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## Pharmacokinetics and $\Delta^1$ -cortienic acid excretion after intravenous administration of prednisolone and loteprednol etabonate in rats

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Detailed pharmacokinetic (PK) studies in rats were performed (i) to compare the PK of prednisolone (PRN) and loteprednol etabonate (LE, a soft corticosteroid) as well as their common inactive metabolite  $\Delta^1$ -cortienic acid ( $\Delta^1$ -CA), (ii) to investigate the excretion of  $\Delta^1$ -CA after PRN and LE administration, and (iii) to investigate the effect of  $\Delta^1$ -unsaturation on the excretion of  $\Delta^1$ -CA versus CA. Following a 10 mg·kg<sup>-1</sup> intravenous bolus dose, the total clearance (CL<sub>tot</sub>) of PRN (27.0 ± 1.4 mL·min<sup>-1</sup> kg<sup>-1</sup>) was significantly lower than that of LE (67.4 ± 11.6 mL·min<sup>-1</sup> kg<sup>-1</sup>) or  $\Delta^1$ -CA (53.8 ± 1.4 mL·min<sup>-1</sup> kg<sup>-1</sup>) indicating that the metabolism/elimination of PRN in the liver (primarily, conjugation) may be less efficient than that of LE (primarily, hydrolysis) or  $\Delta^1$ -CA (unchanged). The volume of distribution (Vd<sub>ss</sub>) of PRN (823 ± 78 mL·kg<sup>-1</sup>) was significantly lower than that of LE (3078 ± 79 mL·kg<sup>-1</sup>) indicating that LE is more distributed to lipophilic tissues. Excretion studies have confirmed that  $\Delta^1$ -CA is indeed a metabolite of PRN. After intravenous injection of 10 mg·kg<sup>-1</sup>, less than 1% of the administered PRN was excreted as  $\Delta^1$ -CA by 4 h (0.38 ± 0.10% in bile and 0.18 ± 0.04% in urine), significantly less than for LE (17.01 ± 2.09% in bile and 2.53 ± 1.17% in urine) indicating that extent of this metabolic transformation can indeed be affected by molecular design. At doses of 100 mg/kg, the proportion of  $\Delta^1$ -CA excreted after PRN administration (0.12 ± 0.03% in bile and 0.19 ± 0.03% in urine) was similar to that of CA excreted after hydrocortisone administration (0.11 ± 0.03% in bile and 0.22 ± 0.04% in urine) indicating that the presence of the  $\Delta^1$  double bond ( $\Delta^1$ -unsaturation) does not affect significantly this metabolic conversion.

### 1. Introduction

The concept of soft drug design for developing drugs with controlled metabolism has been introduced during the late 1970-s (Bodor 1984) and has expanded since then to cover a wide area of pharmaceutical areas as part of the retrometabolism-based drug design strategies (Bodor and Buchwald 2000, 2008). Loteprednol etabonate (LE), a soft corticosteroid, was developed within this framework based on the inactive metabolite approach by introducing a biologically labile 17 $\beta$ -chloromethyl ester function into the structure of the hypothetical inactive metabolite  $\Delta^1$ -cortienic acid ( $\Delta^1$ -CA) to reactivate it (Fig. 1) (Bodor 1993; Bodor and Buchwald 2002, 2006). Accordingly, LE, following administration and exertion of its pharmacological effects, undergoes a rapid systemic hydrolysis to 17 $\beta$ -carboxylate resulting in the intermediate  $\Delta^1$ -cortienic acid etabonate (AE) that is then further hydrolyzed into  $\Delta^1$ -CA; both being inactive and rapidly eliminated from the body (Bodor et al. 1995; Druzgala et al. 1991; Wu et al. 2008). As part of the general soft drug design principles, this predictable, facile metabolic inactivation was introduced to optimize local efficacy and reduce systematic side effects (Bodor 1988; Bodor and Buchwald 2002, 2006). According to the previous reports, prednisolone (PRN) in humans is primarily metabolized in the liver into biologically inactive metabolites (mainly the glucuronide and sulphate) and

