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DETERMINATION OF A PYRIMIDO-BENZAZEPINE ANXIOLYTIC AGENT AND ITS 5-HYDROXY METABOLITE IN WHOLE BLOOD, PLASMA AND URINE BY GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION AND BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Electron-capture gas—liquid chromatographic and reversed-phase high-performance liquid chromatographic assays are described for the quantitation of the compound, 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine, [I], a member of the benzazepine class of compounds undergoing clinical evaluation as anxiolytic agents. Studies on the biotransformation of [I] in the rat and dog showed that the compound was metabolized mainly by hydroxylation to yield the 5-hydroxy compound, [II], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido [5,4-*d*][2]-benzazepin-5-ol (major metabolite), along with the formation of lesser amounts of the N-oxide, [III], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine 3-oxide, and the phenolic analogue, [IV], 3-chloro-4-(9-chloro-5H pyrimido-[5,4-*d*][2]benzazepin-7-yl)phenol. This report describes the quantitation of [I] and [II] (major metabolite) in plasma using the above analytical techniques, both in preclinical studies in the dog and in clinical pharmacokinetic studies in man.

INTRODUCTION

The compound, 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine, [I], Fig. 1, is a member of the benzazepine class of compounds undergoing clinical evaluation as anxiolytic agents [1].

In vitro studies on the biotransformation of [I] [2], using a 9000-*g* microsomal fraction from a rat liver homogenate showed that the compound was metabolized mainly by hydroxylation to yield the 5-hydroxy compound, [II], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepin-5-ol, along with the formation of lesser amounts of the N-oxide, [III], 9-chloro-7-

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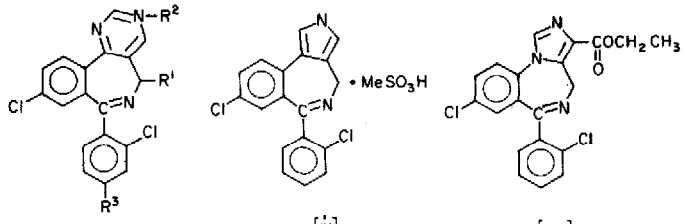

Compound	R^1	R^2	R^3
[I]	H	—	H
[II]	OH	—	H
[III]	H	→O	H
[IV]	H	—	OH

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

(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine 3-oxide, and the phenolic analogue, [IV], 3-chloro-4-(9-chloro-5H-pyrimido[5,4-*d*][2] benzazepin-7-yl)phenol. In vivo biotransformation studies in the rat and in the dog [3] confirmed that unconjugated [II] was the major plasma metabolite. The N-oxide metabolite, [III], and the phenol, [IV], were minor metabolites. The synthesis and pharmacological properties of these compounds are reported elsewhere [4]. The primary focus of this report is on the quantitation of [I] and [II] in plasma using different analytical approaches.

Simultaneous quantitation of compounds [I] and [II] necessitated the silylation of [II] to enhance its response to gas—liquid chromatography with electron-capture detection (GLC—ECD), using the compound, 8-chloro-6-(2-chlorophenyl)-2H,4H-pyrrolo[3,4-*d*][2]-benzazepine methanesulfonate, [V], as the internal standard. The sensitivity limit of this assay is 2 ng of [I] and [II] per ml of plasma.

A reversed-phase high-performance liquid chromatographic (HPLC) assay (UV detection at 254 nm) was developed for the determination of [I] and [II] (sensitivity limit: 50 ng/ml). Although it is less sensitive than the GLC—ECD assay, its simplicity and high sample throughput via automation offers advantages during pre-clinical drug development when sensitivity is not critical [5]. A normal-phase HPLC method (UV detection at 254 nm) was also developed (senstivity limit: 25–50 ng/ml) for the determination of compounds [I], [II], [III], and [IV] using 8-chloro-6-(2-chlorophenyl)-4H-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester, [VI], as the internal standard, and was used to verify the specificity of the GLC—ECD assay. All assays involve extraction of [I] and [II] into benzene from blood, plasma, or urine buffered to pH 6.1 (saturated potassium chloride solution), the residue of which is analyzed using the appropriate methods described.

EXPERIMENTAL

Reagents

All reagents must be of analytical reagent grade (> 99% purity). Saturated potassium chloride, pH 6.1, approximately 4.8 *M*, is prepared by adding 300

g of potassium chloride (Matheson, Coleman and Bell, Reagent Crystals, Norwood, OH, U.S.A.) to 900 ml of distilled deionized water.

The organic solvents (all analytical-reagent grade) used were as follows: benzene, methanol, acetonitrile, and isopropanol were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetone and *n*-hexane (H-301) were from Fisher Scientific (Springfield, NJ, U.S.A.); ethanol (absolute, U.S.P. grade) was from U.S. Industrial Chemicals (Tuscola, IL, U.S.A.).

