Quantitative Determination of Fenclorac in Serum

J. K. LEE ˣ, R. D. SMYTH *, A. POLK, T. HERCZEG, C. T. TSUEI, and N. H. REAVEY-CANTWELL

Abstract □ A spectrophotometric method for the analysis of fenclorac and its metabolite, 3-chloro-4-cyclohexylbenzeneglycolic acid, in human serum was developed. The parent compound represented at least 90% of the total species present in blood; the metabolite was present to the extent of about 10%, primarily in the elimination phase. The basic procedure consists of extraction of both compounds from serum, further extraction to remove interfering substances, alkaline conversion of fenclorac to the α-hydroxy acid metabolite, oxidation of this metabolite to the corresponding benzaldehyde derivative, and spectrophotometric measurement of the absorbance of the aldehyde at 252 nm. A comparison of serum concentrations obtained by this method with concentrations calculated from ^{14}C-data following oral administration of 1-^{14}C-fenclorac to eight normal adult volunteers indicated a 90% correlation between methodologies over a range of 1.4–25.5 μg of fenclorac/ml of serum.

Keyphrases □ Fenclorac and metabolite—spectrophotometric analysis, serum □ Spectrophotometry—analysis, fenclorac and metabolite in serum □ Anti-inflammatory agents—fenclorac and metabolite, spectrophotometric analysis in serum

Fenclorac (α,3-dichloro-4-cyclohexylbenzeneacetic acid) is a potent antiphlogistic, antinociceptive, and antipyretic agent (1, 2). Fenclorac is more potent than phenylbutazone or aspirin in the treatment of developing or established adjuvant arthritis and inhibition of carrageenan paw edema. Fenclorac is more potent than aspirin and indomethacin in reducing fever in rats rendered hyperthermic with brewer's yeast.

This paper describes the development of a quantitative assay for fenclorac (I) and its metabolite, 3-chloro-4-cyclohexylbenzene glycolic acid (II), in serum.

EXPERIMENTAL

Apparatus—A spectrophotometer[1], a recording spectrophotometer[2], and 1-cm quartz semimicro cells[3] were used.

Materials and Reagents—Methanol[4] (certified ACS grade), ceric ammonium sulfate dihydrate[4] (certified grade), sodium hydroxide[4] (certified ACS electrolytic pellets), chloroform[5] (spectrophotometric grade), sulfuric acid[5], hydrochloric acid[6] (analytical reagent grade), hexane[7] (distilled-in-glass UV grade), and normal human serum[8] were used as received.

Compounds I, II, 3-chloro-4-cyclohexylbenzaldehyde (III) and 1-^{14}C-carboxyl-labeled I and II were all synthesized[9].

Ceric reagent solution, 0.2 *M*, was prepared by the following procedure (3). Ceric ammonium sulfate, 126 g, was transferred to a 1-liter glass-stoppered volumetric flask containing a magnetic stirring bar. Approximately 700 ml of distilled water was added, followed by 30 ml of concentrated sulfuric acid. The mixture was stirred briefly, the volume was adjusted to 1 liter, and mixing was continued for about 2 hr. Insoluble material was allowed to settle overnight, and the clear supernate was used.

Serum Samples for Standard Curve—Standard solutions of I in serum were prepared by first mixing 3 ml of a 1.25-mg/ml methanolic solution of the diethylammonium salt of I with 97 ml of normal human serum to yield a stock serum standard equivalent to 30 μg of I/ml (factor for converting diethylammonium salt to free acid = 1.25). Dilutions of this stock serum standard were prepared to yield samples containing 2, 5, 10, 20, and 30 μg of I/ml of serum. Aliquots were dispensed into small tubes and frozen prior to use.

Analytical Procedure—The following procedure was a modification of the method of Cummins and Perry (4).

Methanol (5.0 ml) was added to a 35-ml ground-glass-stoppered conical centrifuge tube containing exactly 1.5 ml of serum sample or serum standard. The contents were mixed[10] during this addition, and the mixing was continued for another 10 sec. The tube was capped, and the contents were mixed[10] for 30 sec, followed by centrifugation for 5 min at 1500 rpm.

The methanol extract was quantitatively transferred to a second tube containing 1 ml of water, 0.2 ml of 8 *N* hydrochloric acid, and 5 ml of chloroform. The sample was mixed[10] for 1 min and centrifuged for 5 min. The lower phase (containing the chloroform extract) was quantitatively transferred to another tube containing exactly 8.5 ml of 1 *N* sodium hydroxide. The sample was again mixed for 1 min[10] and centrifuged for 5 min. Exactly 8 ml of the clear aqueous phase was transferred to a 40-ml glass-stoppered centrifuge tube, and the sample was reextracted with a second 8.5-ml aliquot of 1 *N* sodium hydroxide and centrifuged. The clear aqueous phase was transferred quantitatively to the second tube containing the first alkaline extract, and the combined extracts were mixed thoroughly.

