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Influence of Pentobarbitone on In-vivo Local Disposition of Diclofenac in Rat Liver

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Abstract

Because the liver is the main organ eliminating many drugs from the body and because pentobarbitone and other analogues can inhibit biliary secretion, the influence of pentobarbitone on hepatic local disposition of diclofenac has been investigated. Diclofenac was infused into the portal and femoral veins of non-anaesthetized rats (group A) and rats anaesthetized with pentobarbitone (group B) and the plasma concentration of diclofenac and the total amount of diclofenac excreted in the bile (in both cases intact diclofenac plus its glucuronide) were simultaneously monitored by HPLC at appropriate time intervals. The time-courses of plasma concentration and amount excreted in the bile were evaluated by moment analysis with trapezoidal integration. The hepatic recovery ratio (F_H) was calculated by comparing the area under the curve (AUC) of plasma concentration after intravenous infusion with that after intraportal infusion. The mean biliary transit time ($\bar{t}_b$) was estimated by subtracting the mean residence time (MRT) of the plasma data from the mean biliary residence time (MRT_b) of the biliary excretion data. The F_H values of diclofenac were 0·664 in group A and 0·643 in group B. The biliary excretion ratio (F_b) of total diclofenac after intravenous administration was 27·0% in group A and 14·1% in group B. The $\bar{t}_b$ values for total diclofenac were estimated to be 0·192 h (intravenous) and 0·159 h (intraportal) in group A, and 0·174 h and 0·238 in group B. Analysis of variance showed that differences among these four $\bar{t}_b$ values were insignificant at the 5% level. The differences in the mean residence time (MRT), total clearance (CL) and distribution volume at steady state (V_{SS}) were insignificant between groups A and B.

Whereas total and the hepatic clearance of diclofenac were not affected by pentobarbitone, biliary clearance was extensively reduced. It took a relatively long time for diclofenac to move from the sinusoid into the bile and the time was not affected by pentobarbitone.

The liver is the main organ that eliminates many drugs from the body by metabolism and biliary excretion. An effective method for evaluation of the hepatic local disposition of drugs is, therefore, required for understanding of their disposition. There are many reports of the analysis of hepatic local disposition, but reports on local disposition in-vivo are limited. Intraportal infusion has been adopted as a technique for evaluation of the hepatic first-pass effect in-vivo in the liver (Hori et al 1992; Bramer et al 1993). After bolus intravenous injection there is a lag between the time-course of the plasma concentration of a drug and that of biliary excretion rate. To explain this lag-time, the concept of mean biliary excretion time for the hepatobiliary transport process was proposed by using phenol red as a model substance in anaesthetized rats (Kakutani et al 1992).

Pentobarbitone is used widely as an anaesthetic in animal experiments. Pentobarbitone and other barbiturates inhibit the biliary secretion of organic acids or organic anionic dyes. We have previously shown that pentobarbitone blocked the enterohepatic circulation of diclofenac by inhibition of biliary excretion of diclofenac and its glucuronide by pentobarbitone anaesthesia (Fukuyama et al 1994). A new method of analysis using the portal-venous concentration difference (P-V difference) was proposed independently by Hoffman et al (1995) and Tabata et al (1995) for evaluation of the first-pass effect in-vivo, especially absorption from the intestinal tract into the portal system and local disposition in the liver of the conscious rat.

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The purpose of this investigation was to evaluate the effect of pentobarbitone on hepatic local disposition of diclofenac by use of the clearance concept, by means of intravenous and intraportal infusions into conscious rats and rats anaesthetized with pentobarbitone.

Methods and Materials

Chemicals

Diclofenac sodium was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Heparin was obtained from Novo Industries (Denmark). Sodium pentobarbitone solution (Nembutal for animal injection; Abbott Laboratories, North Chicago, IL, USA) was used to anaesthetize the rats. Other reagents used for the assay of diclofenac were of guaranteed grade or HPLC grade.

