# Dose-Dependent Pharmacokinetics of L-693,612, a Carbonic Anhydrase Inhibitor, Following Oral Administration in Rats

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The disposition of L-693,612, a carbonic anhydrase inhibitor, was examined in rats following oral doses of 0.05 to 25 mg/kg. Area under the blood concentration–time curve (AUC) increased linearly with dose up to 0.25 mg/kg. However, the linear range did not extend to 5 and 25 mg/kg doses; AUC rose only 10-fold overall despite a 500-fold increase in dose. A similar pattern of disproportionality occurring after i.v. administration indicated that the nonlinear behavior after oral doses was not due to dose-limited absorption, but rather it arose because blood clearance increased with dose. Concentration-dependent erythrocyte/plasma partitioning arising from saturation of binding to erythrocyte carbonic anhydrase could explain the dose-dependent blood clearance. At blood concentrations (<25 $\mu M$) achieved in the linear dose range, L-693,612 was extensively sequestered in red blood cells, bound to carbonic anhydrase, with a constant low free fraction in plasma available for elimination. At doses which saturated the binding capacity of carbonic anhydrase, blood clearance increased, since for low hepatic extraction compounds, the rate of elimination is dependent upon the free fraction in blood. Dose-dependent increases in distribution volumes were consistent with the view that high-affinity binding to carbonic anhydrase confined this compound largely to blood volume at low doses, but saturation of binding sites increased availability to peripheral tissues after high doses. Increasing the dose had a minimal effect on terminal half-life because it reflected the concentration–time profile during a period of linear distribution into erythrocytes.

**KEY WORDS:** nonlinear pharmacokinetics; carbonic anhydrase inhibitors.

## INTRODUCTION

L-693,612 [(*S,S*)-4-propylamino-5,6-dihydro-6-(3-methoxy-propyl)-7,7-dioxide-4H-thieno(2,3-b)thiopyran-2-sulfonamide] is a potent inhibitor of human carbonic anhydrase II (CA-II). The compound may have use in glaucoma treatment through inhibition of ocular CA-II, which decreases aqueous humor secretion and lowers intraocular pressure. Although the ocular enzyme is the therapeutic target, more than 90% of CA in the body is located in red blood cells (RBC), where it catalyzes hydration of carbon dioxide to bicarbonate during respiration (1,2).

Previously, MK-417, another CA-II inhibitor of the sulfonamide class, was shown to exhibit nonlinear pharmacokinetics following intravenous administration (3). The disposition of these compounds after oral administration is not well studied and the lower peak blood concentrations that may result after oral administration could alter the magnitude of nonlinearity. In the present studies, the pharmacokinetic behavior of L-693,612 was examined in rats following both oral and i.v. administration.

## MATERIALS AND METHODS

Unlabeled and radiolabeled L-693,612 (hydrochloride salt) were synthesized at Merck Research Laboratories (West Point, PA and Rahway, NJ). The position of the $^{14}C$-label was the terminal methoxy substituent of the methoxypropyl side chain (Fig. 1).

*Pharmacokinetic Studies.* Male Sprague–Dawley rats (225 to 300 g) purchased from Taconic Farms (Germantown, NY) were housed under standard conditions with free access to food and water. On the day preceding drug administration, a silicone rubber tubing/polyethylene cannula was implanted in a jugular vein under pentobarbital (40 mg/kg i.p.) anesthesia (4). After an overnight fast, oral doses of 0.05, 0.25, 5, and 25 mg/kg of L-693,612 (5 mL/kg in distilled water) were administered in solution by oral gavage using steel ball-tipped feeding needles. Different groups of animals received the same doses dissolved in isotonic saline by bolus injection via the jugular vein cannula. Heparinized blood samples (0.5 mL) were drawn prior to and at 0.5, 1, 2, 6, 24, 48, 72, 96, 120, 144, 168, and 240 hr and stored at −20°C until analysis.

