Development of Amoxicillin Enzyme-Linked Immunosorbent Assay and Measurements of Tissue Amoxicillin Concentrations in a Pigeon Microdialysis Model

L.-C. Yeh,*† W.-M. Lee,* B.-W. Koh,* J. P. Chan,* C.-H. Liu,† J.-P. Kao,* and C.-C. Chou*[1]

*Veterinary Medicine Teaching Hospital and Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan, 402; and †The Branch Institute of Animal Drug Inspection, Animal Health Research Institute, Council of Agriculture, Executive Yuan, Taiwan, 350

ABSTRACT A sensitive ELISA was developed for the detection of amoxicillin (AMX) in serum, urine, and milk. The ELISA used an indirect competitive method produced by coating the plate with ovalbumin conjugated with AMX hapten. Antibodies against AMX-BSA were detected by a goat-antirabbit antibody conjugated with peroxidase. Calibration standard curves ranged from 1.28 ng/mL to 20 μg/mL [IC_{50} (inhibition concentration 50%) = 100 ng/mL], and the limits of detection were 1.3, 2.7, and 4.8 ng/mL for urine, milk, and serum, respectively. The intra- and interassay variations were less than 4 and 9.6%. The antibody produced against AMX cross-reacted highly with penicillin G (77%); cross-reacted moderately with ampicillin, oxacillin, and cloxacillin (56.9, 51.4, and 48.8%, respectively); but was considered non-cross-reactive with dicloxacillin (7.4%), cefadroxil (<1%), and cefazolin (<1%). Concentrations of AMX were measured simultaneously in venous blood and muscles by using the developed AMX ELISA in an in vivo microdialysis model designed for pigeons. Following i.m. injection (25 mg/kg), AMX attained a peak blood level of 4.74 ± 0.30 μg/mL and decreased with a half-life of 2.38 ± 0.16 h. In contrast, measurements in pectoral and femoral muscles exhibited delayed appearances, reduced peak concentrations, and prolonged half-lives of 4.07 ± 0.48 (pectoral) and 3.01 ± 0.26 (femoral) that were significantly different from each other and those in the blood ($P < 0.05$). Blood protein binding was calculated to be 27.9 ± 5.7%. This study demonstrated the semiquantitative application of a selective AMX ELISA in the first microdialysis procedure for continuous monitoring of drug levels in specific tissues of pigeons and maybe useful for related studies in other poultry species.

Key words: pigeon microdialysis, amoxicillin, enzyme-linked immunosorbent assay

2008 Poultry Science 87:577–587
doi:10.3382/ps.2007-00167

INTRODUCTION

Amoxicillin (α-amino-*p*-hydroxybenzyl penicillin, **AMX**) is a medium-spectrum β-lactam antibiotic used to treat bacterial infections caused by gram-positive bacteria, including *Haemophilus influenzae*, *Neisseria gonorrhoea*, *Escherichia coli*, pneumococci, streptococci, and certain strains of staphylococci, and a limited range of gram-negative organisms (Handsfield et al., 1973; Neal, 2002). Amoxicillin acts by inhibiting the cross-linkage between the linear peptidoglycan polymer chains that make up the bacterial cell walls (Handsfield et al., 1973). It is usually the drug of choice within the class because it is better absorbed than other β-lactam antibiotics following oral administration. In the pigeon, AMX is effective against *Streptococcus bovis* (Soenens et al., 1998), *E. coli*, and some *Salmonella* (Dorrestein et al., 1987) and *Haemophilus* species (Carter et al., 1991), but has little effect on *Mycoplasma* and *Chlamydia* (Talaber, 2004). The suggested antimicrobial dosage for the pigeon is 150 to 200 mg/L of drinking water (Harrison et al., 1994) or 15 to 20 mg/kg of BW and could be given in breeding and racing seasons (Talaber, 2004). Amoxicillin is absorbed quickly from the gut after oral administration; its bioavailability ranges from 50 to 80% (Dorrestein et al., 1987; Escudero et al., 1998; Soenens et al., 1998).

Various methods have been used for the detection of AMX or antibiotics with the β-lactam ring. Many of the methods have been updated recently, including microbiological assays (Popelka et al., 2005; Jiang et al., 2006), spectrophotometry assays (Duan et al., 2005; Li and Lu, 2006), and chromatographic analyses such as capillary electrophoresis (Perez et al., 2007) and HPLC with different detector modes (Cha et al., 2006; Li et al., 2006; Samanidou et al., 2006). However, very few ELISA have been designed specifically for AMX (Hill et al., 1992; Mayorga et al., 2002); most of the antibodies raised for detection of AMX were

Received April 23, 2007.
Accepted December 4, 2007.
[1]Corresponding author: ccchou@nchu.edu.tw

against various β-lactams and were developed decade(s) ago (Lapresle and Lafaye, 1985; Hill et al., 1992; Klein et al., 1993; Zhang et al., 1996; Benito-Pena et al., 2005). Although each detection method has its own strengths and limitations, the ELISA has the advantage of being simple, sensitive, inexpensive, and capable of detecting metabolites. When a drug is constantly overused, drug resistance may develop, and new drugs similar in structure are synthesized to replace the original drug. Developing a new ELISA is always desirable because each antibody produced against the immunogen may exhibit characteristic spectra of cross-reactivity that could specifically identify certain analogs with improved selectivity and sensitivity.

Microdialysis is an in vivo sampling method that was introduced for continuous monitoring of neurochemical events in the brains of small laboratory animal species (Bito et al., 1966). In view of its capacity to provide a direct measure of the free (unbound) concentration of drugs in extracellular fluid (de la Peña et al., 2000), microdialysis has been used to study the distribution and metabolism of drugs in vivo (Hansen et al., 1999; Chou et al., 2001) and to measure drug binding to serum proteins (Le-Quellec et al., 1994). In theory, dialysate samples may be collected from any tissue on a continual basis from a single animal, thereby reducing both data variability and the required number of experimental subjects. In addition, in a multiple-probe setting, microdialysis allows simultaneous determination and comparison of central and peripheral (tissue) pharmacokinetics (de la Peña et al., 2000). Recently, microdialysis has been increasingly used to investigate the distribution of antibiotics, including β-lactam compounds such as ampicillin, amoxicillin, cefaclor (de la Peña et al., 2001), ceftriaxone (Kovar et al., 1997), cefpodoxime (Liu et al., 2002), cefpirome (Joukhadar et al., 2002), and piperacillin (Tomasselli et al., 2003), in the muscle interstitial fluid of laboratory animals and in human subjects. However, to our knowledge, a proper microdialysis model has not been designed specifically for pharmacokinetic studies in pigeons. The reason microdialysis is increasingly popular in combination with antibiotic studies is that discrepancies usually arise between therapeutic levels of drugs in blood vs. in tissues, where the drugs are most needed to treat the infections. In many cases, the clinical outcome of therapy needs to be determined by the drug concentration in the tissue compartment in which the pharmacological effect occurs, rather than in the plasma. Inadequate tissue penetration of antibiotics can lead to therapeutic failure and bacterial resistance (Brunner et al., 2005). Pharmacokinetic evaluation of antibiotics should therefore include affected tissues rather than only in the serum. Owing to selective access to the target site for most antiinfective drugs, microdialysis provides pivotal information regarding the pharmacokinetic distribution of drugs and might become a reference technique to access the therapeutic effectiveness of an antibiotic drug.

Here we describe a sensitive AMX ELISA that differed in specificity from previously reported immunoassays. In addition, the usefulness of this assay was demonstrated by monitoring immunoreactive AMX levels in the blood and muscles of pigeons with samples obtained from a microdialysis model designed for fowl species.

