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Validation of an HPLC method for the analysis of the charge heterogeneity of the recombinant monoclonal antibody IDEC-C2B8 after papain digestion [1]

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Abstract

An HPLC procedure was validated for determining the purity with respect to the charge variant distribution of the recombinant monoclonal antibody (MAb) IDEC-C2B8 by high-performance ion-exchange chromatography. Papain was used to fragment the molecule into Fab and Fc fragments prior to chromatographic analysis. Fragmentation allowed the resolution of the variants arising from the cyclization of glutamine to pyroglutamate at the amino-terminus of the light and heavy chains (Fab-pE/Q variants) from the variants resulting from the processing of the carboxy-terminal lysine residues of the heavy chains (Fc-Lys variants). The assay demonstrated good linearity, yielding correlation coefficients of >0.99 for total protein, Fc-Lys variants and Fab-pE/Q variants. Recovery of total protein from the column was 95.7%. Sample recovery studies demonstrated a mean accuracy of 102% for a Fab fragment over the range 2–10% of the total protein. The limit of detection was 0.2 μg and 0.1 μg for Fc and Fab variants, respectively. The repeatability of the assay and intermediate precision had relative standard deviation (RSD) values of $<1\%$. Parameters of the papain digest (time, digest stability, reagent stability, pH and papain vendor) and of the chromatography (mobile phase pH, stability, buffer concentration, and column lot and aging) were evaluated for robustness and determined to be acceptable. Data are presented demonstrating the suitability of the assay for determining the product purity of a recombinant MAb. Published by Elsevier Science B.V.

Keywords: Recombinant protein; Monoclonal antibody; Papain; Cation-exchange chromatography; Method validation

1. Introduction

Established methods for the analysis of the charge variants of recombinant proteins include ion-exchange chromatography and isoelectric fo-

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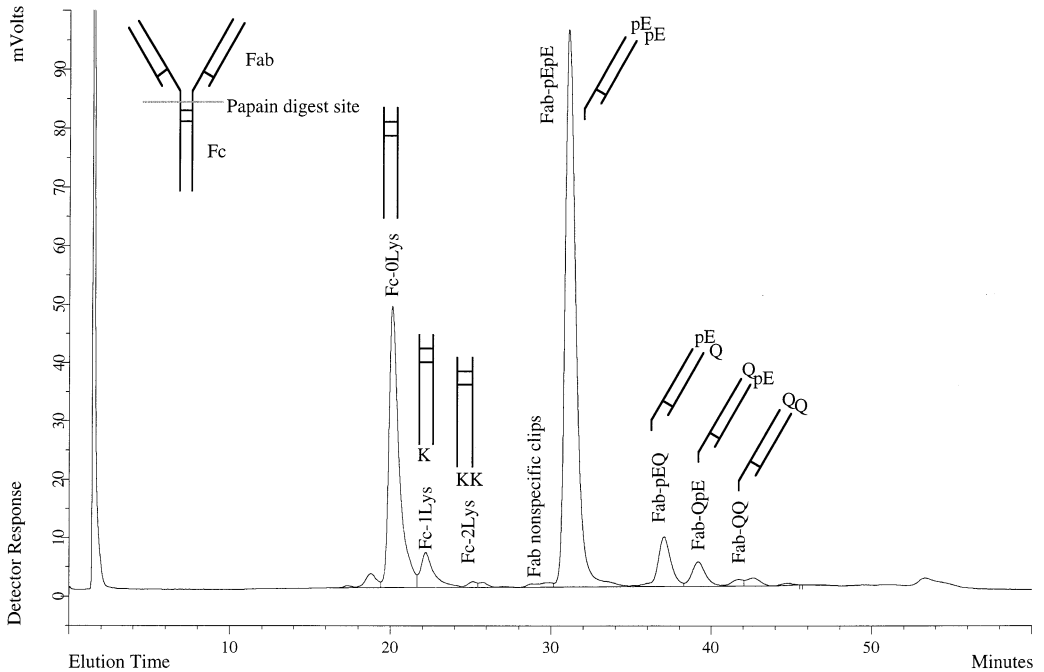

Fig. 1. Typical chromatogram of papain-digested IDEC-C2B8 MAb obtained using a Polymer labs SCX column.

cusing [1]. In ion-exchange chromatography charge variants are separated by differential partitioning on a charged support. Protein charge variants arise from modifications of the amino- and carboxy-termini and amino acid side chains. With small proteins, such as recombinant human growth hormone, resolution of all charge species is possible [2,3]. As molecule size increases the number of possible charge variants increases. As changes in charge from different modifications may be additive or subtractive, ion exchange chromatography becomes more complex while the overall resolution of individual variants may be lost. This is particularly apparent for monoclonal antibodies which are comprised of two light and two heavy chains with overall molecular weights in the region of 150 kDa.

