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Journal of Chromatography B, 779 (2002) 157–162

JOURNAL OF CHROMATOGRAPHY B

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Non-competitive immunoassay for alpha-fetoprotein using micellar electrokinetic capillary chromatography and laser-induced fluorescence detection

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Received 29 January 2002; received in revised form 26 April 2002; accepted 13 May 2002

Abstract

A non-competitive immunoassay based on micellar electrokinetic capillary chromatography (MECC) with laser-induced fluorescence (LIF) detection has been developed for the determination of alpha-fetoprotein (AFP). The anti-AFP antibody was labeled with fluorescein isothiocyanate (FITC) and the product was used as a fluorescent tracer, then AFP was mixed with the labeled antibody. After incubation, the immune AFP–antibody complex was separated from labeled free antibody by MECC. The parameters affecting separation such as the concentration of sodium dodecyl sulfate (SDS), the buffer pH and separation voltage were investigated and the following conditions were selected: 20 m*M* tetraborate containing 100 m*M* SDS at pH 9.50, and 20 kV separation voltage. The detection limit of this assay was 0.1 ng/ml with a linear range spanning two orders of magnitude. This method was applied to determine AFP in human serum.
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Keywords: α-Fetoprotein

1. Introduction

Alpha-fetoprotein (AFP) is an oncofetal glycoprotein frequently used as a tumor marker for hepatocellular and yolk sac tumor. The concentration of serum AFP in healthy adults is very low, typically at the range of 4–13 ng/ml. But in a liver cancer patient, the AFP concentration can attain as high as 10 μg/ml [1]. For a pregnant woman, too high a concentration of AFP in the mother's serum is related to hereditary nephropathy and abnormality [2]. A method of high sensitivity and selectivity is thus greatly needed for the determination of AFP in biofluids such as serum.

Immunoassays are highly selective analytical techniques due to the specific reaction between an antigen and antibody. The high selectivity of immunoassays allows one to measure trace amounts of the compounds of interest in the presence of structurally- or chemically-related compounds [3]. Conventional immunoassays are usually performed using solid-phase techniques and quantification is achieved by measuring either the radioactivity as in radioimmunoassay (RIA) or enzyme-linked immunoabsor-

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PII: S1570-0232(02)00350-1

bent assay (ELISA) [4]. These immunoassays may take hours to complete and require larger sample volumes, in spite of the fact that they can be performed in parallel formats for high throughput. However, this is not desirable when rapid feedback is required.

Capillary electrophoresis (CE) is a powerful separation technique for many compounds, such as amino acids [5,6], drugs [7,8] and proteins [9,10]. The application of CE to protein samples is more difficult due to the adsorptions of sample components to the capillary wall, which affects the reproducibility of the assay. Many methods for overcoming this problem have been tried and one of the effective approaches is using SDS as additive [11]. CE-based immunoassays (CEIA) have also been widely explored because of the advantages of minimal reagent consumption, rapid separations, automation and the ability to simultaneously determine the presence of multiple analytes [12]. The laser-induced fluorescence detection (LIF) of CE allows for high sensitivity. CEIA with LIF combines the high separation efficiency of CE, the high selectivity of immunoassays and the high sensitivity of LIF detection. CEIA-LIF has been applied to a wide range of compounds including human serum albumin [13], hirudin [14], cyclosporine [15], quinines [16], etc. These articles are performed in a competitive format and the labeled antigen (or antibody) should be purified firstly before CE separation.

Compared to competitive immunoassays, noncompetitive assays are more simple and always have larger linear dynamic range. In this work, a noncompetitive immunoassay of MECC and LIF detection was developed for the determination of AFP. This developed method needs no purification process and the labeled free antibody and the immunocomplex could be resolved within 4 min. A good linear curve was obtained and the detection limit was about 0.1 ng/ml. The application of this method was demonstrated by using it to determine the AFP in human serum.