Benzene—acetone—methanol (85:10:5), stored over anhydrous sodium sulphate (Mallinckrodt, St. Louis, MO, U.S.A.) is the solvent for GLC—ECD analysis. Methanol—isopropanol (50:50) is the solvent for HPLC analysis. The silylating agent for the derivatization of [II] was N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) from Regis Chemical (Morton Grove, IL, U.S.A.), mixed with acetonitrile in a 1:10 ratio.

GLC—ECD analysis for compounds [I] and [II] in blood or plasma

Column conditions

The column packing was a pretested phase containing 5% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) packed in a U-shaped, 1.2 m or a 1.8 m × 4 mm I.D. borosilicate glass column. The glass column was treated before packing with a 1% solution of Prosil-28 (PCR Research Chemicals, Gainesville, FL, U.S.A.), for 10—15 sec, thoroughly rinsed with distilled water and dried for 1 h at 100°C. The packed column was conditioned at 325°C under "no flow" conditions for 4 h, then at 275°C for at least 18 h with carrier gas flow-rate of 40 ml/min.

Instrumental parameters for GLC—ECD analysis

A Tracor gas chromatograph, Model 222 equipped with a ^{63}Ni electron-capture detector containing a 15-mCi ^{63}Ni β-ionization source, and an auto-sampler (Model 7671A, Hewlett-Packard, Avondale, PA, U.S.A.) was used. Argon—methane (90:10) from Liquid Carbonic (Harrison, NJ, U.S.A.) was used as the carrier gas and the column head pressure was preset at 275 kPa (40 p.s.i.), with a column flow-rate of 80 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven, 230°C or 250°C for the 1.2-m or the 1.8-m columns, respectively; injection port, 275°C; detector, 350°C.

The electron-capture detector linearizer (Model No. 114460) standing current was adjusted to $0.5 \cdot 10^{-9}$ A, the relative pulse width was adjusted to 0.15 which corresponds to 0.75 μsec and the attenuation was set at 16. The chart speed was 1.27 cm/min and the time constant on the 1.0-mV Honeywell recorder (Model No. 194) was 1 sec (full scale deflection). The conditions of flow-rate and column temperature must be adjusted to obtain retention times of 4.2, 5.4, and 6.8 min on the 1.2-m column or 5.7, 7.2, and 9.1 min on the 1.8-m column for [I], [II]-OTMS, and [V], respectively. Under these conditions, 2.4 ng each of [I] and [II]-OTMS, and 20.0 ng of [V] injected on the column give nearly full scale pen response. The minimum detectable amount of [I] and [II] is 2 ng per ml of blood or plasma.

Reversed-phase HPLC analysis of compounds [I] and [II] in blood, plasma or urine

Column

The column used was a 0.30 m × 3.9 mm I.D. stainless-steel column containing μBondapak C_{18}, particle size 10 μm, generating 10,770 plates/m (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters

A Waters Model ALC/GPC-204 high-pressure liquid chromatograph equipped with a Model 440 absorbance detector, operated at 254 nm, a Model M6000A solvent delivery system, a U6K injector, or a Waters Intelligent Sample Processor (WISP™) Model 710-B was used. The isocratic mobile phase used was a mixture of methanol—water (75:25) at a pressure of 6 MPa (900 p.s.i.) and a constant flow-rate of 1.3 ml/min. Under these conditions, the retention times of compounds [I], [II], and [V] (internal standard) were 5.3, 4.3, and 6.4 min, respectively, with capacity factors (k') of 3.1, 2.2, and 4.0, respectively. The UV detector sensitivity was $1 \cdot 10^{-2}$ absorbance units full scale deflection (a.u.f.s.) and the chart speed on the 10-mV Hewlett-Packard recorder (Model No. 7132A) was 1.27 cm/min. Under these conditions 50 ng each of [I] and [II] and 140 ng of [V] per 10 μl injected gave nearly full scale pen response. The minimum detectable amount of [I] and [II] is 50 ng/ml of blood, plasma or urine.

Preparation of analytical standards

Compounds [I]: $C_{18}H_{11}Cl_2N_3$, MW = 340.21, m.p. = 122—125°C; compound [II]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 174—175°C; compound [III]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 189—190°C; compound [IV]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 208—210°C; compound [V]: $C_{18}H_{12}Cl_2N_2 \cdot CH_4SO_3$, MW = 423.3, m.p. = 239—241°C; and compound [VI]: $C_{20}H_{15}Cl_2N_3O_2$, MW = 400.3, m.p. = 225–228°C, all of pharmaceutical grade purity (> 99%) were used as analytical standards.

GLC—ECD analysis of parent compound [I] and metabolite [II]

Prepare stock solution of compounds [I], [II], and [V] in separate 10-ml volumetric flasks by dissolving 10.00 mg of each compound in 1 ml of ethanol and 1 ml of methanol. Sonicate in an ultrasonic bath if necessary for 5—10 min for complete solubilization and dilute to volume with acetone—*n*-hexane (1:9). These stock solutions (containing 1 mg/ml of [I], [II], and [V] are used to prepare the following mixed standard solutions by suitable dilutions in acetone—*n*-hexane (1:9) to contain the following concentrations:

Working standard solution	Concentration (ng per 0.1 ml)		
	[I]	[II]	[V]* (internal standard)
1	2	2	—
2	4	4	—
3	8	8	—
4	16	16	—
5	24	24	—
6	48	48	—
7	72	72	—
8	—	—	200

*Internal standard [V]: Standard solution No. 8 is prepared by suitable dilution in benzene—acetone—methanol (85:10:5) and is added as the internal standard to all the derivatized residues, prior to the GLC—ECD analysis of all samples.