IDEC-C2B8 is a recombinant mouse/human chimeric monoclonal antibody that binds the human CD20 antigen [4]. Inherent charge variants of this MAb arise during cell culture from the enzymatic removal of the C-terminal lysine from one or both heavy chains to reveal a C-terminal glycine, a common reaction for proteins isolated from cell culture [5]. Additional charge variants arise from the cyclization of the N-terminal glutamine of both the heavy and light chains to produce pyroglutamate, a deamination reaction. These charge variants can occur in random combination at the four N-termini and the two heavy-chain C-termini of the molecule, leading to a possibility of 15 different species [6]. To overcome this complexity, an ion-exchange assay was developed to analyze the charge heterogeneity of the molecule after it has been fragmented by digestion with papain [7].

Papain is a thiol protease that cleaves IgG antibodies at the heavy chain hinge region into three fragments, one Fc and two identical Fab fragments [8,9]. With IDEC-C2B8 papain cleaves the His-Thr bond in the heavy chain hinge sequence -Asp-Lys-Thr-*His*-*Thr*-Cys-Pro-Pro-Cys- so that each Fab fragment consists of the light chain and the N-terminal section of the heavy chain. The Fc fragment consists of the disulfide-linked C-terminal section of the heavy chain. As a

Table 1
Linearity and accuracy of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment		Result
Linearity of response[a]	Fab-pE/Q variants	$R = 1.0$
	Fc-Lys variants	$R = 1.0$
	Total peak area	$R = 1.0$
Column recovery[b]	Total protein	96% Recovery
Sample recovery[c]	2% Variant spike	100% Recovery
	5% Variant spike	103% Recovery
	9% Variant spike	102% Recovery

[a] Linearity was calculated from duplicate injections of papain-digested MAb in the range 10–150 μg. The peak area response was plotted against total protein load and the correlation coefficient (R) calculated.
[b] A single sample of papain digested IDEC-C2B8 was injected in duplicate with the column in and out of line. %Recovery = [(mean peak area with column in line)/(corrected mean peak area with column out of line)] × 100%.
[c] Mixtures of papain-digested IDEC-C2B8 and purified Fab nonspecific clip were prepared at three different levels and analyzed. The measured values from the analysis for the variant were compared to the theoretical values calculated from the protein concentration and purity. % Recovery = (measured/theoretical) × 100%.

result of cleavage, the inherent charge heterogeneity associated with the IDEC-C2B8 Fab fragment can be resolved from the heterogeneity associated with the Fc fragment by cation exchange chromatography. Although the use of papain to produce antibody fragments is well known, the application of this approach as part of a method to monitor and release a marketed recombinant MAb can only occur once the method has undergone a rigorous validation as required by the Food and Drug Administration [10], and other regulatory authorities.

Currently validation studies for recombinant proteins are performed with reference to the guidelines of the International Conference on Harmonization [11,12]. The validation of a method is performed as a series of carefully controlled experiments, each experiment designed to assess a particular aspect of the method, e.g. accuracy, precision (repeatability and intermediate precision), specificity and robustness. The results of each experiment are judged as either acceptable or unacceptable against criteria considered appropriate for the purpose of the method with respect to measuring product purity, potency, efficacy and safety. If the validation results are acceptable the method is considered validated and can be submitted to the appropriate regulatory authority for approval, as part of the product license application. This paper summarizes the validation of the chromatographic analysis of the monoclonal antibody IDEC-C2B8 for charge heterogeneity after digestion with papain.

