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Rapid analytical tryptic mapping of a recombinant chimeric monoclonal antibody and method validation challenges[1]

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Abstract

A rapid and reproducible analytical tryptic mapping method was developed as an identity test for a recombinant chimeric monoclonal antibody for lot release testing. The unfolding, reduction, carboxymethylation, trypsin digestion, and reversed-phase (RP) HPLC steps were optimized to provide a reproducible method. The optimized method requires 30 min for unfolding the protein, 30 min for carboxymethylation, 4 h for digestion with TPCK-trypsin and 140 min for RPHPLC analysis. The total time required is less than 8 h compared to conventional procedures, which must be performed over several days. The optimized method was validated for its precision, recovery, specificity, and robustness. The precision of the method was determined by repeatability and intermediate precision experiments. Relative standard deviation (RSD) values were $\leq 10\%$ for the relative peak areas of marker peaks. The mean recovery of these marker peaks was 88.4%. The specificity was demonstrated by the unique tryptic mapping patterns obtained compared with several other monoclonal antibodies. Robustness was demonstrated by the relative insensitivity of the tryptic map to small deliberate changes in key method parameters. Excessive relative peak area variability observed for one peak (RSD 52%) was traced to adsorption to glass autosampler vials. This variability was substantially reduced (RSD 11%) by substituting polypropylene autosampler vials. The data demonstrate that this method may be applicable to a wide range of pharmaceutically relevant monoclonal antibodies. Published by Elsevier Science B.V.

Keywords: Recombinant protein; Monoclonal antibody; Identity; Tryptic mapping; Reversed-phase HPLC; Method validation

1. Introduction

Tryptic mapping is an identity or 'fingerprinting' assay that has been used extensively as a tool for the structural elucidation of proteins [1,2]. In

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addition, tryptic maps have been used to determine variants [3,4], genetic stability [5], and product consistency [6], and to monitor the stability of protein drugs [7]. The usefulness of this assay stems from the ability of trypsin, a proteolytic enzyme, to cleave specifically after the C-terminal side of lysine and arginine amino acid residues, giving rise to a predictable set of peptides. The resultant peptide mixture is chromatographed on a reversed-phase support to produce a unique profile, or map, of each protein to establish identity. The methods reported in the literature for tryptic mapping are time consuming, typically taking 2 days to complete. Often, separation of the subunits of multimeric proteins is required in order to obtain reproducible results [8]. Because tryptic mapping is an essential component of many biopharmaceutical control systems, a rapid, reliable and easily performed method is desirable. Described in this paper is a rapid tryptic mapping method for a therapeutic monoclonal antibody.

The recombinant chimeric monoclonal antibody (IgG1, kappa) has two 451-residue heavy chains and two 213-residue light chains. The variable regions of both the heavy and the light chains are of mouse origin, whereas the constant regions are of human origin. There are 16 disulfide bonds and one site of N-linked glycosylation at Asn-301 of each heavy chain. Each light chain is linked by disulfide bonds to a heavy chain and two interchain disulfide bonds attach the heavy chains to each other. Based on trypsin's specificity, after reduction of the disulfide bonds, 39 peptides and a free lysine residue are expected from the heavy chain and 17 peptides and a free arginine residue are expected from the light chain. A well-developed tryptic map should be able to account for the entire primary structure.

The tryptic mapping method described in this paper was validated to monitor the quality of monoclonal antibody product based on International Conference on Harmonisation (ICH) guidelines [9,10]. A recent publication addresses the issues involved in the validation of a peptide map [11]. It is estimated that there are more than two dozen monoclonal antibodies in clinical development for various therapeutic indications at the present time. A rapid and reliable tryptic mapping method for such products would be of great value.