Aliquots (0.1 ml) of standard solutions Nos. 1—7 are added to 0.9 ml of the benzene extract of control blood or plasma, derivatized with BSTFA after evaporation (as described under Assay procedure), and are used as the external "matrix" (calibration) standard curve, to verify the linearity and performance of the GLC—ECD system.

Similarly, 0.1-ml aliquots of standard solutions Nos. 1—7 are evaporated to dryness, the residue is reconstituted in 0.5 ml of control blood or plasma, extracted as described under the Assay procedure, the residue of which is derivatized, and used as the processed (recovered) standard calibration curve for the determination of the concentration of [I] and [II]-OTMS in the unknowns.

Calibration of compounds [I], [II]-OTMS, and [V] by GLC—ECD. Calibration (external standard) curves of the peak area ratio of [I] to [V], and of [II]-OTMS to [V] vs. concentration of [I] and [II] added to the extract of control blood or plasma are constructed. Fresh calibration curves of the "matrix" external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the reproducibility of the GLC system.

For reversed-phase HPLC analysis of parent compound [I] and metabolite [II]

Prepare stock solutions of compounds [I], [II], and [V] as previously described for the GLC—ECD assay of compounds [I] and [II]. The stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in acetone—*n*-hexane (1:9) to contain the following concentrations:

Working standard solution	Concentration (ng per 0.1 ml)		
	[I]	[II]	[V] (internal standard)
9	50	50	1400
10	100	100	1400
11	300	300	1400
12	500	500	1400
13	900	900	1400
14	1800	1800	1400
15	2700	2700	1400
16	—	—	1400

Aliquots (0.1 ml) of standard solutions Nos. 9—15 are added to 0.9 ml of the benzene extract of control blood, plasma or urine and evaporated to dryness. The residues are dissolved in 100 μl of methanol—isopropanol (50:50) for reversed-phase HPLC analysis. Appropriate aliquots are injected as the "matrix" external (calibration) standard curve, to establish the parameters for reversed-phase HPLC analysis with UV detection at 254 nm.

Aliquots (0.1 ml) of standard solutions Nos. 9—15 are evaporated to dryness, the residue is reconstituted in 0.5 ml of control blood, plasma or urine, extracted as described and used as the processed (recovered) standard calibration curve for the determination of the concentration of [I] and [II] in the unknowns by reversed-phase HPLC analysis. A separate standard solution, No. 16, containing 1400 ng of [V] per 0.1 ml of acetone—*n*-hexane (1:9) is prepared and added as the internal standard to all unknown samples as per above procedure (i.e., evaporated and unknown sample added), for HPLC analysis.

Calibration of compounds [I] and [II] by HPLC. Calibration (external standard) curves of the peak height ratio of [I] or [II] to [V] vs. concentration of compound added to the extract of control blood, plasma or urine are constructed. Fresh calibration curves of the "matrix" external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Analysis of blood, plasma or urine

GLC—ECD analysis for parent compound [I] and metabolite [II]

The flow diagram of the extraction procedure is shown in Fig. 2. Into a 15-ml conical centrifuge tube (PTFE No. 13 stoppered), add 0.50 ml of heparinized whole blood or plasma, 0.50 ml of pH 6.1, ca. 4.8 *M*, saturated potassium chloride solution (vortex), and extract with 1.0 ml of benzene by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. Along with the unknown samples, process nine 0.50-ml specimens of control blood or plasma, two to be used as controls and seven to be used as "matrix" external standards. In addition, process seven 0.50-ml specimens of control blood or plasma containing 0.1 ml of standard solutions Nos. 1—7 (equivalent to 2, 4, 8, 16, 24, 48, and 72 ng of [I] and [II] per 0.50 ml of blood or plasma) as the processed (recovered)

