Journal of *Chromatography*, 635 (1993) 313-318
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 904

Capillary micellar electrokinetic, sequential multiwavelength chromatographic characterization of a chimeric monoclonal antibody-cytotoxin conjugate

David Emlyn Hughes* and Priscilla Richberg

Bristol-Myers Squibb Company, Pharamaceutical Research Institute, P.O. Box 4755, Syracuse, NY 13221-4755 (USA)

(First received November 17th, 1992; revised manuscript received January 25th, 1993)

ABSTRACT

An analysis of a doxorubicin-conjugated chimeric antibody has been developed by micellar electrokinetic capillary chromatography. The procedure separates the conjugated antibody, the antibody light and heavy chains, antibody fragments, and unconjugated doxorubicin. The protein-derived species are monitored at 280 nm and the doxorubicin bearing species are monitored at 500 nm. Consistency with the proposed *in vivo* mechanism of action of the antibody conjugate in acid media is demonstrated.

INTRODUCTION

The origin of interest in antitumor agents of the anthracycline class (which doxorubicin is a member) began at least as early as the 1950s. Liquid extraction of soil samples collected in India yielded a red solid which was shown to exhibit antitumor activity [1] and was traced to a strain of ***Streptomyces.*** Later work indicated that the microbial metabolite of interest was rhodomycin B which did not improve survival times ***in vitro*** testing but did generate interest in anthracycline anticancer agents [2]. Further studies yielded other anthracycline compounds from ***Streptomyces peucetius*** colonies; in particular, S. ***peucetius*** var. ***caesius*** was found to produce doxorubicin, an antibiotic with potent antitumor activity [3].

While chemotherapy with compounds such as doxorubicin continues to be a major therapeutic approach, there are important limitations. Antineoplastic agents are usually toxic to normal cells which reproduce rapidly; in particular, cells contained in the gastrointestinal tract and bone marrow are susceptible. Chemotherapeutic agents, when traditionally administered, are limited by their systemic toxicity.

Delivery of a cytotoxic agent to the site of the cancerous cells allows high local concentrations of the drug while minimizing toxicity to normal cells. One approach is to synthesize "immunotoxins," ***i.e.,*** IgG antibodies (conjugated with a cytotoxic agent) with a preference or specificity towards tumor cells. Antibodies conjugated with doxorubicin have previously been synthesized via dextran hydrazide-glutaraldehyde derivatization and periodate-oxidized dextran bridges. Although these conjugates were somewhat effective using ***in vivo*** and ***in vitro*** targeting models and yielded locally high doxorubicin concentrations, the cytotoxic activity was modest [4,5].

Other antibody-doxorubicin linkers that have been investigated include N-succinimidyl-3-(2-pyridyldithio) propionate [6] and more recently an acid-labile, 13-acylhydrazone [7]. Although

* Corresponding author.

the overall mechanism of action for 13-acylhydrazone is unclear, it has been postulated that internalization of conjugates into intracellular acidic compartments (such as lysosomes) may release doxorubicin and hence explain the observed cytolytic activity. The structure of doxorubicin and the antibody complex appear in Fig. 1.

The examination of an IgG antibody or antibody-drug conjugate represents a substantial analytical challenge. With an average molecular mass of 150000 and the likelihood of several forms varying only slightly in amino acid and carbohydrate content, examinations based on molecular mass differences alone would not be expected to be useful. Relatively new separatory procedures, principally size-exclusion, ion-exchange, affinity, hydrophobic interaction and hydroxyapatite chromatography, have proven useful for antibodies [8]. Of these liquid chromatographic techniques, ion-exchange chromatography is possibly the most selective procedure for large proteins (such as antibodies), perhaps implying that charge-dependent separations show substantial promise. Although capillary electrophoresis (CE) (which separates species on the basis of molecular mass and net charge) has been more consistently used for analysis of peptides, proteins such as adrenocorticotropin, transferrin, ribonuclease, insulin and growth hormone [9,10] have also been examined. Importantly, protein studies performed by CE have been shown to be able to separate the major species from species arising from degradative deamidation and amino acid and glycan microheterogeneity [11]. Although CE studies on antibodies are not abundant, one combined theoretical and empirical study [12] demonstrated that a single amino acid difference in a chimeric IgG antibody was detectable. Analysis of conjugated antibodies is substantially more complex. An ideal separating procedure for an antibody conjugate would allow examination of the unconjugated antibody, the agent which is to be complexed with the antibody, and the conjugated antibody. In one study [13], a CE procedure was developed that was capable of separating IgG monoclonal antibody (from mouse ascites), alkaline phosphatase (M_r 140 000), and an alkaline phosphatase-IgG conjugate.