2. Materials and methods

2.1. Reagents and materials

The recombinant chimeric monoclonal antibody (IgG1, kappa) was expressed in Chinese hamster ovary (CHO) cell culture. Iodoacetic acid was from Research Organics (Cleveland, OH). ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] and polysorbate 80 were from Sigma Chemical (St. Louis, MO). TPCK-Trypsin was from

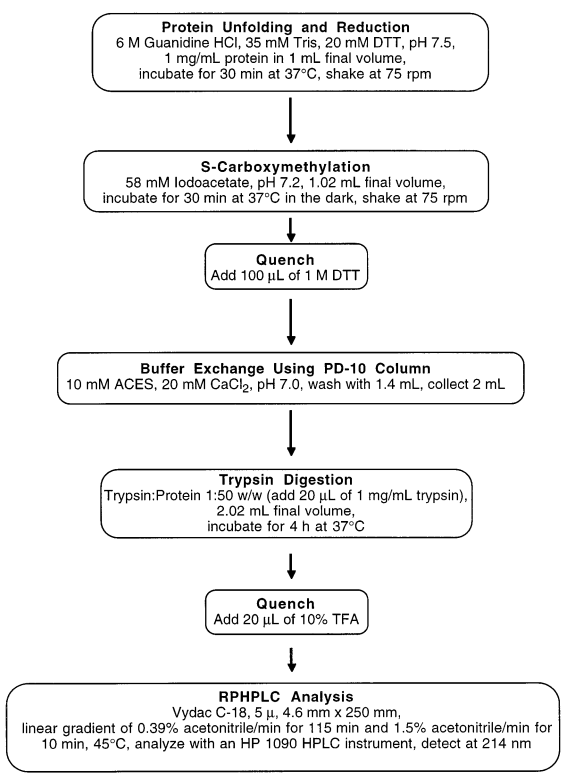

Fig. 1. Outline of the tryptic mapping procedure for the monoclonal antibody.

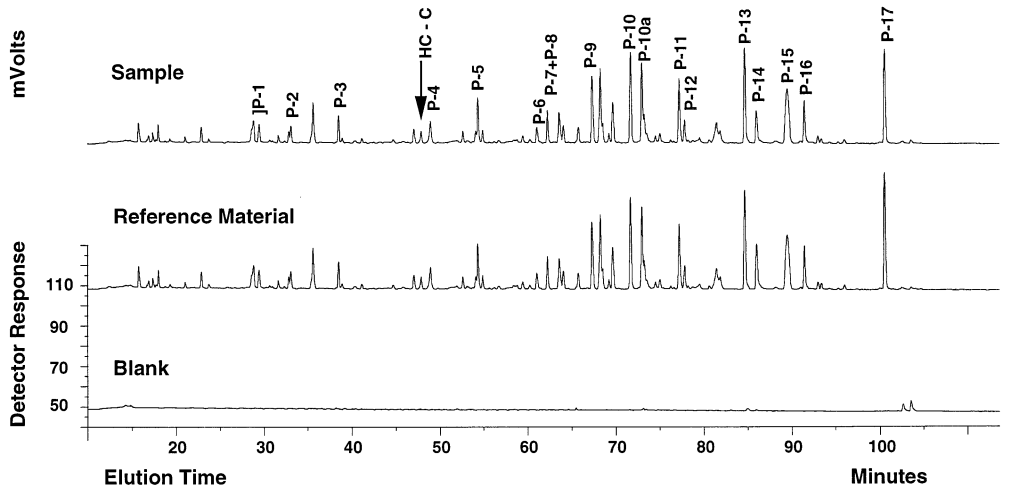

Fig. 2. Typical set of chromatograms obtained for the monoclonal antibody. P-1, Glycopeptide region; HC-C, Heavy chain C-terminus (444–450); P-6, Heavy chain N-terminus; P-10, Internal standard; P-10a, Light chain N-terminus; P-11 and P-12, System suitability peaks; P-14, Variable peak.

Worthington Biochemical (Freehold, NJ). PD-10 columns were from Pharmacia Biotech (Piscataway, NJ). The reversed-phase C-18 column, 218TP54, was from Vydac (Hesperia, CA). The reciprocal shaking water bath was from Precision Scientific (Chicago, IL). Autosampler glass vials were from Alltech (Deerfield, IL) and polypropylene vials were from Sun International (Wilmington, NC). AquaSil™ Siliconizing Fluid was from Pierce Chemical (Rockford, IL). All other chemicals were analytical reagent grade.

