Dye Marked Antibiotics for Lactating Cow Mastitis Therapy

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ABSTRACT

Dye markings of intramammary antibiotic infusions could give a dairy farmer immediate visual warning that milk contains antibiotic residue. Six dye and antibiotic preparations for lactating cows were studied for rates of dye and antibiotic milk-out. Albacillin® containing 1 x 10^5 IU of penicillin plus 150 mg of novobiocin combined with 25, 125, or 250 mg of Food, Drug, and Cosmetic Blue No. 1 or No. 2 per infusion was used. Thirty cows with healthy udders producing 13.6 to 22.7 kg milk per day were treated in all quarters twice in 24 h (0500 and 1500 h). Milk samples from 14 posttreatment twice per day milkings (0500 and 1500 h) were tested for dye and antibiotic residue. Dye content was determined by a visual method and subvisually by an ion exchange resin method. No antibiotic residues were found by the cylinder plate method after the second to fourth posttreatment milkings. Antibiotic residue was detected up to the sixth milking by Delvotest-P®. Depending on the dye type and its concentration, milk was visibly blue for one to four milkings. Subvisual quantities for dye were detectable by the ion exchange resin method for three to five milkings. The preparation showing the most promise for farm use contained 250 mg Blue No. 1 per infusion. Milk from cows treated with this preparation contained visually and subvisually detectable dye through three or four and five milkings, respectively. The dye persistence exceeded or coincided with the maximum antibiotic persistence in nearly all cows treated regardless of dye formulation or method of antibiotic detection.

INTRODUCTION

Mastitis is the most costly disease affecting dairy cows (4, 5). Cows in the average herd contract clinical mastitis 1.5 times per year (14). The minimum of antibiotic treatments per case for clinical mastitis given during lactation is two; with approximately 11 million dairy cows in the United States, the annual minimum number of antibiotic treatments is estimated at 33 million. The Food, Drug, and Cosmetic (FD&C) Act (1) established that milk and milk products containing antibiotics are adulterated; this makes monitoring milk for antibiotics required. Despite these regulations, a 1977 study of the United States milk supply showed that 10.3% of bulk tank milk was contaminated with some type of antibiotic (14).

Detection of antibiotic residues in milk can be accomplished by direct and indirect methods. Direct test methods are conventional microbiological assays. Problems associated with these methods are: 1) they are slow [the Food and Drug Administration (FDA) approved cylinder plate (CP) assay requires 16 to 18 h incubation time], and 2) they require trained laboratory personnel in an appropriately equipped laboratory.

The second form of antibiotic residue detection is by indirect methods. Mastitis remedies containing marker dyes are infused into a cow's udder. For effective indirect control, the dye is excreted in milk with endpoints equal to those of the antibiotic. The potential advantage of dye marking is that it immediately alerts the dairy farmer to antibiotic contamination by visually discoloring milk (15).

A variety of chemicals has been combined with antibiotics to be used for intramammary infusion to detect indirectly antibiotics in milk.
(10). Each compound was tested for ease of use and limits of detection in whole milk. Odorous marker compounds were unsatisfactory (10). Addition of a combination of fat-soluble fluorescein and uranine to penicillin provided a satisfactory milk-out time, but these were not approved additives (10). Certified food dyes have been investigated widely for possible use as markers (10). Incorporating Food Green No. 4 (6), Food Blue No. 3 (9), or FD&C Blue No. 1 (7) resulted in close correlation between excretion of dye and antibiotic.

In September 1962, dye marked intramammary penicillin preparations were introduced for mandatory use in the State of Victoria, Australia. Approved preparations contained 125 mg of FD&C Blue No. 1 for each 1 × 10⁵ units penicillin (8). Feagan (8) reported that after 19 mo of compulsory use of dye-marked intramammary infusions, the incidence of penicillin residues decreased by 74.6 and 77.5% in individual farmer and bulk supplies. In 1977, the South Africa government decreed that all antibiotics for intramammary infusions must contain Blue No. 2, Blue No. 3, or Green No. 4 (15). The same law prohibited milk producers from selling the milk for human consumption for as long as the milk was visibly colored (15). Dye marking has been adopted in Japan (15).

