# Determination of Oxfendazole in Cow Milk by Reversed-Phase High-Performance Liquid Chromatography

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Received August 18, 1980, from the *Department of Analytical and Metabolic Chemistry, Syntex Research, Palo Alto, CA 94304.*
Accepted for publication January 6, 1981.

**Abstract** □ A specific and sensitive high-performance liquid chromatographic method for the analysis of oxfendazole in cow milk is described. Oxfendazole was extracted from milk using a mixture of acetone and chloroform under alkaline conditions. The solvents were evaporated, and the oily residue was purified by hexane–acetonitrile partition and acid–base extraction. The residue obtained after cleanup was redissolved in methanol for chromatographic analysis. Chromatography was performed on a reversed-phase column with acetonitrile–water as the mobile phase. As low as 0.005 μg of oxfendazole/g can be measured by this method using 50 g of milk. The method was applied to measure oxfendazole in the milk of a cow given an oral 5-mg/kg dose.

**Keyphrases** □ Oxfendazole—high-performance liquid chromatographic analysis of cow milk □ High-performance liquid chromatography—analysis of oxfendazole in cow milk □ Anthelmintics—oxfendazole, high-performance liquid chromatographic analysis in cow milk

Oxfendazole, [5-(phenylsulfinyl)-1*H*-benzimidazol-2-yl]carbamate (I), is a new orally effective anthelmintic agent for the control of GI and lung parasites in cattle and sheep (1). The use of oxfendazole as an anthelmintic in dairy cattle may result in its presence in milk at low levels. To protect the general public from exposure to drug residue in milk, a government guideline for the safe use of animal drugs requires a sensitive analytical method for drug level monitoring in marketed milk (2).

## BACKGROUND

Oxfendazole belongs to the benzimidazole class of anthelmintics. The analysis of benzimidazole compounds in animal tissues and biological fluids by colorimetric (3), fluorometric (3–5), high-performance liquid chromatographic (HPLC) (6, 7), and radioimmunoassay (8) methods has been described. The colorimetric and fluorometric methods lack the specificity and sensitivity needed for assay from milk. HPLC and radioimmunoassay are intended for use in plasma, utilizing minimum sample cleanup. Because of the high fat content of raw milk, these methods are not applicable to the analysis of trace drug levels in milk.

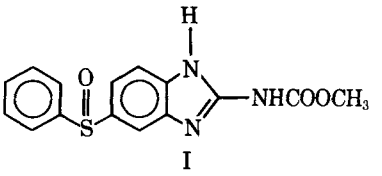
An HPLC method for the analysis of benzimidazole compound in animal tissues and milk based on ion-exchange chromatography also was reported (9). The sensitivity achieved was 0.05 μg/g in cow milk and 0.1 μg/g in cattle tissues. To ascertain the safe use of oxfendazole in food-producing animals, an analytical method capable of measuring the drug level at 0.005 μg/g in milk is required.

This paper describes an analytical method using HPLC and UV detection. The nanogram per gram level of measurement was achieved by a highly efficient extraction and cleanup procedure. The method was used to establish the depletion profile of oxfendazole in milk of a dairy cow following a single oral dose of 5 mg of oxfendazole/kg.

## EXPERIMENTAL

**Reagents and Chemicals**—All solvents were distilled-in-glass grade[1]. Other reagents and chemicals were reagent grade. Phosphate saline buffer (pH 8) was 0.05 *M*, containing 9 g of sodium chloride in 1 liter of buffer. Oxfendazole and the internal standard, [5-(4-methylsulfinylphenoxy)-1*H*-benzimidazol-2-yl]carbamate (II), were obtained in-house[2].

**Apparatus**—Analyses were performed on a high-performance liquid chromatograph[3] equipped with a UV detector set at 254 nm and a 10-mv recorder. A 4-mm × 30-cm reversed-phase column[4] coupled with a precolumn[5] was used for chromatographic separation.



I

**Chromatographic Conditions**—The mobile phase was 24.5% acetonitrile in water at a flow rate of 2 ml/min. The columns were operated at ambient temperature, and column pressure was maintained between 1000 and 2000 psi.

