Redox Mode Electrochemical Detection Approach with Chemically Modified Electrodes for the Measurement of Frenolicin B (Antibiotic) and Related Compounds in Poultry Feed

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An electrochemical detection approach for frenolicin B (quinone antibiotic) in combination with high-performance liquid chromatography is described utilizing two sequential generator/detector electrodes. Initially, frenolicin B is electrolyzed, and then the reaction product is detected electrochemically at the second electrode. The efficiency of reduction for frenolicin B could be increased after chemically modifying the porous carbon electrodes with metallic zinc. The zinc-modified electrodes offered increased sensitivity for the detection of frenolicin B and related compounds compared with unmodified electrodes. Application of the technique for the measurement of frenolicin B in poultry feed is described.

Keywords: *Frenolicin B; electrochemical detection; poultry feed*

INTRODUCTION

Frenolicin B is an important animal feed antibiotic that is particularly effective against chicken coccidiosis and as feed efficiency enhancer in swine. *Streptomyces roseofulvus* produces a group of frenolicin structures (Figure 1) that are converted to frenolicin B postfermentation. Other related isochromanequinone structures synthesized by *Streptomyces* include kalafungin, nanaomycin, medermycin, and actinorhodin (Kakinuma *et al.*, 1990; Kitao *et al.*, 1980; Omura *et al.*, 1981; Cole *et al.*, 1987; Tanaka *et al.*, 1982).

Although frenolicin B and related compounds can be detected spectrophotometrically, the absorption maxima for these compounds are considerably different. This difference makes their detection and quantitation difficult when employing single wavelength UV detectors. Frenolicin B and related compounds all possess the 1,4-quinoid moiety. Therefore, it was considered that electrochemical detection may offer better sensitivity and selectivity for frenolicins.

Electrochemical detection in combination with HPLC has been used for the determination of biogenic quinones (Ikenoya *et al.*, 1979, 1981; Hart *et al.*, 1984, 1985; Haroon *et al.*, 1984, 1987). These methods are often compromised by slow electron-transfer kinetics at the electrode surface. As a consequence, reduction (or oxidation) occurs at potentials that are greater than the expected thermodynamic potential (Hart, 1993; Haroon, 1993). Both the selectivity and limits of detection of the electrochemical measurement are affected to an extent dependent on the magnitude of the applied overpotential. In many instances, the detection of an analyte may require an overpotential that is beyond that of solvent electrolysis, making detection impossible. Chemically modified electrodes (CMEs) can overcome this problem through the use of surface-bound redox mediators. These mediators catalyze the oxidation or reduction of specific solute species at substantially reduced potentials. Various approaches, such as direct adsorption, polymer film coating, and covalent binding, have been developed to immobilize electrocatalysts onto the electrode materials (Baldwin and Thomsen, 1991; Murray *et al.*, 1987; O'Shea and Lunte, 1994). The benefits of using CMEs include fast electron transfer reactions and reduction in electrode fouling.

