

# Liquid Chromatographic Determination of Chlorothiazide and Hydrochlorothiazide Diuretic Drugs in Bovine Milk

Badar Shaikh\* and Nathan Rummel

Center for Veterinary Medicine, U.S. Food and Drug Administration,  
8401 Muirkirk Road, Laurel, Maryland 20708

A liquid chromatographic (LC) procedure was developed for the determination of chlorothiazide (CTZ) and hydrochlorothiazide (HCTZ) in bovine milk. Whole milk was defatted by centrifugation at 4 °C. The top layer was treated with lead acetate and acetonitrile, mixed, and centrifuged. The supernatant was extracted with ethyl acetate to remove acetonitrile. The solvent mixture was further treated with sodium tungstate and mixed. The top layer was removed and evaporated to dryness, the residue was dissolved in the mobile phase, and the final extract was analyzed by LC. The LC employed a polymer column, a mobile phase of acetonitrile/tetrahydrofuran in phosphate buffer, and a UV detector. The average recoveries of CTZ from milk fortified at 35, 70, and 140 ppb were 97, 96, and 99%, respectively, with corresponding coefficients of variation (CVs) of 8, 5, and 5%. The average recoveries of HCTZ at 35, 70, and 140 ppb were 87, 91, and 90%, respectively, with CVs of 5, 6, and 5%. The method was validated by assaying milk obtained from cows dosed separately with the two diuretics. CTZ was detected in 8-h (430 ppb) and 24-h (85 ppb) incurred milk samples and HCTZ (47 ppb) only in the 8-h incurred milk samples.

**Keywords:** Chlorothiazide; hydrochlorothiazide; trichlormethiazide; LC; bovine milk; thiazides; diuretics; incurred residue

## INTRODUCTION

The thiazide diuretics chlorothiazide (CTZ), hydrochlorothiazide (HCTZ), and trichlormethiazide (TCMTZ) are approved for use in cattle as an aid in reduction and for the treatment of postparturient udder edema (*Code of Federal Regulations*, 1991). CTZ is administered as a 2-g bolus once or twice daily for 3–4 days. HCTZ is administered as an injection, containing 125–250 mg, intravenously or intramuscularly, once or twice a day, and treatment may be continued for several days if necessary. However, it is often more convenient to use HCTZ injection to achieve prompt onset of diuresis and maintain this activity for the desired time with oral administration of CTZ boluses (*Veterinary Pharmaceuticals and Biologicals*, 1993/1994). Both CTZ and HCTZ are potent compounds in their influence on electrolyte excretion, and as a result, dosage and administration must be individualized. The safe level of both CTZ and HCTZ has been tentatively set by the U.S. Food and Drug Administration (FDA) to be 67 ppb (Shaikh, 1996). Milk taken from dairy animals during treatment and for 72 h after the latest treatment of both diuretics must not be used for food.

In the literature, almost all analytical methods have been limited to assaying diuretic drugs in plasma and urine. These methods have been reviewed extensively (Shaikh 1996; Hernandez et al., 1992) and indicate that liquid chromatography (LC) is the main technique employed for the analysis of CTZ and HCTZ in plasma and urine, but no methods were reported for their detection in milk. The exception has been a recently reported LC method for the determination of a loop

diuretic, furosemide, in milk (Shaikh, 1995). There is also a report in the earlier literature (Werthmen, 1972) for the determination of CTZ in human milk by liquid–liquid extraction followed by color formation and measurement at 520 nm. However, the method is cumbersome, and the reported sensitivity of 1 ppm is not sufficient for use in residue work.

The procedure described in this paper employs initial defatting of the milk by centrifugation, followed by treatment with lead acetate and acetonitrile (ACN). The ACN is extracted in ethyl acetate from the supernatant, and the organic solvent mixture containing both diuretics (CTZ and HCTZ) is further treated with sodium tungstate. The final extract is evaporated to dryness, reconstituted in mobile phase, and analyzed by LC using a reversed-phase polymer column and UV detection at 225 nm.