2. Materials and methods

2.1. Reagents and materials

IDEC-C2B8 was manufactured by IDEC Pharmaceuticals (San Diego, CA). Papain (from *Car-*

Table 2
Precision of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result	
		Fc-Lys variants	Fab-pE/Q variants
Repeatability[a]	Injection	0.9% RSD ($n = 6$)	0.3% RSD ($n = 6$)
	Digestion	0.8% RSD ($n = 6$)	0.3% RSD ($n = 6$)
Intermediate precision	Day-to-day[b]	0.7% RSD ($n = 3$)	0.7% RSD ($n = 3$)
	Analyst-to-analyst[c]	0.5–1.3% Difference ($n = 3$)	0.3–1.5% Difference ($n = 3$)

[a] Six replicate injections of a single papain digest and six replicate digestions of IDEC-C2B8 were analyzed.
[b] Papain digests of IDEC-C2B8 were prepared and analyzed by a single analyst on three different days.
[c] Two analysts analyzed three different lots of IDEC-C2B8 using separate reagents and equipment. The results are the range of differences seen for the three lots.

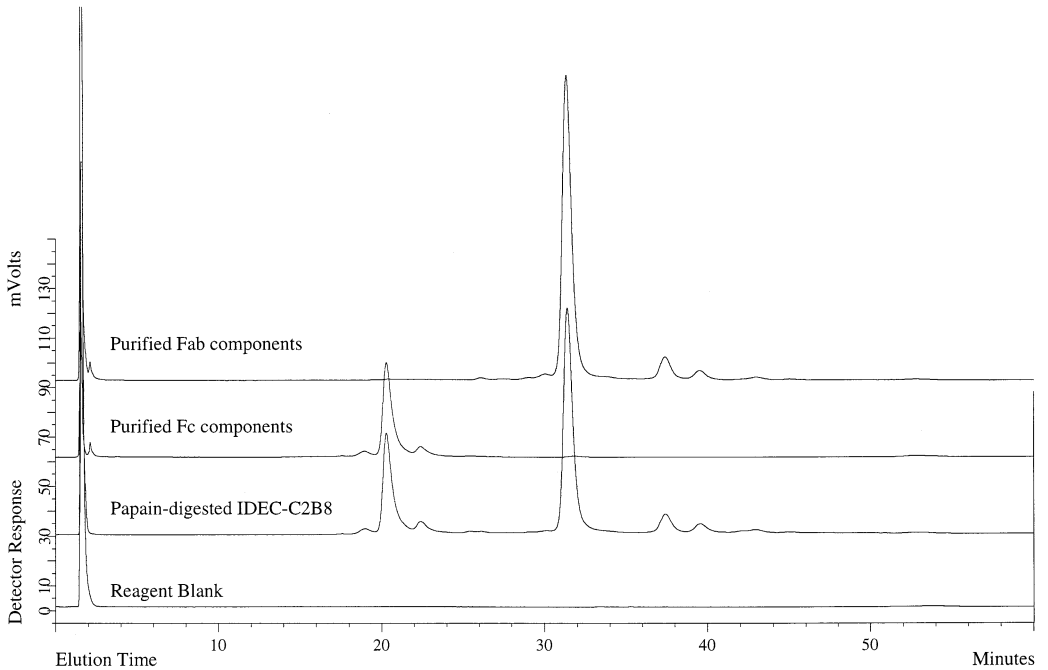

Fig. 2. Specificity of the analysis of papain-digested IDEC-C2B8 MAb.

ica papaya, EC 3.4.22.2) was purchased from Boehringer Mannheim (Indianapolis, IN), Worthington Biochemical (Freehold, NJ) and Serva (Crescent Chemical, Hauppauge, NY). L-Cysteine was from Sigma (St. Louis, MO). 2-(*N*-morpholino)ethanesulfonic acid (MES) was from Research Organic (Cleveland, OH). PD-10 buffer exchange columns were from Pharmacia Biotech (Piscataway, NJ). PL-SCX columns (1000 Å, 8 μm, 150 × 4.6 mm) were from Polymer Laboratories (Amherst, MA), and TSK G3000SW$_{XL}$ columns (7.8 × 300 mm) were from TosoHaas (Montgomeryville, PA). The Poros A/M column (4.6 × 100 mm) was from PerSeptive Biosystems (Framingham, MA). Centrifugal concentrators were from Amicon (Beverly, MA). All other chemicals were of analytical reagent grade.

2.2. Papain digestion

The standard digestion of the recombinant MAb by papain was as follows. IDEC-C2B8 was incubated at a final concentration of 1 mg ml^{-1} in 0.1 M Tris–HCl, 4 mM EDTA, 1 mM cysteine (pH 7.4). The digestion was initiated by the addition of papain (diluted to 0.1 mg ml^{-1} with water) to give a final protein:enzyme ratio of 100:1, the final digestion volume was 1.5 ml. The digestion was carried out for 2–2.5 h at 37°C, then 1 ml of the digested MAb was buffer exchanged into 2 ml of 10 mM MES (pH 6.0) (solvent A) using PD-10 columns.