Objectives were to determine: 1) excretion endpoints of six dye-antibiotic intramammary
infusion products, and 2) effectiveness of each dye type and concentration combination as an indicator of antibiotic(s) in producer milk.

MATERIALS AND METHODS

Test Materials

Six intramammary infusion formulations for lactating cows were studied for determination of dye and antibiotic milk-out rates. Experimental preparations consisted of Albacillin® plus blue dye (The Upjohn Company, Kalamazoo, MI). Albacillin® contains $1 \times 10^5$ IU procaine penicillin-G and 150 mg of sodium novobiocin per 10 ml plaster of 2% glycerol monostearate in peanut oil gel (GlyPOG). Albacillin® containing either FD&C Blue No. 1 (Color Index 42090) or FD&C Blue No. 2 (Color Index 73015) at 25, 125, or 250 mg was administered.

Animal Selection, Treatment, Sampling, and Analyses

Five to seven healthy Holstein cows producing 13.6 to 22.7 kg milk per day in their third or greater lactation were selected for each of the residue studies. Each quarter of every cow was checked for udder health by palpation and by the California Mastitis Test (CMT) prior to treatment. Milk samples from all quarters were negative for antibiotics by Delvotest-P® (GB Fermentation Industries, Inc., Charlotte, NC) before treatment began. Cows were treated by intramammary infusion twice in a 24-h ($T_1 = 0500$ h, $T_2 = 1500$ h) interval with the entire contents of 10-ml infusions into each of four
quarters. Samples for dye and antibiotic residue assays were taken from the total production of all quarters.

All milk samples for 14 posttreatment milkings were tested for antibiotic residues by the Delvotest-P® method. Penicillin was analyzed for 14 posttreatment milkings. Posttreatment milk samples were assayed for novobiocin until at least three consecutive negative results were recorded. Dye was analyzed visually and subvisually for 14 posttreatment milkings.

Analytical Procedures

Visibly blue milk samples were diluted serially with normal milk until the color matched a colored reference solution containing 1.0, .75, .50 or .25 mg of dye per liter (7). A concentration of .25 mg per liter of FD&C Blue No. 1 or FD&C Blue No. 2 was the visual limit of detection. Samples for comparison were placed in white titration cups and examined under cool-white fluorescent lights. Actual dye concentrations were calculated from the amount of dilution.

Visibly normal samples were tested for subvisual dye by thorough mixing of a 100-ml sample of milk for 5 min with .2 g of Dowex® AG 1 × 8, 200 to 400 mesh chloride form ion-exchange resin. To this mixture, 100 ml of boiling deionized, distilled water was added, and the mixture was filtered under partial vacuum through a 3.16-cm milk filter pad. The resin-dye complex was transferred quantitatively to a 10-ml glass vial. A total of 5 ml of deionized water was added to each vial to make a diluted slurry. Standards for subvisual comparison were made with milk containing .125, .0625, .05, .04, .03 and .02 mg (lowest limit of detection) of dye per liter of FD&C Blue No. 1 and .125 to .01 mg (lowest limit of detection) of dye per liter of FD&C Blue No. 2 was the visual limit of detection.