Terminal half-life was determined by linear regression of the log-linear portion of concentration–time profiles. Blood concentrations were measured because results from a preliminary study indicated that concentrations in plasma were very low relative to blood. Blood clearance was calculated as: $CL_{total} = Dose_{i.v.}/AUC$, where AUC is the area under the blood concentration–time curve from time 0 to infinity. Steady-state apparent volume of distribution was calculated as $V_{ss} = [(Dose_{i.v.} \times AUMC)/AUC^2]$ (5). Since kinetic behavior was subsequently shown to be nonlinear obtained parameters are time-averaged values. Areas were calculated by LaGrange interpolation with extrapolation to time infinity (6). Bioavailability was calculated as $F = (AUC_{oral}/AUC_{iv}) \times 100$, measured after oral and i.v. administration of the same dose.

*RBC/plasma Partitioning* ex Vivo *and* in Vitro. Pooled freshly drawn heparinized rat blood was preincubated at 37°C with gentle shaking for 30 min and then L-693,612 was added in final concentration of 6 to 20 μg/mL. After 10 min, 2 mL blood was centrifuged for 1 min and the plasma immediately separated. A preliminary experiment determined that equilibrium was achieved by 3 min after the addition of L-693,612. Blood and plasma concentrations of L-693,612 were determined by HPLC. Concentration in red blood cells was calculated as $C_{rbc} = [C_{blood} - [C_{plasma} \times (1\text{-hematocrit})]/\text{hematocrit}]]$, where $C_{blood}$ and $C_{plasma}$ are the concentration in blood and plasma, respectively.

For *ex vivo* determination of RBC/plasma partitioning, a 25 mg/kg dose of L-693,612 was administered by bolus i.v. injection. Blood samples (0.3 mL each) were drawn 5, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min after the injection, then immediately centrifuged, and the plasma was separated. He-

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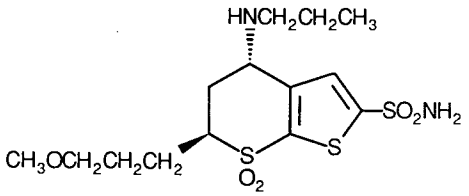


Fig. 1. Structure of L-693,612.

matocrit at each sampling time was determined from an aliquot of the remaining blood. Equal volumes of blood and plasma were pooled from five rats because of plasma assay considerations.

*HPLC Analysis for L-693,612 in Blood and Plasma.* L-693,612 concentrations were determined by a high-pressure liquid chromatographic method. Briefly, after immersion in boiling water to denature proteins, blood or plasma was buffered to pH 6.5 with 0.2 *M* sodium phosphate. L-693,612 and internal standard (L-694,743, a structural analogue) were extracted into an organic phase that consisted of toluene/ethyl acetate/2-propanol, 49/50/1 (v/v/v), then back extracted into aqueous phase at acidic pH (recovery, >85%). After neutralization to pH 6 to 8, aliquots of the aqueous phase were analyzed using a Zorbax Rx-C18 250 × 4.6-mm column (Mac-Mod, Chadds Ford, PA) with UV detection at 256 nm. The mobile phase consisted of 25 m*M* sodium phosphate (pH 6.5)/acetonitrile/triethylamine, 69/31/0.15 (v/v/v) at a flow rate of 1.5 mL/min. After elution of internal standard, acetonitrile content was increased to 67% for 8 min to remove strongly retained endogenous components. Retention time of L-693,612 and internal standard were 10.5 and 15.6 min, respectively. Assay error and variability were <10% ($n$ = 5) over the 25 to 10000 ng/mL range. The limit of quantification was 25 ng/mL using 0.5 mL blood or plasma.

*Plasma Protein Binding.* Ultrafiltration was used to determine protein binding of $^{14}$C-L-693,612 in rat plasma at concentrations observed at these doses. Control plasma was freshly obtained, the pH checked, and the plasma used immediately. L-693,612 was added to plasma at final concentrations of 50, 250, 500, 1000, 2500, and 5000 ng/mL. After incubation at 37°C for 15 min, aliquots of plasma were transferred to Centrifree Micropartition devices (30,000 molecular weight cutoff; Amicon, Danvers, NY) and centrifuged at 1500*g* for 15 min at 37°C. Plasma and ultrafiltrate concentrations were determined by direct addition to scintillation cocktail and assayed for radioactivity by liquid scintillation spectrometry. A preliminary study determined that adsorption to the ultrafiltration device was minimal (<1%).

*Statistical Analysis.* One-way analysis of variance was used to assess the effect of dose on pharmacokinetic parameters. A log transformation of data was made to correct for unequal variances across dose levels. Differences between dose levels were evaluated using the Tukey multiple-comparisons test (7).

