Journal of Food and Drug Analysis, Vol. 15, No. 1, 2007, Pages 71-74　　藥物食品分析　第十五卷　第一期

A New Detection Technique for Fluoroquinolone-Conjugated Proteins by High Performance Liquid Chromatography with UV/Fluorescence Detectors

SHAO-WEN HUNG, CHING-WEN SHIH, BO-RUI CHEN, CHING-YU TU, YU-FEN LING, LI-TSE TSOU, SHU-PENG HO AND WAY-SHYAN WANG*

Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, 250 Kuo Kuang Rd., Taichung 402, Taiwan, R.O.C.

(Received: July 18, 2006; Accepted: November 7, 2006)

ABSTRACT

This study was aimed to develop a simple, fast, and reliable technique to detect fluoroquinolones (FQs) -conjugated bovine serum albumin (BSA). Four tested FQs, enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin, were conjugated with BSA by following the N-hydroxysuccinimide ester method. The technique was designed according to the different absorption characteristics of the FQs and BSA; FQs can be detected by both UV and fluorescence detectors, but BSA can only be detected by UV. The results demonstrated that the developed method was efficient in detecting FQs-BSA conjugates. In addition, the method not only trace the FQs and BSA conjugation responses but also can be used to estimate the level of FQs-BSA conjugation. Therefore, this technique is a valuable tool for the detection drug-carrier-conjugated antigens, especially for FQs-BSA-conjugates during the production of anti-FQs monoclonal antibodies.

Key words: fluoroquinolone, HPLC, N-hydroxysuccinimide ester, bovine serum albumin

INTRODUCTION

Fluoroquinolones (FQs) are currently used for the treatment of invasive and systemic bacterial infections, such as upper and lower respiratory infections, gonorrhea, bacterial gastroenteritis, skin and soft tissue infections, and urinary tract infections that occur in humans and animals[1]. Most of FQs such as enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin are bacteriostatic at low concentrations and bactericidal at high concentrations. They are more active against most Gram-negative pathogens, including *Pseudomonas aeruginosa* and the *Enterobacteriaceae*, than Gram-positive bacteria[2].

Serum albumins are the major proteins in the circulatory system of animals and contribute to the osmotic blood pressure. They play a dominant role in drug disposition and efficacy[3]. Many drugs can bind reversibly to albumins, which then function as carriers. Serum albumins often increase the apparent solubility of hydrophobic drugs in plasma and thus modulate their delivery to cells. Furthermore, the molecular weight (MW) of some drugs, like antibiotics, is too low to raise their antibodies. Therefore, for these low MW drugs, also called haptens, albumins can act as carrier proteins. When hatpens are conjugated with carrier proteins, their MWs become larger and thus can stimulate the production of anti-hatpen antibodies. It is therefore necessary to investigate the conjugation of FQs and bovine serum albumin (BSA)[4] in order to gain a better understanding of the processes involved.

Drug residues in animal tissues and milk pose a potential threat to human health. There is an obvious need for a rapid and accurate method to detect the residual drug in edible animal tissues in order to protect consumers' health. There are many techniques currently being employed to analyze drug residues, including gel-filtration chromatography, electrophoresis, ultracentrifugation, immuno-diffusion, chemical analysis, and isotope labeling analysis[5]. Omura *et al.*[6] reported that HPLC with mass spectrometry and proton nuclear magnetic resonance (^{1}H-NMR) detector was capable of coupling the product of 2'-deoxycytidine, and this method can be efficacious in sorting productive components. Duan and Yuan[1] used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), $FeCl_3$ colorimetry, and UV/VIS scanning methods to ensure that the production of ciprofloxacin-BSA was complete. They were able to detect the conjugated protein. However, these techniques are very complicated and expensive to perform. A cheaper and simpler procedure is demanded.

FQs are readily detected with UV/VIS and fluorescence spectroscopy, which are powerful tools for the study of the reactivity in the chemical and biological systems[4,7]. Therefore, the purpose of this study was to estimate the extent of FQ-BSA conjugations by HPLC with UV/fluorescence detectors.

* Author for correspondence. Tel: +886-4-22840894 ext. 508; Fax: +886-4-22862073; E-mail: wswang@dragon.nchu.edu.tw

MATERIALS AND METHODS

I. *Chemicals*

In this study, four fluoroquinolones (FQs) were used: enrofloxacin (Bayer, Wuppertal, Germany), danofloxacin (Pfizer, New York, NY, USA), ofloxacin (Sigma, Louis, MO, USA), and orbifloxacin (Dainippon Pharmaceutical, Osaka, Japan) (Figure 1). The carrier protein, BSA, and N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and dimethylformamide (DMF) were purchased from Sigma. Trifluoroacetic acid (TFA) was from Bakers (Phillipsburg, NJ, USA).