2.2. Tryptic mapping

The tryptic mapping of the recombinant chimeric monoclonal antibody was performed as outlined in Fig. 1, involving protein unfolding, reduction of disulfide bonds, *S*-carboxymethylation of cysteine residues, trypsin digestion and reversed-phase chromatography. A reference material was prepared from this antibody for use in the assay. To evaluate the identity of a sample, most of the experiments required an analysis of the reference material, the sample, and a co-mix that was prepared by mixing equal amounts of the tryptic digests of the reference material and the sample.

2.3. Precision

Both repeatability and intermediate precision experiments were performed according to ICH guidelines [10]. The repeatability of the trypsin digestion and of the reversed-phase chromatography were determined separately. For digest repeatability, six replicates of the reference material were digested and analyzed. For repeatability of the chromatography, six replicates of a single digest were analyzed. The retention times and relative peak areas of the 18 marker peaks (P-1 to P-17 including P-10a) shown in Fig. 2 were used for the precision calculations.

2.4. Recovery

Recovery was studied at two stages: at the PD-10 column step to assess the recovery of carboxymethylated protein and at the chromatography step to assess the recovery of tryptic peptides present in marker peaks. PD-10 chromatography was performed on six replicates of samples according to the manufacturer's instructions, except that 2.0 ml was used for elution instead of 2.5 ml to minimize the level of guanidine hydrochloride in the protein solution. The recovered protein was

Table 1
Robustness evaluation

	Parameter	Control (R)[a]	Sample (R)[a]
Trypsin digestion	Enzyme:protein ratio (w/w)	1:50 (2.1)	1:45 (2.2) 1:55 (2.2)
	Digestion temperature	37°C (1.7)	35°C (1.7) 39°C (1.7)
	Digestion time	4.0 h (1.8)	3.75 h (1.8) 4.25 h (1.8)
Mobile phase	TFA concentration	0.10% in A/0.08% in B (2.3)	0.09% A/0.07% B (2.3) 0.09% A/0.09% B (2.4) 0.11% A/0.07% B (2.3) 0.11% A/0.09% B (2.3)
	Shelf-life	Fresh (1.6)	One month old (1.7)
Column performance	Column life	5 inj. (1.6)	53 inj. (1.7) 100 inj. (1.8) 256 inj. (1.2)
	Column temperature	45°C (1.7)	47°C (2.0)
Column equivalence	Column-to-column	Column 1 (1.8)	Column 2 (1.7) Column 3 (1.7)
	Column lot-to-lot	Lot 1 (1.8)	Lot 2 (2.0) Lot 3 (1.8)
Reagent Shelf-life		Fresh (1.8)	1 week (1.8) 2 weeks (1.7) 6 weeks (1.8)

[a] R = Resolution. Numbers in parenthesis indicate resolution values determined in the respective experiment.

quantitated according to the Bradford method [12]. For tryptic peptide recovery, marker peaks were collected individually, evaporated to dryness, subjected to 6 N HCl hydrolysis overnight and analyzed for their amino acid content. Recovery was calculated by comparing the recovered amount of peptide with the unchromatographed tryptic digest.

2.5. Robustness

A number of parameters were evaluated to assess the robustness of the method in terms of trypsin digestion, mobile phase composition, column performance, column equivalence, and reagent shelf-life. The various parameters tested for the control method and the deliberately altered method (to demonstrate robustness) are outlined in Table 1. In addition, *S*-carboxymethylation was assessed by quantitation of *S*-carboxymethylcysteine by amino acid analysis.

2.6. Stability of tryptic digests

The stability of the tryptic digest was evaluated in order to determine the best storage conditions. Aliquots of digested monoclonal antibody were stored at ambient, 5, −10 and −60°C and analyzed at 24 h, 48 h, 72 h, and 1 week.