is mostly excreted in the urine as free or conjugated metabolites together with an appreciable amount of unchanged form (Czock et al. 2005; Miyabo et al. 1976). In rats, different metabolic reactions of PRN, such as reduction-oxidation at C-11, reduction at C-20 and C-3, hydrogenation of the cross-conjugation system in ring A, cleavage of the dihydroxyacetone side chain, and, to a lesser extent, hydroxylation on C-6, have also been reported (Bush et al. 1968; Herken and Seeber 1963; Vermeulen and Caspi 1958). However, no evidence has yet shown that  $\Delta^1$ -CA is one of the actual metabolites of PRN. On the other hand, hydrocortisone (HC), which has a closely related structure (Fig. 1), has been reported to undergo a variety of oxidative and reductive metabolic conversions in human including the oxidation of its dihydroxyacetone side chain, which, through a 21-aldehyde (21-dehydrocortisol) and a 21-acid (cortisolic acid), ultimately leads to the formation of cortienic acid (CA) (Monder and Bradlow 1980). On the basis of structural similarity (an unsaturated double bond),  $\Delta^1$ -CA was hypothetically defined as the inactive 17 $\beta$ -carboxylic acid metabolite of prednisolone, and it has been used as a lead compound for developing various soft steroids (Bodor 1984; Bodor and Buchwald 2002, 2006). Accordingly, it was assumed that part of the administered PRN is metabolized to  $\Delta^1$ -CA to some extent and excreted in the urine and/or bile. A main goal of the present work was to prove that  $\Delta^1$ -CA is indeed a common metabolite of PRN and LE. We have also

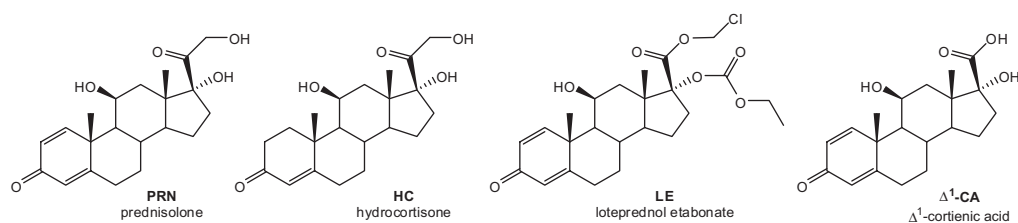


Fig. 1: Structures of compounds included in the present study

compared in detail the pharmacokinetic (PK) characteristics of PRN, LE, and  $\Delta^1$ -CA, and investigated the excretion of  $\Delta^1$ -CA after intravenous administration of PRN and LE. In addition, we also studied the effect the presence of the 1-double bond (i.e., PRN vs. HC) has on the relative excretions of the corresponding metabolites ( $\Delta^1$ -CA vs. CA). The structures of all corresponding compounds are shown in Fig. 1.

## 2. Investigations, results and discussion

### 2.1. Pharmacokinetics

In humans, prednisolone is rapidly absorbed following an oral dose, and peak effects after oral or intravenous administration occur within 1–2 h. PRN is eliminated from the plasma with a half-life of 2–4 h; however, the biological half-life is 18–36 h (Czock et al. 2005). Here, the PK of PRN after intravenous (i.v.) injection in rats at a dose of  $10 \text{ mg}\cdot\text{kg}^{-1}$  has been studied and compared with the previous results of LE and  $\Delta^1$ -CA obtained under the same experimental conditions (Bodor 1988; Bodor 1993; Bodor and Buchwald 2002, 2006). As shown in Fig. 2, PRN exhibited a bi-exponential elimination from blood, and  $\Delta^1$ -CA was undetectable in the blood at any time.