II. *Apparatus*

The HPLC system consisted of a LC1022 Plus (Perkin-Elmer, Bridgeport, CT, USA), Degasys DG-1210 (Uniflows, Tokyo, Japan), a chromatointegrator (PE Nelson 1022; Perkin-Elmer), a UV/VIS detector (LC295) (Perkin-Elmer, Bridgeport, CT, USA) and a fluorescence detector (TEC A4-2100A) (St. John Associates, Beltsville, MD, USA). FQ-BSA conjugates were monitored by absorbance at 280 nm in UV detector or by emission at 450 nm in fluorescence detector while excited at 290 nm.

III. *Preparation of Conjugated Proteins of Fluoroquinolones and BSA*

As shown in Figure 2, the method described by Watanabe *et al.*[8] was used with modification. The haptens, namely enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin, were individually conjugated with the carrier protein, BSA, by NHS ester method. 0.1 mM of each FQ, 0.5 mM NHS and 0.5 mM EDC were solublized in 1 mL of DMF and incubated for 24 hr at room temperature with constant stirring. This solution was labeled as solution A (pH 6.0). One hundred milligrams BSA (H_2N-E-A-H-K-S-E-) was dissolved in 10 mL of 0.01 M PBS and labeled as solution B. Solution A was dripped into solution B very slowly while being mixed. The mixed solution was stirred for 3 hr at room temperature in the dark and then dialyzed against PBS (pH 7.4) at 4°C for six days. The Coomassie plus protein assay reagent kit (Pierce, Rockford, IL, USA) was used to determine the conjugated protein concentration. The conjugated proteins were concentrated using the Speed Vac SC 110-120 (Savant, Holbrook, NY, USA) and stored at -80°C until use.

IV. *Detection of the Fluoroquinolones-BSA Conjugated Proteins*

BSA, four FQs (enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin), and four conjugated proteins, FQ-BSA, were diluted with PBS to the final concentrations 2 mg/mL, 20 μg/mL, and 800 μg/mL, respectively. A volume of 20 μL of each solution above was injected into the HPLC system. With these amounts of BSA, FQ-BSA injected, we could get the best HPLC chromatograms. Furthermore, no derivative procedure was used for the fluorescence analysis. The mobile phase, acetonitrile (with 0.05% TFA) and double distilled water (with 0.05% TFA) at a ratio of 60:40 (v/v) was first degassed using Degasys DG-1210. The sample solutions were eluted and resolved using a Cosmosil 5C18-AR-300 column (4.6 × 250 mm) (Nacalai, Kyoto, Japan). The flow rate was 1 mL/min. The combined detectors, namely UV/VIS and fluorescence detectors, were used to detect the FQs and conjugated proteins of FQs-BSA. Finally, the data was analyzed with a chromatointegrator.

RESULTS

Twenty microliter of BSA at 2 mg/mL, which served as a control, were injected into the HPLC system with