MATERIALS AND METHODS

Preparation of Immunogen and Antibody Production

To prepare for the immunogen, AMX was conjugated to BSA by use of the glutaraldehyde (**GA**) method (Dietrich et al., 1998). A mixture of 40 mg of AMX and 40 mg of BSA was prepared and dissolved in 10 mL of PBS (0.01 *M*, pH 7.4). A 200-μL quantity of 25% GA solution was then added to the above PBS mixture and permitted to react in the dark for 6 h at room temperature before it was dialyzed against PBS (renewed 3 times/d) for 6 d at 4°C. The conjugate was then filtrated, collected, and stored at −20°C. Formation of AMX-BSA conjugate was confirmed by UV spectroscopy (with photodiode array detector) at 214 nm in combination with capillary electrophoresis (Beckman Coulter, Fullerton, CA) by using 20 m*M* sodium tetraborate-20 m*M* phosphate-50 m*M* SDS as the run buffer. Two adult female New Zealand White rabbits were immunized with 1.0 mg of the AMX-BSA conjugate emulsified with an equal volume of Freund's complete adjuvant. Intradermal injections were given along both sides of the neck, and booster immunizations were carried out every 2 wk. Blood samples were collected from the auricular vein 1 wk after each booster injection and tested for the titer of antibodies to AMX until the antibody titer plateau. All chemicals used in this section, including AMX, BSA, GA, GA solution, and Freund's complete adjuvant, were purchased from Sigma Ltd. (St. Louis, MO).

AMX ELISA

To establish the assay, AMX-ovalbumin (**OVA**) conjugate was first prepared as described above for AMX-BSA. Optimal antibody and conjugate concentrations were determined by the checkerboard method (Chard, 1987). In brief, AMX-OVA was diluted (1:500-, 1:1,000-, 1:2,000-, and 1:4,000-fold) with coating buffer (0.5 *M* carbonate-bicarbonate buffer, pH 9.6). A 96-well polystyrene microtiter plate (Nalge Nunc, Naperville, IL) was coated with 100 μL/well of the diluted AMX-OVA conjugate across rows and kept at 4°C overnight or left to react for 2 h at 37°C. Plates were then rinsed 3 times with 400 μL of **PBST** (PBS with 0.05% Tween 20). Amoxicillin antibodies to be assayed were diluted (1:200-, 1:400-, 1:800-, 1:1,600-, 1:3,200-, and 1:6,400-fold) with PBS and 100 μL/well of diluted AMX antibody was added to the plate across columns and incubated for 1 h at 37°C. After washing 3 times with PBST, peroxidase conjugated goat-antirabbit IgG antibody (1:2,000 dilution) was added at 100 μL/well and allowed to incubate at 37°C for an additional hour. The plate was again washed 3 times with 400 μL/well of PBST, and 100 μL/well of 3,3′,5,5′-tetramethybenzidine (**TMB**) was added to each well to initiate a color change. The plate was read at 30 min after color initiation. It was determined

that this was the optimal length of time to allow the color formation reaction to reach equilibrium, eliminating the need for an acid solution to stop the process. A Dynex MRX microplate reader (Dynex Technologies, Billingshurst, West Sussex, UK) was used to measure optical density (**OD**) at 650 nm. The antibody and AMX-OVA combination that gave rise to an OD of 1.5 was chosen as the optimal total binding. Chemicals in this section, including OVA, peroxidase conjugated goat-antirabbit IgG, and TMB, were purchased from Sigma Ltd.; Tween 20 was purchased from Merck Co. (Darmstadt, Germany).

ELISA Standard Curve and Cross-Reactivity

To establish the standard curves, microtiter plates were coated with diluted AMX-OVA (1:1,000) and washed as described above. Amoxicillin stock standards (10 concentrations at 500, 100, 20, and 4 μg/mL; 800, 160, 32, 6.4, and 1.28 ng/mL; and 256 pg/mL, diluted in PBS, milk, normal bovine serum, and normal bovine urine) were added at 50 μL to each well containing 50 μL of diluted AMX antibody (1:1,600). Plates were then incubated at 37°C for 1 h and washed 3 times with 400 μL of PBST, followed by addition of peroxidase conjugated goat-antirabbit IgG antibody (1:2,000 dilution) as described previously for the checkerboard method and allowed to incubate at 37°C for 1 h. The plate was then washed again with 400 μL of PBST, and 100 μL/well of TMB was added to each well to initiate a color change, which was read at 30 min by an ELISA reader measuring OD at 650 nm. Immunologic cross-reactivity between AMX and 9 pharmacologically or structurally related analogs [ampicillin, 6-aminopenillinic acid (6-**APA**), carbenicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G, cefadroxil, and cefazolin] was assessed by the ability of the test compounds to inhibit the reaction between the AMX-OVA and anti-AMX antibody. This was performed by substituting various concentrations of the test analogs (2 mg/mL; 200, 20 and 2 μg/mL; 200, 20 and 2 ng/mL; and 200 pg/mL diluted in PBS) for the AMX standards in the above ELISA procedure. Standard curves for AMX and the various analogs were generated and the cross-reactivity was determined by averaging the calculated IC_{25}, IC_{50}, and IC_{75} (concentration at which 25, 50, 75% of AMX-OVA binding was inhibited) over the AMX concentration required to achieve an equivalent inhibition ($IC_{25/50/75\ drug}/IC_{25/50/75\ AMX}$).

The sensitivity of the assay was determined according to Hayashi et al. (2004). The lowest concentration in the projected standard curve that showed less than 30% variation was determined as the limit of detection (sensitivity) of this assay. To assess the accuracy of the assay, the established ELISA was used to determine AMX at 2 nominal concentrations (10 μg/mL and 800 ng/mL) in PBS, bovine serum, urine, and milk. The accuracy was represented by the recovery calculated by the equation: Recovery (%) = (estimated concentration/nominal concentration) × 100%. Intra- and interday variations were assessed at 5 concentrations (10, 1, 0.1, 0.01, and 0.001 μg/mL). All experiments were performed in triplicate for 5 plates.

Microdialysis Apparatus and Sample Collection

The microdialysis system was assembled entirely from commercially available equipment and supplies (Figure 1). Linear-type microdialysis probes with a 10-mm membrane length (LM-10, molecular weight cut-off = 6 kDa, Bioanalytical System Inc., Lafayette, IN) were used in all subjects. Following implantation of the probes (see below) in the left pectoral and femoral muscles, each probe was connected to a gas-tight microliter syringe (1 mL) with small-bore polyethylene tubing (0.12 mm i.d., CMA Microdialysis Inc., Stockholm, Sweden). Probes were perfused independently with sterile 0.9% (wt/vol) sodium chloride solution (normal saline, Tai-Yu Chemical and Pharmaceutical Co. Ltd., Tao-Yan, Taiwan) by battery-powered syringe pumps (801, Univentor Ltd., Zejtun, Malta). Following the initial washout period of 30 min at 5 μL/min, probes were perfused at a flow rate of 1 μL/min throughout the experiment and presterilized polyethylene tubing (1 m) was used to connect the lines of each probe to a collection vial. A quantity of 25 mg/kg of AMX was administered into the right femoral muscles. Dialysate fractions were collected at preset intervals (10, 20, 30, and 60 min) for 12 h in glass microvials (300-μL capacity) after AMX administration. The delay time associated with dead volume of the tubing was determined and accounted for in all data calculations. Blood samples (50 μL) were collected in parallel to the collection of dialysate via intermittent venipuncture at 10, 20, 30, 40, and 50 min, and at 1, 2, 3, 4, 5, 6, 7, and 8 h post-AMX injection. Blood samples were diluted 10-fold to obtain enough volume for triplicate wells in the assay and for accurate conversion of the drug concentration using the "middle" linear range of the standard curve. Microvials containing dialysate samples were covered tightly and stored at 4°C before analysis on the following day.