The combined alkaline extract was heated in a boiling water bath for 15 min and then cooled to room temperature. Approximately 5 ml of hexane was added, and the sample was mixed for 30 sec[10] and centrifuged. The hexane phase was separated and discarded, and the sample was then reheated in boiling water for another 5 min to remove residual hexane.

After cooling to room temperature and acidification with 3 ml of 8 *N* hydrochloric acid, exactly 5 ml of ceric reagent was added and the contents were mixed briefly and allowed to stand for 30 min in the dark. Approximately 3 ml of 10 *N* sodium hydroxide was added, and the sample was mixed. The aqueous phase, containing the product of oxidation, was then extracted with 2.5 ml of hexane by mixing for 45 sec[10]. After centrifugation, the hexane phase was transferred to a semimicro UV cell. The absorbance was measured at 252 nm with hexane in the reference cell and then compared with the measurements obtained from the serum standard curve. Each standard curve was drawn from linear regression analysis of the individual data points.

Recovery Studies—Although II is a minor metabolite, its recovery from serum must approximate that of I so that the final extract is representative of the original serum sample.

Small aliquots of 1-^{14}C-I and/or 1-^{14}C-II were added to serum, and these samples were then carried through the assay procedure. Recoveries of carbon-14 were determined after each step, and the cumulative percent recovery was determined from these values. To minimize any effect due to volume changes upon sampling, no more than 10% of any extract was sampled for ^{14}C-analysis.

The radioactivity of all samples was determined in a liquid scintillation counter[11] using a modified Bray's solution cocktail (5). All counts were then corrected for background and quench by the external standard channels ratio method and converted to disintegrations per minute. The counting efficiency was 68%.

Extent of Oxidation—The oxidation of II, the final product obtained in the assay procedure just prior to oxidation, was studied by determining the disappearance of chloroform-extractable radioactivity from the acid medium of the ceric reaction mixture. Since the ^{14}C-label was on the carboxyl function of both I and II, its disappearance as ^{14}C-labeled carbon dioxide after oxidation served as an index of the extent of oxidation.

Identification of Oxidation Product—A serum sample containing added fenclorac and a serum sample from a subject who had received

[1] Model DB or DU, Beckman Instruments, Irvine, Calif.
[2] Cary 14, Cary Instruments, Monrovia, Calif.
[3] Precision Glass Products Co., Oreland, Pa.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Mallinckrodt, St. Louis, Mo.
[7] Burdick and Jackson Laboratories, Muskegon, Mich.
[8] Fox Chemical Co., Tucson, Ariz.
[9] Department of Medicinal Chemistry, William H. Rorer, Fort Washington, Pa.

[10] Vortex test tube mixer, Scientific Industries, Springfield, Mass.
[11] Model LS-350, Beckman Instruments, Irvine, Calif.

Table I—Recovery of I and II in the Extraction Procedure

Assay Step	Recovery, %		
	I[a]	II[b]	I[c] and II[c]
Methanol extraction	89.6	81.5	91.0
Chloroform extraction	100	100	100
Alkaline extraction	76.9	88.1	83.0
Cumulative	68.9	71.8	75.5

[a] Original serum sample contained 19.6 μg/ml. [b] Original serum sample contained 23.8 μg/ml. [c] Original serum sample contained 9.8 μg of I and 11.9 μg of II/ml.

Table II—Extent of Oxidation of II to III

Component in Original Serum Sample	Percent of Sample Unoxidized	Percent Oxidized
I[a]	2.4	97.6
II[b]	0.2	99.8
I and II[c]	1.4	98.6

[a] Serum sample contained 19.6 μg of I/ml. [b] Serum sample contained 23.8 μg of II/ml. [c] Serum sample contained 9.8 μg of I and 11.9 μg of II/ml.

fenclorac were carried through the entire procedure. The UV spectra were determined on the product of oxidation and compared with the spectrum of the authentic oxidation product, III.

Correlation with Radioactivity Data—Serum samples were obtained from eight normal adult volunteers following oral administration of 200 mg of 1-^{14}C-I (specific activity of 0.317 μCi/mg). One hundred and twenty samples, representing various time intervals after the administration of the radioactive compound to the eight subjects, were assayed (15 samples/subject).

The levels of I found were compared to those levels obtained from liquid scintillation counting. Since GLC–mass spectrometry studies (6, 7) had shown that I is the major serum component along with small amounts of II, a direct correlation was made between levels obtained by this spectrophotometric method and the ^{14}C-levels.

Reproducibility—Within-day reproducibility was determined by assaying, on the same day, 10 identical serum samples containing exactly 20 μg of I/ml.

Day-to-day reproducibility was determined on a series of standard curves obtained over 3 weeks.

