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Determination of trichlormethiazide in bovine milk by high-performance liquid chromatography

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Abstract

A liquid chromatographic procedure was developed and validated for the quantitative determination of trichlormethiazide (TCMTZ) in bovine milk. Whole milk was defatted by initial centrifugation at 4°C. The resulting skim milk was treated with lead acetate and acetonitrile, vortex mixed, and centrifuged. The acetonitrile from the supernatant was back extracted in ethyl acetate. The organic solvent mixture which contained TCMTZ was further treated with sodium tungstate, vortex mixed, and centrifuged. The top organic layer was removed and evaporated to dryness; the resulting residue was reconstituted in the mobile phase, and the final extract was analyzed by high-performance liquid chromatography (HPLC). The HPLC conditions employed included a polymer column, a mobile phase consisting of 30% acetonitrile or 30% acetonitrile–tetrahydrofuran (2:1, v/v) in a phosphate buffer (pH 3), and a UV detection at 225 nm. The average recoveries of TCMTZ from milk fortified at 7, 14, 35, 70, and 140 ppb were 88, 93, 117, 110, and 99%, respectively, with corresponding C.V. values of 7, 18, 11, 9, and 21%. The method was validated by assaying milk obtained from a cow dosed with Naquasone. TCMTZ concentration was detected only in the 8 h post dose milk samples and was determined to be 6 ppb. © 1998 Elsevier Science B.V.

Keywords: Bovine milk; Trichlormethiazide; Thiazides; Diuretics

1. Introduction

Trichlormethiazide (TCMTZ) is approved for use in cattle as an aid in reduction and for the treatment of a postparturient udder edema [1]. TCMTZ is given in combination with dexamethasone [2] and is an orally administered, highly effective diuretic agent of the benzothiazide series [3]. The combined diuretic activity of TCMTZ and the specific antiinflammatory activity of dexamethasone are complementary in the reduction of a physiological parturient edema of the mammary gland and associated structures in cattle. Because the two drugs are complementary in their action, effects are achieved with a minimum dosage of TCMTZ. Studies in man and experimental animals show that TCMTZ presents a favorable pattern of less potassium excretion than chlorothiazide (CTZ) and hydrochlorothiazide (HCTZ). The clinically determined saluretic potency of TCMTZ is estimated to be 10–20 times that of HCTZ and 100–200 times that of CTZ, resulting in decrease in the incidences of hypokalemic manifestations [4]. Milk taken from dairy animals during TCMTZ treatment and for 72 h after the latest treatment must not be used for food [2,5]. The chemical structure of TCMTZ and other closely

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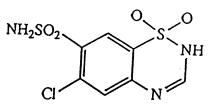
related thiazides, CTZ and HCTZ, approved for use in dairy cattle are shown in Fig. 1.

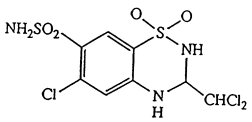
In the literature, almost all analytical methods have been limited to assaying diuretic drugs in plasma and urine. These methods have been reviewed extensively [5,6] and indicate that HPLC is the main technique employed for their analysis. Among the thiazide diuretics, not much work has been reported for the detection of TCMTZ in biological fluids. de Croo et al. [7] studied the liquid chromatographic behavior of diuretic standard compounds including TCMTZ in terms of their selectivity using a different organic modifier in the mobile phase. Their experiments indicate that acetonitrile–water mixtures are suitable for the chromatography of thiazide diuretics, and use of tetrahydrorfuran as a third mobile phase component can be useful when a different selectivity is needed. Smith et al. [8] further evaluated a number of separation conditions on the reproducibility of retention values in HPLC for thiazide standards. Their results confirm that good interlaboratory reproducibility will be achieved, when the temperature of the column is controlled and the same brand of column packing material is used. Cooper et al. [9] developed a comprehensive screening procedure using HPLC for the detection of diuretics, belonging to various pharmaceutical groups, in urine. This was made possible by extracting urine separately under acidic and basic conditions, pooling the two extracts, and assaying on two different reversed-phase columns. The detection limit for TCMTZ in urine in both extractions was determined to be 1 ppm. Jumppanen et al. [10] used capillary zone electrophoresis (CZE) to screen diuretics including TCMTZ in urine and plasma. CZE is a good alternative to HPLC since less sample is required and lower detection limits are achieved. However, resolution problems arise in CZE when proteins and other endogenous compounds are not completely removed from biological fluids.