Probe Implantation

Three adult (1- to 2-yr-old) healthy racing pigeons (*Columba livia*) weighting 380 to 480 g were used in this study. The experimental protocols in this investigation that involved pigeons were approved by the College of Veterinary Medicine Experimental Animal Supervising Committee. To implant microdialysis probes in the pectoral and femoral muscles, the pigeon was temporarily immobilized by a flexible elastic bandage wrapping around the wing area. The pigeon was anesthetized by 3% isofluorane through a face mask to provide a duration of 10 to 20 min for surgical preparation and probe implantation. Small regions measured at 5 × 5 cm^2 and 3 × 3 cm^2 of skin overlaying the pectoral muscle and left femoral muscle were surgically prepared with an antiseptic solution (1% iodine). The linear probe was sutured directly through the pectoral and femoral muscles by a cutting needle and the LM-10 microdialysis probe was pulled through the muscle

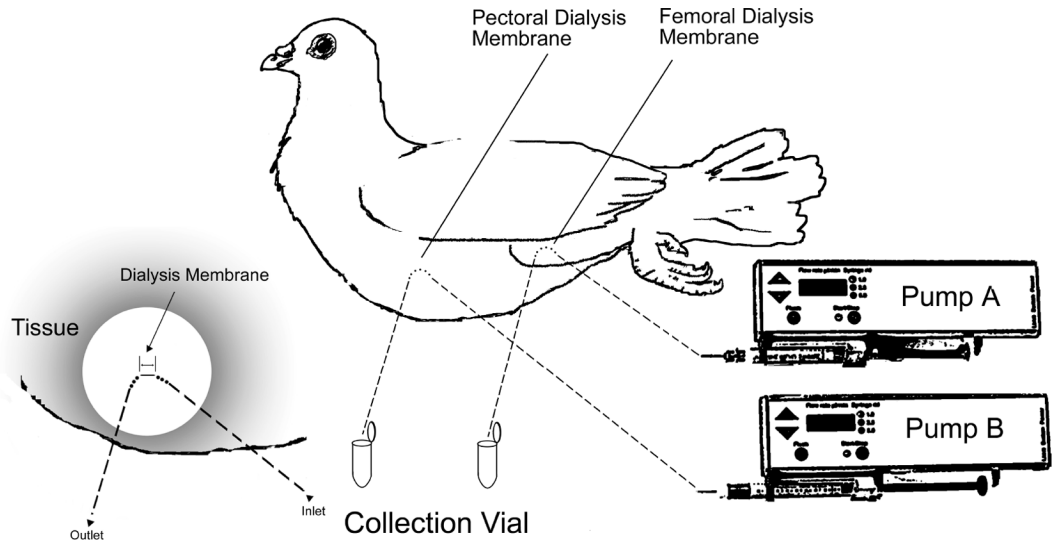

Figure 1. Schematic representation of the instrumental setup and implantation sites for microdialysis probes in pigeons.

and positioned so that the membrane was centered between the entry and exit points (2 cm apart) of the skin, thereby placing the probe directly within the muscle (Figure 1). Inlet and outlet lines were connected, and the probe was flushed as described above. Following implantation, the anesthetization was terminated and microdialysis probes were perfused with sterile saline for a minimum of 1 h to ensure equilibration before AMX administration.

Evaluation of the In Vitro Recovery and AMX Protein Binding

The in vitro recovery of AMX was determined at 3 concentrations (1 μg/mL, 500 ng/mL, and 100 ng/mL in normal saline) and 3 flow rates (0.5, 1, and 2 μL/min) for the LM-10 probe. The probe was immersed in AMX solution in a beaker and perfused with normal saline. The recovery was calculated by dividing the AMX concentration in the perfusate, determined by the developed ELISA, over the designated AMX concentration in the beaker. A brief study was carried out to delineate the extent of AMX binding to pigeon serum proteins. Amoxicillin was spiked in the fresh pigeon serum at a final concentration of 1 μg/mL and incubated (12 h at 4°C) before the estimation of free drug concentrations (C_F) by microdialysis at a flow rate of 1 μL/min. The percentage of drug bound to protein (B) was calculated from the relationship:

$$B = [1 - C_F / C_T] \times 100\%$$

where C_T is the total concentration of drug in serum (1 μg/mL) and C_F is the drug concentration in dialysate divided by fractional drug recovery.

Pharmacokinetic Analyses and Statistics

Pharmacokinetic parameters were determined for AMX in the blood and the pectoral and femoral muscles of the pigeon by using commercialized pharmacokinetic-pharmacodynamic modeling software (WinNonLin version 1.1, Pharsight Corp., Mountain View, CA). Compartmental analysis, which assumes the existence of first-order kinetics, was used to fit data for drug concentrations in blood and muscles vs. time. Data were evaluated for statistical differences between the 2 sampling methods and between pectoral and femoral muscles by using Student's *t*-test. A *P*-value of <0.05 was accepted as a statistically significant difference.

RESULTS

Sensitivity of the Assay

The optimal dilution of the antibody and AMX-OVA conjugate, as determined by the checkerboard method, was 1:1,600 and 1:1,000, respectively. Inhibition binding of 50% (IC_{50}) was attained at AMX concentrations of approximately 100 ng/mL. The sensitivities of the assay were within the range of 1 to 5 ng/mL in different matrices (Table 1), and the working ranges of the assay spanned 4 orders of magnitude (Table 1 and Figure 2). Addition of normal bovine serum, urine, and milk caused no interference; however, addition of normal bovine serum and milk caused a slight left shift in the standard curve (Figure 2). Because the slope of the standard curve was not significantly affected by drug-free urine, serum, or milk, it was

Table 1. The sensitivity and estimated working ranges of the developed ELISA in various matrices

Matrix	Limit of detection (ng/mL)	Working range (ng/mL)
PBS	6.4	6.4~100,000
Serum	4.8	1.3~20,000
Urine	1.3	1.3~20,000
Milk	2.7	0.3~20,000

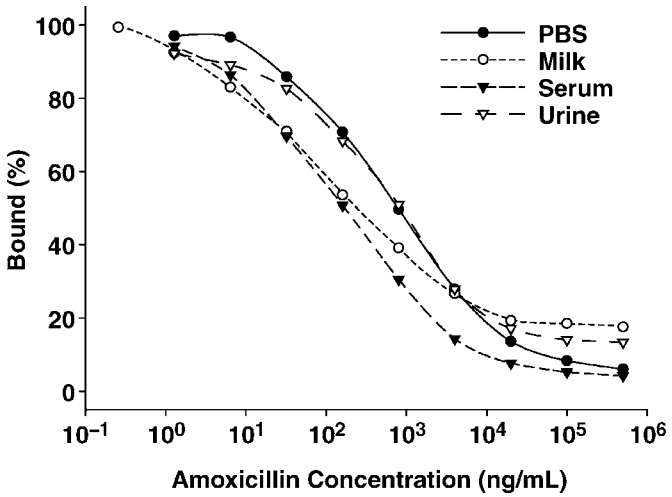

Figure 2. Standard curves for amoxicillin in PBS, normal bovine serum, normal bovine urine, and milk. Data were obtained from triplicate wells for 5 plates (n = 5).

