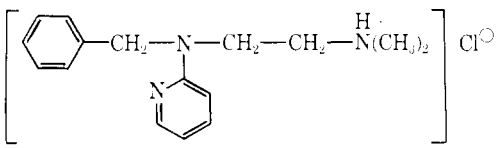
Determination of Pyribenzamine (Tripelennamine) Residues in Bovine Milk

Richard C. Luders,[1] James Williams, Karen Fried, Carl R. Rehm, and Frederick Tishler[2]

A gas chromatographic procedure is described for the determination of Pyribenzamine (PBZ) in bovine milk. The procedure is capable of detecting PBZ levels down to 10 ppb with average recoveries of greater than 80%. The average milk controls gave an apparent PBZ concentration of less than 3 ppb. Although recovery data is only presented for PBZ in muscle (greater than 80%), it is felt that with some modification the procedure should be applicable to other tissues.

PBZ (I) is a drug not only used to control allergic manifestations in small and large animals, but it is also used as a stimulant in cows who refuse to stand because of various disorders.



I

In order to comply with FDA regulations regarding residues in milk, it was necessary to develop methods capable of detecting 10 ppb of the drug. The use of gas-liquid chromatography for the determination of antihistamines has increased through the years and was most recently summarized by Gudzinewicz (1967).

Several researchers (Fontan *et al.*, 1963; MacDonald and Pflaum, 1963, 1964; Rehm, 1964) have found that PBZ can quantitatively be determined with the aid of a flame ionization detector. Fontan *et al.* (1963) have shown that by using Chromosorb W treated with potassium hydroxide and a suitable injector temperature antihistamine, hydrochlorides easily dissociated so that the observed peaks were those of the free base.

In his studies, Rehm (5) observed that the use of a polar liquid phase such as Carbowax or a polyester such as cyclohexanediol succinate permitted the use of a flame ionization detector (together with an electrometer of high sensitivity) for the determination of submicrogram quantities of antihistamines in biological systems.

The procedure described below, although elaborate, is capable of detecting PBZ levels down to 10 ppb with average recoveries of greater than 80%, and gives in the control apparent average PBZ levels of less than 3 ppb.

To determine an appropriate withdrawal time, a study was carried out in five cows receiving a total of three intramuscular injections of PBZ at 4 hr intervals.

EXPERIMENTAL

Pyribenzamine Standard Solution. Accurately weigh 50.0 mg of Pyribenzamine hydrochloride into a 30-ml separatory funnel. Add 10 ml of 1*N* sodium hydroxide. Extract three times with 10 ml portions of chloroform. Drain each chloroform extract through glass wool combining all extracts in a 50-ml round bottom flask. Evaporate the combined extracts to dryness at 35° C under nitrogen.

Add 25 ml of dimethylformamide to the flask and carefully transfer the sample to a 100 ml volumetric flask. Wash the round bottom flask with four successive 5 to 10 ml portions of dimethylformamide, combining all washing into the 100 ml volumetric flask. Dilute the sample to 100 ml with dimethylformamide. Further dilute 2.0 ml of this solution to 100 ml with dimethylformamide. Final concentration = 0.01 mcg per μl as Pyribenzamine hydrochloride.

APPARATUS

A gas chromatograph such as a Barber Colman 5000 for glass columns, on column injection, and a flame ionization detector were used. The following conditions were employed:

Temperature: Column 218° C, cell 240° C, Flash heater 230° C
Carrier Gas: Nitrogen, 60 cc/min
Electrometer: 5×10^{-11} amps full scale
Hydrogen Flow: Approximately 40 cc/minute
Air Flow: Approximately 600 cc/minute
Sample Volume: 2 microliters

Borosilicate U-shaped columns, 8 ft × 4 mm i.d., were packed with Gas-Chrom S, 100-120 mesh, previously washed with base (30 gm of support washed with 150 ml of a 5% methanolic potassium hydroxide solution and dried) and coated with 2% Carbowax 20M. Evaporator-N-Evap, Model 104 Organomation Association or any other suitable evaporator. Tapered tubes, 15 ml, custom-made at CIBA Pharmaceutical Co. (Figure 1) and calibrated to 10 μl. Glassware-Centrifuge tubes, tapered tubes, and syringes must be coated with Desicote (Beckman Instruments, Inc.) by washing with the Desicote, allowing to drain, and then rinsing with a small portion of chloroform and ether.