2.7. Evaluation of the variable peak

To evaluate the cause of the variability of the relative peak area response of peak P-14, the following experiments were performed:

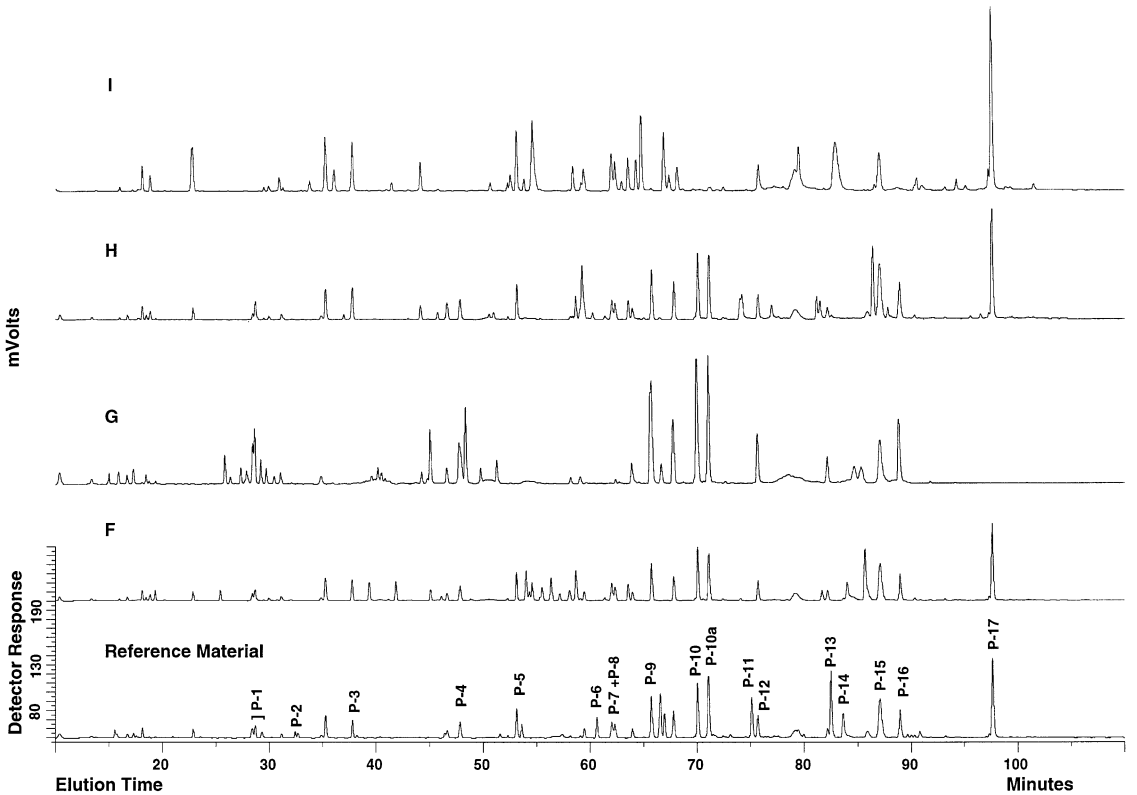

Fig. 3. Tryptic maps of various recombinant monoclonal antibodies. Ref. material, Mouse/human chimeric monoclonal antibody; F and H, Recombinant humanized monoclonal antibodies; G, Recombinant receptor-Fc immunoadhesin; I, Recombinant humanized F(ab′)2 with hinge region.

2.7.1. Autosampler vial

The autosampler glass vials were siliconized with 0.2% AquaSil™ Siliconizing Fluid, air dried and then placed in the autosampler. Polypropylene vials were also evaluated as alternates.

2.7.2. Digest additives

Polysorbate 80 was spiked at a final concentration of 1% into a freshly made tryptic digest and chromatographically analyzed.

3. Results and discussion

For most of the robustness studies, the reference material was evaluated together with the sample and a co-mix of the two. Profile comparisons were made based on visual observation, identification of marker peaks, quantitation of relative peak areas [(normalized peak area of sample ÷ normalized peak area of reference material) × 100%], and evaluation of resolution values (R). A typical set of chromatograms obtained for the monoclonal antibody is presented in Fig. 2.

3.1. Protein unfolding, reduction, S-carboxymethylation and trypsin digestion

A concentration of 6 M guanidine hydrochloride was used for unfolding the monoclonal antibody. The extent of unfolding was monitored by circular dichroism and was found to be complete under the experimental conditions. The use of another common protein unfolding reagent, 8 M urea, resulted in incomplete digestion, most likely due to incomplete unfolding of the antibody.

