ELSEVIER

Journal of Chromatography A, 735 (1996) 357–366

JOURNAL OF CHROMATOGRAPHY A

Analysis of monoclonal antibody and immunoconjugate digests by capillary electrophoresis and capillary liquid chromatography

Jinping Liu[a,*], Huiru Zhao[b], Kevin J. Volk[a], Steven E. Klohr[a], Edward H. Kerns[a], Mike S. Lee[c]

[a] *Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492, USA*

[b] *Roche Diagnostic System, Somerville, NJ 08876, USA*

[c] *Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543, USA*

Abstract

Comparative peptide mapping of a monoclonal antibody chimeric BR96 and corresponding doxorubicin (DOX) immunoconjugate was performed using capillary electrophoresis (CE) and capillary liquid chromatography (CLC). A unique, highly sensitive and selective approach combined with both UV absorbance and laser-induced fluorescence (LIF) detection has been developed and applied to studies including enzymatic digests of antibody and conjugate and related drug and conjugation linker substances. The analytical methodology has been established based on the unique characteristic of the anticancer drug DOX which yields native fluorescence. With an excitation wavelength of 488 nm from argon-ion laser, DOX conjugated to the monoclonal antibody using a hydrazone linker can be determined with a detection limit at the attomole level. Approaches were developed based on the successful conjugation and analysis of a model peptide conjugate. Enzymatic digests of the monoclonal antibody BR96 and its immunoconjugate were mapped by CE and CLC with on-line UV and LIF detection, which results in a unique fingerprint for structural analysis. With a two-dimensional LC–CE approach, conjugated peptide–DOX species from LC were further analyzed by CE with LIF detection. The drug-containing peptide fragments in the mixture were readily detected, which can be further characterized using other complementary analytical techniques.

Keywords: Capillary electrophresis; Peptide mapping; Laser-induced fluorescence detector; Monoclonal antibodies; Doxorubicin; Hydrazone

1. Introduction

Monoclonal antibodies (MAbs) and their conjugates associated with various drugs and toxins are of significant interest in biotechnology and pharmaceutical drug discovery as effective therapeutic agents. The interdisciplinary approach represented by MAb production, cell biology, recombinant technology, and chemistry has resulted in rapid advances in the area of drug targeting and created a new strategy in the treatment of human cancers. The potential usefulness of MAbs in cancer therapy is associated with their increased selectivity to tumor-specific antigens. Conjugation of these macromolecules to cytotoxic agents such as radioisotopes, natural toxins, or antitumor drugs provides a highly selective vehicle for cytotoxic drug delivery to the tumor site [1–6]. It has been described that conjugates with antitumor activity against established tumors were superior to that of an optimal dose of unconjugated

*Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01054-8

drug [7]. The recognition of the target cell and the consequent increase in the local concentration of drug allows for site-directed killing of the cell.

MAbs possess many potential sites for drug attachment. One of the recent approaches in the conjugation procedure is the modification of the thiol groups in the hinge region of MAb BR96, a chimeric (mouse–human) variant of BR96 MAb, for the attachment of drugs to retain the antibody's immunoreactivity [8] and improve antibody-directed cytotoxicity to the human carcinomas [3,7]. This approach has proven to be very effective for tumor selectivity and internalization after binding. One of the major concerns in these studies is the efficiency of the conjugation, or the actual conjugated sites of the drug over the antibody. This has been generally predicted based on the entire antibody structure, but the detailed information regarding the conjugated species and fragments remains somewhat difficult to be determined.

Analysis and characterization of proteins and bioconjugates have recently been accelerated by new analytical methodologies which are primarily based on modern liquid chromatography (LC) and electrophoresis [9–13]. Among the recent trends in analytical instrumentation, microcolumn separation systems, such as microcolumn-LC (micro-LC) and capillary electrophoresis (CE), have achieved considerable attention [14–17]. These emerging analytical techniques appear to be highly promising with regard to separation efficiency, detection sensitivity, analysis speed, simplicity, and automation as compared to conventional approaches. Among their various applications, peptide mapping has been a technique widely utilized as a key analytical tool to serve as a unique fingerprint for structural characterization, detection and identification of amino acid and peptide modification, and monitoring structural consistency in genetically engineered protein products and conjugates [10,12,18,19]. One of the important issues of these new analytical methodologies is the development of highly sensitive detection methods which are essential for applications in the biosciences, where proteins and other biomolecules are frequently only available in small quantities [15,20]. The high-resolution separation techniques combined with highly sensitive detection approaches, such as laser-induced fluorescence (LIF) detection and electrospray-based mass spectrometry (ESI-MS), etc., are powerful tools for the characterization of various biomacromolecules.

The current studies focus on the utilization of capillary separation techniques based on LC and electrophoresis for fingerprinting conjugated peptide fragments resulting from the tryptic digests of MAb chimeric BR96–doxorubicin immunoconjugate. This immunoconjugate consists of the carrier MAb BR96, a chimeric variant of the murine BR96 MAb, the anticancer drug doxorubicin (DOX), and the chemical hydrazone linker which links the antibody and drug via inter-thiol groups of MAbs. We will demonstrate the utility of a LIF system for the highly sensitive and selective detection and identification of BR96 conjugated peptide fragments and related compounds, DOX and DOX–hydrazone (DOX–HDZ). The system also provides the capability of being incorporated with a variety of detection formats such as UV absorbance, diode-array, and MS to provide multi-dimensional information on complex samples. This approach would provide useful information allowing for the optimization of the conjugation process to ensuring the maximum drug–antibody ratio in the reaction.

