AN EVALUATION METHOD FOR NONLINEAR LOCAL DISPOSITION IN RAT LIVER AND KIDNEY

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ABSTRACT:

A two-sampling sites method was developed to separately estimate the nonlinear local disposition in the liver and kidney by sampling blood simultaneously from the hepatic vein and an artery after intravenous administration. Using this method, it was attempted to predict the renal elimination from the systemic and hepatic elimination. Etoposide, a substrate of both P-glycoprotein and CYP3A, was used as a model drug. The blood samples from the hepatic vein and an artery were simultaneously taken from a rat after intravenous administration of etoposide at a dose of 20 or 80 mg/kg. At a dose of 20 mg/kg, the total clearance (CL), hepatic clearance (CLH), and renal clearance (CLR) were almost constant, were 2.82 ± 0.24, 0.742 ± 0.214, and 2.09 ± 0.34 l/h/kg, respectively. At a dose of 80 mg/kg, CL and CLR considerably decreased with an increase in plasma concentration, whereas CLR slightly decreased. By means of the two-sampling sites method, we estimated the local drug disposition in the liver and kidney. The present local pharmacokinetic method would be applicable to assess the local disposition of other drugs that are mainly metabolized in these organs.

Drug transporters as well as drug-metabolizing enzymes play a critical role in the disposition and the elimination of xenobiotics. For example, P-glycoprotein (P-gp) is a prominent ATP-dependent efflux pump that has broad substrate specificity (Gottesman and Pastan, 1993). Cytochrome P450 is a key member of the group of phase I enzymes metabolizing many drugs, and it plays an important role in the first-pass metabolism of drugs after oral administration. P450s are mainly expressed in the liver, intestinal wall, and kidney (Ford and Hait, 1990; de Wildt et al., 1999). It is predicted that their substrates exhibit nonlinear pharmacokinetics in these organs.

Etoposide, a podophyllotoxin derivative, is an antineoplastic agent that acts via inhibition of DNA topoisomerase II activity. Etoposide is commonly used in the treatment of neoplastic diseases such as small-cell lung cancer and Kaposi’s sarcoma (Belani et al., 1994; Clark and Slevin, 1987). It is known that etoposide is a substrate of both P-gp (Kunta et al., 2000; Guo et al., 2002) and CYP3A (Kawashiro et al., 1998). Myelosuppression during clinical treatment is a major side effect of etoposide, which is caused by capacity-limited disposition. This side effect of etoposide tends to occur following coadministration with drugs that influence the expression and function of P-gp and CYP3A.

In a recent study, the three-sampling sites method was proposed to separately estimate the local absorption kinetics and the hepatic first-pass metabolism by sampling blood from the portal and hepatic veins, and an artery (Ueda et al., 2002). In this method, the local intestinal absorption kinetics can be assessed by using the blood concentration difference between the portal vein and the systemic circulation after oral administration. By adding the blood concentration in the hepatic vein, the hepatic elimination of the drug can be simultaneously evaluated in a single rat under nonlinear conditions.

In the present investigation, it was attempted to predict the nonlinear local disposition in the kidney based on the information on the systemic and hepatic disposition in the whole living animal, using etoposide as a model drug.

Materials and Methods

Chemicals. Etoposide was purchased from Sigma-Aldrich (St. Louis, MO) and heparin from Novo A/S (Bagsvaerd, Denmark). Sodium pentobarbital solution (Nembutal; Abbott Laboratories, Abbott Park, IL) was used to anesthetize the rats. The etoposide solution had a concentration of 20 mg/ml and was also dissolved in citric acid (2 ml/g), polyethylene glycol 300 (650 mg/ml), benzyl alcohol (30 mg/ml), Tween 80 (80 mg/ml), and ethanol (30.5%, v/v) (Turner et al., 2000). Etoposide was diluted to the required concentration in physiological saline and was prepared immediately before administration to animals. All chemicals used were of analytical or HPLC grade.

