Evidence for the Involvement of a Pulmonary First-Pass Effect via Carboxylesterase in the Disposition of a Propranolol Ester Derivative after Intravenous Administration

TERUKO IMAI, YASUSHI YOSHIGAE, MASAKIYO HOSOKAWA, KAN CHIBA, and MASAKI OTAGIRI
Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (T.I., Y.Y., M.O.); and Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan (M.H., K.C.)
Received July 3, 2003; accepted August 21, 2003

ABSTRACT
The disposition kinetics of O-butyryl propranolol (butyryl-PL), a model compound containing an ester moiety, after intravenous administration was compared with that of PL in rats and beagle dogs. Rats showed only 30% conversion of butyryl-PL to PL up to 2 h after dosing, whereas dogs showed nearly complete conversion within 10 min after administration. The CL\textsubscript{tot} of butyryl-PL in rats was 5.8 l/h/kg and that in dogs was 65.6 ± 18.6 l/h/kg, both of which were greater than hepatic blood flow. The in vivo conversion from butyryl-PL to PL in the rat could be explained on the basis of the hydrolysis characteristics in the liver and blood. The in vitro hydrolysis data and the in vivo data after intra-arterial administration clearly demonstrated that the extremely high CL\textsubscript{tot} of butyryl-PL in dogs was dependent on first-pass hydrolysis in the lung in addition to hydrolysis at a blood flow-limited rate in the liver and kidney. The availability of butyryl-PL after passage through the lung was 50%. Furthermore, the isoform of carboxylesterase involved in the pulmonary hydrolysis of butyryl-PL in the dog was identified as D1, a CES-1 group enzyme. However, butyryl-PL was not recognized as a substrate by CES-1 family carboxylesterases, which are present at high levels in the rat lung (RH-1) and kidney (RL-1). These findings indicate that extrahepatic metabolism, especially in the lung, is important in the disposition of drugs containing an ester moiety after intravenous administration and that the substrate specificity of carboxylesterase isozyme distinguishes from others.

A number of ester- and amide-containing drugs have been clinically used in oral, cutaneous, and injectable formulations. The main route of detoxification or metabolic activation of these compounds is through enzymatic hydrolysis. Multiple carboxylesterases (E.C. 3.1.1.1) efficiently catalyze the hydrolysis of ester- and amide-containing drugs to their respective free acids, and these carboxylesterases are localized in the endoplasmic reticulum (ER) of a variety of organs and tissues in many mammalian species (Heymann, 1982; Satoh, 1987; Satoh and Hosokawa, 1998). In general, marked species differences are seen in esterase activity. For example, the level of esterase activity in mammalian liver microsomes is in the order dog > human > rat when p-nitrophenylacetate is used as the substrate, but is in the order dog > rat > human for isocarboxazid (Hosokawa et al., 1990). The different spectra of esterase activity for different substrates in various tissues lead to markedly divergent patterns of disposition after the administration of ester- and amide-containing drugs via various routes in mammalian species. Of all the animal tissues studied to date, the highest level of hydrolytic activity is seen in the liver and a moderate level of hydrolytic activity is seen in the proximal tubule of the kidney (Tsujita et al., 1988; Tsujita and Okuda, 1993). Interestingly, significant esterase activity has recently been found in the small intestine (Prueksaritanont et al., 1996; Yoshigae et al., 1998b), the skin (McCracken et al., 1993; Ahmed et al., 1997), the heart (Dean et al., 1995), muscle (Nambu et al., 1987), the blood (Van Lith et al., 1992; McCracken et al., 1993; Ahmed et al., 1997), and the lung (McCracken et al., 1993; Dean et al., 1995; Forkert et al., 2001). Although hydrolytic activity is observed in most tissues, ester- and amide-containing drugs are converted to free acid mainly by the hydrolytic enzyme in the liver. Moreover, further metabolism in various organs complicates the disposition of drugs after administration to animals.

The present study focused on the extrahepatic metabolism by esterase, particularly pulmonary metabolism, as well as species differences in metabolism, using O-butyryl-propranolol (butyryl-PL; Fig. 1), a PL ester derivative, as a model...
ester-containing compound. PL is distributed not only to the liver but also to several other tissues, including the lung, due to its hydrophobicity and basicity. Pang et al. (1982) has reported 50 to 60% first-pass uptake of PL by the lung through a simple diffusion mechanism in dogs with cardiac catheterization. Dollery and Junod (1976) have reported the accumulation of PL in the rat lung. Butyryl-PL is more hydrophobic than PL and its basicity is comparable with that of PL [logP \text{ (octanol/water)} = 4.0 for PL, 0.38; butyryl-PL, 1.54; and pK_a \text{ of PL, 9.44; butyryl-PL, 9.02}]. Therefore, it is possible that butyryl-PL may be easily taken up and hydrolyzed to PL in the lung after intravenous administration.

In the present study, the disposition kinetics of butyryl-PL after intravenous administration was investigated in rats and beagle dogs. The tissues contributory to the hydrolysis of butyryl-PL were also identified in these two species. In addition, the extent of in vivo hydrolyase activity in the lung was determined by comparison of intravenous and intra-arterial administration of butyryl-PL in beagle dogs. Furthermore, the isoform of carboxylesterase involved in pulmonary hydrolysis of butyryl-PL in the dog was identified.

