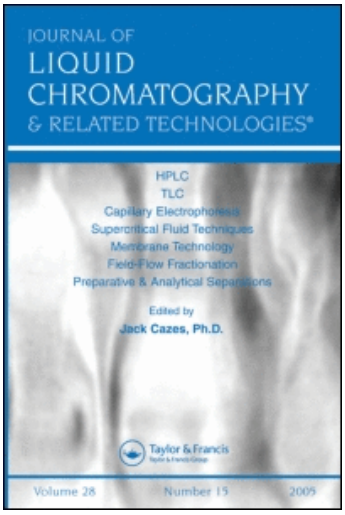

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hwang, Kin-Kai , Battor, Jan , Drees, David and Browne, Ronald(1985) 'High Performance Liquid Chromatograph 1C Method for Determination of Mifobate in Rat Feed', Journal of Liquid Chromatography & Related Technologies, 8: 10, 1915 — 1922
To link to this Article: DOI: 10.1080/01483918508074105
URL: http://dx.doi.org/10.1080/01483918508074105

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(10), 1915-1922 (1985)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF MIFOBATE IN RAT FEED

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ABSTRACT

A high performance liquid chromatographic (HPLC) method is described for the quantitative determination of Mifobate (SR-202) in rat feed. Mifobate is extracted in acetone and isolated from other extractants on a Waters Sep-pak® C_{18} disposable pre-column. The extracted drug and internal standard are chromatographed on a μBondapak™ C_{18} reverse phase column with a mobile phase consisting of water and acetonitrile (55:45, v/v). The eluent is monitored at 225 nm.

The method provided a 101.56 ± 5.1% mean recovery of Mifobate from spiked feed samples ranging in the 22.24 to 433.04 mg/kg concentration range. Standard curves bracketing this concentration range had linear coefficients greater than 0.9998. The average relative standard deviation (%) for the entire concentration range was 4.2%. The critical steps and precision of the method were also evaluated.

INTRODUCTION

Mifobate (SR-202), dimethyl-α-(dimethoxy phosphinyl)p-chlorobenzyl phosphate, is a new drug with anti-atherosclerosis potential (1). Studies in hyperlipidemic animal models suggest that Mifobate alters serum cholesterol levels. These studies further demonstrated that Mifobate

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favorably alters the lipoprotein profile in plasma, specifically increasing HDL (High Density Lipoprotein) free cholesterol (2). This appears to be linked to the cholesterol-clearing activity of Mifobate and associated with the overall mechanism(s) of action. This information suggests that Mifobate might have a therapeutic potential in atherosclerosis (3).
The purpose of this work was to develop a simple, rapid, and accurate HPLC methodology which would increase the specificity and sensitivity for the quantitation of Mifobate in rat feed at the 20 ppm level. This report describes a drug-feed analysis method for samples in the 25 to 800 mg/kg concentration level and includes some performance evaluation data for the method.

MATERIALS AND METHODS

Chemicals and Reagents

The acetonitrile and water were purchased from Burdick and Jackson (High purity solvents, Muskegon, Michigan), and the acetone used for extraction was purchased from JT Baker Chemical Co (Phillipsburg, New Jersey). The Mifobate was provided by Symphar SA (Geneva, Switzerland) and the chlorfenvinphos was purchased from Crescent (Riedel-de Haen Laboratory Chemicals, Hauppauge, New York).

Chromatography System

The analysis was performed on a modular liquid chromatograph equipped with a Model 6000A Solvent Delivery System (Waters Assoc, Milford, MA), a Model 710B Intelligent Sample Processor (Waters Assoc) and a Model 480 Lambda-Max Absorbance Detector with multiple UV wavelength capability (Waters Assoc). The Sep-pak® Cartridge rack and C_{18} cartridges were obtained from Waters Associates. The separation was achieved on a μBondapak™ C_{18} column [3.9 mm (ID) x 30 cm - 10 micron particle size] protected by a guard column packed with μBondapak C_{18}/corasil (37 to 50 microns particle size). Peak areas were measured by a Model 730 Computing Data Module (Waters Assoc). The mobile phase was prepared by mixing water

and acetonitrile (55:45, v/v) which were filtered through a Millipore membrane (pore size 0.45 μm) and degassed prior to use. The flow-rate was 2.0 ml/min.

