$\rangle\ddot{N}-C(=O)- \leftrightarrow \rangle\overset{+}{N}=C(-\overset{-}{O})-$. In general, of the compounds studied, the lactams possess predominantly CNS-stimulant activities. This effect is especially pronounced with 2-azacyclooctanone and 2-azacyclononanone. While many compounds were studied in order to make a generalization with regard to the effects of lipophilic–hydrophilic character and alkyl substitutions on the general biological behavior of these drugs, the majority of the report converges on the most active stimulants, namely 2-azacyclooctanone and 2-azacyclononanone. By various methodologies, sufficient data and information were obtained to conclude that they are CNS stimulants with the predominant site of action located in the midbrain and the medulla, that is, supraspinal and subcortical.

Nikethamide and pentylenetetrazole are two clinically useful stimulant drugs thought to exert their effects also in the midbrain and medulla. Moreover, all penicillins are irritating to the CNS. EEG abnormalities and convulsions have followed the application of penicillin directly to the human cerebral cortex (10). The administration of high doses of penicillin intravenously has produced convulsions in some patients, and convulsions and death have occurred following intraarterial administration of large doses of penicillin to patients with impaired renal function (11). The penicillins, like nikethamide and pentylenetetrazole, also contain moieties that have similar resonating structures to the compounds discussed in this report. These observations, together with the results presented in this report, particularly those obtained with 2-azacyclooctanone and 2-azacyclononanone, demonstrate that the resonating moiety referred to confers upon many substances CNS-stimulant activities.

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GLC Determination of *dl*-2-(3-Phenoxyphenyl)propionic Acid (Fenoprofen) in Human Plasma

J. F. NASH, R. J. BOPP, and A. RUBIN

Abstract □ The quantitative determination of *dl*-2-(3-phenoxyphenyl)propionic acid (fenoprofen) in plasma is described. Fenoprofen, extracted with hexane from 1 ml. of acidified plasma, is converted to the silyl ester and then measured by GLC utilizing a flame-ionization detector. Favorable quantitation is achieved by using *dl*-2-(4-phenoxyphenyl)valeric acid as a mass internal standard. The assay is quantitative above 0.25 mcg./ml. Overall, precision and accuracy of the assay are approximately ±10% (*RSD*) and ±5% (*RE*) in the range of 3–8 mcg./ml. Plasma data are presented to demonstrate the clinical utility of the method.

Keyphrases □ *dl*-2-(3-Phenoxyphenyl)propionic acid—GLC analysis in human plasma □ Fenoprofen—GLC analysis in human plasma □ Plasma levels—fenoprofen, GLC analysis □ GLC—analysis, *dl*-2-(3-phenoxyphenyl)propionic acid (fenoprofen) in human plasma

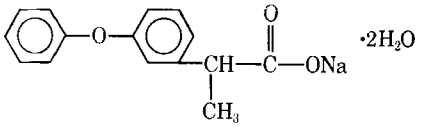
Since Northover (1) showed that aryl- and alkyl-substituted phenoxyacetic acids exhibit anti-inflammatory properties, several structurally related chemicals have been synthesized and marketed. These include ibufenac or 2-(4-isobutylphenyl)acetic acid (2), ibuprofen or 2-(4-isobutylphenyl)propionic acid, and namoxyrate or 2-dimethylaminoethanol salt of 2-(4-biphenyl)butyric acid (3). In general, sensitive, simple, specific, and reproducible methods have not been readily available to determine quantitatively this class of chemical compounds in biological fluids. To determine blood levels of ibufenac, Adams and Cliffe (2) developed a method based upon paper chromatographic separation and reaction of the drug with bromcresol purple. The method was quantitative above 7.5 mcg./ml. serum, with a precision of ±15%; unfortunately, the analysis required 48 hr. Bergen *et al.* (3) used ^{14}C-namoxyrate to study the absorption, distribution, and metabolism of the drug.

Nickander *et al.* (4) reported on the synthesis and pharmacologic effects of a series of structurally related chemicals, one of which is fenoprofen, *dl*-2-(3-phenoxyphenyl)propionic acid. A procedure is described herein for its quantitative determination in plasma using GLC. This method should be useful in quantitating other structurally related compounds in biological media.

