-terminal loss of ammonia leading to pyroglutamic acid residue formation may result in the few acidic variants of F(ab')₂. This was confirmed by direct Edman degradation of the whole mAb after digestion with pyroglutamate aminopeptidase [37,42,43].

3.8. mAbPF2

MAbPF2 CEX profile is heterogeneous for several reasons. IdeS digested charge variants profile were particularly helpful for interpretation. Indeed, basic variants observed on the intact mAb profile are, from one major part, due to C-terminal proline amidation following glycine deletion (as above described), but also to residual glutamine residues that were not fully converted to pyroglutamate. Both modifications affect Fc or F(ab')₂ CEX profiles, respectively and were confirmed thanks to complementary experiments (data not shown). Acidic fractions might have been enhanced by the formulation buffer pH (Tris-HCl 100mM, pH 7.5) and were correlated to deamidation. Deamidation of the Fc part has been previously discussed in section 3.1, and our attention was therefore focused on the acidic fraction of the F(ab')₂ fragment. The chromatographic separation was good enough to collect the corresponding products. We have thus performed tryptic peptide mapping by LC-MS^E. Especially, T11, a CDR peptide of the heavy chain containing an asparagine at position 108, has retained our attention. As depicted in Fig. 4, its extracted ion chromatogram results in three separated peaks which were interpreted as N108, D¹⁰⁸, isoD¹⁰⁸ and confirmed by fragmentation spectra (focus on the area of interest on A & B, Fig. 4). Deamidated forms which represent 28% could not be discriminated by MS since they are isobaric. It is, from our knowledge, the first time that deamidation occurring on a -NY- sequence is described.

4. Conclusions

Thanks to the described workflow, it is possible to discriminate critical and less critical charge variants, according to the CEX charge variants profile of IdeS digested mAbs. This is an important feature since screening of hot spots is an unavoidable part of a developability platform. As mentioned by Jarasch et al. [44], despite the recent progress to predict degradation-prone asparagine and aspartate residues, the functional impact of such degradation can currently only be experimentally assessed. As an example, mAbPF2 highlights a non-described deamidation hot spot, -NY-, which was evidenced after short stress study and investigation of the Fab'2 fragment. As reported in this study, CEX appears as a fast and reliable technique for the characterization of charge variants. However, one of its limitations is the difficulty to identify the observed acidic and basic variants. To solve this issue, it is possible to collect some fractions and then reanalyze the samples with LC-MS, but this procedure is time-consuming. Recently, Stoll et al. have reported the possibility to directly identify CEX peaks using an on-line CEX x RPLC-MS platform [45-48], which could be particularly useful to speed up the identification of charge variants.

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481

482

Figure 1:

IgG charge micro-variants and their importance (from Liu et al. [3]). ++++ indicates the highest level of importance; + indicates the lowest level of importance. Adapted from Beck et al [1]. Modifications that are common to recombinant mAb and endogenous IgG molecules are considered to pose a lower risk of immunogenicity in patients. On the other hand, modifications that are unique to recombinant mAbs could potentially pose higher risk. The focus of this review is the comparison of frequently observed modifications of recombinant monoclonal antibodies to those of endogenous IgG molecules.

492

493 Figure 2:

494 CEX chromatograms of 7 therapeutic monoclonal antibodies. Each antibody was analyzed

495 either intact (chromatogram at the top) or IdeS digested (chromatogram below) within

496 identical analytical conditions (salt or pH gradient mode CEX, depending on the mAb).

498
 499 **Figure 3:**
 500 Sequence alignment of Fc/2 fragments from the studied IgG1 obtained after IdeS digestion.
 501 In green are highlighted differences. Asparagine are red colored.

		1		50
Adalimumab	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
Bevacizumab	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
Rituximab	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
Dalotuzumab	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
Trastuzumab	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
mAbPF1	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
mAbPF2	(1)	GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF	NWYVDGVEVH	N
		51		100
Adalimumab	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
Bevacizumab	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
Rituximab	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
Dalotuzumab	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
Trastuzumab	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
mAbPF1	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
mAbPF2	(51)	AKTKPREEQYNSTYRVVSVLTVLHQDWL	NGKEYKCKVSNKALPAPIE	KTI
		101		150
Adalimumab	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
Bevacizumab	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
Rituximab	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
Dalotuzumab	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
Trastuzumab	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
mAbPF1	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
mAbPF2	(101)	SKAKGQPREPQVYTLPPSR	DELTKNQVSLTCLVKGF	YPSDIAVEWES
		151		200
Adalimumab	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
Bevacizumab	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
Rituximab	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
Dalotuzumab	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
Trastuzumab	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
mAbPF1	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
mAbPF2	(151)	PENNYKTTTPVLDS	SDGSFFLYSKLTVDKSRWQ	QGNVFSCSVMHEALH
		201	211	
Adalimumab	(201)	TQKSLSLSPG	K	
Bevacizumab	(201)	TQKSLSLSPG	K	
Rituximab	(201)	TQKSLSLSPG	K	
Dalotuzumab	(201)	TQKSLSLSPG	K	
Trastuzumab	(201)	TQKSLSLSPG	K	
mAbPF1	(201)	TQKSLSLSPG	-	
mAbPF2	(201)	TQKSLSLSPG	-	

502

Figure 4:

Mass spectrometry analysis of isolated acidic fraction of mAbPF2 from CEX put in evidence deamidation in a non-expected N at position 108 in CDR3 of the heavy chain. On figure 4A, is presented the extracted ion chromatogram (EIC) of the tryptic peptide T11(HC) containing the CDR3 deamidation site. The chromatogram displays 3 peaks: peaks 2&3 represent 28% of the total area. MS spectra corresponding to chromatographic peaks 1, 2 & 3 are displayed on figure 4B showing at mass shift of +1 Da for peaks 2 and 3 when compared to MS spectrum of peak 1. Additional MS/MS spectra (figure 4C) confirm the sequence of T11(HC) peptide: non deamidated for peak 1 ; deamidated for peak 2.

515

516 Table 1

517 Summary of mAbs described in the study and their attributes.

INN (International Nonproprietary Name)	Target name	Indications	Format	Company	Proprietary name	Calculated pI	CEX method
adalimumab	TNF	rheumatoid arthritis, crohn disease and others	hlgG1k	AbbVie Inc.	HUMIRA	8.7	salt gradient
bevacizumab	VEGF	collorectal cancer metastatic and others	hzigG1k	Roche, F. Hoffmann- La Roche Ltd./Genetech Inc.	AVASTIN	8.5	pH gradient
rituximab	CD20	non-hodgkin's lymphoma	clgG1k	Biogen, Inc, Roche, F. Hoffmann-La Roche Ltd./Genetech Inc.	RITUXAN (US, Canada, Japan); MABTHERA (EU)	9.1	salt gradient
dalotuzumab	IGF1R	Solid tumors	hzigG1k	Merck / Pierre Fabre	n/a (Phase II / III)	9.0	pH gradient
trastuzumab	HER2	breast cancer ; gastric cancer	hzigG1k	Roche, F. Hoffmann- La Roche Ltd./Genetech Inc.	HERCEPTIN	8.8	pH gradient
mAbPF1	-	n/a	hzigG1k	Pierre Fabre	n/a	9.2	pH gradient
mAbPF2	-	n/a	hzigG1k	Pierre Fabre	n/a	8.9	pH gradient

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