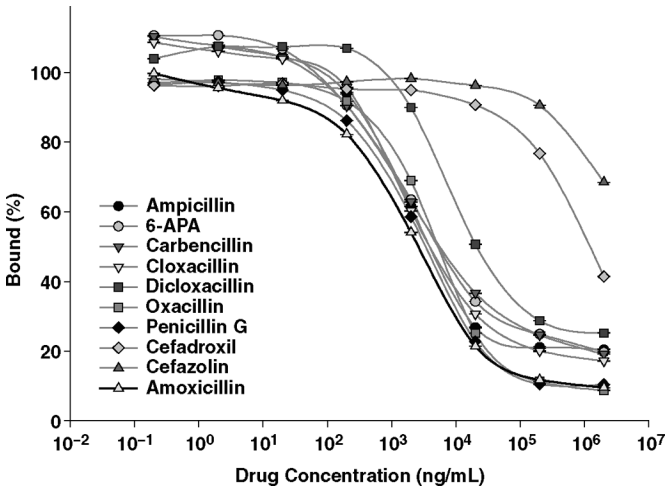

Figure 3. Standard inhibition curves depicting cross-reactivity of amoxicillin and selected pharmacological or structural congeners, using conditions described in the Materials and Methods section (n = 5). 6 APA = 6-aminopenillinic acid.

concluded that the assay was suitable for detection of AMX immunoreactivity in urine, serum, and milk samples.

Intra- and Interassay Variations and Accuracy of the Assay

Mean intra- and interassay variations were determined through repeated analyses of 5 plates with triplicate wells containing AMX at 5 concentrations (10 and 1 μg/mL and 100, 10, and 1 ng/mL) in PBS. The results indicated that the intraassay variations were in the range of 1.4 to 4%, whereas the interassay variations were in the range of 0.6 to 8.9% (Table 2). The accuracy of the assay was expressed by recovery (%). The mean recoveries of AMX in different matrices ranged from 92.7 ± 5.6% in serum to 109.8 ± 6.4% in milk (Table 3).

Cross-Reactivity

Cross-reactivity was calculated as 100 times the concentration ratio of a test drug over the AMX concentration required to achieve an equivalent inhibition ($100 \times IC_{25/50/75\ drug}/IC_{25/50/75\ AMX}$). Standard inhibition curves for each analog are shown in Figure 3. Using this ELISA, we determined that the anti-AMX antibody cross-reacted highly with penicillin G (77.39%), cross-reacted moderately with ampicillin, oxacillin, and cloxacillin (56.87, 51.41, and 48.79%, respectively), and cross-reacted minimally with dicloxacillin (7.4%). Cefadroxil and cefazolin had insignificant (<1%) cross-reactivity with the developed AMX antibody. The chemical structures of AMX and its analogs and their cross-reactivities are summarized in Table 4 and Figure 4.

In Vitro Recovery of the Microdialysis Probe and AMX Protein Binding

The in vitro recoveries of AMX at combinations of 3 concentrations and 3 flow rates are shown in Table 5. The recoveries were found to be inversely related to the flow rate but remained relatively constant over 3 different concentrations. The optimal flow rate of 1 μL/min was chosen thereafter in view of its acceptable combination of recovery and sample volume. In addition, a preliminary study (data not shown) confirmed that drug passage through the dialysis membrane exhibited bidirectional symmetry, such that the fraction of drug recovered from an isotonic medium (recovery mode) was equivalent to the fraction of drug that diffused from the perfusion fluid into the external drug-free medium (delivery mode). This result provided a rational basis for using the delivery method as a

Table 2. Intra- and interassay variations for amoxicillin analyzed by the developed ELISA[1]

Amoxicillin concentration (μg/mL)	Intraassay variation		Interassay variation	
	Average binding (%)	CV%	Average binding (%)	CV%
10^1	16.7 ± 0.7	4.0	15.2 ± 2.4	9.6
1	55.5 ± 1.2	2.1	60.8 ± 5.1	8.4
10^{-1}	85.3 ± 1.9	2.3	88.9 ± 3.4	3.8
10^{-2}	94.3 ± 1.3	1.4	92.9 ± 2.3	2.5
10^{-3}	97.0 ± 1.4	1.4	98.6 ± 0.6	0.6

[1]Variations were tested on 5 concentrations prepared in PBS (n = 5).

Table 3. Accuracy of the amoxicillin ELISA in various matrices[1]

	Nominal concentration (recovery %)			
Matrix	10 μg/mL	%	800 ng/mL	%
PBS	9.51 ± 0.25	95.1 ± 2.7	753 ± 59	94.1 ± 7.9
Serum	9.27 ± 0.51	92.7 ± 5.6	780 ± 32	97.5 ± 4.1
Urine	10.77 ± 0.31	107.7 ± 2.9	780 ± 36	97.4 ± 4.7
Milk	10.97 ± 0.70	109.8 ± 6.4	849 ± 43	106.1 ± 5.1

[1]Numbers under the percentage sign (%) represent the calculated percentage recovery (n = 5).

way to measure AMX recovery in vivo for microdialysis probes placed within the pectoral and femoral muscles of pigeons. The brief study that was carried out to delineate the extent of AMX binding to pigeon serum protein indicated that the binding was not high, at 27.9 ± 5.7%.

Comparison of AMX Concentrations in Venous Blood and Muscles

The results of concentration-time profiles for AMX in blood by venipuncture and for pectoral and femoral muscles by microdialysis are shown in Figure 5. The levels of AMX in intermittent venipuncture exhibited a quick rise to a peak concentration of 4.74 μg/mL approximately 30 min after injection. The peak concentrations of AMX in pectoral (0.97 μg/mL) and femoral (1.6 μg/mL) muscles occurred later, at approximately 60 min. Concentrations of AMX in both the pectoral and femoral muscles appeared to decrease gradually to a concentration of 0.3 to 0.5 μg/mL during the subsequent 8-h monitoring period. Amoxicillin achieved peak concentrations in muscles that were significantly lower than the peak AMX concentration in venous blood. Pharmacokinetic analysis of AMX concentrations in the muscles revealed statistically significant differences in the apparent half-life ($t_{½}$), peak time (T_{max}), and peak concentration (C_{max}) between the 2 muscle locations and from those obtained in blood (Table 6). The lowest blood concentration of AMX (at 8 h) remained above 0.6 μg/mL, indicating that a 10-fold dilution was appropriate to maintain an accurate concentration conversion because the diluted, lowest concentration (60 ng/mL) was well within the linear working range of the standard curve (Figure 2).

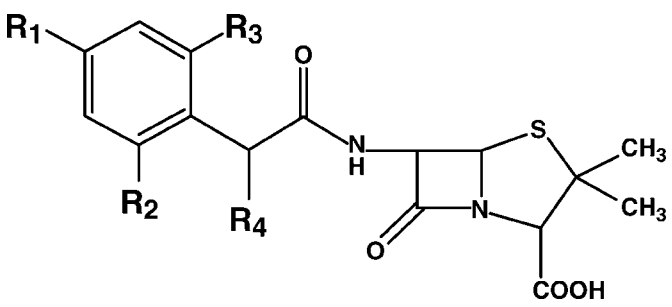

Figure 4. Chemical backbone structure of amoxicillin and substitution sites for 9 pharmacologically or structurally related congeners. Refer to Table 4 for substituents.