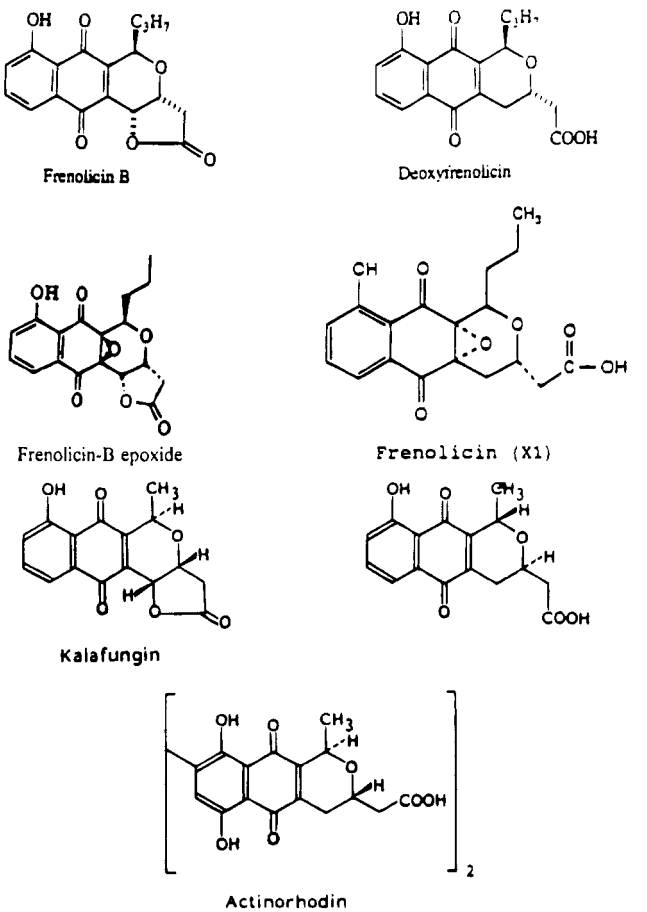

Figure 1. Structural formulas of some isochromanequinones.

The purpose of this paper is to elucidate a sensitive redox electrochemical detection approach with zinc-modified electrodes for the detection of frenolicin B and related compounds.

EXPERIMENTAL PROCEDURES

Chemicals. Frenolicin B was obtained from the Bioprocess Department at Hoffmann La-Roche, Nutley, NJ. Deoxyfre-

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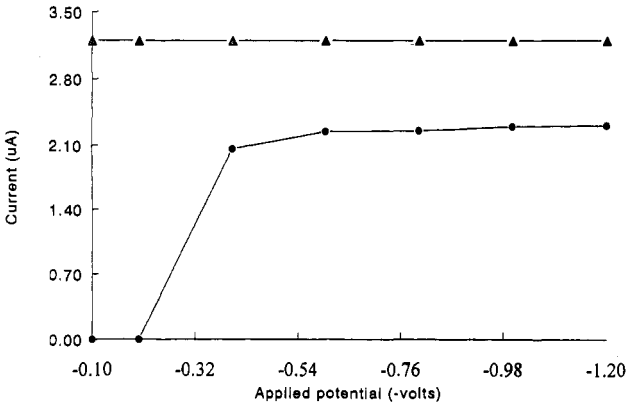

Figure 2. Cathodic hydrodynamic voltammograms for frenolicin B with unmodified (●) and zinc-modified electrodes (▲). Downstream electrode was held constant at +0.4 V.

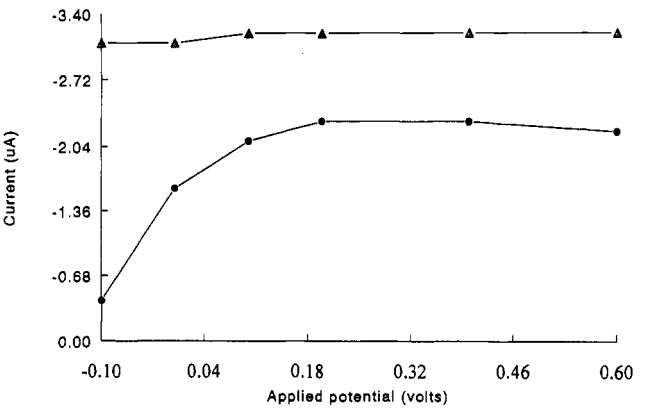

Figure 3. Anodic hydrodynamic voltammograms for frenolicin B with unmodified (●) and zinc-modified electrodes (▲). Upstream electrode was held constant at −1.2 V.

nolicin, α- and β-epoxides, was synthesized by Curtis Adams, Hoffmann La-Roche. Zinc chloride was obtained from Aldrich (Milwaukee, WI). All other chemicals were analytical reagent grade. HPLC-grade solvents were purchased from Burdick and Jackson (Muskegon, MI).

Apparatus. The liquid chromatograph consisted of a Model 510 reciprocating pump (Waters Chromatography, Milford, MA) coupled to a Coulochem II (ESA, Bedford, MA) electrochemical detector via an autoinjector (WISP, Model 717, Waters Chromatography). The electrochemical electrodes consisted of two porous graphite analytical electrodes (ESA; Model 5011) in series. A Zorbax C_{18} R_x column (150 × 4.6 mm i.d.; Mac-Mod Analytical, Chaddsford, PA) was employed during these studies.

