Development and Validation of a Liquid Chromatographic Method for the Determination of Furosemide, a Diuretic, in Bovine Milk

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A rapid and sensitive reversed phase liquid chromatographic (LC) procedure was developed and validated for the quantitation of furosemide, a diuretic, in bovine milk. Whole milk was defatted by initial centrifugation at room temperature. The resulting skim milk was deproteinated with acetonitrile and centrifuged again. The acetonitrile from the supernatant was evaporated, and the remaining aqueous portion was directly analyzed by LC. The LC conditions employed include a Spherisorb 5 ODS 2 column, a phosphate/acetonitrile buffer (pH 3), and a fluorescence detector set at 272 and 410 nm excitation and emission wavelengths, respectively. The average recoveries of furosemide from milk fortified at 5, 10, and 20 ppb were 108, 91, and 85%, respectively, with corresponding CVs of 14, 8, and 6%. The method was validated by assaying milk obtained from a cow dosed intravenously with 500 mg of furosemide. The furosemide concentrations in 8 and 24 h milk samples were determined to be about 150 and 5 ppb, respectively. No furosemide was detected in 32 and 48 h samples.

INTRODUCTION

Furosemide, 4-chloro-*N*-(2-furylmethyl)-5-sulfamoyl-anthranilic acid, is approved for use in cattle for the treatment of physiological parturient edema of the mammary gland and associated structures (*Code of Federal Regulations*, 1991). The drug is administered once daily by the intramuscular or intravenous route at a dosage of 500 mg per animal; the treatment is not to exceed 48 h postparturition. The edible products (milk and meat) must not be used for food within 48 h following the last treatment (*Code of Federal Regulations*, 1991). Furosemide is a potent short-acting diuretic that causes rapid fluid loss and improves the appearance of muscle tone; therefore, it is a common drug of abuse in livestock shows (Ray et al., 1984).

Two-year toxicology and carcinogenesis studies conducted under the sponsorship of the National Toxicological Program showed that there was equivocal evidence of carcinogenic activity of furosemide in male F344/N rats and some evidence of carcinogenic activity in female B6C3F1 mice (National Toxicological Program, 1989).

Because of a potential misuse of furosemide in food-producing animals, this study was undertaken to develop a reliable and reproducible LC method that is sensitive enough to detect low residue concentrations of furosemide in cow's milk.

In the literature, almost all analytical methods employ LC for the analysis of furosemide. These methods have been limited to assaying furosemide in plasma and urine; however, none could be found for its determination in milk. The most commonly used LC conditions include a reversed phase column, an acidic mobile phase with organic modifier, and fluorescence detection (Sidhu and Charles, 1993; Russel et al., 1989; Radek and Heller, 1989; Forthing et al., 1992; Saugy et al., 1991;

Reeuwijik et al., 1992; Vree et al., 1994). The LC analysis is generally preceded by sample cleanup with the exception of direct injection into internal surface reversed phase column (Pinkerton et al., 1986). The sample cleanup involves deproteination with acetonitrile (Sidhu and Charles, 1993; Radek and Heller, 1989; Saugy et al., 1991), methanol (Sood et al., 1987), or acid followed by ethyl acetate extraction (Ray et al., 1984; Reeuwijik et al., 1992). Solid phase extraction alone (Russel et al., 1989; Radek and Heller, 1989) and solid phase extraction with on-line elution via an advanced automated sample processor (AASP) have also been used for sample cleanup of plasma samples (Forthing et al., 1992). Pretreatment of urine samples before LC analysis generally requires less cleanup (Ray et al., 1984; Smith et al., 1980) and in some cases no cleanup (Vree et al., 1994).

The procedure described in this paper employs initial defatting of the milk by centrifugation (Shaikh and Jackson, 1989), followed by deproteination with acetonitrile, and LC analysis using a reversed phase column, an acetonitrile/buffer mobile phase, and fluorescence detection.