Cow No. 3627 3647 3654 3576 3094

Figure 3. Duration of dye (D, solid bar denotes subvisual assay; open bar denotes visual assay), novobiocin [N, CP (cylinder plate) method], penicillin (P, CP method), and antibiotic (A, Delvotest-P®) in milk from cows following two intramammary infusions with Albacillin® (150 mg novobiocin; 100,000 IU penicillin/10 ml infusion) containing 250 mg dye No. 1 (FD&C Blue No. 1).
Figure 4. Duration of dye (D, solid bar denotes subvisual assay; open bar denotes visual assay), novobiocin [N, CP (cylinder plate) method], penicillin (P, CP method), and antibiotic (A, Delvotest-P®) in milk from cows following two intramammary infusions with Albacillin® (150 mg novobiocin; 100,000 IU penicillin/10 ml infusion) containing 25 mg dye No. 2 (FD&C Blue No. 2).

liter for FD&C Blue No. 2. Milk containing no dye also was analyzed and used as zero control. Resin-dye complexes from experimental samples were compared to standards, and the concentration of the standard dye-resin complex that most closely matched the color of the sample was recorded as the subvisual dye concentration.

Novobiocin and penicillin were assayed for by the CP method (11, 12). Penicillin was assayed after selective removal novobiocin (2) from the combination by Dowex® resin incorporated in the agar. Five stainless steel cylinders (penicylinders,® Fisher Scientific Company) were placed aseptically on the agar surface. Pasteur pipettes were used to fill three cylinders with test sample (11). Alternate cylinders were filled with milk containing .05 units/ml penicillin per ml milk as a reference solution. Plates were incubated at 32°C for 14 to 18 h. After incubation, diameters of zones of inhibition were measured to the nearest .5 mm with a divider and millimeter ruler. The sensitivity of the test method is <.0125 IU penicillin.

Novobiocin was assayed by the method described by Barbiers and Smith (3). Sample milk was treated with .5 ml of penicillinase (Bactopenase concentrate,® Difco Laboratories) per 10-ml sample before assay (12), and the mixture was incubated at 37°C for 30 min to inactivate penicillin (12). The penicillin-free milk was transferred into three alternating cylinders on corresponding plates. The remaining cylinders were filled with milk containing .5 μg novobiocin/ml as a reference. Plates were incubated at 32°C for 16 h. After incubation, zone diameters were measured with dividers and a millimeter ruler. The sensitivity of the test method is <.1 ppm novobiocin.

Delvotest-P® was used as a supplement to the CP test. Positive and negative controls were run with each group of samples to ensure
adequate incubation time and proper function of the test system. Delvotest-P® ampules were inoculated with .1 ml of sample, then incubated in a 63 to 66°C water bath for 2.5 h. A yellow color of the whole solid medium indicated a negative test (equivalent to 0 to .003 IU penicillin/ml), whereas a solid purple color of the medium indicated a positive test (>.006 IU penicillin/ml) (16). Samples with inhibition equivalent to .004 to .005 IU penicillin per milliliter caused a blended yellow, purple color reaction. These samples were designated as ± but were considered positive for data interpretation, because penicillin will inhibit *Streptococcus thermophilus* at .004 IU/ml (13).

Standard penicillin concentrations used to establish standard response curves were made following a geometrical progression by a factor of two ranging from .0063 IU/ml to .2 IU/ml. The standard concentrations used to establish standard response curve for novobiocin ranged from .06 µg/ml to 2 µg/ml and were related geometrically by a factor of two. A regression equation was calculated for each standard curve from the log₁₀ of the standard concentrations as y and the corrected zone diameters as x. The equation allowed conversion of corrected average zone diameters of test samples to antibiotic concentrations (12). Corrected zone diameters are obtained by adding or subtracting differences between the zone diameters from the standard curve and those on a test plate to the test sample zones (12).

**RESULTS AND DISCUSSION**

**Antibiotic and Dye Milk-Out Endpoints**

The main objective of this study was to determine extinction endpoints of dye and antibiotics in each of six dye-marked intra-
mammary infusions. Preparations were studied to select an infusion in which the dye concentration in milk from treated quarters would not fall below the visual endpoint, .25 mg dye/liter of milk, or the subvisual endpoint, .02 mg of FD&C Blue No. 1 or .01 mg FD&C Blue No. 2/ml of milk, until the antibiotic concentration had fallen below detectable limits, .004 IU penicillin/ml.