Materials and Methods

Materials. O-butyryl-PL hydrochloride was synthesized from PL hydrochloride (Wako Pure Chemicals, Osaka, Japan) and butyryl chloride (Tokyo Kasei, Tokyo, Japan) according to the methods described previously (Shameem et al., 1993). The identity and purity of the synthesized butyryl-PL were confirmed by infrared, NMR, atomic analysis, and HPLC. All other reagents used were of analytical grade.

Intravenous Administration to Rats and Beagle Dogs. Male Wistar rats (230–270 g, 5 weeks of age) and beagle dogs (9–11 kg, 1–3 years of age) were housed in an air-conditioned room with free access to commercial chow and tap water, and fasted for 15 h before the intravenous administration of PL or butyryl-PL. PL (2.5 mg/400 \mu g/kg for rats, 2.0 mg/400 \mu g/kg for dogs), and equivalent doses of butyryl PL were dissolved in sterilized phosphate buffer (0.05 M, pH 6.0). In rats, the solutions were injected via the femoral vein and blood samples were drawn from the jugular vein under ether anesthesia. In dogs, the solutions were injected via the right cephalic vein and blood samples were drawn from the left cephalic vein without anesthesia.

Intra-Arterial Administration of Butyryl-PL to Beagle Dogs. Before intra-arterial administration to beagle dogs, a catheter (pig-tail angiocatheter; Goodman Co., Ltd. Nagoya, Japan) was advanced via the femoral artery until the tip was placed in the left ventricle. The dogs were anesthetized by the intramuscular injection of atropine (0.005 mg/kg; Fuso Pharmaceutical Industry Ltd., Osaka, Japan), and further intramuscular injection of mixture of ketamine hydrochloride (0.66 mg/kg; Sankyo Co., Ltd., Tokyo, Japan) and Xylazin hydrochloride (0.13 mg/kg; Bayer AG, Leverkusen, Germany). Proper placement of the catheter in the left ventricle was confirmed by monitoring the blood pressure. Four hours after arousal, butyryl-PL was injected into the left ventricle via the intra-arterial catheter. The butyryl-PL solution was the same as that used for i.v. injection (2 mg/400 \mu g/kg equivalent to PL). The catheter was flushed with phosphate-buffered saline solution (pH 6.0) to ensure that no butyryl-PL remained in the catheter before it was removed from the femoral artery.

Determination of Plasma Concentrations of PL and Butyryl-PL. Blood samples (0.45 ml for rats, 1.0 ml for dogs) were drawn using heparinized syringes rinsed with a trace amount of paraoxon, an inhibitor of the hydrolysis of butyryl-PL. The plasma sample (200 \mu l) obtained from each blood sample after centrifugation (12,000 g for 30 s) was added to 0.3 ml of 0.1 M phosphate buffer (pH 4.0) saturated with NaCl. PL and butyryl-PL were then simultaneously extracted twice with 6 ml of ethyl acetate. Less than 1% of the butyryl-PL in the plasma degraded by this procedure. The organic phase was evaporated to dryness and the residual material was redissolved in 50 \mu l of the mobile phase before the injection of a 20-\mu l aliquot into the HPLC system.

Data Analysis for Blood Concentrations of PL and Butyryl-PL. The blood level of each compound was calculated from the plasma concentration based on the blood/plasma ratio (Rb). The Rb was estimated from the drug concentrations in the plasma and blood after incubation of each compound in the blood, which was treated with 1 \mu M paraoxon, for 10 min at 37°C. The mean Rb values of PL and butyryl-PL were constant over the concentration range from 10 to 1000 ng/ml. In addition, the Rb values of PL and butyryl-PL immediately after adding them in the blood were nearly same as those after 10-min incubation. In rats, the Rb of (R)-PL and (S)-PL was 0.55 and 1.2, respectively, and that of butyryl-PL was 0.88 for (R)-isomer and 0.91 for (S)-isomer. In dogs, the Rb of both (R)- and (S)-PL was 0.80, and that of butyryl-PL for both enantiomers was also 0.80. The racemic blood levels of PL and butyryl-PL were calculated as the sum of the blood concentrations of each enantiomer. The AUC from time 0 to infinity (AUC_{\infty}) was calculated as the sum of the AUC of the mean blood concentration-time curve up to final sampling point by the trapezoidal rule and the AUC from the terminal exponential slope obtained by linear regression of the log-linear portion of the blood concentration profile. The apparent total body clearance was calculated as dose/AUC_{\infty}.

Organ clearance (CL_{\text{org}}) for liver and kidney was calculated by following equation: CL_{\text{org}} = Q \cdot f_p \cdot R_m/(Q + f_p \cdot CL_{\text{int}}). Intrinsic clearance (CL_{\text{int}}; litters per hour per kilogram) was estimated from the Michaelis-Menten kinetic parameters and protein content (milligrams per kilogram body) in respective organ according to the following equation: CL_{\text{int}} = (V_{\text{mean}}/K_m) \times (protein content), where protein content was 1.280 mg/kg for rat liver microsomes, 3.218 mg/kg for dog liver S9, 425 mg/kg for dog lung S9, and 423 mg/kg for dog kidney S9. The free fraction in blood (f_p) for butyryl-PL was calculated by f_p/R_b. The free fraction of butyryl-PL in plasma (f_p) treated with 1 \mu M paraoxon was determined by ultrafiltration method using a micropartition system MPS-1 (Amicon, Danvers, MA; a molecular weight cut-off value of 30,000). The f_p value of racemic butyryl-PL was 0.08 for rats and 0.07 for dogs. Then, f_p of butyryl-PL was calculated 0.09 for both dogs and rats. Tissue blood flow rate (Q) listed in Table 3 was used for calculation.

