Table I—Serum Concentrations (Micrograms per Milliliter) and Urinary Excretion of Cefazolin in Three Subjects Given a Single Intramuscular Dose (8 mg/kg) of Drug

Subject	Hours after Dose[a]								Excretion in 0–24-hr Urine, % of Dose
	0.5	1	1.5	2	4	5.5	7	8	
1	25	30	30	28	14	7.7	3.5	1.8	45
2	39	42	31	29	13	6.4	3.5	1.8	41
3	21	35	32	28	16	11	6.0	5.7	65

[a] No cefazolin was detected at zero hour.

given a single intramuscular dose (8 mg/kg) of cefazolin are shown in Table I.

The procedure is specific in subjects known to be taking only cefazolin but has not been checked to determine whether related compounds may interfere.

The method may have general applicability to other cephalosporins that can be extracted from serum and/or urine and that contain a structural moiety absorbing in the UV spectrum.

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GLC Assay of Fenclorac in Human Plasma

A. F. DeLONG ˣ, A. POLK, G. MARTIN, R. D. SMYTH *, and N. H. REAVEY-CANTWELL

Received May 11, 1977, from the *Research Division, William H. Rorer, Inc., Fort Washington, PA 19034.* Accepted for publication November 10, 1977. *Present address: Department of Drug Metabolism, Bristol Laboratories, Syracuse, NY 13201.

Abstract □ A simple, sensitive GLC assay for fenclorac is described. Plasma proteins were precipitated with methanol, and the methanolic extract was refluxed with hydrochloric acid to form the methyl esters of fenclorac and the internal standard. The esters were purified by partitioning into benzene. Aliquots of 1 μl of the concentrated benzene phase were injected into the gas chromatograph and quantitated by a ^{63}Ni-electron-capture detector. Recovery of fenclorac from plasma averaged 82 ± 1.6%.

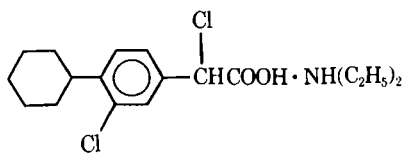
Keyphrases □ Fenclorac—GLC analysis in plasma □ GLC—analysis, fenclorac in plasma □ Anti-inflammatory agents—fenclorac, GLC analysis in plasma

Fenclorac (DL-α,3-dichloro-4-cyclohexylphenylacetic acid) is a potent nonsteroidal anti-inflammatory agent with significant analgesic and antipyretic activities (1–3). In humans and animals, the compound is rapidly absorbed, extensively metabolized, and quantitatively eliminated within 24–48 hr (4, 5). Fenclorac is the primary circulating drug species (>85%) with small amounts of the α-hydroxy metabolite (3-chloro-4-cyclohexylphenylglycolic acid), which has about 0.3 times the anti-inflammatory potency of fenclorac.

A spectrophotometric method (6) was used to quantitate fenclorac in biological samples. However, this method is not specific for the parent drug, and the sensitivity is approximately 1 μg/ml. This report describes a simple, sensitive, specific GLC assay of fenclorac in plasma.

EXPERIMENTAL

Materials—The chemicals and reagents used were: fenclorac diethylammonium salt (I), DL-4′-chloro-α-methyl-4-biphenylglycolic acid (internal standard) (II), GC–spectrophotometric quality benzene and ethyl acetate, and reagent grade methanol and hydrochloric acid.



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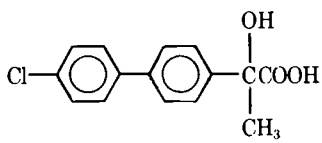

II

Instrumentation—*GLC*—GLC analysis was performed on a gas chromatograph[1] equipped with a ^{63}Ni-electron-capture detector and fitted with a 0.9-m × 2-mm coiled glass column packed with 3% silicone[2] [methyl–cyanopropyl (50:50)] on 80–100-mesh Gas Chrom Q. The column was conditioned at 220° for 18 hr. The methyl esters of fenclorac and the internal standard were formed by refluxing in hydrochloric acid–methanol. Purification and concentration of the resultant product are described later.

The methyl esters of fenclorac and the internal standard were eluted isothermally from the column under the following temperature conditions: column, 220°; injection port, 240°; manifold, 240°; and detector, 300°. Argon (95%)–methane (5%) gas flow was maintained as follows: column, 40 ml/min; and auxiliary, 60 ml/min. Samples (0.5–1.0 μl) were injected manually or with an automatic injector[3].