Animal Experiments. Male Wistar rats, weighing 210 to 270 g, were purchased from Shizuka Agricultural Co-operative Association for Laboratory Animals (Shizuoka, Japan) and maintained on standard chow and water. All animals were starved for 16 h, with free access to water, before the experiments. The animals were anesthetized with pentobarbital (50 mg/kg body weight), and cannulas (PE10; BD Initiative, Sparks, MD) filled with heparinized saline solution (100 U/ml) were inserted into the hepatic vein, and the right femoral artery of each rat after a midline incision of the abdomen. The cannulation of the hepatic vein was achieved by modifying a reported method...
(Nishigaki et al., 1995, 1998). The hepatic vein junction of the left and central lobe was easily identified. Into the cannula, an indwelling catheter and needle (24-gauge; Terumo Co., Ltd., Tokyo, Japan) were inserted upward about 3 mm into the junction of the hepatic vein. The catheter was fixed to the inside of the hepatic vein using a cardiovascular surgical suture (BEAR surgical sutures, Kyowa Precision Instruments Co., Ltd., Chiba, Japan). The catheter was connected to a polyethylene tube, and the free end of the catheter was exteriorized at the back of the abdomen. The right femoral artery of each rat was also cannulated, and the free end of the catheter was subcutaneously exteriorized at the back of the leg. Both cannulas were filled with heparinized saline solution (100 U/ml) and connected to a 1-ml syringe (PLASTIPAK; BD Initiative). Each rat was held in an animal cage (Bollman cage; Natsume Co., Ltd., Tokyo, Japan). Etoposide was given intravenously (20 or 80 mg/kg) to all the operated rats, and blood specimens from the hepatic vein and the femoral artery were taken after administration of etoposide.

**Investigation of Drug Distribution to Erythrocytes.** The whole blood to plasma partition ratio \( R_p \) of etoposide was evaluated using heparinized whole blood. After preincubation of 0.29 ml of blood at 37°C, etoposide solution (10 \( \mu \)l) was added to produce the standard blood solution (5 or 20 \( \mu \)g/ml). The blood samples were incubated for 15 min at 37°C and centrifuged for 10 min at 3000 rpm. The plasma concentrations of etoposide were then measured by HPLC as described below to estimate \( R_p = (C_p/C_b) \), where \( C_p \) and \( C_b \) are the blood concentration and the plasma concentration of etoposide, respectively. The \( R_p \) values were almost constant at the different concentrations, and the \( R_p \) of etoposide was estimated to be 0.519 \( \pm 0.043 \) (\( n = 6 \)).

**Assay Procedure.** To the plasma samples was added 10 \( \mu \)l of methoxsalen solution as internal standard followed by extraction with 1 ml of chloroform. After centrifugation for 8 min at 3500 rpm, the chloroform layer was removed and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 40 \( \mu \)l of methanol, and 10 \( \mu \)l was injected on to a reverse-phase column (5C18-AR Waters; Nacalai Tesque, Kyoto, Japan) using a C-RA4 HPLC system (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of acetonitrile/acetic acid/water (27:1:72) at a flow rate of 1.2 ml/min. Etoposide was monitored at 254 nm and the detection limit was 0.01 \( \mu \)g/ml. Using the peak area ratio of etoposide to the internal standard, ranging from 0.08 to 150 \( \mu \)g/ml, all correlation coefficients were 0.999.

**Data Analysis.** Systemic elimination. The elimination of etoposide after intravenous administration from the body is based on a two-compartment model with Michaelis-Menten elimination. The pharmacokinetic analysis was based on eqs. 1 and 2:

\[
dX/dt = V_d dC/dt = - \left( CL_{12} + \frac{V_{max}^G}{K_m^G + C_t} \right) C_t + CL_{21} C_2
\]

\[
dX/dt = V_d dC/dt = CL_{12} C_1 - CL_{21} C_2
\]

\[
C_1(t) = 20 \cdot Wt/V_1 \text{ and } 80 \cdot Wt/V_1
\]

\[
C_2(t) = 0
\]

