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DETERMINATION OF DICLOFENSINE, AN ANTIDEPRESSANT AGENT, AND ITS MAJOR METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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SUMMARY

A sensitive and selective high-performance liquid chromatographic assay was developed for the determination of diclofensine (I) and its key metabolites in human plasma. The assay involves deproteinization of plasma, overnight glusulase incubation to hydrolyze the major metabolite (I-B-glucuronide), extraction of the parent compound and its deconjugated metabolites (I-A, I-B and I-C) from the alkalinized aqueous phase into diethyl ether—ethanol (95:5), the residue of which (containing compounds I, I-A, I-B and I-C) is alkylated with 2-iodopropane dissolved in acetone, using solid potassium hydroxide as a catalyst. The compounds are extracted from the reaction mixture into diethyl ether, after adding ethanol—water—acetic acid (55:40:5), the residue of which is dissolved in 0.05 *M* sulfuric acid, and reacted with mercuric acetate at 100°C, which oxidizes tertiary tetrahydroisoquinolines to their 3,4-dihydroisoquinoline derivatives, followed by a photochemical reaction in the same solution to form intensely fluorescent isoquinolinium derivatives.

An aliquot of this reaction mixture is injected onto a reversed-phase high-performance liquid chromatography column (5-μm Nova-Pac C_{18} phase in a radial compression cartridge, 10 cm × 8 mm), using the mobile phase 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25). The void volume (V_0) is approximately 1.4 min and the retention times (t_R) of the respective isoquinolium derivatives of diclofensine (I) are ca. 3.5 min, internal standard (II) ca. 4.2 min, nordiclofensine (I-A) ca. 5 min, while the phenolic metabolites I-B and I-C give peaks at 6.4 min and 10.4 min, respectively. The derivatives are detected by fluorescence.

The method was used to determine plasma concentrations of the parent drug-(I) and its major phenolic metabolite I-B (aglycone) in plasma in two normal volunteers following a single oral 45-mg dose and following seven consecutive days of oral dosing of 45 mg three times a day as part of a multiple ascending dose tolerance study.

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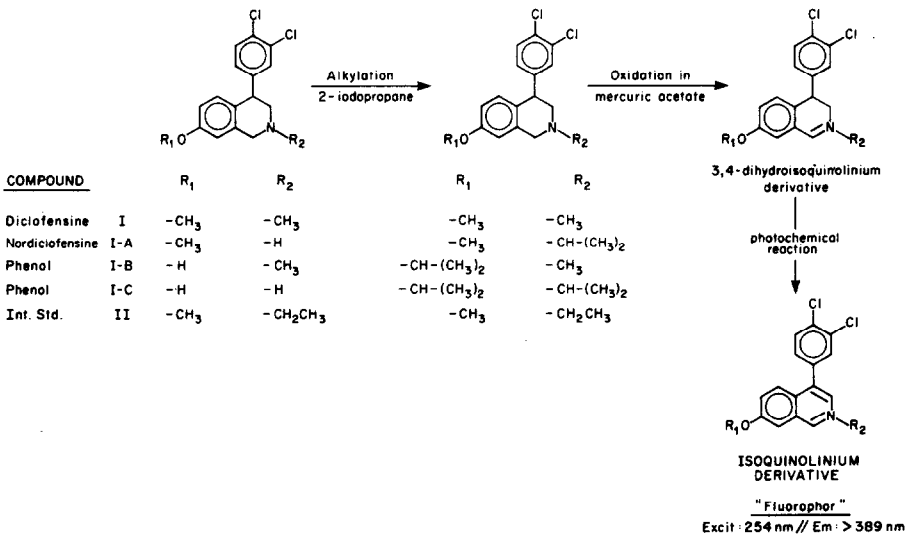
INTRODUCTION

Diclofensine, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-methylisoquinoline (I, Fig. 1), is an antidepressant drug with an unusual profile of pharmacologic activity and a novel chemical structure [1, 2]. It differs chemically from tricyclic antidepressants, such as imipramine, and tetracyclic antidepressants, such as mianserin. Nomifensine (Merital®) is the only antidepressant currently available [3, 4] which contains the basic isoquinoline moiety of diclofensine for which a high-performance liquid chromatographic (HPLC) method using UV detection was recently published [5]. However, diclofensine differs from nomifensine in its central and peripheral nervous system pharmacological properties [6].



COMPOUND		R_1	R_2	R_1	R_2
Diclofensine	I	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
Nordiclofensine	I-A	$-CH_3$	$-H$	$-CH_3$	$-CH-(CH_3)_2$
Phenol	I-B	$-H$	$-CH_3$	$-CH-(CH_3)_2$	$-CH_3$
Phenol	I-C	$-H$	$-H$	$-CH-(CH_3)_2$	$-CH-(CH_3)_2$
Int. Std.	II	$-CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_2CH_3$

Fig. 1. Chemical structures and reactions of the compounds referred to in the text.

Preliminary studies on the pharmacokinetics and pharmacodynamics of diclofensine in man following 25- and 50-mg oral doses [7] showed that the compound was rapidly absorbed but also extensively metabolized by N- and O-demethylation to nordiclofensine (I-A) and the phenolic metabolites O-desmethyldiclofensine (I-B) and O-desmethylnordiclofensine (I-C), respectively (Fig. 1). The half-life ($t_{1/2}$ β) of the parent drug (I) was of the order of 14.9 h (range 8.2—20.2 h). Oral absorption was complete based on the recovery of the major metabolite in urine, viz. O-desmethyldiclofensine (I-B) [7—9].

Plasma concentrations of diclofensine and nordiclofensine are in the low nanogram concentration range as determined by gas chromatography—mass spectrometry (GC—MS) [10] and in metabolic studies in man using ^{14}C-labeled drug [11]. The major metabolite (I-B) accounted for over 30% of the dose in urine as a glucuronide conjugate, therefore it was expected that this metabolite could also be determined in plasma after enzymatic deconjugation.

A sensitive and selective HPLC assay was developed for the determination of diclofensine (I) and its key metabolites in human plasma (Fig. 1). The assay involves deproteinization of plasma, overnight glusulase incubation to

hydrolyze the major metabolite, I-B-glucuronide, and extraction of the parent compound and its metabolites I-A, I-B and I-C. Following evaporation of the organic phase, compounds I-A, I-B and I-C in the residue are alkylated with 2-iodopropane dissolved in acetone. The compounds are extracted from the reaction mixture into diethyl ether after adding ethanol—water—acetic acid (55:40:5), which is then evaporated to dryness. The residue is dissolved in 0.05 *M* sulfuric acid and reacted with mercuric acetate at 100°C to oxidize the tertiary tetrahydroisoquinolines to their 3,4-dihydroisoquinoline derivatives, followed by a photochemical reaction in the same solution to form intensely fluorescent isoquinolinium derivatives [12] (Fig. 1).

