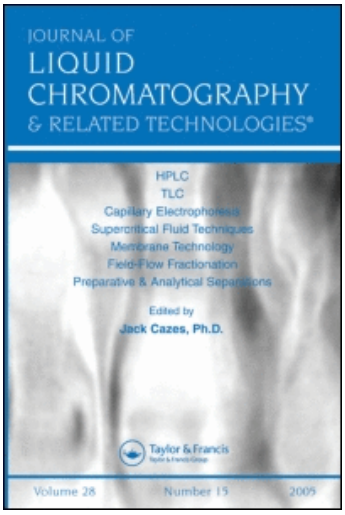

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A Rapid High Pressure Liquid Chromatographic Assay of Benoxaprofen in Plasma

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A RAPID HIGH PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF BENOXAPROFEN IN PLASMA

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ABSTRACT

A rapid high-performance liquid chromatographic assay for the determination of the anti-inflammatory drug benoxaprofen in human plasma, is described. Plasma samples of 1.0 ml, to which benoxaprofen, and warfarin as an internal standard, had been added, were extracted with ether under acidic conditions. The samples were analyzed on a MicroPak CN-10 column using 25% acetonitrile in water (pH 2.5 with phosphoric acid). Detection was made on a variable wavelength UV absorbance detector at 309 nm.

Samples containing 0.5-10 μg benoxaprofen gave a mean extraction recovery from control plasma of 90.6 ± 6.8% (n=18). Stability tests have shown that benoxaprofen in plasma is stable for at least two weeks after freezing.

INTRODUCTION

Benoxaprofen (Structure 1), [2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid] has been shown to possess notable

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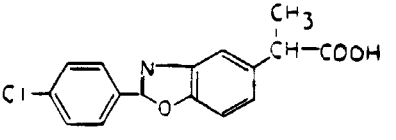
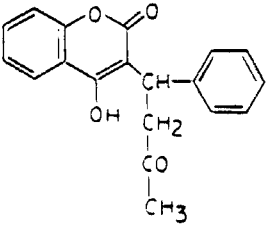
anti-inflammatory activity, being several times more potent than phenylbutazone (1,2). Two gas chromatographic (3,4) methods have been used to determine benoxaprofen in biological fluids, but they are time consuming, involving derivitization procedures. The high pressure liquid chromatographic determination described here is rapid in having only a single extraction step and a retention time for benoxaprofen of 6.0 min. Sodium warfarin (Structure 2), has been found to be an excellent internal standard for this procedure since it has a retention time of 5.0 minutes. The use of sodium warfarin as an internal standard greatly increases the precision and accuracy of the assay.





EXPERIMENTAL

Materials

Sodium benoxaprofen (903.5 mg free acid per gram) and sodium warfarin, were generously supplied by Eli Lilly (Indianopolis, IN)

and Endo laboratories, Inc. (Garden City, NY), respectively. A stock solution of 100 µg/ml benoxaprofen in 0.025 M Na_2HPO_4 was prepared. The internal standard stock solution contained 100 µg/ml warfarin in 0.025 M Na_2HPO_4. Working standards were prepared by appropriate dilutions with deionized water of these stock solutions to give 10 ml samples of 2.5, 5.0, 15, 25, 35 and 50 µg/ml benoxaprofen, each containing 25 µg/ml of the internal standard. Each plasma sample was spiked with 5 to 100 µl volumes of 100 µg/ml benoxaprofen stock solution to give final concentrations of 0.5, 1, 3, 5, 7 and 10 µg/ml.

Ether, phosphoric acid, hydrochloric acid and dibasic sodium phosphate were all analytical grade and supplied by Mallinckrodt Inc. (St. Louis, MO). HPLC grade acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI). All solvents including deionized water, were filtered through 0.45 µm filters (Millipore Corp., Bedford, MA) prior to use in the liquid chromatograph. Sample analyses were carried out on a model 5000 liquid chromatograph equipped with a 10 µl manual loop injector and a Vari-Chrom variable wavelength UV detector (Varian Assoc., Walnut Creek, CA). An alkylnitrile column, MicroPak CN-10 (Varian Assoc.) of medium polarity was used. The mobile phase consisted of 25% acetonitrile in water (pH 2.5 with H_3PO_4). At ambient operating temperature, the flow rate was 1.8 ml/min at a pressure of 160 atm. The column effluent was monitored continuously at 309 nm. All UV spectra of stock solutions were taken on a Cary model 219 UV spectrophotometer (Varian Assoc.)

Procedures

Plasma samples of 1.0 ml, to which 5 µg of internal standard had been added, are acidified with 400 µl of 1N HCl and mixed thoroughly by hand shaking. After the addition of 8 ml of ether, the mixture is vortexed for 30 sec. and then placed on a test tube rotator for 10 min. The sample is centrifuged at 3600 rpm for 10 min. and then the ether layer is transferred to another test tube and evaporated to dryness under a nitrogen stream. The residue is reconstituted in 200 µl of methanol and a 10 µl aliquot injected into the column.

Six point standard curves were obtained from the stock solutions. Spiked plasma samples were compared to the stock solutions to obtain recovery percentages. Data was obtained by plotting peak height ratios versus benoxaprofen concentration and a linear least squares regression program was utilized.

RESULTS

Fig. 1 shows typical chromatograms for control plasma, plasma with benoraprofen and plasma with benoxaprofen and warfarin. The retention times for warfarin and benoxaprofen were 5.0 and 6.0 min respectively. No interfering peaks in the control plasma were noted.