2. Experimental

2.1. Apparatus

2.1.1. Capillary electrophoresis

All capillary electrophoretic experiments were performed in uncoated fused-silica capillaries (obtained from Polymicro Technology, Phoenix, AZ, USA) with 75 μm I.D. and 360 μm O.D. and effective separation length of 42.0 cm. The detection window was placed at about 6.7 cm from the outlet end. The instrument was a Beckman P/ACE Model 2100 CE system equipped with a UV absorbance detector. An alternative 5.0 mW argon-ion laser emitting at 488 nm was also incorporated into the system. The laser beam is transmitted via a fiber optic cable to the detector and illuminates the capillary window. The fluorescence signal was col-

lected through a narrow-band 520-nm emission filter. The capillary chamber was temperature-controlled at 25°C by employing a liquid coolant. Pressure injection mode was employed for all experiments. Data was collected and analyzed with Beckman System Gold software.

2.1.2. Capillary liquid chromatography

The capillary LC system employed in this study is essentially the same modification of Beckman System Gold conventional HPLC system as previously described [21]. Briefly, the solvent gradients were directly delivered into a micromixer obtained from Upchurch Scientific (Oak Harbor, WA, USA) at flow-rates of 0.2–0.4 ml/min. A precolumn splitting device was used to obtain appropriate output flow-rates (approximately 3 μl/min) for packed capillary columns. The split ratio was easily regulated by adjusting the length of restriction line where a fused-silica capillary with 50 μm I.D. was used. The capillary columns with 250 μm I.D. and 375 μm O.D were packed in-house with C_{18}, 5-μm particle of 300-Å pore size, from Vydac (Hesperia, CA, USA) using an ISCO μLC-500 pump. The columns were directly connected into a Valco microinjector with 100-nl and 500-nl internal loops. The transfer line from column outlet consisted of fused-silica capillary with 50 μm I.D. and 190 μm O.D. on-line connected to UV and subsequent detectors. An ABI Model 785A UV detector (Applied Biosystem, Foster City, CA, USA) equipped with Z-shape capillary flow cell obtained from LCpacking (San Francisco, CA, USA) was used in this study. The mobile phase used in this system consisted of 5 m*M* ammonium formate at pH 6.88 (solvent A) and 20% water–80% acetonitrile–5 m*M* ammonium formate at pH 6.88 (solvent B). Gradient elution (0–60% solvent B over 120 min) was performed for all LC separations.

The unique on-line UV and LIF detectors associated with the CE system were specially configured for capillary LC separation and detection in this study. The capillary LC system described above was connected to the CE–LIF system through a piece of Teflon connector constructed to accommodate the capillary from the LC transfer line to the LIF detector with virtually no dead volume. Both UV and LIF signals can be simultaneously acquired and processed using Beckman System Gold software.

2.2. Materials and reagents

Peptide standard and trypsin were purchased from Sigma (St. Louis, MO, USA). UV-grade acetonitrile (J.J. Baker, Phillipsburg, NJ, USA) and HPLC-grade water (Fisher, Fair Lawn, NJ, USA) were used. MAb BR96, an in-house product, is a chimeric (mouse–human) variant of the murine BR96 MAb [22] and was produced by homologous recombination [23]. Anticancer drug DOX and DOX–HDZ were obtained in house. The peptide–DOX conjugate was prepared by incubating a cystine-containing peptide, CGYGPKKKRKVGG, and DOX–HDZ with 1:1 ratio in ammonium formate buffer (pH 6.8) for 24 h. Preparation of MAb–drug conjugate requires special sample treatment and procedures [7]. The CE buffer consisted of 100 m*M* boric acid and 50 m*M* sodium dodecyl sulfate with the pH adjusted to 9.08 with sodium hydroxide.

2.3. Enzymatic digestion

The MAb BR96 and its DOX conjugate at the drug molar ratio 7.4 were subjected to tryptic digestion by modification of the previous method [18]. Two different conditions, native and denaturation, were applied to the digestion of MAb or conjugates. With an enzyme–substrate ratio of 1:10 (w/w), BR96 was digested by trypsin in its native state prior to reduction and alkylation. Under denaturation condition, MAb BR96 or BR96–DOX conjugate (10 mg/ml) in chain-separating buffer (0.5 *M* Tris, 100 m*M* EDTA, 50 m*M* $CaCl_2$, 6 *M* urea, and 25 m*M* DTT) was incubated for 15 min at 55°C under nitrogen to effectively reduce the disulfide bonds in MAb. A 2-fold molar excess of alkylating agent (iodoacetamide) was added to the reaction and the mixture was incubated in the dark for 30 min. The solution was diluted 4-fold by volume with tryptic digestion buffer (0.1 *M* ammonium bicarbonate buffer, pH 8.0). The solution was incubated with trypsin at 37°C for 4 to 24 h. The reaction mixtures were either directly used for electrophoretic and chromatographic analysis on reversed-phase HPLC