Table 2
Precision of the tryptic mapping method

Repeatability	Digest	Retention time SD	≤0.05 min
		Relative peak area RSD	≤7.1%[a]
	Chromatography	Retention time SD	≤0.02 min
		Relative peak area RSD	≤4.1%[a]
Intermediate precision		Retention time SD	≤0.27 min
		Relative peak area RSD	≤10.0%[a]

[a]Calculated for all marker peaks except peak P-14.

The reduction and *S*-carboxymethylation steps were optimized with standard reagents. Short incubations (30 min at 37°C) were sufficient for optimum disulfide reduction and *S*-carboxymethylation of cysteine. Under these conditions, 89% *S*-carboxymethylation was achieved.

Trypsin digestion conditions were chosen to obtain complete digestion without significant nonspecific reactions. Traditionally, longer digestions with higher trypsin concentrations are used [1]. In the case of this particular monoclonal antibody, increased chymotrypsin-like cleavages were observed when a second spike of trypsin at 1:50 (enzyme:substrate) was added with incubation continued for an additional 4 h (data not shown).

3.2. Reversed-phase chromatography (RPHPLC) and identification of peptides

The RPHPLC separation was optimized using a binary gradient of water and acetonitrile with TFA as the ion-pairing reagent. Approximately 60 peaks were resolved. Although the theoretically expected number of peptides was 56, a few of the peptides exhibited chymotrypsin-like cleavage(s) resulting in additional peaks. The key attributes of an optimized gradient are the number of peptide peaks resolved and the precision of the tryptic map in terms of peak retention time and relative peak area. Using on-line liquid chromatography/electrospray mass spectrometry (LC/ESMS) and N-terminal sequencing techniques, 94% of the primary structure of the molecule was accounted for. The unidentified regions of the molecule are primarily small peptides (2–4 residues) or single amino acids, all of which are expected to elute in the injection peak. An earlier version of the method exhibited more chymotrypsin-like cleavages, although 100% of the primary structure (including the flow-through peak) was identified.

It is impractical to quantitate each peak in the tryptic map for its retention time and relative peak area response. To facilitate data evaluation, 18 peaks were chosen for detailed analysis based on peak homogeneity, resolution and relative peak area; these peaks were then designated as marker peaks. Together, these marker peaks represent 69% of the monoclonal antibody primary structure and where feasible, include terminal peptides and the glycopeptide. Peak P-10 (retention time $\sim$70 min) was designated as the internal standard and the peak areas of the other marker peaks were normalized against P-10 to minimize the effects of any sample preparation and instrument variability. Two neighboring peaks (P-11 and P-12) were chosen as system suitability peaks so that the *R* value could be calculated (Fig. 2). This value was used as the primary criterion for column/chromatogram suitability. Among the marker peaks, peak P-14 was unique in its high relative peak area variability and was not included in the relative peak area calculations. It was noticed that the relative peak area response of this peak decreased as a function of time in tryptic digests stored at ambient temperature during chromatography. Investigations leading to identification of the source of this variability and apparent solutions are discussed separately.

3.3. Specificity

The primary use of tryptic mapping is to establish the identity of the product. The tryptic map-