## RESULTS

The area under the concentration–time curve increased proportionally to the dose after oral administration of 0.05 to 0.25 mg/kg (Figs. 2 and 3). However, increases in AUC were less than proportional at higher doses. Overall, as the dose was increased 500-fold, AUC and maximum blood concentration increased only about 10-fold, and administration of 5 and 25 mg/kg oral doses resulted in almost-indistinguishable AUC and maximum blood concentrations (Table I). An identical pattern of disproportionality was observed after i.v. administration of the same doses. Since the elimination was nonlinear, the clearance and volume of distribution parameters obtained represent time-averaged values. The rate of elimination, as measured by time-averaged blood clearance, was unchanged over the 0.05 to 0.25 mg/kg range, however, it increased more than 50-fold over the dose range examined ($P < 0.05$ ANOVA; Table I). The calculated steady-state apparent volume of distribution was 80 mL/kg and constant up to at least 0.25 mg/kg, but increased in a dose-dependent manner to a maximum observed value of 3670 mL/kg at the 25 mg/kg dose. Bioavailability from oral solution after 0.05 and 0.25 mg/kg doses was 72 and 90%, respectively.

Concentration dependence of RBC/plasma partitioning was assessed *in vitro* and *ex vivo* as an explanation for the nonlinear pharmacokinetic behavior. RBC/plasma concentration ratio *in vitro* was relatively constant at blood concentrations up to ca. 12 μg/mL, but thereafter it decreased in a concentration-dependent manner from more than 400 to less than 6 at 20 μg/mL ($P < 0.05$), consistent with the view that the dose-dependent increase in blood clearance arose from

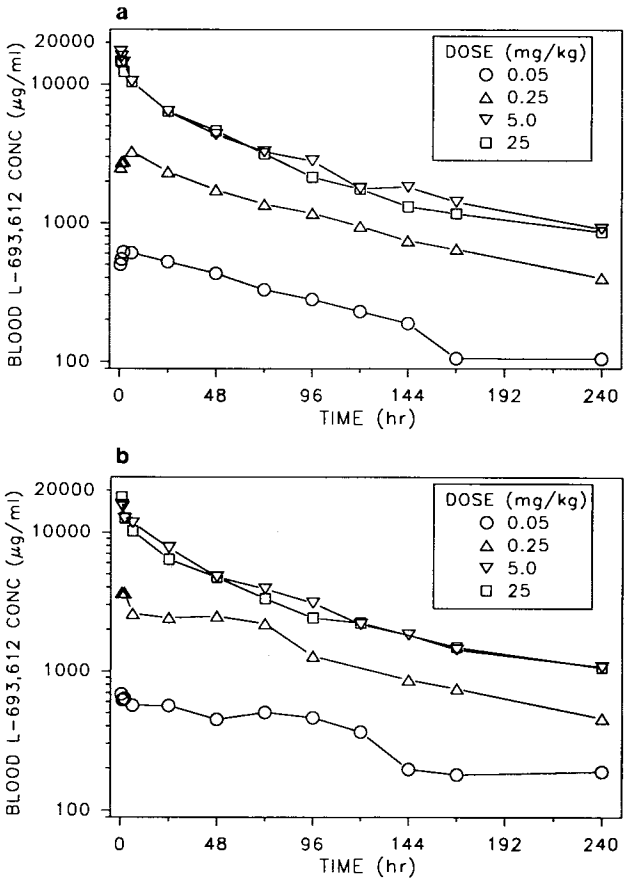


Fig. 2. Blood concentrations after (a) oral and (b) intravenous administration of 0.05 to 25 mg/kg L-693,612. Shown as the mean of four to eight animals.