The paper presented here describes the CE examination of a chimeric antibody conjugated with the cytotoxic agent doxorubicin. The conjugated forms are monitored at 280 nm to observe the proteinaceous component of the complex and at 500 nm to determine the extent of doxorubicin conjugation. Method development optimization studies are presented for the sodium dodecyl sulfate (SDS) concentration, sodium borate concentration and pH of the carrier. Selectivity with respect to the heavy and light antibody chains and the F_{ab} antibody fragment is demonstrated.

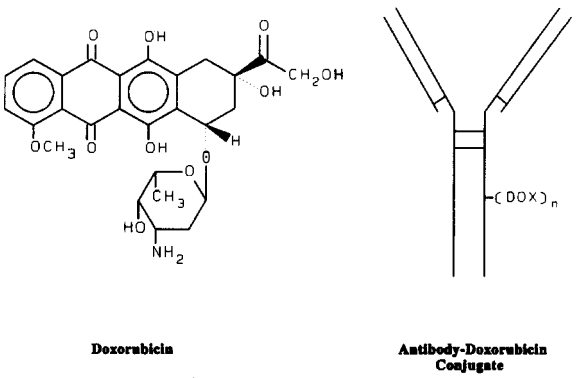


Fig. 1. Chemical structure of doxorubicin and a representation of the structure of the chimeric antibody-doxorubicin conjugate.

EXPEFUMENTAL

Materials

Sodium hydroxide 10 *M* solution and sodium borate crystals were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Dodecyl sulfate, sodium salt was purchased from Aldrich (Milwaukee, WI, USA). Pretreated capillary cartridges were purchased from Beckman Instruments (Palo Alto, CA, USA). The samples were chimeric antibody conjugates (Bristol-Myers Squibb, Syracuse, NY, USA). Water from a Milli-Q filtration system (Millipore, Bedford, MA, USA) was used in the preparation of the buffer solutions.

Capillary electrophoresis system

A Beckman P/ACE 2100 capillary electrophoresis system (Palo Alto, CA, USA) using an IBM PS/2 with P/ACE software and Microsoft Windows interface was used throughout this study.

Capillary electrophoresis procedures

The CE separation was performed using a 12 m*M* borate buffer (pH 9.4) containing 25 m*M* sodium dodecyl sulfate. Samples were transferred to P/ACE micro vials contained in sample holders, and applied to the capillary as described below. Upon completion of each sample analysis, the capillary column was cleaned with a high-pressure rinse of 0.1 *M* sodium hydroxide solution for 0.5 min, followed by a high-pressure rinse of the separation buffer solution for 0.5 min.

A fused-silica capillary cartridge (50 cm × 75 μm I.D., Beckman Instruments, catalog number 338467, rinsed by the manufacturer with 0.1 *M* NaOH solution) was used in the separation. Samples were injected by a positive nitrogen pressure of $6.2 \cdot 10^5$ Pa (90 p.s.i.) for 5 s when 214-nm and 280-nm detection were employed and 10 s for 500-nm detection. The samples were then separated by a voltage of 30 kV at 25°C. The samples were monitored at 214, 280 and 500 nm.

The light and heavy chains were formed in excess of 95% purity by heating the antibody conjugate in 1% SDS at 90°C for 2 min. The F_{ab} fragment was produced by a standard papain digestion [14].

RESULTS AND DISCUSSION

When examined by procedures which rely at least in part on the total charge of the species of interest, IgG antibodies may be separated into several forms. Antibodies show variability due to heterogeneity in the amino acid sequence or carbohydrate (oligosaccharide) content or type, as well as forms due to degradation, such as deamidation. For drug-labelled antibodies, further variation is possible due to different drug–antibody conjugation ratios and conformations.

All of the major species described so far would have molecular masses (*ca.* 150 000) very nearly equal to that of an unconjugated, undegraded antibody. Hence, as was mentioned previously, mass-based methods of analysis (such as size-exclusion chromatography) would not be expected to distinguish between the various antibody-derived species. The proteinaceous species would, however, be expected to vary in net charge and hydrophobicity, hence providing a basis for separation.