ANALYTICAL PROCEDURE

Milk. Pipet a 5-ml portion of the milk sample into a 40 ml glass stoppered centrifuge tube and add 0.5 ml of 6*N* NaOH. Extract two times with 30-ml portions of 20% methylene chloride-ether, combining all organic extracts in a 125-ml separatory funnel. Add 5 ml of the diluted hydrochloric acid solution (1 to 9) to the extracts, shake vigorously for 2 min, and then allow the layers to separate for about 2 min. Allow the bottom layer to drain into a 40-ml glass stoppered centrifuge tube, and repeat the extraction a second time using another 5 ml portion of the diluted hydrochloric acid solution, combining the extracts. Pipet 3 ml of 6*N* NaOH into the sample solution and allow to cool. Add 5 ml of the 20% methylene chloride–ether solution. Shake vigorously, centrifuge, and transfer the organic phase (upper layer) to a 15-ml

Analytical Research and Development Division, CIBA Pharmaceutical Company, Summit, N.J. 07901
[1] To whom correspondence should be addressed.
[2] Present address: Geigy Pharmaceuticals, Ardsley, N.Y. 10502

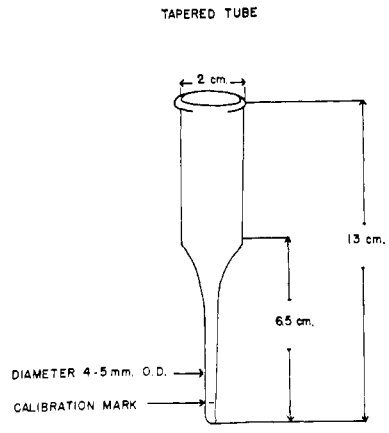


Figure 1. Construction of tapered evaporating tubes

tapered calibrated tube. Extract the sample with two additional 4-ml portions of the methylene chloride-ether solution and combine the extracts in the tapered tube.

Place the tapered tube in the constant temperature bath of the N-Evap maintained at approximately 35° C and evaporate. Periodically during the evaporation, wash down the sides of the tube with small portions (0.2 to 0.5 ml) of the methylene chloride-ether solution. This step is mandatory to prevent sample loss and to insure sample concentration into a minimum volume. When the sample volume reaches about 50 μl, it is helpful to remove the tapered tube and nitrogen line from the N-Evap and manually hold the needle inside the tube simultaneously tapping the walls of the tube. This step facilitates the removal of the solvent in the tube at such small volumes.

When all of the solvent has been evaporated, dissolve the residue with about 6 to 7 μl of dimethylformamide, using a 10 μl syringe for adding the solvent. Place the tapered tube on a mixer and shake for 30 sec. Centrifuge and spin the sample for 1 min at one half maximum speed. Check the volume of the solution in the tapered tube and adjust to the 10 μl mark if necessary. Adjust the gas chromatograph to the conditions described above and use a 2 μl portion of the Standard and sample for analysis.

Calculation: As/Ast × 20 = ppb Pyribenzamine hydrochloride

Where: As = Area of sample peak
Ast = Area of standard peak

MUSCLE

Weigh accurately a 10 g portion of the ground tissue into a 40-ml glass stoppered centrifuge tube and then pipet a 10-ml portion of a 6*N* sodium hydroxide solution into the tube. Cap the tube with a glass stopper and place it in a 60° C water bath for 3 hr, removing the tube every one-half hour and shaking vigorously so that, eventually, all of the tissue comes in contact with the sodium hydroxide. At the end of 3 hr, remove the centrifuge tube from the bath.

