Development of a Monoclonal Antibody-Based Immunoassay To Detect Furosemide in Cow's Milk

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Furosemide is a potent diuretic drug used in both human and veterinary medicine. High-performance liquid chromatographic methods (HPLC) were developed to detect furosemide in blood and urine samples. Recently, immunoassay kits have appeared to measure furosemide; these were developed for the race horse industry where furosemide is used to treat epistaxis. In dairy cattle, furosemide is used for treatment of physiological parturient edema and there is a 48-h withdrawal period before milk from treated animals can be used. We report here the development of a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for detecting furosemide in milk. In addition, we report on the development of an HPLC method for detection of furosemide in milk that is an adaptation of published methods for its detection in serum and blood. Unlike the HPLC method, no sample preparation is necessary for the ELISA. Raw milk is added directly into the assay, or if needed, it is diluted with assay buffer. The immunoassay had a lower limit of quantification of 2 ppb and a lower limit of detection of approximately 0.5 ppb. Good correlations were observed between the HPLC and ELISA methods when samples with both incurred and spiked furosemide residues were analyzed.

Keywords: *Furosemide; immunoassay; residue detection; milk*

INTRODUCTION

The use of immunoassays as analytical tools to measure chemical residues in foods and environmental samples has increased greatly over the past several years. Recent reviews discuss the role of immunoassay in analytical chemistry and provide numerous examples of their application and ability to expand sampling capabilities (Hammock and Gee, 1995; Dixon-Holland, 1992; Morgan et al., 1992; Samarajeewa, 1990; Stanker, 1994). We previously reported on the development of a series of monoclonal antibodies to furosemide (Carlin et al., 1993). Furosemide is a potent diuretic used in both human and veterinary medicine for treatment of edema and ascites. Specifically, in veterinary medicine furosemide is used to treat dogs, cats, and horses for edema and it is used in cattle for the treatment of physiological parturient edema of the mammary gland (Darling, 1993). A 48 h withdrawal period is imposed both for milk taken from cows following the last treatment of furosemide (four milkings), and for cattle used for food (*Code of Federal Regulations*, 1994).

High-performance liquid chromatography (HPLC) methods are commonly used for analysis of furosemide (Farthing et al., 1992; Singh et al., 1990; Voyksner et al., 1990; Singh et al., 1989; Uchino et al., 1984; Swezley et al., 1979). In addition to HPLC methods, immunoassays for detecting furosemide also have been reported. In an early study, Woods et al. (1988) used a polyclonal anti-furosemide antibody in a competition enzyme-linked immunosorbent assay (cELISA) that was able to detect 20 ng/mL furosemide in equine blood. Tobin et al. (1988) reviewed the use of nonisotopic immunoassays for drug testing in horses. Recently, Singh et al. (1990) evaluated the performance of three methods for detecting and quantifying furosemide in equine serum and plasma: an HPLC method, a commercial ELISA test-kit method, and a particle concentration fluorescence immunoassay kit (PCFIA). These authors concluded that the ELISA and PCFIA "lacked quantitative reproducibility" and were not suitable for quantitation of furosemide in equine plasma or for studying the pharmacokinetics of furosemide.

In our initial report we described four monoclonal antibodies to furosemide, and the cross-reactivity of these antibodies was related to energy-minimized three-dimensional molecular models of furosemide and a variety of other related compounds (Carlin et al., 1993). The antibodies were highly specific for furosemide. For example, monoclonal antibody FURO-73 was tested for binding to nine related compounds. Significant inhibition of antibody binding was observed only with furosemide (IC_{50} = 0.02 ng/mL). This antibody weakly bound two other compounds, metolazone and furfuryl benzoate, with a cross reactivity of 0.05 and <0.02%, respectively. No inhibition of binding was observed with the other compounds tested (acetazolamide, bumetanide, furfuryl alcohol, furfurylamine, hydrochlorothiazide, sulfanilamide) at concentrations up to 100 μg/mL (Carlin et al., 1993). Thus, monoclonal antibody FURO-73 was used in the studies described here to develop a sensitive cELISA for this drug in milk. In addition to the cELISA, we report on the development of an HPLC method. Results from both the HPLC and cELISA analyses on the same samples containing either spiked or incurred furosemide residues are presented.