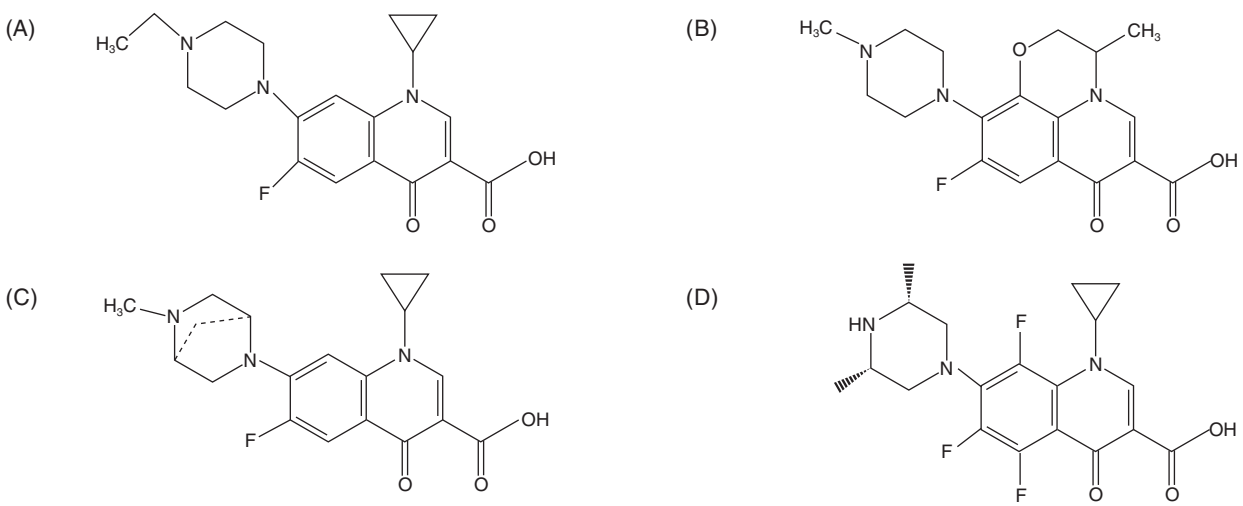

Figure 1. The structures of four fluoroquinolones. (A) enrofloxacin; (B) ofloxacin; (C) danofloxacin; (D) orbifloxacin.

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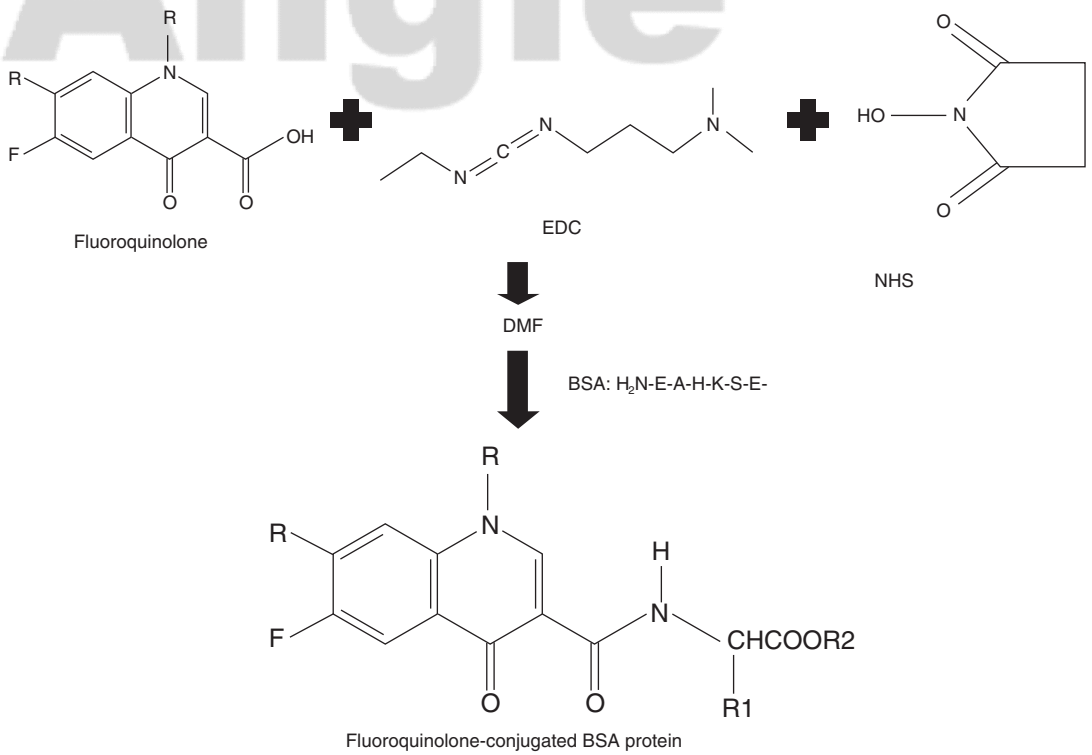

Figure 2. The N-hydroxysuccinimide ester method used for the conjugation of fluoroquinolone with BSA. The fluoroquinolones used included enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin. The abbreviations used are as follows: N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dimethylformamide (DMF), and bovine serum albumin (BSA).

UV/fluorescence detectors. An obvious peak of BSA appeared at 15.693 min under the UV detector, but there was no signal for it under the fluorescence detector at the same time point as shown in Figure 3. Twenty microliter of enrofloxacin, ofloxacin, danofloxacin, or orbifloxacin at 20 μg/mL were analyzed by HPLC with UV/fluorescence detectors. There were obvious peaks of enrofloxacin, ofloxacin, danofloxacin, and orbifloxacin at 12.897, 10.875, 11.978, and 12.235 min, respectively under both UV and fluorescence (Figure 3). By the same method, we could detect the conjugated proteins. Distinct peaks of enrofloxacin-BSA, ofloxacin-BSA, danofloxacin-BSA, and orbifloxacin-BSA were revealed at 16.600, 16.335, 16.235, and 17.335 min, respectively (Figure 3). According to the above results, we concluded that the established HPLC method with UV/fluorescence detectors was useful to detect the conjugated proteins of FQs-BSA.