Animal experiments

Male Wistar rats, 195–240 g, were divided into two groups, non-anaesthetized (group A) and anaesthetized with intraperitoneal pentobarbitone (50 mg kg^{-1}) (group B). The abdomens of the rats were opened by midline incision under a light ether anaesthesia. One cannula was implanted into the bile duct to prevent enterohepatic circulation. The free end of the cannula was led out through the incision in the abdomen.

Simultaneously, the right jugular vessel of each rat was cannulated for venous blood sampling; the free end of this cannula with heparinized (100 int. units mL^{-1}) physiological saline was directed subcutaneously and led out at the top of the neck to prevent its removal by the rat. Rats in each group were

further divided into an intravenous infusion group and an intraportal infusion group. A third cannula, for infusion, was implanted into the right femoral vein in the intravenous infusion group, and implanted in the upper part on the portal system through the pyloric vein in the intraportal infusion group. The free end of the third cannula for the intraportal infusion group was led out through the incision on the abdomen. The rats in group A were held in a Bollman gauge and were left to recover for 30 min from the ether anaesthesia. The rats in group B were held in the Bollman gauge after pentobarbitone administration (50 mg kg^{-1}) and the subsequent experiment started after 30 min. Diclofenac sodium (500 μg $min\ kg^{-1}$) dissolved in polyethylene glycol (30% solution) was infused for 30 min into the third cannula by means of a peristaltic pump. Blood samples (0·11 mL) were drawn from the right jugular vein at 2, 5, 10, 15, 30, 32, 35, 40 and 45 min and 1, 1·5 and 2 h in group A, and at 2, 5, 10, 20, 30, 32, 35, 40 and 50 min and 1, 1·5 and 2 h in group B. After each sampling, the sampled blood was supplemented with an equal volume of saline. After centrifugation for 5 min at 2000 *g*, the separated plasma samples were stored at −20°C until analysis. The bile samples were collected during the time periods 0–0·5 h, 0·5–1·0 h, 1–1·5 h, 1·5–2·5 h and 2·5–3·5 h and were immediately frozen at −20°C.

Assay procedure

Acetonitrile (100 μL) was added to the plasma sample (50 μL) to denature the plasma protein. After precipitation of the protein by centrifugation (5 min at 2000 *g*), supernatant (50 μL) was injected into the HPLC (detection limit 0·01 μg mL^{-1}; n = 3; CV < 20%). Most of the diclofenac was excreted as its glucuronide in the bile. The biliary concentration of total diclofenac (intact diclofenac plus its conjugate) was determined by adding Na_2CO_3 (0·1 M; 100 μL) to the bile sample (100 μL). The mixture was incubated for 2 h at 40°C to hydrolyse completely the glucuronide conjugate of diclofenac (Tsuchiya et al 1980). A mixture of 1% CH_3COOH and methanol (33 : 67, v/v; 300 μL) was then added to the incubated mixture. After shaking for 10 s and centrifuging for 10 min at 2000 *g*, supernatant (50 μL) was injected into the HPLC (detection limit 0·05 μg mL^{-1}; n = 3; CV < 20%). HPLC (LC-10A; Shimadzu, Kyoto, Japan) was performed with a 150 × 4·6 mm i.d. Chemcosorb 5-ODS-H column. For analysis of plasma samples the mobile phase was H_2O-CH_3CN, 1 : 1 (v/v) adjusted to pH 3·3 with CH_3COOH; CH_3OH-CH_3CN-1% CH_3COOH, 55 : 12 : 33 (v/v) was used for the analysis of bile samples. The column temperature was 40°C, the mobile phase flow-rate was 1·0 mL min^{-1} and the detector wavelength was 280 nm. The peak area was recorded with a Chromatopac C-R6A integrator (Shimadzu). Calibration lines for both samples were prepared daily by mixing plasma and bile with diclofenac at five different concentrations in a range assumed to be appropriate to the time-course of the experiment. All calibration line correlation coefficients were > 0.999.

Data analysis

AUC, MRT and MRT_b were calculated by trapezoidal integration with extrapolation (Yamaoka et al 1978). MRT and MRT_b were corrected for the infusion period (T) by use of equations 1 and 2.

$$MRT = MRT^{inf} - T/2 \quad (1)$$

$$MRT_b = MRT_b^{inf} - T/2 \quad (2)$$

where the superscript 'inf' specifies the mean residence times during the infusion period.