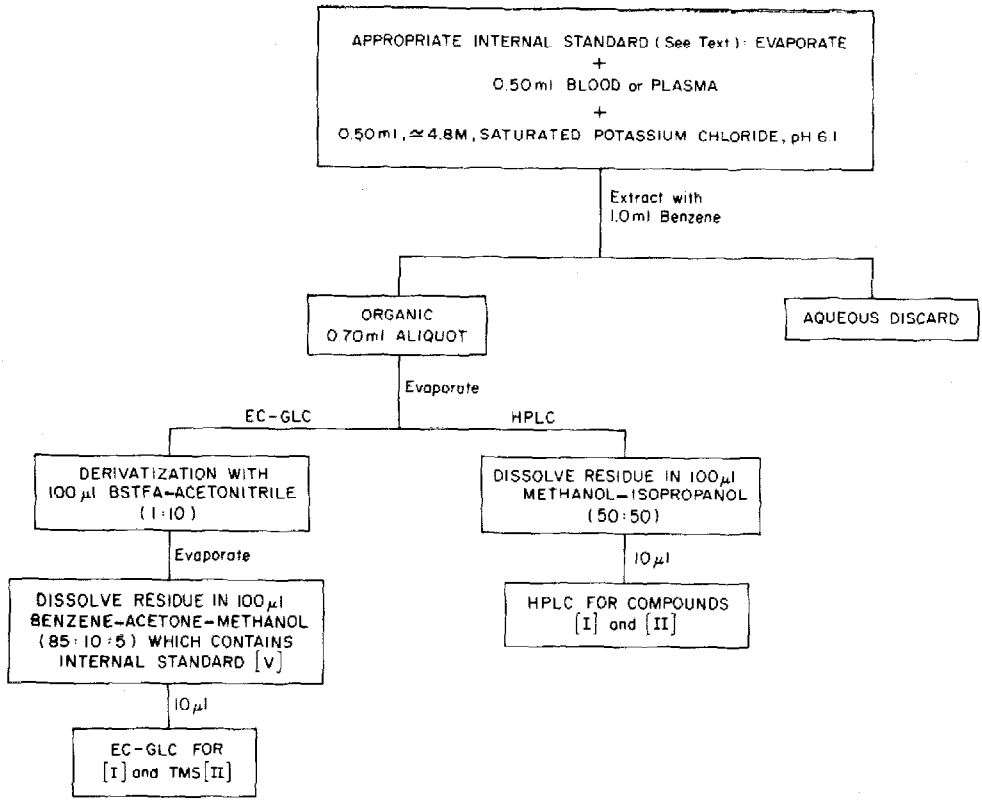

Fig. 2. Flow diagram of the extraction procedure for compounds [I] and [II] from blood or plasma.

standard curve. The solution (100 µl) is evaporated to dryness at 35—40°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen prior to the addition of control blood or plasma. Centrifuge the samples at 1500 *g* in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min. Transfer a suitable aliquot (0.7—0.8 ml) of the upper organic phase into a disposable tube (13 × 100 mm) (which was used in order to eliminate adsorption losses of both compounds) and evaporate the benzene extract to dryness at 35—40°C under nitrogen as before. Immediately add 100 µl of BSTFA—acetonitrile (1:10) to the dry residue, stopper the tubes tightly, vortex, and react at 60°C for 20 min (Multi-Block Heater, Lab-Line Instruments, Melrose Park, IL, U.S.A.), continuing to vortex every 5 min. Evaporate the derivatization mixture to dryness immediately after the reaction and reconstitute the residue in 100 µl of benzene—acetone—methanol (85:10:5) containing 200 ng of the internal standard [V]. Inject a 10-µl aliquot for GLC analysis, either manually or by transferring the reconstituted residue into a 100-µl automatic sampler vial (HP 5080-8779, Hewlett-Packard) for auto-injection.Typical chromatograms of plasma extracts are shown in Fig. 3.

