Quantitation of the Anti-Inflammatory Agent Fenbufen and Its Metabolites in Human Serum and Urine Using High-Pressure Liquid Chromatography

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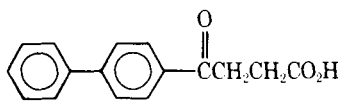
Received December 27, 1977, from *Lederle Laboratories, A Division of American Cyanamid Company, Pearl River, NY 10965.* Accepted for publication March 30, 1978.

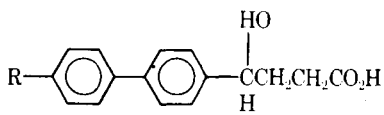
Abstract □ Specific procedures are described for the determination of fenbufen and its metabolites in serum and urine using high-pressure liquid chromatography. Serum or urine extracts were chromatographed on a bonded reversed-phase partitioning column. The sensitivity of the assay for fenbufen was 0.5 μg/ml in serum with 2-ml samples and 1.0 μg/ml in urine with 1-ml samples. The procedures are suitable for bioavailability and pharmacokinetic studies.

Keyphrases □ Fenbufen—high-pressure liquid chromatographic analysis in serum and urine □ High-pressure liquid chromatography—analysis, fenbufen in serum and urine □ Anti-inflammatory agents—fenbufen, high-pressure liquid chromatographic analysis in serum and urine

Fenbufen[1], 3-(4-biphenylylcarbonyl)propionic acid (I), is an orally effective, nonsteroidal, anti-inflammatory analgesic and antipyretic agent (1) and has been shown to be an effective and safe treatment for rheumatoid arthritis in humans (2–4). In a program to determine the structure–activity relationships of a series of analogs (5), two major human serum metabolites of fenbufen, 3-(4-biphenylylhydroxymethyl)propionic acid (II) and 4-biphenylacetic acid (III), were found to possess the same spectrum of activity as fenbufen itself. A TLC assay[2] for fenbufen and these metabolites, based on chromatographic separation followed by quantitation, was reported (6).



I



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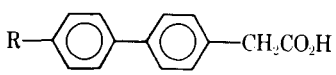
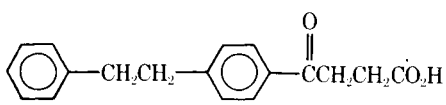
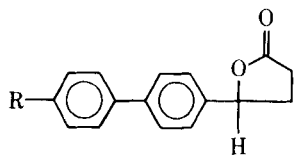
V: R = OH



III: R = H
IV: R = OH



VI



VII: R = H
VIII: R = OH

For bioavailability studies where large numbers of samples must be analyzed, a simpler analytical procedure was required. This paper describes a quantitative assay for I–III as well as two additional fenbufen serum and urinary metabolites, 4′-hydroxy-4-biphenylacetic acid (IV) and 3-(4′-hydroxy-4-biphenylylhydroxymethyl)propionic acid (V), based on high-pressure liquid chromatography (HPLC).

Table I—Retention Volumes, V_R, for I–V on Reversed-Phase HPLC [a]

Compound	V_R, ml
I	40.52
II (VII)[b]	54.00
III	31.38
IV	14.60
V (VIII)[b]	21.72
VI	82.94

[a] Chromatographic conditions are described under *Experimental.* [b] Compounds II and V form lactones VII and VIII, respectively, during the assay.

Table II—Absolute Recovery (Percent ± *SD*) of I–V from Spiked Serum Specimens [a]

Compound[b]	Amount Added, μg/ml[c]			
	2.5	0.625	50.0	12.5
I	93.03 ± 4.38	—	92.11 ± 4.23	—
II	93.60 ± 4.43	—	87.09 ± 4.68	—
III	98.75 ± 5.55	—	93.73 ± 4.08	—
IV	—	69.15 ± 5.87	—	61.42 ± 2.72
V	—	94.59 ± 7.91	—	85.35 ± 3.09

[a] The procedure to determine these values is described under *Experimental.* [b] Absolute recovery of VI was 93.72 ± 2.45%. [c] Five samples at each concentration level.

Table III—Absolute Recovery (Percent ± *SD*) of I–V from Spiked Urine Samples [a]

Compound[b]	Amount Added, μg/ml[c]		
	5	25	200
I	102.36 ± 5.65	107.80 ± 4.12	91.44 ± 1.44
II	76.88 ± 1.92	86.59 ± 5.00	92.45 ± 1.76
III	101.21 ± 9.85	106.10 ± 2.39	94.48 ± 1.22
IV	76.80 ± 0.68	87.62 ± 7.02	99.59 ± 1.47
V	63.09 ± 2.48	69.46 ± 3.60	71.31 ± 1.16

[a] The procedure to determine these values is described under *Experimental.* [b] Absolute recovery of VI was 95.68 ± 2.31% from spiked urine samples. [c] With the exception of I at 5 μg/ml where there were only four determinations, all other values are for five determinations.