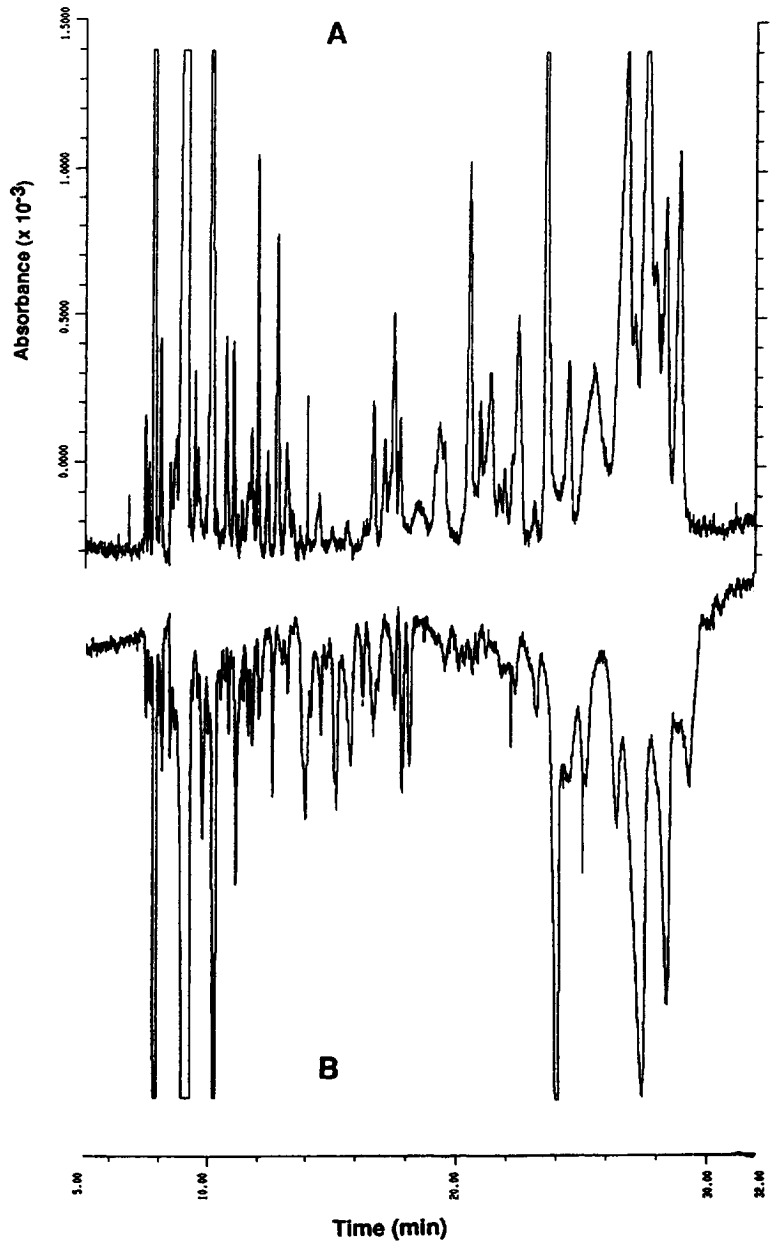

Fig. 1. Comparison of tryptic digests of MAb BR96 (A) and MAb BR96–DOX conjugate (B), separated by capillary electrophoresis with UV detection at wavelength of 214 nm. Both antibody and conjugate were denatured prior to digestion at 37°C for 4 h. Capillary, 48.7 cm×75 μm I.D. (42 cm effective separation length); background electrolyte, 0.1 *M* boric acid–50 m*M* SDS (pH 9.08); pressure injection, 2 s; applied voltage, 10 kV.

or dried and stored at −20°C and redissolved in background electrolyte or mobile phase prior to use. For LC–CE analysis, fractions collected from the LC column are directly used for subsequent CE analysis.

3. Results and discussion

Both native MAb BR96 and DOX-conjugated BR96 were initially used to establish appropriate

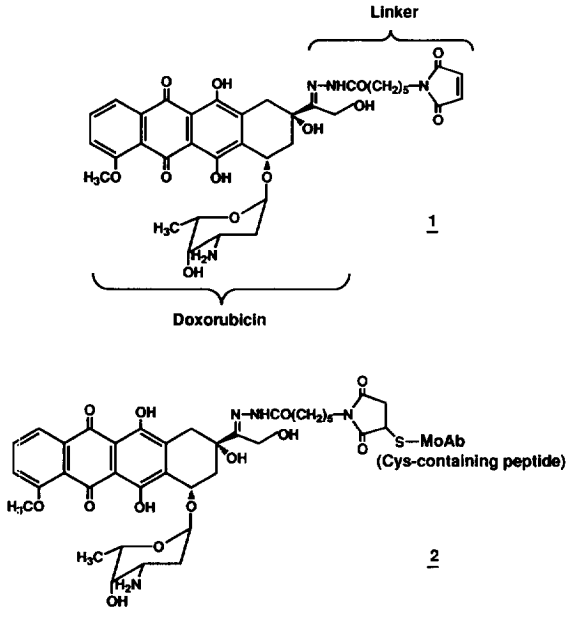

Fig. 2. Representative structures of doxorubicin hydrazone (DOX–HDZ, 1) and the corresponding conjugate (2) with MAb BR96 and cystine-containing peptide.