GLC–Mass Spectrometry—Mass spectra of standards and extracts of plasma were obtained using a GLC–mass spectrometer[4]. A 0.9-m × 2-mm coiled glass column packed with 5% OV-17 on Chromosorb W(HP) was used. The ester fractions were eluted isothermally at 225° with a

[1] Perkin-Elmer model 90013.
[2] Applied Science Laboratories.
[3] Hewlett-Packard model 7076.
[4] Hewlett-Packard model 5930-A.

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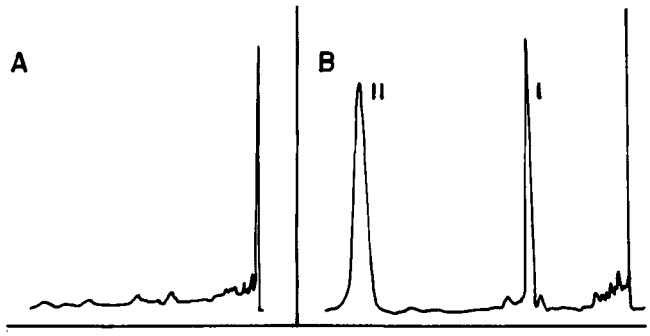

Figure 1—*Chromatograms of derivatized extract of pooled human plasma (A) and derivatized extract of pooled human plasma containing I and II (B).*

helium flow rate of 55 ml/min. The mass spectrometer detector gain was 5, and the ion monitor range was 5 v.

Analytical Procedures—*GLC of Plasma*—Plasma, 200 μl, containing 10 μg of the internal standard was added to a 50-ml, conical, glass-stoppered centrifuge tube. Methanol, 5 ml, was added, and the tube was vortexed for 30 sec and centrifuged[5] for 10 min at 1200×*g*. The methanolic phase was transferred to a 15-ml screw-capped tube, and the plasma residue was reextracted with a second 5-ml aliquot of methanol.

After centrifugation, the methanolic phases were combined, and 200 μl of concentrated hydrochloric acid was added. The tubes were sealed and heated for 1 hr at 100°. The methanolic extract was then transferred to a clean 50-ml, conical, glass-stoppered centrifuge tube containing 5 ml of benzene and 4 ml of water. The tubes were vortexed for 1 min and centrifuged for 20 min at 1200×*g*, and the upper phase was transferred to a 15-ml conical centrifuge tube.

The benzene extract was evaporated to dryness using a 50° water bath and a stream of dry air provided by a manifold apparatus. The dried residue was reconstituted in 100 μl of ethyl acetate, and aliquots were injected into the gas chromatograph. The retention times of fenclorac and the internal standard were 3.3 and 8.0 min, respectively. Plasma standard and unknown samples were processed simultaneously in the manner described.

GLC–Mass Spectrometry of Plasma—Aliquots of 3 ml of plasma, 3 ml of 2 *N* HCl, and 25 ml of ether were added to a 45-ml glass-stoppered centrifuge tube, mixed for 1 min, and centrifuged for 5 min at 2000×*g*. The ether phase was decanted and transferred to a 250-ml round-bottom flask. The remaining residue was reextracted as described, and the ether phases were combined.

The ether extract was evaporated to dryness on a rotary flash evaporator with a 40–50° water bath. The residue was dissolved in 25 ml of methanol with 3 drops of concentrated hydrochloric acid, and the mixture was heated at reflux for 30 min. After cooling, the acid–methanol solution was transferred to a 250-ml separator, the flask was washed with 100 ml of 5% NaCl, and the wash was added to the separator along with 50 ml of benzene. The mixture was shaken for 30 sec and allowed to separate, and the benzene layer was filtered through a 30-g pad of anhydrous sodium sulfate prior to being transferred to a clean 250-ml round-bottom flask.

The aqueous layer was reextracted with benzene as described. The filtered extracts were combined along with 15 ml of benzene, which was used to rinse the sodium sulfate pad. The benzene extract was evaporated to near dryness on a rotary flash evaporator in a 40–50° water bath, and the residue was increased to a final volume of 10 ml with benzene. The concentrated benzene extract was quantitatively applied to a 15 × 450-mm glass column containing 10 g of magnesium silicate[6], which was activated at 130° for 48 hr.

