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DETERMINATION OF THE AGONIST–ANTAGONIST ANXIOLYTIC AGENT 6-(2-CHLOROPHENYL)-4-HYDROXY-4*H*-IMIDAZO[1,5-*a*]-[1,4]BENZODIAZEPINE-3-CARBOXAMIDE AND ITS BENZOPHENONE CARBOXYLIC ACID METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C.V. PUGLISI, F.J. FERRARA and I. BEKERSKY*

Department of Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110 (U.S.A.)

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of 6-(2-chlorophenyl)-4-hydroxy-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxamide (I) and its benzophenone carboxylic acid metabolite, 1-[2-(2-chlorobenzoyl)phenyl]-4-(aminocarbonyl)-1*H*-imidazole-5-carboxylic acid (II) in plasma. The assay involves the extraction of both compounds into benzene from buffered plasma (pH 5.4) and subsequent analysis by reversed-phase HPLC. The overall recovery of I and II is 98.3 ± 9.4 and 59.7 ± 15.7% for dog plasma, 86.0 ± 14.7 and 52.8 ± 15.1% for rat plasma and 98.1 ± 9.3 and 66.9 ± 18.0% for human plasma, respectively. The sensitivity limit of the assay for I and II is 20.0 and 40.0 ng/ml of plasma using ultraviolet detection at 254 nm. The assay was used in studies in dog and rat.

INTRODUCTION

The compound 6-(2-chlorophenyl)-4-hydroxy-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxamide (I, Fig. 1), synthesized by Walser and co-workers [1,2], is a member of the imidazo-1,4-benzodiazepine class of compounds, currently undergoing evaluation as mixed agonist–antagonist anxiolytic agents [3].

Studies on the in vitro biotransformation of I using the 9000 *g* supernate of incubated rat liver prepared from control or phenobarbital-pretreated rats [4] indicated that the compound was metabolized by hydrolysis of the azomethine bond in the diazepine ring to form the benzophenone carboxylic acid, 1-[2-(2-chlorobenzoyl)phenyl]-4-(aminocarbonyl)-1*H*-imidazole-5-carboxylic acid (II, Fig. 1).

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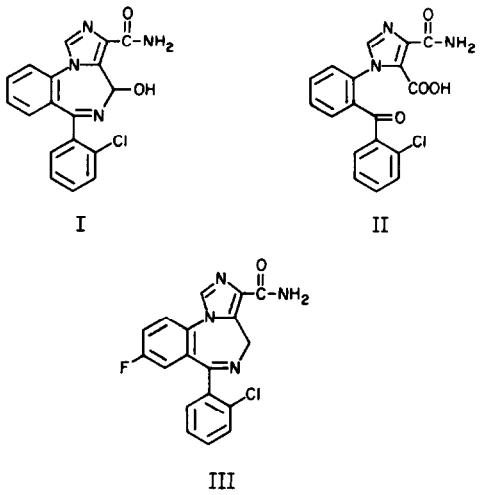

I II III

Fig. 1. Chemical structures of compounds I, II and III (internal standard).

Assay development is an integral part in the preclinical stages of the drug development process. The utilization of a validated assay provides plasma concentration data on drug disposition from toxicological studies, preclinical and clinical pharmacokinetic studies as well as stability studies in biological fluids.

In this context, high-performance liquid chromatography (HPLC) was investigated as a means of quantitation and resulted in the development of a rapid, sensitive and selective assay for the determination of compounds I and II in plasma. The method presented herein determines compounds I and II by reversed-phase HPLC using their ultraviolet (UV) absorbance at 254 nm for quantitation. The analogous compound, 6-(2-chlorophenyl)-8-fluoro-4*H*-imidazo-[1,5-*a*]-[1,4]benzodiazepine-2-carboxamide (III, Fig. 1), is used as the internal standard. The stability of I was demonstrated in human, dog and rat plasma at ambient temperature (24°C) for 24 h and storage in dog plasma at −17°C for 90 days by the reanalysis of previously assayed samples. Long-term storage stability in human, dog and rat plasma at −17 and −70°C for up to six months is currently under investigation.