With the exception of an HPLC method for the determination of a loop diuretic, furosemide, in milk [11], no chromatographic method has been reported in the literature for assaying diuretic drugs including thiazides in milk. Recently, we have reported an HPLC method for the determination of CTZ and HCTZ in milk [12]. The procedure described in this paper is the continuation of our work on methods to detect diuretic drugs in milk [11,12].

Trichlormethiazide (TCMTZ)



Chlorothiazide (CTZ)



Hydrochlorothiazide (HCTZ)

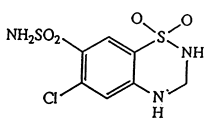


Fig. 1. Structures of thiazide diuretics approved for use in food producing animals.

2. Materials and methods

2.1. Apparatus

The LC consisted of a Hewlett-Packard Model 1050 system (Palo Alto, CA, USA) fitted with a quaternary pump, an auto sampler, a column heater, a solvent bottle holder with a 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 analytical (150 mm×4.1 mm) and guard (25 mm×4.1 mm) columns employed were reversed-phase polymer PRP-1 (Hamilton, Reno, NV, USA) having a packing of 5 μm particle size. Both the analytical and guard columns were placed in a column heater set at 30°C. The temperature was changed to 25°C when recoveries at 7 ppb were determined to allow for greater resolution of the TCMTZ peak from endogenous interferences.

All centrifugations were carried out at 5500 rpm using both an HS-4 swinging Rotor (5810*g*) and SE-200 fixed rotors (4380 *g*) in a Sorvall RC-5

refrigerated centrifuge (Dupont, Wilmington, DE, USA) set at 4°C. Polypropylene tubes (15 and 50 ml) with plug type screw caps were used (Corning Glass Works, Corning, NY, USA). All transfers were made with Eppendorf digital pipettes.

2.2. Reagents

Glass distilled organic solvents (Burdick and Jackson Laboratories, Muskegon, MI, USA) and distilled deionized water, filtered through a 0.2 μm nylon filter, were used. All chemicals were of HPLC grade, except where noted. The diuretic, TCMTZ, was obtained from Sigma (St. Louis, MO, USA).

2.3. The mobile phase

One liter of stock solution of 0.5 *M* potassium phosphate was prepared and refrigerated until used. A 0.05 *M* mobile phase buffer (1 l) was prepared from 0.5 *M* stock solution of phosphate buffer and 250 μl of phosphoric acid (85%) was added to bring the pH to 3. The mobile phase consisted of either 30% acetonitrile or 30% acetonitrile–tetrahydrofuran (2:1, v/v) in 0.05 *M* potassium phosphate buffer (pH 3).

2.4. 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 μl of 5 μg/ml stock solution of TCMTZ to give 35, 70, and 140 ppb fortification levels, respectively. Seven and 14 ppb fortification levels were prepared by spiking 5 ml control milk with 35 and 70 μl of one ppm standard, respectively. The TCMTZ incurred milk was generated from a lactating Holstein cow treated with a bolus of Naquasone containing 200 mg of TCMTZ and 5 mg of dexamethasone for three consecutive days. Control milk was collected prior to initial dosing 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 use in the event that further experiments are warranted. Naquasone was purchased from Merck (Ag. Vet. Div.; Rahway, NJ, USA).