DISCUSSION

In the present study, we described a method for microdialysis-based monitoring of AMX concentrations in skeletal (pectoral and femoral) muscles of the pigeon and for verifying the results by using the developed ELISA. Amoxicillin was selected as a prototype agent for this investigation in view of its frequent use and lack of amoxicillin-specific and more updated immunoassays to detect it; in addition, its pharmacokinetics in the pigeon had never been investigated by using microdialysis. After development of the ELISA, AMX concentrations with time were assessed and the pharmacokinetic parameters of AMX in central (blood) and peripheral (muscles) compartments were compared. Although ELISA is not generally considered a quantitative assay, it has nevertheless been used for various semiquantitative purposes, including pharmacokinetic analysis (Hill et al., 1992; Chou et al., 2001). The incentive for developing an effective ELISA testing procedure was based in part on the speed, sensitivity, and low cost of this technique; the effectiveness of an ELISA is largely associated with the specificity (and thus titers) of the antibody raised against the immunogen. To develop an AMX-specific ELISA, different attempts were made to obtain polyclonal antibodies comparably specific for AMX. Conjugation to a suitable carrier protein was first evaluated because drugs are small molecules, which alone do not induce satisfactory immunogenicity and thus possibly elicit a weak immune response. Many different carrier proteins can be used for coupling to synthetic drugs. The most commonly selected carriers are BSA, keyhole limpet hemacyanin (**KLH**), and human serum albumin (**HSA**). Ovalbumin is another often used carrier protein, usually chosen as a second carrier protein when verifying whether antibodies are specific for the drug alone or also specific to the carrier. In this study, AMX was coupled to different carriers (BSA, OVA, HSA, and 0.85-μm carboxyl-polystyrene particles) to render it immunogenic. The results (data not shown) suggested that AMX-BSA coupled with the GA method was considered more efficacious in producing antibodies than was the carbodiimide method. Therefore, conjugation of AMX with BSA by use of the GA method was used for developing the AMX ELISA in this study. In addition, to achieve optimal selectivity for this indirect ELISA, we found it necessary for the AMX-OVA conjugate to replace AMX-BSA in coating the plate. A literature review indicated that reports regarding ELISA designed specifically for AMX have been very limited (Hill et al., 1992; Mayorga et al., 2002), sug-

gesting that the production of a high-titer AMX-specific antibody might not be readily successful. Previously, Hill et al. (1992) reported an indirect competitive ELISA with detection and quantification limits of 10 and 50 ng/mL, respectively, to measure AMX penetrations into lung secretions. Mayorga et al. (2002) used polyclonal rabbit antiidiotypic antibodies to study antigenic determinants of AMX at a sensitivity of 20 ng/mL. Most of the other AMX-detecting ELISA were studies originating against other β-lactam drugs such as ampicillin (Dietrich et al., 1998), cloxacillin (Usleber et al., 1994), or 6-APA (Benito-Pena et al., 2005), which detects AMX on a cross-reactive basis

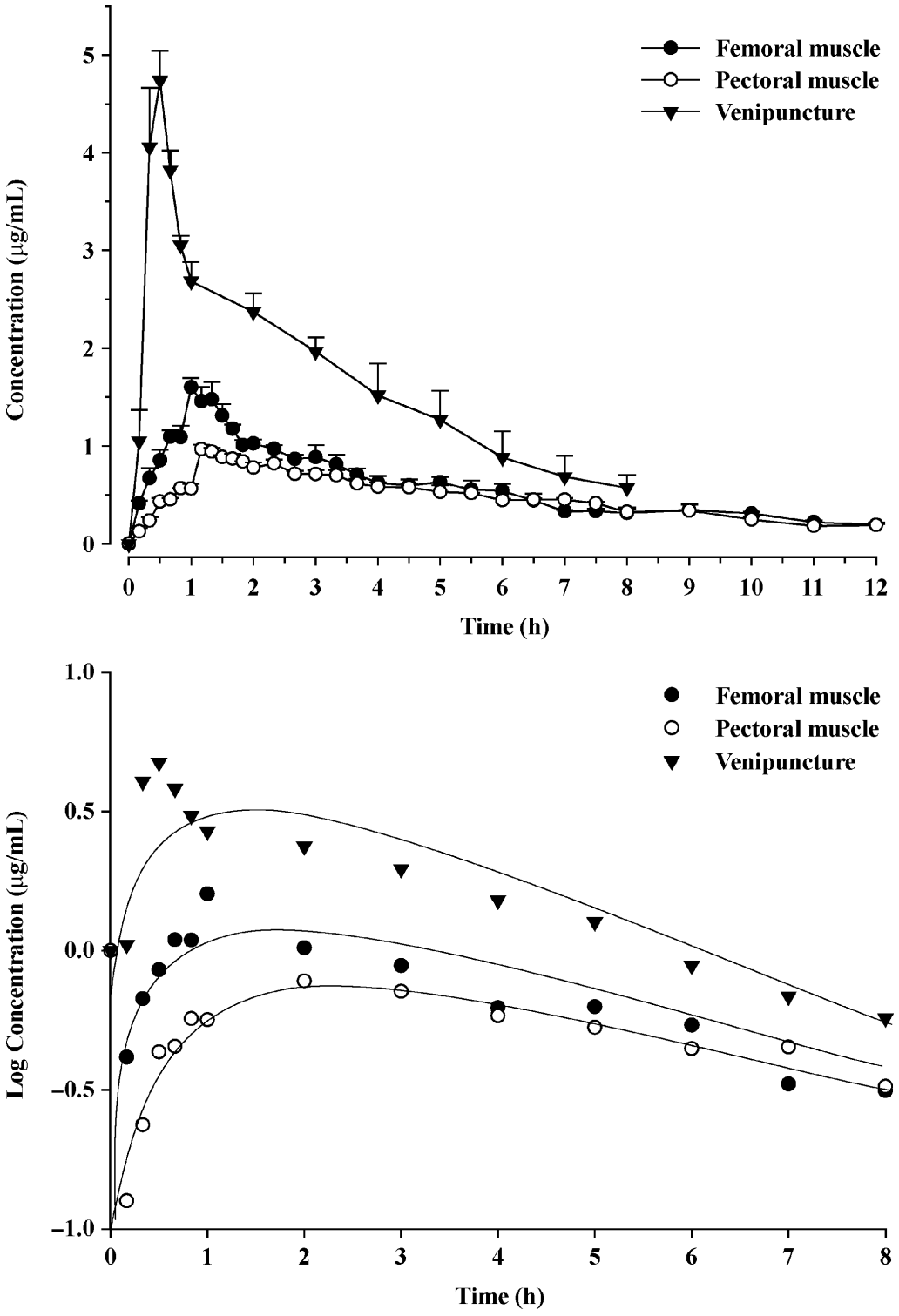

Figure 5. Concentration-time profiles for amoxicillin in blood and in the pectoral and femoral muscles by microdialysis. The lower panel depicts the log-transformed concentration-time profile. Dialysate fractions were collected at various intervals for 12 h, whereas blood samples were collected in parallel via venipuncture for 8 h (n = 3).

Table 4. Chemical substituents and cross-reactivities of amoxicillin and 9 pharmacologically or structurally related congeners[1]

β-Lactam	R_1	R_2	R_3	R_4	Cross-reactivity (%)
Amoxicillin	OH	H	H	NH_2	100.00
Penicillin G	H	H	H	H_2	77.39
Ampicillin	H	H	H	NH_2	56.87
Oxacillin	H	H	H	C_4H_3NO	51.41
Cloxacillin	H	H	Cl	C_4H_3NO	48.79
6-Aminopenillinic acid	—	—	—	NH_2	37.71
Carbenicillin	H	H	H	COOH	36.00
Dicloxacillin	H	Cl	Cl	C_4H_3NO	7.40
Cefadroxil	OH	H	H	NH_2	0.19
Cefazolin	—	H	—	H_2	0.03

[1]Refer to Figure 4 for chemical structures.

and is inferior to the sensitivity of direct AMX assays. Therefore, the ELISA test designed here for AMX offered at least an equal or improved combination of sensitivity and selectivity compared with previously published assays. The sensitivity of the assay was within the range of 5 ng/mL (Table 1), and AMX at low concentrations could be successfully detected in urine, serum, and milk samples. The broad spectrum of drugs detected and the high sensitivity made this method particularly useful as a screening technique for AMX in biological fluids.