The methyl ester of fenclorac was eluted from the column with 50 ml of benzene–ether (90:10). The column eluate was concentrated to 100 μl in a 40° water bath with dry nitrogen, and aliquots (5 μl) were injected into the gas chromatograph–mass spectrometer. The retention time of the fenclorac methyl ester was obtained from the total ion scan of authentic fenclorac methyl ester samples. Fenclorac was identified in biological fluids by mass spectra obtained at the beginning, middle, and tail of the total ion peak, which had a retention time corresponding to standard fenclorac methyl ester. Confirmation was considered positive when

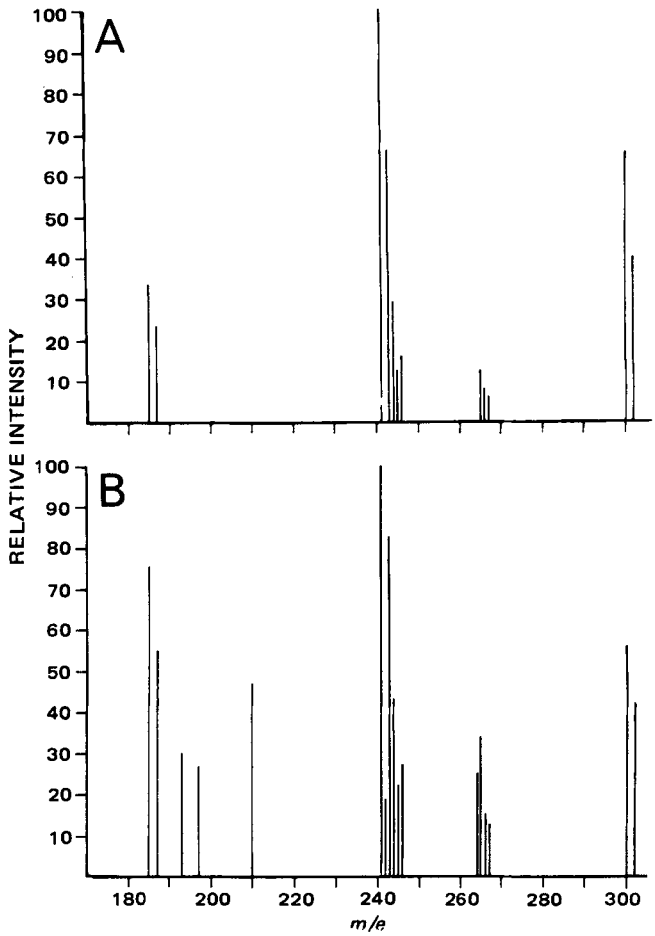

Figure 2—*Mass spectra of methyl ester derivative of fenclorac standard (A) and derivatized extract of plasma obtained from human subjects treated with fenclorac (B).*

two or more prominent ions (*m/e* 300/302 and 241/243) characteristic of fenclorac methyl ester were found to have an intensity greater than twice the intensity observed for the same mass ions in background spectra.

Drug Administration to Humans—Six healthy, normal, male volunteers[7] were administered 200 mg of ^{14}C-fenclorac (specific activity of 0.155 μCi/mg). The subjects, 21–50 years old and 77–86 kg, were fasted for 18 hr prior to receiving a 200-ml dose of a fenclorac solution. Heparinized blood specimens were obtained at appropriate time intervals, and plasma was prepared by centrifugation at 2000×*g* for 10 min. The plasma was frozen immediately and stored at −20° prior to analysis.

Analysis of Radioactivity in Plasma—The determination of radioactivity in plasma samples was described previously (5).

RESULTS AND DISCUSSION

Derivatives of Fenclorac—The trimethylsilyl esters of I and II are readily formed by the use of the derivatizing reagent *N,O*-bis(trimethylsilyl)acetamide[8]. The reaction is complete within 2 min, and the derivatives are stable for up to 8 hr. Peak loss due to nonspecific column adsorption (particularly I) occurs; to obtain linearity, the column must be saturated by multiple injections of I (7).