The corresponding PK parameters derived from compartmental and non-compartmental analyses are shown in Table 1. Blood concentration-time profiles (0–120 min) could be adequately described by an empirical two-compartmental model,  $C = Ae^{-\alpha t} + Be^{-\beta t}$ , with a good correlation coefficient of the compartmental fits ( $r=0.99$ ). The total clearance,  $CL_{\text{tot}}$ , of PRN ( $27.0 \pm 1.4 \text{ mL}\cdot\text{min}^{-1} \text{ kg}^{-1}$ ) was significantly lower than that of LE ( $67.4 \pm 11.6 \text{ mL}\cdot\text{min}^{-1} \text{ kg}^{-1}$ ) and  $\Delta^1$ -CA ( $53.8 \pm 1.4 \text{ mL}\cdot\text{min}^{-1} \text{ kg}^{-1}$ ) indicating that the metabolism and

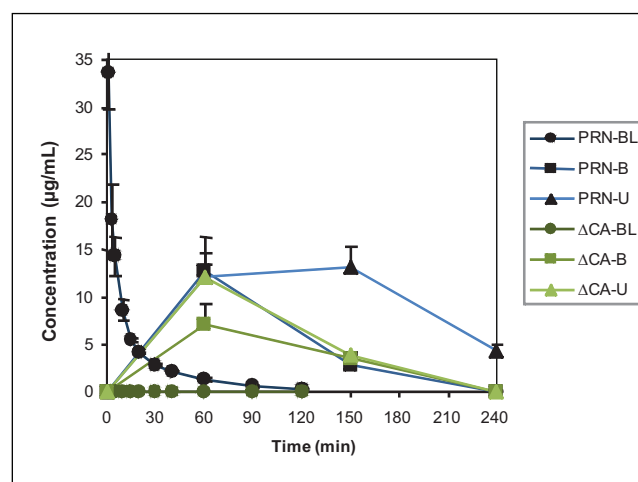


Fig. 2: Concentration-time profiles of prednisolone (PRN) and  $\Delta^1$ -cortienic acid ( $\Delta^1$ -CA) in blood (BL), bile (B), and urine (U) after i.v. administration of  $10 \text{ mg}\cdot\text{kg}^{-1}$  PRN ( $n=3$ )

elimination process of PRN in the liver (primarily conjugation) may be less efficient than that of LE (mainly hydrolysis) and  $\Delta^1$ -CA (excreted unchanged). The clearance of LE is higher than the physiological hepatic blood flow in rats,  $58 \text{ mL}\cdot\text{min}^{-1} \text{ kg}^{-1}$  (Harashima et al. 1985) indicating that for LE, in addition to metabolism in liver, enzymatic degradation also takes place in blood and possibly other organs. The volume of distribution,  $V_{d_{ss}}$ , of PRN ( $823 \pm 78 \text{ mL}\cdot\text{kg}^{-1}$ ) is similar to that of  $\Delta^1$ -CA ( $799 \pm 21 \text{ mL}\cdot\text{kg}^{-1}$ ), but significantly less than that of LE ( $3078 \pm 79 \text{ mL}\cdot\text{kg}^{-1}$ ) indicating that compared to LE, PRN and  $\Delta^1$ -CA are better distributed into the hydrophilic com-

**Table 1: Pharmacokinetic parameters obtained after i.v. injection of  $10 \text{ mg}\cdot\text{kg}^{-1}$  of prednisolone (PRN), loteprednol etabonate (LE), and  $\Delta^1$ -cortienic acid ( $\Delta^1$ -CA) in rats**

