A Rapid Fluorescence Assay for Danofloxacin in Beef Muscle: Effect of Muscle Type on Limit of Quantitation†

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ABSTRACT

A simple, rapid fluorescence screening assay was applied to the analysis of beef muscle for danofloxacin at the U.S. tolerance level of 200 ng/g. Muscle samples were homogenized in acetic acid—acetonitrile, the resultant mixture centrifuged, and fluorescence of the supernatants was then measured. The significant difference between the fluorescence of control muscle sample extracts and extracts of samples fortified at 200 ng/g allowed for successful discrimination between the samples. Setting a threshold level at the average 200 ng/g fortified sample extract fluorescence —3σ allowed for identification of potentially violative samples. Successful analysis of a group of blind fortified samples over a range of concentrations was accomplished in this manner, without any false-negative results. The limits of quantitation for danofloxacin, as well as enrofloxacin, using this assay were determined in three types of beef muscle (hanging tenderloin, neck, and eye round steak), as well as in serum. Significant differences in limits of quantitation were found among the three different muscle types examined, with hanging tenderloin muscle providing the lowest value. This work not only shows the potential for use of the fluorescence screening assay as an alternative to currently used microbial or antibody-based assays for the analysis of danofloxacin in beef muscle, but also suggests that assays using beef muscle may vary in performance depending on the specific muscle selected for analysis.

Fluoroquinolones (FQs) are a class of antibiotics displaying activity against a wide range of gram-positive and gram-negative bacteria, and are used in both human medical and veterinary applications. Use of FQs in food animals and the potential for residues that could lead to increased microbial resistance has led to some concern. Thus, efficient methods are needed to monitor the food supply to ensure that any residues, if present, are at a concentration below the U.S. Food and Drug Administration tolerance level. A large number of beef and dairy cows and steers are slaughtered annually in the United States, yet only a small percentage of these animals are identified as containing violative levels of antimicrobials. In 2005, for example, the number of violations was less than 1.5% of the animals selected by inspectors for testing (10). With such a low level of violations, rapid screening assays can be used to efficiently identify those relatively few samples that may contain problematic levels of antimicrobials, and then only these samples need be sent for further quantitative and confirmatory analysis. Such an approach can save a considerable amount of time, expense, and effort.

Screening assays in use for the FQs typically involve either immunoassays, which necessitate obtaining the correct antibodies (4, 5, 11) or microbial inhibition assays using one to seven agar plates, frequently involving long incubation times (3, 6–8). Variations on the classic microbial inhibition assay that utilize thermophilic bacteria, offer shorter incubation times, but are less sensitive to FQs (1, 2).

We have recently developed a rapid, simple fluorescence screening assay for enrofloxacin (ENRO) in chicken muscle, and have applied this approach to tetracyclines in chicken muscle as well (9). Danofloxacin (DANO) has recently been approved for use in cattle by the U.S. Food and Drug Administration, and it would thus be useful to provide a facile rapid screening method for DANO in beef. In this work, we show that the fluorescence screening approach can be used successfully in beef muscle, in detecting danofloxacin at its tolerance level of 200 ng/g. In the course of this study, we also unexpectedly found that the sensitivity of the assay varies depending on the type of muscle tissue selected for analysis.

MATERIALS AND METHODS

Reagents and solutions. DANO was obtained from Pfizer (Groton, Conn.) and ENRO (99.9%), from Bayer (Kansas City, Mo.). High-performance liquid chromatography—grade acetonitrile was from J.T. Baker (Phillipsburg, N.J.), and USP glacial acetic acid was from Mallinckrodt (Paris, Ky.). Stock solutions (100 μg/ml) of each FQ were prepared in 0.03 M NaOH and stored refrigerated. Stock solutions were prepared freshly after 6 months.

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All aqueous solutions were prepared using deionized water from a Barnstead (Dubuque, Iowa) E-pure system.

**Beef samples.** Beef hanging tenderloin, eye round, and neck muscle samples, as well as beef blood were obtained from local processing plants. Beef muscle samples were initially frozen and then individually thawed for processing. Any major areas of fat were removed, the muscle was cut into small pieces, homogenized in a food processor, and then refrozen at —20°C. Beef blood was allowed to clot and then centrifuged (1,500 × g for 20 min) to yield serum, which was then frozen at —80°C.

**Fortification and extraction of FQs.** Samples of beef muscle or serum (2.0 g) were weighed into 50-ml disposable centrifuge tubes. Fortified samples were prepared by addition of a small volume (100 μl) of a dilution of either DANO or ENRO in methanol in order to achieve the desired fortification level. The same volume of methanol was then added to control samples. A solution of 1% acetic acid in acetonitrile (6 ml) was added to each sample. Muscle samples were homogenized using an Ultra-Turrax homogenizer (Janke and Kunkel, Cincinnati, Ohio), while serum samples were vortex mixed (15 s). After centrifugation (3,696 × g for 20 min), the supernatants were decanted into 15-ml disposable centrifuge tubes. If matrix matched samples were desired, then an identical volume of FQ dilution was added to blank extracts at this point. Fluorescence of the supernatants was then measured.