MATERIALS AND METHODS

Apparatus. The LC consisted of a Hewlett-Packard Model 1050 system (Palo Alto, CA) fitted with a quaternary pump, an autosampler, a column heater, a solvent bottle holder with helium purge, an HP computer (Vectra QS/65) with HP ChemStation software (DOS series), an HP desk Jet 500 printer, and a Perkin-Elmer LC 240 fluorescence detector set at 272 and 410 nm excitation and emission wavelengths, respectively.

The LC column and guard column used were a Spherisorb 5 ODS-2, 15 cm × 4.6 mm (Phase Separations, Inc., Norwalk, CT), and a Spherisorb 5 ODS-2, 1 cm × 4.6 mm cartridge (Alltech Associates, Deerfield, IL), respectively. Both the analytical and guard columns were placed in a column heater set at 35 °C.

All centrifugations were carried out at 4000 rpm (3070*g*) for 15 min in a Sorvall RC-5 refrigerated centrifuge (DuPont

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Co., Wilmington, DE) set at 20 °C and fitted with HS-4 rotor. Polypropylene tubes (15 mL) with graduations down to 1.5 mL and plug-type screw caps were used (Corning Glass Works, Corning, NY). All transfers were made with Eppendorf digital pipetes.

Reagents. Glass distilled organic solvents (Burdick and Jackson Laboratories, Muskegon, MI) and distilled deionized water, filtered through a 0.2 μm nylon filter, were used. All chemicals were of HPLC grade, except where noted. The mobile phase consisted of a 30:70 ratio of acetonitrile and potassium phosphate buffer, pH adjusted to 3 with 150 μL of phosphoric acid (85%). The standard solutions were prepared by weighing 5–10 mg of furosemide reference standard (U.S. Pharmacopeial Convention, Rockville, MD) in a small plastic dish and then transferring it to a 50–100 mL amber glass volumetric flask with methanol. A portion of the stock solution was transferred to a 10 mL amber glass volumetric flask and diluted with methanol to give a working standard solution of 1 μg/mL or as appropriate. All solutions were refrigerated until used.

Milk Samples. Control milk samples were obtained from four lactating Holstein cows, from a bulk milk tank (ARS-USDA, Beltsville, MD), and from a local grocery store (shelf milk). Fortified milk samples were prepared by transferring 3 mL of control milk samples to 15 mL polypropylene centrifuge tubes. They were spiked with 15, 30, and 60 μL of a 1 μg/mL stock solution of furosemide to give 5, 10, and 20 ppb fortification levels. Furosemide incurred milk was generated at this facility from a lactating Holstein cow treated with a single intravenous dose of 500 mg of furosemide (lasix injectable solution, Phoenix Pharmaceutical, Inc., St. Joseph, MO). Milk samples were refrigerated at 4 °C if not analyzed immediately. In addition, portions of the milk samples were frozen for stability and other studies.

Sample Preparation. A 3 mL portion of the milk (control, spiked, or incurred) was transferred to a 15 mL polypropylene centrifuge tube and centrifuged for 15 min. The fat layer that solidified on the top of the milk sample was removed with a metal spatula. To deproteinate, 9 mL of acetonitrile was added to the defatted milk, which was then vortex mixed for 10–15 s and centrifuged as above. Six milliliters of the deproteinated sample was transferred to a polypropylene centrifuge tube with graduation marks. The sample was evaporated to 1.5 mL under a nitrogen stream by placing the vial in an N-Evaporator with water bath temperature set at about 35 °C. An aliquot (~500 μL) of the sample extract was transferred to a 2 mL amber glass autosampler vial, and 100 μL was injected into the LC column.

Standard Curve. The 1 μg/mL furosemide standard stock solution was used to prepare the standard curve. Portions of 50, 100, and 250 μL were transferred to 10 mL amber glass volumetric flasks and diluted to the mark with methanol to give concentration levels of 5, 10, and 25 ng/mL standard solutions. Additional standard solutions covering the concentration ranges 3–25, 5–50, 10–250, and 250–1000 ng/mL were prepared as appropriate. About 500 μL of each of the standard solutions was transferred to 2 mL amber glass autosampler vials and 100 μL of each was injected onto the LC column.

