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Methodology for Quantifying Residues of Chlorhexidine in Raw Dairy Milk

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A residue method was developed as part of a pharmacokinetics study to determine the elimination of chlorhexidine in raw milk after intramammary infusion into dairy cows affected with bovine mastitis. The developed liquid/liquid and solid-phase extraction procedures effectively reduced sources of milk product interferences in the final extract. By optimizing mobile-phase pH buffer/acetonitrile gradient conditions and employing an end-capped reverse-phase polar embedded-phase chromatographic column, excellent peak resolution was achieved without the additional need of mobile-phase amine modifiers or ion-pairing reagents. The combined cleanup and chromatographic method steps reported herein were sensitive and reliable for determining the pharmacokinetic elimination of chlorhexidine following intramammary infusion. The residue method was found to be rugged with a lower detection limit of 0.1 ppm.

KEYWORDS: Chlorhexidine; chromatography; food analysis; milk

INTRODUCTION

Chlorhexidine [CH; 1,1′-hexamethylenebis[5-(4-chlorophenyl)biguanide]] has a wide spectrum of bactericidal and antiviral activity and is a common ingredient in various formulations ranging from skin disinfectants in healthcare products to antiplaque agents in dentistry (*1*, *2*). The presence of two symmetrically positioned basic chlorophenyl guanide groups attached to a lipophilic hexamethylene chain (**Figure 1**) aid in rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmic leakage, and enzyme inhibition (*3*).

Because of its wide range of antimicrobial activity, CH is used in veterinary medicine for preventing the spread of bacteria associated with bovine mastitis. Various chlorhexidine preparations are marketed as topical postmilking teat dips and udder washes for use in commercial dairy milking operations (*4*). These outer-skin treatments are considered nonfood antiseptic uses because CH residues are unlikely to be transferred into milk (5). This antiseptic has also been shown to be efficacious as a therapeutic agent for treating bovine mastitis within inflamed cow mammary quarters (*6*, *7*). This treatment relies on direct intramammary infusion into the udder, so CH residues may potentially be transferred at milking and contribute to human dietary exposure. The acute oral toxicity measured in male and female rats is relatively low (i.e., LD_{50} > 1000 mg/kg of body weight) (*5*). Although the chlorhexidine residues detected in milk following therapeutic cessation of lactation may well be below any probable population adjusted dose, there currently is no dietary exposure data reported for this substance. As a result, CH is not registered for food use and lacks a published tolerance for all food products (*5*).

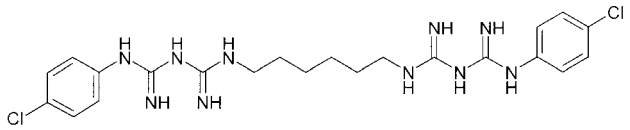

Figure 1. Chemical structure of chlorhexidine.

The use of chlorhexidine as an intramammary therapeutic agent represents an extra-label use falling under the provisions of the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1996. Under this provision, a veterinarian may use a drug or compound in an extra-label fashion (i.e., not in accordance with the approved labeling) in food-producing animals if residues transferred to edible tissues (i.e., milk, eggs, meat, and other edible products) are below levels of toxicological concern. As part of the AMDUCA, verifiable analytical methods must be developed to quantitatively show that CH residues will be below dietary exposure risks prior to the marketing of milk and milk products. This paper describes a quantitative HPLC residue detection method that was developed for assessing the elimination lifetime of CH residues in milk

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following intramammary infusion into dairy cows affected with bovine mastitis.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals used in the extraction procedure and in liquid chromatography were hydrochloric acid (J. T. Baker), glacial acetic acid (J. T. Baker), petroleum ether (J. T. Baker), methylene chloride, a class II carcinogen (Fisher), HPLC grade acetonitrile (Fisher), HPLC grade water (Fisher), methanol (Fisher), HPLC grade sodium acetate trihydrate (Aldrich-Sigma), and octadecyl C-18 disposable extraction cartridges (BakerBond SPE). All solvents were of pesticide grade or better. Chlorhexidine diacetate was purchased from Sigma (St. Louis, MO). Fortification solutions and CH standards for HPLC calibration were prepared in a buffer solution of 0.2 M sodium acetate trihydrate that was adjusted to pH 3.6 with 0.2 M acetic acid.

Sample Preparation. All sample workups and analyses were usually conducted in discrete sets consisting of six to eight individually treated milk samples with two quality control (QC) spike (CH fortified) samples and an untreated milk control sample.