[1] Cinopal.

[2] F. S. Chiccarelli, unpublished data.

0022-3549/78/1200-1662$01.00/0

Table IV—Precision Data for the Determination of I–V in Spiked Serum

Amount Added, I–III, μg/ml	Amount Added, IV and V, μg/ml	Assay Concentration, μg/ml of Serum[a] I	II	III	IV	V
100	25	96.54 ± 2.16	96.17 ± 2.36	93.90 ± 3.01	24.47 ± 1.08	25.53 ± 1.05
50	12.5	47.87 ± 1.96	47.85 ± 2.29	46.88 ± 2.60	12.17 ± 0.39	12.49 ± 0.48
25	6.25	24.42 ± 0.61	25.36 ± 2.58	24.67 ± 1.96	6.37 ± 0.52	6.41 ± 0.41
10	2.5	9.99 ± 0.28	9.97 ± 0.40	9.83 ± 0.26	2.52 ± 0.19	2.49 ± 0.12
5	1.25	5.00 ± 0.11	4.99 ± 0.19	4.93 ± 0.14	1.24 ± 0.08	1.23 ± 0.04
2.5	0.625	2.57 ± 0.07	2.57 ± 0.06	2.42 ± 0.42	0.63 ± 0.06	0.63 ± 0.09
1.25	0.312	1.37 ± 0.08	1.31 ± 0.06	1.42 ± 0.10	0.33 ± 0.06	0.32 ± 0.05
Mean percent recovery ± *RSD*		100.32 ± 4.72	100.06 ± 3.32	99.03 ± 6.85	100.83 ± 2.82	100.85 ± 1.62

[a] Mean ± *SD* for six determinations at each level.

Table V—Precision Data for the Determination of I–V in Spiked Urine

Amount Added, μg/ml	Assay Concentration, μg/ml of Urine[a] I	II	III	IV	V
200	205.95 ± 12.23	200.45 ± 14.12	201.30 ± 8.29	197.25 ± 9.53	200.37 ± 2.69
100	103.12 ± 3.09	99.02 ± 4.75	101.95 ± 1.12	102.10 ± 3.41	101.33 ± 3.43
50	52.60 ± 1.60	50.50 ± 2.26	51.34 ± 1.53	52.40 ± 2.96	51.10 ± 1.99
25	26.32 ± 1.30	25.30 ± 1.37	25.75 ± 0.90	26.45 ± 1.60	25.90 ± 0.98
10	9.68 ± 0.43	10.52 ± 0.84	10.01 ± 0.28	10.02 ± 0.29	10.15 ± 0.31
5	4.98 ± 0.48	5.13 ± 0.56	4.98 ± 0.47	4.78 ± 0.33	4.90 ± 0.42
Mean percent recovery ± *RSD*	102.25 ± 3.36	102.60 ± 2.10	101.33 ± 1.39	101.19 ± 3.79	101.14 ± 1.94

[a] Mean ± *SD* for five determinations at each level.

EXPERIMENTAL

Reagents—Fenbufen, its metabolites, and the internal standard[3] (VI) were used as received[4]. Spectroquality solvents were used in the chromatography. All other solvents were analytical grade.

Standard Solutions—A solution of the internal standard was prepared in methanol (100 μg/ml). For the serum assay, a solution containing I–III, each at a concentration of 100 μg/ml, and IV and V, each at a concentration of 25 μg/ml, was prepared in methanol. A stock solution containing I–V, each at a concentration of 50 μg/ml, was prepared in methanol for the urine assay.

Chromatographic Conditions—The high-pressure liquid chromatograph[5] was equipped with a variable wavelength UV absorbance detector set at 265 nm with a sensitivity of 0.05 aufs. The column used for chromatography of serum and urine extracts was 10-μm porous silica bonded with octadecylsilane for reversed-phase chromatography[6]. The mobile phase was chloroform–methanol–water–acetic acid (0.33:33:66:0.67 v/v) at a flow rate of 2.50 ml/min. The chromatograph oven was maintained at 45°. Retention volumes for fenbufen and its metabolites are shown in Table I.