A deconvolution method (Prueksaritanont et al., 1997) was used to calculate the systemic formation rate of PL after intravenous administration of butyryl-PL. Assuming that the disposition kinetics of PL and butyryl-PL was independent of each other and that their disposition kinetics were linear, the systemic formation rate (Rm) was calculated by following equation:

\[ C_{\text{Bu-PL}}(t) = \int_0^t R_m(t - r) dr \]

where Rm is the systemic formation rate of PL from butyryl-PL, C_{Bu-PL} and C_{PL} are plasma PL concentration after intravenous administration of butyryl-PL and PL.
Preparation of Microsomes and Cytosol from Various Organs. Male beagle dogs (9–10 kg, 4–5 years of age) and male Wistar rats (230–270 g, 8 weeks of age) were used after overnight fasting with free access to water. The rats were exsanguinated under ether anesthesia. A cannula was placed in the inferior vena cava, and the liver, kidneys, and lungs were perfused with ice-cold 0.15 M KCl to remove the blood. These organs were then resected, washed with ice-cold 0.15 M KCl, and homogenized with 3 volumes of 10 mM phosphate buffer (pH 7.4) containing 0.15 M KCl in a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle under ice-cold conditions. The microsomes and cytosol fractions were prepared as described previously (Yoshigae et al., 1997). Briefly, the homogenate obtained (25% wet w/v) was centrifuged at 9000g for 20 min at 4°C to obtain the supernatant (S9) fraction. The S9 fraction was further centrifuged at 105,000g for 1 h at 4°C, and the resulting supernatant was used as the cytosolic fraction. The pellets were washed and resuspended by homogenization in phosphate buffer to obtain the microsomes solution. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein, and the enzyme solutions were stored at −80°C until use.

Hydrolysis Experiments. Hydrolysis experiments were performed using subcellular fractions diluted with Tris-HCl buffer (50 mM, pH 7.4) according to the method described previously (Yoshigae et al., 1997). The hydrolysis reaction was initiated by the addition of butyryl-PL after preincubation of each subcellular fraction for 5 min. The reaction was terminated by the addition of acetonitrile. In experiments involving inhibition by bis-p-nitrophenyl phosphate (BNPP), the enzyme solution was preincubated for 5 min with various concentrations of BNPP (final concentrations, 10−5 to 10−3 M) dissolved in isomethyl sulfoxide (DMSO; 5 μl) or without esterase inhibitor (5 μl of DMSO alone) in controls. DMSO, a solvent for butyryl-PL and BNPP, was added to a final concentration of 1% (v/v) in the enzyme solution. The PL formed was determined by HPLC.

HPLC Assay for Enantiomers of PL and Butyryl-PL. The HPLC assay was performed according to method described previously (Yoshigae et al., 1998a). The HPLC system comprised a Hitachi L-6000 pump with a loop-fitted Rheodine injector (volume 20 μl), a Hitachi L-7480 fluorescence detector, and a Hitachi D-2500 chroomato-integrator (Hitachi Co., Ltd., Tokyo, Japan). In the case of butyryl-PL, an ES-PD column (150 × 6.0-mm i.d.; Shinwa Chemical Industries, Ltd., Kyoto, Japan) was used with a mobile phase of acetonitrile/20 mM KH2PO4 (45:55 v/v) at a flow rate of 0.8 ml/min. A Chiralcel OD column (25 × 0.46 cm inner diameter; Daiso Chemical Industries, Ltd., Tokyo, Japan) was used for PL with a mobile phase of n-hexane/ethanol/diethylamine (85:15:0.6 v/v/v) at a flow rate of 0.6 ml/min. Both PL and butyryl-PL were detected with excitation and emission wavelengths of 285 and 340 nm, respectively.

Inhibition Experiments for Hydrolysis Using anti-D1 Polyclonal Antibody. Anti-D1 polyclonal IgG was purified from anti-D1 rabbit serum using a Protein D column (Funakoshi, Tokyo, Japan) and was used for the immunochemical staining. A rabbit anti-D1 IgG for 1 h, the membrane was rinsed three times with blocking buffer and was then incubated with goat antibody to rabbit IgG for 1 h. After washing with blocking buffer, the membrane was processed using a Konica immuno-detection system for determining peroxidase activity (Konica Co., Ltd., Tokyo, Japan).

Blood Concentrations of PL and Butyryl-PL after Intravenous Administration. The plasma levels of PL and butyryl-PL after intravenous administration of PL and butyryl-PL to rats (2.5 mg/kg, equivalent to PL) and dogs (2.0 mg/kg, equivalent to PL) are shown in Fig. 2. After the intravenous administration of butyryl-PL to rats, the plasma concentrations of both intact butyryl-PL and converted PL were significantly lower than the plasma PL concentration from intravenously administered PL in rats. In contrast, the temporal profile of PL converted from butyryl-PL after the intravenous administration of butyryl-PL in dogs was nearly the same as that of the intravenously administered PL, although the plasma concentration of intact butyryl-PL was markedly lower.

