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RP–HPLC tryptic mapping of IgG1 proteins with post-column fluorescence derivatization

Dipti Gulati *, Jacob Bongers, Sudhir Burman

Analytical Sciences Department, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, PA 19406, USA

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Abstract

Peptide mapping is an important analytical technique widely used to study the primary structure of proteins. In quality control settings, it is employed as an identity test to probe for small changes in protein primary structure. A great challenge in peptide mapping is to minimize the detection limit for peptides due to the low detectability of smaller peptides based on their ultraviolet absorbance. The detection of peptide fragments can be enhanced by pre- or post-column derivatization with fluorescent tags. The use of post-column *o*-pthalaldehyde (OPA) and fluorescamine chemistries for on-line derivatization of peptide fragments from the RP–HPLC tryptic maps of several IgG1 monoclonal antibodies was explored. This paper describes the simple and sensitive peptide mapping technique for structural confirmation of proteins using picomoles of samples by post-column fluorescence derivatization. A comparison of UV and fluorescence detection of a peptide map is also presented. The method includes post column OPA derivatization of tryptic peptides from RP–HPLC tryptic maps with fluorescence detection. The conclusion reached that fluorescence detection gave relative detectability for tryptic peptides that range from 10- to 100-fold better than those observed with UV detection. The sensitivity of the peptide map increased by about 200–500 fold, i.e. peptide maps could be obtained using 2–5 pmol of digest instead of 1 nmol of digest. A roughly equal fluorescence response for all peptides (equal peak areas) was generally observed.

Keywords: Peptide mapping; *o*-Pthalaldehyde (OPA) derivatization; Fluorescent detection

1. Introduction

Rapid advances in recombinant DNA technology have stimulated great interest in proteins as novel therapeutics in the pharmaceutical industry. Structural characterization of these proteins presents a significant analytical challenge. RP–HPLC peptide mapping is a powerful technique for structural elucidation and structure confirma-

Abbreviations: HPLC, high-performance liquid chromatography; DTT, Dithiothreitol; TPCK, Tosyl-L-phenylalanine chloromethylketone; rMAb, recombinant monoclonal antibody; TFA, trifluoroacetic acid; OPA, *o*-pthalaldehyde; PITC, phenylisothiocyanate.

* Corresponding author. Tel: +1-610-270-5616; fax: +1-610-270-5830.

E-mail address: dipti_g_gulati@sbphrd.com (D. Gulati)

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tion of proteins [1–4]. Peptide mapping is commonly used to identify alterations in the primary sequence of a protein. Peptide mapping is capable of detecting small structural modifications derived from oxidation, deamidation or amino acid substitution etc. This technique is also used to locate glycosylation sites [5,6] and disulfide linkages [7]. Today peptide mapping is increasingly used in biotechnology for the quality control of recombinant proteins. Peptide mapping is established as a method to assure that the recombinant proteins has the same primary structure as the native protein. Peptide mapping also provides important information on lot-to-lot product consistency, expression errors and mutation or modification sites [1]. Peptide mapping is accomplished by site specific chemical or enzymatic digestion of proteins [4,8,9]. The digest is then analyzed by reversed phase HPLC and detected by ultraviolet absorbance. The resultant peptide map is a unique fingerprint profile of a protein and may be compared to a reference chromatogram to establish equivalency of the sample to reference protein.

A digest is ideally an equimolar mixture of the various peptide fragments of the protein with each having absorption in the far UV region, roughly proportional to the number of peptide bonds. In a typical peptide map, smaller peptides have a very low relative detectability based on their ultraviolet absorbance. Detection sensitivity of the peptide map can be increased by using narrow-bore and micro-bore columns, instead of regular 4.6-mm i.d. column. However, relative detectability of the smaller peptides in peptide map remains unaltered. Moreover, lower relative detectability of smaller peptides increases the detection limit for the digest.