The HPLC assay was used to monitor the plasma concentration–time profile and urinary excretion profile in three dogs in a three-way cross-over study following single 10.0 mg/kg oral doses and single 2.5 mg/kg intravenous doses of compound I.

EXPERIMENTAL

Column

The column used was a 0.30 m × 3.9 mm I.D. stainless-steel column containing μBondapak C_{18} (10 μm), generating 22 230 plates/m (Waters Assoc., Milford, MA, U.S.A.).

TABLE I

COMPOSITION OF STANDARD SOLUTIONS 1–8

Standard solution	Concentration (ng per 100 μl)		
	I	II	III (internal standard)
1	20.0	40.0	—
2	40.0	60.0	—
3	100	150	—
4	200	300	—
5	400	600	—
6	1000	1500	—
7	2000	3000	—
8	—	—	1200

Instrumental parameters

A Waters Model ALC/GPC-204 high-pressure liquid chromatograph equipped with a Model 440 absorbance detector, a Model M6000A solvent delivery system, a U6K injector or a Waters WISP™ Model 710-B sample processor was used. The isocratic mobile phase used was methanol–0.01 *M* potassium phosphate buffer pH 4.0–tetrahydrofuran (47:50:3) at a pressure of 16.7 MPa (2500 p.s.i.) and a constant flow-rate of 1.5 ml/min. Under these conditions, the retention times of compound I, II and III were 9.3, 6.7 and 14.5 min, respectively, with corresponding capacity factors (k') of 4.1, 2.7 and 7.0 (see Fig. 3). The UV detector was operated at 254 nm at a sensitivity of $5 \cdot 10^{-3}$ a.u.f.s. and the chart-speed on the 10-mV Hewlett-Packard recorder (Model No. 7132A) was 0.64 cm/min. Under these conditions 20.0 ng of I, 30.0 ng of II and 40.0 ng of III per 10-μl injection gave nearly full-scale pen response. The minimum detectable amounts of I and II were 20.0 and 40.0 ng per ml of plasma, respectively.

Preparation of analytical standards

Compound I ($C_{18}H_{13}ClN_4O_2$, MW 352.79, m.p. 305–308°C), compound II ($C_{18}H_{12}ClN_3O_4$, MW 369.76, m.p. 207–210°C) and compound III ($C_{18}H_{12}ClFN_4O$, MW 354.77, m.p. 297–299°C) were obtained from Hoffmann-La Roche, Nutley, NJ, U.S.A. and were of pharmaceutical-grade purity (>99%) for use as analytical standards.

Prepare stock solutions of compounds I, II and III in separate 10-ml volumetric flasks by dissolving 10 mg of each compound in 2 ml of methanol. Sonicate if necessary for 5–10 min for complete solubilization and dilute to volume with methanol. These stock solutions (containing 1 mg/ml) are used to prepare the mixed standard solutions 1–8 by suitable dilutions in methanol to contain the amounts given in Table I.

Aliquots (100 μl) of solutions 1–7 and 50-μl aliquots of solution 8 are added to 5.0 ml of the benzene extract of control plasma and evaporated to dryness. The residues are dissolved in 100 μl of methanol and 10.0-μl aliquots are injected as the matrix external (calibration) standard curve to establish the parameters for reversed-phase HPLC analysis.

Aliquots (100 μl) of standard solutions 1–7 and 50-μl aliquots of standard solution 8 are mixed with 1.0 ml of control plasma 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.