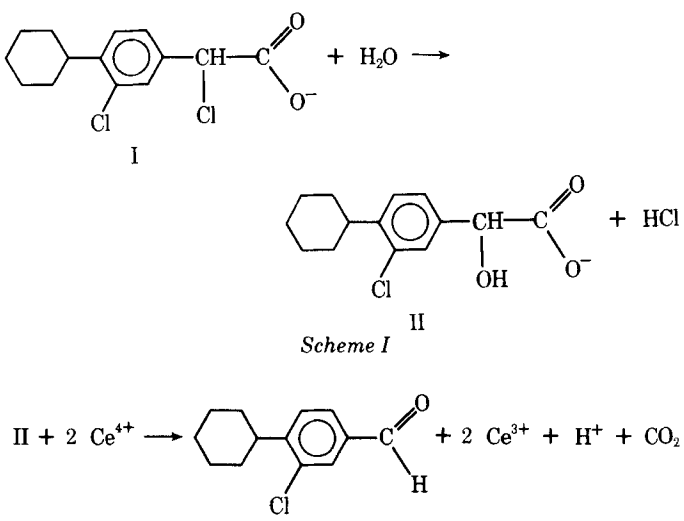
RESULTS AND DISCUSSION

The principal reactions in this assay are (*a*) the conversion of I to II (8, 9) (Scheme I), which occurs at room temperature but is greatly accelerated in an alkaline medium at elevated temperatures (100°); and (*b*) the oxidation of II by ceric sulfate (10) (Scheme II).

Although small amounts of the methyl esters of both I and II are formed in acidified methanol, base-catalyzed hydrolysis at elevated temperatures converted the esters to 3-chloro-4-cyclohexylbenzeneglycolate and eliminated any interferences (11).

Recovery Studies—The cumulative percent recovery of I and/or II from serum was equivalent up to the step immediately prior to oxidation. Therefore, if a serum sample contained I and/or II, the final extract was representative of the composition of the original serum sample. This condition is necessary when two compounds are present in the same sample and when both must be assayed simultaneously. Table I summarizes the complete recovery study.

Extent of Oxidation—Less than 2.5% of radioactivity was recovered into chloroform from the ceric reaction mixture, indicating that the oxidation was essentially quantitative after 30 min (Table II).



Scheme I

Scheme II

Identification of Oxidation Product—The product of ceric oxidation of II or extracts of serum samples from subjects who received I had the same UV spectral characteristics as the predicted product of oxidation, III (Fig. 1).

Correlation of Spectrophotometric and ^{14}C-Analyses—Linear regression analysis of serum concentrations of I determined by spectrophotometric (C_{sp}) or ^{14}C (C_{RI}) procedures indicated a 90% correlation between the two methods ($r^2 = 0.965$; $C_{sp} = 0.90C_{RI} - 0.46$) over a range of 1.4–25.5 μg of I/ml. There was no significant serum blank, and slight hemolysis in serum did not interfere.

GLC–mass spectrometric studies indicated that I was the major species present in serum after oral administration of fenclorac. Only small amounts (<10%) of II were present (6, 7). Also, due to the small difference in molecular weight between I and II (~7%), this assay gives a direct measure of the unchanged parent compound.

Figure 2 is a graphical comparison of the average values obtained on the serum samples of eight subjects by the spectrophotometric method with the ^{14}C-values obtained by liquid scintillation.