Reversed-phase HPLC analysis of [I] and [II] in blood, plasma or urine

The flow diagram of the extraction procedure is shown in Fig. 2. Follow the

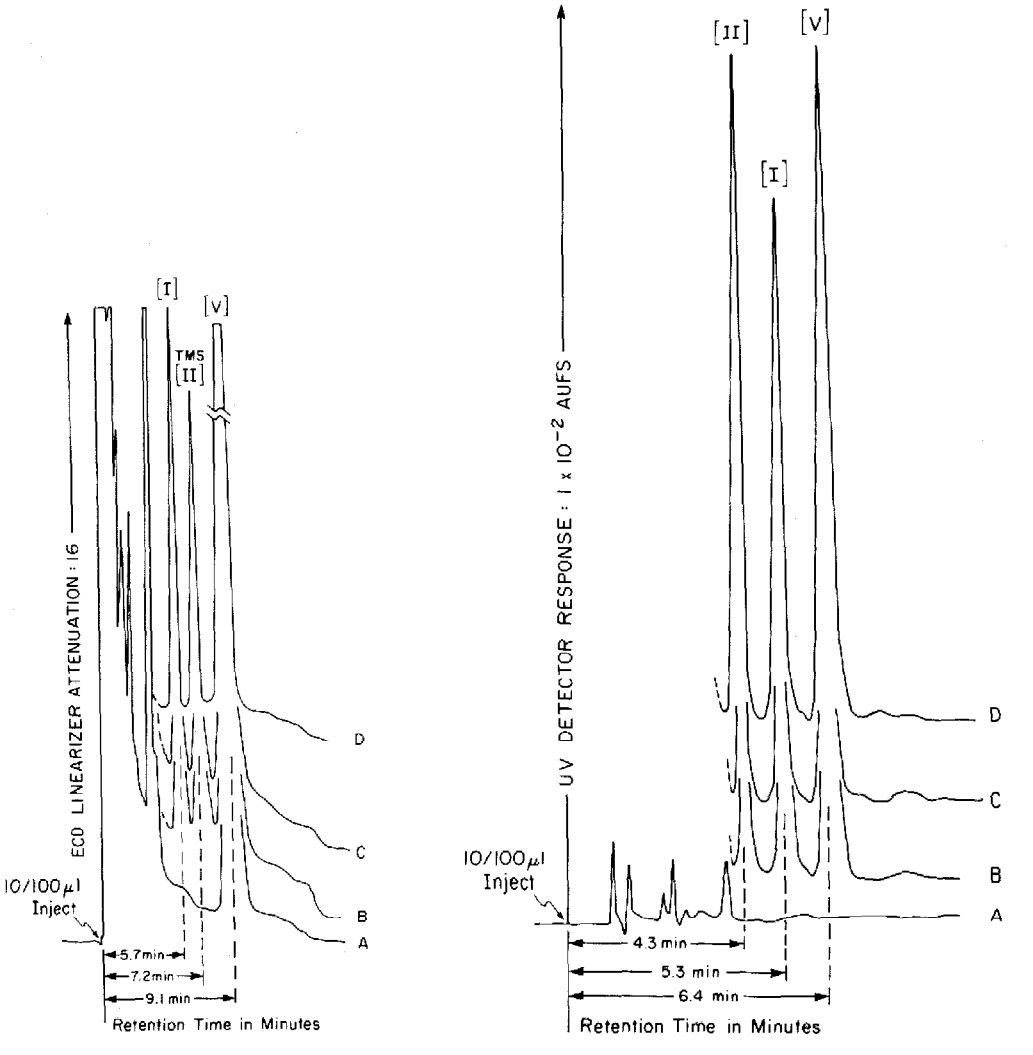

Fig. 3. Chromatograms of the GLC—ECD analysis of derivatized benzene extracts of (A) control human plasma with added internal standard; (B) human plasma following a single 5-mg oral dose of [I]; (C) authentic standards recovered from control human plasma; and (D) authentic standards added to the residue of control human plasma (matrix external standard).

Fig. 4. Chromatograms of the reversed-phase HPLC analysis of benzene extracts of (A) control dog plasma; (b) dog plasma following chronic oral dosing of [I] at 300 mg/kg/day on day 1; (C) authentic standards recovered from control dog plasma; and (D) authentic standards added to the residue of control dog plasma (matrix external standards).

extraction procedure as described for GLC—ECD analysis, adding 100 µl of standard solution No. 16, containing 1400 ng of compound [V], as the internal standard. Along with the unknown samples process nine 0.50-ml specimens of control blood, plasma or urine (as for GLC—ECD), and seven 0.50-ml specimens of control blood, plasma or urine containing 0.10 ml of standard solutions Nos. 9—15 (equivalent to 50, 100, 300, 500, 900, 1800, and 2700 ng of [I] and [II], and 1400 ng of compound [V] (internal standard) per 0.50 ml

of blood, plasma or urine), evaporate to dryness and reconstitute in the appropriate biological specimen. After the extraction step, transfer a suitable aliquot (0.7—0.8 ml) of the upper organic phase into a disposable tube (13 × 100 mm), and evaporate the benzene extract to dryness at 35—40°C under nitrogen. Immediately dissolve the residue in 100 μl of methanol—isopropanol (50:50) and inject a 10-μl aliquot into the liquid chromatograph either manually, or by using the WISP™ auto-injector. A typical chromatogram of plasma extracts is shown in Fig. 4.

Calculations

The concentrations of [I] and [II] in the unknowns were determined by interpolation from a least squares regression equation (power equation: $Y = m\ X^b$) of the calibration data of the recovered standards processed along with the unknowns using peak height or peak area ratios (peak height or peak area of compounds [I] or [II] to peak height or peak area of the internal standard [V] vs. concentration of [I] or [II] per 0.5 ml of plasma, blood or urine). A typical calibration curve as defined by the equation $Y = 0.026X^{0.911}$ is linear from 2 to 72 ng of [I] and [II] per 0.5 ml of plasma, as assayed by the GLC—ECD method for parent compound [I] and metabolite [II]. The correlation coefficient (r) is equal to 0.998 and the average deviation from the line is 6.7%. The same equation is used to define the calibration curves for the reversed-phase HPLC assay. All calibration data were processed by a HP Model 3354B Laboratory Automation System (Hewlett-Packard).

RESULTS AND DISCUSSION

A sensitive and specific GLC—ECD assay was developed for the determination of parent compound [I] and its 5-hydroxy metabolite [II] from 0.50 ml of blood or plasma. The metabolite [II] is converted to its OTMS derivative by reaction with a mixture of BSTFA—acetonitrile (1:10) resulting in a Gaussian shaped symmetrical peak with enhanced ECD response. Although the internal standard, compound [V], has compatible chromatographic behavior to that of [I] and [II]-OTMS, it was introduced in the solvent used to dissolve the sample residue prior to injection, hence, it monitors only the final chromatographic step. This assay was used during preclinical evaluation of the biopharmaceutic/pharmacokinetic profile of [I] in the dog following intravenous and oral administration and during initial clinical evaluation of single 5-mg oral doses of compound [I] in man.