2.5. 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 pipette by puncturing through the fat layer and transferred to a 50 ml polypropylene centrifuge tubes. Two ml of 5% lead acetate solution and 9 ml of acetonitrile were added, vortex mixed for 10 s, and the mixture was centrifuged using the fixed rotor for 30 min. The supernatant (13 to 14.5 ml) was removed and 25 ml of ethyl acetate added, vortex mixed, and centrifuged for 5 min to separate the layers. A 30–32 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 26–29 ml portion of the organic layer was removed into 50 ml centrifuge tubes and evaporated to dryness using an N-evaporator with water bath temperature set at about 30–35°C. The residue was dissolved in 0.25 to 0.5 ml mobile phase; a portion of the final extract was transferred to a glass insert which was placed into an autosampler vial and 50 or 100 μl was injected into the LC column. A relatively high volume (5 ml) of milk was extracted to achieve desired lower limits of detection for TCMTZ.

2.6. Preparation of standard solutions

A stock standard solution of TCMTZ was prepared by weighing 10 mg of solid standard and by transferring with methanol into a 100 ml amber glass volumetric flask. Additional methanol was added to bring the volume to the mark to give a 100 ppm solution. A second stock standard solution of 10 ppm TCMTZ was prepared by transferring 1 ml of the above stock solution into 10 ml amber glass volumetric flasks and by adding additional methanol to bring to the mark. A third stock standard solution of 1 ppm was prepared by transferring 1 ml of 10 ppm stock solution to a 10 ml amber glass volumetric

flask and bringing the volume to the 10 ml mark with methanol. Further dilutions were made as appropriate. All solutions were refrigerated until used.

2.7. Standard curves

A 5 ppm standard was prepared by pipetting 500 μl of the 100 ppm stock standard into a 10 ml amber glass volumetric flask, diluting to 10 ml with mobile phase and mixing. 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 5 ppm standard into four 10 ml glass volumetric flasks, followed by diluting to 10 ml with mobile phase. Additional calibration standards of 0.1, 0.2, 0.3, 0.4, and 0.5 ppm were prepared by transferring 0.1, 0.2, 0.3, 0.4, and 0.5 ml, respectively, of 10 ppm stock standard solution into amber glass volumetric flasks and bringing the volume to the 10 ml mark with a

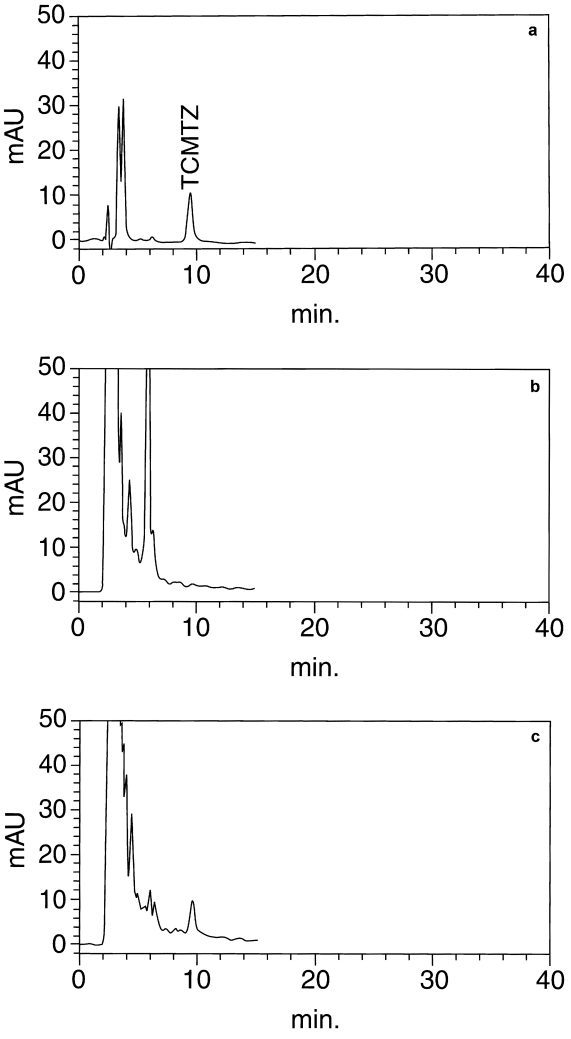


Fig. 2. Liquid chromatograms: (a) 75 ng of TCMTZ standard; (b) control milk extract; (c) 70 ng/ml TCMTZ fortified milk extract.