β-Glucuronidase Treatment. Two vials of the bacterial enzyme β-glucuronidase (Sigma Chemical Co., St. Louis, MO, catalog no. G5897), 1000 units/vial, with added phosphate buffer and bovine albumen as stabilizer, were reconstituted with 10 mL of water to give 100 units/mL. The reconstituted solution contained approximately 4 mM phosphate buffer, pH 6.8. Three milliliters from each of the 8 h, 48 h, and control milk samples was treated with an equal volume of the enzyme solution, and the mixture was incubated for 20 h at 37 °C. The pH of the milk samples was 6.6. The samples were carried through cleanup and LC analysis procedures as described above.

Exhaustive Extraction. After the deproteination step of the incurred milk samples, the protein pellet was rinsed with 1.5 mL of water. The washed pellet was mixed with 6 mL of methanol/acetonitrile/water (1:2:1) to extract any residual furosemide. The mixture was centrifuged, and the organic
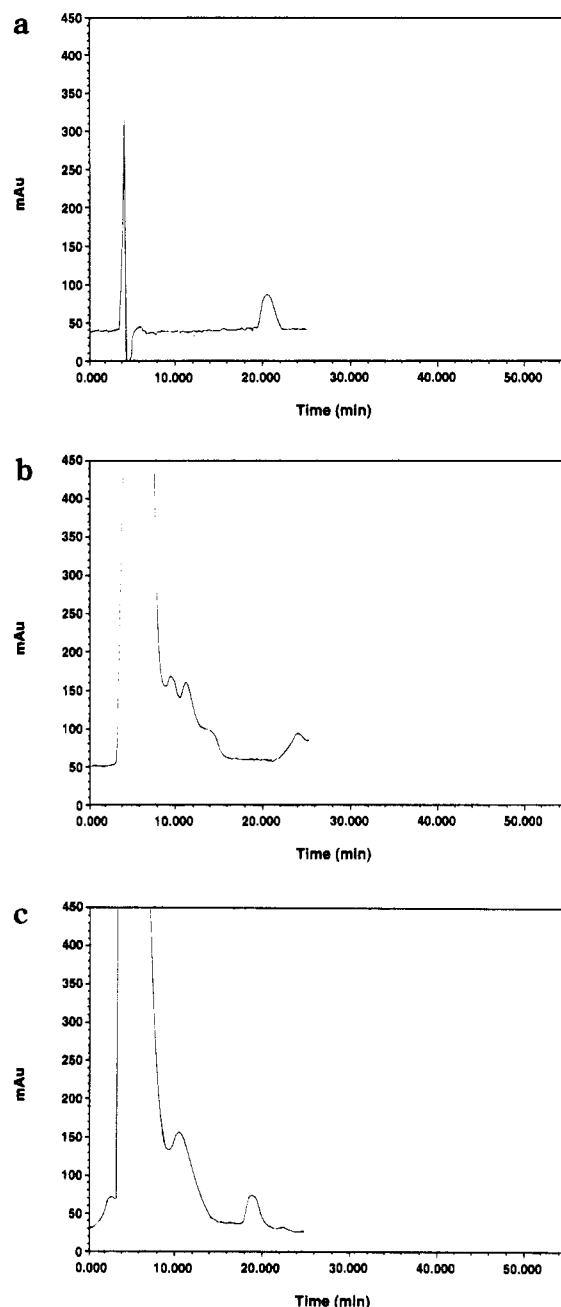

Figure 1. LC chromatograms of (a) 10 ng/mL furosemide standard, (b) control milk extract, and (c) 10 ng/mL fortified milk extract. An injection volume of 100 μL was used in each case.

phase of the supernate was evaporated with nitrogen in an N-Evaporator. The remaining aqueous layer was analyzed according to the LC procedure as described above.