2. Experimental

2.1. Chemicals and reagents

Alpha-fetoprotein (AFP) was purchased from Golden Bridge International (Seattle, WA, USA) (as congelation and dissolved with a small amount of distilled water). Anti-AFP antibody (Rabbit anti-Alpha Fetoprotein) was bought from Beijing Zhongshan Biotechnology (2 μg/ml in PBS). The stock solutions of AFP and anti-AFP antibody were stored at 4 °C. Both fluorescein isothiocyanate (FITC, HPLC grade) and sodium dodecyl sulfate (SDS, electrophoresis reagent) were purchased from Sigma–Aldrich (Steinheim, Germany) and dissolved in 20 m*M* tetraborate. Other chemicals including buffer substances and supporting electrolytes were all of analytical reagent grade from Beijing Chemical Reagent Factory (Beijing, China) and used as received.

2.2. Apparatus

The electrophoretic separations were performed on a Beckman P/ACE 5500 Model CE system (Fullerton, CA, USA) fitted with an LIF detector. The 488-nm line of a 5 mW argon ion laser was utilized as exciting source and the emitted fluorescence collected at 520 nm. An uncoated fused-silica capillary of 57 cm length (7 cm effective length from the cathode to the detector) and 50 μm I.D. (Yongnian Optical Fiber Factory, Heibei, China) was used throughout with the temperature maintained at 20 °C. Samples were injected by voltage (−5 kV for 10 s) at the shorter end of the capillary and separated at −20 kV. To obtain good reproducibility, the capillary was rinsed with 100 m*M* SDS solution (in 20 m*M* tetraborate) for 1 min between two injections and the same buffer was used for separation. A personal computer was used to control the P/ACE instrument and perform data analysis with the P/ACE software (Beckman, USA). A pH-HJ90B pH meter (Aerospace Computer Company, Beijing, China) was used for the pH measurements.

2.3. Preparation of FITC-labeled anti-AFP antibody (Ab*)

The labeling reaction was initiated by the addition of three aliquots of 0.2 μg/ml anti-AFP antibody (diluted from store solution with 20 m*M* tetraborate) to one aliquot of FITC (dissolved in 20 m*M* tetraborate, 3.0×10^{-4} mol/l). The mixtures were then kept in darkness at room temperature overnight. This

labeled anti-AFP antibody was used without further purification.

2.4. Immune reaction of Ab* with AFP

The stock solution of AFP was diluted with PBS to suitable concentration, then 10 μl diluted AFP solution were mixed with 10 μl Ab* in a 0.5-ml centrifuge and incubated in a black box for a certain time before injection for MECC separation. For the calibration curve, 10 μl Ab* were mixed with 10 μl AFP concentrations from 0.5 to 100 ng/ml. Inter-assay variation was defined as the variation in the concentrations of AFP determined in five consecutive runs performed on the same day and intra-assay variation was defined as the variation in the concentrations of AFP determined in five runs performed on separate days.

2.5. Preparation of serum sample

The blood samples were collected from the arm veins of a healthy woman and stored in a clean centrifuge tube without anticoagulant. The blood samples were centrifuged for 20 min or put in air for 3 h to allow for its delamination, then the supernatants were removed to another centrifuge tube and stored in a refrigerator until analysis.