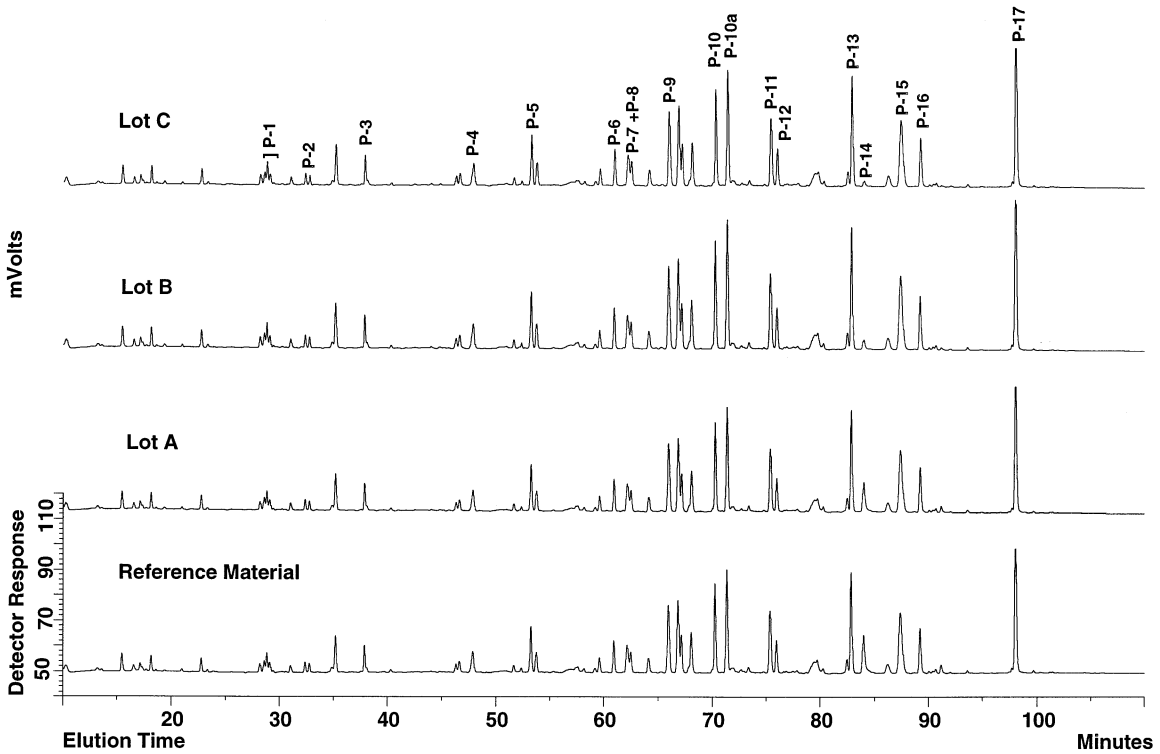

Fig. 4. Comparison of three lots of the monoclonal antibody with the reference material.

ping method described here provides a unique 'fingerprint' for each of the monoclonal antibodies analyzed; these data are presented in Fig. 3. Among the molecules tested were both chimeric and humanized monoclonal antibodies with various antigen specificities.

3.4. Precision

The data shown in Table 2 demonstrate good assay precision given the complexity of the elution profile. The intermediate precision, which was evaluated between two laboratories with two different sets of reagents, instruments and columns, showed a retention time standard deviation (SD) of ≤ 0.27 min and a relative peak area RSD of $\leq 10\%$. A comparison of three sample lots with the reference material is shown in Fig. 4. The profiles all demonstrate a similar pattern and comparable relative peak areas, indicating lot-to-lot consistency of antibody production.

3.5. Recovery

The mean recovery at the PD-10 column step was 77.3% (RSD 5.5%). This recovery was somewhat low and most likely due to the choice of elution volume, which was lower than that recommended by the column manufacturer (Section 2). Consistent performance of PD-10 columns, however, was evident by the acceptable RSD value obtained.

Mean column recovery at the reversed-phase chromatography stage of 15 marker peaks (P-1 through P-17 except P-2, P-13, P-14) was 88.4% with an RSD of 15% for three determinations. The peaks that exhibited chymotrypsin-like cleavages that resulted in lower recovery were not included in the calculation. Recoveries of the internal standard and the two system suitability peaks were 84.3, 98.2, and 99.0%, respectively. Acceptable recoveries observed for a set of peptides with varying lengths and hydrophobicities