RESULTS AND DISCUSSION

Recovery of Furosemide from Fortified and Incurred Milk. Parts a, b, and c of Figure 1 show typical liquid chromatograms of 100 μL injections of a 10 ng/mL furosemide standard, a control milk extract, and a 10 ng/mL fortified milk extract, respectively. Parts a, b, and c of Figure 2 show liquid chromatograms of the extracts from incurred milk samples obtained at 8, 24, and 32 h, respectively, after dosing of a cow with furosemide. A lower injection volume (10 μL) was used for 8 h milk extracts to keep the furosemide peak response on scale. The furosemide peak is well separated from the endogenous compounds in milk extracts under the LC conditions used in this study.
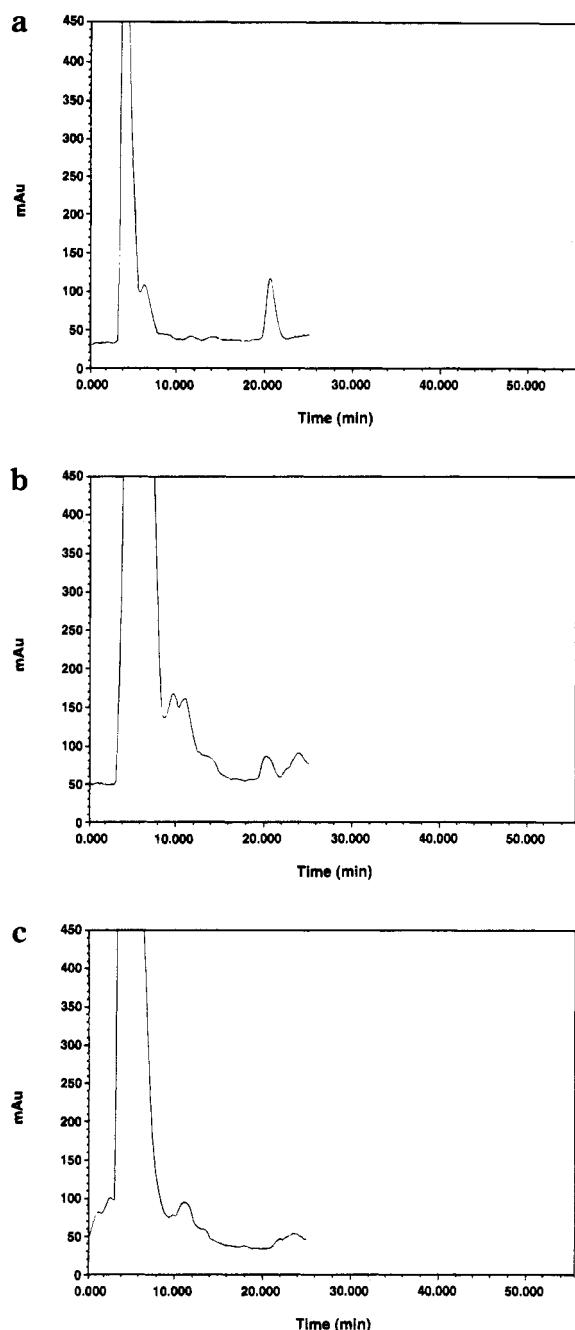
Figure 2. LC chromatograms of incurred milk sample extracts: (a) 8 h milking; (b) 24 h milking; and (c) 32 h milking. Injection volumes of 10 μL for (a) and 100 μL for both (b) and (c) were used.

A linear relationship was obtained for a number of standard curves generated to cover a wide range of concentrations in this study. A correlation coefficient of 0.999 was obtained for the four standard curves covering the concentration ranges 3–25, 5–50, 10–250, and 250–1000 ng/mL. Appropriate standard solutions for standard curves, covering various concentration ranges, were prepared for use in quantitating spiked and incurred samples.

The recoveries of furosemide from fortified milk samples are given in Table 1. The average recoveries of furosemide at 5, 10, and 20 ppb fortification levels were determined to be 108, 91, and 85%, respectively, with corresponding CVs of 13.7, 7.9, and 5.6%. The FDA method guidelines for acceptable recovery and CV when residues are below 100 ppb are 60–110% and equal to or less than 20%, respectively. Both the recoveries and the CVs in this study are well within these guidelines.