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METHODS

Materials. Furosemide and metolazone were analytical samples obtained from Hoechst-Roussel Pharmaceuticals, Inc. (Englewood Cliffs, NJ). Methylene chloride (B&J brand) was obtained from Baxter (Muskegon, MI). Glacial acetic acid (A-38), sodium bicarbonate (S-233), sodium carbonate (S-264), and potassium phosphate (monobasic, P0285) were from Fisher Scientific (Fair Lawn, NJ). The following were from Sigma (St. Louis, MO): Tween 20 (P-5927), sodium chloride (S-9888), potassium chloride (P-8041), sodium phosphate (dibasic, S-0876), Tris hydrochloride (T-3253), Tris base (T-8524), magnesium chloride (M-8266), and goat anti-mouse IgG [whole molecule conjugated to horseradish peroxidase (A-5278)]. K-blue (enzyme substrate) was purchased from ELISA Technologies (Lexington, KY) and used as recommended by the manufacturer. Preparation of furosemide−BSA conjugate used as plate-coating antigen and production of monoclonal anti-furosemide antibody were previously described (Carlin et al., 1993). Nonfat dry milk (NFDM), 2% milk, and half and half milk were purchased from a local grocery store. Fresh raw milk samples were obtained at milking from both the bulk tank and individual animals housed at the Dairy Cattle Research Center, Texas A&M University, College Station, TX. Milk samples with incurred furosemide residues were obtained from animals being treated for parturient edema of the mammary gland.

Buffers. Assay buffer (AB) (pH 7.75) contained the following per liter of water: 11.4 g of Tris-HCl, 3.32 g of Tris base, 8.76 g of sodium chloride, 5 g of NFDM, and 0.05 mL of Tween 20. Coating buffer (pH 9.6) contained the following per liter of water: 1.59 g of sodium carbonate, 2.93 g of sodium bicarbonate, and 0.203 g of magnesium chloride. Blocking buffer (pH 9.0) contained the following per liter of water: 8.76 g of sodium chloride, 1.42 g of sodium phosphate (dibasic), and 30 g of NFDM.

Preparation of Spiked Milk Samples. Fresh raw milk samples obtained from the bulk tank and from individual animals were aliquoted (10-mL aliquots) and fortified with furosemide, resulting in concentrations of 1000, 500, 250, 100, 50, 10, and 5 ppb. Both fortified and blank samples were stored at 4 °C and were analyzed by the ELISA and HPLC methods described below within 48 h of spiking.

ELISA Method. The immunoassay used in these studies was an indirect competition ELISA. The assay was formatted in 96-well microtiter plates using the anti-furosemide monoclonal antibody, FURO-73, as previously described (Carlin et al., 1993). Briefly, 96-well microtiter plates were coated with a furosemide−BSA conjugate (50 ng/well) and incubated for 1 h at room temperature. Unreacted sites on the plates were then blocked for 1 h with blocking buffer. After the wells were washed five times with a solution of 0.05% Tween 20 in water, varying concentrations of a furosemide standard (0.0001−0.05 mg/L) in 100 μL of control milk were then added followed by the addition of 100 μL of antibody diluted in assay buffer to give approximately 50% of the maximal signal when no competitor was present (a 1:1000 dilution of the hybridoma supernatant). The plates were then incubated 1 h at 37 °C, washed five times with a 0.05% Tween 20−water solution, and 100 μL/well peroxidase conjugated goat anti-mouse antibody diluted 1:500 in AB was added to each well. The plates were then incubated for 1 h at 37 °C and washed five times with a 0.05% Tween 20−water solution. Substrate (K-blue) was added and the plates were read in a microtiter plate reader (Bio-Rad Inc., Herculès, CA) using Reader Driver 1.0 software (Bio-Rad).

Both the spiked samples and those containing incurred furosemide were added to a microtiter well and diluted in a 2-fold series in control milk. Data were converted to percent inhibition of control as follows

$$\% \text{ IC} = (1 - (B/B_0)) \times 100 \quad (1)$$

using Excel software (Microsoft Inc., Redmond, WA) where B_0 equals the O.D. when no competitor is present and B is the O.D. when competitor is present. The percent IC values for unknowns that were near the 50% value (in all cases between 40 and 60% IC) were used to calculate the concentration of furosemide in the unknowns by extrapolation to the standard curve.