Preparation of Spiked Feed Standard Solution

Standard spiked solutions of Mifobate were prepared in acetone by diluting the 0.05560 and 0.10826 gm/ml acetone **stock** solution of Mifobate. A proper amount of Mifobate acetone standard solution was then added into 5 gm of feed. The spiked concentrations contained 22.24, 43.30, 55.60, 111.20, 216.52 and 433.04 mg of Mifobate per kg of feed. The commercially available chlorfenvinphos was used as an Internal Standard (IS). The internal standard solution was prepared by diluting the 101.25 mg per 10 ml acetonitrile stock solution to 101.25 μg per 10 μl.

Preparation of Feed Samples

Powdered Purina Rodent Food (ARCS 1873; lot no. Jul 0783A) was mixed with Mifobate in a PK blender according to the standard operation procedure used in the toxicity studies. Duplicate feed samples of the 25, 100, 400, and 800 mg/kg dose levels were taken after mixing from several different regions within each mix to minimize sampling errors.

Extraction Procedure

A 5 gm (± 0.01 gm) aliquot of the spiked standard, feed blank or test sample was placed in a 50 ml round bottom centrifuge tube (screw cap) and extracted with 10 ml of acetone for one hour at room temperature on a Fisher mechanical shaker at a maximum speed of 10. Samples were centrifuged at 25,000 rpm for ten minutes at 20°C to clarify the extracts. The 1 ml aliquots of the extracts from low dose group (25 mg/kg) and the 2 ml aliquots of the extracts from the spiked standard feeds and blank were quantitatively transferred into 150 x 20 mm glass culture tubes. Feed samples from the medium (100 mg/kg) and high dose groups (400 and 800 mg/kg) were diluted to provide concentrations which would fit in the concentration bracket of the spiked standard curve. The 2 ml diluted

aliquots in acetone of the 1:10 dilution (100 mg/kg) and 1:50 dilution (400 and 800 mg/kg) were quantitatively transferred into 150 x 20 mm glass culture tubes. All feed extracts were evaporated to dryness under a stream of nitrogen and redissolved in 2 ml HPLC grade water. Each extract was then passed through a Sep-pak C_{18} cartridge which had previously been activated by washing with five volumes of 1 ml methanol. The cartridge was then washed with 2 ml of water followed by washing with 2 ml of a 40% methanol in water solution. Following the washes, the Mifobate was eluted from the cartridges with 2 ml of acetonitrile. Then 100 µl aliquots of the internal standard stock solution (1.01 mg/ml) were pipetted into each vial, sealed, and shaken by hand for one minute. Then 15 µl aliquots were injected in duplicate into the HPLC system described as above.

Method of Calculation

As indicated, all unknown feed samples were prepared and tested in duplicate. The Mifobate analyses were also performed by making duplicate HPLC injections of the aliquots prepared from each unknown sample, using a randomized order for the standards and samples. Instrument calibration was maintained by making duplicate injections of the standard after every eighth sample.

All determinations were related to the internal standard incorporated into the solutions. Results were calculated using the response ratios computed from the electronically integrated peak areas of the calibration standards.

RESULTS AND DISCUSSION

Chromatographic Separation

Baseline separation was achieved under the experimental conditions with retention times of 3.04 and 17.50 for the Mifobate and the internal standard, respectively. Endogenous material from rat feed caused no interference in the mifobate and the internal standard components. Typical feed chromatogram tracings are shown in Fig. 1.