Table 1 shows the peak height ratios of benoxaprofen to 5 µg warfarin in the 0.5-10 µg range of benoxaprofen extracted from human plasma. Each value represents the average of three samples. A linear regression curve obtained from the data has the equation $y=0.257X + 0.041$ with $r^2=0.9996$, where r^2 is the coefficient of determination.

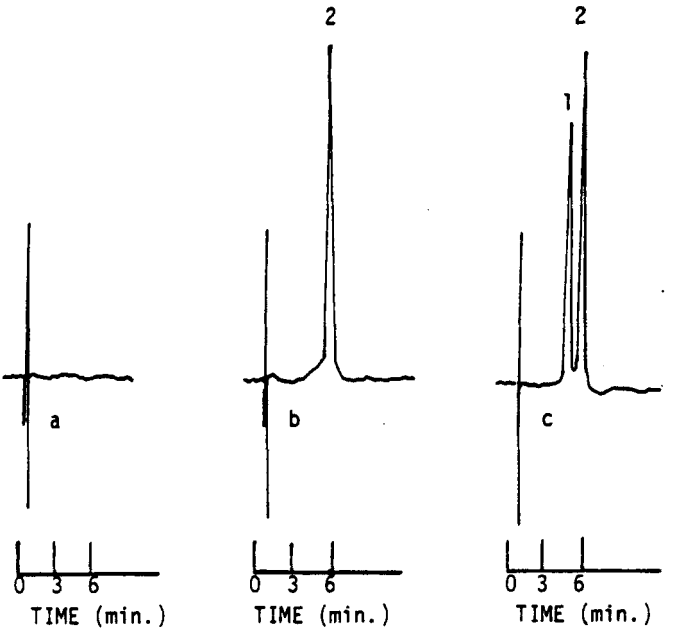


FIGURE 1

Liquid chromatograms of a) control human plasma b) human plasma containg 5 µg benoxaprofen (2) and c) human plasma containing 5 µg warfarin (1) and 5 µg benoxaprofen (2).

TABLE 1

Peak Height Ratios for Benoxaprofen Plasma Calibration Curve (N=3)

Amount Added (µg)	Peak Height Ratio (Mean ± SD)
0.5	0.149 ± 0.004
1.0	0.320 ± 0.030
3.0	0.831 ± 0.035
5.0	1.300 ± 0.064
7.0	1.837 ± 0.145
10.0	2.620 ± 0.093

Extraction recoveries were determined by injecting known concentrations of benoxaprofen with internal standard and comparing them to the extracted plasma samples (Table 2). The extraction recovery was 90.6 ± 6.8% for 18 samples.

Stability tests were performed by adding 0.5, 1, 3, 5, 7 and 10 µg of benoxaprofen to samples of plasma and freezing them. Analyses were performed at 1, 4, 9 and 15 days, the results of which are listed in Table 3. These findings show the samples are stable for this time period.

DISCUSSION

Two G.C. methods (3,4) have been used to determine benoxaprofen levels but no liquid chromatographic method has previously been

TABLE 2

Benoxaprofen Recovery From Human Plasma (N=3)

Amount Added (µg)	Amount Found (µg)	Recovery, % (Mean ± SD)
0.5	0.478 ± 0.015	95.5 ± 2.9
1.0	0.942 ± 0.088	94.2 ± 8.8
3.0	2.820 ± 0.120	94.0 ± 4.0
5.0	4.235 ± 0.205	84.7 ± 4.1
7.0	6.069 ± 0.483	86.7 ± 6.9
10.0	8.870 ± 0.270	88.7 ± 2.7
		Ave. 90.6 ± 6.8

TABLE 3

Effect of Frozen Storage on Benoxaprofen Stability in Human Plasma

Added (μg)	Days				
	0	1	4	9	14
	recovered (μg)				
0.5	0.48	0.38	0.48	0.50	0.61
1.0	0.94	0.85	0.93	1.00	1.04
3.0	2.82	2.46	2.54	2.68	2.85
5.0	4.24	3.94	3.75	4.50	4.22
7.0	6.07	5.47	5.95	6.15	5.35
10.0	8.87	8.46	8.47	8.30	8.33

reported. Radiolabelled [^{14}C] benoxaprofen has also been used in biological fluid assays (4,5,6). The extraction recovery obtained, 90.6 ± 6.8%, is similar to the extraction efficiency obtained in the radiolabelled assay (4).

Though not structurally similar to benoxaprofen, warfarin has proved to be an excellent internal standard. Retention times were one minute apart, namely 5.0 min. for warfarin and 6.0 min. for benoxaprofen. Peak height ratios exhibited excellent linearity in the concentration range studied (0.5-10 μg/ml). A variable wavelength UV detector was used to monitor benoxaprofen levels at 309 nm, its absorbance maximum in the solvent system used. Warfarin has an absorbance maximum of 308 nm in the same system. The signal-

to-noise ratios obtained were on the order of 2-3 times greater than when a 254 nm filter detector was used.

Stability studies have shown that benoxaprofen was stable for at least two weeks in frozen plasma. It was found, however, that stock solutions of benoxaprofen were not stable at room temperature during this time interval. Preliminary studies in this laboratory have shown that aqueous solutions of benoxaprofen undergo extensive photodegredation. This is especially apparent when a sample is placed in an intense UV light source, as in a fluorimeter. New stock solutions were prepared when necessary and concentrations were confirmed by UV spectra.

The lowest level of benoxaprofen detected in this study was 0.5 µg. Lower levels can be detected by decreasing the reconstituting volume or using a higher detector sensitivity. However, the concentration range studied was within the expected therapeutic range [3,6].

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