digestion conditions with trypsin. Various peptide maps were obtained with respect to digestion time, protease-to-substrate ratio, and denaturation status. A 1:10 (w/w) protease-to-substrate ratio, slightly higher than normal for tryptic digestion and denaturating conditions for at least an 8-h digestion, appears to be an appropriate combination to generate reproducible maps. As expected, the nondenatured protein and immunoconjugate were somewhat difficult to completely digest. Fig. 1 shows two typical CE maps of peptide fragments generated from the native MAb BR96 (Fig. 1A) and the BR96–DOX conjugate (Fig. 1B). The map obtained from a conjugated MAb BR96 appears to exhibit differences compared to that of the native MAb in some regions. This is not unexpected since the migrating mobility of DOX-conjugated peptides may differ from that of the original peptide fragments due to their charge status resulting from the attachment of the drug. The direct comparison of these peptide maps provides a fingerprint for monitoring the consistency of the conjugation process. This approach is also considered to be the basis for further structural analysis using other analytical techniques such as mass spectrometry

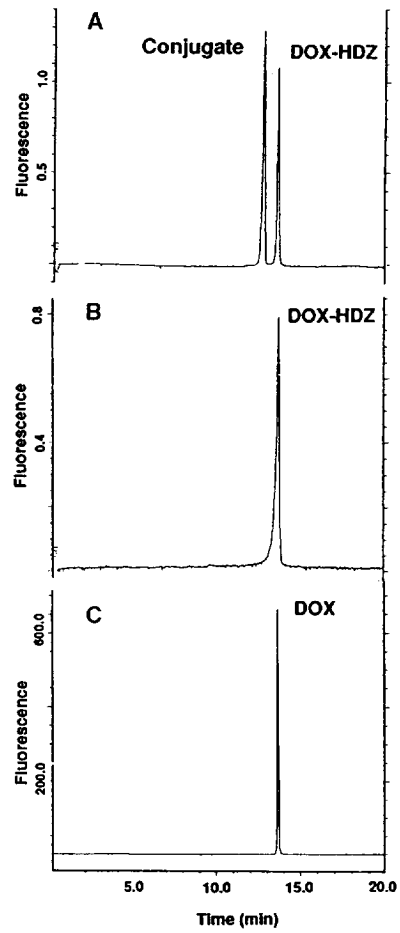

Fig. 3. Electropherograms of (A) a cystine-containing peptide (CGYGPKKKRKVGG) and doxorubicin conjugate, (B) doxorubicin hydrazone (DOX–HDZ), and (C) doxorubicin, separated by capillary electrophoresis with laser-induced fluorescence detection. Excitation wavelength, 488 nm; emission filter, 550 nm; capillary, 56.7 cm×75 μm I.D. (50 cm effective length); background electrolyte, 50 m*M* boric acid–50 m*M* SDS (pH 9.10); applied voltage, 20 kV.

[21]. One of the difficulties frequently encountered in peptide mapping is the complication of peptide fragments due to unexpected and non-specific cleavage and even enzyme autolysis. An alternative labeling approach and selective monitoring method may often simplify the mapping process.

MAbs are known to possess a wide range of reactive groups that can be utilized for coupling purposes. These functional groups generally involve the use of bifunctional crosslinkers, direct binding

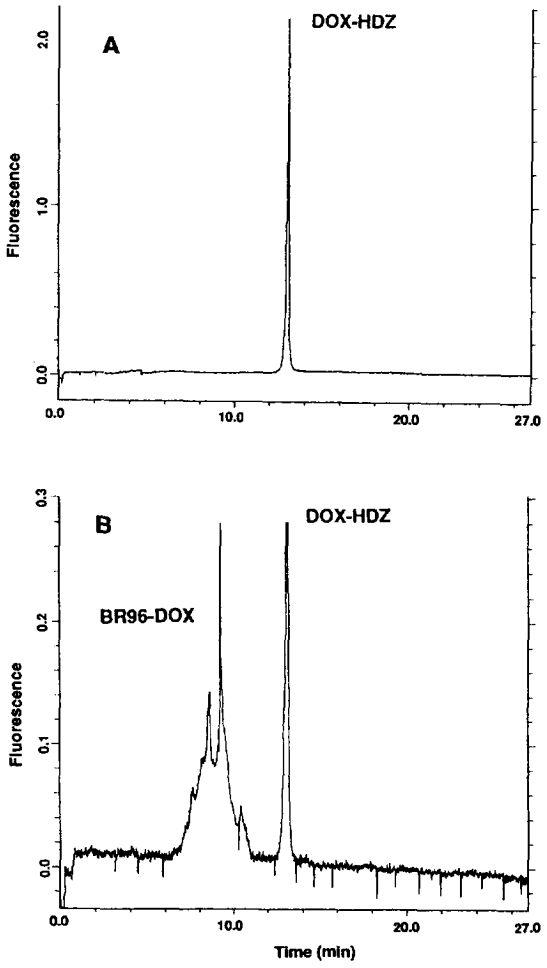

Fig. 4. Electropherograms of (A) doxorubicin hydrazone (DOX–HDZ) and (B) MAb BR96 and doxorubicin conjugate, separated by capillary electrophoresis with laser-induced fluorescence detection. Applied voltage, 25 kV. Other conditions as in Fig. 3.

via periodate oxidation to carbohydrate moieties, and reduction of existing disulfide bridges of the antibody to expose free sulfhydroxyl groups. In the current studies, the MAb chimeric BR96–DOX immunoconjugate was constructed such that DOX was incorporated via a thioether linker utilizing free thiol-containing groups on the MAb after reduction with dithiothereitol and an acid-labile hydrazone bond [7] as displayed in Fig. 2. Detecting the conjugated peptides upon digestion is selectively straightforward since DOX can be readily detected with our laser-based fluorescence system.