Extraction of Frenolicin B from Feed. Ground feed (10 g) was weighed into a 500-mL volumetric flask. Anhydrous alcohol (200 mL) was added to the feed and the mixture was sonicated for 5 min. After sonication, the samples were agitated for 1 h on a mechanical shaker and allowed to soak in the extractant for ~12−14 h. In later experiments, frenolicin B was extracted from feed by sonicating in a water bath for 30 min at 40 °C. After extraction, the samples were filtered through a 0.45-μm glass microfiber filter (Whatman, Clifton, NJ) and 3−4-mL sample was transferred to a WISP vial.

Standard Solution. Frenolicin B (0.12−1.5 μg/mL) and related compounds (0.5 μg/mL) were dissolved in anhydrous alcohol and injected.

Electrode Modification. Unmodified electrodes were used as supplied by ESA. Zinc-modified electrodes were prepared by pumping 10 mM zinc chloride added to a mobile phase of acetonitrile−water (1:1 v/v) containing 0.1 M sodium acetate−acetic acid buffer (pH 4.5). During the electrode modification procedure, the potential of the generator electrode was set at −1.0 V for 6−8 h. Electrodes modified in this manner could be operated at a potential of −0.2 V for several weeks without loss of sensitivity or electrode passivation.

RESULTS AND DISCUSSION

Optimization of Redox Mode LCEC Conditions. To determine the optimum potential to apply to the two electrodes, two hydrodynamic voltammogram were constructed (Figures 2 and 3). The reductive voltammogram indicated that the maximum current (signal) was reached at −0.6 V. During reoxidation of frenolicin B hydroquinone back to frenolicin B quinone, the limiting plateau was observed at +0.4 V.

During redox-mode detection of frenolicin B, it was not necessary to remove dissolved oxygen from the mobile phase and the samples. The negative applied potential at the generator electrode was sufficient to induce reduction of frenolicin B and oxygen, while the applied detector electrode potential was sufficiently positive to oxidize frenolicin B hydroquinone but not peroxide or water.

Although the high surface area of the "coulometric" cells used in this assay usually maintain their sensitivity over several days of continued use, a problem encountered during electrochemical detection was the dramatic loss in sensitivity with time. This loss of reduction efficiency and electrode passivation is thought to occur via adsorption of metal species onto the electrode surface at the high negative potential used for the reduction of frenolicin B at the generator electrode. Although it was possible to inhibit electrode passivation by the addition of 0.1 mM EDTA to the mobile phase, the reduction efficiencies eventually decrease to unacceptable levels.

To regain sensitivity, one of the two procedures was adopted. The first is to reverse the polarity of the generator electrode to +1.2 V, while continuing to run the mobile phase. This procedure will oxidize the absorbed metals off the electrode surface *in situ*. When this "burn-off" procedure was ineffective, the cells had to be removed from the system and washed with 2 M sodium hydroxide, followed by 6 M nitric acid, and finally methanol. While the above procedures were acceptable in the research surroundings, when large numbers of samples needed to be processed on a routine basis, they were found to be cumbersome and time-consuming. The strategy developed to overcome the technical difficulties associated with electrode passivation was to modify the electrodes by depositing zinc metal onto the electrode surface. The porous graphite electrode was modified with zinc metal by adding zinc chloride (10 mM) to the mobile phase and allowing the solvent to flow through the detector for 6−8 h while maintaining the generator electrode potential of −1.0V. The E_0 for zinc reduction is −0.762 V. With the electrodes modified in this manner, it was possible to reduce frenolicin B at considerably lower potentials (−0.1 to −0.2 V). Because these zinc-modified electrodes could be operated at lower negative potentials (Figure 2), they were less susceptible to electrode passivation and more acceptable during routine analysis. A prerequisite when zinc-modified electrodes are employed is the necessity of removing oxygen from the mobile phase. Oxygen dissolved in the mobile phase can react with zinc (MacCrehan and May, 1984), leading to poor reduction efficiencies. Oxygen removal was achieved either by sparging the mobile phase with helium or *in situ* by inserting a precolumn between the pump and the injector to scavenge oxygen (MacCrehan and May, 1984).