The selectivity of an ELISA is characteristic of the specific antibody raised against the target compounds and is therefore of great importance insofar as it reflects the nature of the substances that are able to be detected by ELISA tests. On the basis of our results, it appeared that the developed polyclonal AMX antibody recognized some key multiple epitopes on the penicillin molecules (Table 4 and Figure 4). For instance, ampicillin, which differed in only 1 oxygen molecule at the R_1 substituent with AMX, shared only 56.87% cross-reactivity with AMX, suggesting that the R_1 position was a critical determinant for antibody recognition. On the other hand, the R_4 position showed significant but differential effects in cross-reactivity based on the substitution group. Penicillin G, oxacillin, cloxacillin, and carbenicillin, which also had 1 hydrogen substitute at the R_1 position like ampicillin but differed in the R_4 position, had variable cross-reactivities, ranging from 77 to 36%. The significant cross-reactivity drop of dicloxacillin from oxacillin and cloxacillin suggested that the R_2 substituent with a chloride significantly reduced the antibody recognition (from 49% to 7%), whereas the R_3 substituent of the same molecule (chloride) posed virtually no change to the cross-reactivity (Table 4 and Figure 4). These results pointed to a greater recognition of the R_1 and R_2 groups for this antibody in terms of cross-reactivity to AMX. Although cefadroxil, the first generation of cephalosporin, shared identical groups of R_1, R_2, R_3, and R_4 with AMX, the 6-membered dihydrothiazine ring (instead of the thiazolidine ring in penicillins) that diffused with the β-lactam ring resulted in a significantly reduced cross-reactivity (to only <1 %). A similar result was found with cefazolin, which could lead to the conclusion that first-generation cephalosporins should exhibit low cross-reactivity to this AMX antibody because of replacement of the thiazolidine ring. Although other newer generation cephalosporins were not tested in this study, on the basis of the current analysis of structure-reactivity, it seemed plausible that drugs such as ceftiofur, cephalothin, or cephaloridine, which all have a significant modification at the R_1 position and replacement of the thiazolidine ring, should have low cross-reactivity to the developed AMX antibody, making this ELISA comparatively selective to the most often used β-lactams. Nevertheless, it should be noted that the overall cross-reactivity is the result of multiple antibody recognition at multiple sites with different cross-reactive significances. Therefore, it may not be possible to explain or predict all cross-reactivities by substitutions at 1 or 2 sites. Nevertheless, compared with the 2 previously developed antibodies against AMX, both studies failed to provide suitable characterization of their antibodies in terms of the cross-reactivity to structurally similar β-lactam analogs. For those antibodies produced against ampicillin, cloxacillin, and 6-APA that also cross-reacted to AMX, their cross-reactivity patterns were very different from ours. For example, the ampicillin-KLH antibody (Dietrich et al., 1998), although detecting AMX completely (108% at more than 100% cross-reactivity), detected penicillin G (30% cross-reactivity) and oxacillin (14% cross-reactivity) at much

Table 5. In vitro recoveries of amoxicillin at combinations of concentrations and 3 flow rates (0.5, 1, and 2 μL/min)[1]

Amoxicillin concentration (ng/mL)	Recovery (%)		
	0.5 μL/min	1 μL/min	2 μL/min
1,000	77.37 ± 8.18	57.17 ± 6.04	30.13 ± 8.29
500	74.33 ± 11.10	50.11 ± 8.88	39.33 ± 6.35
100	74.54 ± 8.09	51.67 ± 1.25	29.80 ± 0.69

[1]Values represented mean ± SEM for 4 trials (n = 4).

Table 6. Derived pharmacokinetic parameters for amoxicillin in venous blood and in the pectoral and femoral muscles[1]

Parameter	Venipuncture	Pectoral muscle microdialysis	Femoral muscle microdialysis
$t_{1/2}$ (h)	2.38 ± 0.16	4.07 ± 0.48[a]	3.01 ± 0.26[a,b]
T_{max} (h)	0.62 ± 0.17	1.94 ± 0.14[a]	1.17 ± 0.10[a,b]
C_{max} (μg/mL)	3.82 ± 0.3	0.81 ± 0.02[a]	1.45 ± 0.05[a,b]

[a]Different from venipuncture ($P < 0.05$).

[b]Different from pectoral muscle ($P < 0.05$).

[1]Values are presented as mean (±SEM) for 3 independent trials in pigeons administered i.m. amoxicillin (25 mg/kg).

lower sensitivities; the cloxacillin-HSA antibody (Usleber et al., 1994) cross-reacted only to oxacillin and dicloxacillin, but not to other β-lactams; and the 6-APA-KLH antibody (Benito-Pena et al., 2005) detected AMX and oxacillin-like compounds at much lower sensitivities (2 to 12% cross-reactivity). These comparisons indicate that the antibody raised and the ELISA developed here exhibited great sensitivity and a characteristic spectrum of selectivity, and therefore represented a novel addition to the existing antibody-based assays for semiquantitative analysis of AMX and certain β-lactams.

Although microdialysis is a well-established technique, its application has been mostly focused on laboratory animals and mammals. Very few studies have used microdialysis to evaluate muscle or other tissue concentrations of AMX until recently (Marchand et al., 2005; Sawchuk et al., 2005). This study described and validated the first microdialysis model for continuous sampling of drugs in vivo from the soft tissue (skeletal muscles) of pigeons. The capacity to measure levels of drugs and drug metabolites simultaneously by in vivo microdialysis in multiple tissues afforded investigators with a powerful and less invasive approach for conducting pharmacokinetic studies in pigeons and possibly other poultry species. In the present study, the pigeon was injected with a dose (25 mg/kg, i.m.) of AMX, and drug concentrations in the blood were measured by the developed ELISA. In view of the semiquantitative nature of ELISA and the small number of animals used, pharmacokinetic analysis of the drug in the pigeon was not the main purpose of the study. Nevertheless, estimates of pharmacokinetic parameters and the peak blood concentration and time (5 μg/mL at 20 to 30 min) agreed closely with results (5.8 μg/mL at 24 min) published previously for pigeons after i.m. injection of AMX at the same 25 mg/kg dosage (Escudero et al., 1998). At a higher i.m. dosage (100 mg/kg), a higher peak concentration (28.8 μg/mL) at a similar peak time (30 min; Dorrestein et al., 1987) or a similar peak concentration (5.04 μg/mL) at a slightly delayed peak time (54 min) after an oily suspension (Dorrestein et al., 1986) were reported previously. Both of these agreed reasonably well with the current results because a higher peak concentration from a higher dosage and a reduced peak concentration or delayed peak time from an oily suspension were expected in those situations. On the other hand, the serum elimination half-life ($t_{1/2}$) of 2.3 h in this study was longer than the 1.5 h in the study by Escudero et al. (1998) and the 0.55 h in other i.m. studies using a higher dosage (100 mg/kg). The difference in elimination half-life could be partially explained by the difference in dosages or dosage formations (Dorrestein et al., 1986) and the different models used for pharmacokinetic analysis. A terminal β $t_{1/2}$ could be longer than if the elimination were to be presented as a single $t_{1/2}$, which discounted the distribution phase (α $t_{1/2}$). Although it has been suggested that the number of animals needed in preclinical pharmacokinetic studies can be substantially reduced by applying the microdialysis technique (Fettweis and Borlak, 1996; de la Peña et al., 2000), larger sample sizes are always desirable to further validate these similarities and differences.