The apparent hydrolysis ratio from butyryl-PL to PL after intravenous administration was analyzed using the deconvolution method. The plasma levels of PL after the administration of butyryl-PL and after the administration of PL were used as the output and weight functions, respectively. The results of the analysis showed that only 30% of the butyryl-PL was converted to PL up to 2 h after administration in rats, whereas nearly complete conversion was observed within 10 min in beagle dogs (Fig. 3).

The AUCblood and CLtotal of PL and butyryl-PL after intravenous administration in rats and dogs are listed in Table 1. In both species, the CLtotal for PL was nearly the same as the hepatic blood flow due to high clearance via hepatic P450 metabolism, which in agreement with the findings of previous reports (George et al., 1976; Iwamoto and Watanabe, 1985). However, the CLtotal of butyryl-PL in rats was about 1.5-fold greater than hepatic blood flow, and dogs showed a significantly increased CLtotal, that was about 35-fold greater than hepatic blood flow and about 9-fold greater than cardiac output. In both species, particularly in the dog, the CLtotal exceeded hepatic blood flow, indicating that extrahepatic metabolism contributed to the elimination of butyryl-PL.

In Vitro Hydrolysis in Rat and Dog Tissues. In general, the liver is the most efficient organ for drug metabolism, but other organs such as the lung and kidney also often exhibit considerable drug metabolism activity. Moreover, high esterase activity is also present in the plasma. Therefore, the hydrolysis activity for butyryl PL in these tissues was assayed to clarify the extrahepatic metabolism of butyryl-PL. As shown in Table 2, the highest esterase activity for butyryl-PL in rats was observed in the liver microsomes, in contrast to significantly lower activities in the liver cytosol and in the lung and kidney preparations. The hydrolysis activity of lung and kidney microsomes was more than 60
times lower than that of liver microsomes. However, high levels of hydrolase activity for p-nitrophenylacetate (PNPA), a substrate for carboxylesterase, were observed in liver, kidney, and lung microsomes. These findings suggest that butyryl-PL is not easily recognized by the esterases in the rat lung and kidney.

On the other hand, dogs showed high hydrolase activity not only in the liver but also in the lung and the kidney, as shown in Table 2. The hydrolase activity in several organs (except for the plasma) was much higher in dogs than in rats. Although the order of hydrolase activity for butyryl-PL was liver > kidney > lung, that for PNPA was liver > lung > kidney. These findings suggest that substrate specificity varies in different tissues, possibly due to the expression of different esterases.

In addition, the hydrolase activity of rat plasma for butyryl-PL was 1.102 ± 11.4 pmol/min/mg protein, which seemed quite low compared with that of liver microsomes. This may have been due to a high protein content of plasma proteins such as albumin. In fact, the hydrolysis half-life of butyryl-PL (100 μM, 43.5 μg/ml) was about 4 min in rat plasma and 2.8 min in rat blood. In contrast, the hydrolase activity for butyryl-PL in dog plasma was 18.8 ± 2.71 pmol/min/mg protein and its half-life was about 70 min. In addition, the hydrolysis half-life of butyryl-PL in the blood was 31 min, which was much shorter than that in plasma due to contribution of red blood cells to hydrolysis. However, the hydrolysis half-life of butyryl-PL in dog blood is markedly longer than the in vivo elimination half-life of butyryl-PL.

Estimation of Organic Clearance from in Vitro Hydrolysis Parameters. The enzyme kinetic parameters, $K_m$ and $V_{max}$, for hydrolase activity in tissue microsomes were estimated, and the organic clearance was then calculated from the $K_m$ and $V_{max}$ values based on a well stirred model (Table 3). The hepatic clearance of butyryl-PL in rat was nearly the same as the hepatic blood flow, indicating that butyryl-PL was hydrolyzed at a hepatic blood flow-limited rate in the rat liver. However, hepatic clearance accounted for only 60% of the $CL_{total}$ after intravenous administration in the rat. The remaining 40% of the $CL_{total}$ may have been due mainly to hydrolysis in the blood, showing a short half-life of 2.8 min, because hydrolase activity is quite low in the rat kidney and lung.

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Dosing Compound</th>
<th>Rat*</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL AUC$_{blood, PL}$ (ng · h/ml)</td>
<td>649</td>
<td>1052 ± 219</td>
</tr>
<tr>
<td>CL$_{total, PL}$ (l/h/kg)</td>
<td>3.85</td>
<td>1.90 ± 0.61</td>
</tr>
<tr>
<td>Butyryl-PL AUC$_{blood, Bu}$ (ng · h/ml)</td>
<td>433</td>
<td>30.5 ± 8.60</td>
</tr>
<tr>
<td>AUC$_{blood, Bu}$ (ng · h/ml)</td>
<td>289</td>
<td>1103 ± 310</td>
</tr>
<tr>
<td>CL$_{total,Bu}$ (l/h/kg)</td>
<td>5.77</td>
<td>65.6 ± 18.6</td>
</tr>
<tr>
<td>Blood flow (l/kg)</td>
<td>3.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Liver</td>
<td>17.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* Parameters were calculated from mean blood concentration after intravenous administration of PL and butyryl-PL in rats (2.5 mg/kg for rats, equivalent to PL). AUC of drugs after administration in rats was calculated from mean plasma concentrations from time zero to infinity. AUC$_{blood} = AUC_{plasma} \times R_b$. AUC$_{blood, PL}$ means the AUC$_{blood}$ of PL after administration of PL, AUC$_{blood, Bu}$ and AUC$_{blood, Bu}$ PL mean the AUC$_{blood}$ of butyryl-PL and PL after administration of butyryl-PL, respectively. CL$_{total, PL}$ and CL$_{total, Bu}$ mean the total clearance of PL and butyryl-PL after administration of these drugs.
TABLE 2
Hydrolysis activity of plasma, liver, lung, and kidney