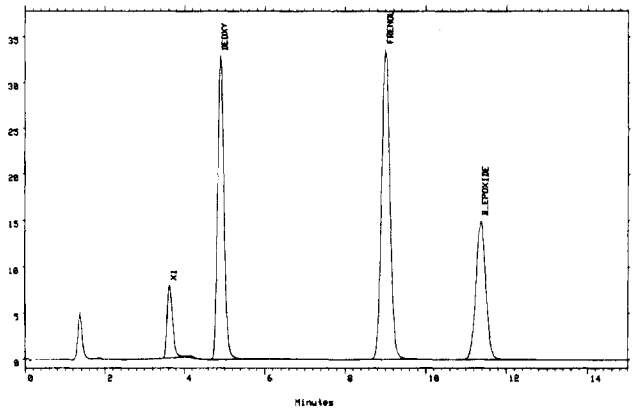

Figure 4. Redox mode detection of frenolicin B and related compounds.

Table 1. Extraction Efficiencies for Frenolicin B from Feed (Poultry) Employing Different Solvent Systems and Conditions

solvent system	frenolicin B (ppm)	% of claim	n	% CV
anhydrous alcohol	54.0	90.0	6	2.4
anhydrous alcohol (soak)	60.0	100	6	1.3
anhydrous alcohol (sonicate at 40 °C for 30 min)	61.6	103	2	2.6
acetonitrile (soak)	54.0	90.0	6	1.7
propan-2-ol (soak)	44.4	74.0	2	4.2
0.1% HCl in methanol (soak)	52.1	87.0	2	3.5

An added advantage that was gained with zinc-modified electrodes was the increased sensitivity for the detection of frenolicin B compared with unmodified electrodes (Figures 2 and 3).

Chromatography of Frenolicin B and Related Compound Standards. During initial experiments, when chromatography was performed with unmodified electrodes, it was only possible to detect frenolicin B and deoxyfrenolicin. Epoxide derivatives of frenolicin compounds, such as the α- and β-epoxides and X_1, were detected with extremely poor sensitivities. Similar detection efficiencies for frenolicin B, deoxyfrenolicin, and their epoxide derivatives could be obtained after modification of the porous graphite electrodes with metallic zinc. A representative chromatogram illustrating the detection of frenolicin B and related compounds with zinc-modified electrodes is shown in Figure 4.

Preliminary results indicate that these zinc-modified electrodes are also useful for the detection of other quinones, such as menadione (feed additive), K vitamins (phylloquinone and menaquinones), and ubiquinones (Haroon, 1993). Additionally, quinone derivatives of anthraquinone, which are added during the modern wood pulping process to enhance deligninification, could also be detected electrochemically with the zinc-modified electrodes.

Assay for Frenolicin B in Poultry Feed. *Extraction.* The extraction solvents and conditions that were evaluated to develop an efficient extraction system for frenolicin B from poultry feed is shown in Table 1. In earlier experiments, frenolicin B was extracted with 0.1% HCl in methanol because this solvent system was effective for extracting lasalocid from feeds. Extraction of poultry feed with acidic methanol led to poor recoveries for frenolicin B (Table 1). The major reason for the low recoveries was the instability of frenolicin B in methanol. Currently, available data suggest that frenolicin B participates in hydrogen abstraction reaction (reduction) with methanol, which is the hydrogen atom donor (HAD) compound (Poulsen and Birks, 1989). The products of this reaction are deoxyfrenolicin and the methyl ester of deoxyfrenolicin:

$$Q + CH_3OH \rightarrow {}^0QH + {}^0CH_2OH \quad (1)$$

$${}^0CH_2OH + Q \rightarrow {}^0QH + CH_2O \quad (2)$$

$$2\,{}^0QH \rightarrow Q + QH_2 \quad (3)$$

$$QH_2 + O_2 \rightarrow dQ + H_2O \quad (4)$$

Q represents frenolicin B quinone, ^{0}QH, frenolicin B semiquinone, QH_2 frenolicin B hydroquinone, and dQ, deoxyfrenolicin quinone. Evaluation of the results shown in Table 1 suggested that the best extractant for frenolicin B in poultry feed was anhydrous alcohol.

