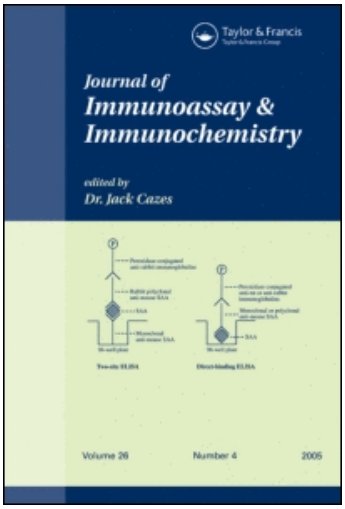

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DEVELOPMENT OF AN IMMUNOASSAY FOR MONITORING THE LEVELS OF CIPROFLOXACIN IN PATIENT SAMPLES

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ABSTRACT

It has been traditional to measure drug concentrations using high pressure liquid chromatography (HPLC). While this method is highly accurate, it is time consuming and requires the use of appropriate standards for identification of the compound. In addition, identification and quantification of drugs from patient samples requires significant manipulation to remove protein. In contrast, enzyme-linked immunoassays (EIA) are able to assay samples with a high degree of specificity, and are able to process multiple samples at a time. In addition, serum proteins do not interfere with sample quantification and samples may be tested without significant preparation. We describe the development of an EIA for the detection of ciprofloxacin in serum and dialysate samples. The immunoassay is specific for ciprofloxacin and is sensitive for picogram amounts of the antibiotic.

(KEY WORDS: immunoassay, antibiotic, ciprofloxacin)

INTRODUCTION

Since their inception in the early 1970's, enzyme-linked immunoassays (EIA) have been recognized for their sensitivity and specificity in detecting a

specific molecule (1-3). In the last 10 years, detection of hapten analytes by EIA has increased dramatically as these assays have become an important tool for drug abuse screening (4-8). Clinically, it is becoming increasingly important to monitor patients to assure that therapeutic levels of drugs are actually achieved or to prevent toxic levels of drugs. To this end, EIA assays have been developed to detect not only drugs of potential abuse, but also anti-cancer agents (9-11). These assays allow for easy *in vivo* detection and pharmacological analysis of the drug (9,12). As patient problems become more complex, there will be an increased demand for analyzing patient samples for numerous drugs. Currently, analysis of patient samples for quinolone antibiotics is performed by high pressure liquid chromatography. This requires purification of the sample in the absence of proteins (to which the antibiotics bind) and is a time consuming process.(13) To this end, we have developed an EIA for ciprofloxacin, a quinolone antibiotic, and examined its ability to detect and quantify the drug in serum samples.

MATERIALS AND METHODS

Preparation of conjugates

Ciprofloxacin was conjugated to either keyhole lymphocyte hemocyanin (KLH) or bovine serum albumin (BSA) as carrier proteins using the heterobifunctional reagent 1-ethyl-3-(dimethylaminopropyl)carbo-diimide HCl (EDC, Pearce Chemicals Co., USA). In brief, the ciprofloxacin was prepared by dissolving 2 mg ciprofloxacin HCl powder (Miles, Inc, West Haven, CT.) in

0.5 mL of conjugation buffer (0.1 M 2-[N-morpholino]ethane sulfonic acid), 0.9 M NaCl, 0.02% NaN_3, pH 4.7) and added to 0.2 mL of carrier protein (either KLH or BSA) dissolved in deionized water. For the KLH conjugation, 50 µl of EDC (10 mg/mL deionized water) was added to the ciprofloxacin/KLH solution and for the BSA conjugation, the ciprofloxacin/BSA solution was added to 10 mg of EDC and the EDC dissolved by gentle mixing. The solutions were incubated at room temperature for 2 hours to allow conjugation to occur. The conjugate was then purified by gel filtration using a 5 mL desalting column which had been equilibrated with 0.083 M $NaPO_4$, 0.9 M NaCl at pH 7.2 before use. The conjugate was added to the column and the column washed with 0.083 M $NaPO_4$, 0.9 M NaCl at pH 7.2. Column fractions containing protein were identified by absorbance at 280 nm. Samples containing protein were pooled, dialyzed to remove the salt and lyophilized. The fractions were then stored at -20°C until use.