Property	PRN <sup>a</sup>	LE <sup>a,b</sup>	$\Delta^1$ -CA <sup>a,b</sup>
Dose, $\text{mg}\cdot\text{kg}^{-1}$	10	10	10
AUC, $\mu\text{g}\cdot\text{min mL}^{-1\text{c}}$	$372.9 \pm 18.0$	$159.2 \pm 31.3$	$186.3 \pm 5.0$
$CL_{\text{tot}}$ , $\text{mL}\cdot\text{min}^{-1} \text{ kg}^{-1\text{c}}$	$27.0 \pm 1.4$	$67.4 \pm 11.6$	$53.8 \pm 1.4$
MRT, $\text{min}^{\text{c}}$	$30.42 \pm 0.33$	$48.72 \pm 8.95$	$14.87 \pm 0.44$
$V_{d_{ss}}$ , $\text{mL}\cdot\text{kg}^{-1\text{c}}$	$823 \pm 78$	$3078 \pm 79$	$799 \pm 21$
A, $\mu\text{g}/\text{mL}^{-1}$	$26.01 \pm 2.30$	$8.65 \pm 2.75$	$33.50 \pm 5.75$
B, $\mu\text{g}/\text{mL}^{-1}$	$5.76 \pm 0.13$	$1.92 \pm 0.25$	$6.08 \pm 0.66$
$\alpha$ , $\text{min}^{-1\text{d}}$	$0.23 \pm 0.02$	$0.28 \pm 0.15$	$0.56 \pm 0.07$
$\beta$ , $\text{min}^{-1\text{d}}$	$0.024 \pm 0.001$	$0.017 \pm 0.003$	$0.048 \pm 0.003$
$t_{1/2(\beta)}$ , $\text{min}^{\text{d}}$	$29.27 \pm 0.63$	$43.41 \pm 7.58$	$14.62 \pm 0.46$
$V_{d_c}$ , $\text{mL}\cdot\text{kg}^{-1\text{d}}$	$318 \pm 24$	$1092 \pm 264$	$307 \pm 31$
$V_{d_{\text{area}}}$ , $\text{mL}\cdot\text{kg}^{-1\text{d}}$	$1177 \pm 47$	$3987 \pm 289$	$1139 \pm 64$
$K_{\text{el}}$ , $\text{min}^{-1\text{d}}$	$0.088 \pm 0.003$	$0.071 \pm 0.024$	$0.182 \pm 0.021$
$r^{\text{e}}$	$0.989 \pm 0.003$	$0.998 \pm 0.001$	$0.998 \pm 0.001$

<sup>a</sup> Data represent mean  $\pm$  SD of 3–5 trials

<sup>b</sup> Data adapted from previous studies (Bodor et al. 1995)

<sup>c</sup> Parameters obtained by non-compartmental analysis of the blood concentration-time profiles

<sup>d</sup> Compartmental analysis for the estimation of parameters using a body two-compartment model

<sup>e</sup> Correlation coefficient of the compartmental fits

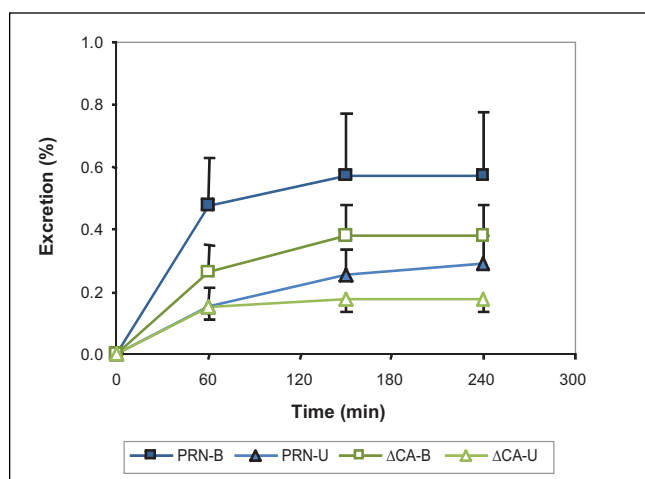


Fig. 3: Excretion-time profile of PRN and  $\Delta^1$ -CA ( $\Delta$ CA) after i.v. bolus administration of  $10 \text{ mg}\cdot\text{kg}^{-1}$  PRN ( $n = 3$ ; B-bile, U-urine)