An aliquot of this reaction mixture is injected onto a reversed-phase HPLC column in a radial compression cartridge. The derivatives are detected by fluorescence. Diclofensine (I) per se is determined separately following selective extraction, as its isoquinolinium derivative (omitting the alkylation step) with pre-concentration prior to HPLC analysis.

Plasma concentrations of the parent drug (I) and its major phenolic metabolite I-B (aglycone) were determined by the separate procedures in two normal volunteers following a single oral 45-mg dose and following seven consecutive days of oral dosing of 45 mg three times a day (t.i.d.), as part of a multiple ascending dose tolerance study.

EXPERIMENTAL

Reagents

All inorganic reagents were analytical-reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered through a 0.2-μm filter (Type DS System, Hydro-Service and Supplies, Durham, NC, U.S.A.). The inorganic reagents include: 0.1 *M* and 10 *M* sodium hydroxide, solid potassium hydroxide pellets, 0.05 *M* sulfuric acid, and mercuric acetate reagent [dissolve 32.8 g of sodium acetate, anhydrous (0.4 mol), 1.5 g of mercuric acetate and 3 ml of glacial acetic acid in 100 ml of distilled water (final pH ca. 6); this solution should be made fresh weekly]. Molar (pH 5.4) phosphate buffer was prepared by mixing 1.0 *M* potassium dihydrogen phosphate (136.1 g/l) and 1.0 *M* dipotassium hydrogen phosphate (174.2 g/l), then titrating the former solution to pH 5.4 with the latter.

The following organic solvents and reagents were also used: ethanol (200 proof, Pharmco, Publicker Industries, Philadelphia, PA, U.S.A.), diethyl ether [anhydrous, analytical reagent (ACS), Mallinckrodt, Paris, KY, U.S.A. washed with 0.05 *M* sulfuric acid immediately before use], acetone (ACS, Fisher, Fair Lawn, NJ, U.S.A.), 2-iodopropane, 97% (Aldrich, Milwaukee, WI, U.S.A.), glacial acetic acid (ACS), Glusulase® (an enzyme preparation containing 100 000 U of glucuronidase and 50 000 U of sulfatase per ml, available from Endo Labs., Garden City, NY, U.S.A.), and 0.25 *M* (pH 2.5) triethylammonium phosphate (TEAP) (Regis Chemical, Morton Grove, IL, U.S.A.).

Analytical standards

The following compounds, all of pharmaceutical-grade purity (>99%), were used as the analytical standards: compound I · HCl, diclofensine, (±)-4-(3,4-

TABLE I

PREPARATION OF WORKING SOLUTIONS 1—10

Working solution No.	Aliquots (μl) of standard solution									Final concentration (ng/ml of solution)				
	A-2	A-3	B-2	B-3	C-2	C-3	D-2	D-3	E-2	I	I-A	I-B	I-C	II
1	1000	—	500	—	200	—	2000	—	200	1000	500	200	2000	200
2	500	—	1000	—	—	—	200	—	200	500	1000	—	200	200
3	200	—	200	—	1000	—	—	—	200	200	200	1000	—	200
4	100	—	—	—	—	—	1000	—	200	100	—	—	1000	200
5	—	—	—	500	500	—	100	—	200	—	50	500	100	200
6	—	500	100	—	100	—	500	—	200	50	100	100	500	200
7	—	200	—	100	—	500	—	500	200	20	10	50	50	200
8	—	100	—	200	—	100	—	200	200	10	20	10	20	200
9	—	50	—	50	—	200	—	100	200	5	5	20	10	200
10	—	—	—	—	—	—	—	—	200	—	—	—	—	200

dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-methylisoquinoline · hydrochloride, $C_{17}H_{17}ONCl_2$ · HCl, molecular weight (MW) = 358.70, m.p. = 273—275°C; compound I-A · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxyisoquinoline · hydrochloride, MW = 344.68, m.p. = 240—242°C; compound I-B · HBr, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-2-methyl-7-isoquinolinol · hydrobromide, MW = 389.14, m.p. = 284—285°C; compound I-C · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-isoquinolinol · hydrochloride, MW = 330.66, m.p. = 273—275°C; and compound II · HCl, (±)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-ethylisoquinoline · hydrochloride (internal standard), MW = 372.72, m.p. = 247—249°C.

Stock solutions of I · HCl, I-A · HCl, I-B · HBr, I-C · HCl and II · HCl were prepared as follows (amberized 10.0-ml volumetric flasks were used for all standard solutions).

Solution A-1. Dissolve 1.11 mg of I · HCl (equivalent to 1.0 mg of I, free base) in 10.0 ml of absolute ethanol in a 10-ml amberized volumetric flask to yield 111 μg of I · HCl per ml.

Solution B-1. Dissolve 1.12 mg of I-A · HCl (equivalent to 1.0 mg of I-A, free base) in 10.0 ml of absolute ethanol.

Solution C-1. Dissolve 1.26 mg of I-B · HBr (equivalent to 1.0 mg of I-B, free base) in 10.0 ml of absolute ethanol.

Solution D-1. Dissolve 1.12 mg of I-C · HCl (equivalent to 1.0 mg of I-C, free base) in 10.0 ml of ethanol.

Prepare separate 1:10 serial dilutions of each stock solution to yield standard solutions A-2, A-3, B-2, B-3, C-2, C-3 and D-2, D-3 containing 10 μg/ml or 1.0 μg/ml, respectively.

Solution E-1. Dissolve 1.11 mg of II · HCl (equivalent to 1.0 mg of II, free base) in 10.0 ml of ethanol (internal standard for HPLC assay).

Solution E-2. Dilute 1.0 ml of solution E-1 to 10.0 ml in ethanol to yield a 10 μg/ml solution.

Working standard solutions 1—10 were prepared by diluting various aliquots of the standard solutions above to 10.0 ml in ethanol as given in Table I. These working solutions (1—10) were used for the determination of total (free and bound) compounds in plasma (procedure A).

Analysis of parent drug (I) per se

A separate set of working solutions a—g were prepared by diluting various aliquots of the standard solutions of I and II to 10 ml of ethanol as given in Table II. These working solutions (a—g) were used for the determination of free (unconjugated) I in plasma (procedure B).

The primary stock solutions and mixed standard solutions are stable for two months when stored at 5°C.

Equipment and instrument parameters

The photolytic reaction was conducted with a PyroLux R-57 lamp (Luxor, NY, U.S.A.). A Model PR-J refrigerated centrifuge with a No. 253 rotor (Damon/IEC Division, Needham, MA, U.S.A.) was used for all centrifugations.

Column. The column used for reversed-phase HPLC was a pre-packed, 10 cm

TABLE II

PREPARATION OF WORKING SOLUTIONS a–g

Working solution	Aliquots (μl) of standard solution			Final concentration (ng per 20 μl of solution)	
	A-2	A3	E2	I	II
a	1000	—	200	20	4
b	500	—	200	10	4
c	200	—	200	4	4
d	100	—	200	2	4
e	—	500	200	1	4
f	—	200	200	0.4	4
g	—	—	200	—	4

× 8 mm, Radial-Pak cartridge containing Nova-Pak® C_{18} (a C_{18} phase bonded to 5-μm spherical silica particles) (Waters Assoc., Milford, MA, U.S.A.).