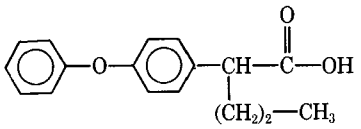
EXPERIMENTAL

Reagents—Sodium fenoprofen dihydrate and *dl*-2-(4-phenoxyphenyl)valeric acid were synthesized[1]. The latter compound served as a mass internal standard (MIS). All solvents were analytical reagent quality, except spectroquality hexane, which was used at the concentration stage of the procedure. Diatoport-S (80–100 mesh)

[1] At Eli Lilly and Co., Indianapolis, Ind.



sodium *dl*-2-(3-phenoxyphenyl)propionate dihydrate



dl-2-(4-phenoxyphenyl)valeric acid

Structural formulas of phenoxyphenyl alkanoic acids

and methylvinyl silicone gum rubber (UCCW-982) were purchased[2]. Dimethyldichlorosilane and hexamethyldisilazane were also used[3].

Apparatus—An F&M[4] model 402 dual-column gas chromatograph, equipped with hydrogen flame-ionization detectors and −0.2 to 1.0-mv. Honeywell Electronik 16 recorders, was used. Samples were evaporated to dryness under nitrogen on a model 106 N-Evap[5]. Dissolution of the dried samples in a solution of hexamethyldisilazane in carbon disulfide was aided by ultrasonic vibration[6]. Tapered glass centrifuge tubes (12- and 15-ml.), fitted with Teflon-lined screw caps, were used[7].

Chromatographic Conditions—The Honeywell Electronik recorder was operated at a chart speed of 0.63 cm./min.

Helium carrier gas, flowing at 90 ml./min., was filtered through a 1.83-m. × 1.27-cm. (6-ft. × 0.5-in.) copper tubing filled with pellets of molecular sieve 5A. The filter was preconditioned overnight at 200° while flushing with dry nitrogen.

A glass column, 0.92-m. (3-ft.) × 3.0-mm. i.d., which was packed with 3.8% UCCW-982 (80–100 mesh) on Diatoport-S and operated at a temperature of 175°, was used in the analyses. To condition newly packed columns, carrier gas flow was turned on for 2–5 min. and then turned off. The oven temperature was raised to 250° without carrier flow for 1 hr. and then cooled to room temperature. The oven was reheated to 250° with carrier gas flowing overnight. The columns were then "loaded" with repeated injections of 3 mcg. of fenoprofen as the trimethylsilyl ester and 0.5 mcg. of the trimethylsilyl ester of the MIS in a chloroform solution until: (*a*) peak

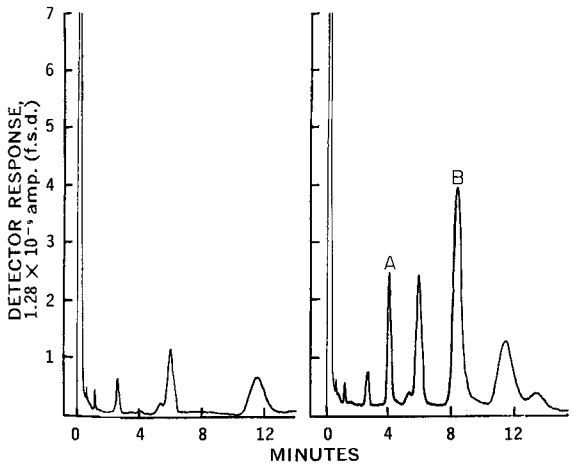


Figure 1—*Gas chromatogram of human plasma assayed as described. Left: chromatogram from normal plasma. Right: chromatogram from normal plasma with 5 mcg./ml. fenoprofen (A) plus 12 mcg./ml. MIS (B) added.*

[2] F&M Scientific Co., Avondale, Pa.
[3] From Pierce Chemical Co., Rockford, Ill.
[4] Hewlett Packard, Skokie, Ill.
[5] Organomation Associates, Shewsbury, Mass.
[6] Using a model D-100 Sonogen, Cole-Parmer, Chicago, Ill.
[7] Matheson Scientific Co., Chicago, Ill.

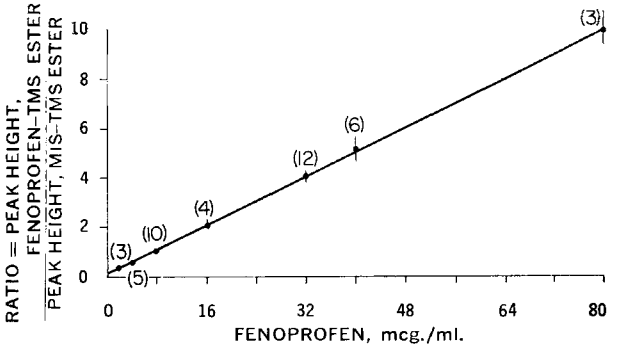


Figure 2—*Calibration curve. Relationship between the ratio of peak height of fenoprofen–trimethylsilyl ester/MIS–trimethylsilyl ester and fenoprofen concentration in plasma. Parentheses enclose the number of observations. The bar represents the range of values.*