## 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 486/66XM with HP ChemStation software (DOS series), an HP Laser Jet 4 plus printer, an HP Interface 35900, and an HP variable-wavelength detector set at 225 nm.

The LC (150 × 4.1 mm) and guard (25 × 4.1 mm) columns employed were reversed-phase polymer PRP-1 (Hamilton Co., Reno, NV) having a packing of 5-μm particle size. Both the analytical and guard columns were placed in a column heater set at 30 °C.

All centrifugations were carried out at 5500 rpm using both an HS-4 swinging rotor (5810g) and an SE-200 fixed rotor (4380g) in a Sorvall RC-5 refrigerated centrifuge (Dupont Co., Wilmington, DE) set at 4 °C. Polypropylene tubes (15 and 50 mL) with plug-type screw caps were used (Corning Glass

\* Author to whom correspondence should be addressed [e-mail: bshaikh@bangate.fda.gov; fax (301) 827-8170].

Works, Corning, NY). All transfers were made with Eppendorf digital pipets.

**Reagents.** Glass-distilled organic solvents (Burdick and Jackson Laboratories, Muskegon, MI), distilled deionized water, filtered through a 0.2- $\mu$ m nylon filter, and water-saturated ethyl acetate were used. All chemicals were of HPLC grade, except where noted. The diuretics, CTZ, HCTZ, and TCMTZ, were obtained from Sigma Chemical Co. (St. Louis, MO).

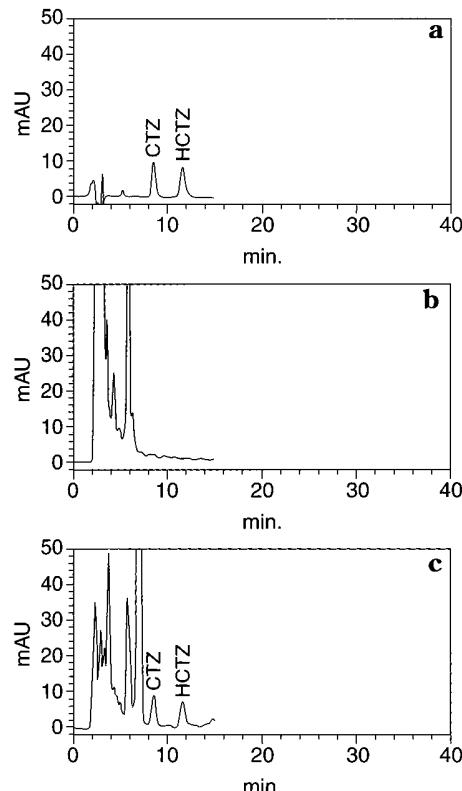
**Mobile Phase.** A stock solution of potassium phosphate, 0.5 M, was prepared by weighing 68 g of  $\text{KH}_2\text{PO}_4$  and transferring with water into a 1-L glass volumetric flask; additional water was added to the mark and mixed. This stock buffer was refrigerated until used. A mobile phase buffer, 0.05 M, was prepared by transferring 100 mL of 0.5 M phosphate buffer into a 1-L glass volumetric flask, additional water was added to reach the mark, and 250  $\mu$ L of phosphoric acid (85%) was added to bring the pH to 3. The mobile phase consisted of a 1:1 ratio of 14% ACN/tetrahydrofuran (THF) in 0.05 M potassium phosphate buffer (pH 3).