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total Activity for R- and S-Butyryl-PL (nmol/min/mg protein)</th>
<th>Activity for PNPA (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat R/S Ratio</td>
<td>Dog R/S Ratio</td>
</tr>
<tr>
<td>Plasma</td>
<td>187 ± 11.4</td>
<td>1.13</td>
</tr>
<tr>
<td>Liver</td>
<td>172 ± 25.4</td>
<td>0.39</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.47 ± 0.26</td>
<td>0.49</td>
</tr>
<tr>
<td>Lung</td>
<td>1.93 ± 0.08</td>
<td>2.39</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.64 ± 0.17</td>
<td>1.59</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.78 ± 0.19</td>
<td>0.40</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.36 ± 0.09</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Organic clearance in dogs was estimated from the hydrolyase activity in the S9 fraction due to the presence of hydrolyase activity in both the microsome and cytosol fractions. The CL\textsubscript{organ} of the liver was the same as the hepatic blood flow, indicating that butyryl-PL was hydrolyzed at hepatic blood flow-limited rate in the dog liver. The CL\textsubscript{organ} values of the lung and kidney were also high at 60 and 70% of the blood flow rate, respectively. Interestingly, the lung showed the highest CL\textsubscript{organ} among these three organs. However, the in vivo CL\textsubscript{total} (65.6 l/h/kg) was much higher than the sum of the CL\textsubscript{organ} values of these three organs (7.3 l/h/kg). In general, an intravenously administered drug first enters the lung and is then distributed to the other organs via arterial blood flow. The above-mentioned findings suggest that butyryl-PL is hydrolyzed by the first-pass effect through the lung before entering the systemic circulation in beagle dogs.

**Intra-Arterial Administration of Butyryl-PL to Dogs.** To demonstrate the extent of first-pass hydrolysis in the dog lung, butyryl-PL was administered intracardially as a route of administration that bypasses the lungs. Figure 4 shows that the plasma concentrations of PL converted from butyryl-PL after intra-arterial administration were significantly lower than those after intravenous administration. The CL\textsubscript{int} of intact butyryl-PL after intra-arterial and intravenous administration of butyryl-PL were 67.1 ± 28.7 and 30.5 ± 8.6 ng·h/ml, respectively, and their ratio (indicating availability of butyryl-PL after passage through the lung) was about 50%. These findings indicate that butyryl-PL undergoes first-pass hydrolysis in the lung in beagle dogs.

**Stereoselective Hydrolysis and Disposition of Butyryl-PL.** The R/S ratios of the hydrolyase activities in various tissues in rats and dogs are listed in Table 2. Butyryl-PL was (R)-preferentially hydrolyzed in dog plasma, liver, lung, and kidney. In rats, the enantiospecificity of the hydrolysis of butyryl-PL differed in various tissues. In particular, (S)-preferential hydrolysis and nonenantioselective hydrolysis were observed in the liver and in the plasma, respectively.

Table 4 shows the AUC of each enantiomer of PL and butyryl-PL after the intravenous administration of racemic PL or racemic butyryl-PL to rats (2.5 mg/kg, equivalent to PL) and dogs (2.0 mg/kg, equivalent to PL). In rats, the AUC\textsubscript{PL} of (R)-PL after the intravenous administration of racemic PL was greater than that of its antipode, in agreement with the findings of previous reports (Vermeulen et al., 1992). However, the AUC\textsubscript{PL} of PL was equivalent for the two enantiomers after correction based on the blood/plasma ratio [Rb: R-PL, 0.55 ± 0.10; (S)-PL, 1.21 ± 0.25]. In addition, both the AUC\textsubscript{PL} and AUC\textsubscript{PL} of (R)-butyryl-PL were 1.4-fold greater than those of the (S)-isomer after the intravenous administration of racemic butyryl-PL to rats. This may have been due to (S)-preferential hydrolysis of butyryl-PL in the rat liver. Moreover, the AUC\textsubscript{PL} of converted (R)-PL after the administration of butyryl-PL was higher in rats than that of PL after intravenous administration of racemic PL.

![Fig. 4. Plasma concentrations of PL and butyryl-PL after intra-arterial and intravenous administration of butyryl-PL (2.0 mg/kg, equivalent to PL) in dogs. The circles and triangles indicate intravenous and intra-arterial administration, respectively. Open and closed symbols indicate the concentrations of converted PL and intact butyryl-PL, respectively. * p < 0.05 compared with intra-arterial administration. Values are mean ± S.D. (n = 4).](image-url)
than that of its antipode, possibly due to subsequent P450 metabolism of (S)-PL after (S)-preferential hydrolysis in the liver.