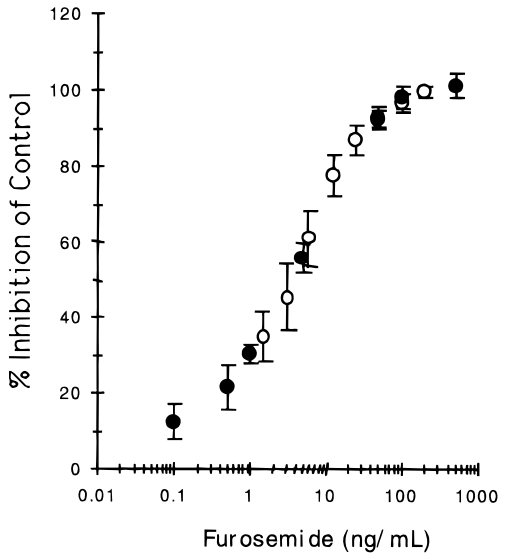

Figure 1. Results from a cELISA analysis of furosemide standards in buffer (open circles) and spiked raw milk samples (filled circles). Error bars represent ± one standard deviation.

HPLC Method. The HPLC method was a modification of the method published by Singh et al. (1990) for the analysis of furosemide in horse plasma. Briefly, 1 mL of milk sample was added to a screw-cap 15-mL conical centrifuge tube followed by 100 μL of 2 ppm bumetanide (internal standard, IS). The solution was vortexed and acidified with 1 mL of 0.5 N HCl followed by 1 mL of 5% NaCl (w/v). The mixture was again vortexed and 10 mL of methylene chloride was added to the tube. The tube was shaken on a wrist action shaker for 10 min and centrifuged for 10 min at 1000*g*. A portion of the organic layer (4 mL) was removed and added to a 4-mL sample vial, the solvent removed under N_2, and the residue reconstituted in 0.41 mL of acetonitrile−phosphoric acid (0.02%, pH 2, 35:65). Aliquots of this were injected onto the HPLC system.

The HPLC system consisted of a Dionex (Sunnyvale, CA) gradient pump, fluorescence detector (excitation wavelength, 235; emission wavelength, 410), and an AI-450 chromatography workstation. The column was a 4.6 mm × 25 cm, 5-μm Supelcosil LC-18 (5 μm) (Supelco, Bellefonte, PA). The solvent flow rate was 1.5 mL/min. The solvent system consisted of 65% phosphoric acid (0.02%, pH 2; solvent A) in acetonitrile (solvent B) maintained for 6 min postinjection. A linear gradient was initiated to 40% solvent A at 7 min and maintained for 10 min. The initial solvent conditions were reached at 11 min. The column was reequilibrated for 4 min prior to subsequent injection. Sample concentrations were derived from a standard curve made in acetonitrile−phosphoric acid (35:65) using furosemide/IS peak area ratios.

RESULTS AND DISCUSSION

ELISA Characterization. We previously described the production and cross-reactivity of a set of anti-furosemide monoclonal antibodies (Carlin et al., 1993). One of these antibodies, FURO-73, was used in this study to develop a cELISA for detection and quantification of furosemide in milk samples. Shown in Figure 1 (open circles) is the response observed in the cELISA when furosemide standards, diluted in assay buffer, were used as the competitor. The data shown represent the average of seven determinations, each performed in duplicate over a 6-week period. Thus, the error bars (± one standard deviation) represent the interassay

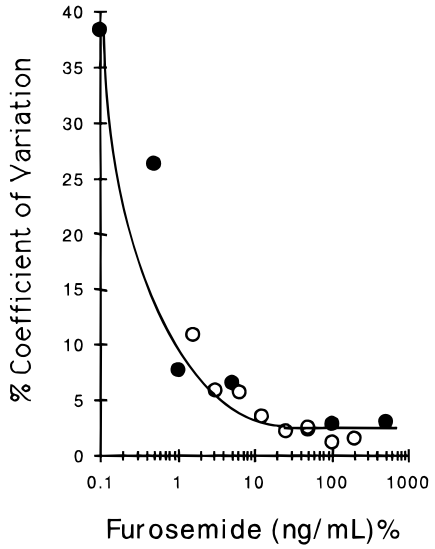

Figure 2. Analysis of the interassay percent coefficient of variation observed for the furosemide buffer standard (open circles) and furosemide spiked-milk samples (filled circles).

variation observed over this time period. Shown in Figure 2 (open circles) are the changes in the coefficients of variation for this set of data. As expected, smaller coefficients of variation were observed at the higher competitor levels.