Table 1. Recovery of Furosemide from Fortified Milk

sample	calcd value (ppb)	% recovery
5 ppb spike 1	5.3	106
5 ppb spike 2	6.0	120
5 ppb spike 3	6.3	126
5 ppb spike 4	4.6	92
5 ppb spike 5	4.8	96
av	5.4	108
CV	13.7	13.7
10 ppb spike 1	8.7	87
10 ppb spike 2	8.1	81
10 ppb spike 3	9.3	93
10 ppb spike 4	10.0	100
10 ppb spike 5	9.3	93
av	9.1	91
CV	7.9	7.9
20 ppb spike 1	16.3	82
20 ppb spike 2	18.3	92
20 ppb spike 3	16.0	80
20 ppb spike 4	16.9	85
20 ppb spike 5	17.6	88
av	17.0	85
CV	5.5	5.6

Table 2. Furosemide Concentrations in Incurred Milk Samples[a]

sample	concn (ppb)
8 h milk	144.9
8 h milk	159.2
8 h milk	153.1
8 h milk	144.9
8 h milk	150.0
av	150.4
CV (%)	4.0
24 h milk	5
24 h milk	6.8
24 h milk	3.9
24 h milk	4.3
24 h milk	4.4
av	4.9 (4.4, $n = 4$, exclude sample 2)
CV (%)	23.3 (10.3, $n = 4$, exclude sample 2)
8 and 24 h mix milk	17.8
8 and 24 h mix milk	18.0
8 and 24 h mix milk	17.4
8 and 24 h mix milk	18.9
8 and 24 h mix milk	19.3
av	18.3
CV (%)	4.3

[a] Five replicates of milk samples obtained at 8 and 24 h postdosing were analyzed.

Table 2 indicates furosemide concentrations in milk collected at 8 and 24 h after the intravenous administration of a single dose of 500 mg of furosemide to a cow. The average furosemide concentrations of five replicates of 8 and 24 h milk were determined to be about 150 and 5 ppb, respectively. No furosemide was detected in 32 and 48 h milk samples. The low furosemide residue concentration in the 24 h milk and the undetectable levels in 32 and 48 h milk samples suggest rapid depletion of furosemide from lactating cows; however, it must be emphasized that the data from a single cow may not reflect the rate of depletion of furosemide from the milk of dairy cows in general. The 8 and 24 h furosemide incurred milk samples were mixed in a 1:9 ratio and carried through the cleanup

and analysis procedure. The furosemide concentration was determined to be 18.3 ppb or 96% of the expected concentration (19 ppb; based on the 1 to 9 mixture of the two milk samples) with a 4.3% CV, indicating an excellent precision.

A number of deproteination techniques were evaluated to obtain clear extracts and acceptable recovery of furosemide from milk samples. Initially, deproteination of the milk with trichloroacetic acid (Shaikh and Jackson, 1989) followed by LC analysis of the supernatant was carried out. Endogenous interfering peaks at or near the elution position of furosemide were present, and therefore, this approach was abandoned. Acid deproteination followed by ethyl acetate extraction of the furosemide has been frequently reported in the literature to determine furosemide in plasma samples. In this study a number of acids, such as phosphoric acid, perchloric acid, trichloroacetic acid, and hydrochloric acid, were evaluated to deproteinate milk samples. The acid deproteination was followed by ethyl acetate extraction of the furosemide and LC analysis. In all cases, the furosemide recoveries were poor, inconsistent, and irreproducible. This is perhaps due to instability of furosemide under light and acidic conditions (Smith et al., 1980; Rowbotham et al., 1976)). Therefore, this approach was also abandoned. Acetonitrile deproteination provided consistently higher and reproducible furosemide recoveries from milk. Initial defatting of the milk at both centrifuge temperatures of 4 °C (cold) and 20 °C (room) was satisfactory. Centrifugation at room temperature was selected, since nonrefrigerated centrifuges are less costly and readily available in most laboratories.