Initial CE optimization studies with the chimeric antibody studied here suggested that an additional separator-y mechanism was required since net charge differences (and hence, temporal separation) between species was insufficient when the ionic strength and pH of the carrier were varied. The addition of SDS to the carrier in concentrations above the critical micellar concentration has been shown to aid separation of neutral or a similar net charge species with smaller molecules. The precise mechanism of separation when analytes of similar mass to SDS micelles such as the IgG antibody are electrophoresed is unclear. Whether separatory enhancement is due to SDS ion-pairing with hydrophobic protein regions or simply differential migration of negatively charged analyte through a zone of negatively charged micelles is not easily determined empirically. Independent of the precise mechanism, the conjugated antibody was found to separate into several species as the SDS concentration increased above the critical micellar concentration (*ca.* 8 m*M*). As in most CE applications, the pH and buffer concentration of the carrier were also found to be critical to the efficient separation of the various species. Method development studies were performed in which the SDS concentration, pH and sodium borate concentration were varied (Figs. 2-4). For these studies, the resolution equation $R_s = 16(t/w)^2$ was employed where R_s is the resolution, t is the migration time of the peak and w is the peak width measured at the baseline. The critical separation was determined to be between three early-migrating species (Fig. 5, peaks 1, 2 and 3) which were present in all samples of antibody conjugate examined and a thermally-induced peak (produced between peaks 2 and 3, Fig. 5). The optimized conditions were 12 m*M*

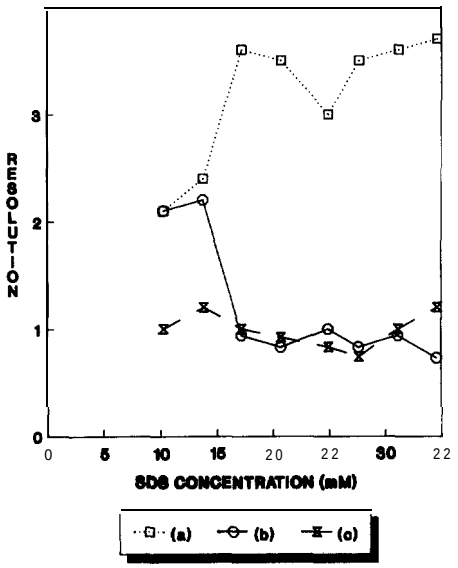


Fig. 2. Resolution of electrophoretic species as a function of SDS concentration at a concentration of 12 m*M* sodium borate and pH 9.4: (a) resolution between peaks 1 and 2, (b) resolution between peaks 2 and a thermally-induced species, and (c) resolution between peaks 2 and 3. Refer to Fig. 5 for peak identification. The thermally-induced peak, appearing between peaks 2 and 3 is not represented.

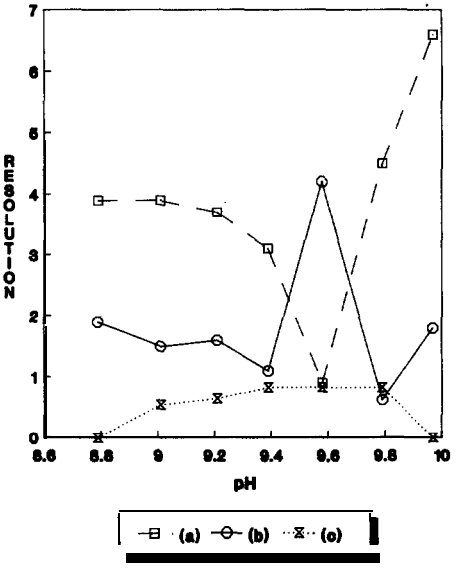


Fig. 3. Resolution of electrophoretic 'species as a function of pH at an SDS concentration of 25 m*M* and a borate concentration of 12 m*M*: (a) resolution between peaks 1 and 2, (b) resolution between peaks 2 and a thermally-induced species, and (c) resolution between peaks 2 and 3. Refer to Fig. 5 for peak identification. The thermally-induced peak, appearing between peaks 2 and 3 is not represented.

sodium borate, 25 m*M* SDS at a pH of 9.40. Although Figs. 2-4 would suggest that a higher borate concentration would produce a better separation, the antibody is not stable under these conditions and forms measurable amounts of light and heavy chains and antibody fragments.

The final separation appears in Fig. 5 with 280- and 500-nm detection. Detection at 280 nm would be expected to visualize all proteinaceous species whereas 500-nm detection would detect only those species containing doxorubicin. Referring to Fig. 5, the electropherogram represents three antibody conjugate species (1, 2, 3) and doxorubicin (7). Standard techniques for generating the light and heavy chains and the F_{ab} fragment were employed. The resulting species migrated in a third temporal zone, *i.e.*, F_{ab} (4), heavy chain (5) and light chain (6). Hence the separation appears to be capable of detecting degradation of the antibody conjugate to heavy and light chains and the F_{ab} fragment.