Results from cELISA experiments using furosemide spiked raw milk samples also are shown in Figure 1 (filled circles). In these latter experiments, B/B_0 was calculated using an unspiked raw milk sample as the control to obtain a value for B_0. Likewise, the coefficients of variation for these spiked milk samples is shown in Figure 2 (solid circles) and are similar to those seen in the buffer experiments (open circles). These data suggest that the cELISA for furosemide is highly reproducible and that the standard curves generated in assay buffer or in raw milk are identical. Furthermore, these data demonstrate that the coefficient of variation is less than 12% for all samples spiked at levels greater than 1 ppb. Some samples containing furosemide at less than 1 ppb are on the linear portion of the curve (Figure 1). However, in these samples the coefficient of variation at concentrations below 1 ppb was observed to increase rapidly (Figure 2). Thus, the limit of quantitation (LOQ) for furosemide in raw milk was set at 2 ppb (because of the increased CV at lower spike levels), and the limit of detection (LOD) for the assay is 0.5 ppb. Comparable cELISA results were obtained in spiking experiments using commercially obtained whole commercial milk, 2% milk, and half and half milk (data not shown).

HPLC Analysis. In a separate set of experiments, raw milk from the bulk tank at the dairy, as well as 2% milk, and half and half milk obtained from local food stores were spiked with furosemide and incubated for at least 24 h at 4 °C. The samples were then spiked with an internal standard (bumetanide), acidified, and extracted with methylene chloride; the extract was dried and reconstituted in acetonitrile−phosphoric acid and analyzed by HPLC. Shown in Figure 3 are representative chromatograms obtained using a furosemide standard (trace A), a spiked raw milk sample (trace B), and a nonspiked control milk sample (trace C). A retention time of approximately 7.4 min was observed for furosemide, whereas bumetanide (the internal standard) had a retention time of 10.25 min (Figure 3). Analysis

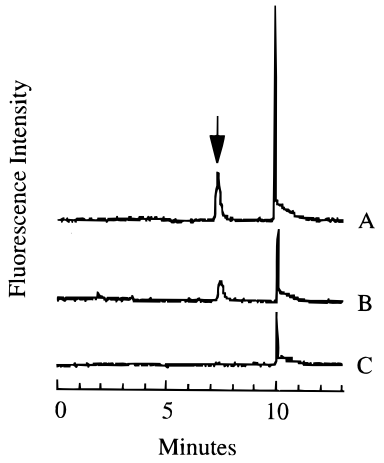

Figure 3. HPLC chromatograms of (A) furosemide standard; (B) furosemide-spiked raw milk; and (C) nonspiked raw milk. Furosemide (arrow) had a retention time of approximately 7.4 min while bumetanide, the internal standard, had a retention time of approximately 10.25 min.

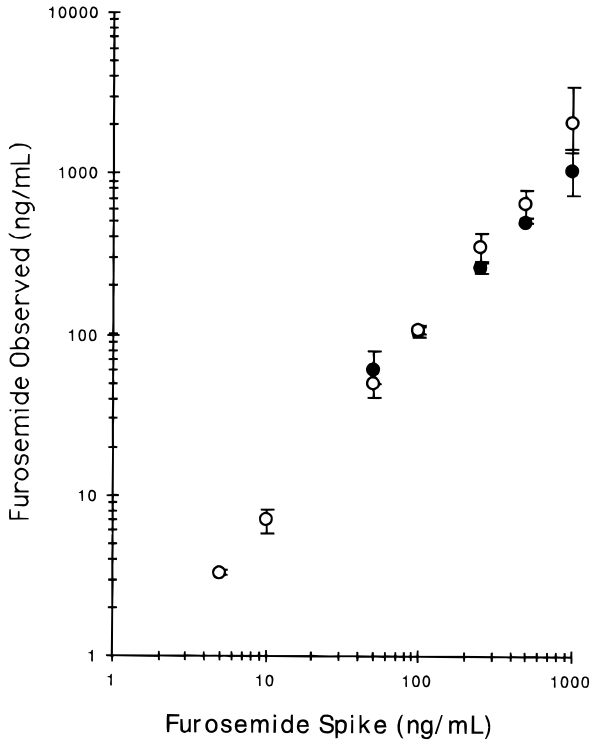

Figure 4. Analysis of furosemide-spiked raw milk samples using the cELISA (open circles) and the HPLC method (filled circles). Error bars represent ± one standard deviation.