partment, whereas the more lipophilic LE (Buchwald 2008) is better distributed to, and entrapped into more lipophilic local tissues, where it can exert its anti-inflammatory effects before being hydrolyzed to its inactive metabolites. The shorter  $\beta$  phase half-life of  $\Delta^1$ -CA ( $14.62 \pm 0.91 \text{ min}$ ) compared to those of PRN ( $29.27 \pm 1.10 \text{ min}$ ) and LE ( $43.41 \pm 13.14 \text{ min}$ ) indicates a faster elimination of this inactive metabolite from the body, which is consistent with the more hydrophilic nature of  $\Delta^1$ -CA.

## 2.2. Excretion of $\Delta^1$ -CA after i.v. injection of PRN and LE

The excretion study was performed to confirm that  $\Delta^1$ -CA is indeed a metabolite of PRN. The appearance of  $\Delta^1$ -CA in blood, bile, and urine after administration of PRN was quantified, and the relative amounts in these media were calculated and compared to that of the parent compound (PRN). Fig. 2 shows the concentration time-profiles of PRN and  $\Delta^1$ -CA in blood, bile, and urine after i.v. injection of PRN at a dose of  $10 \text{ mg}\cdot\text{kg}^{-1}$ . Results indicate that following a rapid disappearance of PRN from blood, some of the administered dose is excreted unchanged and only a small part is metabolized to  $\Delta^1$ -CA and excreted in the bile and urine.

Fig. 3 shows excretion time-profiles for PRN and  $\Delta^1$ -CA in bile and urine after PRN injection ( $10 \text{ mg}\cdot\text{kg}^{-1}$ , i.v.). Results indicate that excretion of unchanged PRN and  $\Delta^1$ -CA are both less than 1% suggesting that the oxidation of PRN into  $\Delta^1$ -CA is not a major metabolic pathway. Also, the amount of  $\Delta^1$ -CA excreted in the bile is about two-fold higher than in the urine indicating that the liver is a main site of this metabolic transformation.

The metabolism of PRN to  $\Delta^1$ -CA was also investigated at a higher,  $100 \text{ mg}\cdot\text{kg}^{-1}$  dose. The cumulative excretions of unchanged PRN and  $\Delta^1$ -CA in bile and urine at 2.5 h after 10 and  $100 \text{ mg}\cdot\text{kg}^{-1}$  PRN (i.v.) are shown in Fig. 4. Results indicate that the cumulative excretion of unchanged PRN increases with increasing dose (from 10 to  $100 \text{ mg}\cdot\text{kg}^{-1}$ ), however, the percent excretion of  $\Delta^1$ -CA in bile is largely reduced (approximately 3-fold) suggesting that a saturation of PRN metabolism in the liver might take place at higher doses and that the metabolism of PRN into  $\Delta^1$ -CA in the liver is dose-dependent, in a manner similar to that previously reported for LE (Bodor et al. 1995).

The cumulative excretions (% of dose) of PRN, LE, and their common metabolite  $\Delta^1$ -CA at 4 h after i.v. administration of PRN or LE ( $10 \text{ mg}\cdot\text{kg}^{-1}$ ) are compared in Table 2 and Fig. 5. After PRN administration, relatively low levels (<1%) of both unchanged PRN and  $\Delta^1$ -CA metabolite were found in bile and