Extraction. For each analytical set of samples, 10 g of treated or control milk was weighed into 250 mL centrifuge bottles. The QC spikes were fortified at concentrations ranging from 0.4 to 10 ppm (w/w) using a 1 mg/mL solution of CH prepared in pH 3.6 acetate buffer solution. After addition of the milk, 1 mL of concentrated HCl was carefully added to each bottle and swirled before centrifugation at 10000 rpm (14000*g*) for a period of 10 min. After centrifugation, the acidified supernatant was carefully decanted from each bottle into a 125 mL separatory funnel through a glass-filter funnel lined with glass wool to capture suspended milk solids. The residual pellet that consisted of precipitated milk solids was rinsed with 5 mL of a 3% solution (v/v) of acidified HCl in HPLC grade water. This rinse was carefully transferred using a Pasteur pipet back into the 125 mL separatory funnel.

Analyte Partition. The acidified aqueous supernatant containing the ionized CH was partitioned several times with organic solvents to remove residual fats and other possible organic coextractables. First, 50 mL of petroleum ether was added to the 125 mL separatory funnel, which was then gently swirled for ~2 min with periodic venting. The lower aqueous phase containing the ionized CH was retained and repartitioned with 50 mL of petroleum ether. The aqueous phase was then transferred into a second 125 mL separatory funnel followed by the addition of 15 mL of methylene chloride. The separatory funnel was then gently swirled for ~2 min with periodic venting. An emulsion layer usually formed between the two phases. The emulsion was transferred to a 25 mL glass centrifuge tube and centrifuged at 10000 rpm (12500*g*) for 10 min. After centrifugation, the upper aqueous phase was recombined with the aqueous phase remaining in the 125 mL separatory funnel. The methylene chloride phases were discarded. The partitioned aqueous solution was then transferred to a 50 mL evaporator flask and placed in a Zymark Turboevaporator under nitrogen gas at 40 °C for 5 min to dispel any codissolved methylene chloride from the solution.

Analyte Isolation. Octadecyl C-18 disposable solid-phase extraction cartridges (500 mg; BakerBond SPE) were prepared for isolating CH from the aqueous solution. The C-18 cartridge was first conditioned under negative pressure with 1 column volume of methanol followed by 2 column volumes of a 3% HCl solution (v/v) in HPLC grade water. The cartridge was not allowed to dry out during conditioning. The aqueous sample extract was then poured into the cartridge and evacuated at a flow rate of ~5 mL/min. The cartridge bed was then rinsed with 5 mL of 3% HCl and air-dried under negative vacuum for ~10 min. CH was eluted with 2 mL of HPLC grade methanol into a 15 mL graduated centrifuge tube. For each sample, the solvent volume was reduced to near dryness under nitrogen gas at 35 °C and then adjusted with pH 3.6 acetate buffer solution to a volume suitable for residue analysis (usually 2 mL). Each sample was then filtered through a 0.45 μm poly(tetrafluoroethylene) (PTFE) filter (Whatman) into a 2 mL autosampler vial for subsequent analysis by HPLC with photodiode array detection.

HPLC Analysis. A Supelco Discovery RP-Amide C16 (15 cm × 3 mm, particle size = 5 μm) with matching guard cartridge was used for chromatography of CH residues. Fifty microliters of each sample was injected for quantitation using a Varian 9012 solvent delivery system, a 9100 autosampler, and a 9065 Polychrom photodiode array detector. CH was eluted at a flow rate of 1 mL/min using a programmed gradient of pH 3.6 acetate buffer and acetonitrile (**Table 1**). After analyte elution, the remaining gradient program was required to elute from the column remaining matrix coextractables. To avoid changes in retention time, an equilibrium time of 1 min was required for re-establishing stable mobile-phase conditions before the next analytical run. The detector was set to scan each run at wavelengths of 200−320 nm, and CH residues were monitored specifically at 258 nm. Chromatographic data were integrated using a Varian Star Chromatography Workstation ver. 5.3. CH residues were quantified using a multipoint calibration with external CH standards prepared in acetate buffer (pH 3.6).