Instrument. The HPLC system consisted of a Model 6000A reciprocating piston pump (Waters Assoc.), a Waters Intelligent Sample Processor (WISP™) Model 710B, a Waters Z-module™ radial compression system (to compress and hold the Radial-Pak cartridge) and a Schoeffel Model FS-970 LC fluorometer operated at 254 nm for excitation and emission at wavelengths greater than 389 nm (Kratos Analytical Instruments, Westwood, NJ, U.S.A.). The fluorescence detector range was set at 0.2 μA full scale and the photomultiplier sensitivity was 580. The chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP auto-injector was programmed for a 15-min run time per sample using methanol as the rinse solvent.

The isocratic mobile phase consisted of 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25), operated at a constant flow-rate of 1.8 ml/min which resulted in a pressure of ca. 3 MPa (ca. 500 p.s.i.).

The void volume, V_0, was ca. 1.4 min, and the retention times (t_R) of the respective isoquinolinium derivatives of diclofensine (I) were ca. 3.5 min, internal standard (II) ca. 4.2 min, nordiclofensine (I-A) ca. 5 min, while the phenolic metabolites I-B and I-C gave peaks at 6.4 min and 10.4 min, respectively (Fig. 2).

Procedure A: analysis for total (free and bound) I and metabolites in plasma

Extraction. For each unknown, a 300-μl aliquot of working solution 10 (equivalent to 60 ng of II, the internal standard) was pipetted into a separate 100 × 13 mm disposable borosilicate culture tube. The organic solvent was then evaporated at 20—30°C in an N-EVAP. A 0.1-ml aliquot of unknown plasma was then added to each tube.

A calibration curve of ten standards was processed with each set of unknowns. Aliquots (300 μl) of solutions 1—9 were evaporated separately in 100 × 13 mm culture tubes and 0.1 ml of control plasma was added to each. The tenth standard is a 0.1-ml sample of control plasma without any compound added.

To each culture tube containing 0.1 ml of plasma were added 2 ml of 1 *M*

phosphate buffer pH 5.4. The contents were mixed, then heated at 90°C for 3—5 min (with mixing, every 30 sec) to denature and precipitate the proteins. The samples were centrifuged at 1100 *g* (2100 rpm) for 10 min at 10—15°C. The clear supernate was transferred to a 50-ml round-bottom glass-stoppered centrifuge tube. The precipitate was re-eluted, as above, with 1 ml of 1 *M*

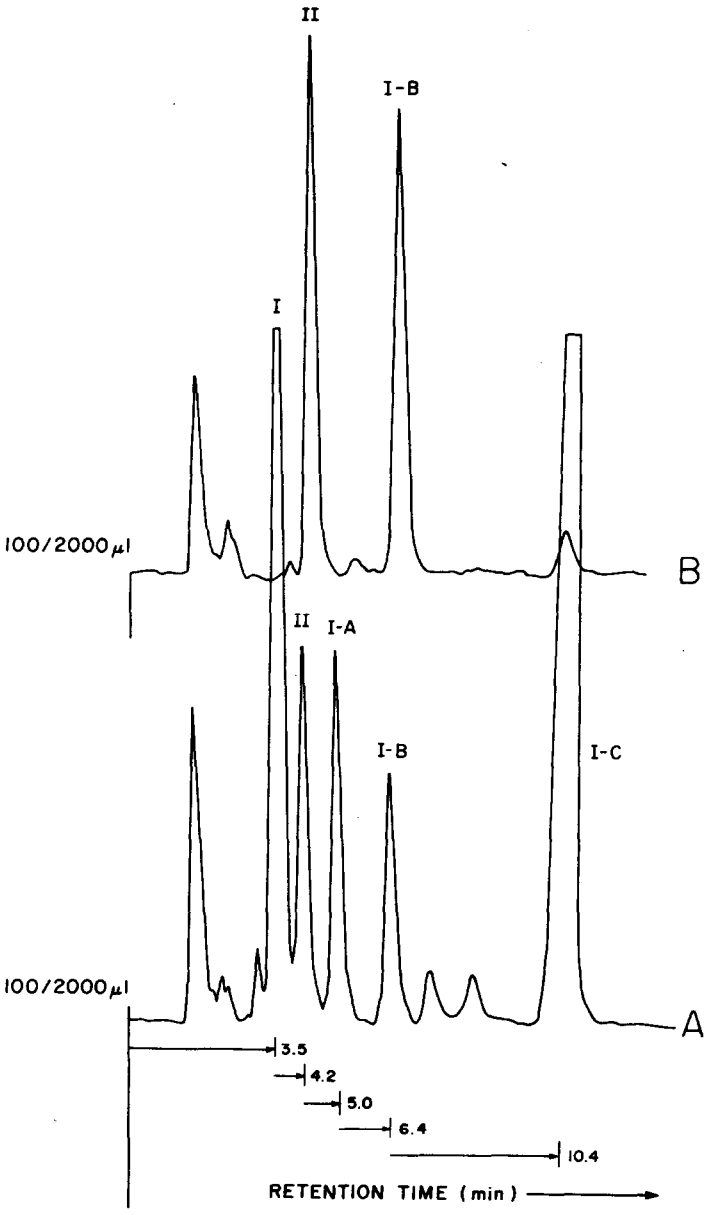


Fig. 2. Chromatograms showing (A) the analysis of compounds I, I-A, I-B, I-C and the internal standard (II) recovered from plasma as their respective alkylated and/or isoquinolinium derivatives; (B) the analysis of I-B (aglycone), the major metabolite in human plasma following the oral administration of a 45-mg dose of I · HCl.

phosphate buffer, pH 5.4, centrifuged, and the supernates were combined. To each sample, 150 μl of Glusulase were added using a 1-ml glass hypodermic syringe fitted with a stainless-steel needle. The samples were then placed in a rack, loosely stoppered with cotton and incubated overnight (about 16 h) at 37°C in a mechanical incubation shaker (Dubnoff, Precision Scientific, Chicago, IL, U.S.A.).

Following incubation, the samples were removed from the water bath, adjusted to alkaline pH by the addition of 0.4 ml of 10 *M* sodium hydroxide, mixed well, and allowed to equilibrate to room temperature. The samples were then extracted twice with 5-ml portions of a freshly prepared mixture of diethyl ether—absolute ethanol (950:50) (sealing the tubes using PTFE stoppers) by shaking for 5 min on a reciprocating shaker, and then centrifuging for 10 min at 1100 *g* (2100 rpm) at 15—20°C. Following each centrifugation, the organic extract was transferred to a 125 × 16 mm culture tube with a PTFE-lined screw cap. If an emulsion occurred, then 0.5 ml of additional ethanol was added to each sample, mixed by manual inversion, then centrifuged again. The organic extracts were evaporated to dryness at 40°C under a gentle stream of nitrogen in an N-EVAP (Organomation Assoc., Worchester, MA, U.S.A.) water bath.