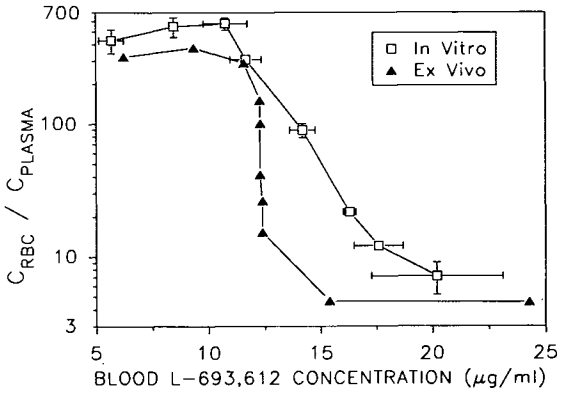


Fig. 3. RBC/plasma partitioning of L-693,612 determined *in vitro* and *ex vivo*. *In vivo* determinations ($n$ = 4) are shown as mean ± SD. *Ex vivo* determinations were made with equal volumes of blood and plasma pooled from five rats.

an increased fraction in blood available for elimination (Fig. 3). Similarly, RBC/plasma partitioning measured following a 25 mg/kg dose was concentration dependent. Although *ex vivo* and *in vitro* measures were comparable at blood concentrations below 12 and greater than 16 μg/mL, at intermediate concentrations, the *ex vivo* parameter was as much as 10-fold lower than that seen *in vitro* at equivalent blood concentrations (Fig. 3).

L-693,612 was moderately bound to plasma proteins. The free fraction was 41 to 54% and appeared to be independent of concentration over the 50 to 5000 ng/mL range.

## DISCUSSION

In the present studies with L-693,612, the maximum blood concentration and AUC achieved after oral administration increased much less than dose proportionally (Table I). Such observations are typical when absorption is dose limited because of limited drug solubility in gastrointestinal fluids or saturation of intestinal transport pathways that lower the fraction of dose absorbed (8–10). However, in the present case the nonlinear behavior was not due to dose-dependent absorption since an identical nonlinear pattern was observed after i.v. administration (Table I).

Rather the lack of a proportional increase in systemic exposure arose because blood clearance increased with increasing dose. The dose-dependent blood clearance was consistent with concentration-dependent RBC/plasma partitioning of L-693,612 arising from saturation of binding to carbonic anhydrase in red blood cells. Although erythrocyte partitioning was in the nonlinear range for a comparatively brief period (up to 4 hr postdose; Fig. 3), it had a profound effect on AUC. Partitioning of L-693,612 into erythrocytes was markedly nonlinear at concentrations greater than 30 μ*M* (ca. 12 μg/mL) *in vitro* and *ex vivo* (Fig. 3). This is consistent with the view that the binding capacity of CA-II was the primary determinant of pharmacokinetic behavior because blood enzyme concentrations have been previously estimated at ca. 30 μ*M* and the enzyme possesses a single binding site (1,11). At blood concentrations (<25 μ*M*) achieved in the linear dose range, L-693,612 is extensively sequestered in RBC, bound to CA-II, with a constant low free fraction in plasma available for elimination (Fig. 3). However, at higher concentrations the binding capacity of CA-II was exceeded, leading to an increased free fraction in blood. Clearance increased since for low-hepatic extraction compounds, the rate of elimination is dependent upon the free fraction in blood. Indeed the increase in clearance was such that blood concentration–time profile was similar after the 5 and 25 mg/kg doses (Fig. 2). Instantaneous elimination rates during the initial 2-hr period after drug administration probably varied much more than the 55-fold increase in time-averaged clearance measured here since fractional distribution in plasma during this time varied approximately two orders of magnitude (Fig. 3 and Table I). The terminal $t_{1/2}$ was unchanged with dose because it reflected the concentration–time profile occurring after 48 hr, at which time concentrations were less than 13 μ*M* (5 μg/mL), well below the physiological concentration of CA-II.

Dose-dependent increases in clearance are also observed with drugs that exhibit concentration-dependent plasma protein binding. In the linear concentration range, the intravenously administered antibiotic ceftriaxone is extensively bound to plasma proteins, but at high concentrations protein binding is saturated and the peak free fraction in plasma increases from 3 to 15% (13). Saturable plasma protein binding did not contribute to the results seen here with L-693,612 since binding was moderate and independent of concentration, further support for the view that binding to carbonic anhydrase was the primary determinant of the free fraction in blood.

Reflecting the high affinity for CA-II, the steady-state distribution volume was constant up to doses of 0.25 mg/kg at 80 to 95 mL/kg, which is approximately equal to the blood volume in the rat. Distribution volume increased after higher doses, suggesting that L-693,612 was confined largely to blood after the lower doses, but there was increased distribution to peripheral tissues once blood enzyme sites were saturated. The change in tissue distribution may not have been as great as the change in $V_{ss}$ since the calculation assumes linear clearance (5).

