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Note

High-performance liquid chromatographic measurement of (*R*)-4-[3-[(2-hydroxy-2-phenethyl)amino]-3-methylbutyl]benzamide monohydrochloride (LY195448) and its *p*-hydroxy metabolite in human plasma and urine

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(*R*)-4-[3-[(2-Hydroxy-2-phenethyl)amino]-3-methylbutyl]benzamide monohydrochloride, LY195448, I (Fig. 1), is a phenethanolamine derivative that inhibits tumor cell growth in vitro when incubated with a number of established cell lines [1]. This compound has some structural and pharmacological properties in common with adrenergic drugs. For example, both the parent compound and its *p*-hydroxy metabolite (II, LY135114, Fig. 1) bind to β-adrenergic receptors and cause a decrease in blood pressure in rats [2]. As part of a Phase 1 trial in patients with cancer we developed a reversed-phase paired-ion high-performance liquid chromatography (HPLC) assay which we used to measure plasma and urine levels of I and II.

EXPERIMENTAL

Reagents and chemicals

I, II and the internal standard (I.S., Fig. 1) were supplied by Lilly Research Labs. (Indianapolis, IN, U.S.A.). 1-Heptanesulfonic acid, sodium salt (98%

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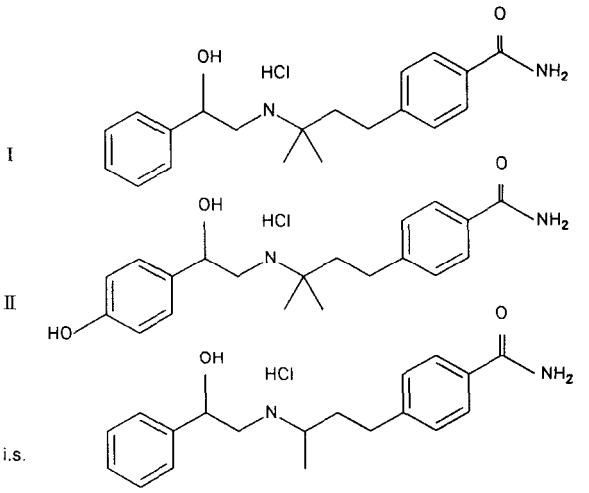

Fig. 1. Structures of I, its *p*-hydroxy metabolite (II) and the internal standard (i.s.) for the HPLC assay.

pure) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Monobasic potassium phosphate was from Sigma (St. Louis, MO, U.S.A.). Orthophosphoric acid (85%), hydrochloric acid and sodium carbonate were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). HPLC-grade acetonitrile and ethyl acetate were purchased from American Burdick and Jackson (Muskegon, MI, U.S.A.). HPLC-grade water was obtained by filtration of dionized water through a Milli-Q water system from Millipore (Milford, MA, U.S.A.).

Apparatus

We used a 6000A solvent delivery system, a WISP Model 712 automatic injector, a Model 481 Lambda-Max spectrophotometer and a Model 740 data module, all from the Waters Chromatography Division of Millipore. An Ultrasphere IP (150 mm×4.6 mm I.D., 5 μm particle size) column (Beckman Instruments, San Ramon, CA, U.S.A.) and a Guard-Pak pre-column module with a μBondapak C_{18} insert were used.

Chromatographic conditions

The mobile phase consisted of 0.05 *M* heptanesulfonic acid and 0.05 *M* monobasic potassium phosphate (pH 3.5) in acetonitrile–water (25:75, v/v) and was delivered isocratically at a flow-rate of 1 ml/min. The injection volume was 50 μl. The detection wavelength used was 233 nm.