In dogs, the AUC<sub>plasma</sub> and AUC<sub>blood</sub> of PL after the intravenous administration of racemic PL were not significantly different for the two enantiomers. In addition, the AUC<sub>plasma</sub> and AUC<sub>blood</sub> of (S)-butyryl-PL were greater than those of (R)-butyryl-PL after the administration of racemic butyryl-PL in dogs, reflecting (R)-preferential hydrolysis in various tissues. However, the AUCs for the plasma and blood concentrations of PL converted from butyryl-PL were not enantioselective due to the nearly complete hydrolysis of both enantiomers within 10 min in various tissues, including the lung.

**Inhibition of Hydrolysis of Butyryl-PL by BNPP.** It has previously been demonstrated in inhibition experiments using several inhibitors for esterases that the hydrolysis of butyryl-PL in rat plasma and liver microsomes is catalyzed by carboxylesterase (Yoshigae et al., 1999). To identify the esterase involved in the hydrolysis of butyryl-PL in the dog liver and lung, BNPP was used as a selective inhibitor of carboxylesterase. The relative activity against various BNPP concentrations (10 nM–1 mM) is shown in Fig. 5. High concentrations of BNPP completely inhibited the hydrolysis of either enantiomer of butyryl-PL in microsomal fractions of the liver and lung, indicating that carboxylesterases are involved in the hydrolysis of butyryl-PL in these tissues. Moreover, BNPP inhibited the hydrolytic activity of cytosol in both tissues.

**PAGE Electrophoresis of Subcellular Fraction of the Dog Liver and Lung.** It is well known that the carboxylesterase activity present in microsomes is due to the binding of carboxylesterase to the ER (Satoh and Hosokawa, 1998). Despite the low hydrolase activity in the cytosol of any tissues in the rat, a relatively high cytosolic esterase activity was observed in the liver, lung, and kidney in the dog. To distinguish cytosol esterases from microsomal esterases, non-denaturing gel electrophoresis was performed. As shown in Fig. 6, non-denaturing gel electrophoresis of liver microsomes revealed two electrophoretically distinct esterases with activity toward 1-naphthylacetate. Dog liver cytosol also showed two bands at the same positions as for microsomes, indicating the presence of esterases similar to those in microsomes. The kidney also showed two bands, whereas the lung showed only the upper band in both the cytosol and microsomal fractions. These findings indicate that the dog liver and kidney express two types of esterase but the lung expresses only one. Furthermore, it is suggested that the cytosolic esterases are the same as the microsomal esterases in each tissue. Microsomal carboxylesterase that is loosely

---

**TABLE 4**

<table>
<thead>
<tr>
<th>Administration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-Isomer</td>
<td>S-Isomer</td>
</tr>
<tr>
<td>PL</td>
<td>610</td>
<td>255</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;plasma&lt;/sub&gt;, PL</td>
<td>336</td>
<td>309</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;blood&lt;/sub&gt;, PL</td>
<td>3.71</td>
<td>4.02</td>
</tr>
<tr>
<td>CL&lt;sub&gt;total, Bu&lt;/sub&gt;</td>
<td>281</td>
<td>199</td>
</tr>
<tr>
<td>Butyryl-PL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;plasma&lt;/sub&gt;, Bu</td>
<td>250</td>
<td>180</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;blood&lt;/sub&gt;, Bu</td>
<td>272</td>
<td>89.1</td>
</tr>
<tr>
<td>Butyryl-PL, Bu–PL</td>
<td>150</td>
<td>108</td>
</tr>
<tr>
<td>CL&lt;sub&gt;total,Bu&lt;/sub&gt;</td>
<td>5.01</td>
<td>6.80</td>
</tr>
</tbody>
</table>

<sup>a</sup> AUC of drugs after administration in rats was calculated from mean plasma concentrations from time zero to infinity. Rb values in the rat were (R)-PL, 0.55; (S)-PL, 1.2; (R)-butyryl-PL, 0.88; and (S)-butyryl-PL, 0.91. Rb values in the dog were (R)-PL, 0.80; (S)-PL, 0.86; (R)-butyryl-PL, 0.81; and (S)-butyryl-PL, 0.80. AUC<sub>plasma</sub> and AUC<sub>blood</sub> were calculated by AUC<sub>plasma</sub> multiplied by Rb. CL<sub>total</sub> was calculated from dose/AUC<sub>blood</sub>.

---

**Fig. 5.** Inhibition of the hydrolysis of each butyryl-PL enantiomer in dog liver and lung microsomes by BNPP. BNPP concentrations were 10 nM to 1 mM. Remaining activity values [K, (R)-isomer; ●, (S)-isomer] were plotted against the logarithm of mol of BNPP per gram of protein of microsomes. Values are mean ± S.D. (n = 3).

**Fig. 6.** Non-denaturing gel electrophoresis of dog tissue microsomes and cytosol. Staining for esterase activity was based on the formation of a red insoluble complex between Fast Red TR and 1-naphthol, which was released enzymatically from 1-naphthylacetate. Lanes 1 to 6 contained 35 µg of protein except lane 4 (130 µg of protein). Lane 1, lung cytosol; lane 2, lung microsomes; lane 3, kidney microsomes; lane 4, kidney cytosol; lane 5, liver microsomes; and lane 6, liver cytosol.
bound to the ER may leak into the cytosolic fraction during the preparation of homogenates.

