Journal of Chromatography, 559 (1991) 385–390
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 488

High-performance capillary electrophoresis as a fast in-process control method for enzyme-labelled monoclonal antibody conjugates

SARAH J. HARRINGTON*, RUDOLF VARRO and THOMAS M. LI
Syva Company, 900 Arastadero Road, M/S S3-3, Palo Alto, CA 94303 (USA)

ABSTRACT

High-performance capillary electrophoresis (HPCE) was evaluated as a method for characterizing enzyme–antibody conjugates. Alkaline phosphatase (AP) and immunoglobulin G (IgG), individually and in a mixture, were used as standards to identify the components of an AP–IgG conjugate separation. 0.5% Methyl cellulose was added to the running buffer to separate and identify the conjugate, unreacted AP and unreacted IgG. Due to its speed and resolution, HPCE appears to be a good candidate for in-process evaluations of conjugates for immunoassays.

INTRODUCTION

The purpose of this study was to evaluate high-performance capillary electrophoresis (HPCE) as a quality control instrument for the characterization of enzyme–antibody conjugates. The results of a conjugation process may include unwanted material such as unreacted antibody and enzyme, and protein aggregates. The conjugate is usually purified using liquid chromatography after the conjugation procedure [1].

This study utilized capillary zone electrophoresis (CZE) for the separation of proteins. The separation mechanism of CZE is based on electrophoretic mobilities of the sample components which are affected by solvent characteristics including pH, ionic strength, and viscosity.

Alkaline phosphatase (AP) and immunoglobulin G (IgG) were the enzyme and antibody compounds to be conjugated. The relative molecular mass of AP is very close to that of IgG. Their mass–charge ratios may also be similar making CZE separation of an unreacted mixture of AP and IgG difficult. A linear polymer, methyl cellulose, was introduced into the running buffer to facilitate separation. Methyl cellulose (MC) has been previously added to HPCE buffers to enhance separations [2], but it has not been reported for monoclonal antibody separations or as an in-process method for diagnostic product development. HPCE could be used as a quick procedure for evaluating the success of the conjugation (independent of immunoassay performance).

0021-9673/91/$03.50

EXPERIMENTAL

Materials

The Beckman P/ACE 2000 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. IgG monoclonal antibody (produced in-house) from mouse ascites (3G1, IgG_1 subclass) and alkaline phosphatase (from calf intestine, lot 12056526–08, Boehringer Mannheim, Mannheim, Germany) were used as standards and in the conjugation procedure. Methyl cellulose (15 cP at 2% solution in water; Aldrich, Milwaukee, WI, USA) was used as an additive in the running buffer to create a molecular sieve [2].

The sample buffer was 0.1 *M* borax with 0.5 m*M* sodium dodecyl sulfate (SDS) at pH 10. At this pH, all proteins are negatively charged and are repelled from the wall of the uncoated capillary [3]. The running buffer consisted of 0.5% MC in 0.1 *M* borax with 0.5 m*M* S.D.S. (pH 10).

Methods

Samples were injected using a one second pressure injection into a 27 cm × 75 μm I.D. capillary. The proteins were separated at a current of 5 kV and the temperature was reduced to 15°C. A 280-nm wavelength detector filter was used.

At the beginning of each day, the capillary was pre-rinsed for 5 min. After separation, post-run rinses were used to clean the capillary. This procedure included a 1-min sodium hydroxide rinse, followed by a 1-min water rinse, and a final 3 min rinse using the running buffer.

The IgG was thiolated with S-acetylthioglycolic acid N-hydroxysuccinimide ester (Sigma, St. Louis, MO, USA). The AP was maleimidated with succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL, USA). The conjugate was prepared by reacting the thiolated IgG with maleimidated alkaline phosphatase. This method [4] produces a conjugate consisting of one enzyme and one antibody molecule.