To determine whether the electrophoretic separation would be capable of demonstrating

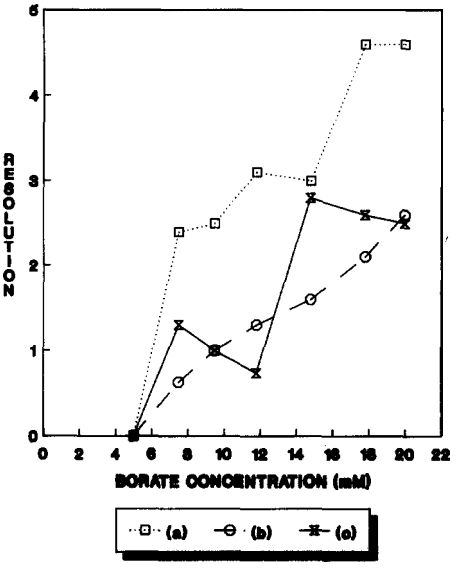
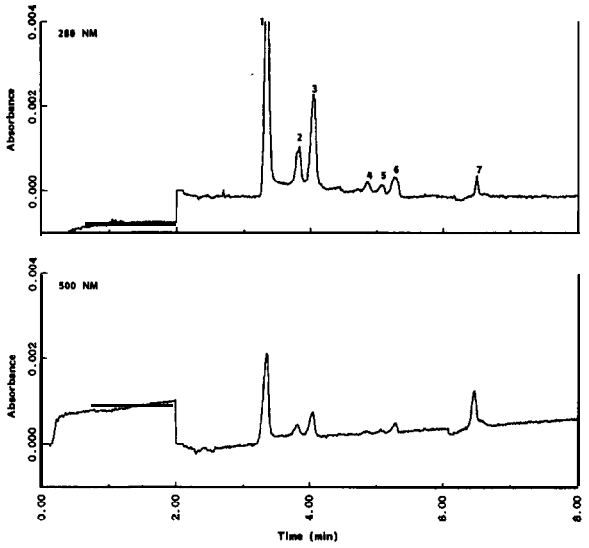


Fig. 4. Resolution of electrophoretic species as a function of sodium borate concentration at an SDS concentration of 25 m*M* and pH 9.4: (a) resolution between peaks 1 and 2, (b) resolution between peaks 2 and a thermally-induced species, and (c) resolution between peaks 2 and 3. Refer to Fig. 5 for peak identification. The thermally-induced peak, appearing between peaks 2 and 3 is not represented.



'Fig. 5. Electropherogram of the final separation (12 m*M* sodium borate, 25 m*M* SDS, pH 9.4) with detection at 280 nm and 500 nm. Peaks: 1, 2, 3 = antibody conjugate; 4 = F_{ab}; 5, 6 = heavy and light chains; 7 = doxorubicin.

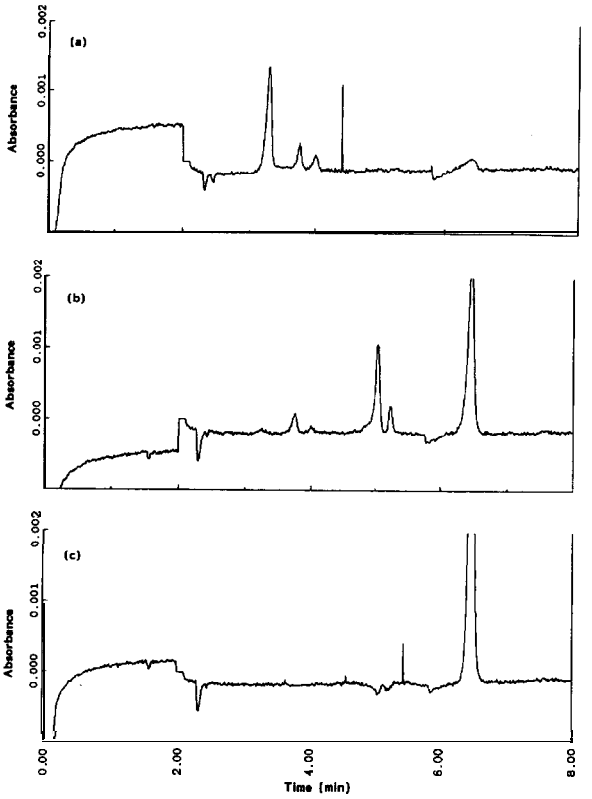


Fig. 6. Acid hydrolysis at pH 1.5 monitored at 500 nm. (a) Initial sample; (b) after 3 min; (c) after 15 min.