Alkylation. The residues were reconstituted in 1 ml acetone and 0.1 ml of 2-iodopropane was added to each tube. Three pellets (approx. 300 mg) of solid potassium hydroxide (washed with 2 vols. of acetone just prior to use) were added and the tubes were tightly capped. The tubes were placed in a rack in a 70—75°C bath for 30 min, then taken out and cooled to room temperature.

After alkylation, the samples were first diluted with 0.5 ml of a mixture of ethanol—water—glacial acetic acid (55:40:5) and then extracted twice with 4.5-ml portions of acid-washed diethyl ether by mixing on a vortex mixer, and centrifuging at 20°C for 5 min at 1100 *g* (2100 rpm). Following each centrifugation the clear supernate was transferred to a clean 125 × 16 mm culture tube and the organic extracts were evaporated to dryness at 40°C under a gentle stream of nitrogen in an N-EVAP water bath. The residues were vacuum dried (over Drierite) in a vacuum desiccator for 10 min to insure the removal of less volatile organic solvents which would otherwise contaminate the sample.

The samples were then processed for oxidation and photolysis as described below.

Oxidation and photolysis. To the residue in each tube 0.3 ml of 0.05 *M* sulfuric acid was added and the contents were mixed on a vortex mixer. Then 0.3 ml of the mercuric acetate reagent was added and the culture tubes were tightly capped with PTFE-lined screw caps. The culture tubes were placed in a rack in a boiling water bath (100°C) and allowed to react for 30 min to complete the oxidation to the intermediate. The samples were cooled to room temperature and then exposed directly to high-intensity light from a Pyro-Lux R-57 lamp contained in a 75 cm × 75 cm × 60 cm (approximately) wooden box lined with aluminum foil. The samples must be arranged in a single row in a suitable rack and placed 30 cm from the light source. The samples were exposed for 20 min to complete the photochemical reaction to the final fluorophore.

HPLC procedure. The tubes were cooled to room temperature and 1.4 ml of

mobile phase added and the solution was vortex-mixed. The solution was transferred to a standard Waters Assoc. 4-ml glass vial (Part No. 72711) and each vial was sealed with a cap with a self-seal septum (Part No. 73010). The vials were placed in an autoinjector (WISP 710B) which was programmed to inject 100 μl (out of a total volume of 2.0 ml) for HPLC analysis using the chromatographic parameters previously described.

Procedure B: analysis for parent drug, diclofensine (I) per se

For each unknown to be analyzed, a 20-μl aliquot of standard solution g (equivalent to 4 ng of II, the internal standard) was pipetted into a separate 100 × 13 mm disposable borosilicate culture tube (Cat. No. 14-962-10C, Fisher). These aliquots were then evaporated at 20—30°C in an N-EVAP evaporator under a stream of clean, dry nitrogen. A 1.0-ml aliquot of unknown plasma sample was then added to each culture tube containing the internal standard (II).

A calibration curve of seven recovered standards was processed with each set of unknowns. Aliquots of 20 μl of the appropriate solutions a—f were evaporated separately in 100 × 13 mm culture tubes and 1.0 ml of control plasma was added to each to yield plasma standards containing 20, 10, 4, 2, 1, 0.4 or 0 ng/ml I, each containing 4 ng/ml II (internal standard). Metabolites I-A, I-B and I-C in the unconjugated form are not quantitated in this procedure due to the extremely low concentrations present in vivo [11].

To each culture tube containing plasma were added 1.0 ml of 0.1 *M* sodium hydroxide and 4.5 ml of acid-washed diethyl ether, then the tubes were stoppered with polyethylene caps (Plugtite Cat. No. 127-0019-100, Elkay Products, 800 Boston Turnpike, Shrewsbury, MA, U.S.A.) and mixed for 10—15 sec on a vortex mixer. The caps were loosened momentarily to release the ether vapor pressure, stoppered, and shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 50—80 strokes per min for 5 min. The samples were centrifuged at 1100 *g* (2100 rpm) for 10 min at 10—15°C in a refrigerated centrifuge and the ether phase was transferred into a 125 × 16 mm borosilicate culture tube with a PTFE-lined screw cap (Corning 9826-16X). The aqueous mixture was extracted again with an additional 4.0 ml of diethyl ether. The ether extracts were combined as before and evaporated at 20—30°C in an N-EVAP evaporator under a stream of clean, dry nitrogen. (Note: the alkylation step is omitted.)

The residues are processed for chemical/photochemical oxidation as previously described to yield the final isoquinolinium derivatives, which are extracted once into 5 ml of methylene chloride. After 10 min of shaking and 5 min of centrifugation, the lower methylene chloride phase was transferred by pipet to a clean 100 × 13 mm disposable culture tube and evaporated to dryness at 25°C under a stream of nitrogen. The samples were re-dissolved in 400 μl of mobile phase (using a vortex mixer) and sonicated in an ultrasonic cleaner bath (to insure complete dissolution), then capped and centrifuged for 1—2 min (to precipitate solids). An aliquot of 300—350 μl was transferred into a Waters plastic low-volume insert (Part No. 72030) held in a standard 4-ml glass vial (Waters Part No. 72711) by a compression spring (Part No. 72708). Each vial was sealed with a cap containing self-seal septum (Part No.

73010) and the vial was tapped to remove any air bubbles trapped at the bottom of the low-volume insert. The autoinjector (WISP 710B) was programmed to inject up to 200 μl (50% of the total sample) for HPLC analysis (Fig. 3).

Calculations

The major component quantitated in procedure A was metabolite I-B whose concentrations far exceeded those of the parent drug (I) and metabolites I-A and I-C which were diluted out in order to keep the peak for I-B on scale. The parent drug (I) per se was quantitated by procedure B. The concentration of either I or I-B in the unknowns was determined by interpolation from a least-squares regression equation (weighted linear equation: $y = ax + b$) of the calibration data (processed by a Hewlett-Packard Model 3357 Laboratory Automation System) of the respective recovered standards processed along with the unknowns using peak height ratios [peak height of either compound I or I-B to peak height of internal standard (II)] versus concentration of either I (0.4—20 ng/ml) using a 1.0-ml specimen per assay or I-B (0.06—3.0 μg/ml) using a 0.1-ml specimen per assay.

RESULTS AND DISCUSSION

Sensitive and selective reversed-phase HPLC assays with fluorescence detection were developed for the determination of directly extractable diclofensine (I), I-A, I-C, and the major metabolite (I-B, aglycone) in human plasma. Compound II was chosen as the internal standard due to its structural similarity to compound I.