Compared to UV detection, fluorescence detection provides the additional advantage of enhanced selectivity and lower limits of detection. Several studies suggest that the detection of smaller peptide fragments can be enhanced by pre- or post-column derivatization with fluorescent tags and fluorescence detection [10–12]. Pre-column and post-column fluorescent detection methods are routinely used for amino acid composition analysis of proteins and peptides [10,13–16]. In contrary to amino acid analysis, very few reports are available for post-column fluorescence detection of peptide maps [10]. Therefore, to improve the detectability of smaller peptides in the peptide map and increase the detection sensitivity of the peptide map, the possibility of labeling the peptides of tryptic digest with fluorophores, using

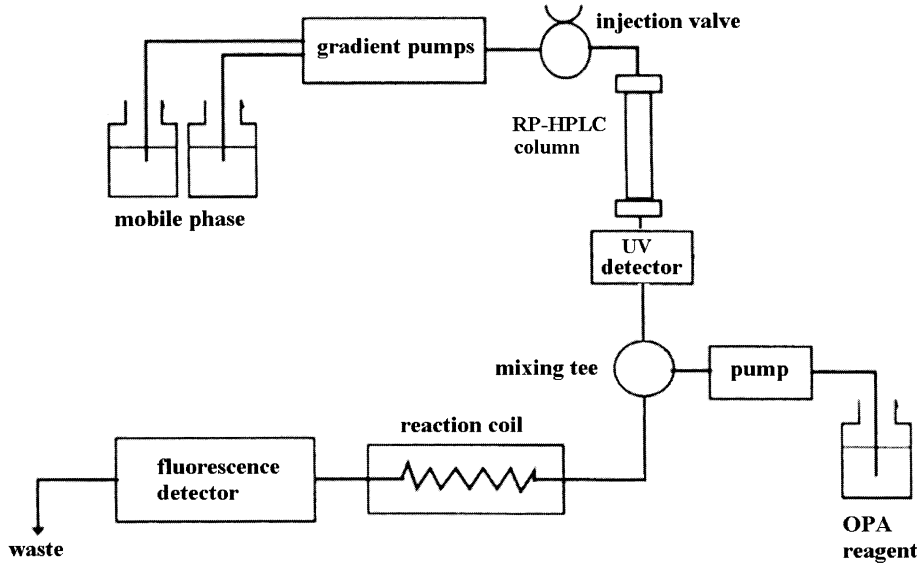

Fig. 1. System for post-column *o*-phthalaldehyde (OPA) derivatization and fluorescence detection of peptides.

