Determination of Furaltadone in Milk

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A spectrophotometric method for determining furaltadone in milk has been developed based on a double extraction and concentration procedure using chloroform and 0.1*N* hydrochloric acid. The method is sensitive to 1 mg. per 100 liters (0.01 p.p.m.) of furaltadone with an accuracy of ±10% at the 95% confidence level. Background material due to milk is equivalent to 0.0033 p.p.m. of furaltadone or less. The method was applied to milk from cows medicated with furaltadone in a mastitis preparation. It was concluded that a 48-hour withdrawal period following medication is sufficient to allow for depletion of furaltadone residues in milk to the zero level.

THE EFFECTIVENESS of furaltadone [5-morpholinomethyl-3-(5-nitrofurfurylideneamino)-2-oxazolidinone], against the micrococci associated with mastitis (*3*) has emphasized a need for information pertaining to drug levels in the milk at various intervals following medication, and an analytical method capable of measuring trace amounts of this drug. Analytical methods for the nitrofurans, such as the phenylhydrazone procedure for plasma (*1*) and the phenylhydrazone procedure followed by chromatography (*2*), lack the needed sensitivity. A direct spectrophotometric method sensitive to 0.01 p.p.m. has been developed for determining furaltadone in milk. This method is based on the favorable distribution coefficients (Table I) of furaltadone between chloroform and water at various pH's. Using these favorable distribution coefficients, a double extraction procedure was devised which concentrated the compound and minimized the nonspecific absorbance due to milk. This method was applied to milk from cows medicated with furaltadone to determine the drug's disappearance from milk.

Experimental

Reagents and Apparatus. The reagents include *N,N*-dimethylformamide (b.p. 150° to 154° C.) and crystalline standard furaltadone (Eaton Laboratories). Instruments include a Beckman Model DU or other suitable spectrophotometer.

Analytical Method. Place 200-ml. milk samples in 250-ml. centrifuge bottles and warm in a water bath to 40° C. Add 2 ml. of glacial acetic acid (reagent grade) dropwise with stirring. Continue stirring for about 5 minutes or until all casein flocculates. Centrifuge at 2000 r.p.m. for 30 minutes, remove 150 ml. of the aqueous phase by aspiration or by decanting and filtering through a glass-wool plug. Care should be taken not to contaminate the aqueous phase with any of the flocculated casein or fatty material. Adjust the pH of the aqueous solution to 6.0 with 2*N* potassium hydroxide using a pH meter, and transfer to 250-ml. centrifuge bottles. Add 50 ml. of chloroform (USP), stopper the centrifuge bottles, shake vigorously for 1 minute, and centrifuge at 2000 r.p.m. After centrifuging for 15 minutes, break the gel interphase with a stirring rod and centrifuge at 2000 r.p.m. for 30 minutes. Remove 35 ml. of the chloroform phase using a 50-ml. syringe fitted with a 6-inch No. 15 needle with a square-cut tip. Care must be taken not to contaminate the chloroform phase with any of the aqueous or gel material. Transfer the 35 ml. of chloroform extract to 50-ml., glass-stoppered centrifuge tubes containing 5 ml. of 0.1*N* hydrochloric acid. Stopper the tubes, shake vigorously for 1 minute, and centrifuge for approximately 10 minutes to hasten separation of phases. Determine the absorbance of the hydrochloric acid extract on a spectrophotometer from 320 to 400 mμ in 10-mμ increments against 0.1*N* hydrochloric acid.

Determine the concentration of furaltadone from the control-corrected absorbance (absorbance of furaltadone milk sample minus absorbance of drug-free milk sample) at 360 mμ and the standard curve. The control-corrected absorbance will show a maximum at 360 mμ if furaltadone is present.

This method is applicable to milk containing from 0.01 to 2.00 p.p.m. of furaltadone. If concentrations of furaltadone exceed 2.00 p.p.m., dilute the 0.1*N* hydrochloric acid extract using 0.1*N* hydrochloric acid to determine the absorbance. All furaltadone solutions must be protected from fluorescent light and sunlight to avoid decomposition.