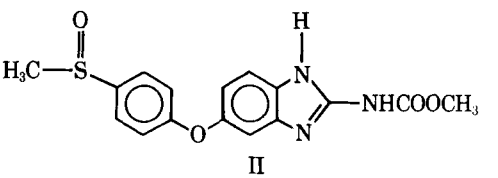
**Extraction**—Raw milk (50 g) was weighed into a 250-ml polypropylene bottle, and a solution of internal standard (1 ml of a 1-μg/ml solution in 0.1 *N* HCl) was added. The bottle contents were swirled, and 150 ml of acetone was added. The bottle was capped and mechanically shaken for 10 min.

After centrifugation, the acetone extract was poured into a 500-ml separator. Then 10 ml of pH 8 phosphate buffer and 300 ml of chloroform were added to the acetone extract. The mixture was shaken for 10 min, and the layers were allowed to separate over 10 min. The lower organic layer was drained into a 500-ml erlenmeyer flask containing 80 g of granular anhydrous sodium sulfate, and the flask was stoppered and shaken briefly. The mixture was filtered through a 80-mm i.d. polypropylene powder funnel fitted with a glass wool plug.

The filtrate was collected into a 1-liter round-bottom evaporation flask. The erlenmeyer flask was rinsed with 50 ml of chloroform–acetone (2:1), and the rinse solvent was filtered through the same funnel and combined with the first extract in the 1-liter flask. The organic extract was evaporated to an oily residue, using a vacuum rotary evaporator with the water bath set at 37°. The residue obtained was transferred to a 125-ml separator with the aid of 50 ml of hexane and 50 ml of acetonitrile, and the separator was capped and shaken for 5 min. The separated acetonitrile layer then was drained into another separator and washed twice with 25 ml of hexane.

The final acetonitrile extract was drained into a 250-ml round-bottom flask and evaporated to a residue on a rotary evaporator. The residue obtained was transferred quantitatively to a 125-ml separator with the aid of 50 ml of ethyl acetate. The ethyl acetate was extracted with 0.2 *N* HCl (3 × 10 ml). The acid was combined in another 125-ml separator and washed with hexane (2 × 10 ml). All ethyl acetate and hexane layers were discarded. Then 6 ml of 1 *N* NaOH was added to the acid extract, and the solution pH was adjusted to 8 by the addition of 300 mg of sodium bicarbonate.

The basic solution was extracted with ethyl acetate (2 × 25 ml). The ethyl acetate extract was dried over a bed of anhydrous sodium sulfate (5 g) and evaporated to a residue. The residue was redissolved in 10 ml of ethyl acetate and filtered through a 0.5-μm filter[6] into a 15-ml conical test tube. The filtrate was evaporated to dryness and redissolved in 200



II

[1] Burdick & Jackson Laboratories, Muskegon, Mich.
[2] Syntex Research, Palo Alto, Calif.
[3] Model ALC/GPC-204, Waters Associates, Milford, Mass.
[4] μBondapak $C_{18}$, Waters Associates, Milford, Mass.
[5] Co-Pell ODS guard column, Whatman, Clifton, N.J.
[6] Fluoropore filter FHLP 1300, Millipore Corp., Bedford, Mass.



*0022-3549/81/0800-0858$01.00/0*

**Table I—Percent of Radioactivity Present at Each Step of Extraction**

| Extraction Step | Recovery[a], % |
|---|---|
| Acetone extract | 93.21 |
| Chloroform–acetone (2:1) extract | 92.12 |
| Aqueous layer | 1.27 |
| Hexane | 1.02 |
| Ethyl acetate after 0.2 *N* HCl extraction | 4.84 |
| Hexane wash of 0.2 *N* HCl | NM[b] |
| Base after ethyl acetate extraction | 0.57 |
| Final extract for HPLC | 79.23[c] |
| HPLC assay (μg/g) | 0.01 |

[a] From raw milk spiked with 0.01 μg of [$^{14}$C]oxfendazole/g. [b] Not measurable. [c] About 6% of the radioactivity was not accounted for, probably due to adsorption onto glassware.

μl of methanol. An aliquot (25 μl) was injected onto the chromatograph for analysis.

**Extraction Efficiency and Total Recovery**—A known amount of $^{14}$C-labeled oxfendazole was spiked into drug-free raw milk. Extraction efficiency and total recovery of oxfendazole were established by monitoring the radioactivity present in every fraction at each step.