heights were stable, and (*b*) injection of the ester of the internal standard 10 min. after injection of the ester of fenoprofen did not produce a peak of the ester of fenoprofen. This latter observation is important because occasionally injections of the trimethylsilyl ester of the internal standard appear to displace residual amounts of the ester of fenoprofen from column binding sites if the columns are not conditioned and repeatedly "loaded" as described. This "knock-off" phenomenon may reappear after 5–7 months of column usage; in this case, it is convenient to replace the column because the problem has not been eliminated in aged columns.

The glass column and glass wool used to prepare the column were siliconized with dimethyldichlorosilane before use (5). The injection port and detector block were at 220°. Under these conditions, the retention times of the trimethylsilyl esters were 4 min. (19.1 methylene units) for fenoprofen and 8.5 min. (20.8 methylene units) for the MIS (Fig. 1)[8]. At a sensitivity of 1.28×10^{-9} amp./mv., 1 mcg. of fenoprofen as its trimethylsilyl ester produced a full-scale deflection.

Procedure—The glassware was washed with chromic acid cleaning solution, rinsed with water, dried, and siliconized using a 1% solution of dimethyldichlorosilane in toluene[9].

Heparinized (or citrated) blood was centrifuged to separate the plasma[10]. Plasma samples from fenoprofen-treated subjects and control samples for calibration curves were analyzed similarly, except that fenoprofen was added in known amounts to the latter. Calibration curves were prepared daily as follows: 1 ml. of human plasma was pipeted into each of five 12-ml. screw-cap centrifuge tubes. By using a 50-μl. syringe, 0.0, 5.0, 10.0, 20.0, or 40.0 μl. of a standard aqueous solution of fenoprofen sodium dihydrate (1 mcg. equivalent acid/μl.) was added in separate tubes. To each tube was added 5 μl. (15 mcg.) of MIS dissolved in chloroform. The mixture was shaken to ensure uniform distribution of its constituents.

Two milliliters of an aqueous solution of trichloroacetic acid (10%) was added to each tube, and the contents were mixed and allowed to stand for 10 min. Five milliliters of hexane was added to the tubes; each tube was shaken vigorously for 2 min. and centrifuged at 2000 r.p.m. for 2 min. Approximately 4 ml. of the hexane extract was transferred to a 15-ml. screw-cap centrifuge tube. After redispersing the protein precipitate, the hexane extraction was repeated; approximately 5 ml. of hexane extract was withdrawn and added to the first hexane extract[11].

To the combined hexane extract was added 5 ml. of 0.1 *N* sodium hydroxide. The tubes were shaken for 2 min. and centrifuged, and the organic phase was discarded by aspiration. After adjusting the

[8] The extraction procedure effectively separates fenoprofen and the internal standard from normally appearing plasma constituents and from a primary metabolite of fenoprofen, 4′-hydroxyfenoprofen.
[9] No experimental evidence is available to indicate that siliconization of the glassware is necessary. Glassware is routinely siliconized in this laboratory, unless the procedure is specifically contraindicated. If glassware requires additional cleaning after siliconization, rinse with chloroform before use.
[10] Samples of fresh plasma as well as plasma frozen at −20° for 3–4 weeks were successfully used. However, blood bank plasma that has been frozen for more than 3 months was unsatisfactory for this assay due to interference from silylatable contaminants.
[11] Samples may be stored in a refrigerator for at least 3 days at this stage.

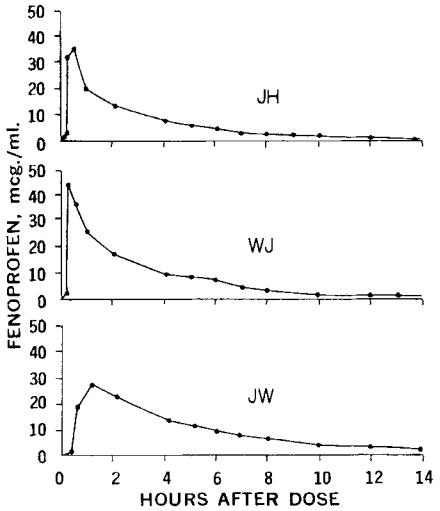


Figure 3—*Plasma concentrations of fenoprofen in three male subjects administered 250 mg. p.o. fenoprofen.*

pH of the aqueous phase to approximately 3 with 1.2 ml. of glacial acetic acid, extraction was performed again using 8 ml. of spectroquality hexane. The hexane phase[11] was transferred to a 12-ml. centrifuge tube and was concentrated to dryness on the N-Evap at 50° under a gentle stream of dry nitrogen. The sides of the tubes were washed with 0.5 ml. of spectroquality hexane, which was again evaporated to dryness[11]. The washing and evaporating were repeated. To the residue[12] was added 20 μl. of 1:20 hexamethyldisilazane in carbon disulfide to form the trimethylsilyl esters of fenoprofen and of internal standard, thus facilitating their chromatography. The samples were sonicated for 30 sec. One microliter of each sample was injected onto the GLC column.