Table I. Distribution Coefficients of Furaltadone

pH	K_p = (Furaltadone) Chloroform Phase / (Furaltadone) Aqueous Phase
1	0.06
4	2.14
5	11.08
6	19.25
7	20.74
8	24.00

Preparation of Standard Curve. Prepare a standard solution by dissolving 50 mg. of crystalline furaltadone in 5 ml. of *N,N*-dimethylformamide and making to volume, using distilled water, in a 100-ml. volumetric flask. Dilute this solution, using distilled water, to obtain 200-ml. aqueous samples containing from 0.01 to 2.00 p.p.m. of furaltadone. Include a reagent blank with the standards and treat all samples as previously described for milk. Construct a standard curve by plotting reagent blank-corrected absorbances at 360 mμ versus concentration. The standard curve follows Beer's law from 0 to 2.0 p.p.m.

Results and Discussion

Method Development. During the preliminary work an attempt was made to extract whole milk with chloroform. This produced a solid gel which would not separate on centrifugation. This problem was eliminated by precipitation and removal of the casein before extracting with chloroform. The casein was pre-

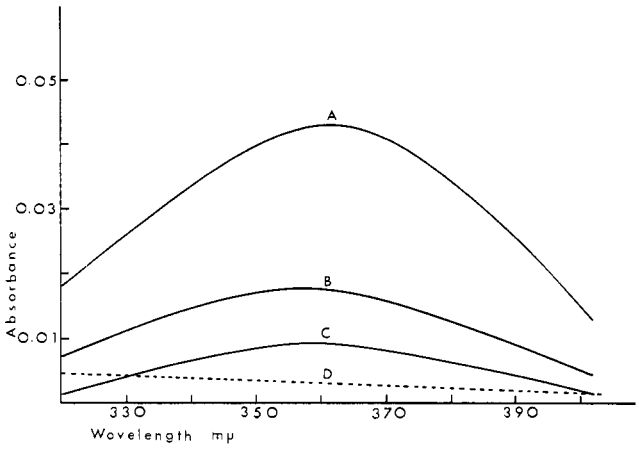

Figure 1. Absorbances of furaltadone

Control-corrected absorbances for (A) 0.05 p.p.m., (B) 0.02 p.p.m., (C) 0.01 p.p.m., (D) maximum absorbances for control milk

Table II. Recovery of Furaltadone from Milk

P.P.M. Furaltadone Added to Milk	Furaltadone Recovered			
	P.P.M.		Percent	
	Meon	Std. dev.	Mean	Std. dev.
2.00	1.95	0.05	97.5	2.5
1.00	0.97	0.02	97.0	2.0
0.01	0.010	0.001	100	10.0
0[a]	0.0019	0.0007	...	...

[a] Control milk as equivalent p.p.m. of furaltadone.

Table III. Levels of Furaltadone in Milk (P.P.M.)

Cow No.	24 Hr. Post-Med.	32 Hr. Post-Med.
1	0.07	0.022
2	0.20	0.012
3	0.49	0.190
4	2.56	0.022
5	0.04	0
6	0.74	0.120
8	0.26	0.015
34	0.21	0
66	0.41	0.030
80	0.054	0.013

cipitated by heating 200 ml. of milk to 40° C. in a water bath and adding 2 ml. of glacial acetic acid dropwise with continued stirring until the casein was completely flocculated. The aqueous portion of the milk was then separated from the flocculated casein and fat by centrifuging.

In early experiments, a colloidal precipitate formed when the aqueous phase was adjusted to pH 7.0, but this precipitate did not appear at pH 6.5 or below. There was only a small difference in the distribution coefficient of furaltadone between chloroform and water at pH 6.0 and 7.0—19.25 as compared to 20.74. The pH of the aqueous portion of the milk was therefore adjusted to 6.0 with 2*N* potassium hydroxide, using the pH meter, before extracting with chloroform since this pH was low enough to avoid the formation of a colloidal precipitate and well within the pH range for favorable extraction of furaltadone with chloroform.