Table 1. Gradient Elution Program for Separation of Chlorhexidine in Milk

operation	time	%A	%B	gradient curve
elution	initial	80	20	
	1 min	80	20	linear
	5 min	30	70	linear
	8 min	30	70	(held at 30/70 for 3 min)
	10 min	0	100	linear
	17 min	0	100	(held at 0/100 for 7 min)
return	20 min	80	20	linear
equilibration	21 min	80	20	(held at 80/20 for 1 min)

RESULTS AND DISCUSSION

CH is a dicationic base and should be expected to exist in an ionic state over a wide pH range (**Figure 1**). As such, a complex set of equilibria can govern its physiochemical behavior and affect matrix extraction efficiency and chromatographic separation. One prior study reported the use of HPLC for the quantitation of CH residues in milk following its topical application as a teat dip (*8*). This method relied on acidifying the milk sample before sequential organic/aqueous liquid/liquid extractions were performed followed by HPLC determination using a traditional reverse-phase octadecyl C-18 column with UV−vis detection. We first attempted to validate the above method for evaluating intramammary infusions of CH residues in raw dairy milk. Although the above procedures provided adequate cleanup for quantitation at the mid-to-high parts per million level, coeluting matrix interferences prohibited needed quantitation at lower residue levels. We also found that the choice of a traditional C-18 column when using the reported mobile-phase pH 3 acetate buffer gradient conditions was not best suited for resolving trace-level CH residues from the background owing to excessive peak tailing. This observation was not unexpected given that under acidic mobile-phase buffer conditions basic amine compounds can interact with available stationary-phase acidic silanol groups and result in poor chromatographic resolution (*9*).

Because of the need for greater method sensitivity and chromatographic resolution, we initiated various cleanup and HPLC chromatographic steps to improve CH residue quantitation in raw dairy milk. To improve upon isolating CH from milk fats and other possible matrix interferences, we initially chose strong cation exchange (SCX) over more traditional liquid/liquid partitioning procedures. Unfortunately, the complex ionic behavior of CH made it difficult to select an appropriate elution solvent for consistent and quantitative extraction from the ion-exchange resin bed. A series of liquid/liquid partitions using petroleum ether followed by methylene chloride was found to be a reasonable alternative for cleaning up fats and other products from the acidified aqueous matrix. These partitions