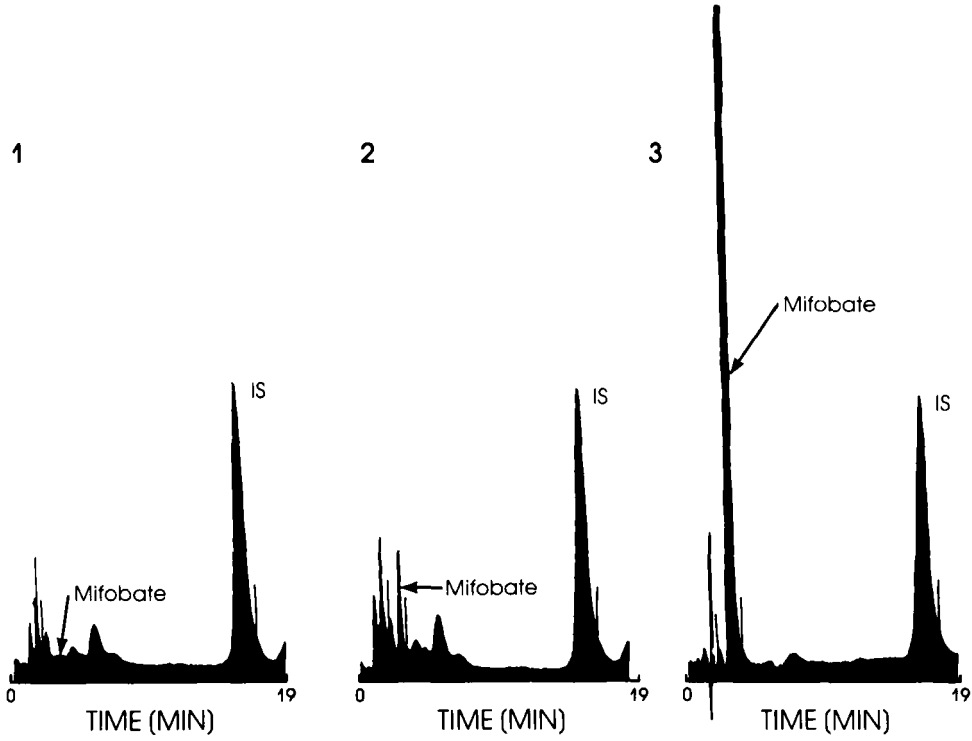

Figure 1 Chromatograms of Extracted Mifobate Feed Samples

1. Feed blank with internal standard (IS).

2. A 15 µl aliquot of Mifobate extract at the dosage concentration of 10 mg per kilogram of feed.

3. A 15 µl aliquot of Mifobate extract at the dosage concentration of 433 mg per kilogram of feed.

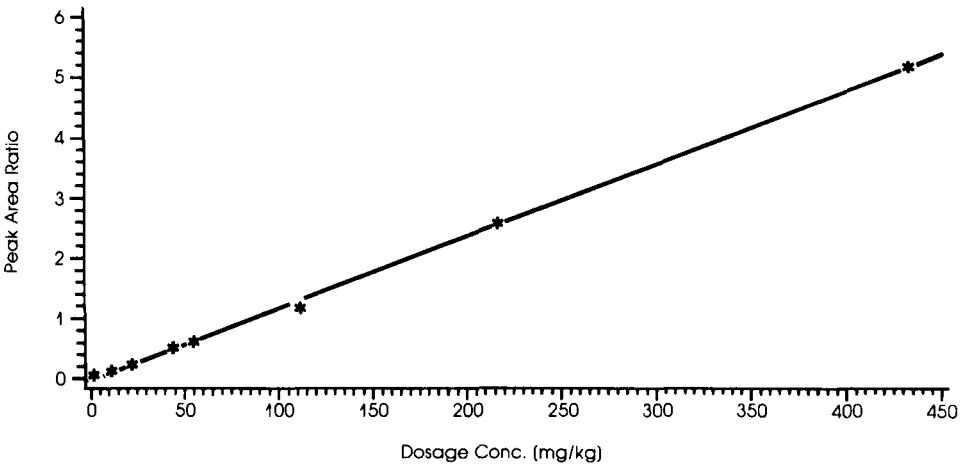

Figure 2: Linear regression analysis of Mifobate in rat feed.