**Milk Samples.** Control milk samples were obtained from four different lactating Holstein cows. Fortified milk samples were prepared by transferring 5 mL of control milk samples to 15-mL polypropylene centrifuge tubes. They were spiked with 35, 70, and 140  $\mu$ L of 5  $\mu$ g/mL stock solution of CTZ and HCTZ to give 35, 70, and 140 ppb fortification levels. These levels represent 0.5, 1, and 2 times the safe concentration level (67 ppb), which is rounded to 70 ppb. The HCTZ incurred milk was generated from a lactating Holstein cow treated with an intramuscular dose of 10 mL of injectable solution (25 mg/mL) of Hydrozide for 3 consecutive days. The CTZ incurred milk was also generated from a lactating Holstein cow treated with a 2-g bolus of Diuril for 3 consecutive days. Control milk was collected prior to initial dosing in both cases, and all samples were refrigerated at 4 °C if not analyzed immediately. In addition, portions of the milk samples were frozen at -80 °C for other studies. Both Hydrozide and Diuril were purchased from Merck & Co., Inc. (Ag. Vet. Div.), Rahway, NJ.

**Sample Preparation.** A 5-mL portion of the milk (control, spiked, or incurred) was transferred to a 15-mL polypropylene centrifuge tube and centrifuged for 15 min using a swinging bucket rotor. The defatted milk (4.75 mL) was removed from the tube with a Pasteur glass pipet by puncturing through the fat layer, and the defatted milk was transferred to a 50-mL polypropylene centrifuge tube. Two milliliters of 5% lead acetate solution and 9 mL of ACN were added and vortex mixed for 10 s, and the mixture was centrifuged using the fixed rotor for 30 min. The supernate (12–14 mL) was removed and 25 mL of water-saturated ethyl acetate added, vortex mixed, and centrifuged for 5 min to separate the layers. A 27–30-mL portion of the organic layer was removed, and 4 mL of 10% sodium tungstate solution was added, vortex mixed, and centrifuged for 5 min as before. A 25–27-mL portion of the organic layer was removed into a 50-mL centrifuge tube and evaporated to dryness using an N-evaporator with water bath temperature set at about 35 °C. The resulting residue was reconstituted in 0.25–1.0 mL of mobile phase. A portion of the final extract was transferred into a glass insert, which was placed into an autosampler vial, and 50 or 100  $\mu$ L was injected into the LC column.

**Preparation of Standard Solutions.** The standard solutions were prepared by weighing 10.1 and 10.4 mg of CTZ and HCTZ solid standards, respectively, and by transferring with methanol into two 100-mL amber glass volumetric flasks. Additional methanol was added to bring to the mark to give 101 and 104 ppm for CTZ and HCTZ, respectively. A secondary standard stock solution of ~10 ppm of each thiazide was prepared by transferring 1 mL of each diuretic into two separate 10-mL amber glass volumetric flasks and by adding additional methanol to bring to the mark. Further dilutions were made as appropriate. All solutions were refrigerated until used.

**Standard Curves.** A 5 ppm CTZ/HCTZ combined standard was prepared by pipetting 500  $\mu$ L each of the 101 and 104 ppb stock standards of CTZ and HCTZ in a 10-mL amber



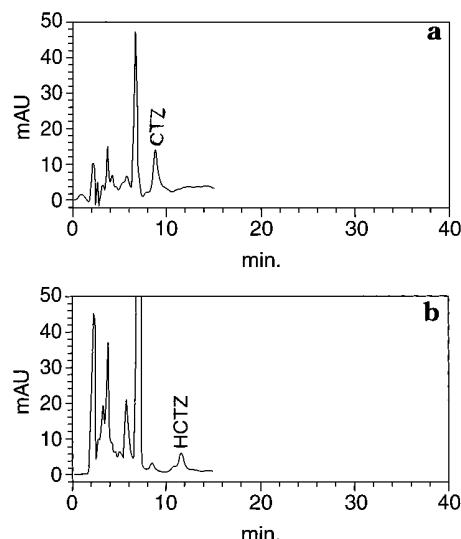
**Figure 1.** Liquid chromatograms of (a) 50 ng each of CTZ and HCTZ standards, (b) control milk extract, and (c) 70 ng/mL fortified milk extract.

glass volumetric flask and diluted to 10 mL with mobile phase. Calibration standards, 0.25, 0.5, 0.75, and 1.0 ppm, were prepared by pipetting 0.5, 1.0, 1.5, and 2.0 mL, respectively, of the mixed 5 ppm standard into four 10-mL glass volumetric flasks, followed by diluting to 10 mL with mobile phase. Injections of 50 or 100  $\mu$ L were made into the LC column.

