Determination of Furaltadone and Nitrofurazone in Milk

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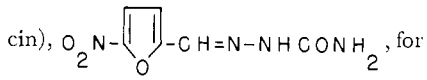
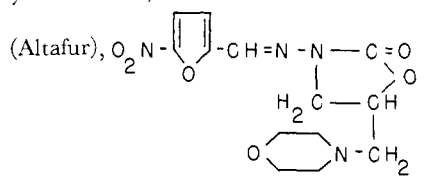
A method for determining nitrofurazone and furaltadone in raw milk is based on the formation and colorimetric estimation of 5-nitro-2-furaldehyde phenylhydrazone. Chromatographic separation of the phenylhydrazone from interfering milk pigments permits quantitative determination of these nitrofurans in milk at the 0.25 to 5 p.p.m. levels.

THE use of nitrofurazone, 5-nitro-2-furaldehyde semicarbazone (Furacin),  , for the treatment of mastitis (*4*, *6*) and the effectiveness of furaltadone, 5-morpholinomethyl - 3 - (5 - nitrofurfurylideneamino) - 2 - oxazolidinone (Altafur),  against the micrococci associated with mastitis (*5*) have emphasized the need for an analytical method to determine low levels of these drugs quantitatively in milk. The colorimetric method for the determination of nitrofurans in plasma, based on the formation of 5-nitro-2-furaldehyde phenylhydrazone (*2*), was not adequate for direct application to milk because of variable, nonspecific colorimetric interference of the milk pigments. A similar interference due to liver pigments was remedied by the use of column chromatography (*3*). Modifications of the phenylhydrazone chromatography method enable the quantitative determination of nitrofurazone and furaltadone in raw milk at levels as low as 0.25 p.p.m.

Materials and Methods

Reagents and Instruments. The reagents include reagent aluminum oxide, Merck (Catalog No. 71707); phenylhydrazine hydrochloride, 1.5% solution in water, prepared daily and refrigerated when not in use; 5*N* hydrochloric acid; *N*,*N*-dimethylformamide (b.p. 150–4° C.), toluene, reagent grade, purified by passing through an alumina column to remove interfering materials; ethyl acetate, anhydrous; and crystalline standards of the nitrofurans (Eaton Laboratories). Instruments include a Rinco rotary flash evaporator and a Beckman Model DU spectrophotometer.

Preparation of Standards. Dissolve an accurately weighed sample of about 60 mg. of the crystalline standard nitrofuran in 4 ml. of *N*,*N*-dimethylformamide in a 100-ml. volumetric flask and dilute to volume with distilled water. Dilute 5 ml. of this solution to 500 ml. with distilled water to obtain a solution containing 6 μg. of the nitrofuran per ml. Protect dilute nitrofuran solutions from direct sunlight or fluorescent lighting at all times to avoid decomposition. Prepare standards fresh daily.

Procedure for Milk. Fresh raw milk or frozen stored milk which has been thawed can be used. Place 12-ml. samples in 50-ml. glass-stoppered centrifuge tubes. To each tube add 4 ml. of 1.5% phenylhydrazine hydrochloride and 4 ml. of 5*N* hydrochloric acid. Analyze standards of the appropriate nitrofuran simultaneously with the milk samples. Standard curves for nitrofurazone and for furaltadone are reproducible at the 95% confidence level with a deviation of ±1.5 and ±3.8%, respectively. It is therefore not necessary to include a standard curve with each determination for routine analysis. Include a reagent blank and a control milk sample with each determination. For a standard curve add 0.5, 1, 2, 4, and 5 ml. of the 6 μg. per ml. standard solution to 50-ml. glass-stoppered centrifuge tubes containing 4 ml. of 1.5% phenylhydrazine hydrochloride, 4 ml. of 5*N* hydrochloric acid, and enough water to make the final volume 20 ml. Mix the contents and heat in a water bath at 70° C. The heating period for nitrofurazone is 15 minutes, that for furaltadone is 25 minutes. After heating, cool the tubes in an ice bath for 5 minutes. Extract with 20 ml. of toluene by shaking vigorously for 1 minute and centrifuge to separate phases. Break up the gel interphase with a stirring rod and centrifuge again, if necessary, in order to have at least 10 ml. in the toluene layer. Remove 10 ml. of the toluene layer and chromatograph on an aluminum oxide column prepared as follows:

Pack the chromatographic column, 26 × 1 cm., dry with aluminum oxide to a height of 6 to 7 cm. on a glass wool plug. Wash the column with toluene and insert a second glass wool plug at the top. Before addition of the samples, equilibrate the column with toluene. After addition of the 10-ml. sample, pass 10 ml. of toluene through the column. At this point the red phenylhydrazone band is retained just below the surface of the column with a faint yellow pigment from the milk above it. Some milk samples also produce a violet band which can be detected above the yellow band. After the toluene has passed through the column, develop the chromatogram with 10 ml. of toluene–ethyl acetate (3 to 1); this reverses the position of the yellow and violet milk pigment bands and slowly moves the phenylhydrazone band down the column ahead of the milk pigments. Change the solvent to a 1 to 1 mixture of toluene and ethyl acetate and develop until the phenylhydrazone band is near the bottom of the column. Then use pure ethyl acetate (approximately 20 ml.) as the eluting agent and elute and collect the phenylhydrazone.

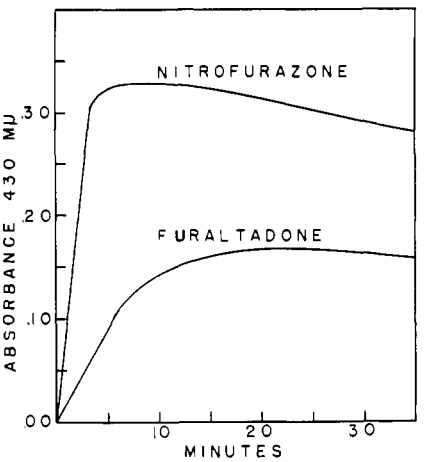


Figure 1. Conversion at 70° C.

Evaporate the ethyl acetate eluate to dryness with a rotary flash evaporator (using a water aspirator pump and a lukewarm bath as a source of heat) and make up to 5 ml. with toluene. Determine the absorbance from 400 to 460 mμ against a reagent blank (containing all reagents and carried through the entire procedure, including chromatography) using a spectrophotometer.

Construct a standard curve for each nitrofuran by plotting the absorbance at 430 mμ of the six concentrations of the standard nitrofuran used against the amount of that nitrofuran originally present (3, 6, 12, 24, 48, and 60 μg. per tube). Subtract the absorbance at 430 mμ of the control milk sample (nitrofuran-free milk) from the absorbance at 430 mμ of the milk containing the nitrofuran and read the amount of nitrofuran present in the sample directly from the standard curve. Obtain the nitrofuran concentration in the original milk sample by dividing the micrograms found per tube by 12, the volume in milliliters of the initial milk sample.

The criteria for positive identification of the nitrofuran in milk are a positive difference curve (absorbance of experimental milk samples minus those of control milk samples) that shows an absorption maximum at 430 mμ, plus visual identificaton of the red phenylhydrazone band on the chromatographic column.

Experimental Results

Conditions for Phenylhydrazone Formation. Standard curves obtained during the earlier work showed wide variations from one determination to the next, indicating that conditions for the formation of the phenylhydrazone needed to be more specific as to time and temperature. Time conversion curves were determined at 70° C. for each nitrofuran (Figure 1). The time chosen for each nitrofuran extended past the maximum absorbance point, to ensure that all samples were well over the sharp crest of the curve (15 minutes for nitrofurazone and 25 minutes for furaltadone). After these conditions were established, standard curves could be reproduced with a high degree of accuracy.

Toluene extracts of 5-nitro-2-furaldehyde phenylhydrazone prepared from nitrofurazone and furaltadone display an absorption peak at 430 mμ. The specificity of the reaction has been shown (*1*, *2*). Previous work in this laboratory has shown dilute solutions of the nitrofurans, including 5-nitro-2-furaldehyde phenylhydrazone, to be sensitive to ultraviolet light. Therefore all nitrofuran-containing solutions should be protected from fluorescent light and sunlight.