Production of polyclonal antisera

Two female New Zealand white rabbits were immunized with ciprofloxacin-KLH at CoCalico Biologicals Inc. (Reamstown, PA.). The initial immunization was 100 µg of ciprofloxacin-KLH in complete Freund's adjuvant, given intramuscularly. Booster immunizations of 50µg of ciprofloxacin-KLH in incomplete Freund's adjuvant were administered on days 14 and 21. A test bleed was performed on day 35 and the rabbits received an additional booster immunization on day 49. Production bleeds were obtained approximately 8 weeks after the initial immunization.

EIA tests

For detection and quantitation of antibodies to ciprofloxacin, the ciprofloxacin-BSA conjugate was used. Microtiter plates (Immulon IV, Dynatech, Chantilly, VA.) were coated with ciprofloxacin-BSA by adding 100 µl/well ciprofloxacin-BSA (6 µg/mL) in bicarbonate-saline (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, 0.15 M NaCl, pH 9.6) and incubating the covered plates overnight at 4°C. Following the incubation, the plates were blocked by adding 100 µl/well 2% BSA in phosphate-buffered saline (PBS, 0.02 M phosphate, 0.15 M NaCl, pH 7.0) and incubating for an hour at 4°C. The plates were washed 5 times with PBS/Tween (PBS with 0.05% Tween 20 added). Primary antibody (rabbit anti-Ciprofloxacin-KLH diluted in 1% BSA in PBS/Tween) was added to the plates and the plates were incubated for 2 hours at 37°C with shaking. Following incubation the plates were washed 5 times with PBS/Tween and 100 µl/well of secondary antibody (horseradish peroxidase labelled goat anti-rabbit IgG diluted 1:1500 with 1% BSA in PBS/Tween) was added. The plates were protected from light and incubated with shaking for 1 hour at 37°C. The plates were washed 5 times with PBS/Tween and 100 µl/well of substrate (4 mg/mL o-phenylenediamine dihydrochloride in 0.1 M citrate buffer, pH 4.5 and 0.4 µl/mL 30% H_2O_2) plates were incubated at room temperature for 10 minutes and the optical density of the solution was measured at 450 nm in a microtiter plate reader (Ceres UV900, Biotek Instruments, Winoski, VT.).

EIA inhibition tests

The ciprofloxacin-BSA conjugate was adsorbed to the wells and the

plates blocked as described above. Unconjugated ciprofloxacin was added to the plates at the concentrations indicated (ranging from 1 pg/mL to 1 μg/mL) with the primary antibody, and incubated as described above. The plates were washed and secondary antibody added as described above. After incubation, the plates were washed again and 100 μ/well of substrate was added. The plates were incubated at room temperature for 10 minutes and the optical density of the solution read at 450 nm. Using the resulting curve, the concentration of ciprofloxacin in spiked biological samples was determined. Stock solutions of ciprofloxacin were made up at 2 mg/mL in PBS.

In addition, specificity of antibody binding to ciprofloxacin was determined using a metabolite of ciprofloxacin 7-(2-aminoethylamino)-1-cyclopropyl-6-flouro-1,4-dihydro-4-oxo-3 quinolinecarboxylic acid (M1) (13).

Detection of ciprofloxacin in serum

To test the ability of the EIA to detect ciprofloxacin in body fluids, ciprofloxacin solution (2 mg/mL)was added to human serum to achieve a concentration of 1-10 μg/mL (the therapeutic range) and the samples were coded for determination of ciprofloxacin concentration by a blinded investigator. The concentration of ciprofloxacin in each sample was determined by EIA assay and was compared to the actual concentration. Because the detection range for ciprofloxacin by EIA was 10 pg/mL to 10 ng/mL and the therapeutic range was 1-10 μg/mL, all samples were diluted 100 and 1000 times before assay.

The coefficient of variation was within 10% for all assays run on a given day.