Assay—*Serum*—The internal standard (2 ml) containing 200 μg of VI was added to a 20-ml screw-capped test tube. After removal of the methanol with a nitrogen stream, the standard was redissolved in 0.25 ml of 0.1 *N* NaOH. To this alkaline solution was added 2 ml of human serum, and the contents of the tube were thoroughly mixed for 45 sec. Then a 2.0-ml aliquot of concentrated hydrochloric acid was carefully added, and the tube was shaken for 30 sec. After standing for 5 min, the acid solution was extracted with 15 ml of cyclohexane–ether (7:3 v/v).

Following centrifugation, as much as possible of the organic phase was transferred to a new tube, and the solvent was evaporated to dryness under a stream of clean dry nitrogen. The residue was redissolved in 1.5 ml of chloroform–methanol–acetic acid–water (0.95:94.05:0.05:4.95 v/v), and the solution was filtered through a 0.5-μm filter[7] into a clean, dry tube. A 36-μl aliquot of the final extract was injected into the chromatograph.

The concentrations of fenbufen and its metabolites were determined from standard curves, employing peak area ratios of drug-related material to internal standard *versus* concentrations in normal serum. A laboratory computer system[8] was used for these calculations.

Urine—The procedure for extraction of urine (1 ml) aliquots was exactly as for serum, except that only 100 μg of the internal standard and 1 ml of concentrated hydrochloric acid were used and the extraction solvent was chloroform–ether (8:3 v/v) in two portions, 15 and 10 ml.

Absolute Recovery of Fenbufen and Metabolites—Aliquots of serum (2 ml) and urine (1 ml) were spiked with known quantities of fenbufen and its metabolites to give drug concentrations as detailed in Tables II and III, respectively. After the samples were treated as described under *Assay*, the peak areas for the respective components were compared to peak areas from the same amount of material[9] that did not go through the extraction procedure to give absolute recoveries.

Precision Determinations—Aliquots of normal human serum and urine were spiked with known amounts of fenbufen and its metabolites to give several concentrations of drug-related material (Tables IV and V, respectively). The samples were assayed using the analytical conditions

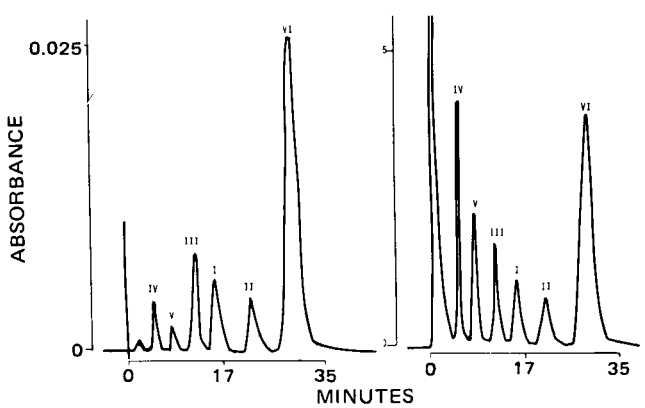


Figure 1—*Representative chromatograms of serum (left) and urine (right) extracts containing I–V plus an internal standard (VI).*

[3] 3-[4-(2-Phenylethyl)benzoyl]propanoic acid.
[4] Supplied by Lederle Laboratories.
[5] Model 7114-24 chromatograph, model 785 UV absorbance detector, and model 725 autoinjector, Micromeritics Instrument Corp., Norcross, Ga.
[6] Partisil-10 ODS column (4.6 mm i.d. × 25 cm), Whatman, Inc., Clifton, N.J.
[7] Fluoropore, FHLP 01300, Millipore Corp., Bedford, Mass.
[8] Model 3352 data system, Hewlett-Packard, Cupertino, Calif.
[9] Instead of II and V, respectively, the respective lactones VII and VIII were substituted. As described later, the lactones are actually the species chromatographed in the assay.

Table VI—Serum Concentrations of I–V following a Single Oral Dose of 600 mg of I [a]

Hours after Dose	Concentration in Serum, μg/ml				
	I	II	III	IV	V
0	0	0	0	0	0
0.16	<0.50	<0.50	<0.50	<0.50	<0.50
0.33	<0.50	<0.50	<0.50	<0.50	<0.50
0.50	3.48	5.98	<0.50	<0.50	<0.50
0.75	8.69	27.97	0.65	<0.50	<0.50
1	6.21	39.25	0.91	<0.50	<0.50
2	6.97	71.47	2.45	<0.50	1.00
4	2.97	61.86	4.91	<0.50	1.27
6	1.98	49.73	5.60	0.59	0.85
12	1.58	33.64	6.29	0.50	0.50
24	1.02	19.43	5.31	0.56	<0.50
36	0.76	13.36	4.34	<0.50	<0.50
48	<0.50	6.27	2.13	<0.50	<0.50