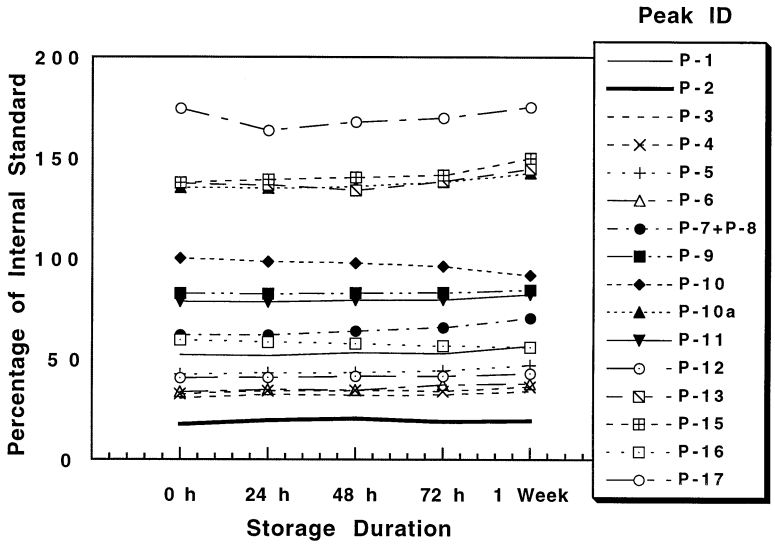

Fig. 5. Stability of the tryptic digest of the monoclonal antibody stored at ambient temperature.

indicate that the RPHPLC column is suitable for routine quality control use.

3.6. Robustness

There were no significant pH effects observed in the *S*-carboxymethylation of the antibody. Values of 87.9 ± 3.1, 87.7 ± 1.4, and 91.3 ± 3.5% of theoretical *S*-carboxymethylcysteine were obtained at pH 7.4, 7.2 (control) and 7.0, respectively, demonstrating that the method is robust. Evaluation of the robustness of the remaining portion of the method was performed in three ways:

Table 3
Reducing peak P-14 variability

	Treatment	Relative peak area RSD (%)[a]
Autosampler vial	Glass vial-control	52.0
	Siliconized glass vial	27.8
	Polypropylene vial	11.1
Digest additives	None-control	52.0
	Polysorbate 80	47.7

[a]Variability of peak P-14 was monitored using consecutive HPLC runs from the same digest.

3.6.1. Visual observation of the chromatograms of deliberately altered conditions compared to control

This entailed confirmation of a consistent overall map, the internal standard, system suitability peaks and marker peaks. Overall, all the parameters studied demonstrated that the method is robust.

3.6.2. Quantitative relative peak area comparison of marker peaks of the altered conditions compared to control

Relative peak areas of marker peaks were determined for the chromatograms generated under both altered and control conditions. These values were within the target range of 100 ± 20%, showing that the method is robust.

3.6.3. Comparison of R values of the altered conditions compared to control

Finally, as an example, the resolution between two system suitability peaks P-11 and P-12 was compared under both conditions. Only one of the parameters studied to evaluate the robustness of the method had any significant effect on the *R*