where \( V_{max}^G \) is the maximum elimination rate from the whole body, \( C_1 \) is equal to the plasma concentration \( C_t \) in the systemic circulation, \( K_m^G \) is the Michaelis-Menten constant in the whole body, and \( Wt \) is the mean body weight of rats (0.235; 20 mg/kg, 0.233; 80 mg/kg, respectively). It is noted that the coefficient \( V_{max}^G/(K_m^G + C_t) \) in eq. 1 represents the total plasma clearance. This model was simultaneously fitted to the plasma concentration of etoposide in the systemic circulation after intravenous administration of 20 and 80 mg/kg by MULTI/RUNGE (Yamaoka and Nakagawa, 1983).

**Hepatic metabolism.** The hepatic recovery ratio \( F_{HI} \) depends on the concentration of etoposide in the blood entering liver, and etoposide is eliminated from the liver by a process described by the Michaelis-Menten equation. In this assumption, the hepatic elimination is described by eq. 3:

\[
VD_{out}/dt = Q_{dR}(C_t - C_{ma}) - \frac{V_{max}^H}{K_m^H + f_{out}^C \cdot C_{out}} \cdot C_{out}
\]

where \( f_{out}^C \) represents the concentration in the hepatic tissue, \( V_{HI} \) is the hepatic distribution volume, \( Q_{dR} \) (19.1 ml/min per 250-g rat) is the blood flow rate of the hepatic vein (Ito et al., 1997), and \( C_{ma} \) is generally defined by the flow-weighted average of the input concentration of etoposide entering the liver, where the flow rate of portal vein to that of hepatic artery = 4:1 (Bischhoff et al., 1971), and is given by

\[
C_{ma} = \frac{Q_{pR} C_{por} + Q_{lar} C_P}{Q_{pR} + Q_{lar}}
\]

where \( Q \) is blood flow rate, \( C \) is concentration, and the subscripts por and har specify portal vein and hepatic artery.

When there is no concentration difference between the portal vein and an artery, \( C_{ma} \) coincides with \( C_{out} \) is the plasma concentration of etoposide in the hepatic vein, \( f_{out}^C \) is the protein unbound fraction in plasma, \( K_m^H \) is the Michaelis-Menten constant in the liver, and \( V_{max}^H \) is the maximum elimination rate in the liver. It is noted that the coefficient \( V_{max}^H/(K_m^H + f_{out}^C \cdot C_{out}) \) in eq. 3 represents the plasma intrinsic clearance in the liver. \( f_{out}^C \) in rat is reported to be 0.520 (Fleming et al., 1991), whereas \( f_{out}^C \) in human is 0.15 to 0.07 (Tofolli et al., 2001; Wurthwein et al., 2002). The change in blood concentration in the global process is slow compared with that in the single-pass process through the liver (Fukumura et al., 1999; Higashinori et al., 2000). Thus, the transient difference (\( \Delta C \)) in the drug concentration can be approximated to be zero for a short time interval (\( \Delta t \)) in the liver (i.e., pseudosteady state condition) (Ueda et al., 2002). This concept is based on the rectangular approximation of \( C_{ma}(t) \) and \( C_{out}(t) \) to \( C_{ma,n} \) and \( C_{out,n} \) (i = 1 to n) where \( n \) is number of sampling points:

\[
Q_{dR}(C_t - C_{ma}) - \frac{V_{max}^H}{K_m^H + f_{out}^C \cdot C_{out}} \cdot C_{out} = 0
\]

\[
F_{HI} = \frac{C_{out}}{C_t}
\]

**To estimate \( K_m^H/f_{out}^C \) and \( V_{max}^H \).** eq. 5 was fitted to \( F_{HI} \) versus \( C_{ma} \) by MULTI (Yamaoka et al., 1981). When \( C_{ma} \) approaches zero, the hepatic recovery ratio is reduced to eq. 6,

\[
F_{HI \rightarrow 0} = \frac{Q_{dR} R_p}{Q_{dR} R_p + f_{out}^C \cdot V_{max}^H/K_m^H}
\]

Eq. 6 is well known in the linear pharmacokinetics, when \( V_{max}^H/K_m^H \) is replaced by the intrinsic clearance (\( CL_{ma} \)) in the linear system.