## RESULTS AND DISCUSSIONS

**Recovery of CTZ and HCTZ from Fortified Milk.** Parts a, b, and c of Figure 1 show typical liquid chromatograms of 100  $\mu$ L injections of a 70 ng/mL standard of CTZ and HCTZ, a control milk extract, and a 70  $\mu$ g/mL fortified milk extract, respectively. CTZ and HCTZ peaks are well separated from the endogenous interfering peaks as well as from each other, affording a multiresidue method for the two thiazides in milk. A standard curve in the range of 25–100 ng CTZ and HCTZ standards was constructed from the LC analysis and was found to be linear with correlation coefficients of 0.999 for both diuretics. These curves were used to quantitate fortified milk samples (regression equations: for CTZ,  $y = 0.2004x - 0.31$ ,  $R^2 = 0.9995$ ; for HCTZ,  $y = 0.1628x - 0.05$ ,  $R^2 = 0.9995$ ).

The recoveries of both CTZ and HCTZ from fortified milk are given in Table 1. The average recoveries of CTZ at 35, 70, and 140 ppb fortification levels were determined to be 97, 96, and 99%, respectively, with corresponding coefficients of variation (CVs) of 8, 5, and 5%. For HCTZ, the average recoveries at 35, 70, and 140 ppb were 87, 91, and 90% respectively, with corresponding CVs of 5, 6, and 5%. The FDA guidelines (Center for Veterinary Medicine/FDA, 1986) for acceptable recovery and CV when residues are below 100 ppb are 60–110 and  $\leq 20\%$ , respectively; for  $\geq 100$  ppb



**Figure 2.** Liquid chromatograms of incurred milk sample extracts: (a) 8-h CTZ post-dose milking; (b) 8-h HCTZ post-dose milking.

**Table 1. Recovery of CTZ and HCTZ from Fortified Milk**

sample	% recovery of thiazides	
	CTZ	HCTZ
35 ppb spike	87.8	88.0
35 ppb spike	108.3	81.1
35 ppb spike	92.3	89.0
35 ppb spike	97.7	84.2
35 ppb spike	97.7	92.7
av	96.8	87.0
SD	7.7	4.5
%CV	7.9	5.1
70 ppb spike	94.0	86.0
70 ppb spike	95.2	88.4
70 ppb spike	104.3	99.0
70 ppb spike	96.5	92.0
70 ppb spike	91.6	89.2
av	96.3	90.9
SD	4.8	5.0
%CV	5.0	5.5
140 ppb spike	97.9	87.9
140 ppb spike	97.8	94.0
140 ppb spike	93.2	88.0
140 ppb spike	99.4	84.5
140 ppb spike	106.4	94.5
av	98.9	89.8
SD	4.8	4.3
%CV	4.8	4.8

residues, they are 80–110 and  $\leq 10\%$ , respectively. Both the recoveries and the CVs in this study at the three fortification levels are well within these guidelines.

**Analysis of Incurred Milk Samples.** The incurred milk samples were assayed to validate the overall procedure for the detection of CTZ and HCTZ. Parts a and b of Figure 2 show the liquid chromatograms of the extracts from the incurred milk samples obtained 8 h after dosing of cows with CTZ and HCTZ, respectively. Again the CTZ and HCTZ peaks are well separated from the endogenous compounds in the incurred milk extracts. The chromatogram from HCTZ incurred milk sample shows a small peak at the retention time of  $\sim 8$  min. This peak probably is either an endogenous