The preparation containing 25 mg of FD&C Blue No. 1 proved to be unsatisfactory as a visual indicator of antibiotics in milk because of rapid extinction of dye (Figure 1). Milk was not discolored visibly after the second (cows 3637, 3644, and 3669) or third (cows 3355 and 3197) milkings. Resin detectable quantities of dye were seen at third or later milkings. Milk from cow 3197 contained no detectable dye at the fourth milking, skipped one milking subvisually, but was positive subvisually on the fifth milking. Antibiotics were detectable until the fourth milking of cow 3197. Milk from cows 3355, 3637, 3644 and 3669 were depleted of dye at the third milking. Antibiotic excretion was equal to or less than subvisual dye extinction in milk from four cows; both antibiotic and subvisual dyes were detectable until the third or fourth milking.

Dye extinction from cows treated with infusions containing 125 mg of FD&C Blue No. 1 was also more rapid than penicillin or novobiocin (Figure 2). Milk was visibly blue until the third milking. Antibiotics were detectable by the CP method for at least three milkings, and by Delvotest-P® for as many as six milkings (cow 3355). Resin analyses of dye extinction paralleled Delvotest-P® results in milk samples from four of the seven cows treated. Milk from cow 3669 was depleted of dye by the third milking.

Results from analysis of milk from cows infused with 250 mg of FD&C Blue No. 1
show close agreement between visual dye and antibiotic extinction times (Figure 3). In three of the five cows treated (cows 3627, 3654, and 3576), both antibiotics and visually detectable dye were excreted by the third milking. The remaining two cows (3647 and 3094) excreted visually discolored milk for one milking after the antibiotic concentration was below detectable limits by all methods. Subvisual dye was detected until the third milking in milk from all cows. Milk from cow 3654 showed no detectable visible dye at the fourth milking but contained .125 mg dye per liter at the fifth milking (Figure 3). This preparation was the only dye and antibiotic combination in this limited study that met established criteria for a visual method of detecting antibiotics in producer milk by visually discoloring milk until antibiotic had fallen below detectable limits. In other cows for different formulations antibiotic was detected longer than dye was visible.

Milk from cows infused with preparations containing 25 mg of FD&C Blue No. 2 was visibly blue for one milking in four cows and for two milkings from cow 3654 (Figure 4). No dye was detectable in milk from the third milking. Antibiotics were present until the fourth milking in four cows; milk from cow 3630 had no detectable antibiotic after the third milking.

Infusions containing 125 mg of FD&C Blue No. 2 were also unsatisfactory as a visual indicator of antibiotics in producer milk because of rapid dye excretion (Figure 5). In milk from all five cows treated, dye was visible through the second milking. Subvisual dye was detected through the third milking for four cows; milk from cow 3637 contained no subvisual quantities of dye at the third or greater milking (Figure 5). Antibiotic endpoints agreed closely with subvisual dye endpoints in milk from four cows; both antibiotic and dye were detectable through the third milking. Milk from cow 3637 gave a -+ Delvotest-P® result on the fourth milking.

The 250 mg of FD&C Blue No. 2 was visually detectable in milk for two milkings after infusion; in cow 3576, subvisual amounts were present for one milking after visual concentrations had been excreted (Figure 6). Milk from the other cows showed no subvisual dye after the second milking. Antibiotics were detected until the fourth milking. This preparation was judged unsatisfactory by either detection method.

CONCLUSIONS

Further research to determine correlation between dye and antibiotic milk-out times is warranted for several preparations screened. The 25 mg of FD&C Blue No. 1 and 125 mg of FD&C Blue No. 2 in Albacillin® have potential as subvisual indicators of antibiotics in producer milk. Albacillin® containing 250 mg of FD&C Blue No. 1 has potential as a visual indicator of antibiotics in producer milk. Any changes of vehicle, treatment regimen, antibiotic type or activity, and test method sensitivity most likely would change endpoints of this study. Therefore, each variation of these variables would have to be evaluated with respect to type and concentration that would have endpoints equal to those antibiotics.

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