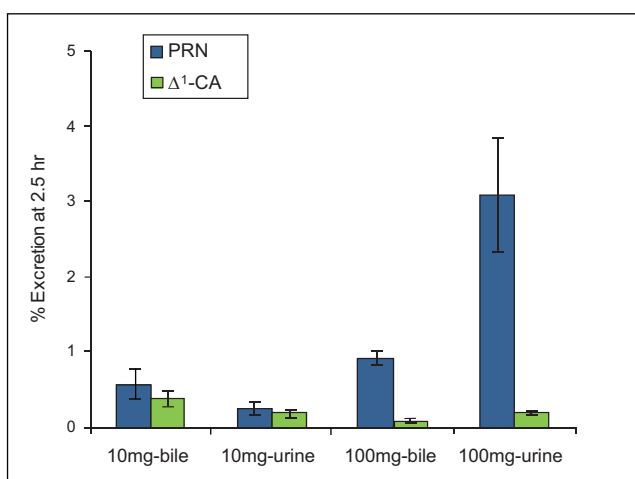


Fig. 4: Cumulative excretion (%) of PRN and  $\Delta^1$ -CA at 2.5 h after 10 and  $100 \text{ mg}\cdot\text{kg}^{-1}$  i.v. administration of PRN

**Table 2: Cumulative excretion (% of total dose) of PRN, LE, and their common metabolite  $\Delta^1$ -CA at 4 hr after i.v. injection of  $10 \text{ mg}\cdot\text{kg}^{-1}$  in rats**

	PRN injection		LE injection	
	PRN	$\Delta^1$ -CA	LE	$\Delta^1$ -CA
Bile	$0.58 \pm 0.20$	$0.38 \pm 0.10$	$0.10 \pm 0.01$	$17.01 \pm 2.09$
Urine	$0.29 \pm 0.09$	$0.18 \pm 0.04$	$1.82 \pm 0.21$	$2.53 \pm 1.17$

Data represent mean  $\pm$  SD of 3 trials

urine. The percent excretion of  $\Delta^1$ -CA in bile ( $0.38 \pm 0.10\%$ ) and urine ( $0.18 \pm 0.04\%$ ) are both significantly lower than following LE administration ( $17.01 \pm 2.09\%$  and  $2.53 \pm 1.17\%$  in bile and urine, respectively). These results suggest that whereas in the metabolism of PRN, the oxidative transformation into  $\Delta^1$ -CA is relatively less important than other metabolic pathways (e.g., reduction and conjugation), in the metabolism of LE, the hydrolytic transformation into AE and then  $\Delta^1$ -CA in the liver and other organs play a primary role, confirming the designed, controllable metabolism of soft drugs.

The cumulative excretions (% of dose) of  $\Delta^1$ -CA and CA after PRN and HC administrations, respectively, are compared in Table 3. Results indicate that at 2.5 h after  $100 \text{ mg}\cdot\text{kg}^{-1}$  i.v. injection, the excretion patterns are similar, and the total excretion of

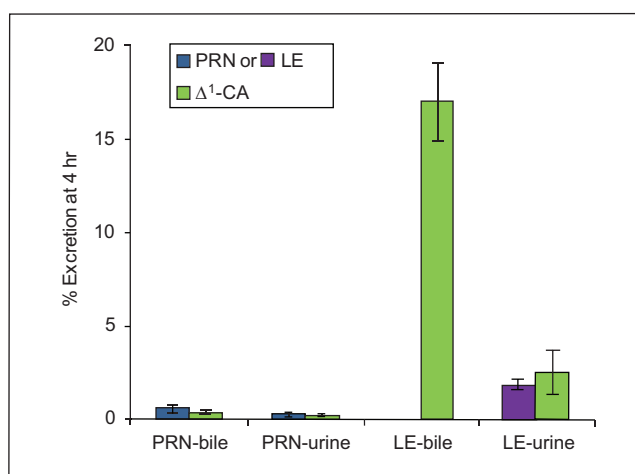


Fig. 5: Cumulative excretion (%) of PRN, LE, and  $\Delta^1$ -CA at 4 h after i.v. administration of LE or PRN at a dose of  $10 \text{ mg}\cdot\text{kg}^{-1}$

**Table 3: Cumulative excretion (% of total dose) of PRN, HC, and their corresponding metabolites,  $\Delta^1$ -CA and CA, at 2.5 h after i.v. injection in rats at a dose of 100 mg kg<sup>-1</sup>**