[a] Chromatographic conditions are described under *Experimental.*

Table VII—Total I–V (Milligrams) Excreted over 2 Days in Urine of an Adult Male after a Single Oral Dose of 600 mg of I [a]

Day[b]	I	II	III	IV	V	Total Percent of Dose[c]
1	19.3	7.5	28.6	68.0	103.4	38.87
2	3.0	—	8.1	30.3	26.0	11.79
Total	22.3	7.5	36.7	98.3	129.4	50.66

[a] Chromatographic conditions are described under *Experimental.* [b] Values noted are sum of individual results for fractional urines collected during Days 1 and 2. [c] Values were collected for stoichiometric differences.

detailed under *Assay.*

Clinical Study—One adult male volunteer was given two 300-mg capsules of I. Serum samples were obtained at 0, 10, 20, 30, and 45 min and 1, 2, 4, 6, 12, 24, 36, and 48 hr after the dose. Urine samples were collected for 48 hr after the dose. The urine volume of each collection was measured, and an aliquot was frozen at −10° until analyzed.

RESULTS AND DISCUSSION

Fenbufen and its metabolites, II–V, have similar chemical properties, and all are strongly bound to serum proteins. In this procedure, treatment of serum specimens with concentrated hydrochloric acid breaks the protein binding and converts II and V essentially quantitatively to their corresponding lactones, 5-(4-biphenylyl)-2(3*H*)-dihydrofuranone (VII) and 5-(4′-hydroxy-4-biphenylyl)-2(3*H*)-dihydrofuranone (VIII). Metabolite III and lactone VII exhibit strong maxima in the UV at 254 nm while the absorption maximum for fenbufen occurs at 280 nm. Since the isosbestic point for I, III, and VII is at 265 nm, which is the maximum for UV absorption of IV and VIII, the lactone of V, this wavelength was chosen to monitor the chromatographic effluent. Typical chromatograms are shown in Fig. 1.

The HPLC conditions and the selection of the internal standard were established through several experiments. Initial efforts to develop an HPLC method led to a procedure based on absorption chromatography. Although this method was adequate for the determination of fenbufen and the major serum metabolites, II and III, it was not suitable for the determination of IV and V, which are the major urine metabolites. In addition, salicylic acid interfered. Neither deficiency is present in the procedure based on reversed-phase HPLC. Comparative retention volumes for I–VI[10] are presented in Table I. Representative chromatograms are shown in Fig. 1. No interfering peaks from normal serum or urine extracts were observed.

Absolute recoveries of I–V from spiked serum and urine are presented in Tables II and III, respectively. The recoveries from serum ranged from 69.15 to 98.75% with standard deviations less than 10%. Absolute recoveries from urine ranged from 63.09 to 107.80% with standard deviations less than 10%.

Precision data for the determination of fenbufen and its metabolites in serum are presented in Table IV. The mean relative accuracies were ±1% of the amount of I–III added over the 1.25–100-μg/ml range with relative standard deviations less than 7%. For IV and V, the relative accuracies observed were ±1% of the compound added for concentrations in the 0.312–25-μg/ml range; relative standard deviations were less than 3%. The lower limit of sensitivity for all components in the assay was 0.5 μg/ml with a 2-ml serum sample.

Precision data for the assay of normal urine specimens spiked with known concentrations of I–V are presented in Table V. The mean relative accuracies were ±3% of the amount added with relative standard deviations less than 5% over the 5–200-μg/ml range. The lower limits of sensitivity for the assay were 1.0 μg/ml for I–IV and 2.0 μg/ml for V with 1.0 ml of urine.

To demonstrate the utility of this method, serum and urine concentrations of fenbufen and its metabolites were measured in a normal male volunteer after oral administration of a 600-mg dose of fenbufen (Tables VI and VII). Serum and urine concentrations of I–V were readily measured during the 2-day period after drug ingestion.

The described reversed-phase HPLC method for fenbufen and its metabolites is both rapid and sensitive and can serve as a useful means for studying the pharmacokinetics and bioavailability of fenbufen.

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ACKNOWLEDGMENTS

The authors thank Dr. R. G. Kelly, Lederle Laboratories, for advice and support.

[10] Retention volumes quoted for II and V are actually those of their lactones VII and VIII, respectively.