Previous studies dealing with DOX indicated that this compound exhibits a strong native fluorescent emission at a wavelength of 550 nm with the application of an excitation at wavelength 490 nm. This specific fluorescent characteristic of MAb BR96–DOX conjugate provides a useful diagnostic mechanism to locate those peptides with DOX following enzymatic digestion. To take advantage of this native fluorescence, an argon-ion-based laser system was employed with a fixed excitation wavelength of 488 nm with the emission wavelength set at 550 nm for monitoring DOX attached peptides.

A model cystine containing peptide, CGYGPKKKRKVGG, was profiled/mapped with respect to the conjugation process and subsequent development of detection methodologies. The peptide conjugate with DOX was formed under moderate pH conditions (pH 6.80). Lower pH, especially with acidic conditions, will result in drug release from the conjugate. As shown in Fig. 3, the conjugated peptide has been identified as the first peak (Fig. 3A) at a migration time of 12.8 min and the peak at 13.6 min indicated the unreacted DOX–HDZ while Fig. 3B indicates the drug–linker complex and Fig. 3C indicates the original drug. DOX has the same migration behavior as DOX–HDZ since they are generally neutral species under moderate pH conditions. We have observed the decrease in fluorescent intensity of the first peak (Fig. 3A) while the second peak increases. This phenomenon is most likely due to the release of free DOX from the peptide conjugate after storage for a period of time which further supports the existence of the peptide conjugate. The sensitivity has been determined to be about 30.0 attomole injected for detecting DOX–HDZ with a concentration of $2.4 \cdot 10^{-9}$ *M*.

Similar results were also obtained with the entire antibody BR96 as shown in Fig. 4. The disulfide bridges which hold the light and heavy chains together in the Fab and hinge regions of the immunoconjugate were reduced via dithiothereitol (DTT) to generate free thiol groups for subsequent conjugation. The drug–antibody conjugate is actually held together by hydrophobic forces between the light and heavy chains. The free DOX–HDZ was clearly separated from intact BR96 conjugate, where the resulting broad peaks are most likely due to the existence of glycoforms associated with the antibody, which were not well separated under current

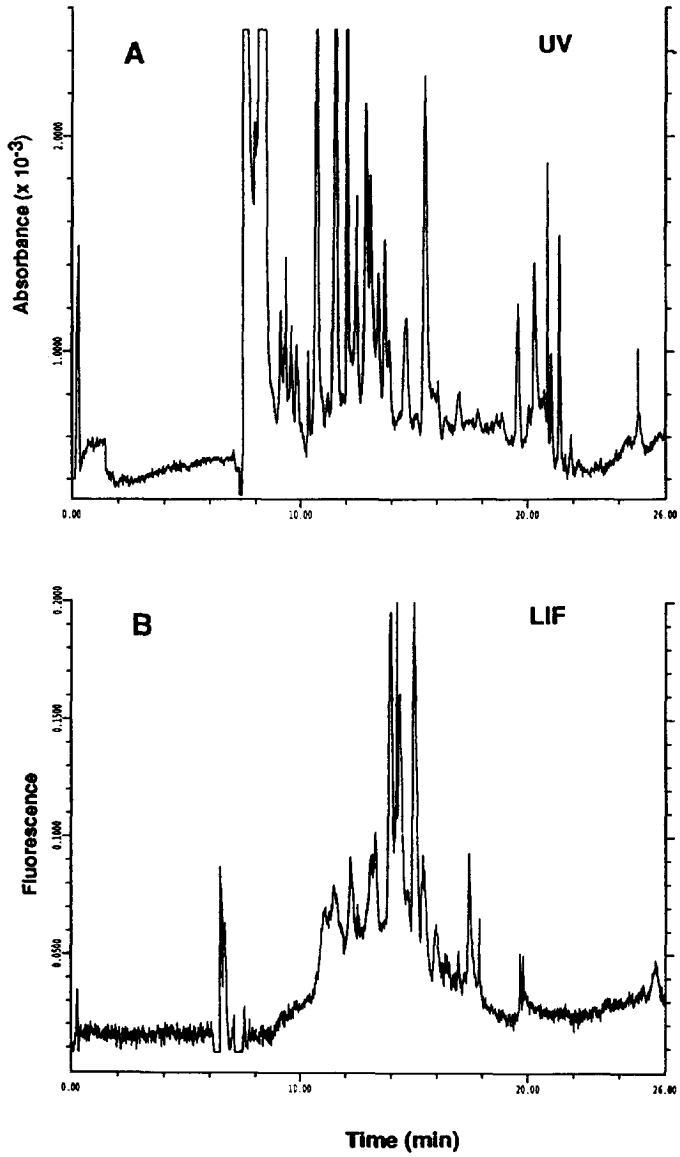

Fig. 5. Comparison of tryptic digests of a MAb BR96–DOX conjugate separated by capillary electrophoresis with (A) UV at wavelength of 214 nm and (B) laser-induced fluorescence detection. Capillary, 75 μm×57.4 cm (50.7 cm effective separation length); background electrolyte, 20 m*M* phosphate–20 m*M* borate–50 m*M* SDS (pH 8.5); applied voltage, 15 kV.

experimental conditions. It is not uncommon to observe broad CE peaks especially for large molecules such as antibodies and other macrobiomolecules. The heterogeneity of carbohydrate moiety in glycoproteins further complicates the CE separations. The complete resolution of these glycoforms is generally difficult due to the insignificant contribution of the carbohydrate moieties to the charge and size of the entire antibody molecule. Other CE modes such as capillary gel electrophoresis and capillary isoelectric focusing may be an alternative approach to enhance the CE resolving power.