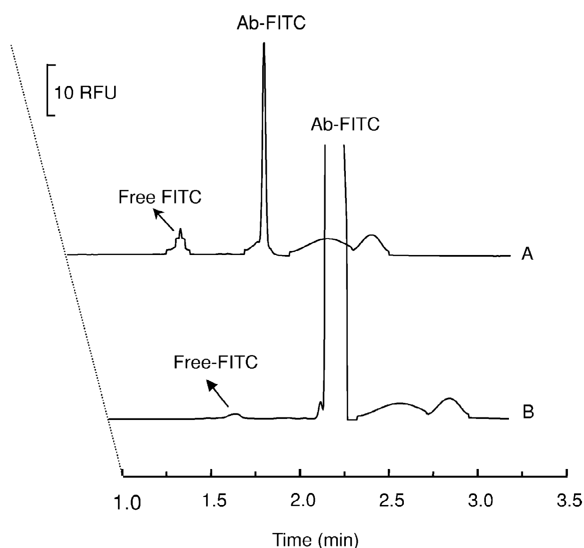

Fig. 1. Electropherograms of anti-AFP labeled with FITC. Samples: (A) 100 ng/ml and (B) 1 μg/ml anti-AFP labeled with FITC, respectively. Buffer: 20 m*M* tetraborate and 100 m*M* SDS at pH 9.30; injection: −5 kV, 10 s; applied voltage: −20 kV; capillary: 57 cm length (7 cm effective length), 50 μm I.D., 360 μm O.D., at 20 °C.

3. Results and discussion

3.1. Derivatization of anti-AFP antibody and its immuno-capacity with AFP

Anti-AFP was firstly diluted with water and labeled with FITC. But only anti-AFP of high concentration antibody (>1.0 μg/ml) could be labeled and detected. When the antibody was diluted by 20 m*M* tetraborate (pH 9.30), it was easily labeled with FITC and ng/ml concentrations of antibody could also be detected. Fig. 1 shows the electropherograms of labeled anti-AFP (Ab*) at different concentrations, which can also be used for the identification of Ab*.

Fig. 2 shows the electropherograms of Ab* with different amounts of AFP. It can be seen that the peak height of Ab* is remarkably reduced and a new peak appears when 30 ng/ml AFP is added to 75 ng/ml Ab*. The reduction of the Ab* peak height is due to the immunoreaction between Ab* and AFP, and the new peak is assigned to the Ab*–AFP complex. This is confirmed by the fact that the height of the new peak increases while, concomitantly, the Ab* peak height decreases as the concentration of AFP increases. When the AFP concentration is up to 80 ng/ml, the peak of Ab* disappears because all of the Ab* molecules may bind with AFP. These results indicated that the FITC-labeled anti-AFP had good immuno-capacity with AFP.

3.2. Effect of time on the immune reaction

The immunoreaction time of AFP and Ab* in our experiment was much shorter compared to that in conventional ELISA or RIA because the reaction occurred in the solution. The peak height of the immunocomplex increased rapidly with immune reaction time up to 10 min, then increased slowly to 40 min, and changed little after 40 min, so 40 min was selected as the suitable reaction time and was used in the later experiments.

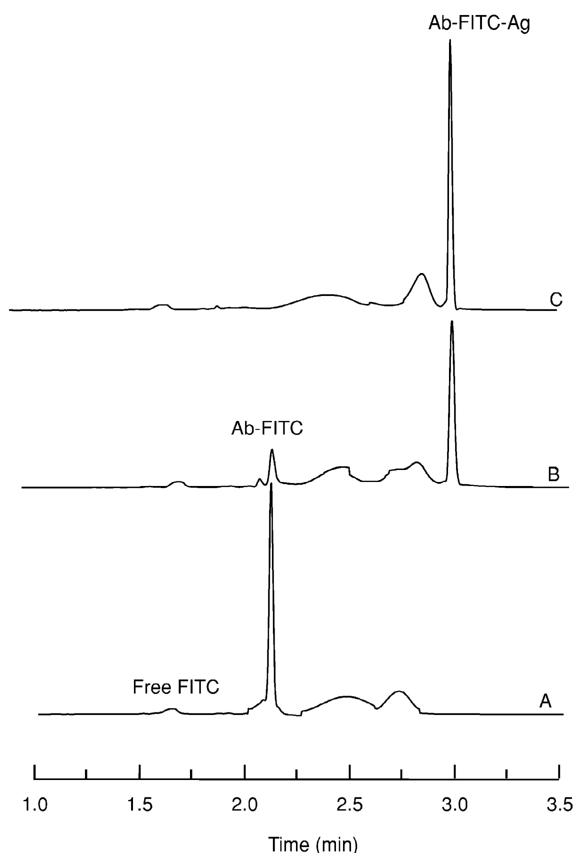

Fig. 2. Immunoreaction of FITC-labeled anti-AFP (Ab*) with (A) no AFP, (B) 30 ng/ml AFP, and (C) 80 ng/ml AFP. Separation conditions are the same as in Fig. 1.