	PRN injection		HC injection	
	PRN	$\Delta^1$ -CA	HC	CA
Bile	0.92 ± 0.08	0.12 ± 0.03	0.31 ± 0.02	0.11 ± 0.03
Urine	3.09 ± 0.76	0.19 ± 0.03	3.60 ± 0.15	0.22 ± 0.04

Data represent mean ± SD of 3 trials

the metabolites ( $\Delta^1$ -CA and CA) is less than 0.5% of the administered dose suggesting that the presence of the  $\Delta^1$ -unsaturation ( $\Delta^1$ -double bond) does not significantly affect the oxidative metabolic conversion of PRN and HC into  $\Delta^1$ -CA and CA, respectively.

In conclusion, this study has demonstrated (i) that  $\Delta^1$ -CA is indeed a metabolite of PRN and the metabolism of PRN to  $\Delta^1$ -CA in the liver is dose dependent, (ii) that the *in vivo* metabolism of LE into its predicted metabolite,  $\Delta^1$ -CA, is significantly higher than that of PRN, in agreement with the inactive-metabolite based soft drug design principle used in the design of LE, and (iii) that  $\Delta^1$ -saturation in the structure of PRN versus HC does not affect significantly the amount of the metabolism into their corresponding metabolites,  $\Delta^1$ -CA and CA.

### 3. Experimental

#### 3.1. Materials and animals

Prednisolone anhydrous (PRN) was from Upjohn Company (Kalamazoo, MI), hydrocortisone (HC) was from Sigma Co., (St. Louis, MO), and loteprednol etabonate (LE) was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Cortienic acid (CA) and  $\Delta^1$ -cortienic acid ( $\Delta^1$ -CA) were from Xenon Vision Inc., (Alachua, FL). 2-Hydroxypropyl- $\beta$ -cyclodextrin (HPCD) was obtained from Pharmatec, Inc. (Alachua, FL). All other chemicals were commercially available products of special reagent grade. Male Sprague Dawley rats (300 ± 25 g) were obtained from Charles Rivers (Wilmington, MA). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health.

#### 3.2. Pharmacokinetics

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg·kg<sup>-1</sup>), and jugular veins were exposed. PRN (10 mg·kg<sup>-1</sup>) was dissolved in a vehicle of 50% HPCD containing 5% DMSO, and injected in the tail vein over one minute (1 mL·kg<sup>-1</sup>). Blood (0.12 mL) was taken from the jugular vein at 0, 1, 3, 5, 10, 15, 20, 30, 40, 60, 90, and 120 min. A 0.1 mL of each sample was mixed with 5% DMSO in acetonitrile solution (0.2 mL), centrifuged, and the supernatant (20  $\mu$ L) was analyzed by HPLC. The obtained concentration data were used for non-compartmental and compartmental pharmacokinetic analyses. For non-compartmental analysis, the area under the curve, AUC, of the plasma concentration versus time was calculated using the linear trapezoidal rule with the area from the last measurement,  $C_t$ , to infinity calculated as  $C_t/\beta$ , where  $\beta$  is the terminal disposition rate constant. AUMC, the area under the first moment curve, was calculated using the trapezoidal rule from the curve of blood concentration  $\times$  time - time, with the area from the last time point,  $t$ , to infinity calculated as  $C_t/\beta + C_t/\beta^2$ . The total body clearance ( $CL_{tot}$ ) was calculated as dose/AUC. Mean resident time (MRT) was calculated as AUMC/AUC. The volume of distribution at steady state ( $V_{d_{ss}}$ ) was determined as the product of  $CL_{tot}$  and MRT. Compartmental PK analysis was performed with WinNonlin<sup>®</sup> (Pharsight, Mountain View, CA.). Best fit was obtained with an empirical two-compartment model,  $C = Ae^{-\alpha t} + Be^{-\beta t}$ , where  $C$  is drug concentration in blood,  $A$  and  $B$  are exponential multipliers, and  $\alpha$  and  $\beta$  are hybrid constants in the central and peripheral compartment, respectively. The volume of distribution of the central compartment ( $V_{d_c}$ ) and the volume of distribution during the elimination phase ( $V_{d_{area}}$ ) were calculated as Dose/( $A + B$ ) and  $CL_{tot}/\beta$ , respectively. The AUC was determined as  $A/\alpha + B/\beta$ , the half-life of the terminal phase,  $t_{1/2}$ , was calculated as  $\ln 2/\beta$ , and the elimination rate constant ( $K_{el}$ ), was calculated as  $CL_{tot}/V_{d_c}$ .