Mobile phases consisting of various combinations of phosphoric acid and acetonitrile (Sidhu and Charles, 1993; Radek and Heller, 1989) were evaluated; however, they produced irreproducible chromatographic separation of furosemide. Therefore, this approach was abandoned. The mobile phase combination of acetonitrile and phosphate buffer was satisfactory and provided adequate separation from endogenous background compounds in milk. The organic modifier (acetonitrile) portion could be varied from 25 to 30% to achieve optimal resolution of furosemide. Various combinations of excitation and emission wavelengths were also evaluated, but the combination of 272 and 410 nm excitation and emission wavelengths, respectively, provided the most sensitive chromatographic response for furosemide. Two columns of different lots were used in this study; both provided adequate resolution of furosemide under the LC conditions employed.

Six control milk samples from various sources (see Materials and Methods) were evaluated for potential endogenous interfering compounds. All milk samples were found to contain no peaks that chromatographically interfered with the elution of furosemide.

A glucuronide conjugate metabolite of furosemide has been reported to be present in the urine of animals treated with furosemide (Ray et al., 1984; Vree et al., 1994; Smith et al., 1980). In this study no increase in furosemide concentration was noted in the milk after treatment with β-glucuronidase enzyme, indicating the absence of glucuronide metabolite in milk. No additional furosemide was detected during exhaustive extraction of the protein pellet of the incurred milk, indicating that the first deproteination step is sufficient to dissociate furosemide from milk solids.

Stability. Stability of furosemide in methanol (standard solutions) and in milk extracts (incurred) was evaluated under a number of storage conditions. Furosemide standard solutions stored in amber glass vials, under refrigeration (4 °C), were evaluated over a 10 month period and found to be stable. However, at room temperature, they were stable for only 8 days. When stored in clear glass vials, they were stable for 8 days at both refrigerator and room temperatures. Incurred milk samples were carried through the cleanup process, and a portion of the final milk extract was assayed by LC, a portion was stored in an amber glass vial at room temperature, and the rest was stored in polypropylene tubes in the refrigerator. The refrigerated samples were analyzed by LC after 1 and 3 days of storage and were found to be stable (recovery >90%). The stability of the samples over 3 days will allow the analyst to store the milk extracts in the refrigerator over the weekend, without the fear of sample deterioration. The sample stored in amber glass for 1 day and analyzed by LC showed no deterioration of furosemide. This will enable the analyst to utilize an autosampler to assay a large number of samples overnight. Incurred milk was also stored at −20 °C and analyzed after 42 days of storage. No deterioration of furosemide was noted and the recovery was >90%.

Specificity of the Method. The following drugs and other diuretics were tested for their potential interference with the analysis of furosemide: tetracycline, chlortetracycline, oxytetracycline, chloramphenicol, penicillin G, cloxacillin, tylosin, sulfamethazine, sulfaquinoxaline, dexamethasone, propranolol, naproxin, saluamine (a degradation product of furosemide), amloride, bumetanide, bendroflumethiazide, benzthiazide, trichlormethiazide, chlorothiazide, and hydrochlorothiazide. Most of these compounds are used in dairy cattle and did not interfere with the elution of the furosemide.

Accuracy, Precision, and Sensitivity. The average recovery for 5−20 ppb fortified samples was 95% with a CV of 9%. The inter- and intraday (over 3 days) CVs of incurred samples (8 and 24 h mix) were 3.4 and 3.7%, respectively. The signal to noise ratio for 1 ng of furosemide standard (comparable to 10 ppb) was 1:16 and for 0.3 ng (comparable to 3 ppb) was 1:4. These results suggest that the method is sufficiently sensitive to determine accurately and precisely the low residue concentrations of furosemide in bovine milk.

Conclusions. An accurate, precise, and sensitive method for the determination of furosemide in bovine milk has been developed. The method distinguishes furosemide from other diuretics, drugs, and antibiotics used in dairy cattle. The method was validated by quantitating furosemide concentrations in milk obtained at various intervals from a cow dosed with furosemide.

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