Sample preparation

Plasma and urine samples were stored at −20°C until analyzed. We then used a three-step extraction procedure employing disposable borosilicate glass

tubes. I.S. (1 μg; 10 μl of a 100 μg/ml solution), sodium carbonate (1 ml, 1 *M*) and ethyl acetate (5 ml) were added to 1 ml of plasma or urine, and the mixture was extracted for 5 min on an Eberbach (Ann Arbor, MI, U.S.A.) reciprocal shaker. The organic phase was separated by centrifugation at 500 *g*, with refrigeration at 4°C. Hydrochloric acid (1 ml, 1 *M*) was added to the organic phase, and the mixture was extracted and centrifuged as before. The organic phase was then discarded, and saturated sodium carbonate (1 ml) and ethyl acetate (5 ml) were added to the aqueous phase. After extraction and centrifugation, 4.5 ml of the organic phase were evaporated under nitrogen at 50°C (N-evap Model 112, Organomation Assoc., South Berlin, MA, U.S.A.). The residue was reconstituted in 200 μl of mobile phase solution.

Standard curves

Stock solutions (1 mg/ml) of I, II and I.S. were prepared in methanol. Each was diluted to 100 and 10 μg/ml with water. The concentration ranges used for the standard curves in plasma were 0.05–20 μg/ml for I and 0.025–2 μg/ml for the *p*-hydroxy metabolite. Standard curves in urine were prepared with concentrations ranging from 0.05 to 20 μg/ml for both compounds. At least five standards and one control sample were used for each standard curve.

Patient samples

Compound I was administered intravenously as a 5-min infusion. Plasma samples were obtained at 30 min following each of thirteen doses in eight patients. In three of these patients (one at each dose level), a more extensive plasma sampling and urine collection schedule was followed for 24 h after drug administration.

Pharmacokinetics

Non-linear regression analysis was performed on plasma concentration versus time data using the computer program, PCNONLIN [3]. Initial estimates were obtained from a curve-stripping program (ESTRIP [4]). Pharmacokinetic parameters were calculated using the formulae for multi-compartment models [5].

RESULTS

Chromatograms

Typically, retention times were approximately 6, 12 and 15 min for the *p*-hydroxy metabolite, I.S. and I, respectively. The extraction efficiency from saline for all three compounds was approximately 80%. Figs. 2 and 3 show chromatograms of the three compounds in extracted plasma and urine samples. There were no peaks interfering with analyses of either I or II due to endogenous urine or plasma components.

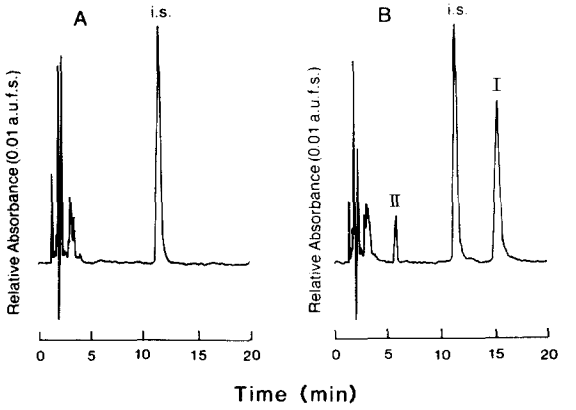

Fig. 2. Chromatograms of I and its *p*-hydroxy metabolite (II) in extracted plasma samples from a patient given 50 mg/m^2 I as a 5-min infusion. Samples were spiked with 250 ng internal standard (i.s.). (A) Pre-injection sample; (B) sample taken 11 min after the end of infusion.

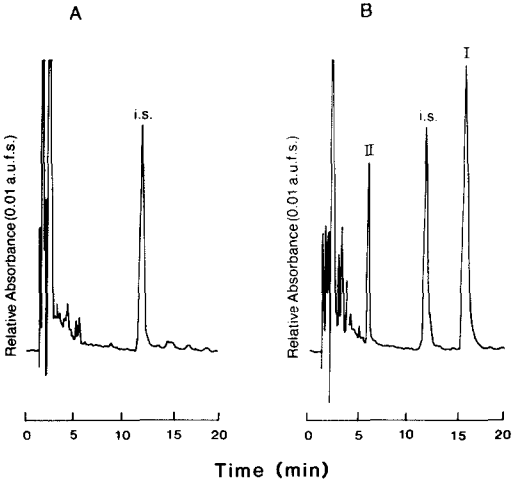

Fig. 3. Chromatograms of I and its *p*-hydroxy metabolite (II) in extracted urine samples from a patient given 75 mg/m^2 I as a 5-min infusion. Samples were spiked with 250 ng internal standard (i.s.). (A) Pre-injection sample; (B) urine collected from 6 to 12 h after infusion.