**Identification of Carboxylesterase Isozyme Involved in the Hydrolysis of Butyryl-PL.** The microsomes from rat and dog tissues were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose for immunostaining. Anti-D1 polyclonal antibody, a specific antibody for dog liver carboxylesterase (D1), was used to detect the presence of the enzyme. In the results of immunostaining (Fig. 7), 60-kDa proteins were expressed in the lung and kidney but not in the small intestine in dogs. The cytosol showed an immunoblotting pattern similar to that of microsomes in dog tissues (data not shown), which supports the idea that carboxylesterase leaks from the microsomes during the preparation of homogenates. The 60-kDa protein was also expressed in the rat liver and lung but not in the rat small intestine. This protein might be a D1 cross-reactive carboxylesterase. Moreover, a protein of higher molecular weight was observed in the rat kidney.

The inhibition profiles of hydrolase activities for each enantiomer of butyryl PL by anti-D1 polyclonal antibody are shown in Fig. 8. The control rabbit IgG did not have any influence on the hydrolase activity for butyryl-PL of the dog liver or lung microsomes, but specific anti-D1 polyclonal antibody inhibited 70% of the hydrolysis of both enantiomers of butyryl-PL in the liver and showed nearly complete inhibition in the lung. These findings indicate that D1 or cross-reactive carboxylesterases catalyze the hydrolysis of butyryl-PL in dog liver and lung. However, no inhibition by anti-D1-antibody was observed in the hydrolysis of butyryl-PL in rat liver microsomes.

**Discussion**

Carboxylesterases play an important role in the metabolism of endogenous compounds and exogenous substances such as drugs (including prodrugs), pesticides, and herbicides. Carboxylesterases are widely distributed in the microsomes of the several tissues such as liver, kidney, brain, and lung, where they are loosely bound to the luminal surface of the ER. The highest concentration of carboxylesterases is found in the liver microsomes (Morgan et al., 1994). Furthermore, it has been reported that secretory form such as serum carboxylesterase is highly expressed in rodent (Yan et al., 1995b). Therefore, the in vivo hydrolysis of drug containing ester moiety depends on hydrolase activity in the tissues as well as the blood.

Taking into account the drug disposition after administration, hydrophilic drugs are mainly distributed to the systemic blood circulation, whereas hydrophobic drugs distribute in several tissues. The hydrophobicity and basicity of butyryl-PL strongly suggest that butyryl-PL is distributed to several tissues, including the lung. Therefore, the blood concentrations of PL and butyryl-PL may be regulated by the hydrolytic activity in the respective tissues in each species. In fact, the markedly low blood levels of butyryl-PL observed after the intravenous administration of racemic butyryl-PL in dogs could not explain the hydrolysis in the blood but the rapid hydrolysis of butyryl-PL in several tissues (Table 2).

It has been reported that PL is taken up into the lung by simple diffusion in the dog and that the lung has a capacity for 50 to 60% first-pass uptake of PL (Pang et al., 1982). About 25% of administered PL accumulates in the isolated rat lung during a 5-min perfusion period through simple diffusion and a saturable pathway (Dollery and Junod, 1976). Therefore, butyryl-PL might also be taken up into the lung due to its higher hydrophobicity and similar basicity compared with PL. However, the intravenous administration of butyryl-PL resulted in marked differences in disposition in rats and dogs (Fig. 2). This divergence was mainly due to differences in pulmonary hydrolase activity in rats and dogs. In particular, dogs showed pulmonary first-pass hydrolysis in which about 50% of the administered butyryl-PL was hydrolyzed (Fig. 4). Furthermore, the hydrolysis of butyryl-PL in the liver and kidney at nearly same rate as tissue blood flow led to the rapid and complete conversion of butyryl-PL to PL in the dogs. However, rats showed the only 30% of conversion of butyryl-PL to PL up to 2 h after intravenous administration, and the plasma concentrations of both butyryl-PL and PL were much lower than the plasma PL concentration when PL was administered to rats. These observations suggest that, in the rat, butyryl-PL is distributed to various tissues, including the lung, where esterase activity for butyryl-PL is low (Table 2). After distribution to various tissues in the rat, butyryl-PL can remain in tissues with little hydrolase activity until it diffuses back into the blood and/or is detoxified to other metabolites. Furthermore, if butyryl-PL flows into the liver, which expresses esterase and P450, it may be sequently metabolized to PL-metabolites after hydrolysis. The intrinsic clearance of PL for its P450 metabolism in the rat liver has been reported to be 810 l/h/kg (Ishida et al., 1992) and 1,690 to 2,260 l/h/kg (Gariépy et al., 1992) in in vitro experiments using rat liver microsomes and isolated perfused liver, respectively. Because these values are much larger than the intrinsic clearance by the hydrolysis of butyryl-PL in the rat liver (Table 3), PL is readily metabolized after the hydrolysis of butyryl-PL. The low PL concentration after the intravenous administration of butyryl-PL to the rat could be explained by retention in nonmetabolizing tissues followed by sequential metabolism in the liver, which is the only major hydrolizing tissue for butyryl-PL.