Calibration of compounds I and II by HPLC

Calibration (external standard) curves of the peak-height ratio of I or II to III versus concentration of compound added to the extract of control plasma are constructed. Fresh calibration curves of the matrix external standards are prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Reagents

All reagents must be of analytical-reagent grade (>99% purity). Potassium phosphate buffer (1 *M*, pH 5.4) is prepared by mixing 820 ml of 1 *M* monobasic potassium phosphate (KH_2PO_4) with 180 ml of 1 *M* dibasic potassium phosphate (K_2HPO_4), adjusting to pH 5.4 with 0.1 *N* phosphoric acid or 0.1 *M* potassium hydroxide as required; potassium phosphate buffer (1 *M*, pH 4.0) is prepared by mixing 985 ml of 1 *M* KH_2PO_4 with 15 ml of 1 *M* phosphoric acid. This solution is diluted with distilled, deionized water to obtain a concentration of 0.01 *M*, adjusting to pH 4.0 as required. Benzene, methanol and tetrahydrofuran were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Analysis of plasma

Into a 15-ml conical centrifuge tube (PTFE No. 13 stoppered) add 600 ng of compound III (50 μl of solution 8) as the internal standard, 1.0 ml of plasma (vortex), 2 ml of 1 *M* potassium phosphate buffer pH 5.4 (vortex) and extract with 6.0 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 samples process eight 1.0-ml specimens of control plasma; one to be used as a control blank and seven to be used for the preparation of the matrix external standards. In addition, process seven 1.0-ml specimens of control plasma containing 100 μl of standard solutions 1–7 and 50 μl of standard solution 8 containing 600 ng of III (internal standard) as the processed (recovered) standard curve. Centrifuge the samples at 2100 rpm (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 and transfer a 5.0-ml aliquot of the upper organic layer into another 15-ml conical centrifuge tube. Evaporate the organic layer to dryness at 35–40°C in an N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of methanol and transfer into a standard Waters glass insert (Waters Part No. 72704) for auto-injection. Program the auto-injector (WISP 710B) to inject 10 μl for HPLC analysis. Typical chromatograms of plasma extracts are shown in Fig. 2.

Calculations and assay validation

The concentration of I and II in the unknowns was determined by interpolation from a least-squares regression equation [weighted ($1/y$) linear equation:

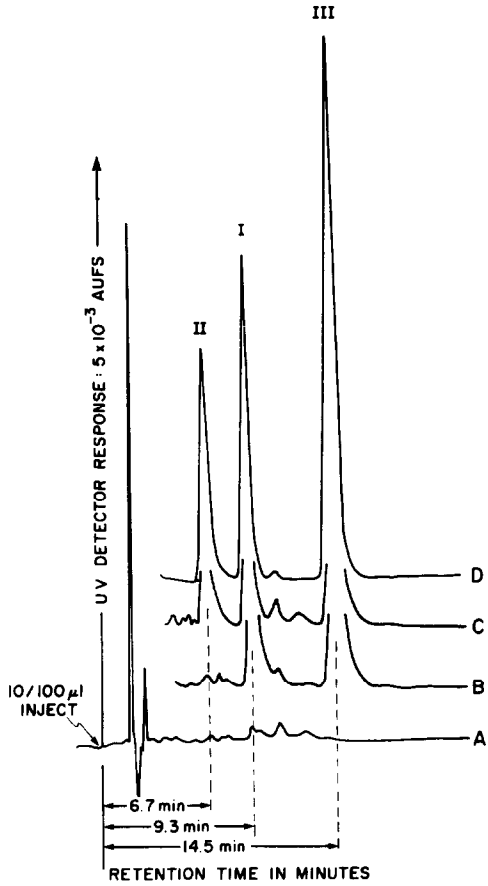

Fig. 2. Chromatograms of HPLC analysis of benzene extracts of (A) control dog plasma, (B) dog plasma following chronic 20 mg/kg oral doses of I, (C) authentic standards recovered from control dog plasma and (D) authentic standards of I, II and III.