Cool the digested sample solution to about 15° C. Pipet a 15-ml portion of ether into the tube, stopper, and shake. Centrifuge and transfer the ether layer to a 15-ml glass stoppered centrifuge tube. Place the centrifuge tube in the constant temperature bath of the N-Evap maintained at approximately 35° C and evaporate. In the meantime, repeat the ether extraction of the sample with a second 15-ml portion of ether. When the ether extract in the 15-ml centrifuge tube has been evaporated to about 1 ml transfer the ether layer of the second ether extraction to the same 15-ml centrifuge tube and continue to evaporate the liquid to dryness. Pipet a 2-ml portion of diluted hydrochloric acid solution and a 3-ml portion of hexane into the tube. Stopper the tube, shake, and then centrifuge. Remove the hexane layer and discard. Repeat the hexane was with a second 3-ml portion of hexane, discarding the second hexane extract once more. Pipet a 2-ml portion of the 6*N* sodium hydroxide solution into the aqueous solution, stopper, and shake the sample gently for about 10 sec. Allow to cool to room temperature and then add 5-ml of a 25% methylene chloride-ether solution. Shake vigorously and then centrifuge. Transfer the organic phase (upper layer) to a 10-ml tapered tube calibrated to 50 μl. Repeat the extraction with a second 5-ml portion of the methylene chloride-ether mixture, combining the organic extracts in the 10-ml tapered tube. Place the tapered tube in the constant temperature bath of the N-Evap maintained at approximately 35° C. Proceed in a similar manner as under "Milk," only dilute the final solution to 50 μl. Using the changes listed below, inject 2 μl of a standard PBZ solution (0.05 μg per μl) and the sample solution into the gas chromatograph.

Temperature: Column 200° C, cell 210° C, flash heater 210° C
Carrier Gas: Nitrogen, 65 cc/min
Electrometer: 9×10^{-11} amps full scale

Calculation:
As/Ast × 250 = ppb Pyribenzamine hydrochloride

Where:
As = Area of sample peak
Ast = Area of standard peak

Table I. Recovery of PBZ from Milk and Muscle Tissue

PPB Added	PPB Found[a]	Mean Recovery %
	Milk	
0	2.3 ± 2.1[b] (8)[c]	...
10	10.0 ± 1.8 (9)	100
20	16.4 ± 2.2 (7)	82
50	53.5 ± 3.1 (4)	107
	Muscle	
0	9.7 ± 5 (4)	...
100	88.2 ± 11 (9)	88
200	159.8 ± 13 (5)	80
400	372.7 ± 15 (6)	93

[a] All recoveries corrected for their respective controls. [b] Standard deviation. [c] Numbers in parentheses represent the number of analyses run.

Table II. PBZ Milk Levels (PPB) in Cows Receiving Intramuscular Injections of the Drug[a]

Dairy Cow No.	Pretreatment	Time, Hr[b] 1	12	24	36
3	N.D.[c]	175	16	5.6	7.3
9	5.8	218	25	15	6.8
J-2	N.D.	226	5.5	5.8	1.1
29	2.2	368	19	56	15
04	3.4	246	27	11	3.6

[a] Intramuscular injections administered three times at 4 hr intervals and each consisting of 25 mg PBZ/20 Kg bovine weight. [b] Time following last (third dosage of Pyribenzamine) dose. In all cases, there were no detectable levels of PBZ in 48 hr samples. [c] N.D.—Not detected—less than 2 ppb.