To our knowledge, tissue pharmacokinetic data of AMX were not available for the pigeon and were also rare for avian species. Therefore, despite small sample sizes, the muscle kinetic data in the current study represented 1 of very few studies available regarding the disposition and elimination of AMX in pigeon tissue and could be a useful reference for other avian species. In view of the limited published information, tissue pharmacokinetic comparisons based on a similar dosage and administration route were difficult. After daily oral applications of AMX (20 mg/kg) to chickens for 6 d, Lashev and Semerdzhiev (1983) suggested that tissue levels of AMX did not surpass the serum levels and that the therapeutic levels of AMX were retained better in the liver, kidney, lungs, and muscles than in the spleen, heart, and brain. After 4 consecutive daily subcutaneous injections of AMX (20 mg/kg) to turkeys, AMX concentrations were greatest in muscle 1 d after treatment ceased, with a subsequent rapid decline. The drug was undetectable in the liver and kidney 10 d after the final dosing (Tomasi et al., 1996). The available results, although fragmented, suggested good tissue (muscle) penetration of AMX in avian species and shorter muscle residual time than in the liver and kidney. In the current study, AMX concentrations rose quickly in blood following i.m. administration of 25 mg/kg of AMX, whereas muscle concentrations of AMX exhibited significant delays (at 60 min) and lower peak concentrations (0.9 μg/mL in pectoral muscle and 1.6 μg/mL in femoral muscle; Figure 5). The tissue penetration and distribution of antibiotics is of great importance, because many infections occur in the tissue. Amoxicillin concentrations obtained from femoral muscle were significantly higher than concentrations in pectoral

muscle. The apparent half-lives of AMX in pectoral and femoral muscles were increased by 26 and 71%, respectively. A significant pharmacokinetic difference was found between the 2 muscle sites (Table 6). A potential explanation for the plasma-to-tissue and femoral-to-pectoral gradient of AMX was that for certain analytes, unrestricted diffusion across capillaries cannot be taken for granted. Physiological factors that determine blood-to-interstitium transfer, such as local blood flow, local capillary density, and capillary permeability, may all have influences. Site-related differences in probe recovery should always be considered (Chou et al., 2001). In addition, local changes secondary to implantation of the probe could promote unequally elevated drug concentrations in muscle extracellular fluids. Placement of the probe within the muscle could cause local irritation and inflammation that change capillary permeability or the muscle microenvironment, leading to altered drug delivery or retention. Although the sample size in the current study was rather small and the basis for the differences between the 2 muscle sites is unknown, our results underscored the importance of monitoring drug levels directly at the potential tissue sites of action. Measurement of AMX concentration in muscle tissue may be necessary to provide better insight into the overall therapeutic effectiveness of this drug and its residual period. In developing the in vivo microdialysis model, its suitability to carry out measurements of AMX in pigeon skeletal muscles was tested. The pectoral and femoral muscles were chosen in an attempt to simulate the study of the 2 most frequently edible tissues in avian species. Anatomical factors also supported selection of these muscles, including the parallel orientation of myofibrils as well as the superficial location of the muscle, which facilitates reliable straightforward insertion (suturing) of the probe. A previous study suggested that many technical problems (reduced dialysate flow, probe breaks, etc.) could be avoided if probes were inserted parallel rather than orthogonal to the orientation of the muscle myofibrils (Chou et al., 2001). Although the reason for this difference is not entirely clear, we assume that probes oriented parallel to the myofibril long axis experience less physical stress associated with changes in muscle length. On the basis of these findings, we concluded that the microdialysis procedure described here represents a suitable method for the performance of pharmacokinetic studies in poultry species. Furthermore, microdialysis-based measurements offer a distinct advantage over traditional venipuncture techniques insofar as interactions with the experimental subject are greatly reduced and multiple tissues may be sampled simultaneously. These advantages could be truly significant for studies designed to elucidate effects wherein interactions between the experimenter and experimental subjects must be minimized. In theory, dialysate samples may be collected from any tissue on a continual basis from a single animal, thereby reducing both data variability and the required number of experimental subjects while maintaining satisfactory statistical significance. This is especially desirable for studies in rare animal species. Because of the fragile nature and small diameter of pigeon blood vessels, microdialysis in the blood was not performed. Although the current design prevented us from direct assessment of free AMX in the blood, it should be noted that in theory, significant differences between direct venipuncture and blood microdialysis are likely with a drug that is highly bound to plasma proteins. In such cases, the free drug concentration in the microdialysate would be expected to differ substantially from the total drug concentration in venipuncture samples because of the inability of bound drug molecules to cross the dialysis membrane (Bailey, 1998; Chou et al., 2001). Our protein-binding study indicated that AMX is not highly bound to pigeon plasma proteins (28%); therefore, it is more likely that the AMX concentration-time curves by microdialysis would be similar to the current results from venipuncture.

In conclusion, an immunoassay was developed for the detection of AMX as well as other β-lactam analogs in serum, urine, and milk samples. The ELISA exhibited great sensitivity and a characteristic spectrum of selectivity. In addition to AMX detection, we developed and validated a reliable in vivo sampling technique for pigeons based on tissue microdialysis. Results from this investigation demonstrated the practicality of using the first in vivo microdialysis in pigeons and revealed significant time-dependent differences in the free concentrations and pharmacokinetics of AMX in skeletal muscles and in the blood. The procedure allowed for continuous measurement of free AMX levels in soft tissues and underscored the advantages of this method for evaluating drug kinetics in muscles. This approach should be readily adaptable for use in other fowl species and could improve our understanding of clinical pharmacokinetics for effective therapy as well as for the capacity to estimate drug residues in specific tissues of interest.

REFERENCES

Bailey, D. N. 1998. Relative binding of therapeutic drugs by sera of seven mammalian species. J. Anal. Toxicol. 22:587–590.

Benito-Pena, E., M. C. Moreno-Bondi, G. Orellana, A. Maquieira, and A. van Amerongen. 2005. Development of a novel and automated fluorescent immunoassay for the analysis of beta-lactam antibiotics. J. Agric. Food Chem. 53:6635–6642.

Bito, L., H. Davson, E. M. Levin, M. Murray, and N. Snider. 1966. The concentrations of free amino acids and other electrolytes in cerebrospinal fluid, in vivo dialysate of brain, and blood plasma of the dog. J. Neurochem. 13:1057–1067.

Brunner, M., H. Derendorf, and M. Muller. 2005. Microdialysis for in vivo pharmacokinetic/pharmacodynamic characterization of anti-infective drugs. Curr. Opin. Pharmacol. 5:495–499.

Carter, G. R., M. M. Chengappa, G. W. Clause, and Y. Rikihisia. 1991. *Haemophilus* and *Taylorella*. Pages 187–190 in Essentials of Veterinary Bacteriology and Mycology. 4th ed. G. R. Carter, and M. M. Chengappa, ed. Lea & Febiger, Philadelphia, PA.

Cha, J. M., S. Yang, and K. H. Carlson. 2006. Trace determination of beta-lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry. J. Chromatogr. A. 1115:46–57.

Chard, T. 1987. An introduction to radioimmunoassay and related techniques. Pages 4–15 in Laboratory Techniques in Biochemistry and Molecular Biology. 4th ed. T. Chard, ed. Elsevier Science, New York, NY.

Chou, C. C., A. I. Webb, M. P. Brown, R. Gronwall, and T. W. Vickroy. 2001. Continuous measurement of caffeine and two

metabolites in blood and skeletal muscle of unrestrained adult horses by semi-automated in vivo microdialysis. J. Vet. Pharmacol. Ther. 24:405–414.

de la Peña, A., T. Dalla Costa, J. D. Talton, E. Rehak, J. Gross, U. Thyroff-Friesinger, A. I. Webb, M. Müller, and H. Derendorf. 2001. Penetration of cefaclor into the interstitial space fluid of skeletal muscle and lung tissue in rats. Pharm. Res. 18:1310–1314.

de la Peña, A., P. Liu, and H. Derendorf. 2000. Microdialysis in peripheral tissues. Adv. Drug Deliv. Rev. 45:189–216.

Dietrich, R., E. Usleber, and E. Märtlbauer. 1998. The potential of monoclonal antibodies against ampicillin for the preparation of a multi-immunoaffinity chromatography for penicillins. Analyst 123:2749–2754.