Standard curves

Standard curves for I were constructed by plotting peak-area ratios of the drug and I.S. versus the concentration of drug in the standard. Similar methods were used for the *p*-hydroxy metabolite. Standard curves for I in plasma and urine were curvilinear in the concentration range used. The standard curve was therefore divided into two linear segments, 0–1 and 1–20 μg/ml. Typical equations describing standard curves (based on peak area) of I extracted

from plasma are $y=1.68\cdot10^4+6.82\cdot10^5x$ (0.01–1.0 μg/ml) and $y=-6.54\cdot10^4+7.74\cdot10^5x$ (1.0–20 μg/ml). Representative standard curves of I extracted from urine are $y=4.11\cdot10^4+2.11\cdot10^6x$ (0.01–1.0 μg/ml) and $y=3.84\cdot10^4+1.76\cdot10^6x$ (1.0–20 μg/ml). Correlation coefficients for standard curves of I extracted from both plasma and urine always exceeded 0.99. Concentrations of the *p*-hydroxy metabolite never exceeded 1 μg/ml; thus, only the lower portion of the standard curve was required. However, higher metabolite concentrations were seen in urine and the standard curves were fitted to two linear segments as for the parent compound, $y=2.21\cdot10^6+3.66\cdot10^6x$ (0–1 μg/ml) and $y=3.41\cdot10^6+1.99\cdot10^6x$ (1–20 μg/ml), based on peak areas. Correlation coefficients for II extracted from either plasma or urine were greater than 0.98. Calculated values for quality control standards were consistently within 10% of actual concentrations. When control samples were assayed repeatedly on seven different days, inter-assay coefficients of variation of 4.8 and 3.9% were obtained for I at 0.05 and 5 μg/ml, respectively. Interassay coefficients of variation obtained for II were 2.1 and 5.3% at 0.025 and 5.0 μg/ml, respectively. The sensitivity of the assay for I was 50 ng/ml; for II, the sensitivity was 25 ng/ml. These lower limits were always obtained at signal-to-noise ratio of 5 or greater.

Patient samples

Fig. 4 shows concentrations of I in patient samples at doses of 50 and 100 mg/m^2. At the low dose, I levels were below the assay sensitivity at 8 h while at the higher dose, levels could be measured for 24 h. Plasma I versus time curves were biphasic and were best described by a two-compartment model. *p*-Hydroxy metabolite formation varied considerably between patients. Fig. 5 shows concentrations of the parent compound and the *p*-hydroxy metabolite in plasma samples from two patients given the intermediate dose (75 mg/m^2). Peak levels of the *p*-hydroxy metabolite were generally seen 15 min after the

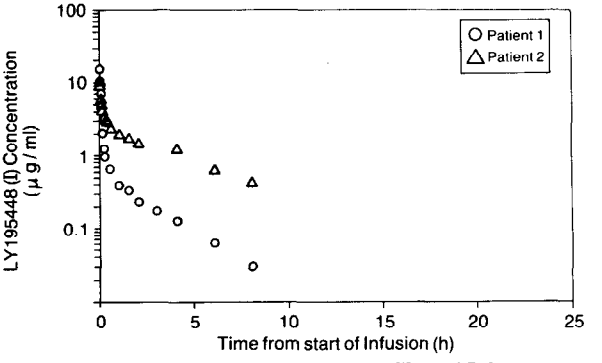

Fig. 4. Plasma concentration–time profiles of I from two patients following 5-min intravenous infusions of 50 or 100 mg/m^2 I.