**Renal elimination (nonhepatic elimination).** The total clearance (CL) is generally represented by the sum of the hepatic clearance (\( CL_{ma} \)) and renal clearance (\( CL_{ur} \)),

\[
CL(C_p(t)) = CL_{ma}(C_p(t)) + CL_{ur}(C_p(t))
\]

Thus, \( CL = CL_{ur} \) was assumed to be \( CL_{ur} \). In the case of nonlinear elimination, the total and hepatic clearances are calculated by eqs. 8 and 9.

\[
CL(C_p(t)) = \frac{V_{max}^G}{K_m^G + C_p(t)}
\]

\[
CL_{ur}(C_p(t)) = Q_{dR}(1 - F_{HI}(C_p(t)))
\]

**Mean clearances.** In this study, the global pharmacokinetics of etoposide is capacity-limited and the local disposition of etoposide is nonlinear. Therefore, an index is needed to characterize the nonlinear hepatic and renal disposition in the whole body (in loci). We define the mean clearances \( CL, CL_{ma}, \) and \( CL_{ur} \) by eq. 10 from the standpoint of statistics.
The elimination of etoposide in the systemic circulation after intravenous administration was based on a two-compartment model with Michaelis-Menten elimination. Based on this model, the Michaelis-Menten constant in the whole body \( K_{\text{m, whole}} \) and the maximum elimination rate from the whole body \( V_{\text{max, whole}} \) were 14.6 \( \mu g/ml \) and 205 \( \mu g/min \), respectively. Figure 2 shows the time courses of the systemic plasma concentration divided by the dose (20 or 80 mg/kg). The time courses must coincide in the linear system. As shown in Fig. 2, the elimination rate at a high dose was slower than that at a low dose. Akaikes information criterion was 56.3 in the nonlinear two-compartment model described by eqs. 1 and 2, whereas Akaikes information criterion was 76.1 in the linear two-compartment model (Yamaoka et al., 1978). Thus, it was confirmed that etoposide disappears from the systemic circulation according to a capacity-limited elimination process. The experimental points and the theoretical lines were in good agreement, demonstrating that the selected pharmacokinetic model is reasonable. The pharmacokinetic parameters of etoposide after intravenous administration at 20 mg/kg and 80 mg/kg are summarized in Table 1. With an increase in dose, the mean residence time (MRT) was prolonged and the total clearance (CL) was reduced, whereas the distribution volume at steady state \( V_{ss} \) remained almost constant, although MRT, CL, and \( V_{ss} \) are apparent parameters in the nonlinear system (Jusko, 1989).

Figure 3 presents \( F_H \) versus \( C_{in} \) in individual rats after intravenous administration of a 20 mg/kg and 80 mg/kg dose of etoposide. The closed circle is the experimental value \( (C_{out}/C_{in}) \) and the line shows the theoretical value according to eq. 5. As shown in Fig. 3A, at a 20 mg/kg dose, it was regarded that \( F_H \) was almost constant, although a slight increase was noticed with an increase in \( C_{out} \) and the mean \( F_H \) value was 0.661 \( \pm \) 0.106. In contrast, as shown in Fig. 3B, at a 80 mg/kg dose, \( F_H \) considerably increased with an increase in concentration, demonstrating that the hepatic elimination is obviously saturated at a high dose of etoposide. The Michaelis-Menten constant in the liver \( K_{m, liver}/f_a \) and the maximum elimination rate from the liver \( V_{\text{max, liver}} \) were estimated by eq. 5, and \( F_{H\rightarrow G} \) is calculated by eq. 6 using \( K_{m, liver}/f_a \) and \( V_{\text{max, liver}} \). The predicted \( F_{H\rightarrow G} \) values are in good agreement with the experimental values, indicating that etoposide was eliminated in the liver.
agreement with the experimental initial values. $F_{H}$ was in good agreement with $F_{H}$ at 20 mg/kg. It was regarded that the elimination of etoposide from the systemic circulation and the liver was almost independent of the concentration at 20 mg/kg, whereas it was dependent on the concentration at 80 mg/kg. Figure 4 presents CL, CL$_{H}$, and CL$_{R}$ versus $C_{p}$, where CL was calculated using eq. 8, CL$_{H}$ using eqs. 5 and 9, and CL$_{R}$ using eq. 7. Figure 4 demonstrates that CL, CL$_{H}$, and CL$_{R}$ were reduced as an increase in $C_{p}$ at a dose of 80 mg/kg, although the dose dependence of CL$_{H}$ is minor. All clearances were reduced with an increase in $C_{p}$. CL$_{R}$ was quite low, particularly at the high concentration of etoposide.