$y = mx + b$] of the calibration data (processed by a Hewlett-Packard Model 3357B laboratory automation system) of the recovered standards processed along with the unknowns using peak-height ratios (peak height of compound I or II to peak height of internal standard III) versus concentration of I or II per ml of plasma. Typical calibration curves for I and II ($y = 0.0035x + 0.015$ and $y = 0.0011x + 0.019$, respectively) were linear from 20.0 to 2000 ng of I and from 40.0 to 3000 ng of II per ml of dog plasma, respectively, using a 1.0-ml specimen. The correlation coefficients (r) were 0.999 and 0.998 and the average deviations from the line were 3.2 and 10.1% for I and II, respectively. The mean intra- and inter-assay coefficients of variation (C.V.) for dog plasma were 3.1 and 4.5% for I and 9.1 and 8.1% for II, respectively. The mean intra- and inter-assay C.V. for rat plasma were 7.2 and 3.4% for I and 5.0 and 5.3% for II, respectively. The mean intra- and inter-assay C.V. in human plasma were 4.0 and 3.3% for I and 5.8 and 6.8% for II, respectively. Intra- and inter-assay validation data for human plasma are tabulated in Table II.

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY IN HUMAN PLASMA

Concentration added (ng/ml)	Intra-assay variability			Inter-assay variability		
	Concentration found (mean ± S.D.) (ng/ml)	*n*	C.V. (%)	Concentration found (mean ± S.D.) (ng/ml)	*n*	C.V. (%)
Compound I						
20.0	18.5 ± 1.9	4	10.2	19.7 ± 1.3	4	6.5
30.0	29.0 ± 1.2	4	4.1	30.0 ± 0.8	4	2.8
40.0	4.2 ± 1.1	4	2.5	41.4 ± 1.0	4	2.4
100	100 ± 6.9	4	6.9	97.5 ± 3.4	4	3.5
200	200 ± 7.8	4	3.9	199 ± 1.1	4	0.5
400	446 ± 5.7	4	1.3	412 ± 32.6	4	7.9
1000	990 ± 23.8	4	2.4	982 ± 10.4	4	1.1
2000	1970 ± 9.4	4	0.5	2010 ± 39.8	4	2.0
Mean			4.0			3.3
Compound II						
40.0	37.8 ± 4.4	8	11.6	44.7 ± 4.3	3	9.6
60.0	59.3 ± 7.6	4	12.8	64.8 ± 4.5	4	7.0
150	169 ± 10.8	4	6.4	152 ± 13.7	4	9.0
300	298 ± 11.9	4	4.0	287 ± 12.5	4	4.4
600	644 ± 19.2	4	3.0	592 ± 57.0	4	9.6
1500	1470 ± 24.9	4	1.7	1470 ± 64.7	4	4.4
3000	2980 ± 39.4	4	1.3	3050 ± 115	4	3.8
Mean			5.8			6.8

The determination of the recovery (%) was calculated (with each analytical experiment) by comparing the absolute response (peak height) of the processed (recovered) standards to the absolute response (peak height) of the matrix external standards.

RESULTS AND DISCUSSION

A sensitive and selective HPLC assay was developed for the determination of compounds I and II from 1.0 ml of plasma using UV detection at 254 nm for quantitation. This method enabled the rapid and accurate quantitation of compounds I and II for routine analysis of the large number of samples obtained during preclinical pharmacokinetic and toxicological studies. The major UV absorption band of compounds I, II and III occurs at 245–260 nm as shown in Fig. 3. The Waters Model 440 absorbance detector used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp allowed for quantitation of I, II and III in the nanogram concentration range.