A sensitive and specific reversed-phase HPLC assay was developed for the determination of compounds [I] and [II] from 0.5 ml of blood, plasma or urine using UV detection at 254 nm for quantitation. The major UV absorption bands of both compounds [I] and [II] occur at 235—240 nm and at 270—280 nm, while the major absorption band for the internal standard [V] occurs at 268—276 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 [I] and [II] in the nanogram range. Compound [V] was selected as the internal standard because of its compatible extraction and

chromatographic behavior to that of compounds [I] and [II]. This assay was used for biopharmaceutic studies in the dog [5].

Recovery, sensitivity limits, and statistical validation

The determination of percent recovery requires that the recovered standards be compared to the external standard curve comprising authentic standards which have been added to the residue of extracted control blood or plasma. This is necessary due to a chromatographic (GLC—ECD and HPLC) enhancement or "matrix effect" which these compounds exhibit; i.e., the peak response is 10—20% greater when they are chromatographed in the residue of a biological extract, than when chromatographed in organic solvents as pure authentic standards.

GLC—ECD method for parent compound [I] and metabolite [II]

The overall recoveries of parent compound [I] and metabolite [II] are 98.5 ± 8.9% (S.D.) and 99.8 ± 5.2% (S.D.), respectively. The sensitivity limit of detection is 2 ng/ml of plasma for both compounds. The intra-assay precision of [I] and [II] over the concentration range of 2—72 ng/ml of plasma showed mean coefficients of variation of 2.8% and 1.8%, respectively. The inter-assay precision of [I] and [II] over the same concentration range showed mean coefficients of variation of 5.1% and 4.2%, respectively (Table I).

TABLE I

INTER-ASSAY PRECISION OF THE GLC—ECD ASSAY FOR COMPOUNDS [I] AND [II]-OTMS (FOLLOWING DERIVATIZATION), RECOVERED FROM CONTROL DOG PLASMA

Compound	Amount added (ng)	Amount found (ng)	*n*	Coefficient of variation (%)
[I]	2.0	2.2 ± 0.1	3	2.5
	4.0	3.9 ± 0.2	3	5.2
	8.0	7.5 ± 0.5	3	6.1
	16.0	14.7 ± 1.0	3	6.7
	24.0	23.7 ± 0.9	3	3.7
	48.0	49.3 ± 3.6	3	7.4
	72.0	77.0 ± 3.4	3	4.4
			Average =	5.1
[II]	2.0	2.1 ± 0.1	3	2.9
	4.0	3.9 ± 0.2	3	5.3
	8.0	7.8 ± 0.5	3	6.2
	16.0	15.6 ± 0.4	3	2.6
	24.0	24.8 ± 1.2	3	4.8
	48.0	48.5 ± 2.4	3	4.9
	72.0	72.0 ± 2.0	3	2.8
			Average =	4.2

TABLE II

INTER-ASSAY PRECISION OF THE REVERSED-PHASE HPLC ASSAY FOR COMPOUNDS [I] AND [II], RECOVERED FROM CONTROL DOG PLASMA

Compound	Amount added (ng)	Amount found (ng)	n	Coefficient of variation (%)
[I]	50.0	50.4 ± 1.4	3	2.8
	100.0	101.0 ± 2.2	3	2.1
	300.0	289.0 ± 8.7	3	3.0
	600.0	611.0 ± 47.5	3	7.8
	900.0	893.0 ± 53.0	3	5.9
	1800.0	1820.0 ± 156.0	3	8.6
	2700.0	2710.0 ± 95.0	3	3.5
		Average =		4.8
[II]	50.0	51.2 ± 2.5	3	4.8
	100.0	101.0 ± 1.7	3	1.7
	300.0	288.0 ± 14.7	3	5.1
	600.0	586.0 ± 23.1	3	3.9
	900.0	888.0 ± 11.4	3	1.3
	1800.0	1880.0 ± 87.2	3	4.6
	2700.0	2710.0 ± 40.4	3	1.5
		Average =		3.3

TABLE III

INTER-ASSAY PRECISION OF THE REVERSED-PHASE HPLC ASSAY FOR UNCONJUGATED COMPOUNDS [I] AND [II], RECOVERED FROM CONTROL DOG URINE

Compound	Amount added (ng)	Amount found (ng)	n	Coefficient of variation (%)
[I]	50.0	46.6 ± 5.0	3	10.6
	100.0	97.3 ± 5.6	3	5.7
	200.0	203.0 ± 9.5	3	4.7
	300.0	302.0 ± 7.7	3	2.5
	400.0	396.0 ± 8.3	3	2.1
	1000.0	1010.0 ± 20.6	3	2.0
		Average =		4.6
[II]	50.0	46.6 ± 5.2	3	11.2
	100.0	102.0 ± 7.1	3	7.0
	200.0	200.0 ± 6.2	3	3.1
	300.0	303.0 ± 8.5	3	2.8
	400.0	396.0 ± 6.1	3	1.5
	1000.0	1000.0 ± 6.1	3	0.6
		Average =		4.4