**Calibration Curve Preparation**—Oxfendazole levels in unknown samples were determined with a calibration curve prepared in the following manner. Known amounts of oxfendazole and the internal standard were spiked into drug-free raw milk in the range of 0.005–0.05 μg of oxfendazole/g and 0.02 μg of the internal standard/g. The samples were processed as described and subjected to HPLC analysis. The calibration curve was constructed by plotting the ratio of the chromatographic peak heights of oxfendazole and the internal standard *versus* micrograms of oxfendazole spiked per gram.

Spiking solutions of oxfendazole, 0.25, 0.5, 1, 1.5, 1, and 2.5 μg/ml, were prepared by diluting the stock solution of oxfendazole with 0.1 *N* HCl. Oxfendazole stock solution was prepared by dissolving 10 mg of oxfendazole in 100 ml of methanol–0.1 *N* HCl (1:1). The internal standard spiking solution (1 μg/ml in 0.1 *N* HCl) was prepared by the dilution of a stock internal standard solution of 50 μg/ml with 0.1 *N* HCl. One milliliter of each oxfendazole spiking solution was added to 50 g of raw milk to yield spiking levels of 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 μg of oxfendazole/g. One milliliter of the spiking internal standard solution then was added to the raw milk (50 g) already fortified with oxfendazole to obtain 0.02 μg of the internal standard/g.

**Analysis of Milk Samples Containing Oxfendazole Levels of >0.05 μg/g**—Milk samples containing oxfendazole levels higher than the calibration curve range were diluted with appropriate amounts of blank milk obtained from an untreated animal. The samples after dilution were treated in the same manner as described under *Extraction*. A dilution factor then was included in the final calculation against the calibration curve.

**Table II—Statistical Parameters of Milk Calibration Curves**

| Curve Number | Correlation Coefficient | Linear Regression Intercept, μg/g | Linear Regression Slope[a] |
|---|---|---|---|
| I | 0.9995 | 0.0003 | 51.16 |
| II | 0.9997 | 0.0004 | 49.17 |
| III | 0.9993 | 0.0007 | 49.84 |
| IV | 0.9998 | 0.0002 | 50.06 |

[a] The mean was 50.06 ± 0.83 (*SD*).

## RESULTS AND DISCUSSION

Oxfendazole, a benzimidazole carbamate, was chromatographed using a reversed-phase microparticulate column and a mobile phase of 24.5% acetonitrile in water. Baseline resolution of oxfendazole and its structural analog used as an internal standard was achieved. The UV spectrum of oxfendazole in methanol indicated that its $\lambda_{max}$ was at 228 nm with a molar extinction coefficient of 45,565. Due to the ready availability of a 254-nm fixed-wavelength UV detector, detection at 254 nm was preferred. The molar extinction coefficient at 254 nm was 11,025 and was adequate for the sensitivity requirement of this assay.

Oxfendazole extraction from milk using various solvents was investigated. The extraction of milk directly with ethyl acetate caused an unbreakable emulsion. Emulsion formation also was observed when chloroform was used. To eliminate this problem, acetone was added to milk to precipitate the milk protein and also to act as an extracting solvent. The acetone extract then was partitioned with chloroform to remove water. The organic layer obtained was cleaned up further *via* hexane–acetonitrile partitioning and acid–base extraction. Until the acetone–chloroform extraction step, ~92% of the spiked [$^{14}$C]oxfendazole was extracted. The overall recovery of [$^{14}$C]oxfendazole spiked in milk that went through the entire extraction procedure was 79%. A step-by-step account of loss and recovery throughout the assay is summarized in Table I.