2.3. Chromatography

Chromatographic analysis by cation exchange chromatography was carried out using a PL-SCX column. 200 μl (approximately 100 μg) of the digested protein was injected onto the column. The gradient for elution was 5 min at 95% solvent A, 5% solvent B (0.2 M NaCl in 10 mM MES pH 6.0) followed by a linear gradient of 5–85% solvent B over 40 min. The flow rate was 1 ml min^{-1}. After elution the column was washed with 100% solvent B for 2 min and re-equilibrated with 95% solvent A. The total run time was 60 min. The column temperature was ambient and elution was monitored at 280 nm (except as noted).

Table 3
Robustness of the papain digestion of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result[a]	
		Fc-Lys variants (% difference)	Fab-pE/Q variants (% difference)
Digest pH	pH 6.8–7.4	1.4	0.8
Time of digest	1–>5 h	1.0 (after 1 h)	0.5 (after 1 h)
Sample stability	Fresh vs. 1 week at 2–8°C	0.3	0.3
Cysteine stability	Fresh vs. 1 day old	0.1	0.2
Papain[b]	Lot 1–3	0.1	0.1

[a] Values are the maximum differences seen for the Fc-Lys and Fab-pE/Q values calculated from a single lot of IDEC-C2B8 analyzed according to the conditions indicated.
[b] Three different lots from Boehringer Mannheim.

Chromatographic analysis by size exclusion chromatography was carried out using a TSK G3000SW$_{XL}$ column. The mobile phase was 0.2 M potassium phosphate, 0.25 M KCl, (pH 7.0). The flow rate was 1 ml min^{-1} and the elution was isocratic. The column temperature was ambient and elution was monitored at 280 nm.

2.4. Preparation of Fab and Fc fragments

Fab and Fc fragments of IDEC-C2B8 were prepared from several pooled digests using protein A affinity chromatography. Digested MAb was injected onto a protein A column equilibrated in 10 mM sodium phosphate, 0.15 M NaCl (pH 7.4). The Fab fragments were collected as the flow-through peak and the Fc fragments eluted with 2% (v/v) acetic acid, 0.1 M glycine, the column was washed with 20% (v/v) acetic acid before re-equilibration with buffer and the next injection. Fc and Fab fragment pools were buffer exchanged into solvent A by dialysis with at least three changes of buffer. Finally the protein was concentrated using centrifugal concentrators prior to chromatographic analysis.

Fc-0Lys, Fab-pEpE and a Fab nonspecific clip variant (see Section 3.1 for full terminology) were purified from papain digested MAb by cation exchange chromatography described above with minor modifications. After collection the purified variants were concentrated and buffer exchanged into solvent A using centrifugal concentrators.

3. Results and discussion

3.1. Method characterization

A typical elution profile is shown in Fig. 1. The isoelectric points (p*I*) of the Fc and Fab fragments were estimated from slab gel isoelectric focusing to be pH 7.1 and 9.1, respectively. At pH 6.0 both fragments have a positive charge and adhere to the negatively charged sulfonate groups of the cation ion-exchange matrix and are eluted by a salt gradient in the order of their increasing isoelectric points. The nature of the separated species was determined by mass spectrometry with or without tryptic digestion (manuscript in preparation). Fc variants elute in order of increasing p*I* resulting from the sequential removal of the lysine residues from each carboxy-termini to reveal glycine. Fc peaks are identified using the following terminology. Fc-0Lys (an Fc fragment without lysine residues at either C-terminus), Fc-1Lys (an Fc fragment with a lysine residue at one C-terminus) and Fc-2Lys (an Fc fragment with lysine residues at both C-termini) collectively are termed the Fc-Lys variants.