HPLC method (reversed-phase) for parent compound [I] and metabolite [III]

The overall recoveries of compounds [I] and [II] from plasma are 103.6 ± 8.8% (S.D.) and 106.1 ± 11.5% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of plasma for both compounds, using UV detection at 254 nm. The intra-assay precision of [I] and [II] over the concentration range of 50—2700 ng/ml of plasma showed mean coefficients of variation of 4.8% and 2.7%, respectively. The inter-assay precision of [I] and [II] over the same concentration range showed mean coefficients of variation of 4.8% and 3.3%, respectively (Table II).

The overall recoveries of unconjugated compounds [I] and [II] from urine are 94.6 ± 8.7% (S.D.) and 101.9 ± 7.7% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of urine for both compounds, using UV detection at 254 nm. The intra-assay precision of [I] and [II] over the concentration range of 50—2000 ng/ml of urine showed mean coefficients of variation of 5.3% and 6.0%, respectively. The inter-assay precision of [I] and [II] over the concentration range of 50—1000 ng/ml of urine showed mean coefficients of variation of 4.6% and 4.4%, respectively (Table III).

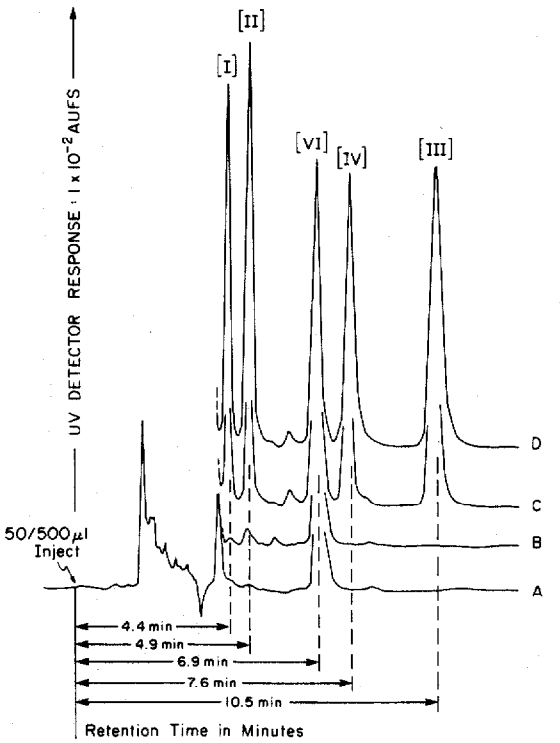

Fig. 5. Chromatograms of the normal-phase HPLC analysis of benzene extracts of (A) control human plasma with added internal standard; (B) human plasma following a single 5-mg oral dose of [I]; (C) authentic standards recovered from control human plasma; and (D) authentic standards added to the residue of control human plasma (matrix external standard).

Normal-phase HPLC analysis of compounds [I], [II], [III], and [IV] in blood or plasma

A sensitive and specific normal-phase HPLC assay was also developed for the determination of compounds [I], [II], [III], and [IV] from 0.5 ml of blood or plasma using UV detection at 254 nm. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing 10-μm Partisil silica, generating 31,000 theoretical plates/m (Whatman, Clifton, NJ, U.S.A.).

The Waters HPLC system was used as before. The isocratic mobile phase used was a mixture of hexane—methanol—tetrahydrofuran—ammonium hydroxide (71.85:8:20:0.15) at a pressure of 4 MPa and a constant flow-rate of 1.5 ml/min.

The retention times of compounds [I], [II], [III], [IV], and internal standard [VI] were 4.4, 4.9, 10.5, 7.6, and 6.9 min, respectively, with capacity factors (k') of 3.23, 3.61, 9.30, 6.46, and 5.77, respectively (Fig. 5). Reversed-phase HPLC was unable to adequately resolve all components of interest.

TABLE IV

PLASMA CONCENTRATIONS OF COMPOUNDS [I] AND [II] IN THE DOG FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION OF COMPOUND [I]

ND = not detectable, NM = non-measurable (< 2 ng/ml), NST = no sample taken.