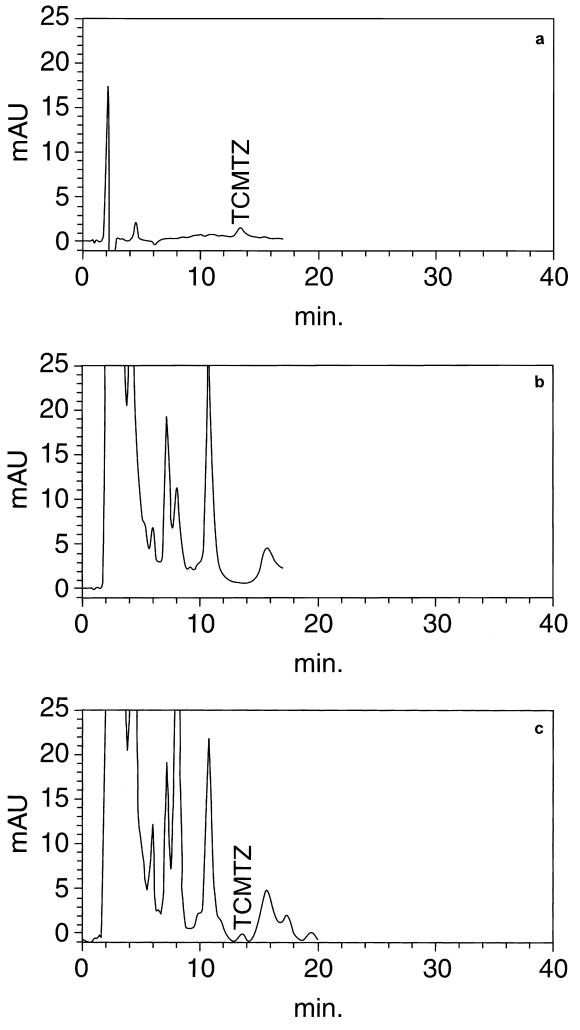


Fig. 3. Liquid chromatograms: (a) 10 ng of TCMTZ standard; (b) control milk; (c) 7 ng/ml fortified milk sample.

mobile phase. Injections of 100 μl were made into the LC column.

3. Results and discussions

3.1. Recovery of TCMTZ from fortified milk

Fig. 2a–c show typical liquid chromatograms of 100 μl injections of a 0.75 ppm standard, a control milk extract, and a 70 ng/ml (70 ppb) fortified milk extract, respectively. The TCMTZ peak is well separated from the endogenous peaks. A standard curve in the range of 25–100 ng standards injected was constructed from the HPLC analysis, found to be linear with an average correlation coefficient of 0.999, and was used to quantitate 35–140 ppb fortified milk samples. Fig. 3a–c also show typical chromatograms of the 100 μl injection of 0.1 ppm standard, control milk extract, and a 7 ng/ml fortified milk extract, respectively. A standard curve in the range of 10–50 ng standards was constructed, found to be linear with an average correlation coefficient of 0.999, and was used to quantitate TCMTZ concentrations from 7–14 ppb fortified milk samples. The peak heights were used in all calculations.

The recoveries from fortified milk samples are given in Table 1. The average recoveries of TCMTZ at 7, 14, 35, 70, and 140 ppb fortification levels were determined to be 88, 93, 117, 110, and 99%, respectively, with corresponding C.V. values of 7, 18, 11, 9, and 21%. The %C.V. for 140 ppb fortification concentration level samples appears to be relatively high. However, if the recovery for the second sample (66.9%), which is outside the range of 1 standard deviation, is not included, the %C.V. is lowered to a reasonable value of 14.3%.