Following chromatography, the baseline was drawn and the heights of the peaks corresponding to the fenoprofen ester and internal standard ester were measured[13]. The ratio of the peak heights was plotted on the ordinate of Cartesian graph paper, and the concentration in micrograms of fenoprofen per milliliter plasma was plotted on the abscissa. Using the peak height ratio of an unknown sample and the calibration curve (Fig. 2), the concentration of fenoprofen in a sample was determined.

RESULTS AND DISCUSSION

To obtain the precision and accuracy of the assay method, plasma samples, prepared to contain known amounts of fenoprofen, were randomized and assayed with the collected plasma samples (Table I).

Using 1-^{14}C-fenoprofen, 4.2 μc./mg., the extraction efficiency for the drug from plasma was studied. The data show that the drug was extracted quantitatively after an initial loss in the protein precipitate fraction (Table II).

A 1:20 dilution of hexamethyldisilazane in carbon disulfide was optimal to provide quantitation of fenoprofen below 200 mcg./ml. plasma[14]. Reacting 1-^{14}C-fenoprofen with hexamethyldisilazane in carbon disulfide yielded a product which, when chromatographed by thin layer, had an R_f 0.07 and contained 90% of the radioactivity. A standard of fenoprofen had an R_f 0.30. The silation reaction appears to have proceeded quantitatively. The F-254 plate was developed in a vapor-saturated tank of benzene–ethanol (95:5 v/v) until the solvent front ascended 15 cm. The small amount of radioactivity at the R_f of the fenoprofen may have been due to an on-the-plate hydrolysis of the silyl ester.

Table I—Precision and Accuracy in Measurement of Fenoprofen Added to Human Plasma

Added, mcg./ml.	Measured[a], mcg./ml.	*RSD*	*RE*
3.3	3.2 (2.9–4.1)	±12.0%	−4.5%
4.9	5.0 (4.8–5.4)	±5.5%	±2.2%
8.2	8.4 (8.0–9.6)	±7.9%	±1.6%

[a] Mean of five replicate samples (range).

Table II—Recovery of 1-^{14}C-Fenoprofen from Human Plasma

Added[a], mcg./ml.	Percent of Radioactivity Remaining in Fractions after Extraction of 1-^{14}C-Fenoprofen: Protein Precipitate	Supernatant after TCA Precipitation	Combined Hexane Extracts of Supernatant	Acidified Aqueous Phase	Percent 1-^{14}C-Fenoprofen in Final Hexane Extract
12.5	—[b]	<1	<0.5	3	71
25	14	<1	—[b]	4	77

[a] Means of three replicate determinations. [b] Not analyzed.

Application of GLC Assay to Biological Samples—The plasma concentrations of fenoprofen in three human subjects following oral administration of 250 mg. fenoprofen (312 mg. sodium fenoprofen dihydrate) illustrates the clinical utility of this method (Fig. 3). The results indicate that the drug is absorbed rapidly from the gastrointestinal tract and disappears rapidly from the plasma, with only trace amounts present after 10 hr.[15].

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[12] This residue was analyzed in a TLC system using glass, precoated silica gel plates (20 × 20 cm., Brinkmann F-254). A solvent system of benzene–glacial acetic acid–methanol (95:5:25) was allowed to develop 10 cm. Visual observation in the short UV range revealed a spot at R_f 0.53, corresponding to a similar spot obtained using authentic fenoprofen chromatographed concurrently.

[13] Results were virtually unaffected when relative peak areas were used instead of relative peak heights.

[14] By diluting hexamethyldisilazane, interference with the GLC analysis by the "pure" silylating agent was eliminated. Silation was also attempted using bis(trimethylsilyl)acetamide, trimethylchlorosilane, and bis(trimethylsilyl)trifluoroacetamide. These reagents were unsatisfactory, causing interference with the GLC analysis.

[15] A pharmacodynamic profile of fenoprofen in man will be published in the future.