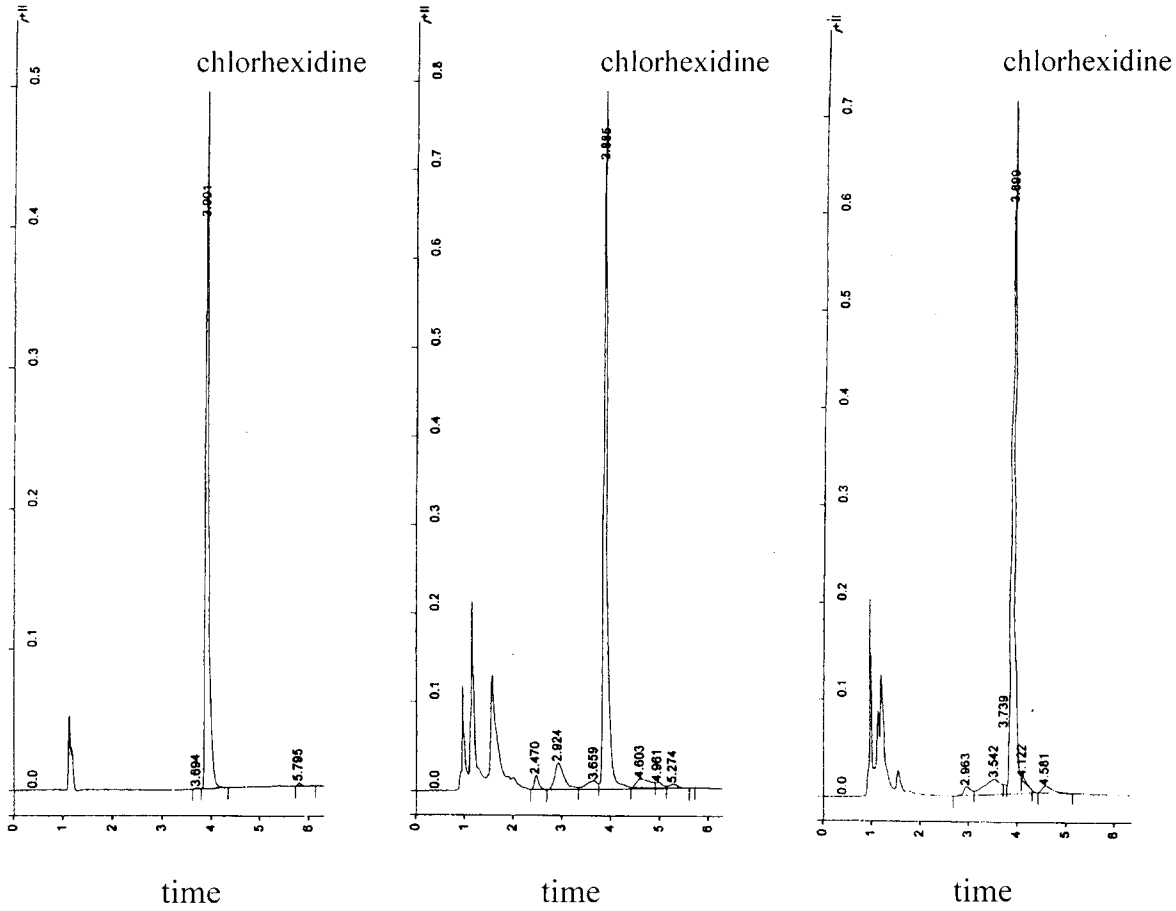

Figure 2. Liquid chromatograms (DAD 258 (m) of chlorhexidine (RT ≈ 3.9 + 1%): (A) 2 μg mL^{-1} standard solution; (B) raw milk fortified at 2 ppm; (C) actual raw milk sample at 8 days post-intramammary infusion.

followed by C-18 SPE analyte isolation/elution, volume adjustments into a pH 3.6 acetate buffer solution, and sample filtration provided sufficient sample cleanup to follow the dissipation lifetime of this antiseptic up to 42 days after intramammary infusion (*10*). To improve peak resolution of basic amines under acidic mobile-phase conditions, we took advantage of recent advances in inert HPLC silica packing and embedded polar-phase column technology. By choosing a highly inert end-capped reverse-phase embedded polar-phase amide column with matching guard cartridge, we were able to appreciably minimize CH peak tailing, thus eliminating the need for mobile-phase ion-pairing reagents or amine modifiers. Chromatographic separation and sensitivity were found to be optimal when using a pH 3.6 acetate buffer−acetonitrile gradient system (**Table 1**). **Figure 2** shows typical chromatograms of CH in standard solution, in fortified raw milk samples, and in intramammary infused treated raw milk samples.

Any variation in the mobile-phase gradient conditions during the analytical run can appreciably alter the retention time and peak resolution of basic amine compounds such as CH. With the mobile-phase acetate buffer−acetonitrile gradient system (**Table 1**), retention times for all standards and samples were stable and fell within 2.5% of the retention window over the experimental time frame without diminishing resolution. For each analytical run, a multipoint linearity was also performed with calibration standards ranging between 1 and 100 μg mL^{-1}. The diode array detector response was found to be linear with a coefficient of determination (r^2) ≥ 0.995 for all standard curves over the course of this study.

To evaluate the precision and sensitivity of the method, replicate control raw milk samples were fortified at concentrations ranging from 0.4 ppm (i.e., our lowest practical limit of quantiation measured as 5 times the method background) to 10 ppm (**Table 2**). The various fortification levels were chosen to represent expected residues in real raw milk samples after intramammary infusion. We found that fortifications performed at the lower levels were more variable with consistently greater recoveries than at the other higher levels of fortification. This was presumably due to trace-level carry-over of UV-detectable coextractables. During our preliminary range-finding evaluations we found that appreciable carry-over in the final milk extract prevented reliable quantitation at or below 0.1 ppm, even after introducing various sample cleanup and chromatographic improvements. This also explains the trend of slightly higher recovery values for the 0.4 ppm fortifications (**Table 1**). Because of the variable background between milk samples, we conservatively chose 0.1 ppm (1−2 times the method background) as our limit of detection. Chlorhexidine residues in raw milk were also found to be stable under cold storage conditions. The average CH residue concentration from replicate fortified milk samples stored for 5 months at −15 to −20 °C was 116% of the 10 ppm time-zero fortification level.

Table 2. Method Recoveries of Chlorhexidine from Milk

level of fortification (ppm)	av recovery[a] (%)	range	SD[b]
0.4 (n = 3)	100	90–122 (n = 3)	18
2 (n = 13)	88	74–103 (n = 13)	11
5 (n = 5)	73	61–87 (n = 5)	11
10 (n = 11)	74	62–88 (n = 11)	7

[a] Overall average recovery: 83%. [b] Overall standard deviation: 15.

The sample preparation, isolation, determination procedures, and limits of quantitation reported herein provided a reproducible and reliable approach for pharmacokinetically assessing CH residue dissipation after intramammary infusion in cows affected with bovine mastitis. The lower limit of quantitation of 0.4 ppm was sufficiently sensitive to follow the elimination of CH over 4 half-lives after intramammary infusion (*10*). Although developed to provide post-treatment dairy cow residue information for human avoidance, the utility of this residue method should be valuable for assessing the presence of CH residues in milk for regulatory enforcement purposes.

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