Total plasma clearance (CL) and distribution volume at steady state (V_{SS}) were calculated using equations 3 and 4 using the data from the intravenous group.

$$CL = D/AUC_{i.v.} \quad (3)$$

$$V_{SS} = D - MRT_{i.v.}/AUC_{i.v.} \quad (4)$$

The hepatic recovery ratio (F_H) was calculated by means of equation 5:

$$F_H = AUC_{i.p.}/AUC_{i.v.} \quad (5)$$

where AUC is the area under the curve and subscripts 'i.p.' and 'i.v.' specify intraportal and intravenous infusion, respectively.

The hepatic plasma clearance (CL_H) was calculated by use of equation 6:

$$CL_H = Q_b(1 - H_t)(1 - F_H) \quad (6)$$

where H_t is haematocrit (0·46) and Q_b is the blood flow-rate (15 mL min^{-1}/350 g). These values were obtained from the literature (Daemen et al 1989; Davies & Morris 1993).

The biliary clearance CL_b was calculated using equation 7:

$$CL_b = CL \cdot F_b \quad (7)$$

where CL is total plasma clearance and F_b is the recovery ratio into the bile.

The mean biliary transit time (t_b) was calculated using equation 8 (Kakutani et al 1992):

$$t_b = MRT_b - MRT \quad (8)$$

where MRT and MRT_b are mean residence time and the mean biliary residence time, respectively.

All experimental results were expressed as the arithmetic mean and standard deviation of three or four replicate determinations. Statistical analysis was performed by analysis of variance at a significance level of 5%.

Results and Discussion

The time-courses of diclofenac plasma concentration after intravenous and intraportal infusion into non-anaesthetized rats (group A) and rats anaesthetized with pentobarbitone (group B) are shown in Fig. 1. Table 1 presents the moment characteristics (AUC, MRT, CL and V_{SS}) and hepatic recovery ratio (F_H) of diclofenac, calculated from the time-courses of the plasma concentrations. The result that pentobarbitone has no effect on CL and V_{SS} agreed with previous reports (Fukuyama et al 1994).

The time-courses of the cumulative biliary excretion ratio of total diclofenac (intact diclofenac plus its glucuronide) in groups A and B are shown in Fig. 2. Table 2 presents F_b, MRT_b and t_b calculated from the biliary excretion data.

The in-vivo hepatic first-pass effects of diclofenac were previously evaluated by using the portal-venous concentration difference (Tabata et al 1995). In this alternative study, the in-vivo hepatic first-pass effect was evaluated by administering diclofenac by intravenous and intraportal infusion. That the