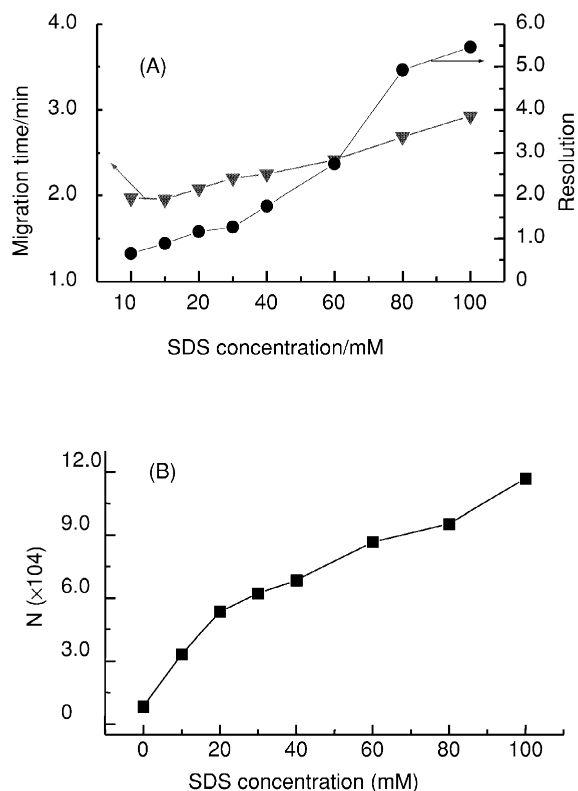

Fig. 3. Effect of SDS concentration on the migration time and resolution (A), and theoretical plates (B). Separation conditions are the same as in Fig. 1.

3.3. Optimization of the separation conditions

Proteins normally absorb onto the silica capillary wall [17,18]. Adding SDS to the buffer has been proven to be an effective way to solve this problem [11]. The influence of SDS concentration on separation was studied initially. Resolution and the theoretical plates increased greatly with SDS concentration, with migration time slightly prolonged. Better peak shape and better reproducibility of the migration time and peak height were also obtained when high concentration SDS was used; on the other hand, high SDS concentration resulted in high separation current that would affect the efficiency and resolution. The effects of SDS concentration on separation are shown in Fig. 3 and 100 m*M* SDS is selected.

The buffer pH is an important parameter in immunoassays based on CE. In our experiments, when the pH changed from 8.0 to 10.5, the immunocomplex was stable within the separation time, but the resolution and the theoretical plates obtained the highest value at pH 9.50 (see Fig. 4). pH 9.5 was chosen for this reason and for the excellent buffer capacity of tetraborate buffer.

Higher separation voltage leads to shorter analysis time and higher efficiency in theory in CE, but too high voltage has an adverse effect on separation when serious joule heating occurred. According to the curve between current and voltage (not shown), 20 kV voltage was suitable.

3.4. Validation of the method

Under the optimal conditions for the immunoreaction and separation, different amounts of AFP