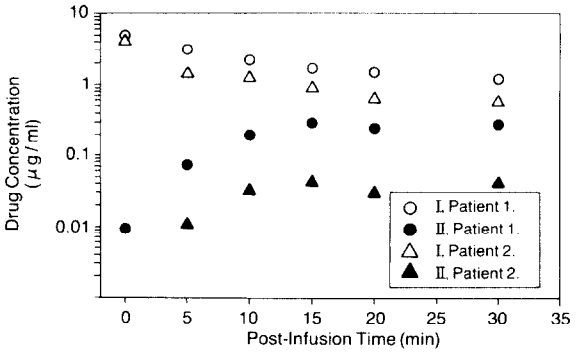

Fig. 5. Plasma concentration–time profiles of I and its *p*-hydroxy metabolite (II) in two patients following a 5-min intravenous infusion of 75 mg/m^2 I.

TABLE I

PHARMACOKINETIC PARAMETERS OF I

Parameter	Patient 1	Patient 2	Patient 3	Mean ± S.D.
Dose (mg/m^2)	50	75	100	
Initial half-life (min)	1.28	0.93	0.93	1.05 ± 0.20
Final half-life (h)	0.92	1.61	2.38	1.64 ± 0.73
Volume of central compartment (l/m^2)	1.56	2.95	3.00	2.50 ± 0.81
Apparent volume of distribution (l/m^2)	26.6	32.2	29.9	29.6 ± 2.8
Volume at steady state (l/m^2)	16.8	29.2	28.2	24.7 ± 6.9
Total clearance (l/[min·m^2])	0.33	0.23	0.14	0.24 ± 0.10

end of infusion. At this time, metabolite concentrations were between 5 and 20% of those of the parent compound, except in one patient whose plasma contained no detectable amount of the metabolite. The extent of metabolite formation was consistent when the same patient was given another course of the drug.

Pharmacokinetics

Table I shows the pharmacokinetic parameters of I in the three patients whose plasma samples were taken for 24 h. Urinary excretion in 24 h was 18 ± 10% of dose for the parent compound. Less than 2% of the administered dose was excreted as the *p*-hydroxy metabolite.

DISCUSSION

The HPLC assay we described enabled us to measure I and its *p*-hydroxy metabolite simultaneously. This would allow the monitoring of both com-

pounds when the parent drug is administered to patients. Other related compounds, such as the dihydroxy, *m*-hydroxy and *p*-hydroxy–*m*-methoxy metabolites, have also been detected when I is administered to rodents [6], but none of these were detected in our patient samples. Thus, the metabolism of this compound appears to be species dependent.

Standard curves were fitted to two linear segments: 0–1 and 1–20 μg/ml. Another approach would have been to dilute samples that had drug concentrations greater than 1 μg/ml. However, since levels of the *p*-hydroxy metabolite in plasma were less than 1 μg/ml, the concentration of this metabolite in diluted samples would have been below the sensitivity of the assay, and each sample would have had to be assayed twice. The calculation method used in our assay gives results comparable to the dilution method and has the advantages of being less time-consuming and more cost-effective. Three-step extraction, although cumbersome, was necessary since a one-step extraction would not remove all the interfering peaks in plasma or urine samples.

Studies done by Ho et al. [2] have shown that the *p*-hydroxy metabolite shares the hypotensive potential of I. We found that levels of this metabolite varied considerably among patients. An attempt was made to correlate metabolite formation with various measures of hepatic function; however, no obvious association was found.

Pharmacokinetics of I in humans were best described by a two-compartment model. The drug's initial half-life was only 1 min, which indicates that drug distribution was very rapid. The final half-life was 1.6 ± 0.7 h, reflecting rapid excretion of the drug. However, only 20% of the administered dose was recovered in the urine by 24 h. Total clearance of the drug exceeded the glomerular filtration rate [7]. Thus, most of the compound is excreted via extra-renal routes or as metabolites that have not been identified.

The apparent volume of distribution and the volume of distribution at steady state were similar and were equivalent to the volume of total body water [8]. The volume of the central compartment was similar to plasma volume [8]. This pattern of distribution, together with the short half-lives, suggests that the drug may not be bound to any particular tissue in humans and is rapidly excreted. Thus, cumulative toxicity is unlikely to occur.

The HPLC assay described herein allows simultaneous measurement of I and its *p*-hydroxy metabolite. Monitoring levels of these compounds in patients undergoing further trials of I may be necessary because of their hypotensive potential.

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