With the methodologies described above, the determination of drug-containing peptides can be

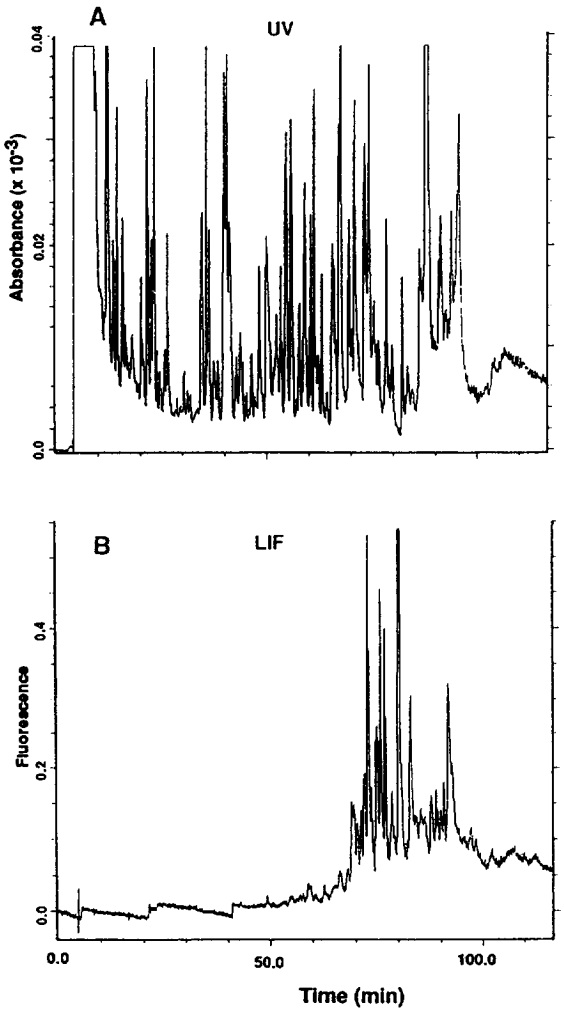

Fig. 6. Comparison of tryptic digests of a MAb BR96–DOXO conjugate separated by capillary LC with on-line UV (A) at 214 nm and laser-induced fluorescence (B) detection. Capillary column, 254 μm I.D. packed with C_{18} (5 μm, 300 Å); solvent gradient, 0–60% solvent B over 120 min.

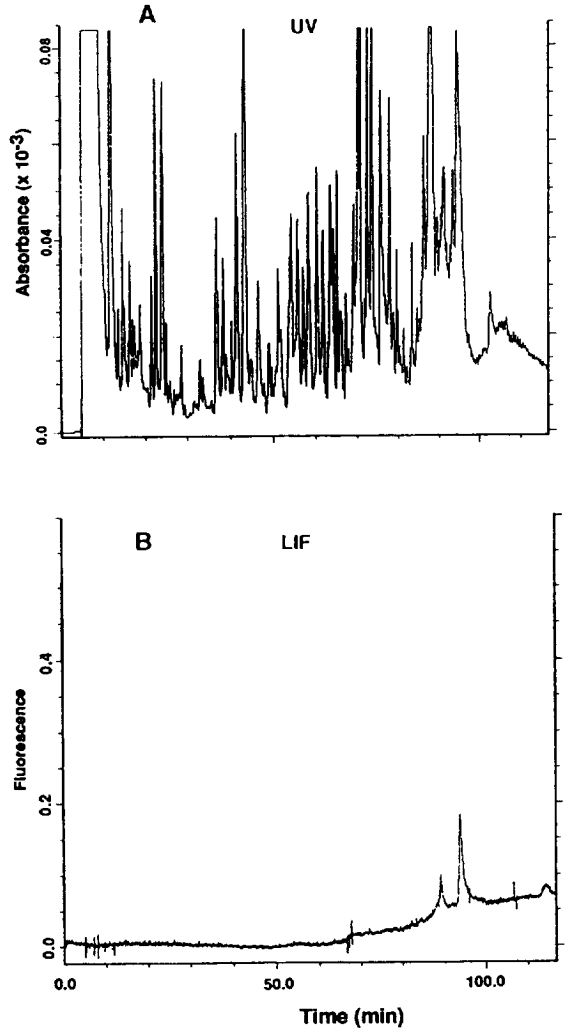

Fig. 7. Comparison of tryptic digests of a MAb BR96 separated by capillary LC with on-line UV (A) at 214 nm and laser-induced fluorescence (B) detection. Capillary column, 254 μm I.D. packed with C_{18} (5 μm, 300 Å); solvent gradient, 0–60% solvent B over 120 min.

accomplished by direct comparison of the peptide maps acquired from tryptic digests of the BR96–drug conjugate with both UV absorbance and LIF detection. Fig. 5 demonstrates the peptide mapping of the immunoconjugate using this approach. Use of the LIF detector revealed all conjugated components, as shown in Fig. 5B, corresponding to those peptide fragments linked with the drug from the BR96–DOX digest as compared to the UV trace displayed in Fig. 5A. Peptide fragments without DOX attachment will not be detected by LIF. These results demonstrate an alternative approach for simple, fast, and especially, the highly sensitive analysis of immunoconjugate and related compounds. It should be mentioned that with our current CE system configuration, UV and LIF detection was limited to alternate use only instead of dual approach. Multiple injections are generally needed to generate reproducible and comparable UV and LIF maps.