Fab variants elute in order of increasing p*I* resulting form the sequential cyclization of the basic amino terminal glutamine to the neutral pyroglutamate at each of the four Fab amino-termini. Fab peaks are identified using the following terminology. Fab-pEpE (a Fab fragment with pyroglutamate at both N-termini), Fab-pEQ (a Fab fragment with pyroglutamate at the heavy-chain

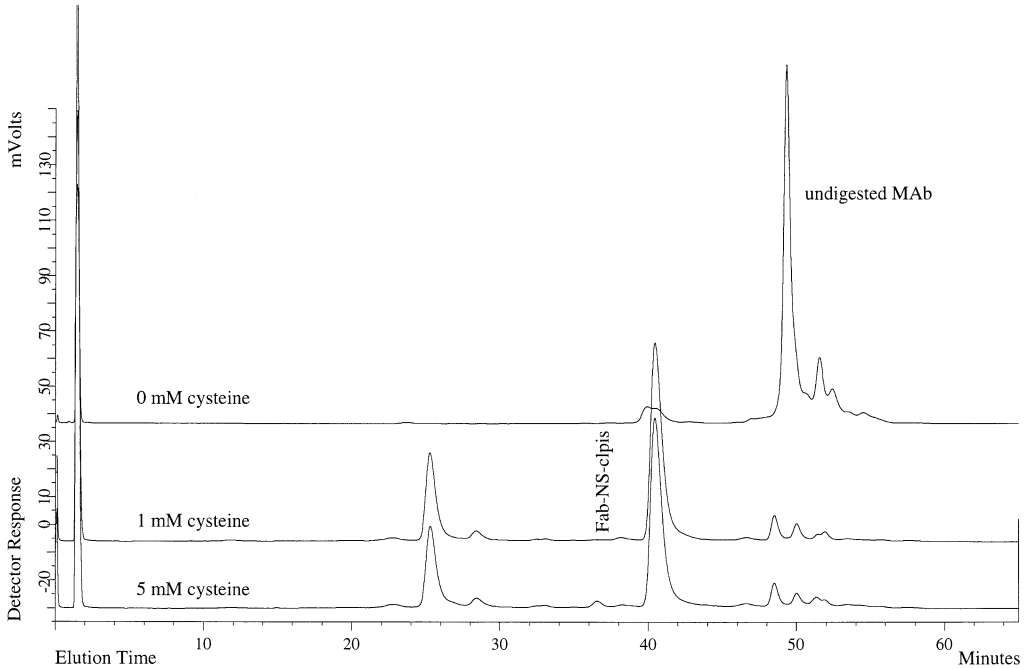

Fig. 3. Effect of cysteine concentration on the papain digestion of IDEC-C2B8 MAb.

N-terminus and glutamine at the light-chain N-terminus) and Fab-QpE (a Fab fragment with glutamine at the heavy-chain N-terminus and pyroglutamate at the light-chain N-terminus) and Fab-QQ (a Fab fragment with glutamine at both N-termini) collectively are termed the Fab-pE/Q variants. In addition to the cleavage producing the Fc and Fab fragments, papain also exhibited a low level of exopeptidase activity resulting in the sequential removal of the histidine and threonine from the new C-terminus of the Fab heavy chain fragment. These further degraded fragments are apparent as peaks eluting just prior to the Fab-pEpE peak and are termed Fab nonspecific clips (Fab-NS-clips). Quantitation was performed using peak area normalization.

3.2. Linearity and accuracy

The results of the linearity and accuracy studies are presented in Table 1. There was a linear response for the analysis of Fc-Lys and the Fab-pE/Q variants and for the sum of all peak areas as demonstrated by correlation coefficients of >0.99. Accuracy was determined in three parts by, column recovery, sample recovery and, by the determination of the limit of detection and quantitation. Column recovery was calculated at 95.7% based on a comparison of peak areas determined by making duplicate injections of digested MAb with the column in and out of line. The mean recovery of a purified Fab-NS-clip variant from experimental mixtures of this Fab variant and papain-digested IDEC-C2B8 (corresponding to variant levels of 2–10% of the total protein by mass) was 102%. The detection limit, of serially diluted samples of purified Fc-0Lys and Fab-pEpE variants was 0.2 and 0.1 μg (0.2% and 0.1% based on a 100 μg load), respectively. The limits of quantitation for the same samples were 2 and 0.5 μg, respectively (2% and 0.5% based on a 100 μg load). The limit of quantitation was somewhat less for the Fc variant as these species showed considerably more peak broadening than the Fab variants.