The major UV absorption bands of diclofensine (I), of its metabolites (I-A, I-B, I-C) and of the internal standard (II) occur at about 210—215 nm, with shoulders at 230 ± 3 nm while the minor UV absorption bands occur at 270—290 nm. The Waters Model 440 absorbance detector, used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp, permitted quantitation of all of these compounds in the μg/ml concentration range. The use of an extended-wavelength module (EWM) (Waters Assoc.) with a 229-nm deuterium lamp and wavelength kit permitted quantitation of these compounds in the approx. 100 ng/ml concentration range. These underivatized compounds did not exhibit intrinsic fluorescence in aqueous buffers or in the HPLC mobile phase used. Due to the complex biotransformation of I, chromatographic separation was essential to ensure specificity of analysis. Initially, the intact (underivatized) compounds were chromatographed on a Waters Nova-Pak (5 μm, C_{18}) Rad Pak cartridge in the Z-module with a mobile phase consisting of 0.25 *M* triethylammonium phosphate (pH 2.5)—water—methanol—acetonitrile (100:400:150:350), pumped at a constant flow-rate of 2.0 ml/min. The void volume was 1.3—1.4 min and the respective retention times were as follows: diclofensine (I), ca. 5.5 min, I-A ca. 5.3 min, I-B, ca. 3.1 min, I-C ca. 3.2 min and II (internal standard) ca. 6.4 min. The extraction recovery of I, I-A and II from plasma was shown to be quantitative after extraction into diethyl ether at alkaline pH, using the above system. Extractions from higher salt concentrations showed lower recoveries of I-A. Ethanol was necessary to prevent foaming in the deconjugated samples. UV detection was not sensitive enough for the

determination of the low concentrations expected.

Derivatization to yield a fluorescent product was examined as a means for sensitive detection. The intact compounds (Fig. 1) are substituted tetrahydroisoquinolines and were expected to undergo a previously published two-step reaction to form fluorescent derivatives [12]. The first step is an oxidative dehydrogenation reaction in aqueous mercuric acetate solution at 100°C to form moderately fluorescent 3,4-dihydroisoquinoline derivatives. The second step is a photochemical oxidation (dehydrogenation) which inserts a second double bond to form the highly fluorescent isoquinolinium derivatives.

The HPLC system used above for the intact compounds was also used for the optimization of the mercuric acetate oxidation reaction to the fluorescent intermediate. The mercuric acetate oxidation (at 100°C) yielded maximum conversion of intact I and II when conducted for 30—45 min. Nordiclofensine (I-A) did not produce significant fluorescent peaks. The retention times of the major derivatives of the initial oxidation were: I ca. 4.8 min, II ca. 5.6 min. The reaction mixture was diluted with mobile phase and injected directly. The fluorescence detector was set at λ_{ex} = 254 nm, and λ_{em} = > 389 nm (UV cut-off filter).

The HPLC system developed for the determination of the intact compounds was also used for the optimization of the photolysis reaction after chemical oxidation. The optimum photolysis time was determined to range between 15 and 25 min. The final products were identified by the disappearance of the

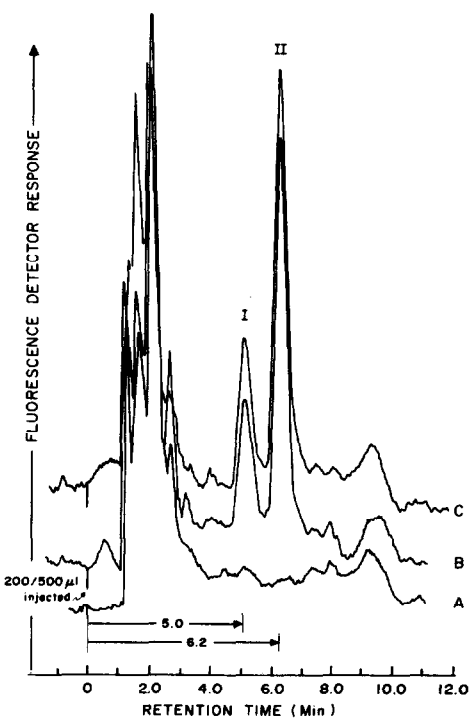


Fig. 3. Chromatograms of (A) control human plasma; (B) analysis of I and II (internal standard) recovered from human plasma; and (C) analysis of diclofensine (I) in human plasma following the oral administration of a 45-mg dose (three 15-mg capsules) of I • HCl.

3,4-dihydroisoquinolinium derivative peaks from the chemical oxidation (100°C) and the appearance of the fully aromatized isoquinolinium derivative peaks due to photolysis. The reaction products were chromatographed at 2.0 ml/min and gave retention times of ca. 5.7 min for I and ca. 7.2 min for II. The metabolites I-A, I-B and I-C, however, did not form strongly fluorescent derivatives in these initial experiments.

Alkylation of metabolites I-A, I-B, and I-C

Since the alkylated compounds I and II yielded the best results it was apparent that the O- and N-dealkylated metabolites should be alkylated to favor the overall derivatization reactions.

Initial experiments with alkylhalide (C_2H_5I) catalyzed by quaternary ammonium salts as suggested by several workers [13, 14] yielded inconsistent results although corroborating the feasibility of the reaction. Other workers [14, 15] used strongly basic inorganic salts as catalysts under anhydrous conditions to provide more vigorous reaction conditions, thus our investigation of the use of potassium hydroxide pellets in acetone heated at 70—75°C (water bath) gave encouraging yields of the alkylated products.

Several alkyl halides, including 1-iodopropane, 2-iodopropane, 1-iodobutane, 2-iodobutane and 1-iodo-2-methylpropane, were investigated as alkylation reagents. The highest conversion efficiency and optimal retention times were obtained with 2-iodopropane. The primary advantage of this reagent was that all the alkylated compounds, viz. I-A, I-B and I-C were chromatographically resolved from I and II (internal standard) after the oxidation steps. Although ethyliodide yielded better alkylation of I-A and I-B than did 2-iodopropane, I-A, however, reacted to yield II (internal standard). An internal standard suitable for use with ethyliodide as the alkylation reagent was not available.

The 2-iodopropane derivatives of I-A, I-B and I-C had retention times of 8.6, 12.3 and 11.7 min, respectively, in this HPLC system.

Extraction of the alkylated products from the reaction mixture also presented problems with respect for recovery, chromatographic cleanliness and overall yield of the respective fluorophors.

When the alkylation mixture was eluted with anhydrous solvents, small particles of potassium hydroxide and condensation products from acetone under alkaline conditions were carried over into the culture tubes used for the mercuric acetate oxidation reaction. This resulted in a final solution (after photolysis) which sometimes was yellow to tan in color, probably due to the precipitation of mercuric salts, which gave low and non-reproducible reaction efficiencies to the respective fluorophores. Addition of ethanol—acetic acid—water to the elution solvents resulted in an effective extraction procedure which yielded clean oxidation reactions with good recoveries. Washing the diethyl ether with 0.05 *M* sulfuric acid and rinsing the potassium hydroxide pellets with acetone immediately prior to use significantly improved the reproducibility of quantitation and the cleanliness of the chromatograms. The alkylation procedure is essential for the sensitive determination of the metabolites. However, the sensitivity for the determination of I was reduced to 2 ng/ml of plasma with the alkylation procedure, whereas without the alkylation the sensitivity for I was about 0.4 ng/ml, following extraction and pre-concentration of the isoquinolinium derivatives (see Table IV).