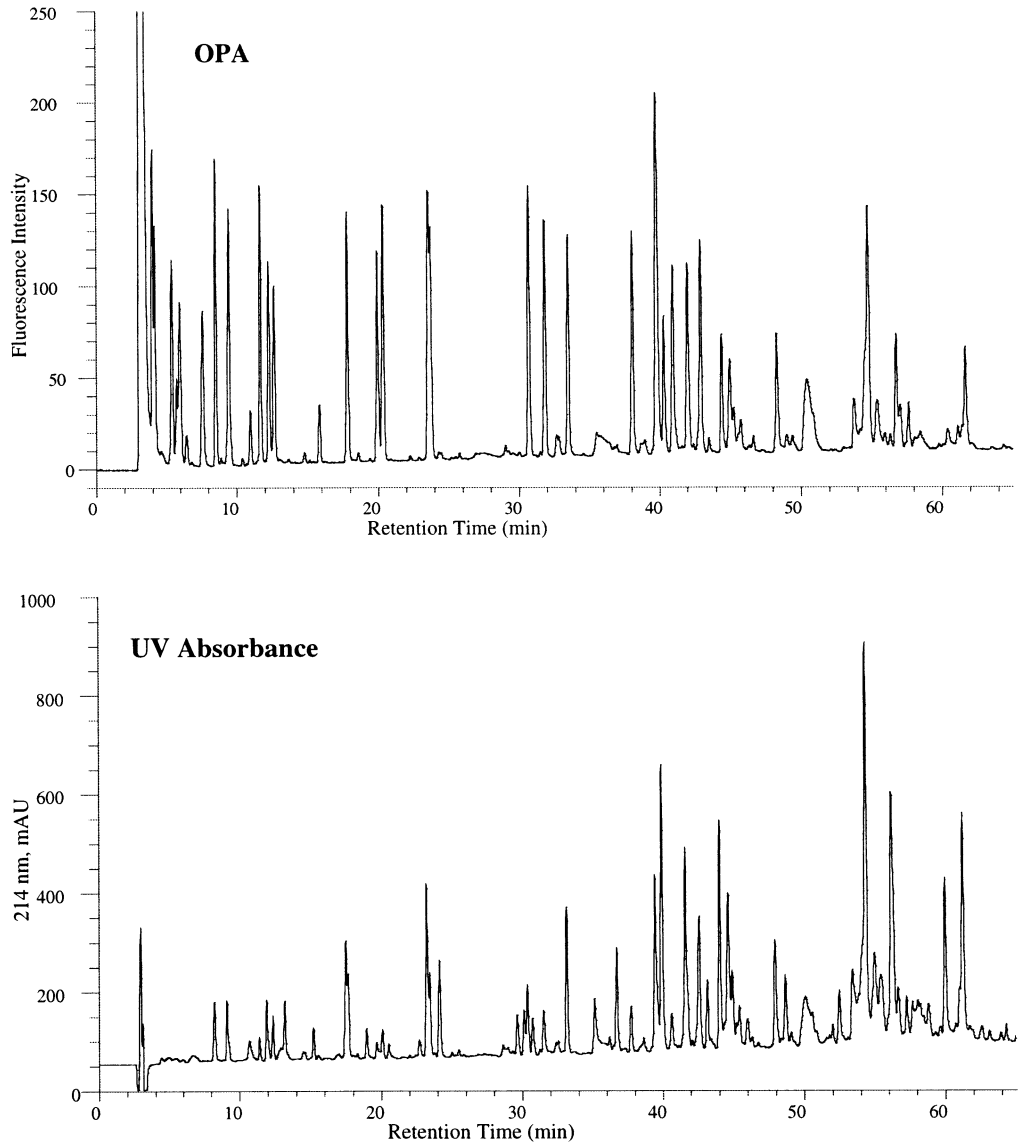

Fig. 2. RP–HPLC tryptic map of recombinant monoclonal antibody showing traces for UV detection (lower) and fluorescence map for post column OPA derivatives (upper).

on-line post-column derivatization was explored. Post-column derivatization technique was preferred due to its several advantages and the main disadvantage of pre-column derivatization, i.e. the formation of side products which influences the chromatographic analysis or reproducibility of the derivatization reaction [17].

A number of derivatizing agents for primary amines have been reported including OPA, ninhydrin, fluorescamine, PITC etc. [18–21]. Since, OPA and fluorescamine are commonly used sensitive reagents for amino acids, peptides and protein detection [22,23], the use of OPA and fluorescamine for on-line post-column derivatization of peptide fragments of tryptic digest for several recombinant monoclonal antibodies was investigated.

Table 1
Sequences of peptides in the tryptic map of *anti*-RSV [24] (Fig. 3)