**Fluorescence analysis.** Fluorescence was measured with a Cary Eclipse fluorescence spectrometer (Varian, Walnut Creek, Calif.), with Cary Eclipse software controlling the instrument operation and signal processing. Fluorescence of samples was measured in 3-ml quartz cuvettes, which were washed between samples with deionized water and acetone. DANO fluorescence in muscle was monitored at excitation (λ_εx) = 288 nm and emission (λ_εm) = 440 nm, while ENRO in muscle was monitored at λ_εx = 320 nm and λ_εm = 440 nm. DANO fluorescence in serum was measured using λ_εx = 285 nm and λ_εm = 440 nm, while ENRO used λ_εx = 278 nm and λ_εm = 440 nm in serum. In each case, emission and excitation slits were set at 10 nm.

**Preparation of blind samples.** A protocol was established in which concentrations of DANO were randomly selected with each of the following parameters: 20 samples having concentrations >200 ng/g, 20 samples having concentrations >0, but <200 ng/g, and 10 control samples with no DANO present. Individual hanging tenderloin samples were randomly assigned to each concentration. The appropriate hanging tenderloin samples were then fortified according to the protocol, with 10 samples being prepared daily. The samples were extracted and their fluorescence measured in blind fashion by another analyst.

**RESULTS AND DISCUSSION**

The initial goal of this work was to determine if fluorescence could be used as a rapid chemical assay method for DANO in beef muscle at the 200 ng/g tolerance level. A preliminary experiment comparing the fluorescence of control muscle extracts with that of extracts of muscle fortified with DANO at 200 ng/g looked promising, with an approximately sevenfold difference in fluorescence response between the control and fortified samples.

The next step was to determine the reproducibility of this initial result, using muscle from different individual animals. A local beef processing plant was able to provide samples of hanging tenderloin muscle from each of 18 different steers. Fortification and extraction of these samples, which was then followed by fluorescence analysis, led to the results illustrated in Figure 1. As can be seen in the figure, a significant fluorescence response difference observed between control and DANO-fortified (200-ng/g) samples was maintained among the different animals. A mixed-model analysis of variance (ANOVA) confirmed this difference as significant (P < 0.001). The fluorescence of control sample extracts was quite low, and displayed surprisingly low relative standard deviations (RSDs), both within three replicates of one sample (<3%) and between samples (7.7%). Fortified samples displayed somewhat larger RSDs (<5% within three replicates, 8.5% between samples). In Figure 1, the average control and fortified fluorescence response values are identified (x̄_0 and x̄_{200}, respectively), as well as these values ±3 SDs (i.e., x̄_0 ± 3σ, x̄_{200} ± 3σ). The considerable fluorescence response difference between the upper boundary of the control area (x_0 + 3σ) and the lower boundary of the fortified area (x_{200} - 3σ) indicates this approach should provide a useful screening method for DANO. In a monitoring situation, 99.8% of the samples containing DANO would display a fluorescence level greater than the upper boundary set for the con-
control area ($x_0 + 3\sigma$) in a random distribution profile. Similarly, 99.8% of the samples with a fluorescence level greater than the upper boundary set for the 200-ng/g fortified region would be expected to be violative and would need further investigation. Setting a decision boundary at this higher level would result, however, in a somewhat lower detection rate of violative samples, as samples in the area between $x_{200} - 3\sigma$ and $x_{200} + 3\sigma$ may also have DANO concentrations greater than the tolerance. Setting a decision boundary at the $x_{200} - 3\sigma$ level would be expected to increase the number of false-violative samples, but would reduce the number of false-negative results. This combination might lead to a few additional samples requiring further analysis, but given the low rate of violations typically found, should not pose an undue burden, while optimizing detection of violative samples.

In order to determine the recovery of DANO from the extraction procedure used in this study, matrix matched samples were analyzed as well for each of the 18 samples represented in Figure 1. Recoveries based on fluorescence responses were calculated as \((\text{fortified} - \text{control})/(\text{matrix matched} - \text{control})\) and ranged from 61 to 70%.

The fluorescence assay was tested using blind fortified samples. The results are listed in Table 1. The first test was to determine if the assay would successfully discriminate between samples containing DANO and the control samples. The assay performed well, successfully detecting all DANO-containing samples, including the lowest sample concentration used, 11 ng/g. No false-positive results were obtained with this test. The second test of the assay results was to determine whether the assay would successfully detect violative samples, given the tolerance of 200 ng/g for DANO in beef muscle. Here again, the assay performed well. As discussed, one could choose different limits. If the highest limit is chosen, $>x_{200} + 3\sigma$, then 16 of the 20 violative samples are identified, with four false-negative results. If the lower limit, $>x_{200} - 3\sigma$ is chosen, then all violative samples are identified, with an accompanying four false-violative results (positives with concentrations <200 ng/g). This lower limit, as discussed above, would be the best for ensuring violative samples are identified. In an actual monitoring situation, the number of accompanying false-violative results would be expected to be low, as the total number of veterinary drug violations typically represent a low percentage of animals processed.