Luminescence spectral characteristics

Individual (100 μg/ml) solutions of each compound were taken through the total derivatization steps of alkylation (where applicable), chemical and photochemical reactions to yield the final alkylated isoquinolinium derivatives. The reaction mixtures were diluted to 1 μg/ml concentrations with 80% ethanol—water and scanned in this solution for their excitation/emission spectral characteristics. The corrected spectra of each compound determined at ambient temperature show very similar characteristics (Fig. 4) with the major excitation maximum at 250—254 nm and a minor one at 365 nm, whereas the emission spectrum is broad with a maximum at 425 nm. Alkylation is essential, not only to enhance the efficiency of fluorophor formation of the isoquinolinium derivative but also to yield products with similar spectral characteristics, i.e. excitation/emission maxima and fluorescence quantum yield. The non-alkylated derivatives of I-A, I-B and I-C not only gave lower fluorescence quantum yields but also exhibited widely different emission maxima, the derivatives of the phenolic compounds I-B and I-C having emission maxima at 580 nm instead of 425 nm for their respective alkylated products.

The two-step nature of the oxidation reaction was demonstrated by reacting I and II at 100°C in the mercuric acetate oxidation reaction per se, diluting the reaction mixture as described above, and obtaining corrected fluorescence spectra. In this case, the non-photolyzed oxidation products of I and II (3,4-dihydroisoquinolinium derivatives) each exhibited major excitation maxima at 250 and 280 nm with a minor peak at 365 nm. The fluorescence emission

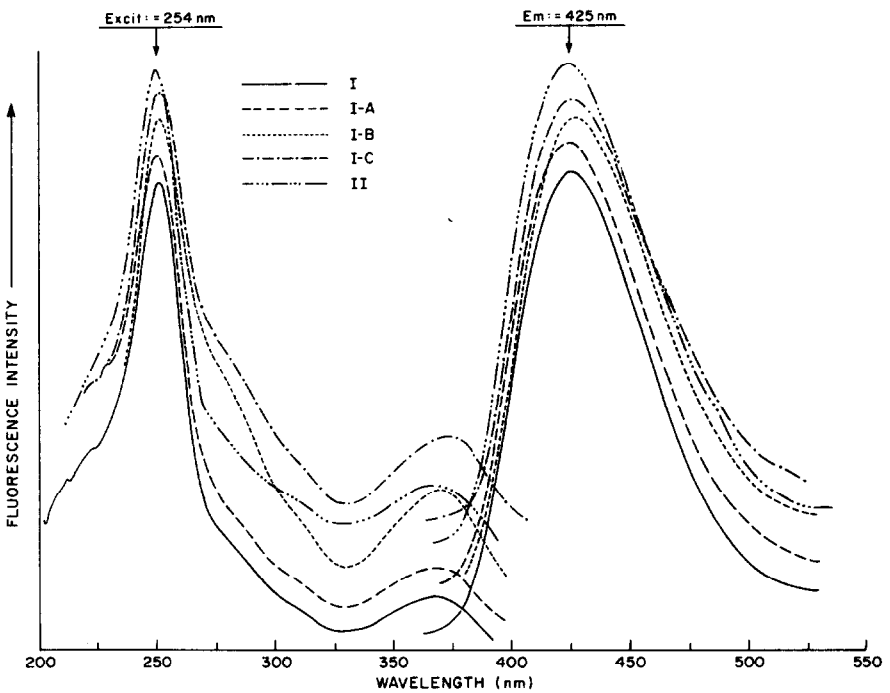


Fig. 4. Corrected ambient temperature excitation/emission spectra of the isoquinolinium derivatives of the respective alkylated compounds I, I-A, I-B, I-C and II (internal standard) in ethanol—0.05 *M* sulfuric acid.

maxima were very broad, with a peak at 475 nm. The fluorescence intensity of these derivatives were one third to one tenth that of the completely derivatized compounds. The alkylated isoquinolinium derivatives exhibited a major excitation maximum at 250—254 nm, disappearance of the 280-nm peak, retention of the minor peak at 365 nm, while the fluorescence emission spectrum showed a hypsochromic (blue) shift from 475 to 425 nm owing to the significant increase in the aromaticity of the compound.

Chromatographic behavior of the alkylated isoquinolinium derivatives

The fully derivatized products of I-A, I-B and I-C exhibited broad, tailing peaks when chromatographed in the HPLC mobile phase developed for the intact, non-derivatized parent compounds. Modifications to this mobile phase resulted in a mixed phase composed of 0.25 *M* triethylammonium phosphate (pH 2.5)—0.25 *M* acetic acid—methanol—acetonitrile—tetrahydrofuran (150:350:125:375:25) pumped at a flow-rate of 1.8 ml/min which improved the peak symmetry, shortened the analysis time, and resulted in the overall resolution of the derivatives (Fig. 2). The volume of tetrahydrofuran may be adjusted to ca. 15 ml to optimize resolution/analysis time.

The HPLC system is flushed initially with methanol, followed by methanol—water (50:50), to remove deposits from the column accumulated from previous use which can have adverse effects on the chromatography of the derivatives. The analytical mobile phase is allowed to recycle through the system for at least 2 h at a flow-rate of 1.8 ml/min to equilibrate the system. Non-equilibration will result in changes in the capacity factor (k') during chromatography.

Studies on the feasibility of the assay

Initial studies for the determination of the parent drug (I) per se without the use of either the alkylation step or the extraction of the isoquinolinium derivative yielded sensitivity and linearity over the concentration range 0.50—20.0 ng/ml of plasma. Although the precision at 0.50 ng/ml was poor (20%), the precision over the concentration range 1.0—20.0 ng/ml was significantly better than 10%. Plasma concentrations of I in man following a 45-mg oral dose (three 15-mg capsules) indicated very low concentrations, in the range 0.6—0.9 ng/ml over the 0.5—2.0 h post-dosing period. Extraction of the isoquinolinium derivatives of I and II (internal standard) from the reaction mixture into methylene chloride and their pre-concentration prior to HPLC analysis enabled their quantitation down to 0.4 ng/ml (400 pg/ml) with acceptable precision and reproducibility (Table IV). Analysis of I and I-A (nordiclofensine) using the alkylation step and without the extraction of the derivatives reduced the overall sensitivity of the assay for both compounds to 2.0 ng/ml with an overall inter-assay coefficient of variation of 14% for I and 16% for I-A. Based on plasma concentrations reported for I using a GC—MS assay with selected-ion monitoring [10, 11], it appeared very probable that I could also be quantitated using the HPLC assay.

Attempts at quantitating the directly extractable metabolite fraction using Procedure A but with alkylation, extraction of the isoquinolinium derivatives and pre-concentration prior to HPLC analysis was unsuccessful due to the carry-over of impurities from the alkylation step which interfered significantly with the chromatographic resolution of these compounds, hence was

abandoned. Thus the parent drug (I) and its major metabolite (I-B) were the two compounds quantitated in the clinical study reported herein.