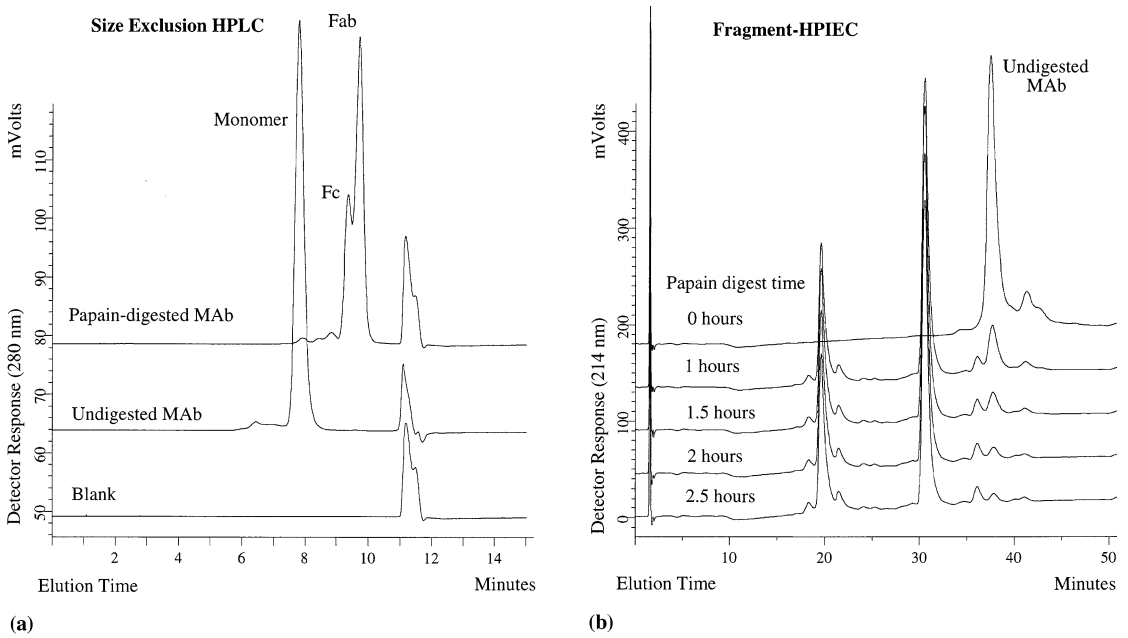

Fig. 4. Completeness of papain digestion of IDEC-C2B8 MAb.

3.3. Precision

Precision was evaluated in two parts, by repeatability and by intermediate precision studies. The results are presented in Table 2. Repeatability studies were performed for the chromatography by evaluating multiple injections and for the digestion by evaluating multiple digests. In both cases for the calculated Fc-Lys and Fab-pE/Q values the RSD values were $< 1\%$. Intermediate precision was determined with three studies. In the first study a second analyst repeated the repeatability studies and again obtained RSD values of $< 1\%$ for both digest and chromatography. In the second study a single analyst prepared and analyzed a single sample of IDEC-C2B8 on 3 separate days. Again RSD values of $< 1\%$ were obtained. In the third study two analysts each analyzed three different lots of IDEC-C2B8. The results of the two analysts showed differences of 0.5–1.3% for the Fc-Lys variants, 0.3–1.5% for the Fab-pE/Q when the three lots were compared. The values represent the highest level of precision testing as the two analysts were using different reagents, columns and instruments. Overall the results of the precision studies were acceptable for a chromatographic procedure.

Reproducibility by an interlaboratory trial was not assessed for the assay as this aspect of precision is now only considered in the case of the standardization of an analytical procedure, such as for inclusion in a pharmacopoeia [12].

3.4. Specificity

Fab fragments were isolated from Fc fragments by affinity chromatography using protein A. These individually prepared fragments were used to show the specificity of the analysis. The profiles for purified variants and for a blank digest which contained all components except the MAb are shown in Fig. 2. The chromatograms clearly demonstrate that all earlier eluting peaks result from Fc fragments and that they are resolved from the later eluting Fab fragments. Injection of individual components after the PD-10 buffer exchange step indicated that the peak eluting at 2 min resulted from the Tris–EDTA components of the digest buffer.

Table 4
Robustness of fragment-HPIEC of the recombinant MAb IDEC-C2B8

Experiment	Condition	Result[a]	
		Fc-Lys variants (% difference)	Fab-pE/Q variants (% difference)
Mobile phase	pH (pH 5.9–6.3)	0.8	1.6
	Molarity (5–20 mM)	0.3	0.1
Column	Column lot 1a–1c[b]	1.5	0.5
	Column lot 1–3[c]	1.8	1.0

[a] Values are the maximum differences seen for the Fc-Lys and Fab-pE/Q values calculated for a single lot of IDEC-C2B8 analyzed according to the conditions indicated.
[b] Three different columns from the same column lot.
[c] Three different columns from three different column lots.