**Table 2. CTZ and HCTZ Levels in Incurred Milk**

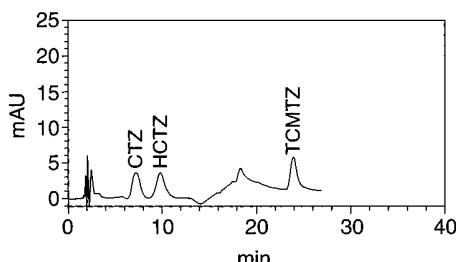
sample	CTZ (ppb)	HCTZ (ppb)
8 h		
1	426.5	41.0
2	424.7	46.4
3	438.0	51.9
4	403.5	47.2
5	449.4	47.2
6	438.0	
av	430.0	46.7
SD	15.8	3.9
%CV	3.7	8.3
24 h		
1	85.9	
2	66.8	
3	97.4	
4	85.9	
5	90.0	
6	85.5	
av	85.2	ND <sup>a</sup>
SD	10.1	
%CV	11.9	

<sup>a</sup> ND, not detected.

compound or a metabolite of HCTZ. Nevertheless, this peak is completely resolved from the HCTZ peak. Table 2 indicates CTZ and HCTZ concentrations in milk collected at 8 and 24 h after the administration of the thiazides to cows. New standard curves covering the range from 12.5 to 50 ng for CTZ and from 25 to 100 ng for HCTZ were prepared and used to quantitate unknown concentrations of the thiazides in the incurred milk sample extracts.

Eight-hour post-dose CTZ samples contained significant amounts of CTZ residue (430 ppb), declined to 85 ppb at 24 h, and were below the detection limit (12.5 ng, equivalent to  $\sim 22$  ppb in milk) in the 32-h and beyond milk samples. HCTZ was detected only in 8-h milk samples (47 ppb), well below the safe level of 67 ppb (Shaikh, 1996). The withdrawal time for both CTZ and HCTZ is reported to be 72 h, and this study suggests that both thiazides are rapidly depleted from the milk, well before their withdrawal time. However, it must be noted that these data reflect the results from a single cow for each drug and may not reflect the rate of depletion of CTZ and HCTZ from the milk of dairy cows in general.

Attempts were made to develop a multiresidue LC method for the separation of the three thiazide diuretics, CTZ, HCTZ, and TCMTZ using LC conditions employed previously for the determination of furosemide in milk (Shaikh, 1995). The mobile phase combination containing 30% ACN used for furosemide was adequate to elute TCMTZ but required much less organic modifier to provide sufficient retention for CTZ and HCTZ. However, the lower percentage of the organic modifier increased the retention of TCMTZ; therefore, a gradient mobile phase was developed to separate the three thiazides (Figure 3). These LC conditions consisted of a reversed-phase polymer column; an initial mobile phase of 11% ACN in 0.05 M phosphate buffer/0.015 M propylamine hydrochloride (pH 3) for 10 min, which was increased to 25% in 15 min and held for 28 min; a temperature of 35 °C; and a wavelength of 225 nm. Initially, propylamine hydrochloride (0.015 M) was added to the mobile phase; however, later it was determined that its use was not necessary to achieve optimal mobile phase combination for the separation of



**Figure 3.** Gradient elution liquid chromatogram of a mixture of 33.3 ng each of CTZ, HCTZ, and TCMTZ standards.

the three thiazides. A number of reversed-phase columns ( $C_{18}$ ,  $C_8$ , and polymer) were evaluated for the optimal separation of the three thiazides. They all gave satisfactory separations for the three; however, the polymer (PRP-1) column appears to provide the desired resolution. Therefore, a polymer reversed-phase column was adopted for the analysis of thiazides in milk. On the basis of the column selected, the gradient profile was modified, including the initial percentage of ACN, to achieve satisfactory separation of the three diuretics. Subsequently, the mobile phase containing ACN was modified to 1:1 ACN and THF to provide improved resolution of CTZ from an endogenous interfering peak in the milk extract that occasionally shouldered with CTZ. The inclusion of THF in the mobile phase caused increased baseline shift during the gradient run. Therefore, the use of the gradient was abandoned, and isocratic mode of analysis was adopted for the determination of thiazides in milk. CTZ and HCTZ were analyzed together using 14–15% ACN/THF (1:1), and TCMTZ was assayed separately using 25–30% ACN/THF (2:1). The overall analysis procedure for TCMTZ in milk will be reported in a separate paper (Shaikh and Rummel, 1997).