During the development of the method, 40 ml. of the chloroform extract was removed for extraction with 0.1*N* hydrochloric acid instead of 35 ml. as described in the procedure. Because of the gel interphase formed during the chloroform extraction, it was difficult to obtain 40 ml. of the chloroform extract without entraining small particles of this gel interphase. By following this procedure, control milk absorbances were equivalent to 0.003 to 0.010 p.p.m. of furaltadone. These wide variations in controls produced large errors in determining the concentration of furaltadone at low levels. There seemed to be a relationship between control milk absorbance and the amount of contamination of the chloroform extract from the gel interphase. The possibility of lowering and maintaining a more constant absorbance value for control milk by using 35 ml. of chloroform extract instead of 40 ml., in order to eliminate the gel interphase contamination, was investigated. The incorporation of this change into the procedure resulted in control milk absorbances equivalent to 0 to 0.0033 p.p.m. of furaltadone, approximately equal to the reagent blank. In Table II are presented the mean and standard deviations for recoveries of furaltadone from milk at 0.01, 1.00, and 2.00 p.p.m., and the mean and standard deviations of control milk as equivalent p.p.m. of furaltadone. The recovery of furaltadone added to milk samples which were stored in a freezer for one month was comparable to that of the freshly prepared samples.

The variation of control milk was checked using both fresh and frozen milk from cows of the Guernsey and Holstein breed and Holstein milk with 1, 2, and 4% butterfat added. Control absorbances (0 to 0.003 at 360 mμ) were equivalent to 0 to 0.0033 p.p.m. of furaltadone. There was as much variation between duplicate samples as between different types of milk or fresh and frozen milk. It was concluded from these results that various sources of control milk will have little or no effect on the background absorbance. The control-corrected absorbances for 0.01, 0.02, and 0.05 p.p.m. of furaltadone in milk are demonstrated in Figure 1 along with the maximum absorbance for control milk over this spectrum.

Medication of Lactating Cows. To determine the persistence of furaltadone in secretion of medicated bovine udders, an experiment was designed using 10 lactating Holstein cows. These cows, maintained on a farm where good management practices were used, were milked at 8:00 A.M. and 4:00 P.M. daily. Starting at 8:00 A.M. on the first day of the study, each cow received an intramammary injection into each quarter of 15 cc. of Valsyn Gel (a mastitis product of The Norwich Pharmacal Co. containing 500 mg. of Valsyn, brand of furaltadone, in peanut oil gelled with aluminum monostearate). Each quarter was injected after each consecutive milking for a total of three injections. A 450-ml. sample of milk from each individual cow was collected from the total production of the udder pre-injection and at 8, 24, 32, 48, 56, 72, 80, 96, 140, and 120 hours after the third injection. The milk samples were frozen and stored at −10° C. until time of assay to prevent any possible decomposition of furaltadone. Milk samples from each cow were assayed, using the previously described procedure, until two consecutive negative assays were obtained. The milk samples collected at 8 hours after treatment were not assayed since furaltadone was visibly present at high levels.

Milk Levels after Medication. The results of milk assays after three consecutive medications of furaltadone are shown in Table III. The first milking at 8 hours postmedication is not included in the table. Since furaltadone levels were obviously high, these samples were not assayed. After the third milking following final medication, or at 48 hours postmedication, furaltadone levels were down to zero or less than 0.01 p.p.m. It is concluded that a 48-hour withdrawal period after the last treatment with Valsyn Gel, at levels described in the experiment, is sufficient to allow for depletion of furaltadone residues in milk to the zero level.

Acknowledgment

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FRUIT PIGMENTS

The Carotenoids of *Diospyros Kaki* (Japanese Persimmons)

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A survey has been made of carotenoid distribution in 40 varieties of persimmon or kaki, *Diospyros Kaki*. The major constituent is cryptoxanthin which amounts to 30 to 35% of the total. The hydrocarbon fraction is highly variable, particularly the lycopene, 0 to 30% of the total. The low lycopene varieties show marked increases in the mono- and di-epoxy diol fractions.