Calibration Curve

A calibration curve was prepared with each batch of unknown samples. The calibration curve consisted of a blank and six single standards as follows: 22.24, 43.30, 55.60, 111.20, 216.52, and 433.04 mg/kg of Mifobate. The correlation coefficient for the peak area ratio vs the Mifobate concentration (by linear regression), as shown in Table 1 and Fig. 2, is usually 0.999 or better indicating a high degree of linearity in the calibration curve over the concentration range studied.

Sensitivity

Under the experimental conditions, this procedure can routinely quantitate Mifobate concentrations in feed as low as 10 ppm with a 5 g sample. When a 10 g sample is used, the minimum concentration that can be quantitated is 5 ppm. Concentrations lower than 5 ppm can be detected but quantitation was not established.

TABLE 1

Summary of Linearity, Recovery, and Accuracy of Mifobate in Rat Feed (n=4)*

Spiked Dose Level (mg/kg)	Mean Mifobate Found in Rat Feed (mg/kg)	Mean Recovery (%)	Standard Deviation	Relative Standard Deviation (%)
Feed Blank	-	-	-	-
22.24	24.57	110.47	1.95	7.9
43.30	44.60	103.00	2.04	4.5
55.60	55.82	100.39	3.43	6.14
111.20	105.41	94.80	4.26	4.03
216.52	217.89	100.63	0.89	0.41
443.04	433.56	100.10	11.23	2.59
		101.56 ± 5.1		4.2

Correlation Coefficient = 0.9998
Y-intercept = -0.0912
Slope = 0.0121

TABLE 2

Summary of the Analysis Results of Mifobate in Feed at Four Dose Levels

Dose Groups (mg/kg)		Theoretical Conc. (gm/kg)	Calculated Conc. (gm/kg)	Mean ± Standard Deviation	Coefficients Variation (%)	Recovery (%)
25	M	0.596	0.6572	0.6339 ± 0.030	4.70	110.2
			0.6106			102.4
	F	0.407	0.4042	0.4092 ± 0.007	0.17	99.3
			0.4142			101.7
100	M	2.308	2.350	2.369 ± 0.027	1.13	101.8
			2.388			103.5
	F	1.623	1.694	1.662 ± 0.045	2.71	104.4
			1.630			100.4
400	M	8.703	8.980	9.248 ± 0.378	4.08	103.2
			9.510			109.3
	F	6.339	7.120	6.783 ± 0.417	6.59	112.3
			6.445			101.7
800	M	15.164	16.235	16.193 ± 0.060	0.37	107.1
			16.150			106.5
	F	11.680	12.785	12.185 ± 0.848	6.96	109.4
			11.585			99.2

Method Validation

To establish the utility of the method, the Mifobate feed mixtures at four different dose levels were assayed. The results are given in Table 2. The assay results of feed samples show average recoveries of 103.5%, 102.5%, 106.6%, and 105.6% for 25, 100, 400, and 800 mg/kg levels, respectively. Precision was achieved using two assays per dosage concentration. Coefficients of variation of 4.7%, 0.17%, 1.13%, 2.71%, 4.08%, 6.59%, 0.37%, and 6.96%, respectively, were attained using this methodology.

CONCLUSION

The method described offers a rapid, easy, and accurate technique for the analysis of Mifobate in rat feed. The advantages of using the disposable pre-column for the quantitation of Mifobate in rat plasma samples has been reported (4). In this study we concluded that the use of Sep-pak C_{18} cartridge for the clean-up technique has significant advantages of speed and simplicity for the assay. Current work is under way employing these techniques to monitor the homogenicity of Mifobate in feed mixing in the ongoing chronic toxicity study.

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