Reversed-phase HPLC analysis was the method of choice, since it is a simple three-step operation which involves selective extraction, sample concentration and direct analysis by HPLC which insures optimum resolution, peak symmetry

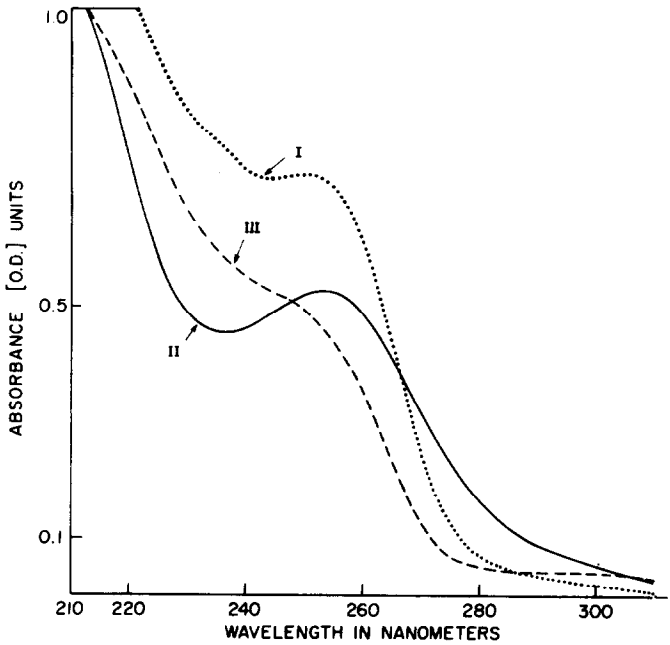

Fig. 3. UV absorption spectra of compounds I, II and III in methanol.

and sensitivity of compounds I, II and III. The order of retention times of compounds I and II was found to vary depending upon the individual Waters μBondapak C_{18} column used, as well as the specific composition of the mobile phase employed. For this reason, it is imperative to positively identify the retention times of the compounds of interest for each individual Waters μBondapak C_{18} column.

Compound III, the fluoronated dehydroxy analogue of I, was chosen as the internal standard in the assay, due to its similar extraction and chromatographic behavior as compounds I and II. Compound III has not been identified as a metabolite of compound I.

Percentage recovery and sensitivity limits

The overall recovery of compounds I and II was of the order of 98.3 ± 9.4 and 59.7 ± 15.7% for dog plasma, 86.0 ± 14.7 and 52.8 ± 15.1% for rat plasma and 98.1 ± 9.3 and 66.9 ± 18.0% for human plasma, respectively. The sensitivity limit of I and II is 20.0 and 40.0 ng per ml of plasma, respectively, using UV detection at 254 nm.

Stability studies on compound I

The stability of compound I was demonstrated at ambient temperature (24°C) for 24 h following its addition to human, dog and rat plasma. The mean changes in recovered compound from human, dog and rat plasma were ±7.8, ±3.6 and ±5.8%, respectively (Table III). Compound I is stable under the sample preparation techniques described with no special precautions required.

TABLE III

STABILITY OF COMPOUND I IN HUMAN, DOG AND RAT PLASMA AT AMBIENT TEMPERATURE (24°C)

Amount added: 200 ng per 0.5 ml; $n=3$. Percentages change compared to 0 h.

Time (h)	Human		Dog		Rat	
	Mean concentration found (ng/ml)	Percentage change	Mean concentration found (ng/ml)	Percentage change	Mean concentration found (ng/ml)	Percentage change
0	199	—	177	—	190	—
1	235	+18	175	−1.1	166	−12.6
4	194	−2.5	162	−8.5	183	−3.7
24	205	+2.9	179	+1.1	192	+1.1
Mean		±7.8		±3.6		±5.8

The stability of compound I was also demonstrated by the reanalysis of previously assayed dog plasma samples stored over a three-month interval at −17°C. The mean changes observed (±8.7 and ±7.4%) indicated no change in the stability of compound I over the study interval (Table IV). A stability study in human, dog and rat plasma stored at −17 and −70°C for up to six months is currently under investigation.

Application of the method to biological specimens

The HPLC method was applied to determine the plasma concentration–time profile of I and II in three dogs receiving doses of I, as either a single 2.5 mg/kg intravenous dose in PEG-400, a 10.2 mg/kg oral solution in PEG-400 or a 10.0 mg/kg oral capsule formulated with 10% Primojel. Plasma concentrations of compound I (Table V) as low as 0.021 μg/ml were determined. Compound II was

TABLE IV

STABILITY OF COMPOUND I IN DOG PLASMA AT −17°C

Percentages change compared to initial concentration found.