IgG (160 000 relative molecular mass) and AP (140 000 relative molecular mass) were tested alone and in equal amounts as a standard mixture. The electropherogram of the standard AP + IgG mixure was compared to the migration pattern of the conjugate.

The conjugate was tested before and after purification by fast protein liquid chromatography (FPLC) using a Superose 6 column (Pharmacia LKB, Piscataway, NJ, USA). The purification procedure separates the conjugate from unreacted IgG, unreacted AP and from large protein aggregates.

After the conjugate was purified by FPLC, absorbance readings at 280 nm (Perkin-Elmer Lambda 4B spectrophotometer, Norwalk, CT, USA) were cross-matched with enzyme-linked immunosorbent assay (ELISA) results to test for the location of the conjugate fractions. Based on ELISA and absorbance readings, two different fractions selected from the FPLC separation were tested. One fraction represented the main conjugate peak, while another fraction consisted mostly of unreacted IgG and AP.

RESULTS

Figs. 1 and 2 show the electropherograms of IgG and AP, respectively. The migration time for IgG was 4 min as shown in Fig. 1, while AP has a longer migration time of 4.5 min (Fig. 2). The small peak prior to the first major peak (found in all

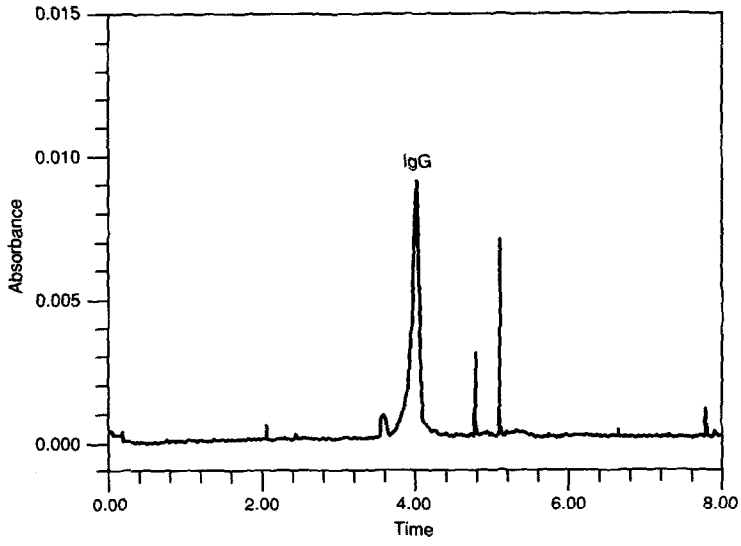

Fig. 1. Analysis of IgG (160 000 relative molecular mass) in 0.5% methyl cellulose in 0.1 *M* borax buffer in 0.5 m*M* SDS at pH 10. Conditions as given under Experimental. For all figures, time (*x*-axis) is in min, and absorbance (*y*-axis) is at 280 nm.

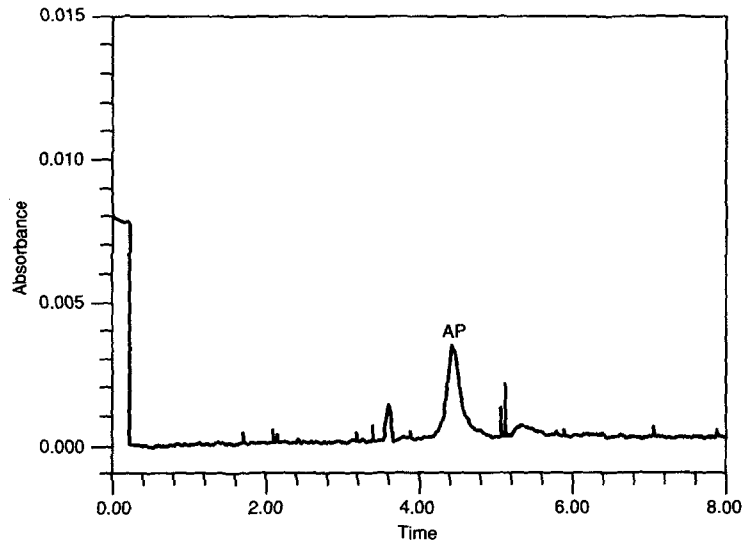

Fig. 2. HPCE of alkaline phosphatase (AP). Relative molecular mass is 140 000. Conditions are as in Fig. 1.