Dorrestein, G. M., H. Van Gogh, J. D. Rinzema, and M. N. Buitelaar. 1986. Bioavailability and pharmacokinetics of ampicillin and amoxycillin from tablets, capsules and long-acting preparations in the homing pigeons (*Columba livia*). J. Vet. Pharmacol. Ther. 9:394–401.

Dorrestein, G. M., H. Van Gogh, J. D. Rinzema, and M. N. Buitelaar. 1987. Comparative study of ampicillin and amoxycillin after intravenous, intramuscular and oral administration in homing pigeons (*Columba livia*). Res. Vet. Sci. 42:343–348.

Duan, Y. L., L. M. Du, and C. P. Chen. 2005. Study on extractive spectrophotometry for determination of amoxicillin with ionic associate. Guang Pu Xue Yu Guang Pu Fen Xi 25:1865–1867.

Escudero, E., M. S. Vicente, and C. M. Carceles. 1998. Pharmacokinetics of amoxicillin/clavulanic acid combination after intravenous and intramuscular administration to pigeons. Res. Vet. Sci. 65:77–81.

Fettweis, G., and J. Borlak. 1996. Topics in xenibiochemistry—Application of microdialysis technique in pharmacokinetic studies. Xenobiotica 26:473–485.

Handsfield, H. H., H. Clark, F. James, K. K. Holmes, and M. Turck. 1973. Amoxicillin, a new penicillin antibiotic. Antimicrob. Agents Chemother. 3:262–265.

Hansen, D. K., M. I. Davies, S. M. Lunte, and C. E. Lunte. 1999. Pharmacokinetic and metabolism studies using microdialysis sampling. J. Pharm. Sci. 88:14–27.

Harrison, G. J., L. R. Harrison, and B. W. Ritchie. 1994. Principles and application. Pages 997–1006 in Avian Medicine. L. Bauck, ed. Wingers Publishing Inc, Lake Worth, FL.

Hayashi, Y., R. Matsuda, T. Maitani, K. Imai, W. Nishimura, K. Ito, and M. Maeda. 2004. Precision, limit of detection and range of quantitation in competitive ELISA. Anal. Chem. 76:1295–1301.

Hill, S. L., D. Burnett, A. L. Lovering, and R. A. Stockley. 1992. Use of an enzyme-linked immunosorbent assay to assess penetration of amoxicillin into lung secretions. Antimicrob. Agents Chemother. 36:1545–1552.

Jiang, X., Z. Zhang, M. Li, D. Zhou, F. Ruan, and Y. Lu. 2006. Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50:2990–2995.

Joukhadar, C., N. Klien, B. X. Mayer, N. Kreischitz, G. Delle-Karth, P. Palkovits, G. Heinz, and M. Müller. 2002. Plasma and tissue pharmacokinetics of cefpirome in patients with sepsis. Crit. Care Med. 30:1478–1482.

Klein, G. F., U. Stanzl, P. O. Fritsch, and J. M. Varga. 1993. Enzyme-linked immunosorbent assay for detection of antibodies to the unmodified beta-lactam ring. Allergy 48:151–157.

Kovar, A., T. Dalla Costa, and H. Derendorf. 1997. Comparison of plasma and free tissue levels of ceftriaxone in rats by microdialysis. J. Pharm. Sci. 86:52–56.

Lapresle, C., and P. Lafaye. 1985. Enzyme-linked immunosorbent assay for measurement of penicilloyl groups. Ann. Inst. Pasteur Immunol. 136C:375–382.

Lashev, L., and V. Semerdzhiev. 1983. Comparative studies of amoxicillin resorption and distribution in poultry. Vet. Med. Nauki 20:22–28.

Le-Quellec, A., S. Dupin, and A. E. Tufenkji. 1994. Microdialysis: An alternative for in vitro and in vivo protein binding studies. Pharm. Res. 11:835–838.

Li, W., F. Tan, and K. Zhao. 2006. Simultaneous determination of amoxicillin and ranitidine in rat plasma by high-performance liquid chromatography. J. Pharm. Biomed. Anal. 41:594–598.

Li, Y., and J. Lu. 2006. Chemiluminescence flow-injection analysis of beta-lactam antibiotics using the luminol-permanganate reaction. Luminescence 21:251–255.

Liu, P., M. Müller, M. Grant, A. I. Webb, B. Obermann, and H. Derendorf. 2002. Interstitial tissue concentrations of cefpodoxime. J. Antimicrob. Chemother. 20:19–22.

Marchand, S., M. Chenel, I. Lamarche, and W. Couet. 2005. Pharmacokinetic modeling of free amoxicillin concentrations in rat muscle extracellular fluids determined by microdialysis. Antimicrob. Agents Chemother. 49:3702–3706.

Mayorga, C., M. J. Torres, A. Romano, F. Moreno, E. Sanchez-Sabater, C. Juarez, and M. Blanca. 2002. Monoclonal antibodies to amoxicillin express different idiotypes determined by anti-idiotype antibodies production. Allergy 72:45–51.

Neal, M. J. 2002. Medical Pharmacology at a Glance. 4th ed. Blackwell Science, Oxford, UK.

Perez, M. I., L. C. Rodriguez, and C. Cruces-Blanco. 2007. Analysis of different beta-lactams antibiotics in pharmaceutical preparations using micellar electrokinetic capillary chromatography. J. Pharm. Biomed. Anal. 43:746–752.

Popelka, P., J. Nagy, R. Germuska, S. Marcincak, P. Jevinova, and A. De Rijk. 2005. Comparison of various assays used for detection of beta-lactam antibiotics in poultry meat. Food Addit. Contam. 22:557–562.

Samanidou, V. F., E. N. Evaggelopoulou, and I. N. Papadoyannis. 2006. Development of a validated HPLC method for the determination of four penicillin antibiotics in pharmaceuticals and human biological fluids. J. Sep. Sci. 29:1550–1560.

Sawchuk, R. J., B. W. Cheung, P. Ji, and L. L. Cartier. 2005. Microdialysis studies of the distribution of antibiotics into chinchilla middle ear fluid. Pharmacotherapy 25:140S–145S.

Soenens, J., H. Vermeersch, K. Baert, B. Vermeulen, H. Nelis, P. Butaye, P. De Herdt, J. P. Remon, and P. De Backer. 1998. Pharmacokinetics and efficacy of amoxycillin in the treatment of an experimental Streptococcus bovis infection in racing pigeons (*Columba livia*). Vet. J. 156:59–65.

Talaber, Z. 2004. General information about medicines. Pages 66–102 in Pigeons and Their Economical Health Care. 2nd ed. Z. Talaber, ed. Vital Kontrol Ltd., Kecskemet, Hungary.

Tomasi, L., L. Giovannetti, A. Rondolotti, G. Della Rocca, and G. L. Stracciari. 1996. Depletion of the residues of colistin and amoxicillin in turkeys following simultaneous subcutaneous administration. Vet. Res. Commun. 20:175–182.

Tomasselli, F., P. Dittrich, A. Maier, M. Woltsche, V. Matzi, J. Pinter, S. Nuhsbaumer, H. Pinter, J. Smolle, and F. M. Smolle-Jüttner. 2003. Penetration of piperacillin and tazobactam into pneumonic human lung tissue measured by in vivo microdialysis. Br. J. Clin. Pharmacol. 55:620–624.

Usleber, E., M. Lorber, M. Straka, G. Terplan, and E. Martlbauer. 1994. Enzyme immunoassay for the detection of isoxazolyl penicillin antibiotics in milk. Analyst 119:2765–2768.

Zhang, L., Y. M. Wang, G. Shen, and B. Y. Chen. 1996. Enzyme-linked immunosorbent assay for detection of IgG against penicillins in children. Zhongguo Yao Li Xue Bao 17:274–277.