3.2. Analysis of incurred milk samples

Fig. 4a–c show the liquid chromatograms of the 15 ng of the TCMTZ standard, control milk extract, and TCMTZ incurred milk sample obtained from a cow 8 h after dosing. Again the TCMTZ peak is well-separated from the endogenous compounds in the incurred milk extracts. Table 2 indicates TCMTZ concentrations in milk collected at 8 h after the administration of the thiazide to a cow. A linear relationship was obtained for standard curves covering the range from 10–50 ng for TCMTZ with an average correlation coefficient of 0.997, which were used to quantitate incurred milk samples.

Eight hour post dose incurred milk samples contained small amounts of TCMTZ residues (6 ppb), close to its safe concentration level [4], and no TCMTZ was detected in 24 h post dose incurred milk samples. The withdrawal time for TCMTZ is reported to be 72 h and this study suggests that TCMTZ is rapidly depleted from the milk, well

Table 1
Recovery (%) of trichlormethiazide from fortified milk samples

Sample No.	7 ppb spike	14 ppb spike	35 ppb spike	70 ppb spike	140 ppb spike
1	82.3	72.1	138.3	114.8	113.6
2	86.1	80.4	115.6	94.4	66.9
3	98.5	96.7	102.1	128	110.1
4	88	102.5	102.6	104	117.3
5	87.3	112.3	117.3	112.7	79.6
6			122.7	109.6	104.1
7			119.1	106.3	
Ave.	88.4	92.8	116.8	110.0	98.6
S.D.	6.0	16.4	12.4	10.4	20.5
%C.V.	6.8	17.6	10.6	9.4	20.8

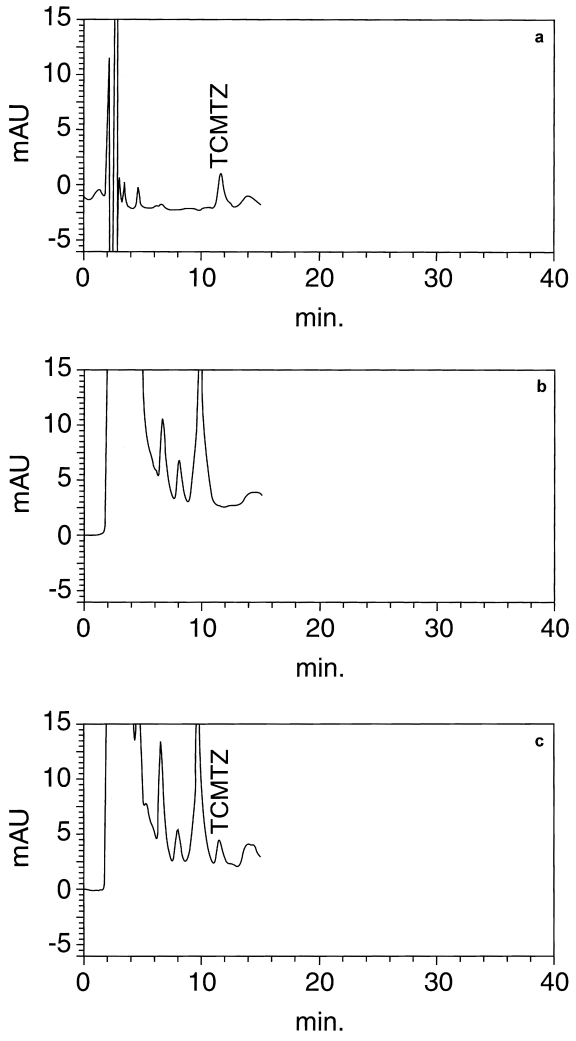


Fig. 4. Liquid chromatogram: (a) 15 ng TCMTZ standard; (b) control milk extract; (c) 8 h post dose TCMTZ incurred milk.

before its withdrawal time period. However, it must be noted that this data reflects the results from a single cow for this drug and may not reflect the rate of depletion of TCMTZ from the milk of dairy cows in general. In this study only one cow was used since the objective of this study was to validate the method performance in incurred milk samples.