r_t (min)[a]	Tryptic fragment[b]
6.5	VDK
6.5	VSNR
8.3	TKPR
8.3	GQPR
11.5	EYK
13.2	AK
14.8	QPPGR
15.1	TISK
15.4	VEPK
16.1	ADYEK
17.4	**FQGR**
19.5	DELTK
22.0	VEIK
22.2	**EEQYNSTYR** (glycopeptides)
23.5	**SFNRGEC**
24.5	LTVDK
27.8	VQWK
27.8	VTITCR
28.1	FSGVPSR
28.9	**VDNALQSGNSQESVTEQDSK**[c]
34.1	**NQFSLR**
34.9	**SLSLSPG**
35.2	ALPAPIEK
35.5	**DTLMISR**
36.3	VTMLVDTSK
37.4	HKVYACEVTHQGLSSPVTK
38.0	VYACEVTHQGLSSPVTK
40.0	**LLIYR**
41.0	EPQVYTLPPSR
41.8	**DIQLTQSPSSLSASVGDR**
42.6	STSGGTAALGCLVK
44.2	GPSVFPLAPSSK
44.2	DSTYSLSSTLTLSK
44.7	SSQTLVHTDGNTYLEWYQQKPGK
45.5	NQVSLTCLVK
46.3	FNWYVDGVEVHNAK
47.4	TPEVTCVVVDVSHEDPEVK
48.6	WQQGNVFSCSVMHEALHNHYTQK
49.3	GFYPSDIAVEWESNGQPENNYK
52.6	TTPPVLDSDGSFFLYSK
55.0	TVAAPSVFIFPPSDEQLK
58.0	LSSVTAADTAVYFCNSWGSDFD-HWGQGTTVTVSSASTK
59.0	GLEWIGWIDPENDDVQYAPK
59.0	SCDKTHTCPPCPAPELLGGPSVFLFPP-KPK
59.8	THTCPPCPAPELLGGPSVFLFPPKPK
60.1	SGTASVVCLLNNFYPR
60.9	**VVSVLTVLHQDWLNGK**
64.5	**pGluVQLQESGPGLVRPSQTLSLTCTVS-GFTFSDYYMHWVR**
65.8	DYFPEPVTVSWNSGALTSGVHTFPAVLQSS-GLYSLSSVVTVPSSSLGTQTYICNVNHKP-SNTK

[a] Retention times refer to peaks in the 215 nm UV trace in Fig. 3.
[b] Those peptides indicated in bold did not give a fluorescence signal.
[c] This peptide gives a very small response with OPA and a good response with fluorescamine.

This paper will discuss some of the applications and advantages of post-column fluorescent derivatization by OPA in peptide mapping to increase detectability of smaller peptides and the overall detection sensitivity of the peptide map and compare it with fluorescamine detection system. These studies demonstrate that sensitivity of peptide mapping was increased by about 200–500 fold, by using post-column fluorescent derivatization on 4.6 mm i.d. column. The fluorescent method enhanced the relative detectability of smaller tryptic peptides by 10–100 fold as compared to the UV absorbance method. This method was able to detect some of the additional small peptides that were undetectable by UV detection.

2. Experimental

2.1. Materials

Recombinant monoclonal antibodies (rMAb) were produced in Chinese hamster ovary cells using a propriety technology. All the chemicals and reagents used were of highest purity and are commercially available. TPCK treated trypsin was from Worthington (Freehold, NJ). Disposable gel-filtration columns were obtained from Bio-Rad. *o*-Phthalaldehyde reagent kit for post column derivatization was from Pickering Laboratories. The OPA solution was prepared according to manufacturer's instruction.

2.2. Reduction, S-carboxymethylation and trypsin digestion of rMAb

Reduction, carboxymethylation and digestion was performed as described earlier [24]. Briefly, 5.0 mg (33.5 nmol) of rMAb was dissolved in 0.5 ml of Tris buffer, pH 8.4, containing 6 M guanidine hydrochloride. The solution was reduced by adding a 40-fold molar excess of freshly prepared dithiothreitol (1.34 μmol) and heating the reaction mixture at 65°C for 60 min. The solution was brought to ambient temperature and 2.9 μmol of sodium iodoacetate was added. The *S*-carboxymethylation reaction was carried out in dark for 40 min at ambient temperature. An additional 3.5 μmol of DTT solution was added to terminate the alkylation reaction. The reaction mixture was passed through 10DG Bio-gel (Bio-Rad) column, pre-equilibrated with 0.1 M ammonium bicarbonate, pH 8.1, to remove the reagents. Final product was digested with 0.1% (w/v) trypsin at 37°C for 24 h. The reaction was terminated by adding 40 μl of 1.0 M HCl.