the mechanism of action which has been proposed *in vitro,* an acid-degradation study was performed. According to the proposed [7] mechanism, the chimeric antibody-doxorubicin conjugate releases unconjugated doxorubicin in the presence of acid media. If this transformation is monitored at 500 nm, which is selective for doxorubicin and doxorubicin-conjugated species, we would expect a decrease in absorbance of proteinaceous doxorubicin species and an increase in unconjugated doxorubicin. As Fig. 6 illustrates, CE examination of acid hydrolysis at a pH 1.5 is consistent with this model. The electropherogram of the initial sample (a) contains peaks for the conjugate and a low level of unconjugated doxorubicin whereas intermediate exposure to an acidic medium (b) yields predominately conjugated heavy and light chains and doxorubicin. After sufficient time, the antibody is no longer conjugated and substantial doxorubicin is observed (c). Studies were also performed at more biologically-assessable conditions (*ca.* pH 5) with similar but predictably less dramatic results.

In summary, a CE procedure has been developed which allows examination of a doxorubicin-linked chimeric antibody. The proteinaceous species are monitored at 280 nm and the doxorubicin-related species are monitored at 500 nm. The method separates three forms of the antibody, the light and heavy antibody chains, the F_{ab} fragment, and unconjugated doxorubicin. Monitoring of acid hydrolysis of the antibody conjugate revealed the formation of unbound doxorubicin and simultaneous loss of protein-bound doxorubicin in agreement with the proposed *in vivo* mechanism of action.

ACKNOWLEDGEMENTS

The authors acknowledge technical assistance in the antibody fragment selectivity studies by Lt. (jg) Christopher Kolanko, National Naval Medical Center, Bethesda, MD, USA. The authors also acknowledge valuable technical discussions with Barbara Root, David Kirkley, John

Woolever, and Kirk Leister, Bristol-Myers Squibb Company, Analytical Research and Development, Syracuse, NY, USA.

REFERENCES

1 F. Arcamone, A. DiMarco, M. Gaetani and T. Scotti, G. *Microbiol., 9* (1961) 83.
2 F. Arcamone, ***Doxorubicin Anticancer Antibiotics,*** Academic Press, New York, 1981, p. 17.
3 A. DiMarco, M. Gaetani and B.M. Scarpinato, Cancer *Chemother. Rep., 53* (1969) 33.
4 R.A. Reisfeld and S. Sell, ***Monoclonal Antibodies and Cancer Therapy,*** Alan R. Liss, New York, 1985, pp. 244-246, 254.
5 S. Sell and R.A. Reisfeld, ***Monoclonal Antibodies in Cancer,*** Humana Press, Clifton, NJ, 1985, p. 11.
6 G.L. Wright, Jr., ***Monoclonal Antibodies and Cancer,*** Marcel Dekker, New York, 1984, p. 197.
7 G.R. Braslawsky, K. Kadow, J. Knipe, K. McGoff, M. Edson, T. Kaneko and R.S. Greenfield, Cancer *Immunol. Immunother., 33* (1991) 367.
8 K.M. Gooding and F.E. Regnier, ***HPLC of Biological Macromolecules,*** Marcel Dekker, New York, 1990, pp. 487-528.
9 A. Guttman, A. Paulus, A.S. Cohen, B.L. Karger, H. Rodrigues and W.S. Hancock, ***Electrophoresis 88,*** VCH Publishers, New York, 1988, pp. 151–160.
10 P.D. Grossman, J.C. Colbum, H.H. Latter, R.G. Nielson, R.M. Riggin, G.S. Sittampalam and E.C. Rickard, *Anal. Chem., 61 (1989)* 1186.
11 S.-L. Wu, G. Teshima, J. Cacia and W.S. Hancock, *J. Chromatogr., 516* (1990) 115.
12 B.J. Compton, *J. Chromatogr., 559* (1991) 357.
13 S.J. Harrington, R. Varro and T.M. Li, *J. Chromatogr., 559* (1991) 385.
14 E. Harlow and D. Lane, ***Antibodies,*** Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, p. 628.