Time (h)	Concentration (μg/ml plasma)			
	Intravenous dose (5 mg/kg)		Oral dose (20 mg/kg)	
	[I]	[II]	[I]	[II]
0	ND*	ND	ND	ND
0.017	15.2	ND	NST	NST
0.042	8.7	ND	NST	NST
0.083	7.11	0.18	NST	NST
0.17	6.12	0.26	0.24	0.11
0.25	5.40	0.40	NST	NST
0.33	3.70	0.27	2.52	0.88
0.50	2.56	0.22	2.83	1.21
0.75	1.73	0.15	3.48	1.26
1.0	1.11	0.11	3.20	1.16
1.5	0.64	0.033	4.32	1.33
2	0.35	0.020	3.98	1.12
3	0.20	0.016	3.14	0.72
4	0.11	0.01	1.14	0.28
6	0.07	0.007	0.47	0.10
8	0.04	0.005	0.29	0.04
11.5	0.03	NM	NST	NST
12	NST**	NST	0.15	0.03
24	0.015	NM	0.08	0.006
30	0.009	NM	0.03	NM
48	0.007	ND	0.025	ND
72	NM***	ND	ND	ND

The extraction procedure is the same as that described for reversed-phase HPLC analysis, except that compound [VI] is used as the internal standard and the final residue was dissolved in the mobile phase for adsorption chromatography. This assay was used to monitor the possible presence in human plasma of compounds [III] and [IV], which were reported to be minor metabolites in the urine of rat and in the dog [3]. The N-oxide, [III] (if present), would undergo thermal degradation in the injection port during

TABLE V

MEAN CONCENTRATION OF COMPOUNDS [I] AND [II] IN MAN FOLLOWING THE ORAL ADMINISTRATION OF A SINGLE 5-mg DOSE OF COMPOUND [I]

Time (h)	Mean concentration ± S.D. (ng/ml)			
	[I]	*n*	[II]	*n*
0	N.D.	4	N.D.	4
0.5	5.0 ± 3.1	2	5.2 ± 4.6	2
1.0	15.1 ± 6.9	4	24.4 ± 14.0	4
1.5	15.9 ± 4.4	4	26.4 ± 9.3	4
2.0	17.0 ± 2.8	4	28.9 ± 10.9	4
3.0	15.5 ± 6.7	4	20.2 ± 10.0	4
4.0	12.2 ± 4.6	4	18.3 ± 7.0	4
6.0	8.8 ± 1.8	4	12.8 ± 3.4	4
8.0	6.2 ± 1.3	3	8.5 ± 1.5	3
12.0	4.9 ± 1.0	4	6.2 ± 2.8	4
18.0	2.9 ± 0.5	3	2.8 ± 0.5	4
24.0	2.5 ± 0.7	2	2.0 ± —	1

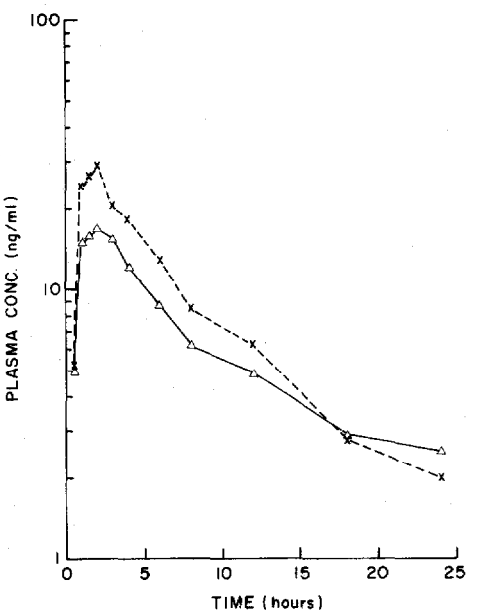

Fig. 6. Mean plasma concentration of compounds [I] (△) and [II] (X) in man following the oral administration of a single 5-mg dose of compound [I].

GLC—ECD analysis to yield [I] thereby impairing the accuracy of its determination.

The overall recoveries of compounds [I], [II], [III], and [IV] from plasma are 99.5 ± 9.8%, 103 ± 12.4%, 102 ± 8.0%, and 58.8 ± 6.6%, respectively. The sensitivity limit of detection is 25 ng/ml of plasma for compounds [I] and [II], and 50 ng/ml of plasma for compounds [III] and [IV].

Application of the methods to biological specimens

The GLC—ECD procedure and the reversed-phase HPLC procedure were used to monitor the plasma concentration—time profiles of compounds [I] and [II] in the dog during biopharmaceutic/pharmacokinetic evaluation following single 5 mg/kg intravenous and 20 mg/kg oral doses of compound [I] (Table IV). The GLC—ECD method was also used to determine the plasma concentrations of compounds [I] and [II] in four human subjects following a single 5-mg oral dose of compound [I] (Table V and Fig. 6).

The reversed-phase HPLC assay was used to determine the bioavailability of oral dosage forms of [I] in the dog [5] and in the evaluation of the stability of compound [I] in dog plasma determined over a 19-month storage interval at −25 to −30°C. The mean change observed (± 5.4%) was within the experimental error of the method, indicating stability of the compound.

The normal-phase HPLC assay for parent compound [I] and metabolites [II], [III], and [IV] was used to monitor the plasma concentration—time profiles of these compounds in two human subjects following a single 5-mg oral dose of compound [I]; however, none of these were detectable. The effluent volume fractions of [III] were collected, evaporated under nitrogen and analyzed by GLC—ECD. No peak for [I] was obtained, indicating the absence of [III] in any significant amounts in human plasma.

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