3.5. Robustness

The robustness of the assay was evaluated to show the reliability of the analysis with respect to deliberate variations in method parameters. This aspect of validation is especially important for assays where an enzyme reaction is included as part of the analysis procedure.

After optimization of the digest for the MAb and papain concentration, for the ease of sample preparation and to give sufficient chromatographic response, the following parameters of the papain digest were studied; cysteine and EDTA concentration, time, digest stability, reagent stability, digest pH, and papain vendor. The results are summarized in Table 3. EDTA was included in the incubation buffer as the presence of divalent cations inhibit papain activity [9], however, in this case EDTA concentrations (0–10 mM) had no effect on the digestion. The cysteine concentration had a significant effect on the digestion as seen by the lack of digestion which occurred at 0 mM cysteine and by the increase in the relative peak area of the Fab nonspecific clips which occurred with increasing concentration (Fig. 3). For this reason the cysteine concentration was optimized to maximize the completeness of digestion while minimizing the production of the Fab nonspecific clips. Cysteine concentrations of 1 mM resulted in nonspecific clip production of less than 1%. The completeness of digestion at 1 mM cysteine was then monitored by the loss of the intact peak which co-migrates with the Fab-QpE peak by cation exchange chromatography and by size exclusion HPLC by the loss of the monomer peak (Fig. 4a and b). Both studies indicated that at least 96–97% digestion was achieved. At 1 mM cysteine the production of Fab nonspecific clips was still observed within the 2–2.5 h digestion time but overall their levels were so low they did not affect the results. This was confirmed with extended digestion times where even after >5 h the Fab nonspecific clips represented no more than 1.2% of the total peak area. The stability of the cysteine reagent was assessed as the –SH group in the papain active site must be in the reduced form for activity. The ability of cysteine to activate the papain after storage was evaluated by comparing the results from a sample of IDEC-C2B8 which had been digested with cysteine that (a) had been freshly prepared and (b) stored for 1 day at 2–8°C. The results indicated that any oxidation of cysteine to cystine that occurred within 24 h did not effect the overall activity of the papain.

The pH of the digest was evaluated with no effect seen over the range pH 6.8–7.4. The papain activity and completeness of digestion were probably not affected by pH as the digestion was carried out under conditions of excess enzyme activity. The stability of the digest was evaluated as the papain was not removed by the buffer exchange step. Residual exopeptidase activity was seen on storage by an increase in the area percent of Fab nonspecific clips of digested samples over time. However, storage experiments indicated that

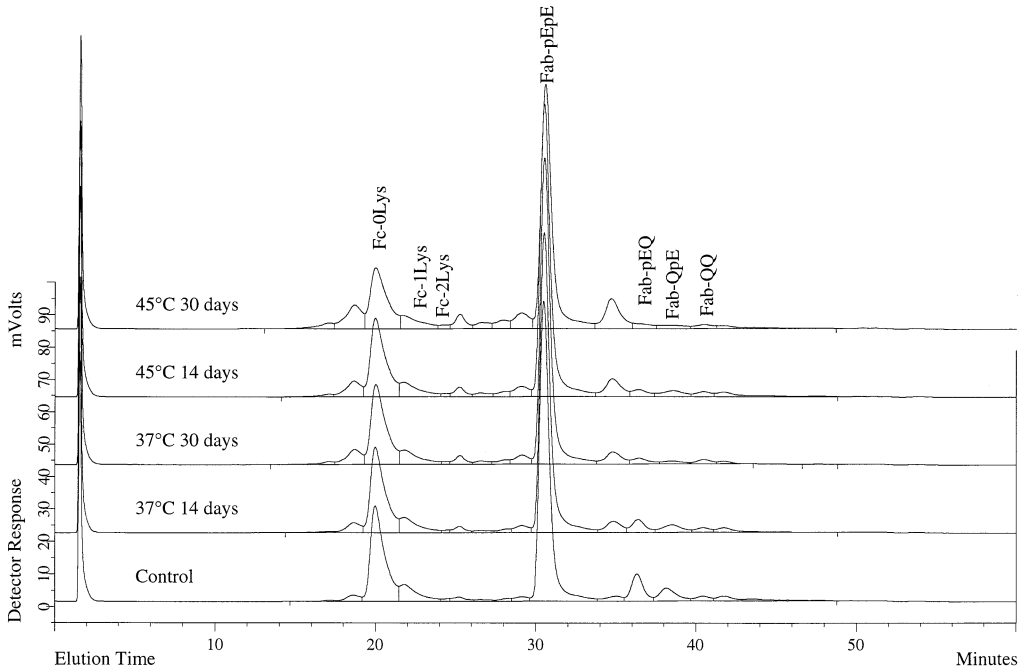

Fig. 5. Thermal degradation of IDEC-C2B8 MAb following storage at elevated temperatures.