#### 3.3. Excretion after intravenous administration

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg·kg<sup>-1</sup>). The urinary tract was closed to prevent urination, and urine samples were collected directly from the urinary bladder through a 26 gauged needle. The common bile duct was cannulated using polyethylene tubing (PE 10, Clay Adams). PRN dissolved in previously described vehicle was administered i.v. through the tail vein at a dose of 10 or 100 mg·kg<sup>-1</sup>. Total bile and urine were collected at varying time intervals (e.g., 1, 2.5, and 4 h). The bile and urine samples (0.1 mL) were added with 0.1 mL of 0.1 N HCl and 1 mL of ethyl acetate and mixed vigorously by a vortex mixer for 3 min. After centrifugation at 10000 rpm for 1 min, 0.8 mL of the upper organic layer was separated. Subsequently, the organic solvent was evaporated in a vacuum centrifuge, and the residue was reconstituted in 0.3 mL of 30% acetonitrile solution. The resulting solution was filtered through a 0.45  $\mu$ m membrane filter, and the filtrate (20  $\mu$ L) was injected in the HPLC for analysis. Selected samples were also analyzed by HPLC-MS for compound identification. The results of LE excretion were adapted from a previously published study (Bodor et al. 1995) performed by the same personnel and following the same experimental design in terms of animals (body weight, age, sex), sampling (blood, bile, and urine), and sample preparation.

#### 3.4. Analytical methods

High performance liquid chromatography (HPLC) operating at ambient temperature was used for quantitative determination of the compounds in the biological fluids. A Phenomenex Luna C8 analytical column (5  $\mu$ , 4.6 mm  $\times$  15 cm) was connected to a component system (Spectra-Physics) consisting of a SP 8810 precision isocratic pump, Rheodyne 7125 injector (injection volume 20  $\mu$ L), SP 8450 UV/VIS variable wavelength detector (operated at 254 nm), and SP 4290 integrator. At a flow rate of 1 mL·min<sup>-1</sup>, the mobile phase consisting of acetonitrile, acetic acid, and water (30:0.2:70) eluted PRN and  $\Delta^1$ -CA at 5.99 and 6.76 min, and HC and CA at 6.12 and 6.92 min, respectively. The detection limit was less than 0.05  $\mu$ g·mL<sup>-1</sup> for all compounds. Standards were developed by adding known amounts of the compounds to blank blood, urine, or bile, and prepared by the same method as described previously for the samples. For further confirmation of the HPLC peaks, selected bile and urine samples were injected (20  $\mu$ L) into an isocratic HPLC-MS system consisting of the previously described HPLC system and a Waters-Micromass Quattro-LC-Z (Beverly, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source.

#### 3.5. Statistical analysis

All data were obtained as three to five replicates. Differences in the PK parameters of the three compounds (Table 1) have been compared using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as a suitable *post-hoc* test for individual differences (Jones 2002). Differences in the cumulative eliminations (bile vs. urine) for PRN at various doses (10 and 100 mg) as well as those between compound pairs (PRN vs. LE or PRN vs. HC) in Tables 2–3 have been compared using the same method. A significance level of  $p < 0.05$  was used in all cases. Statistical analysis was performed using GraphPad Prism 5.01 (La Jolla, CA).

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