To extend the detection capability of the laser-

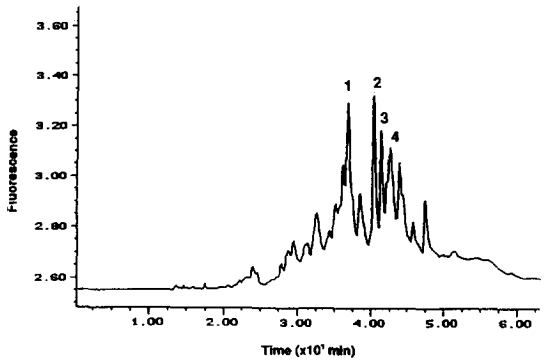

Fig. 8. Chromatogram of a BR96–DOX conjugate digest with fluorescence detection. Excitation wavelength, 495 nm; emission wavelength, 550 nm. HPLC separation was performed on Vydac RP-C_{18} column with ammonium formate–acetonitrile gradient elution. Fractions were collected for subsequent CE analysis.

based CE system, an in-house capillary LC system has also been successfully coupled to the CE–LIF unit, allowing on-line UV and laser detection. Similar to the CE maps described previously, Fig. 6 shows the upper UV trace from a BR96–drug conjugate digest (Fig. 6A), indicating all possible tryptic fragments including those peptides conjugated with DOX, and the lower LIF trace exhibits only those DOX-containing peptide fragments which yield fluorescent emission at 550 nm. These two maps were acquired simultaneously with a single injection assuring an accurate mapping process. As a comparison, the separation and detection of native MAb BR96 digest with both UV (Fig. 7A) and LIF (Fig. 7B) on-line detection was also conducted. As expected, no evidence of conjugated species was indicated with LIF detection although the UV chro-

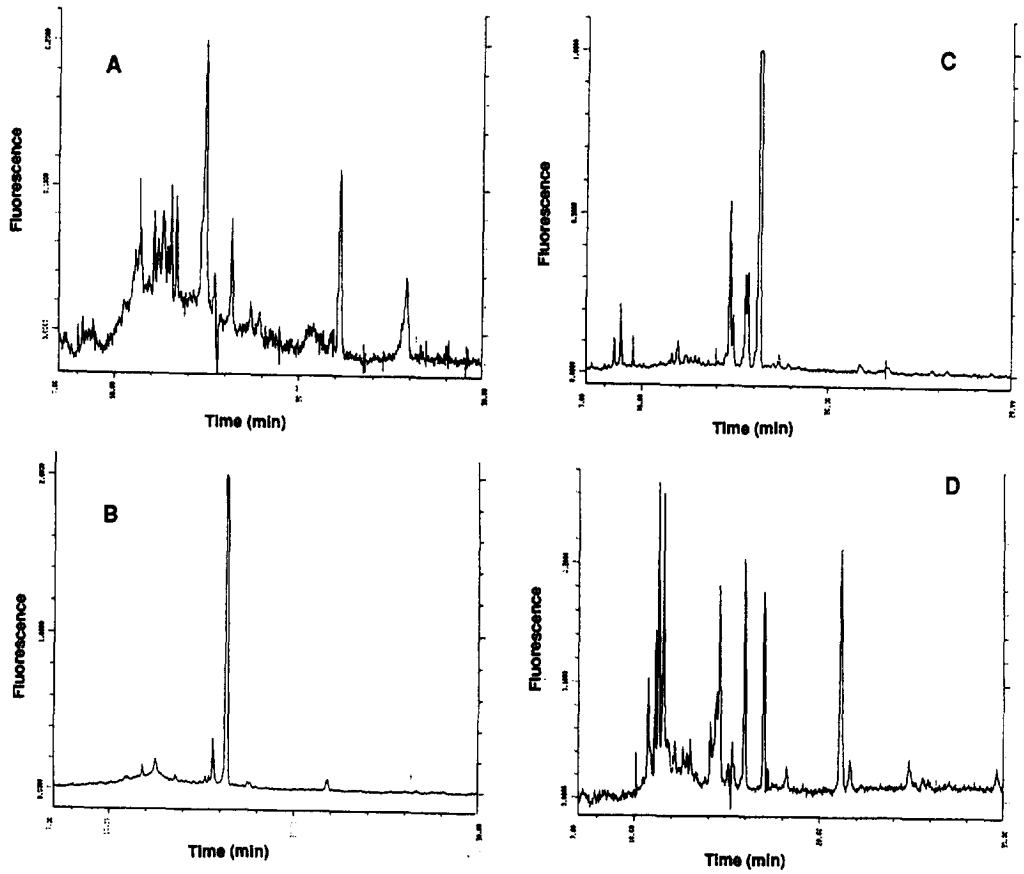

Fig. 9. Capillary electrophoretic separations of selected peptide fragments from HPLC fractions with LIF detection. Each electropherogram indicated as A, B, C, and D was from a single HPLC peak detected with conventional fluorescence. Experimental conditions as in Fig. 5.

matogram is similar to that generated from conjugate.