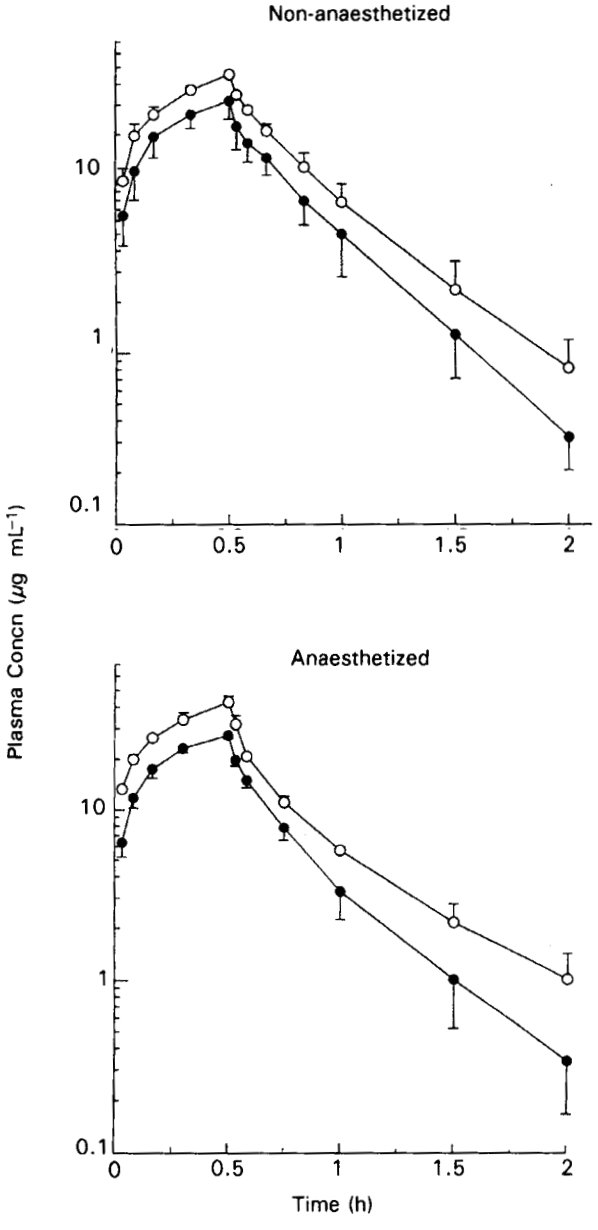

FIG. 1. The time-courses of diclofenac plasma concentration after intravenous (○) and intraportal (●) infusion into non-anaesthetized rats and rats anaesthetized with pentobarbitone (group B).

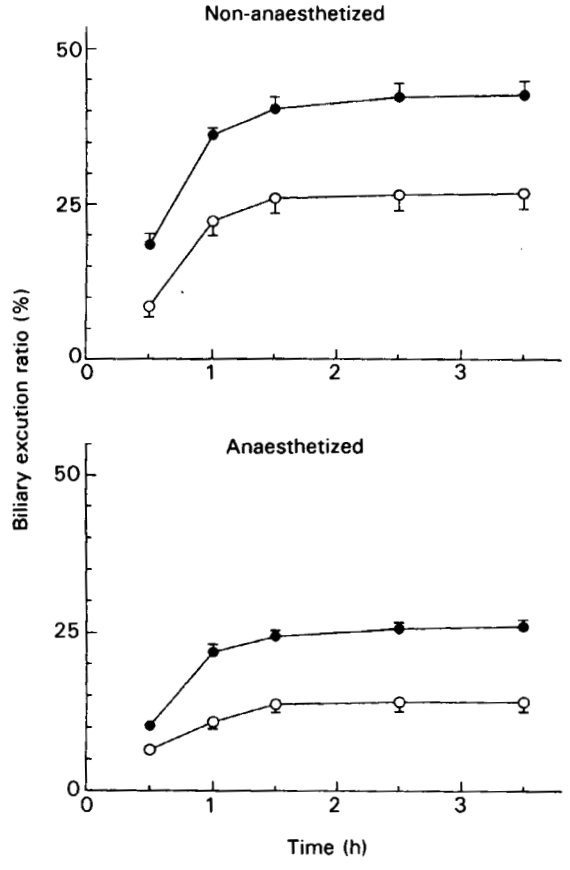

FIG. 2. The time-courses of the cumulative biliary excretion ratio of total diclofenac (intact diclofenac plus its glucuronide) after intravenous (○) and intraportal (●) infusion in non-anaesthetized rats and rats anaesthetized with pentobarbitone.

plasma concentrations in the intraportal group were always lower than those in intravenous group in Fig. 1 is expected from the hepatic first-pass effect. Hepatic recovery ratio (F_H) was estimated to be about 0·65 in both group A and group B, which almost coincided with that previously reported (Tabata et al 1995). There was no difference in MRT between the intravenous and intraportal groups, which shows that the mean hepatic transit time ($\bar{t}_H$) was too short to estimate in this experiment. CL, MRT and V_{SS} were almost the same in groups A and B.

Table 1. Global moment characteristics and disposition parameters from the plasma concentration time-courses of diclofenac.