of spiked milk standards indicated that the HPLC method had a lower limit of quantitation of 50 ppb and a lower limit of detection of 25 ppb. In a separate set of experiments, furosemide-fortified raw milk samples were split into two aliquots and analyzed by both the cELISA and the HPLC methods. The results from these experiments are shown in Figure 4. Results from identical analyses using 2% milk and half and half milk are summarized in Table 1. These data clearly demonstrate the accuracy of the HPLC method over the entire concentration range of furosemide spikes. The cELISA method also appeared to be highly accurate, especially at furosemide levels below 100 ppb. Thus, the cELISA is a useful tool for extending the lower limit of detection beyond that of the HPLC. However, at furosemide levels between 100 and 1000 ppb, the cELISA method was observed to overestimate the level

Table 1. Analysis of Identical Samples by HPLC and cELISA

	raw milk		2% milk		half & half milk	
spike (ppb)	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA
1000	1037 ± 203[a]	2084 ± 1660	927.3 ± 38.6	1621 ± 416	1010 ± 13	1698 ± 182
500	493 ± 7.9	650 ± 156	414 ± 88	716 ± 92	527.5 ± 31.8	896 ± 231
250	260.3 ± 58.5	363 ± 109	236 ± 12.5	368 ± 42.3	262 ± 30.8	464 ± 73
100	104.1 ± 7.4	107 ± 8.3	102 ± 10.3	99.5 ± 0.5	106 ± 11.7	134 ± 31
50	59.9 ± 11.8	41.4 ± 14.6	34.9 ± 8.7	46.8 ± 4.5	45.1 ± 7.5	46.4 ± 4.2
10	nd[b]	7.1 ± 1.5	nd	9.5 ± 2.3	nd	7.9 ± 2.1
5	nd	3.3 ± 0.2	nd	4.8 ± 1.2	nd	3.7 ± 1.7
0	nd	nd	nd	nd	nd	nd

[a] ± one standard deviation. cELISA's performed in triplicates and HPLC in duplicates. [b] nd, not detected. The limit of detection is 50 and 2 ppb for the HPLC and the cELISA, respectively.

Table 2. cELISA Analysis of Fortified Raw Milk Samples Obtained from Individual Animals

	spike level (ppb)			
animal no.	100.0	50.0	10	0
1	102.4	41.6	7.0	nd[a]
2	99.2	32.0	16.8	nd
3	99.2	38.4	8.0	nd
4	99.0	49.6	12.4	nd
5	112.0	56.0	11.2	nd
6	100.0	50.1	23.2	nd
7	100.0	67.2	7.0	nd
8	100.0	50.0	9.0	nd
9	105.0	45.6	10.4	nd
mean	101.9 ± 4.0	47.8 ± 9.6	11.7 ± 5.0	

[a] nd, not detected.

of furosemide in the samples. The cELISA for furosemide reported here is linear between 1 and 50 ppb (Figure 1). Thus, samples containing furosemide levels at 100−1000 ppb required dilution in order to put them on scale. Such dilutions can easily contribute to the large CV observed in the samples spiked with these high levels of furosemide.

The above fortification experiments were conducted using raw milk obtained from the bulk tank at the dairy and commercially obtained milk. To evaluate the ability of the cELISA to detect furosemide in samples from individual cows, raw milk samples from individual animals were obtained, spiked, and analyzed using the cELISA. As in the above experiments, these samples were stored for 24 h at 4 °C after spiking before they were analyzed. A single control milk sample was used for this set of experiments, and the results are summarized in Table 2. These data suggest that while a greater variation was observed when samples from individual animals were analyzed, the cELISA was able to accurately measure furosemide in each sample tested. Equally important, the cELISA did not detect furosemide in the nonspiked samples.