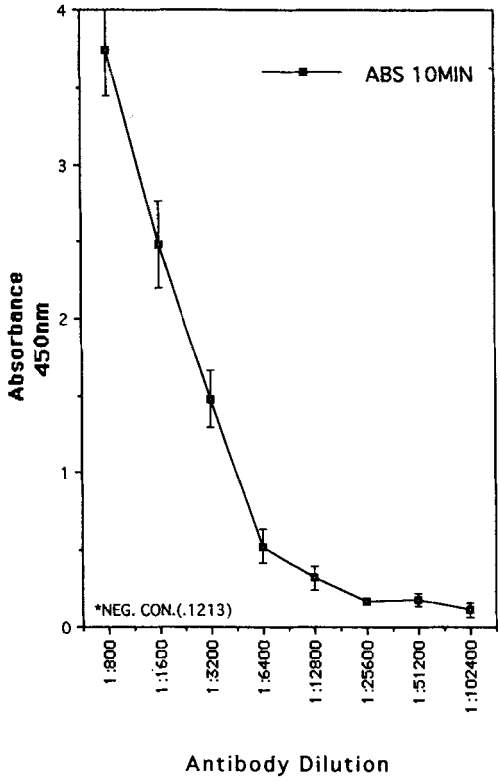

FIGURE 1. Absorbance of titer of antiserum after 10 minutes of incubation with substrate *versus* dilution of antibody.

RESULTS

In order to quantitate the antibody response of the rabbits to ciprofloxacin, we performed EIA tests as described above using ciprofloxacin-BSA as the antigen bound to the microtiter plates. The antiserum was tested in two-fold dilutions to determine both the titer and working titer of the antiserum (Figure 1). The titer was defined as the dilution of antiserum where the absorbance was twice the background absorbance. Therefore, the titer of the

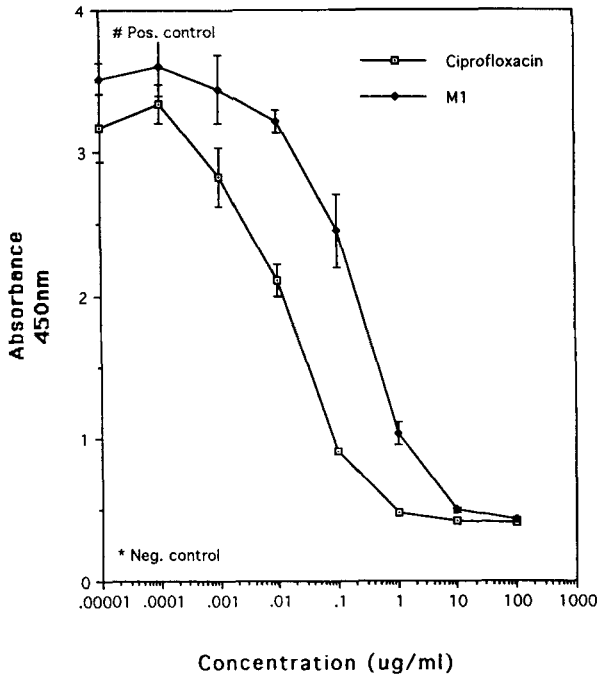

FIGURE 2. Inhibition of binding of 1:1500 dilution of antibody by ciprofloxacin and MI metabolite.

anti-ciprofloxacin antibody was 1:12,800. For the inhibition experiments, a working titer of antiserum was used. The working titer was defined as the titer which gave an absorbance of approximately 1.000 after 10 minutes of reaction with the substrate. For experiments reported here, the working titer was generally 1:3200, with several exceptions (noted below).

Figure 2 demonstrates that when the positive control antiserum was added to the plates at a dilution of 1:1500, the absorbance at 10 minutes was 3.9 $\pm$ 0.1. However, when free ciprofloxacin was added at concentrations from 10 pg/mL to 100 μg/mL, the binding of antibody to ciprofloxacin-BSA was