Procedure A

Statistical validation of the assay. The quantitation of the major metabolite (I-B) was validated over the concentration range 0.06—3.0 μg/ml using weighted ($1/y$) linear regression analysis of the calibration data. Typical calibration curves over the above concentration range were linear ($y = 1.583x + 0.0035$). The correlation coefficient (r) was 0.9969 and the average deviation from the line was 9.6%.

Inter-assay validation data over the linear concentration range of I-B yielded a mean coefficient of variation of 18% (Table III). The poor precision is probably due to the absence of a suitable internal standard to monitor the variation incurred in the alkylation step.

TABLE III

STATISTICAL VALIDATION OF PROCEDURE A (INTER-ASSAY VARIABILITY) OF THE HPLC ASSAY FOR I-B (AGLYCONE)

Number of replicates	Concentration added (μg/ml)	Concentration found (± S.D.) (μg/ml)	Coefficient of variation (%)
5	3.00	2.77 ± 0.40	14.4
7	1.50	1.54 ± 0.18	11.9
4	0.60	0.68 ± 0.10	14.5
4	0.30	0.38 ± 0.07	19.3
3	0.15	0.13 ± 0.03	19.1
3	0.06	0.05 ± 0.01	26.8
Mean			17.7

Percentage recovery and sensitivity limits. The overall recovery of I-B from plasma was 86 ± 18% over the concentration range 0.06—3.0 μg/ml.

The sensitivity limit of the assay for the deconjugated compounds was approx. 30—60 ng/ml for diclofensine (I), 300 ng/ml for nordiclofensine (I-A) and I-C and 60 ng/ml for I-B (major metabolite) using 0.1 ml of plasma and 60 ng of II added as internal standard.

Procedure B

Statistical validation of the assay. The quantitation of diclofensine (I) was validated over the concentration rang 0.4—20 ng/ml of plasma using weighted ($1/y$) linear regression analysis of the calibration data. Typical calibration curves were linear over the above concentration range ($y = 0.1813x + 0.0145$). The correlation coefficient (r) was 0.9961 and the average deviation from the line was 9.6%. Intra- and inter-assay validation data over the linear concentration range of I yielded mean coefficients of variation of 6.2% and 9.1%, respectively (Table IV).

Percentage recovery and sensitivity limits. The overall recovery of I from plasma was 80 ± 13%. The sensitivity limit of the assay for I was 0.4 ng/ml,

TABLE IV

STATISTICAL VALIDATION OF PROCEDURE B OF THE HPLC ASSAY FOR FREE I (DICLOFENSINE) ONLY (NO ALKYLATION)

Number of samples	Concentration added (ng/ml)	Concentration found (± S.D.) (ng/ml)	Coefficient of variation (%)
A. Intra-assay variability			
4	20.0	21.05 ± 0.64	3.0
4	10.0	9.46 ± 0.75	8.0
4	4.0	3.92 ± 0.09	2.2
4	2.0	1.89 ± 0.06	3.5
4	1.0	0.98 ± 0.05	5.3
4	0.4	0.51 ± 0.08	15.1
Mean			6.20
B. Inter-assay variability			
8	20.0	21.02 ± 1.47	7.0
10	10.0	9.95 ± 1.22	12.3
11	4.0	3.65 ± 0.26	7.1
12	2.0	1.97 ± 0.24	12.4
8	1.0	0.99 ± 0.04	4.1
7	0.4	0.53 ± 0.06	11.6
Mean			9.1

following extraction and pre-concentration of the isoquinolinium derivative of I.

Application of the method to biological specimens

Plasma concentrations of diclofensine (I) and its major phenolic metabolite (I-B) were determined in two normal volunteers following the administration of a 45-mg (three 15-mg capsules) oral dose t.i.d. at 0 h, 8 h and 16 h (total daily dose = 135 mg) over seven consecutive days, with a final 45-mg dose at 0 h on day 8. The wash-out of the drug was monitored through days 8, 9 and 10. Blood samples were collected at 0 h, 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 24 h on day 1, and at 24 h thereafter on days 2—8. On day 8 blood samples were drawn at the same time points as on day 1 with the addition of a 12-h sample. Plasma was separated and stored frozen at −20°C until analyzed.

Plasma concentration—time profiles of I and I-B in two subjects from the above study are shown in Fig. 5A and B and Fig. 6A and B, respectively. Plasma concentrations of I in both subjects peaked at 0.95 and 2.65 ng/ml on day 1, between 45 and 60 min after the first 45-mg dose and declined rapidly thereafter to 0.4 ng/ml at 6 h before the second 45-mg dose was administered. The plasma concentrations showed a progressive increase thereafter over the seven days of t.i.d. treatment reaching apparent steady state by day 3, peaked at 3.4 and 7.8 ng/ml, 1 h after the last 45-mg dose on day 8, declining rapidly thereafter to 1.5 and 0.9 ng/ml, respectively at 24 h. The relatively low plasma concentrations of I following total daily dose of 135 mg (45 mg, three times)

suggested either extensive tissue distribution and/or extensive biotransformation and elimination of the drug.

Quantitation of the major metabolite (I-B) present in plasma and urine as a glucuronide conjugate [7, 11] was feasible due to the relatively high concentrations present. Procedure A which describes the quantitation of I-B after enzymatic deconjugation was used to determine plasma concentrations of I-B in the same two subjects.

Plasma concentrations of I-B were sufficiently high as to dilute out the quantitation of any minor components present in the sample, viz. I, I-A and I-C.

Plasma concentrations of I-B following the first 45-mg dose of diclofensine in both subjects peak between 1 and 2.5 μg/ml, indicating rapid biotransforma-