Analysis of Incurred Residues. Milk samples from animals that received a single 500-mg dose of furosemide for treatment of physiological parturient edema of the mammary gland were analyzed using both the cELISA and HPLC methods. Two groups of animals were analyzed. Data from the first group of three animals are shown in Table 3. These animals received a single dose of furosemide within 1 h postpartum. The pretreatment sample was obtained immediately before furosemide treatment. These data suggest that the peak levels of furosemide appear within 12 h posttreatment and that by 48-h residues were not detected. Similar results were obtained with both the cELISA and HPLC methods. We noticed that in the early postpartum samples, where the colostrum levels are high, there was poorer recovery of the internal standard (bumentanide) for the HPLC analyses, particularly in cows 2 and 3. This may in part explain the difference in levels recorded by the two methods for these two samples. Loss of recovery of the IS would result in an overestimation of the analyte. Nevertheless, our results, unlike those of Singh et al. (1990), suggest that the cELISA is a sensitive method that can be used for metabolic studies. Possibly the polyclonal antibody immunoassay used by Singh et al. (1990) was not as specific as the monoclonal-based cELISA we report here. The results obtained for cow 4 are shown separately in Table 4. Three postpartum but pretreatment samples at 12, 24, and 36 h were obtained. Immediately after obtaining the 36-h postpartum sample, the animal was administered a single ip dose of furosemide and additional samples were collected at 12, 36, 48, and 60 h posttreament. Again, there is good correlation between the HPLC and cELISA methods. Only the 36-h posttreatment samples had any appreciable level of residue. Since the treatment was administered at 36 h postpartum, the dynamics of the mammary gland are different from in the group 1 animals. Clearly by 36 h postpartum, the colostrum levels have dropped, indicating changes in transport into the gland. These changes may be responsible for the furosemide peak being observed at 36 h posttreatment. However, both the ELISA and the HPLC re-

Table 3. Analysis for Furosemide in Milk Samples at Various Times Following a Single Injection of Furosemide for Control of Physiological Parturient Edema of the Mammary Gland

	cow 1		cow 2		cow 3	
posttreatment (h)	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC
0 (pretreatment)			nd[a]	nd	nd	nd
12	88.6 ± 5.4	77.0 ± 11.5	144 ± 12	281 ± 12.7	153.5 ± 7.0	223.3 ± 33.8
24	−	−	15.4 ± 1.0	nd	15.7 ± 0.4	nd
36	39.6 ± 0.4	nd	−	−	−	−
48	−	−	nd	nd	nd	nd
60	6.8 ± 0.4	nd	−	−	−	−
72	−	−	nd	nd	nd	nd
84	9.2 ± 0.4	nd	−	−	−	−

[a] nd, not detected. A limit of detection of 50 ppb for the HPLC and 2 ppb for the ELISA was used. [b] −, sample not available for analysis.

Table 4. Analysis of Furosemide in Milk Samples from a Single Cow Given Furosemide at 36 h Post Calving

	ELISA	HPLC
postcalving (h)		
12	nd[a]	nd
24	nd	nd
36	nd	nd
posttreatment (h)		
12	1.4 ± 0.12	nd
36	101.2 ± 6.1	125.9 ± 2.0
48	3.9 ± 0.5	nd
60	2.8 ± 0.17	nd

[a] nd, not detected.

corded the peak in the same sample, at approximately the same level, 101 versus 125 ppb for the cELISA and the HPLC method, respectively.

CONCLUSIONS

The data presented here clearly demonstrate that the cELISA developed here is a useful analytical method for analysis of furosemide levels in milk samples. Using spiked milk samples, the cELISA method was observed to have a LOQ of 2 ppb, where as the HPLC method had a LOQ of 50 ppb. The cELISA method and the HPLC method were applied to aliquots of the same incurred furosemide residue samples. In these studies furosemide was observed only in the early times post furosemide injection and all positive samples identified by HPLC were positive by cELISA. In some samples the cELISA recorded levels of furosemide that were below the LOQ of the HPLC method. All samples having levels less than the LOQ for residues were scored as negatives using either the cELISA or the HPLC, and all spiked samples were measured as positive by both methods. The sample preparation methods needed for the HPLC were cumbersome and relied on organic solvents. No sample preparation, other than simple dilution of the samples, was needed for the cELISA method. Thus, this cELISA should be useful as a screening tool as well as a quantitative method and represents an aid in animal management.

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