2.3. Reversed phase HPLC and post column OPA derivatization of protein digest

The reversed phase HPLC of the digest of rMAb was performed on Vydac C18 reversed phase column (Part # 218TP54, 4.6 × 250 mm), using a gradient of 0.1% (v/v) TFA in water and 80% acetonitrile. Reversed phase HPLC was done on Hewlett Packard 1100 LC. Approximately 1.3 nmol of the digest was injected to achieve UV maps and approximately 60 pmol of the digest was injected to obtain the fluorescent plot. The

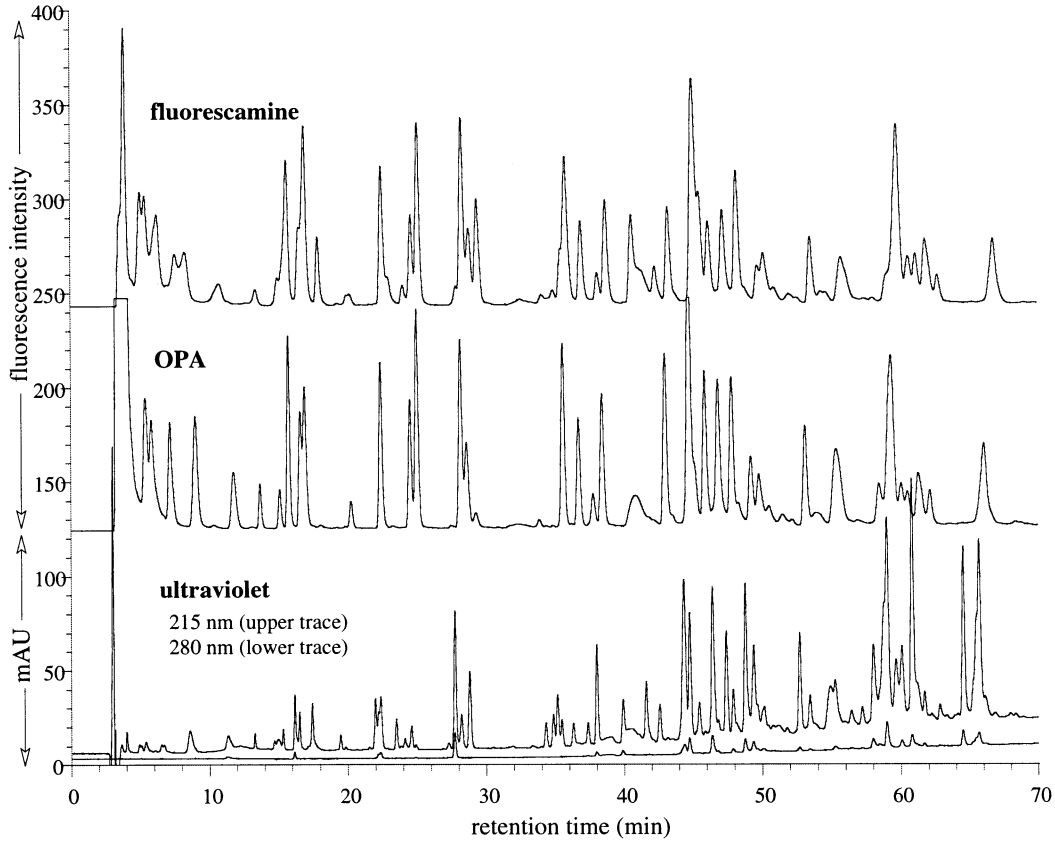

Fig. 3. RP–HPLC tryptic map of reduced/carboxymethylated *anti*-RSV monoclonal antibody [1,17] showing traces for UV detection (lower) and fluorescence traces for post-column OPA (middle) and fluorescamine derivatives (upper).

column was maintained at 45°C and the effluent monitored at 215 and 280 nm. Post-column derivatization of peptides was obtained by directing the effluent from UV detector to a mixing tee into which OPA reagent was pumped (Beckman 114 pump) at flow rate of 0.5 ml/min. The reaction mixture was then passed through 1 ml reaction coil and into Perkin–Elmer LC 240 fluorescence detector (excitation wavelength of 330 nm and emission wavelength of 465 nm).