During the course of analysis of milk extracts, an impurity peak on the LC chromatogram was noted that interfered with the determination of TCMTZ from milk extracts. It was determined that this impurity resulted due to use of ethyl acetate from a different manufacturer in the extraction process. Therefore, the mobile phase was modified to resolve TCMTZ from the interfering peak. The recoveries at 35, 70, and 140 ppb fortification levels were determined using 30% acetonitrile in 0.05 *M* phosphate bluffer (pH 3), while recoveries at 7 and 14 ppb fortification levels and all incurred samples were assayed using acetonitrile–tetrahydrofuran (2:1, v/v) in 0.05 *M* potassium phosphate buffer (pH 3). In order to achieve maximum sensitivity, the final extracts for incurred, 7 and 14 ppb fortification samples were reconstituted in 0.25 ml of mobile phase.

Table 2
Trichlomethiazide concentrations in incurred milk

Sample	TCMTZ (ppb)
8 Hour-1	6.2
8 Hour-2	5.5
8 Hour-3	6.6
8 Hour-4	5.3
8 Hour-5	6.3
Average	6.0
S.D.	0.55
%C.V.	9.3

Note: No TCMTZ residue was detected from 24 h post dose milk samples.

A two component mobile phase of acetonitrile–phosphate buffer gives analysts an option to use a simpler mobile phase when higher concentration levels of TCMTZ are to be determined. Both of the above isocratic mobile phase systems were not suitable to separate and elute the other two thiazides (CTZ and HCTZ), since they required less organic modifier [12]. However, a gradient mobile phase of acetonitrile–phosphate buffer was found to separate the three thiazides, CTZ, HCTZ and TCMTZ, from each other [12] and perhaps this gradient may also be useful to resolve other possible metabolites of TCMTZ and other thiazide diuretics.

3.3. Accuracy, precision, and sensitivity

The average recovery of TCMTZ for 7–140 ppb fortified milk samples was 101%, with an average C.V. of 13%. The accuracy in this study represents a closeness of the recovered value to the amount of TCMTZ spiked in milk. A total of 30 analyses were

carried out for 5 spiking levels resulting in an average recovery of 101%, an excellent closeness to the true values. The interday C.V. values of TCMTZ fortified samples at 14 (2 days) and 35 (4 days) ppb were 18 and 12%, respectively. The intraday C.V. of TCMTZ for 8 h post dose incurred milk samples ($n=5$) was 9%. The signal-to-noise ratio (S/N) for 10 ng of standard of TCMTZ was greater than 5 and the limit of quantitation is about 8 ng injected. These results suggest that the method is sufficiently sensitive to determine accurately and precisely the low residue concentrations of TCMTZ in bovine milk.

3.4. Specificity of the method

The following drugs were tested for their potential interference with the analysis of TCMTZ: chlorothiazide, hydrochlorothiazide, furosemide, dexamethasone, tetracycline, chlortetracycline, oxytetracycline, sulfamethazine, beta-lactam antibiotics (penicillin G, cloxacillin, ampicillin, amoxicillin, penicillin V). These compounds are used in dairy cattle, and they did not interfere with the chromatographic elution of TCMTZ under the HPLC conditions employed.

These drugs were assayed individually. The CTZ and HCTZ require less organic modifier and were eluted early [12], whereas, furosemide was retained longer [11] than TCMTZ. Sulfamethazine and dexamethasone were also retained less than TCMTZ; whereas, the beta-lactams and tetracycline antibiotics appear not to be retained and were eluted in the void volume.

4. Conclusions

An accurate, precise, and sensitive method for the determination of TCMTZ have been developed. The method distinguishes this thiazide from other diuretics, drugs, and antibiotics used in dairy cattle. The method was validated by quantitating TCMTZ concentration in milk obtained from a cow administered with approved doses of this thiazide.

Acknowledgements

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