A multidimensional approach for fine-tuning the analysis of drug-containing peptides can be implemented with the use of CE as additional dimension for the separation of LC fractions detected with fluorescence. Fig. 8 shows a LC chromatogram obtained from tryptic digest of a BR96–DOX conjugate. The mapping profile was monitored with fluorescence detection, indicating those peptide fragments conjugated with the drug. LC fraction with different retention times were collected for subsequent CE analysis. Various CE separation patterns were obtained and some representative electropherograms are displayed in Fig. 9, where the LC fractions were further separated by CE and detected with LIF. Electropherograms A, B, C, and D in Fig. 9 were obtained from their corresponding peaks 1, 2, 3, and 4, respectively, in Fig. 8. It is interesting to note that more conjugated peptide fragments were observed for some of the LC fractions. The complexity of these CE separation patterns is most likely due to the complex mixture generated from either partial or over-digestion of the conjugate, which conventional LC failed to resolve. With enhanced resolving power, those peptides with DOX attached were clearly separated and detected by CE–LIF. This approach would further simplify the analysis and identification of the interesting fragment species, especially when combining CE with other analytical tools such as mass spectrometry. This methodology may provide an alternate and useful approach for fingerprint mapping of the complex conjugate digests.

The comparative peptide mapping approaches demonstrated in this studies provide useful information for the study of monoclonal antibodies and their corresponding immunoconjugates. This unique instrumental configuration is highly sensitive and selective and also holds tremendous potential for other biological applications. In addition, the current configuration can be further combined with electrospray mass spectrometry to provide mass-selective analysis, structural characterization, and confirmation via profiling protocols which will be reported elsewhere.

References

[1] G.A. Pietersz, Bioconjugate Chem., 1 (1990) 89.
[2] H.M. Yang and R.A. Reisfeld, Proc. Natl. Acad. Sci. USA, 85 (1988) 1189.
[3] P.A. Trail, D. Willner, S.J. Lasch, A.J. Henderson, R.S. Greenfield, D. King, M.E. Zoeckler and G.R. Braslawsky, Cancer Res., 52 (1992) 5693.
[4] R.M. Sharkey, R.D. Blumenthal, H.J. Hansen and D.M. Goldenberg, Cancer Res., 50 (1990) 964.
[5] I. Pastan, M.C. Willinggham and D.J. Fitzgerald, Cell, 47 (1986) 641.
[6] P.N. Friedman, D.F. Chace, P.A. Trail and C.B. Siegall, J. Immunol., 150 (1993) 3054.
[7] P.A. Trail, D. Willner, S.J. Lasch, A.J. Henderson, S. Hofstead, A.M. Casazza, R.A. Firestone, I. Hellström and K.E. Hellström, Science, 261 (1993) 212.
[8] H. Zhao, D. Willner, J.C. Cleaveland, G.R. Braslawsky and J.P. Brown, Bioconjugate Chem., 3 (1992) 549.
[9] R.C. Chloupek, R.J. Harris, C.K. Leonard, R.G. Keck, B.A. Keyt, M.W. Spellman, A.J.S. Jones and W.S. Hancock, J. Chromatogr., 463 (1989) 375.
[10] R.L. Garnick, N.J. Solli and P.A. Papa, Anal. Chem., 60 (1988) 2546.
[11] N. Takahashi, Y. Takahashi, N. Ishioka, B.S. Blumberg and F.W. Putnam, J. Chromatogr., 359 (1988) 181.
[12] S. Borman, Anal. Chem., 59 (1987) 969A.
[13] R.J. Harris, S.M. Chamow, T.J. Gregory and M.W. Spellman, Eur. J. Biochem., 188 (1990) 291.
[14] M.V. Novotny, J. Microcolumn Sep., 2 (1990) 7.
[15] M.V. Novonty, K.A. Cobb and J. Liu, Electrophoresis, 11 (1990) 735.
[16] B.L. Karger, A.S. Cohen and A. Guttman, J. Chromatogr., 492 (1989) 585.
[17] Z. Deyl and R. Struzinski, J. Chromatogr., 569 (1991) 63.
[18] G.F. Lee and D.C. Anderson, Bioconjugate Chem., 2 (1991) 367.
[19] W.S. Hancock, Chromatogr. Forum, 1 (1986) 57.
[20] K.A. Cobb and M.V. Novotny, Anal. Chem., 61 (1989) 2226.
[21] J. Liu, K.J. Kevin, E.H. Kerns, S.E. Klohr, M.S. Lee and I.E. Rosenberg, J. Chromatogr., 632 (1993) 45.
[22] I. Hellström, H.J. Garrigues, U. Garrigues and K.E. Hellström, Cancer Res., 57 (1990) 2183.
[23] H.P. Fell, S. Yarnold, I. Hellström, K.E. Hellström and K.R. Folger, Proc. Natl. Acad. Sci. USA, 86 (1989) 8507.