The chromatograms of control milk and milk spiked with oxfendazole

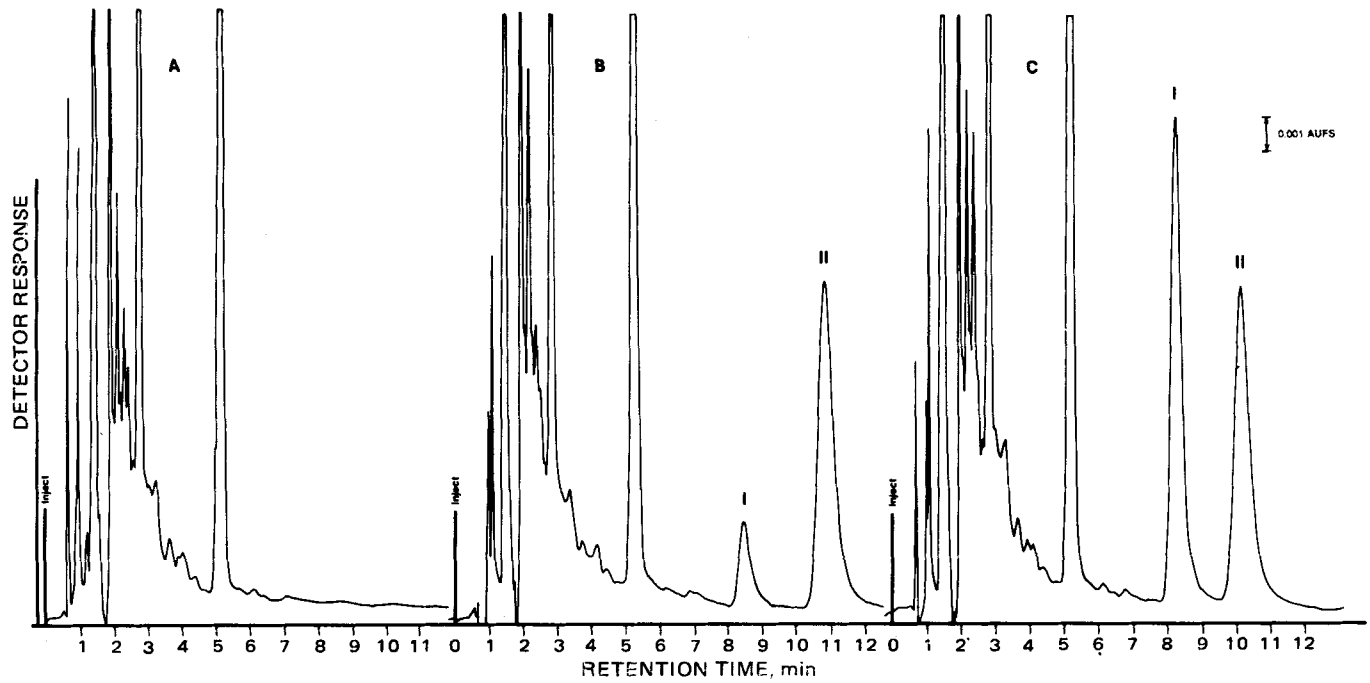



**Figure 1**—*A, high-performance liquid chromatogram obtained from the analysis of control milk processed through the procedure; B, control milk spiked with 0.005 μg of oxfendazole/g and 0.02 μg of internal standard/g; and C, control milk spiked with 0.03 μg of oxfendazole/g and 0.02 μg of internal standard/g. Chromatographic conditions were: mobile phase, 24.5% acetonitrile in water; flow rate, 2 ml/min; temperature, ambient; and detection, 254 nm at 0.01 aufs. Key: I, oxfendazole; and II, internal standard.*

Table III—Analysis of Oxfendazole Level in Spiked Cow Milk

| Oxfendazole Spiked, μg/g | Oxfendazole Found, μg/g | Mean | Ratio of Assayed to Spiked Milk |
|---|---|---|---|
| 0.005 | 0.0051 | 0.0051 | 1.02 |
| | 0.0050 | | |
| | 0.0052 | | |
| | 0.0052 | | |
| 0.01 | 0.010 | 0.0105 | 1.05 |
| | 0.010 | | |
| | 0.011 | | |
| | 0.011 | | |
| 0.02 | 0.021 | 0.0205 | 1.03 |
| | 0.020 | | |
| | 0.021 | | |
| | 0.020 | | |
| 0.03 | 0.030 | 0.030 | 1.00 |
| | 0.030 | | |
| | 0.030 | | |
| | 0.030 | | |
| 0.04 | 0.040 | 0.0405 | 1.01 |
| | 0.040 | | |
| | 0.042 | | |
| | 0.040 | | |
| 0.05 | 0.052 | 0.0503 | 1.01 |
| | 0.049 | | |
| | 0.050 | | |
| | 0.050 | | |

and the internal standard are shown in Fig. 1. The chromatogram obtained from the analysis of drug-free milk showed no interference peak at the retention time of oxfendazole and the internal standard. Chromatograms from milk spiked with 0.005 or 0.03 μg of oxfendazole/g along with 0.02 μg of the internal standard/g indicated adequate peak response for both compounds at these concentrations.

The calibration curve obtained from the analysis of blank milk spiked with oxfendazole in the 0.005–0.05-μg/g concentration range was linear. The statistical parameters showing linear regression fits of four calibration curves are given in Table II. Table III summarizes the data obtained from the repeat analysis of milk samples spiked with oxfendazole.