The stability of CTZ and HCTZ standard solutions, stored in amber and clear glass vials, was evaluated at room and refrigerated temperatures. No degradation of either thiazide diuretic was noted in either amber or clear glass vials over 5 days at both temperature conditions.

Initially, the furosemide extraction and cleanup procedure (Shaikh, 1995) to defat, deproteinate, and isolate the thiazides from milk was employed. Although the defatting procedure was adopted, the other cleanup procedures did not provide sufficiently clean extracts. Therefore, additional acidic and basic extraction procedures were evaluated (Cooper et al., 1989). Milk was deproteinated with trichloroacetic acid, phosphoric acid, and potassium dihydrogen phosphate and centrifuged. The supernatant was extracted with ethyl acetate, evaporated to dryness, reconstituted in mobile phase, and analyzed by LC. The recoveries in most cases were low and widely variable. Using the above cleanup procedure, the recoveries of HCTZ, CTZ, and TCMTZ from fortified milk samples were 60–93, 24–77, and 16–31%, respectively. Basic extraction with sodium bicarbonate/potassium carbonate mixture and potassium monohydrogen phosphate followed by extraction with ethyl acetate as above also gave poor recoveries in most cases. Using bicarbonate/carbonate, the recoveries for CTZ, HCTZ, and TCMTZ were 0.0–10, 108, and 82%, respectively; with potassium monohydrogen phosphate, they were 10, 57, and 46% for CTZ, HCTZ, and TCMTZ, respectively. It appears that a significant degradation of CTZ occurred under the basic conditions. Solid-phase

extraction (SPE) was also evaluated for its potential to achieve the improved cleanup. Thiazide standards or defatted and deproteinated milk extracts were transferred to SPE cartridges ( $C_{18}$  and  $C_8$ ), washed with water or water/methanol, and eluted with methanol (Campins-Falco et al., 1991). The recoveries in this case were also poor. The results of poor recovery for SPE and acidic and basic extractions are consistent with those reported in the literature (Campinas-Falco et al., 1991; Cooper et al., 1989). Therefore, these cleanup procedures were abandoned.

Use of both the lead acetate and the sodium tungstate in the cleanup procedure was essential; they removed a number of endogenous interfering peaks and provided cleaner extracts. The former is known to precipitate pigments and a number of amino acids and the latter to bind with alkaloids and other products. These compounds may have entered the milk perhaps via diet and/or as endogenous byproducts.

The cleanup procedure described under Materials and Methods proved to be the best for recovering thiazides from the fortified milk samples as evidenced in Table 1. Initially, pure ethyl acetate to back extract thiazides was used; however, on occasion some peak shouldering with CTZ showed up. This problem was reduced or disappeared with the use of water-saturated ethyl acetate. In some cases, anhydrous sodium sulfate was used to remove any traces of moisture in the organic phase before the drying step, but later with the use of water-saturated ethyl acetate, it was found to be unnecessary.