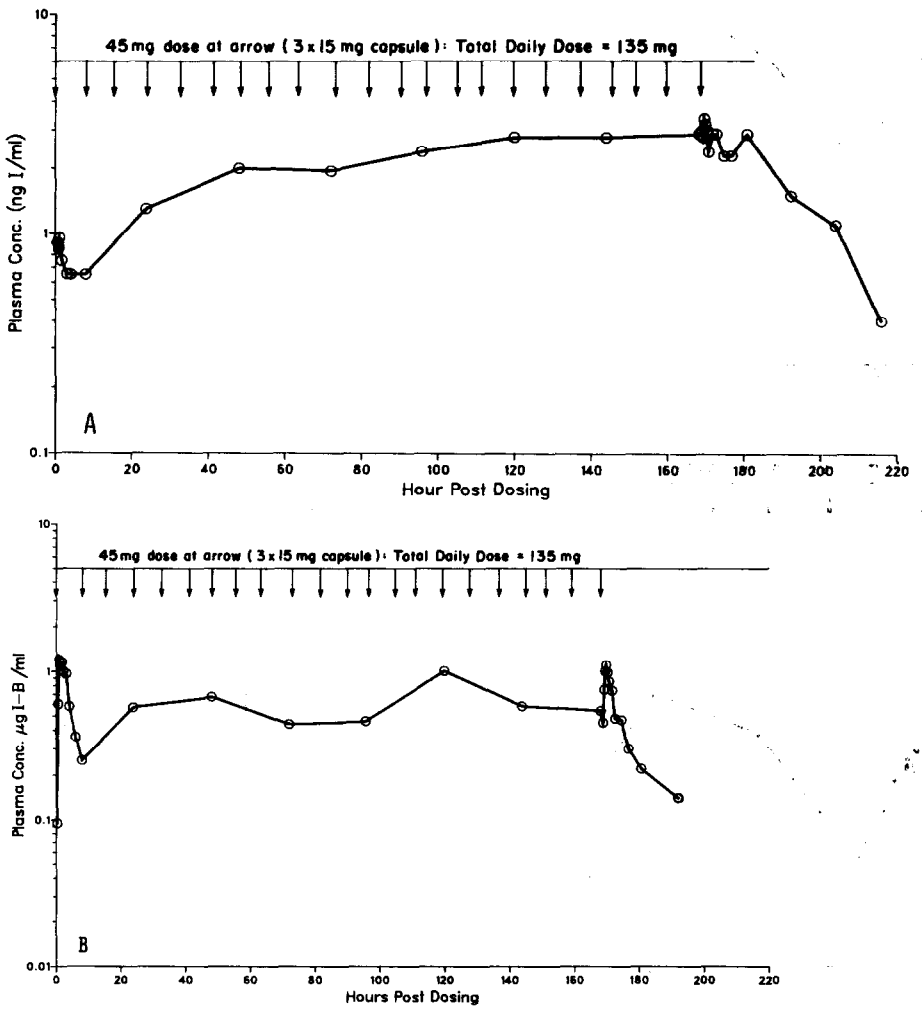


Fig. 5. Plasma concentration—time profiles of (A) diclofensine (I) in subject A following a 45-mg t.i.d. regimen for seven consecutive days followed by a single 45-mg dose on day 8; (B) metabolite I-B (aglycone) in subject A over seven consecutive days of a 45-mg t.i.d. dosing regimen and a final 45-mg single dose on day 8.

tion, decline gradually thereafter and are measurable at 24 h post-dose (100—200 ng/ml). The plasma profiles over the eight-day period in both subjects are shown in Fig. 5B and 6B, respectively. The data suggest that steady-state concentrations of I-B-glucuronide are maintained over the seven-day period of multiple daily dosing (45 mg t.i.d.), and that on day 8 after the last 45-mg dose the plasma concentrations of I-B decline rapidly from a peak of 1.0—2.0 μg/ml

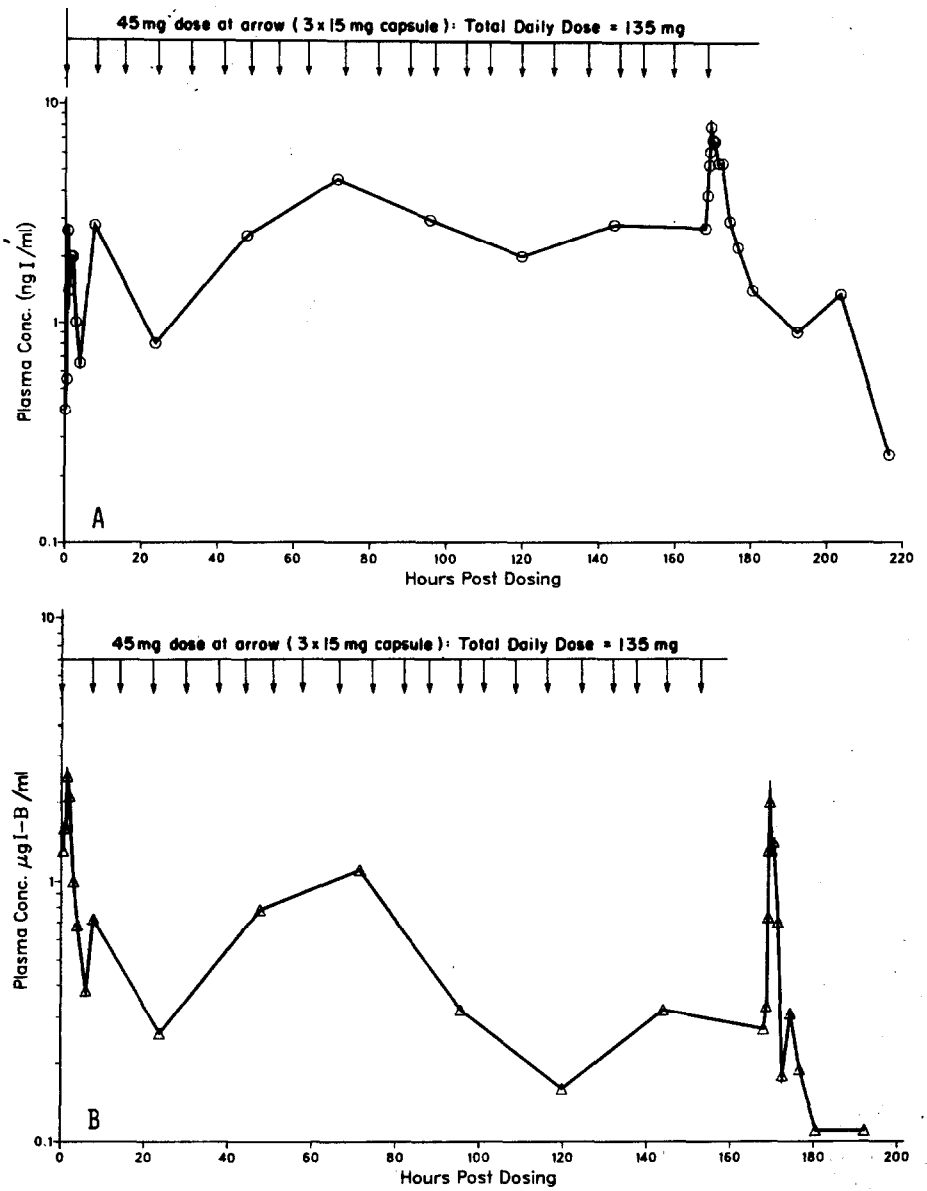


Fig. 6. Plasma concentration—time profiles of (A) diclofensine (I) in subject B following a 45-mg t.i.d. regimen for seven consecutive days followed by a single 45-mg dose on day 8; (B) metabolite I-B (aglycone) in subject B over seven consecutive days of a 45-mg t.i.d. dosing regimen and a final 45-mg single dose on day 8.

at 1 h to around 0.15 μg/ml at 24 h in a parallel manner to that seen on day 1 after the first 45-mg dose. The data suggest that the plasma profile of the major metabolite appears to mimic that of the parent drug in each subject and that, although steady-state concentrations of the metabolite were maintained during therapy, both the parent drug and the metabolite were cleared rapidly upon cessation of therapy.

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