It has been demonstrated that the esterase responsible for pulmonary hydrolysis is D1 based on Western immunoblotting and the inhibition by anti-D1 antibody (Figs. 7 and 8). Carboxylesterase D1 is grouped in the CES-1 family according to the classification system proposed by Satoh and Hosokawa (1998). D1 is also expressed in the dog liver and

Fig. 7. Immunoblots of microsomes from various tissues of the rat and dog probed with anti-D1 IgG. Microsomes from rat and dog tissues were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose for immunostaining. Lanes 1 to 8 contained 5 µg of protein. Lane 1, dog liver; lane 2, dog lung; lane 3, dog kidney; lane 4, dog small intestine; lane 5, rat liver; lane 6, rat lung; lane 7, rat kidney; and lane 8, rat small intestine.
kidney and catalyzes the hydrolysis of butyryl-PL to PL (Table 3). Western immunoblotting showed the presence of a cross-reacting enzyme against anti-D1 antibody in rat tissues. RH-1, RL-1, and hydrolase S, which are in the CES-1 family, are found in the rat lung. Particularly RH-1 is abundantly expressed in the lung (Gaustad et al., 1991; Yan et al., 1995a; Barr et al., 1998). On the other hand, the high levels of RL-1 in the kidney make it possible to evaluate its activity from the hydrolase activity in kidney microsomes. It has been reported that the molecular weight of RL-1 is 61 kDa, in contrast to 58 kDa for RH-1 (Hosokawa et al., 1987). In fact, the cross-reacting protein with anti-D1 antibody in the rat kidney showed a band at a higher molecular weight in Western immunoblotting (Fig. 7). Interestingly, butyryl-PL could not be hydrolyzed in the rat lung and kidney, indicating that RH-1 and RL-1 do not recognize butyryl-PL as a substrate despite their 78 and 66% homology with D1, respectively (Hosokawa et al., 2001). Actually, it has been reported that the hydrolysis of butyryl-PL in rat liver microsomes is not inhibited by anti-RH-1 antibody (Yoshigae et al., 1999).

Hosokawa et al. (1990) have reported that RL-2 presents as a CES-1 isozyme in the rat liver and that RL-2 is characterized by its specific hydrolysis of 1-acyl glycerols (monoglycerides). A possible explanation for the hepatic hydrolysis of butyryl-PL in rat liver microsomes is not inhibited by anti-RH-1 antibody (Yoshigae et al., 1999). Hosokawa et al. (1990) have reported that RL-2 presents as a CES-1 isozyme in the rat liver and that RL-2 is characterized by its specific hydrolysis of 1-acyl glycerols (monoglycerides). A possible explanation for the hepatic hydrolysis of butyryl-PL in rat liver microsomes is that it is catalyzed by a CES-2 enzyme (Yoshigae et al., 1999). However, the major enzyme for hydrolysis of butyryl-PL is not CES-2 enzyme, because the enantioselectivity of the hydrolysis of butyryl-PL in the rat small intestine homogenate was opposite that observed in the liver (Yoshigae et al., 1998b). Further experiments are necessary to identify the CES isozyme responsible for the hydrolysis of butyryl-PL in the rat liver.

Similarly, the CES-2 isozyme is expressed in the dog liver and kidney. As shown in Fig. 6, the lower band in PAGE electrophoresis corresponds to the CES-2 enzyme. The finding that the hydrolytic activity for butyryl-PL in the dog liver showed 70% inhibition by anti-D1 antibody despite complete inhibition by BNPP could be explained by the presence of D2, a dog CES-2 family carboxylesterase, contributory to the hydrolysis of butyryl-PL (Figs. 5 and 8). Moreover, D2 was also observed to contribute to the hydrolysis of butyryl-PL when the hydrolytic activities for butyryl-PL and PNPA were compared in dog lung and kidney microsomes. The ratios of the hydrolysis of PNPA to that of butyryl-PL were 47 and 10 for lung microsomes and kidney microsomes, respectively (Table 2). Because the kidney, which contains both D1 and D2, was found to have a smaller value than the lung, which contains only D1, D2 might extensively catalyze the hydrolysis of butyryl-PL. With regard to the overlap in substrate specificity, it is interesting that butyryl-PL is not hydrolyzed by RH-1 and RL-1, which are grouped in the CES-1 family, despite the fact that CES-2 enzymes, which have less than 50% homology with the CES-1 family, also recognize butyryl-PL as a substrate.
The findings of the present study have demonstrated that pulmonary esterase activity is influenced by the disposition of drugs containing an ester bond and that the species differences in pulmonary esterase activity are based on substrate specificities. A possible carboxylesterase that is present in the human lung is HU-1, a member of the CES-1 family, which has 78% homology with D1 (Munger et al., 1991; Satoh and Hosokawa, 1998). It has been demonstrated that butyryl-PL is a good substrate for HU-1, which is found extensively in the human liver (Satoh and Hosokawa, 1998). These findings suggest the presence of first-pass metabolism of butyryl-PL in the human lung.

In addition to hydrolyase activity in the liver and plasma, pulmonary metabolism is important in the elimination of drugs, but has rarely been studied due to problems inherent in the experimental methodologies currently available. Although in vivo methods have been applied to directly assess pulmonary first-pass metabolism in experimental animals, pulmonary metabolism can also be determined indirectly by conducting in vitro metabolic experiments using lung homogenates. Moreover, commercially available human lung preparations are useful for evaluating pulmonary metabolism and for predicting the disposition of ester-containing drugs in human. Further kinetic studies concerning pulmonary metabolism are required to establish a suitable model for describing pulmonary metabolism in vivo.

References


Address correspondence to: Dr. Teruko Imai, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: iteruko@gpo.kumamoto-u.ac.jp