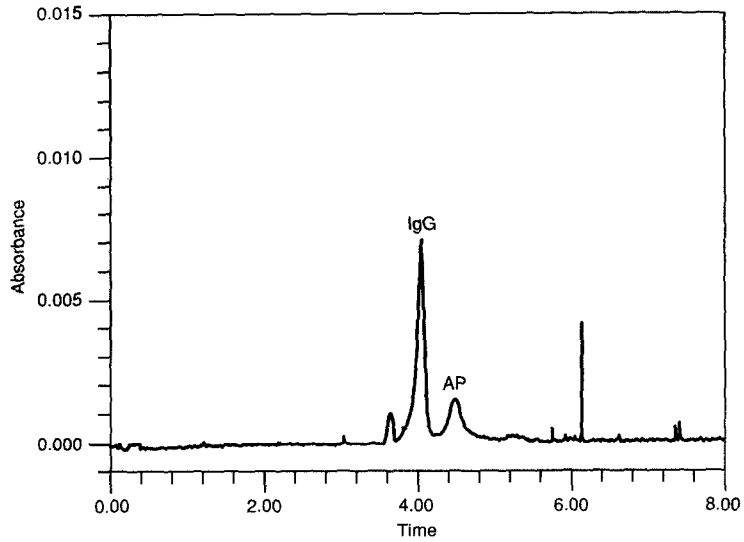

Fig. 3. Separation of the standard mixture of AP+IgG (1:1).

figures) may be due to a refractive index change caused by the front edge of the sample buffer.

By comparing the AP + IgG standard mix in Fig. 3 to the individual runs (Figs. 1 and 2), the first major peak can be identified as IgG (4 min) and the second major peak is AP (4.5 min). The electropherogram of the AP + IgG standard mix was a single peak when MC was not added to the running buffer (data not shown).

Fig. 3 can also be used to identify the components of the unpurified conjugate in

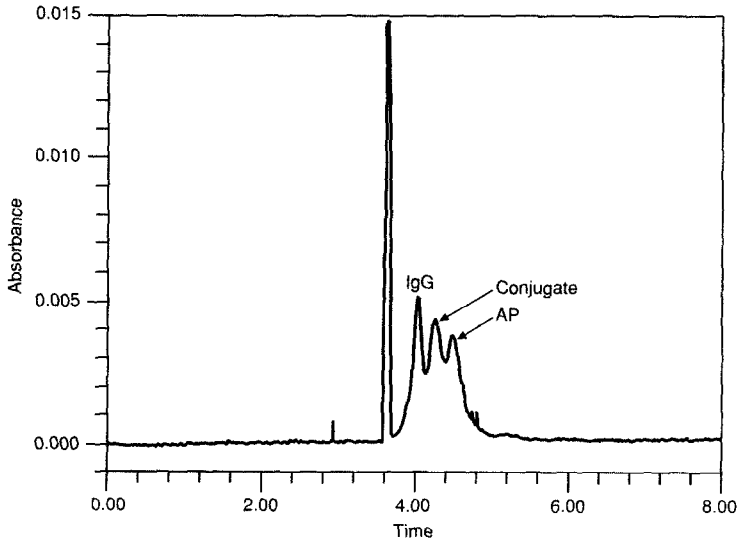

Fig. 4. Separation of unpurified AP–IgG conjugate containing the conjugate and unreacted AP and IgG.