PERSIMMONS belong to the genus *Diospyros*, 190 species of which are distributed throughout the world. The Chinese-Japanese species *D. Kaki* has been extensively improved in Japan, so that it is commonly known as the Japanese persimmon. Colors vary from yellow-orange to red, and according to Bailey (*1*) the same variation is found in *D. Virginiana*, native to the United States.

High sugar, low astringency, and red color are attributes of some of the best Japanese varieties, whereas those of Chinese origin tend to be more astringent and yellow-orange in color. The pigments responsible for these colors are carotenoid in nature. Black blotches which may cover as much as 20% of the area are frequently seen on the surface of Hachiya in markets. The pigment involved is not anthocyanin as it is insoluble in methanol-HCl. Simple tests suggest the presence in kaki of leucoanthocyanin, proved recently by Ito and Oshima (*4*). There is, however, no evidence that the color of different varieties is directly influenced by anthocyanins.

The carotenoids of kaki were first examined by Karrer *et al.* (*5*), who isolated and crystallized lycopene and zeaxanthin from the fruit. They noted that the zeaxanthin was esterified, and they also detected carotene and presumably observed other components: "In den roten Kaki-Fruchten . . . sind verschiedene Carotinoide enthalten." Schön (*6*) crystallized cryptoxanthin, lycopene, and β-carotene, epiphase, and zeaxanthin and a small quantity of violaxanthin from the hypophase. He detected α-carotene spectroscopically and observed little if any unesterified xanthophyll before saponification. He obtained values, colorimetrically determined, in mg. per kg. of fresh fruit as follows: α-carotene, 0.3; β-carotene, 1.0; lycopene, 0.3; cryptoxanthin, 5; zeaxanthin, 12.

Tsumaki *et al.* (*7*) studied variation in carotenoid contents of two varieties, Fuyugaki and Kagoshimasaijo-gaki. They noted that the rapid increases in the quantities of lycopene and of esterified xanthophyll were responsible for the striking color in the Fall. Both varieties were reddish in color and had substantially higher proportions of lycopene (and neolycopene) relative to the xanthophylls than were found by Schön.

Using his combination of countercurrent distribution and chromatographic techniques, Curl (*2*) made a detailed study of the Hachiya variety, and separated 26 components not counting cis- isomers. The mixture was comprised of hydrocarbons 20.6%, (major components—lycopene 7.7% and β-carotene 6.8%); monols 39.7% (cryptoxanthin 38%); diols 21.9% (zeaxanthin 18%, lutein 3.9%); monoepoxide diols 11.7% (antheraxanthin 10%); diepoxide diols 3.5% (violaxanthin 2.7%); polyols 2.7%.

Experimental

In 1959, the carotenoids of two varieties, Hachiya and Fuyu (Fuyugaki) were compared, and as a result of major differences, a systematic study was made of 26 varieties maintained by the University of California at Winters, Calif. Estimates were made of total carotenoids and lycopene in all varieties, and three were selected for detailed study: Fuyu, high in lycopene, deep orange-red in color throughout, typical weight 160 grams; Honan Red, intermediate in color, although the peel was bright red, typical weight 85 grams; Tamopan, yellow-orange throughout, with little or no lycopene, weight 350 grams, described, however, by Bailey (*1*) as bright orange-red in color.

In 1961, twelve varieties from Okitsu, Japan, were examined, as well as fruit of the small mamegaki (pea-Kaki) and of black lotus, *D. Lotus*.

Portions of 50 to 100 grams from fully ripe fruit were blended and exhaustively extracted with acetone and petroleum ether. The residue was rewetted, if necessary, and the extraction repeated until the residue was colorless. The pigments were transferred to petroleum ether, and the solution was dried with Na_2SO_4. Absorbances were determined from 350 mμ to 530 mμ at 5-mμ intervals. Fruits with high lycopene content gave extracts with prominent maxima at 500, 470, and 445 mμ. As the proportion of lycopene diminished, the maximum at 500 mμ became progressively

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