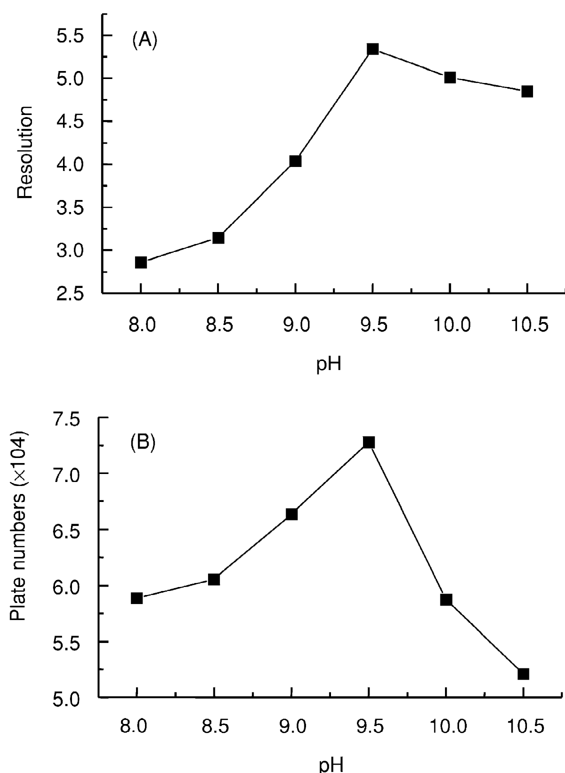

Fig. 4. Effect of the buffer pH on the resolution (A) and theoretical plates (B). Other conditions are the same as in Fig. 1.

(concentration ranged from 0 to 100 ng/ml) were adding to 75 ng/ml Ab* solutions. After 40 min incubation, each sample was analyzed with MECC-

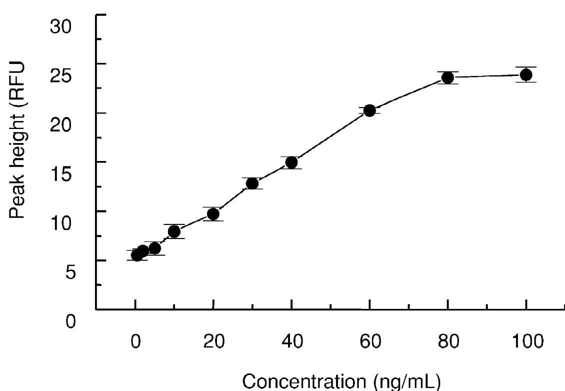

Fig. 5. Plot of the peak height of the immunocomplex versus the concentration of AFP. Buffer: 20 m*M* tetraborate and 100 m*M* SDS at pH 9.50, other conditions are the same as in Fig. 1. Data are shown as mean±SD ($n=3$).

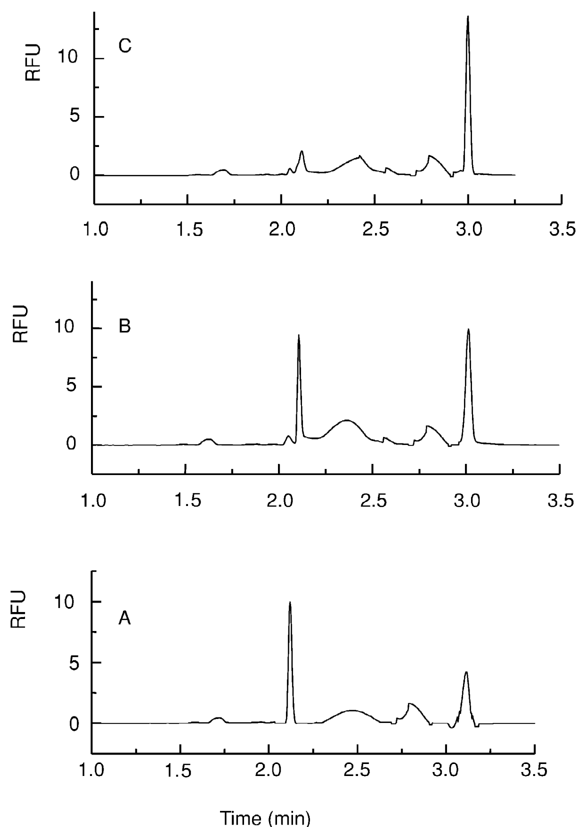

Fig. 6. Determination of AFP in human serum. Samples: (A) without, (B) with 10 ng/ml AFP, and (C) with 25 ng/ml AFP spiked in human serum. Conditions are the same as in Fig. 5.