One interesting observation made during early work on this project was that an extract of control beef sirloin muscle produced notably higher background fluorescence (greater than two times) than what had been observed with hanging tenderloin muscle. In order to determine whether the choice of muscle type would have an impact on the sensitivity of this rapid fluorescence assay, a study was undertaken to determine limits of quantitation for comparison purposes. In this study, DANO, as well as ENRO, was used for fortification purposes. Beef hanging tenderloin, eye round steak, and neck muscle were chosen as matrices for the study. Beef serum was also chosen to determine if this easier to extract matrix would provide comparable sensitivity to the muscle matrices. A local processing plant was able to provide additional samples of eye round steak, neck muscle, and blood, each from six different steers, for the study. Matrix matched standard curves were generated by spiking each of the six samples of each matrix with DANO (0, 50, 100, 200, and 300 ng/g), as well as with ENRO (0, 100, 300, 500, and 700 ng/g), which was followed by extraction and fluorescence analysis of the extracts. The standard curves were linear over the ranges examined, with $R^2 \geq 0.997$. Limits of quantitation (LOQs) were calculated for each analyte associated with each matrix as $10 \times SD$ of fluorescence response of the control samples/slope of the standard curve. The results, shown in Table 2, were subjected to ANOVA, and a mean separation test using the Bonferroni least-significant difference technique.

One of the first observations was that LOQs for ENRO were significantly higher than were those for DANO. This was expected, as ENRO typically displays a weaker fluorescence response than DANO does at a given concentration, as do many of the common FQs. In a routine monitoring screen, if a sample displayed a fluorescence signal $>x_{200} - 3\sigma$ in Figure 1, then it would either likely be due to DANO or a very high concentration of another FQ. In either case, the sample would need further analysis.

For both DANO and ENRO, there are clearly differences in LOQ due to matrix muscle type. All muscle types are not going to work equally well in the assay. It is interesting that the muscle selected for use in the initial study, hanging tenderloin, was the muscle type with the lowest LOQ. Hanging tenderloin was a logical choice at the time, as it is frequently the muscle selected by inspectors when samples are required. For ENRO, the LOQs increased going from hanging tenderloin to neck muscle and finally to eye round steak ($P < 0.05$). For DANO, no significant difference was observed between LOQs in hanging tenderloin and neck muscle, but these values were significantly lower than those for eye round steak ($P < 0.05$). With both DANO and ENRO, serum provided LOQs different from those observed in muscle ($P < 0.05$). Serum LOQs were

**TABLE 1. Analysis of DANO-fortified blind samples, using rapid fluorescence assay**

<table>
<thead>
<tr>
<th>Test</th>
<th>Limit set</th>
<th>Assay positives</th>
<th>Actual positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples &gt; 0 ng/g DANO</td>
<td>$&gt;x_0 + 3\sigma$</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>All samples &gt; 200 ng/g</td>
<td>$&gt;x_{200} - 3\sigma$</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>DANO</td>
<td>$&gt;x_{200}$</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>$&gt;x_{200} + 3\sigma$</td>
<td>16</td>
<td>10</td>
</tr>
</tbody>
</table>

**TABLE 2. Limits of quantitation for rapid fluorescence assay**

<table>
<thead>
<tr>
<th>Matrix (n = 6)</th>
<th>DANO (ng/g) (% RSD)</th>
<th>ENRO (ng/g) (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanging tenderloin</td>
<td>18 (9.5)</td>
<td>111 (3.3)</td>
</tr>
<tr>
<td>Neck muscle</td>
<td>24 (7.0)</td>
<td>219 (5.8)</td>
</tr>
<tr>
<td>Eye round steak</td>
<td>65 (6.1)</td>
<td>575 (1.5)</td>
</tr>
<tr>
<td>Serum</td>
<td>112 (11)</td>
<td>411 (9.8)</td>
</tr>
</tbody>
</table>
relatively high for both DANO and ENRO, suggesting that it may not provide a particularly sensitive matrix for analysis, although it could still be sensitive enough to detect DANO at 200 ng/g.

In conclusion, a rapid fluorescence assay has been shown to provide a promising new screening method for DANO in beef hanging tenderloin muscle. The method has displayed good recovery and excellent RSDs when tested with samples fortified at the tolerance level of 200 ng/g and has successfully analyzed blind fortified samples at concentrations above and below the tolerance level. Such a screening method has the potential to save analysis time by rapidly identifying the relatively few samples that contain potentially violative levels of DANO, and thus reducing the number of samples that would require more extensive quantitative and confirmatory analysis. Interestingly, the assay was found to be variably less sensitive in eye round steak and in serum. When used for ENRO analysis, the assay was less sensitive in neck muscle as well. The variation in sensitivity dependent on muscle type is a novel finding and suggests that other assay methods with fluorescence-based detection should be investigated for a similar effect. It would also be useful in the future for those employing beef muscle in analytical method development to specify exactly what type of muscle was used, in the event this variation is a more frequent occurrence than has been noted to date.

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REFERENCES