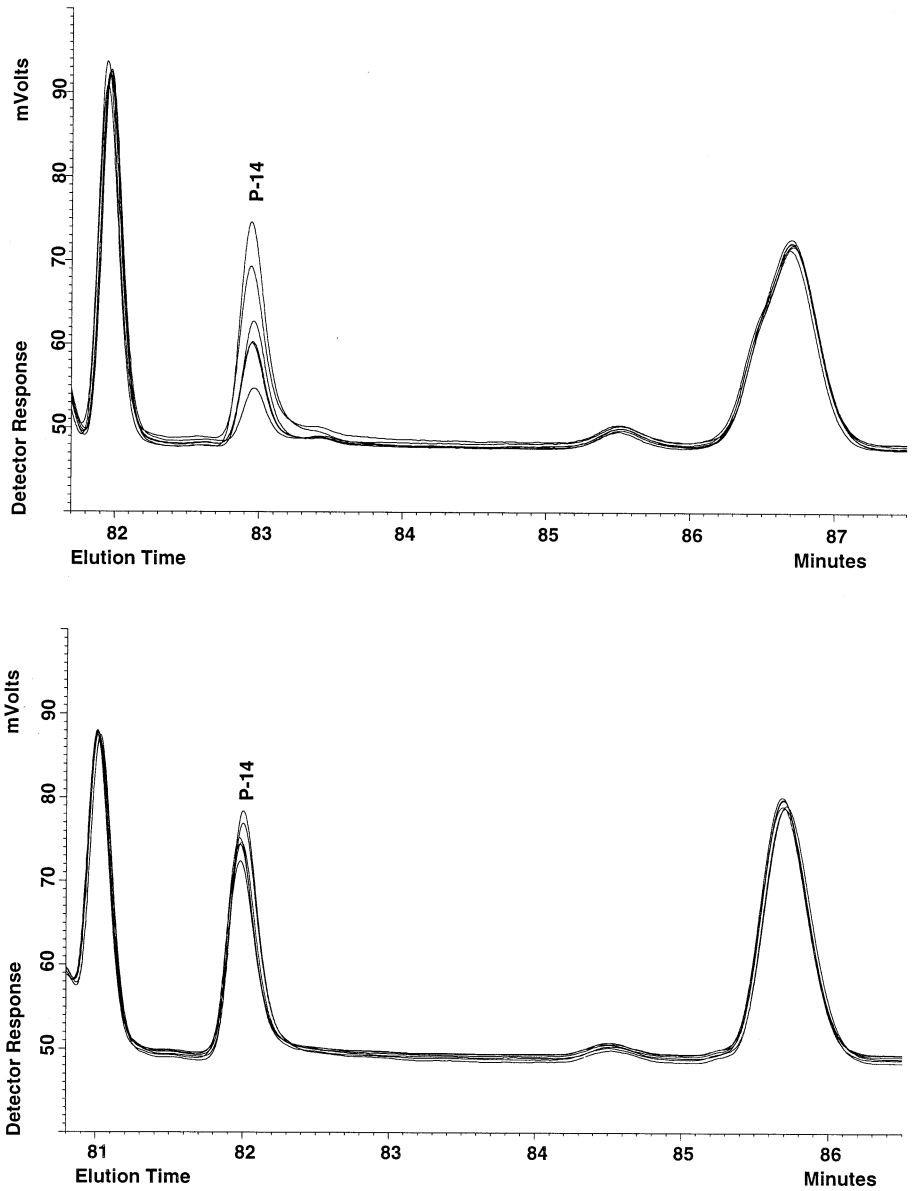

Fig. 6. Overlay plots of six consecutive RPHPLC runs showing the relative peak area variability of peak P-14 with glass autosampler vials (top figure) and with polypropylene autosampler vials (bottom figure).

values (Table 1). This was the temperature of the chromatography, which resulted in R values of 1.5, 1.7 and 2.0 at 43, 45 and 47°C, respectively. This observation demonstrates that the method is sensitive to the temperature of the chromatography, which must be controlled accurately.

3.7. Stability of tryptic digests

The relative peak areas of the marker peaks were comparable for tryptic digests stored at different temperatures. The data are shown for a tryptic digest that was stored at ambient tempera-

ture for 1 week (Fig. 5). These data demonstrate that the digests are stable at least for 1 week when stored at ambient temperature. Similar results were obtained at the other temperatures evaluated.

3.8. Evaluation of the variable peak

In terms of relative peak area precision, peak P-14 demonstrated high variability (52% RSD). In general, the relative peak area for peak P-14 decreased as a function of digest storage time, whereas all other peaks exhibited good storage stability. Peak P-14 contains a peptide that has 27 amino acid residues, six of which are aromatic. It was postulated that this variability was due to low solubility, leading to aggregation and/or surface adsorption because of the size and relative hydrophobicity of the P-14 peptide. Adding polysorbate 80 to a final concentration of 1% did not appreciably reduce the variability compared to that observed from storage in normal glass vials (48% RSD compared to 52%; see Table 3). Using siliconized glass vials did result in significantly lower variability (28% RSD), although it was still higher than the variability observed for other marker peaks. However, when polypropylene autosampler vials were used, there was a dramatic reduction (to 11% RSD) in the observed peak area variability (see Table 3), leading to the conclusion that surface adsorption was the main source of peak P-14 variability. The profiles of peak P-14 stored in glass and in polypropylene vials are presented in Fig. 6.

4. Conclusions

This report describes a tryptic mapping method for a chimeric monoclonal antibody that requires less than 8 h to perform. The validation data demonstrate that the temperature of the chromatography column needs to be accurately controlled and appropriate autosampler vials must be used for reproducible chromatograms. It is also recommended that the sample and reference material be tested together with a co-mix to confirm the identity of the antibody. The number of marker peaks for routine monitoring can be minimized by careful evaluation of the tryptic map. Overall, the method was shown to be specific, precise and robust for routine lot testing for identification in a quality control environment, and may be applicable to a wide range of monoclonal antibodies.

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