2.4. Reversed phase HPLC and post column fluorescamine derivatization of peptide mixture

Five μl of digest (2 mg/ml) was injected on 2.1×250 mm (Vydac 218TP52) column at 45°C with flow rate of 0.2 ml/min. Post column derivatization was achieved by pumping the fluorescamine reagent at 0.1 ml/min. Post column derivatization with fluorescamine was performed by mixing effluent from the UV detector with borate buffer, pH 10.4 at 0.05 ml/min followed by mixing of fluorescamine in acetonitrile (excitation at 390 nm and emission at 475 nm) at 0.1 ml/min.

3. Results and discussion

Fig. 1 shows the system diagram for post-column *o*-pthaldehyde (OPA) derivatization and fluorescent detection of peptides. The effluent from the UV detector was mixed with OPA reagent in a mixing tee and were directed towards the fluorescent detector through a 1 ml reaction coil. Fig. 2 shows the RP–HPLC analysis of rMAb tryptic digest (A) detected at 215 nm (B) derivatized with OPA and detected by fluorescence detector with 330 nm excitation and 465 nm emission wavelength. Most of the peaks in fluorescent map are of equal intensity because each peptide is labeled with one fluorescent tag. Intensities of peaks in UV chromatogram vary depending on the size of the peptides. UV absorbance method requires ≈ 1 nmol of protein digest whereas OPA fluorescent method requires approximately 2–5 pmol of protein digest for detection of peaks on 4.6-mm i.d. column. This indicates that fluorescent labeling method is about 200–500 fold more sensitive than the UV absorbance method. The relative intensities of most of the smaller peptides in the fluorescent map were enhanced by about 10–100 fold. On the contrary, some of the peaks, including glycopeptides were undetectable (Table 1). This may be either due to failure of derivatization of these peptides or quenching of fluorescence signal by the peptides. Moreover, OPA detection system demonstrated its ability to detect very small peptides, which were undetectable by UV detection system (Fig. 2). Since only free amino groups are required for OPA labeling, the size of peptides is not a crucial factor for this detection system. The small peptides derived from the tryptic digest of rMAb were clearly visible in the first 10 min of the chromatogram with post-column OPA detection but were difficult to visualize with UV detector due to limited number of peptide bonds. Thus, the use of OPA detection system, with UV detection system, has additional advantages in peptide mapping.

Fig. 3 shows the RP–HPLC analysis of rMAb tryptic digest on 2.1×250 mm column (A) detected at 215 nm (B) derivatized with OPA (C) derivatized with fluorescamine (excitation at 390 nm and emission at 475 nm). These results demonstrate that peaks of the UV map that are undetectable by OPA derivatization, were also undetectable by fluorescamine derivatization. It was also observed that the narrowbore (2.1 mm i.d.) column (Fig. 3) generates a peptide map with broader and less resolved peaks when comparison to that achieved by using 4.6-mm i.d. column (Fig. 2).

This method is capable of performing the peptide mapping at picomole levels, therefore it is useful in quality control settings for confirmation of protein structure by mapping when a limited amount of sample is available. Also, it opens up the possibility of performing peptide mapping using different fluorescent tags, where all the peaks can be detected.

4. Conclusions

Fluorescent labeling methods require approximately 2 pmol of digest and are about 500 fold

more sensitive than the UV absorbance method. Relative intensities of a few smaller peaks in the fluorescent map are approximately 10–100 fold higher than the UV map. Most of the peaks in the fluorescent method are of equal intensities because each peptide is labeled with one fluorescent tag. OPA fluorescence methods fail to detect several peptides. This may be either due to no derivatization of certain peptides with fluorescent tag or quenching of fluorescence of derivatized peptides.

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