LIF. The antigen–antibody complex was well separated from the free FITC and FITC-Ab*. There were also additional peaks that were not identified. The reason might be that there were impurities in the antibody, and these impurities could also be labeled with FITC, but did not affect the determination of AFP antigen. A curve was obtained by plotting the concentration of AFP versus the peak height of immunocomplex (Fig. 5). There was a good linear relationship from 0.5 to 60 ng/ml of AFP ($Y = 0.381 + 0.247X$, $R^2 = 0.999$). At higher AFP concentration, the peak height of complex obtained a plateau, which meant that the Ab* was saturated by AFP.

The reproducibility of the migration time, peak height and detection limit were tested, respectively. The relative standard derivations (RSD) of 10 ng/ml

and 50 ng/ml AFP for migration time was 1.04–2.87% (inter-day) and 3.57–5.01% (intra-day), and for peak height, 4.58–4.99% (inter-day) and 6.95–7.01% (intra-day). According to the signal-to-noise ratio of 3:1, the limit of detection of AFP was about 0.1 ng/ml.

3.5. Determination of AFP in serum

The developed method was used to detect the AFP in female serum with and without spiking standard AFP. Under the optimum separation conditions, the Ab* and immunocomplex were both completely separated and detected from other components in serum. Typical electropherograms of serum without and with addition of AFP are shown in Fig. 6.

4. Conclusion

A noncompetitive immunoassay based on MECC with LIF detection has been developed for the determination of AFP in human serum. The adsorption of the proteins to the capillary walls was prevented and the analytes were stable in the selected conditions. This assay had good selectivity, high separation efficiency and sensitivity with very little sample and buffer required. The results indicated that the developed method allowed for rapid and quantitative determination of analytes in complex samples such as serum. This method could also be applied to quantify AFP in other biofluids.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 29875012 and 30070209) and Teacher Foundation of Chinese Education Ministry (no. 2000000352).

References

[1] X.W. Wang, H. Xie, Life Sci. 64 (1999) 17.
[2] S. Zhao, in: Medical Test and Disease Diagnosis, Sichuan Science and Technology Press, Sichuan, China, 1991, p. 225.
[3] J. Sowell, P. Parihar, G. Patonay, J. Chromatogr. B 752 (2001) 1.
[4] T.B. Yang, in: Immunology and Immunoassay, Pepple Health Press, Beijing, China, 1998, p. 127.
[5] K. Arlt, S. Brandt, J. Kehr, J. Chromatogr. A 926 (2001) 319.
[6] D.M. Zhang, J.M. Zhang, W.Y. Ma, D.Y. Chen, H.W. Han, H.J. Shu, G.Q. Liu, J. Chromatogr. B 758 (2001) 277.
[7] A. Radschuweit, H.H. Ruttinger, P. Nuhn, J. Chromatogr. A 937 (2001) 127.
[8] R.Y. Wang, W.Y. Ma, D.Y. Chen, Anal. Lett. 34 (2001) 1889.
[9] K.C. Yeung, A.G. Kiceniuk, L. Li, J. Chromatogr. A 931 (2001) 153.
[10] S.X. Peng, J. Chromatogr. B 964 (2001) 59.
[11] M.A. Strege, A.L. Lagu, Electrophoresis 16 (1995) 642.
[12] J.J. Bao, J. Chromatogr. B 699 (1997) 463.
[13] S. Miki, T. Kaneta, T. Imasaka, J. Chromatogr. B 759 (2001) 337.
[14] E. Ban, H.S. Nam, Y.S. Yoo, J. Chromatogr. A 924 (2001) 337.
[15] L.W. Ye, X.C. Le, J.Z. Xing, M.S. Ma, R. Yatscoff, J. Chromatogr. B 714 (1998) 59.
[16] S. Zaugg, W. Thromann, J. Pharm. Biomed. Anal. 24 (2001) 785.
[17] R.J. Xu, C. Vidal-Madjar, B. Sébille, J.C. Diez-Masa, J. Chromatogr. A. 730 (1996) 289.
[18] B. Verzola, C. Gelfi, P.G. Righetti, J. Chromatogr. A 868 (2000) 85.