L-693,612 was well absorbed from oral solutions, as shown by the bioavailability of 72–90% measured after the 0.05 and 0.25 mg/kg doses (Table I). Bioavailability was not calculated for the 5 and 25 mg/kg doses, because in the presence of nonlinear RBC/plasma partitioning, blood concentrations are not proportional to the fraction absorbed. At the highest doses of L-693,612, blood AUC was unchanged despite a fivefold higher dose (Table I).

One feature of the erythrocyte partitioning behavior merits further comment. At concentrations less than 10 and greater than 16 μg/mL, *ex vivo* and *in vitro* measures of RBC/plasma ratio were in reasonable agreement. However, at intermediate concentrations the RBC/plasma ratio measured after an i.v. dose of L-693,612 was as much as an order of magnitude lower than the *in vitro* parameter. Potentially this may reflect the formation of active metabolites which compete for binding sites on blood CA-II. At early time points (5 and 15 min following the i.v. dose), which correspond to the highest L-693,612 concentrations, metabolite concentrations would be low relative to that of parent drug. However, as metabolite concentrations rise, they may be able to displace parent drug from CA-II binding sites, effec-

Table I. Dose Proportionality of L-693,612 After Oral and Intravenous Administration of 0.05, 0.25, 5, and 25 mg/kg[a,*]

| | Oral administration | | | Intravenous administration | | | |
|---|---|---|---|---|---|---|---|
| Dose (mg/kg) | AUC (μg * hr/mL) | $C_{max}$ (μg/mL) | Terminal $t_{1/2}$ (hr)[b] | AUC (μg * hr/mL) | $\overline{CL}_{total}$ (mL/hr/kg) | $\overline{V}_{ss}$ (mL/kg) | Terminal $t_{1/2}$ (hr)[b] |
| 0.05 | 71.4 (2.9) | 0.64 (0.02) | 79.4 (9.0) | 99.4 (15.9) | 0.517[a,b] (0.100) | 80.2[a,b] (26.5) | 78.1 (44.5) |
| 0.25 | 339 (73) | 3.32 (0.51) | 87.4[a] (6.8) | 376 (40) | 0.671[c,d] (0.072) | 95.1[c,e] (18.4) | 84.7 (22.3) |
| 5.0 | 797 (51) | 16.7 (1.1) | 80.5 (8.9) | 877 (68) | 5.73[b,d,e] (0.46) | 584[b,d,e] (42) | 63.1 (3.1) |
| 25 | 732 (170) | 15.2 (1.6) | 64.3[a] (7.9) | 867 (18) | 28.8[a,c,e] (0.6) | 3670[a,c,d] (660) | 92.1 (14.9) |

[a] Shown as mean (SD); $n = 4$ to 6 per dose level. Following log-transformation, $\overline{CL}_{total}$, $\overline{V}_{ss}$, and terminal $t_{1/2}$ were evaluated by one-way ANOVA and Tukey's multiple-comparison test. AUC, area under the concentration–time curve from time 0 to infinity; $\overline{CL}_{total}$, time-averaged blood clearance; $\overline{V}_{ss}$, time-averaged steady-state volume of distribution.

[b] Values for half-lives are harmonic means. Standard deviation was calculated using the jackknife method (15).

* Groups bearing the same superscript were judged to be different at $P < 0.05$.

tively lowering the parent drug RBC/plasma ratio. Later, as drug concentrations decline to less than ca. 25 $\mu M$, the available enzyme binding capacity can accommodate both parent drug and metabolites, so there is minimal influence on the partitioning behavior of parent drug. Preliminary studies of metabolic pathways indicate that L-693,612 forms two active metabolites by N-deethylation and O-demethylation (14).

In summary, following oral administration in rats, systemic exposure of L-693,612 increased much less than proportionally to dose. Blood AUC was insensitive to a fivefold increase in dose in the nonlinear range. A similar pattern of disproportionality was observed after i.v. administration, indicating that the nonlinear behavior was due not to dose-limited absorption but, rather, to blood clearance, which increased with dose. Concentration-dependent erythrocyte partitioning arising from saturation of blood CA could explain the dose-dependent blood clearance.

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