Figure 5 compares the mean clearances between 20 mg/kg and 80 mg/kg. CL and CL$_{R}$ at 80 mg/kg were significantly smaller than those at 20 mg/kg, whereas CL$_{H}$ was not significantly different between 20 mg/kg and 80 mg/kg, demonstrating that the hepatic elimination of etoposide is practically linear.

At 20 mg/kg, CL$_{H}$ was 1.24 l/h/kg which was greater than the glomerular filtration rate (0.27 l/h/kg for rats) (Patel et al., 1989). Thus, it was demonstrated that the tubular secretion mediated by a transporter (P-gp) is the main eliminating pathway of etoposide in the kidney. It is reported that approximately 20 to 45% of etoposide is excreted in the urine unchanged (Clark and Slevin, 1987), and etoposide is mainly removed via excretion by the kidney.

The concentration difference of etoposide was not observed between the portal vein and an artery, despite the presence of both P-gp and CYP3A in the intestinal wall. It is known that levofloxacin and ciprofloxacin are moved from basal side to apical (mucosal) side of Caco 2 cells in a Transwell experiment (Gavet et al., 1997). However, no difference was noticed between the portal and the systemic concentration when there was no absorption from the GI tract into the portal system (Fujieda et al., 1996; Moriwaki et al., 2002). There is a monolayer (Caco 2 cell) between apical and basal sides in the experiment in vitro, whereas there are many layers in addition to epithelium cell between the GI tract and portal blood in the experiment in vivo. Therefore, it is predicted that these layers make a barrier which blocks the movement of etoposide from the portal vein to the GI tract.

In conclusion, the two-sampling sites method offers a means of evaluating the local disposition in the liver and kidney, which mainly metabolize drugs. The proposed mean clearances (CL$_{H}$ and CL$_{R}$) are indices to characterize nonlinear local hepatic and renal disposition in the whole body.

![Figure 2](image2.png)

**FIG. 2.** Arterial plasma concentration-time profiles of etoposide after intravenous administration at 20 mg/kg (○) or 80 mg/kg (□), and the lines are predicted by MULTI.

Each point represents the mean ± S.D. (n = 3).

![Table 1](image1.png)

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 mg/kg</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.639 ± 0.096</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>2.82 ± 0.24</td>
</tr>
<tr>
<td>$V_{SS}$ (l/kg)</td>
<td>1.37 ± 0.18</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n = 3). * p < 0.05, significantly different from a dose of 20 mg/kg.

![Figure 4](image3.png)

**FIG. 4.** The relationship between total clearance (CL), hepatic clearance (CL$_{H}$), renal clearance (CL$_{R}$), and plasma concentration ($C_{p}$) in the individual rat after intravenous administration at 80 mg/kg etoposide.

- CL, □ CL$_{H}$, ▲ CL$_{R}$, respectively.

![Figure 5](image4.png)

**FIG. 5.** Comparison of 20 mg/kg (○) and 80 mg/kg (■) in CL, CL$_{H}$, and CL$_{R}$.

Each bar represents the mean ± S.D. (n = 3), * p < 0.05, significantly different from a dose of 20 mg/kg.
References