Time (h)	Initial concentration found (μg/ml)	Concentration at six weeks at −7°C (μg/ml)	Percentage change	Concentration at three months at −17°C (μg/ml)	Percentage change
0	—	—	—	—	—
0.75	3.5	3.1	−11.4	2.9	−17.1
1.0	3.6	3.2	−11.1	3.2	−11.1
1.5	3.4	2.8	−17.6	2.8	−17.6
2.0	2.8	2.8	±0.0	2.8	±0.0
3.0	2.2	2.1	−4.5	2.2	±0.0
4.0	1.6	1.7	+6.3	1.7	+6.3
6.0	1.0	0.9	−10.0	1.0	±0.0
Mean			±8.7		±7.4

TABLE V

PLASMA CONCENTRATIONS OF COMPOUND I IN THREE DOGS FOLLOWING THE INTRAVENOUS AND ORAL ADMINISTRATION OF COMPOUND I

N.S.T. = no sample taken; N.M. = non-measurable: < 0.020 μg/ml.

Time	Concentration (μg/ml)		
	Dog 1 (2.5 mg/kg intravenously)	Dog 2 (10.2 mg/kg oral solution)	Dog 3 (10.0 mg/kg oral capsule)
2.5 min	5.3	N.S.T.	N.S.T.
5 min	4.8	N.S.T.	N.S.T.
10 min	4.2	2.7	N.M.
20 min	3.8	2.7	N.M.
30 min	3.9	3.0	N.M.
45 min	3.5	3.3	N.M.
1 h	3.6	3.4	0.031
1.5 h	3.4	3.5	0.028
2 h	2.8	3.3	0.047
3 h	2.2	2.7	0.044
4 h	1.6	2.7	0.040
6 h	0.97	2.0	0.042
8 h	0.41	1.5	N.M.
10 h	0.21	0.86	N.M.
24 h	N.M.	N.M.	0.11
28 h	N.M.	N.M.	0.021
32 h	N.M.	N.M.	N.M.
48 h	N.M.	N.M.	N.M.
56 h	N.M.	N.M.	N.M.
72 h	N.M.	N.M.	N.M.

observed only at 2 h (0.092 μg/ml) following the intravenous dose of I and at 1 h (0.040 μg/ml) following the administration of I as an oral solution. A distinct absorption, distribution and elimination phase can be seen in the plasma concentration data from the solution. In contrast, the plasma concentration–time profile with the capsule is suggestive of a prolonged absorption that may be a consequence of a poor dissolution rate for this dosage form.

The plasma method was also applied for the determination of I in urine samples from the same study. The correlation coefficient (r) of the recovered standards was 0.999 and the average deviation from the line was 1.3% over the concentration range 30.0–2000 ng/ml of urine. The urinary excretion of unconjugated I was insignificant (less than 1% of the total dose) for all the doses administered. Attempts at determining the concentrations of unconjugated II and of conjugated I and II were hindered by extracted endogenous impurities.

Due to the fact that compound II was shown not to be present in vivo in significant amounts, a second HPLC method was developed for the determination of compound I per se from 1.0 ml of plasma using UV detection at 254 nm for quantitation. This method was identical to the previously mentioned HPLC method which was used for the quantitation of both compounds I and II, except for the substitution of 4.8 *M* saturated potassium chloride pH 6.1 as the buffering

agent during the extraction. This procedure was shown to selectively extract compounds I and III from dog plasma in the presence of compound II. The overall recovery of compound I from dog plasma is 89.6 ± 4.4% and the sensitivity limit is 20.0 ng/ml of plasma. The intra- and inter-assay variability of I over the concentration range 20.0–2000 ng/ml of plasma showed a mean coefficient of variation of 5.8 and 7.1%, respectively, using least-squares (power: $y = mx^b$) regression analysis.

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