	Group A (non-anaesthetized, n = 4)		Group B (anaesthetized, n = 3)	
	Intravenous	Intraportal	Intravenous	Intraportal
AUC ($\mu g\ mL^{-1}\ h^{-1}$)	28·3 (1·8)*	18·8 (4·5)	25·4 (1·6)*	16·2 (17)
MRT (h)	0·313 (0·059)	0·279 (0·028)	0·313 (0·023)	0·265 (0·038)
CL ($L\ h^{-1}\ kg^{-1}$)	0·532 (0·036)		0·591 (0·036)	
V_{SS} (L)	0·165 (0·023)		0·185 (0·015)	
F_H	0·664		0·643	

Mean (± s.d.). *Significantly greater than that of intraportal group.

Table 2. Biliary moment characteristics and mean residence time into the bile of diclofenac.

	Group A (non-anaesthetized, n = 4)		Group B (anaesthetized, n = 3)	
	Intravenous	Intraportal	Intravenous	Intraportal
F_b (%)	27.0 ± (2.6)*	42.8 ± (2.2)*	14.1 ± (1.5)	26.1 ± (1.1)
MRT_b (h)	0.506 ± (0.017)	0.439 ± (0.029)	0.487 ± (0.038)	0.503 ± (0.084)
$\bar{t}_b$ (h)	0.192 ± (0.066)	0.159 ± (0.042)	0.174 ± (0.038)	0.238 ± (0.049)

Mean ± s.d. *$P < 0.05$ compared with anaesthetized group.

Q_b in rats anaesthetized with pentobarbitone was reported to be the same as that in conscious rats (Hadengue et al 1988), and we therefore conclude that the disposition of diclofenac was not affected by pentobarbitone. The major part of diclofenac is metabolized in the hepatocytes, and mainly excreted into the bile and the urine as its several metabolites (Riess et al 1978; Stierlin et al 1979). Thus, it is expected that diclofenac is exclusively metabolized by the liver. The estimated hepatic clearances (CL_H) in groups A ($0{\cdot}48 \pm 0{\cdot}03$ L h kg^{-1}) and B ($0{\cdot}51 \pm 0{\cdot}04$ L h kg^{-1} almost coincided with the total clearance; whereas administration of pentobarbitone had no effect on CL and CL_H it reduced biliary clearance (CL_b) by half. In this experiment bile flow-rates were approximately 20 μL min^{-1}; these were not affected by pentobarbitone. The decrease in CL_b is, therefore, not explained by the bile flow-rate. Pentobarbitone anaesthesia has also been associated with a decrease in the cellular concentration of uridine diphosphate-glucuronic acid, the essential cofactor for glucuronidation (Bailey et al 1975; Daemen et al 1986). Many studies have, moreover, demonstrated reduced biliary secretion of many organic acids or organic anionic dyes under pentobarbitone anaesthesia, raising the possibility of competition between pentobarbitone and other administered compounds for the hepatobiliary transport systems available. The excretion of total diclofenac into the bile was severely inhibited by pentobarbitone, whereas the mean biliary excretion time (t_b), was almost unaffected by pentobarbitone. It is reported that diclofenac forms a glucuronide (40%) and hydroxy derivatives (30%) in rat hepatocytes (Stierlin et al 1979). Most of the hydroxy derivatives return from the hepatocytes in the hepatic blood and are finally excreted into the urine. The fact that CL_H was not affected by pentobarbitone whereas CL_b was extensively reduced may be because the diclofenac blocked by administration of pentobarbitone was eliminated through a hepatic metabolic pathway other than biliary excretion. Biliary excretion of intact diclofenac and its glucuronide were reduced by administration of pentobarbitone, but the ratio of intact diclofenac to its glucuronide in the bile was not affected (Fukuyana et al 1994). Glucuronide formation from diclofenac is, therefore, predicted to be unaffected by administration of pentobarbitone.

In conclusion, we have shown that hepatic in-vivo disposition of diclofenac, estimated by the method of intravenous and intraportal infusion, almost coincided with that found by the method of portal-venous concentration difference. About 35% of diclofenac was removed by the hepatic first-pass effect, which was unaffected by administration of pentobarbitone. The amount of total diclofenac excreted in the bile was reduced from 27 to 14% by administration of pentobarbitone.

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