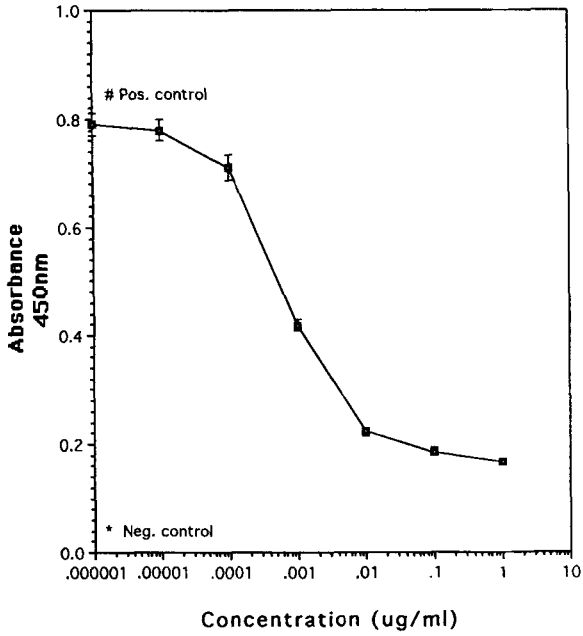

FIGURE 3. Inhibition of binding of 1:3200 dilution of antibody by ciprofloxacin.

reduced from 14% to 90%. Furthermore, concentrations of free ciprofloxacin ranging between 100 pg/mL and 1 μg/mL produced a linear decrease in antibody binding as measured by absorbance. This linear decrease in binding may be used to quantitate free ciprofloxacin in body fluids.

The antibody raised in rabbits to ciprofloxacin-KLH was specific for ciprofloxacin. While the antibody can bind M1, a metabolite of ciprofloxacin, the ability of M1 to competitively inhibit the binding of antibody to ciprofloxacin-BSA was less than 10% that of ciprofloxacin. The standard curve

TABLE 1.
Actual Concentrations of Ciprofloxacin in Serum *versus* Concentrations Detected by Polyclonal Antibody Assay, and Percent Accuracy of Assay

Sample Number	Concentration (μg/mL)		% Accuracy
	Actual	Detected	
1	1	0.48	48
2	1	0.26	26
3	10	1.53	15
4	8	1.80	23
5	5	2.00	40
6	10	2.00	20
7	7	1.70	25

of inhibition required 10 to 100 times as much M1 to achieve the same degree of inhibition as observed with free ciprofloxacin (Figure 2).

At greater dilutions of antibody (1:3200) the amount of free ciprofloxacin required to inhibit the binding of antibody was decreased to 10 pg/mL and was linear to 10 ng/mL (Figure 3).

The ability of a polyclonal antibody to detect ciprofloxacin in human serum was assayed using serum samples which had been spiked with ciprofloxacin. We added ciprofloxacin at concentrations from 1-10 mg/mL to human serum and compared the results to aqueous ciprofloxacin. Since ciprofloxacin binds to serum proteins (14), we were only able to detect free ciprofloxacin. The EIA was able to detect approximately 28% of the ciprofloxacin present (range 15-48%) (Table 1).

DISCUSSION

As demonstrated in this paper, it is possible to detect a quinolone antibiotic using immunoassay techniques. The assay is specific for ciprofloxacin, the polyclonal antibody having less than 10% cross-reactivity with a metabolite of ciprofloxacin, M1. In addition, this assay is able to detect ciprofloxacin present in biological samples at therapeutic concentrations. Approximately 19% of ciprofloxacin is metabolized of which 2% is M1.(13) Cross-reactivity was not determined for the other metabolites due to lack of availability of those species.

Unfortunately the ciprofloxacin detected is less than that actually present, the potential reasons for this include the ability of ciprofloxacin to bind serum proteins, or the breakdown of ciprofloxacin to M1 or other metabolites (M2-4). Since some 16 and 43% of ciprofloxacin is bound to serum albumin and about 19% is metabolized (total 35-62% unavailable for assay detection), and since we are able to detect approximately 25% of the ciprofloxacin known to be in the sample, we conclude that this EIA detects only free ciprofloxacin, present as 38-65% of the ciprofloxacin added to the serum. Furthermore, degradation of ciprofloxacin to M1 significantly decreases the ability of the antibody to detect the material. Since M1 retains biological activity (14) detection would be decreased while efficacy of treatment remains the same.

EIA tests hold the potential for easily assaying the concentration of antibiotics delivered to the site of infection.

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