DISCUSSION

FQs are extensively used in veterinary medicine, so it is very important to detect their residues in edible animal tissues due to public health concerns. However, currently no simple and reliable detection kits are available for residual FQs. Because FQs are haptens, they need to be conjugated with high MW carrier proteins to raise anti-FQs antibodies[9]. Therefore, a detection method for monitoring the conjugation of FQs and BSA during the combination stage is needed.

Pauillac *et al.*[10] reported that 200 to 300 nm UV absorbance scanning from 200 to 300 nm was suitable for the detection of the tested hapten 4-acetyl benzoic acid (ABA) and the carrier protein BSA. Their results showed that ABA absorbs UV in a range from 210 to 254 nm while BSA absorbs UV in a range of 210 to 280 nm. Furthermore, Duan and Yuan[1] indicated that the absorption peaks for ciprofloxacin-BSA and BSA were 271 nm and 275 nm, respectively. In the present study, our results suggest that the appropriate absorption wavelength range for tested FQs, FQs-BSA, and BSA located between 280 nm and 450 nm.

In this study, the conjugation of FQs with BSA was successfully established by N-hydroxysuccinimide ester method and analyzed by HPLC with both fluorescence and UV detectors. Compared with the method employed by Duan and Yuan[1], our technique was much easier and cheaper for detecting FQs-BSA conjugated proteins and can be used to estimate the degree of conjugation according to integrated areas in the chromatograms. The technique would be of value in the production of anti-FQs antibodies and the development of a commercial detection kit for FQs.