**Accuracy, Precision, and Sensitivity.** The average recoveries for 35–140 ppb fortified milk samples were 97 and 89% for CTZ and HCTZ, respectively, with average CVs of 6 and 5% for CTZ and HCTZ, respectively. The intraday (3 days) CVs of HCTZ and CTZ fortified samples at 70 ppb were 6 and 5%, respectively. The interday CVs of HCTZ and CTZ of 8-h post-dose incurred milk samples ( $n = 3$ ) were 12 and 2%, respectively. The signal-to-noise ratio ( $S/N$ ) for 25 ng of standards of HCTZ and CTZ was  $>10$ . The  $S/N$  ratios of CTZ and HCTZ in 35 ppb fortified milk were 8 and 6, respectively. The limit of detection and the limit of quantification were estimated to be 22 and 35, respectively, for both CTZ and HCTZ. These results suggest that the method is sufficiently sensitive to determine accurately and precisely the low residue concentrations of CTZ and HCTZ in bovine milk.

**Specificity of the Method.** The following standard drug compounds, at relatively high concentrations, were tested for their potential interference with the analysis of CTZ and HCTZ: TMCTZ, furosemide, dexamethasone, tetracycline, chlortetracycline, oxytetracycline, sulfamethazine, and  $\beta$ -lactam antibiotics (penicillin G, cloxacillin, ampicillin, amoxicillin, penicillin V). These compounds are used in dairy cattle, and, with the possible exception of sulfamethazine, they did not interfere with the chromatographic elution of CTZ or HCTZ. Sulfamethazine (200 ng on column) appears to be partially resolved from HCTZ. However, if present at low levels ( $<50$  ppb) in milk, sulfamethazine may not be detectable under the LC conditions employed.

**Conclusions.** An accurate, precise, and sensitive method for the determination of CTZ and HCTZ has been developed. The method distinguishes these thiazides from other diuretics, drugs, and antibiotics used in dairy cattle. The method was validated by quantitating CTZ and HCTZ concentrations in milk obtained at

various intervals from cows administered with approved doses of these diuretics.

#### ACKNOWLEDGMENT

We are grateful to Mr. Herbert F. Righter of the Division of Animal Research, CVM-FDA, for providing CTZ and HCTZ incurred milk samples.

#### LITERATURE CITED

Campins-Falco, P., et al. Solid-Phase Extraction Techniques for Assay of Diuretics in Human Urine Samples. *J. Liq. Chromatogr.* **1991**, *14*, 3575–3590.

Center for Veterinary Medicine/FDA. *General Principles for Evaluating the Safety of Compounds Used in Food Producing Animals: Guidelines for Approval of a Method of Analysis for Residues*; FDA: Rockville, MD, Sept 1986.

*Code of Federal Regulations*. Food and Drugs, Parts 520.420 and 522.1150; U.S. GPO: Washington, DC, 1991.

Cooper, S. F., et al. Comprehensive Screening Procedure for Diuretics in Urine by High-Performance Liquid Chromatography. *J. Chromatogr.* **1989**, *489*, 65–88.

Herraez-Hernandez, R., et al. Estimation of Diuretic Drugs in Biological Fluids by HPLC. *Chromatographia* **1992**, *33*, 177–185.

Shaikh, B. Development and Validation of a Liquid Chromatographic Method for the Determination of Furosemide, a Diuretic, in Bovine Milk. *J. Agric. Food Chem.* **1995**, *43*, 2117–2121.

Shaikh, B. Diuretic Drugs Used in Food Producing Animals. In *Veterinary Drug Residues*; ACS Symposium Series 636; Moats, W. A., Medina, M. B., Eds.; American Chemical Society: Washington, DC, 1996; pp 161–168.

Shaikh, B.; Rummel, N. Determination of Trichlormethiazide in Bovine Milk by High Performance Liquid Chromatography. *J. Chromatogr. B, Biomed. Sci. Appl.* **1997**, in press.

*Veterinary Pharmaceuticals and Biologicals*, 8th ed.; Veterinary Medicine Publishing: Lenexa, KS, 1993/1994; pp 729–730.

Werthmann, Jr., M. W.; Krees, S. V. J. Extraction of Chlorothiazide in Human Breast Milk. *Pediatrics* **1972**, *81*, 781–783.

Received for review July 24, 1997. Revised manuscript received January 6, 1998. Accepted January 14, 1998.

JF970637A