Reproducibility—The standard curves used for the radioisotope

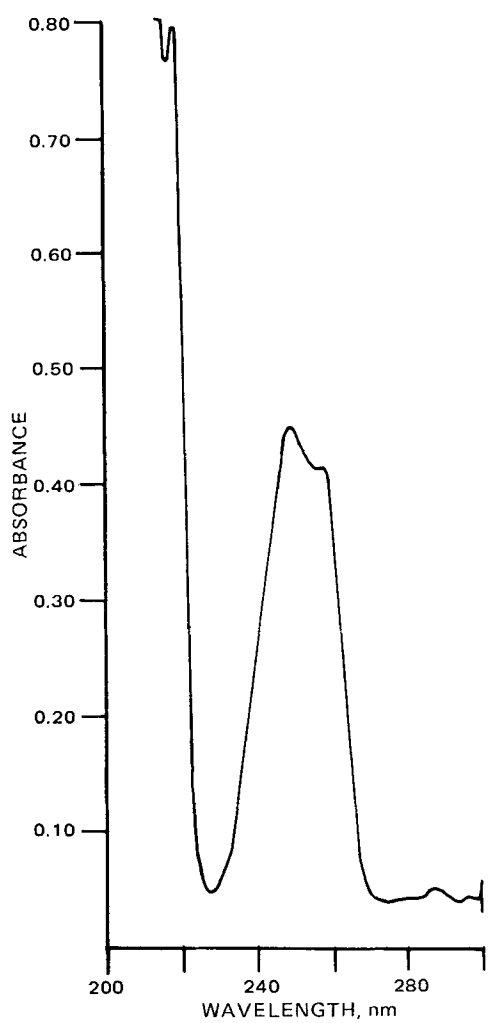


Figure 1—*UV spectrum of III in hexane.*

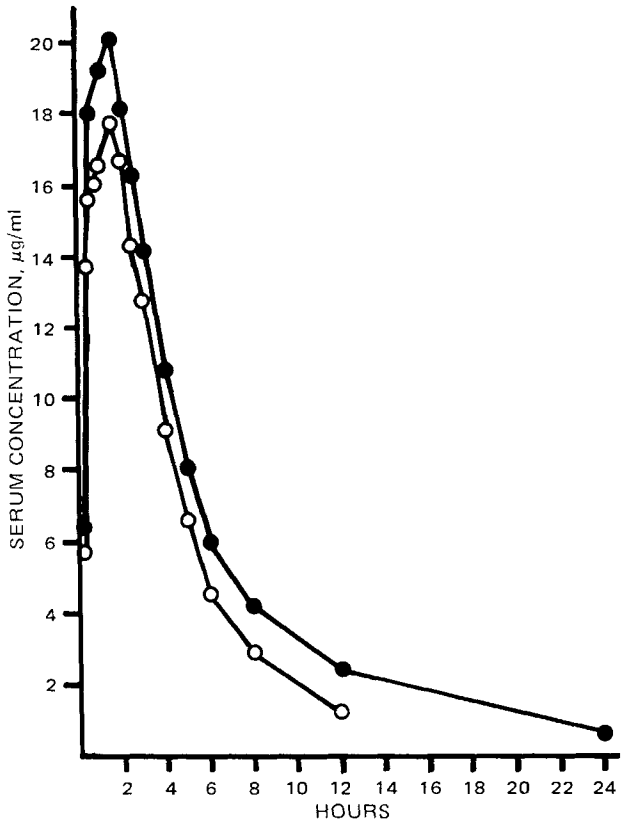


Figure 2—*Serum I concentration after oral administration of 200 mg of I in an aqueous medium. Key (mean values of eight subjects): ●, ^{14}C-assay; and ○, UV spectrophotometric assay.*

study indicated good day-to-day reproducibility on samples assayed over 3 weeks.

The composite linear regression standard curve had a y-intercept of 0.024 absorbance unit (blank), a slope of 0.018 absorbance unit/μg of I/ml, and $r^2 = 0.995$ ($y = 0.018x + 0.024$), where x is micrograms of I per milliliter of serum. The coefficient of variation was 5% with serum samples containing 2, 5, 10, 20, and 30 μg of I/ml on 12–14 determinations of each concentration over 3 weeks.

Replicate determinations of I in 10 identical serum standards, each containing 20 μg of I/ml, on the same day resulted in a coefficient of variation of 2.8% and a mean of 0.321 ± 0.009 absorbance unit at 252 nm.

Sensitivity—As mentioned previously, linear regression analysis of absorbance *versus* standard concentration data yielded a slope of 0.018 absorbance unit for 1 μg of I/ml of serum. When using 1.5 ml of serum per test, the minimum detectable quantity of I was approximately 1 μg/ml.

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Quantitation of the Antimalarial Agent, Mefloquine, in Blood, Plasma, and Urine Using High-Pressure Liquid Chromatography

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Abstract □ Sensitive and specific assays are described for the quantitation of mefloquine in whole blood, plasma, and urine specimens using high-pressure liquid chromatography. Specimens were extracted with ethyl acetate and concentrated before chromatography. Whole blood and plasma extracts were chromatographed on a polar bonded phase partitioning column, and urine extracts were chromatographed on a bonded reversed-phase partitioning column. The sensitivity of the assays for mefloquine was 0.05 μg/ml of whole blood or plasma and 0.25 μg/ml of urine using 5-ml samples. The assays are suitable for studying mefloquine pharmacokinetics in humans.

Keyphrases □ Mefloquine—high-pressure liquid chromatographic analysis, whole blood, plasma, and urine □ High-pressure liquid chromatography—analysis, mefloquine in whole blood, plasma, and urine □ Antimalarial agents—mefloquine, high-pressure liquid chromatographic analysis in whole blood, plasma, and urine

Mefloquine hydrochloride[1] (I) is a radical curative agent for the treatment of drug-resistant falciparum malaria (1, 2). Mefloquine also provided suppressive prophylaxis against mosquito-induced infections with *Plasmodium vivax* and *P. falciparum* in human volunteers (3, 4). The disposition of mefloquine in rats was studied (5), but its fate in humans has not been determined due to the lack of

[1] DL-*erythro*-α-2-Piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride (WR 142,490 hydrochloride), Cordova Chemical, Sacramento, Calif.