ACKNOWLEDGEMENTS

We would like to thank the Council of Agricul-

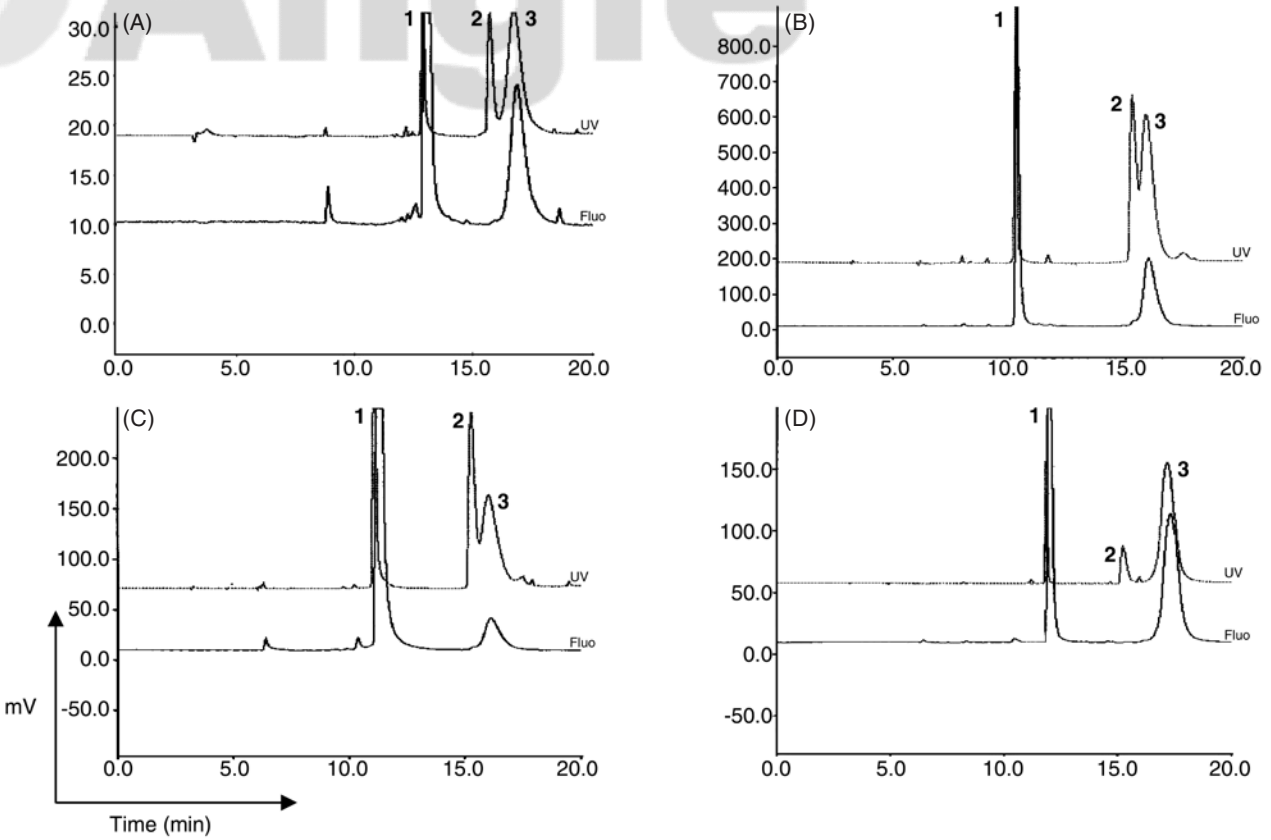

Figure 3. Detection of the fluoroquinolones-BSA conjugated proteins by using HPLC with UV and fluorescence detectors. Twenty μg/mL of fluoroquinolones respectively included enrofloxacin^{A1}, ofloxacin^{B1}, danofloxacin^{C1}, and orbifloxacin^{D1}, 2 mg/mL of BSA$^{A2, B2, C2, D2}$, and 800 μg/mL of conjugated proteins of enrofloxacin-BSA^{A3}, ofloxacin-BSA^{B3}, danofloxacin-BSA^{C3}, and orbifloxacin-BSA^{D3} were injected into HPLC system and detected with UV (upper curve) and fluorescence (lower curve) detectors.

ture, Executive Yuan, Taiwan, for its financial support (91AS-1.2.2-BQ-B2 and 92AS-3.3.1-BQ-B1(1)).

REFERENCES

1. Duan, J. and Yuan, Z. 2001. Development of an indirect competitive ELISA for ciprofloxacin residues in food animal edible tissues. J. Agric. Food Chem. 49: 1087-1089.
2. Angulo, F. J., Johnson, K. R., Tauxe, R. V. and Cohen, M. L. 2000. Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: implications for the use of fluoroquinolones in food animals. Microb. Drug Resist. 6: 77-83.
3. Ming, G. U. O., Zou, J. W., Yi, P. G., Shang, Z. C., Hu, G. X. and Yu, Q. S. 2004. Bind interaction of gatifloxacin with bovine serum albumin. Anal. Sci. 20: 465-470.
4. Kamat, B. P. 2005. Study of the interaction between fluoroquinolones and bovine serum albumin. J. Pharm. Biomed. Anal. 39: 1046-1050.
5. Wong, S. S. 1993. Reactive groups of proteins and their modifying agents. In "Chemistry of Protein Conjugation and Cross-linking". 1st ed. pp. 7-45. CRC Press, Inc. Boca Raton, FL, U.S.A.
6. Omura, K., Hirose, K., Itoh, M., Akizawa, T. and Yoshioka, M. 1997. Development of enzyme immunoassay of 2'-deoxycytidine. J. Pharm. Biomed. Anal. 15: 1249-1256.
7. Wang, W. S., Shih, C. W., Hung, S. W., Ling, Y. F., Tu, C. Y., Chen, P. R., Tsou, L. T. and Ho, S. P. 2006. Development and application of the enzyme-linked immunosorbent assay residual detection kits for fluoroquinolones. Taiwan Vet. J. 32: 391-403.
8. Watanabe, H., Satake, A., Kido, Y. and Tsuji, A. 1999. Production of monoclonal antibody and development of enzyme-linked immunosorbent assay for kanamycin in biological matrices. Analyst 124: 1611-1615.
9. Szurdoki, F., Bekheit, H. K. M., Marco, M. P., Goodrow, M. H. and Hammock, B. D. 1995. Important factors in hapten design and enzyme-linked immunosorbent assay development. In "New Frontiers in Agrochemical Immunoassay". pp. 39-63. Kurtz, D. A., Skerritt, J. H. and Stanker, L. eds. AOAC International. Arlington, VA, U.S.A.
10. Pauillac, S., Naar, J., Branaa, P. and Chinain, M. 1998. An improved method for the production of antibodies to lipophilic carboxylic hapten using small amount of hapten-carrier conjugate. J. Immunol. Methods 220: 105-114.