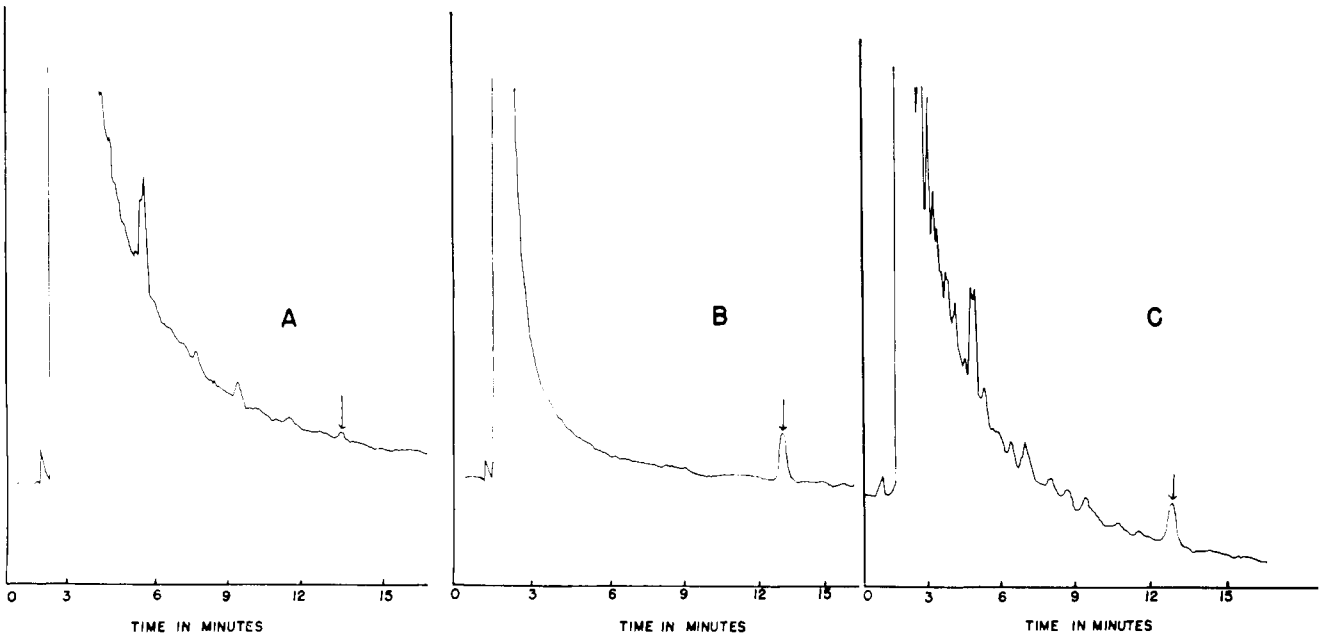


Figure 2. Chromatograms of (A) control bovine milk from cow No. 3, (B) PBZ standard 0.02 mcg, and (C) milk from Cow No. 3 1 hr following last treatment

RESULTS AND DISCUSSION

Table I tabulates the recovery of PBZ from milk and muscle tissue at various levels. As can be seen from the data, the procedure is capable of detecting PBZ levels down to at least 10 ppb with average recoveries of 80% or better at this level and at higher levels. The average milk controls gave an apparent PBZ concentration of less than 3 ppb indicating the specificity of the procedure. Figure 2 shows a typical milk control chromatogram as well as a standard and sample. Although no recovery data is given for PBZ in milk below 10 ppb, the procedure can easily detect 2 ppb. Other tubes for concentrating solutions have been evaluated. However, in order to keep sample loss at a minimum and to eliminate dilution errors, the tube shown in Figure I was constructed.

Some data with recoveries of 80% or greater are presented for the determination of PBZ in muscle at levels usually required by the Food and Drug Administration for regulatory purposes only to show that the methodology for milk, with minor modifications, probably can be applied to other biological systems.

In order to determine an appropriate withdrawal time based on the recommended dosage, a PBZ depletion study was carried out in five cows and is summarized in Table II. As can be seen from the data, no detectable PBZ levels were found in any cows after 48 hr.

ACKNOWLEDGMENT

The authors thank John Stoner, Squibb Agricultural Research Center, Three Bridges, N.J., for his assistance in carrying out the depletion study.

LITERATURE CITED

Fontan, C. R., Smith, C. W., Kirk, P. L., *Anal. Chem.* **35,** 591 (1963).
Gudzinowicz, B. J., *Gas Chromatographic Analysis of Drugs and Pesticides*, Marcel Dekker Inc., N.Y., p. 329 (1967).
MacDonald, A. J., Jr., Pflaum, R. T., *J. Pharm. Sci.* **52,** 816 (1963).
MacDonald, A. J., Jr., Pflaum, R. T., *J. Pharm. Sci.* **53,** 887 (1964).
Rehm, C. R., *Barber-Colman Chromatogram* **4,** No. 1 (1964).

Received for review September 25, 1969. Accepted July 17, 1970.