To obtain efficient extraction of frenolicin B from feed, it was necessary to soak the feed sample in alcohol overnight prior to analysis. The data shown in Table 1 indicate that if the soaking step was omitted, ~10% less frenolicin B was recovered. It was later found that if feed samples were extracted by sonication and heating at 40 °C for 30 min, recoveries similar to that found for samples which were soaked overnight were obtained (Table 1). This procedure was therefore adopted for routine analysis of poultry feed samples.

Precision, Accuracy, and Linearity. The within-run precision of the assay procedure for poultry feed samples, containing 60 ppm of frenolicin B, was ~3.0% for six replicate analysis. The between-run precision was 5.0%. The accuracy of the assay was evaluated by adding known amounts of frenolicin B to control feed and analyzing the samples by the entire assay procedure. The results, summarized in Table 2, indicate that there was good agreement between the levels of frenolicin B recovered and those added. The linearity for injections of standard solutions of frenolicin B is shown in Figure 5.

Analysis of Frenolicin B in Feed Samples. A representative chromatogram for the analysis of frenolicin B in feed sample is shown in Figure 6. A similar

Table 2. Accuracy and Recovery for Frenolicin B from Poultry Feed

frenolicin B added to feed[a]	frenolicin B recovered[b]	% recovery
30.31	30.52	100.7
59.75	60.20	100.7
120.23	120.23	99.8

[a] Frenolicin B added to control feed containing no drug. [b] Values are mean of six replicates.

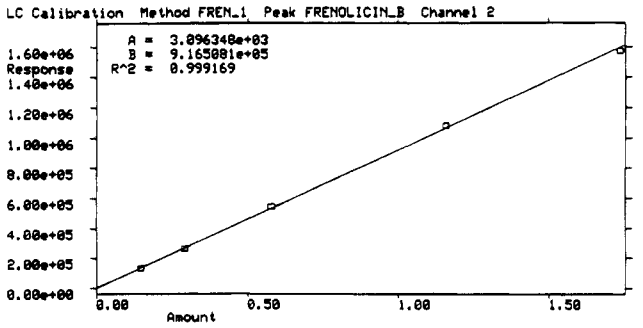

Figure 5. Linear curve for frenolicin B.

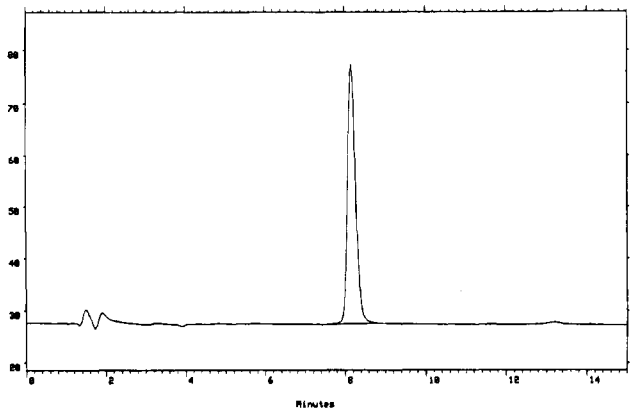

Figure 6. Chromatogram of poultry feed obtained by LCEC.

analysis of control feed indicated that there were no interfering peaks co-eluting with frenolicin B.

CONCLUSIONS

The results reported here indicate that electrochemical detectors based on chemically modified porous carbon electrodes can be an attractive mode for detecting frenolicin B and related compounds. These zinc-modified electrodes were stable and reproducible in performance, while providing enhanced sensitivity for some frenolicin-related compounds. Although the method proved useful for the analysis of frenolicin B in feed, the major advantage of the method will be realized during the analysis of frenolicin B and its metabolic products in tissue samples.

The electrochemical detection approach presented here was not limited to the analyses of frenolicins. Currently available data suggest that other biogenic quinones and quinone derivatives added in the wood pulping process could also be detected, in the redox mode, on zinc-modified electrodes.

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