a sample could be kept for up to 24 h at ambient or 4 days at 2–8°C before the production of nonspecific clips would affect the results. The source of the papain was evaluated to ensure that if papain was not available from the primary vendor (Boehringer Mannheim), then alternative sources could be used. Papain from Worthington and Serva were evaluated for their ability to digest the MAb on both a weight and activity corrected basis by comparison with the primary vendor. Digestion profiles and data analysis for each vendor were consistent with values for the Fc-Lys and Fab-pE/Q variants within 2% of that seen for the primary vendor.

The robustness of the chromatography section was performed by evaluating changes in mobile phase pH (pH 5.9–6.3), concentration (5–20 mM) and column manufacture (both differences between columns from the same lot and columns from different lots). The results, summarized in Table 4, indicated that none of the parameters studied affected the results.

3.6. Stability indicating properties

Finally, the utility of the assay was assessed by its ability as an indicator of product degradation. Four samples which had been subjected to accelerated stability studies at elevated temperatures of 37 or 45°C for 2 or 4 weeks were analyzed. The chromatographic analysis is presented in Fig. 5; the results are summarized in Table 5. There was both a time- and temperature-dependent decrease in the percent peaks areas for both the Fc-Lys and Fab-pE/Q variants. The relative distribution of Fc-0Lys and Fc-1Lys variants did not change, which is consistent with an enzymatic rather than chemical processing of this C-terminal. There was an increase in the area of a more acidic peak eluting prior to Fc-0Lys with both time and temperature. The nature of this peak was not determined but its position is consistent with a degradation reaction associated with an increase in negative charge, such as deamination. The change in peak areas of the individual Fab-pE/Q variants was more complex. N-terminal glutamine

Table 5
Utility of fragment-HPIEC analysis of the recombinant MAb IDEC-C2B8

Conditions[a]	Percent peak area		
	Fc-Lys variants	Fab-pE/Q variants	Fc-Lys + Fab-pE/Q variants
Control, 2–8°C	28.2	63.0	91.2
37°C, 14 days	26.4	60.7	87.1
37°C, 30 days	25.8	59.2	85.0
45°C, 14 days	24.7	57.3	82.0
45°C, 30 days	22.1	50.2	72.2

[a] Samples of IDEC-C2B8 were stored as described before analysis.

cyclization appeared to be a temperature-dependent reaction as there was a change in the distribution of the Fab-pE/Q variants consistent with conversion of the partially cyclized species Fab-pEQ and Fab-QpE to the fully cyclized Fab-pEpE at the lower temperature. At the higher temperature there were significant increases in the areas of peaks not related to cyclization. As exposure to elevated temperatures is known to decrease activity for the MAb (data not shown), the changes seen in the chromatographic profile indicate that this method can be used to monitor product quality on a stability basis.

4. Conclusion

A comprehensive validation was performed on an HPLC based assay for the recombinant MAb IDEC-C2B8 which first required the digestion of the protein with papain. The method showed acceptable recovery and appeared rugged and precise with respect to operating conditions. The validation and optimization of the papain-digest step was evaluated and found to be robust.

Cysteine was required in the digest step for papain activity but increasing cysteine concentrations also increased the nonspecific exopeptidase activity of the enzyme. Other parameters including papain and antibody concentrations, digest time, pH and reagent shelf life were chosen such that 96–97% digestion occurred while the production of nonspecific clips was less than 1% of the total peak area. The utility of the assay as both an indicator of product quality and consistency for lot release and for stability studies was shown by the analysis of thermally degraded samples, which exhibited changes in the chromatographic profile.

The method as validated satisfied all of the rigorous criteria for use as a lot release method and may also be applicable to other recombinant monoclonal antibodies. The success of the method with other antibodies will depend on the relative p*I* values of the Fc and Fab fractions. Replacement of soluble papain with immobilized papain, which would be removed during the buffer exchange step, may increase assay robustness by decreasing the production of nonspecific exopeptidase-related Fab peaks during storage.

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