Coefficient of variance (standard deviation per mean) was calculated to assess assay variability. The coefficients of variance for the analysis at 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 μg/g ($n = 4$) were 1.95, 4.84, 2.94, 0.66, 1.98, and 1.99%, respectively.

Oxfendazole levels in the milk of a cow following the administration of 5 mg of oxfendazole/kg were determined by the described method. The cow was milked at 0 (predose), 4, 8, 12, and 24 hr after dose on the 1st day. For the next 6 days, milking was carried out every 12 hr. The oxfendazole levels in milk 4, 8, 12, 24, 36, 48, 60, and 72 hr following dose administration were 0.097, 0.212, 0.334, 0.460, 0.325, 0.173, 0.058, and 0.009 μg/g, respectively. Oxfendazole was not detected beyond 72 hr after dose.

## REFERENCES


(1) E. A. Averkin, C. C. Beard, C. A. Dvorak, J. A. Edwards, J. H. Fried, J. G. Kilian, and R. A. Schiltz, *J. Med. Chem.*, **18**, 1164 (1975).

(2) M. K. Perez, *J. Toxicol. Environ. Health*, **3**, 837 (1977).

(3) H. L. Pease and J. A. Gardiner, *J. Agric. Food Chem.*, **17**, 267 (1969).

(4) H. L. Pease and R. F. Holt, *J. Assoc. Off. Anal. Chem.*, **54**, 1399 (1971).

(5) "Food Additives Analytical Manual," rev. ed., Food and Drug Administration, Washington, D.C., 1973, Sec.121.260.

(6) K. B. Alton, J. E. Patrick, and J. L. McGuire, *J. Pharm. Sci.*, **68**, 880 (1979).

(7) J. A. Bogan and S. Marriner, *ibid.*, **69**, 422 (1980).

(8) C. Nerenberg, R. A. Runkel, and S. B. Matin, *ibid.*, **67**, 1553 (1978).

(9) J. J. Kirland, *J. Agric. Food Chem.*, **21**, 171 (1973).


## ACKNOWLEDGMENTS


Presented in part at the APhA Academy of Pharmaceutical Sciences, Washington, D.C. meeting, April 1980.

The authors thank Dr. Colin Beard and Dr. Charles Dvorak for the synthesis of the internal standard. The authors are indebted to Marian Fass for technical assistance, and thanks also are extended to Leigh Ann Wolfe for typing the manuscript.


# GLC Determination of Itanoxone in Biological Fluids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Received August 11, 1980, from the *P. Fabre S.A. Research Center, 17 Avenue Jean Moulin, 81106 Castres, France.* Accepted for publication January 6, 1981.



**Abstract** □ A sensitive and reliable method for the quantitative determination of itanoxone, 4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid, in biological fluids is described. A quantitative ethyl acetate extraction of the plasma samples is followed by reduction and methylation of itanoxone. Quantification is achieved by GLC using electron-capture detection and an internal standard. The minimum concentration of itanoxone detected in plasma is 0.1 μg/ml. Recovery of the titrated compound added to human plasma averaged 100.9 ± 2.92% (*RSD*).



**Keyphrases** □ Itanoxone—GLC determination in biological fluids □ GLC determination, electron-capture detection—analysis, itanoxone, biological fluids □ 4-[4′-(2-Chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid—itanoxone, GLC determination in biological fluids


Itanoxone (1), 4-[4′-(2-chlorophenyl)phenyl]-4-oxo-2-methylenebutanoic acid (I), is used in the treatment of metabolic disorders (2–10). The impurities likely to be found in this raw material were studied (11), as was a purification process (12). The present study perfected a sensitive and reliable method for studying the absorption, metabolism, and excretion of I in animals and humans at relatively low doses.

TLC methods were described for the determination of I in pharmaceutical formulations[1] (13). Quantitation was achieved using the UV-absorbing properties of I. The method described in this paper used GLC followed by electron-capture detection for the determination of small quantities of I in biological fluids and pharmaceutical formulations.

## EXPERIMENTAL

**Reagents and Materials**—Itanoxone[2] (I) and internal standard (II) were obtained by a Friedel–Crafts reaction between itaconic anhydride and the corresponding aromatic derivative (14). The standard solutions

[1] A. Boucherle, University of Grenoble, Grenoble, France, expert report.

[2] F 1379.




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