Chromatographic Separation of Phenylhydrazone Derivative. To detect small quantities of nitrofurans in milk by the phenylhydrazone method it is necessary to reduce the absorbance of the analytical sample from unmedicated milk to a minimum. This is done by column chromatography. The absorbance of control milk samples from various sources after chromatography ranged from 0.001 to 0.016 at 430 mμ. The average absorbance plus two standard deviations at 430 mμ was 0.017, approximately equivalent to the absorbance of 0.25 p.p.m. for furaltadone or 0.17 p.p.m. for nitrofurazone. When chromatography was omitted, the adsorbance for control milk samples from various sources ranged from 0.008 to 0.084 at 430 mμ. The average absorbance plus two standard deviations at 430 mμ was 0.069, approximately equivalent to the absorbance of 1.1 p.p.m. for furaltadone or 0.67 p.p.m. for nitrofurazone.

The milk pigments detected on the alumina column vary. A faint yellow band is detected in all milk samples, while some milk samples also produce a violet band. The intensities of these pigments also vary. As the phenylhydrazone band is eluted down the column, it spreads into a broader band and is followed by the milk pigments, which also have a tendency to spread. This causes some overlapping of the bands. When the milk pigments are of low intensity, they are not visible after they start spreading on the way down the column. Because of the overlapping and low intensity of the milk pigments it is necessary to use columns of equal height and nearly equal flow rates, and to elute all columns, including reagent blanks and control milk samples, with equal volumes of solvents. About 20 ml. of the ethyl acetate eluate must be collected in order to displace all the phenylhydrazone from the column.

When alumina columns were washed and equilibrated with reagent grade toluene during the preparation of the columns, a green pigment was detected. This pigment is eluted readily by ethyl acetate. Therefore, in all procedures where toluene was used, it was first passed through a large alumina column to remove this interfering pigment.

Absorbance of Eluates. The absorbances of the eluates from samples, standards, and controls were determined on a spectrophotometer against a reagent blank. The absorbance of the reagent blank when measured against toluene is small (0.004 to 0.011 at 430 mμ), but readings of samples should be made against the former in order to obtain an accurate standard curve which intercepts the origin. The slope of the standard curve for furaltadone is lower than that of the standard curve for nitrofurazone, because of the greater molecular weight of furaltadone.

Recovery of Nitrofurans from Milk. The accuracy of this method was demonstrated by recovery experiments on each nitrofuran from fresh raw milk (Table I). Known amounts of nitrofurazone or furaltadone were added to milk and the concentration was determined using the procedure described. The low absorbance and the lower standard curve contribute to the variable furaltadone recoveries at the 0.25 p.p.m. level.

The recovery of furaltadone and nitrofurazone added to milk samples which were stored in a freezer for one month was comparable to that of the freshly prepared samples.

Table I. Recovery of Nitrofurazone and Furaltadone from Milk

Milk, μg./Ml. Added	Found	Recovered, %
	Nitrofurazone	
0.25	0.242	96.8
0.25	0.250	100.0
0.25	0.258	103.2
0.25	0.250	100.0
0.25	0.242	96.8
0.25	0.250	100.0
0.25	0.242	96.8
0.25	0.250	100.0
	Av.	99.2
	Std. dev.	±2.3
1.00	0.942	94.2
1.00	0.950	95.0
1.00	0.958	95.8
1.00	1.050	105.0
1.00	0.925	92.5
1.00	0.933	93.3
1.00	0.933	93.3
1.00	0.900	90.0
	Av.	94.9
	Std. dev.	±4.4
5.00	5.083	101.7
5.00	4.992	99.8
5.00	5.033	100.7
5.00	5.000	100.0
5.00	4.792	95.8
5.00	4.858	97.2
5.00	4.633	92.7
5.00	4.708	94.2
	Av.	97.8
	Std. dev.	±3.3
	Furaltadone	
0.25	0.333	133.2
0.25	0.325	130.0
0.25	0.250	100.0
0.25	0.250	100.0
0.25	0.333	133.2
0.25	0.192	76.8
0.25	0.317	126.8
0.25	0.367	146.8
	Av.	118.3
	Std. dev.	±23.5
1.00	1.000	100.0
1.00	1.108	110.8
1.00	1.167	116.7
1.00	1.108	110.8
1.00	1.217	121.7
1.00	1.167	116.7
1.00	0.883	88.3
1.00	1.083	108.3
	Av.	109.2
	Std. dev.	±10.6
5.00	5.292	105.8
5.00	5.083	101.7
5.00	5.183	103.7
5.00	5.217	104.3
5.00	5.783	115.7
5.00	5.583	111.7
5.00	5.025	100.5
5.00	4.975	99.5
	Av.	105.4
	Std. dev.	±5.6

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