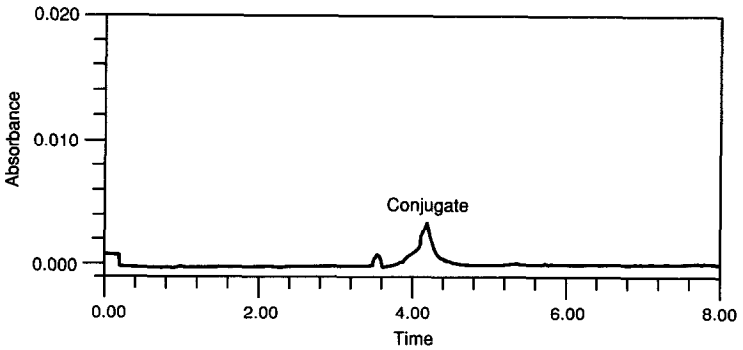

Fig. 5. Electropherogram of a sample from the FPLC purification of AP–IgG conjugate. Represents the fraction with the highest concentration of conjugate.

Fig. 4. Of the three main peaks in Fig. 4, the AP − IgG conjugate appears to be the middle peak located between the unreacted AP and IgG.

The results of the ELISA and absorbance readings correlated well with the migration times of samples from the FPLC fractions obtained during the purification of the conjugate. The electropherogram of a sample containing the main conjugate peak is shown in Fig. 5. This profile can be compared to the one in Fig. 6 showing a sample from a FPLC fraction containing unreacted AP and IgG.

CONCLUSIONS

The high resolution capabilities of HPCE were demonstrated with the separation and identification of components of a conjugate mix and the purified conjugate fractions. Using MC in the running buffer, separation of proteins with similar mass–charge ratios and relative molecular masses was possible. The electropherogram of the conjugate mix (Fig. 4) reveals a triple peak separation. The AP–IgG conjugate was identified as the middle peak between the smaller, unreacted conjugate components. Therefore, MC seems to improve resolution between compounds with similar mass–charge ratios by decreasing electroosmotic flow and enhancing differences in electrophoretic mobilities.

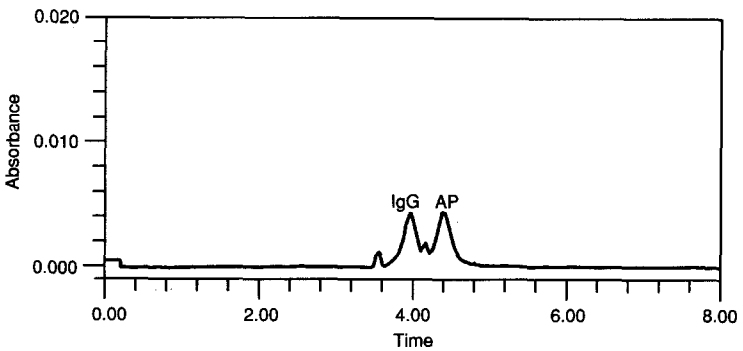

Fig. 6. Profile of a sample from the FPLC purification of AP–IgG conjugate. Fraction primarily contains unreacted AP and IgG.

Along with MC, other factors, such as temperature and SDS concentration enhanced resolution. Decreasing the temperature to 15°C increases migration times, but may also increase resolution.

The capillary electrophoresis system was able to verify the success of the conjugate preparation and purification procedure. Due to its speed and resolution, HPCE appears to be a good candidate for in-process evaluations of conjugates for immunoassays.

ACKNOWLEDGEMENTS

We would like to thank Beckman Instruments for the loan of the P/ACE 2000 capillary electrophoresis system used in this study.

REFERENCES

1 E. Ishikawa, M. Imagawa, S. Hashida, S. Yoshitake, Y. Hamaguchi, and T. Ueno, *J. Immunol.*, 4 (1983) 209.
2 M. Zhu, D. L. Hansen, S. Burd, and F. Gannon, *J. Chromatogr.*, 480 (1989) 311.
3 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
4 R. J. S. Duncan, P. D. Weston and R. Wrigglesworth, *Anal. Biochem.*, 132 (1983) 68.