The methyl ester derivatives of I and II were evaluated by use of the derivatizing reagents boron trifluoride–methanol, hydrochloric acid–methanol, and diazomethane. Under the conditions investigated, the hydrochloric acid–methanol and diazomethane procedures resulted in quantitative production of the methyl esters of I and II, while nonquantitative conversion was obtained with the boron trifluoride–methanol procedure. The kinetics of ester formation were followed using

[5] I.E.C. model K.
[6] Florisil.

[7] Study conducted by Medical and Technical Research Associates at the Massachusetts Correctional Institute, Norfolk, Mass. Informed written consent was obtained.
[8] Pierce Chemical Co., Rockford, Ill.

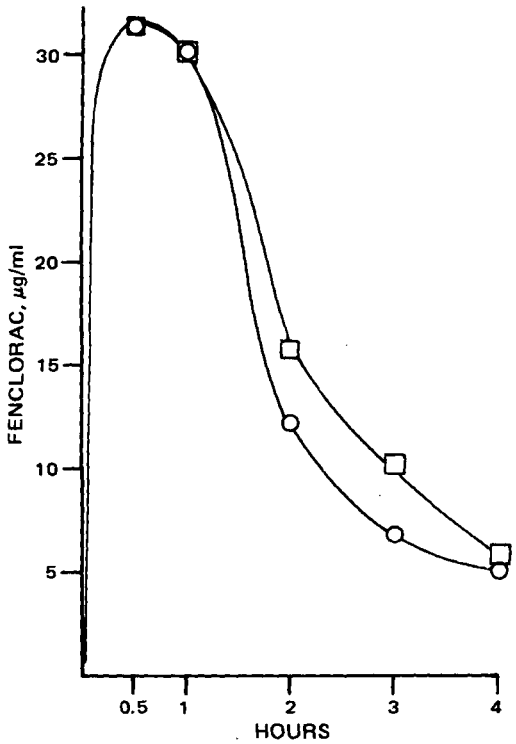

Figure 3—*Plasma fenclorac levels in humans measured by the radioactive (□) and GLC (○) methods.*

^{14}C-labeled I and II and radiochemical TLC. For simplicity, the hydrochloric acid–methanol reflux was used to derivatize I and II for sample analysis.

Analysis of Plasma—The GLC analysis of control human plasma that was carried through the derivatization and extraction procedures is presented in Fig. 1A; very little endogenous plasma constituents could be detected. Figure 1B is a gas chromatogram of the methyl esters of fenclorac (I) and the internal standard (II). The chromatogram was prepared by adding fenclorac and the internal standard to control plasma, which was then carried through the derivatization and extraction procedures. Both fenclorac and the internal standard methyl ester peaks were symmetrical and had retention times of 3.3 and 8.0 min, respectively, at 225°. Plasma samples prepared in this manner were stable for at least 1 week at room temperature.

Assay Sensitivity and Specificity—The standard curve is linear over a plasma concentration range of 0.2–32 μg of fenclorac free acid/ml. Adequate day-to-day reproducibility was indicated by the consistency of the slopes of the standard curves over 4 weeks. The coefficient of variation (*CV*) ranged from 5 to 8%. GLC–mass spectrometric analysis of plasma from drug-treated subjects confirmed that peak I was the fenclorac methyl ester (Fig. 2).

Table I—Recovery of ^{14}C-Fenclorac from Plasma

Plasma Concentration, μg/ml	Recovery, %
0	0
2	84
4	82
8	85
16	81
32	82
Mean ± *SD*	82 ± 1.6

Recovery of Fenclorac from Plasma—Known concentrations of ^{14}C-fenclorac in ethanol were added to pooled normal human plasma. The samples were derivatized, extracted, and concentrated as described under *Experimental*. The mean recovery of added ^{14}C-fenclorac was 82 ± 1.6% over a range of 2–32 μg of compound/ml (Table I).

Analysis of Human Plasma Samples—GLC and analysis of radioactivity were performed on plasma samples obtained from human subjects who received 200 mg of fenclorac. The GLC analysis and total ^{14}C-fenclorac (acid) equivalents were nearly identical for up to 1 hr after oral dosing. From 1 to 4 hr postdose, the GLC analysis was consistently lower than the total radioactivity. This discrepancy could be due to small quantities of circulating metabolite (5) or experimental error.

A typical analysis of human plasma level data is presented in Fig. 3. Analysis of human urine for fenclorac indicated the absence of intact drug. This result was expected since fenclorac is completely biotransformed prior to excretion (5).

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