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Inhibitory Effect of Pentobarbital on Biliary Excretion of Diclofenac in a Rat Liver Perfusion System

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Abstract

The effect of pentobarbital on the biliary excretion of diclofenac was investigated in a rat liver perfusion system following a pulse input of the drug.

Without albumin in the perfusate, a trace amount of diclofenac was detected in the outflow from the liver (< 0·1%). The total biliary excretion of diclofenac (intact diclofenac plus its glucuronide) decreased from 23·8% (diclofenac 6·01, glucuronide 17·8%) to 16·3% (diclofenac 5·09, glucuronide 11·2%) with an increase in the perfusate concentration of pentobarbital from 0 to 2·5 $\mu g\ mL^{-1}$. At pentobarbital concentrations exceeding 2·5 $\mu g\ mL^{-1}$, the biliary excretion of diclofenac and its glucuronide (14% total diclofenac) was not reduced further. The mean local excretion times of both diclofenac and its glucuronide were approximately 17 min and were unchanged at all pentobarbital concentrations tested. The ratios of biliary excreted diclofenac and its glucuronide to total diclofenac were 22 and 78%, respectively, and these values were virtually constant at all concentrations of pentobarbital in the perfusate.

These results suggest that the glucuronidation of diclofenac and the biliary excretion of its glucuronide are rapid processes and that pentobarbital blocks a step before glucuronidation.

Some anaesthetics that are commonly used in animal experiments can affect the global disposition of a drug. The liver is the most important organ in the elimination of drugs; this elimination occurs by means of chemical transformation and biliary excretion. The local hepatic disposition of some drugs in the anaesthetized animal is often significantly different from that in the conscious animal. When the local hepatic pharmacokinetics of such drugs is investigated, the use of a conscious animal or the choice of a suitable anaesthetic is required. However, such experiments are usually performed with the anaesthetized animals. It is therefore important to investigate the effects of commonly used anaesthetics on the disposition of various drugs in the liver. Some barbiturates, such as phenobarbital and pentobarbital, inhibit the biliary excretion of organic anions. For example, the biliary excretion of probenecid (Klaassen 1971), succinylsulphathiazole (Bailey et al 1975), iopanoic acid (Cooke & Cooke 1983) and indocyanine green (Daemen et al 1986) were shown to be decreased in rats anaesthetized with pentobarbital in experiments carried out to determine the global disposition of these drugs. The inhibitory effect of phenobarbital on the biliary excretion of acetaminophen glucuronide was extensively investigated in experiments in-situ, in-vitro and in-vivo (Studenberg & Brouwer 1992; Studenberg & Brouwer 1993; Brouwer 1993).

There are few investigations on the effect of pentobarbital on drug disposition in hepatic perfusion systems. It has been reported that pentobarbital had no effect on the local disposition of lidocaine and its glucuronide in a single-pass perfusion system using rat isolated liver (Ngo et al 1995). Diclofenac sodium, a nonsteroidal anti-inflammatory drug, is an organic anion which is excreted significantly into the rat bile (Riess et al 1978; Stierlin et al 1979). In a previous study, we found that the enterohepatic circulation of diclofenac was extensively blocked in rats anaesthetized with pentobarbital (Fukuyama at al 1994). In the present investigation, we have evaluated the effect of pentobarbital on the biliary excretion of diclofenac in a rat liver perfusion system following the pulse input of diclofenac.

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Materials and Methods

Chemicals

Diclofenac sodium was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Pentobarbital sodium was obtained from Nakalai Tesque Inc. (Kyoto, Japan). The other reagents used for the Krebs-Ringer bicarbonate buffer and the mobile phase of HPLC were of guaranteed reagent or HPLC grade.

Animal experiments

Male Wistar rats, 205–268 g, were used. A single-pass perfusion experiment in-situ using a rat isolated liver was performed according to the method of Mortimore & Tieze (1959). The rats were lightly anaesthetized with ether, the abdomen was incised and the common bile duct was cannulated with a polyethylene tube (100 mm PE-10; Becton Dickinson & Co., Parsippany, USA). Pentobarbital sodium was dissolved in Krebs-Ringer bicarbonate buffer (pH 7·4) containing 10 mM glucose to make perfusates of appropriate concentrations (0, 1, 1·5, 2·5, 25 and 250 $\mu g\ mL^{-1}$). Albumin and red blood cells were not added to the perfusate, to avoid their influence on diclofenac disposition in the liver. The perfusate was saturated with 95% O_2–5% CO_2 and was maintained at 37°C during the experiment. After the completion of ether inhalation, the portal vein was rapidly cannulated with a polyethylene tube (1·67 mm o.d., Hayashi R.K. Co. Ltd, Kyoto, Japan) and the perfusate was delivered through the cannula into the liver by a peristaltic roller pump (RP-N3, Furue Science Co. Ltd, Tokyo, Japan) at a constant flow rate ($15 \pm 0{\cdot}4$ mL min^{-1}). It has

been demonstrated that, at this flow rate, the hepatic uptake and metabolism of many drugs in the perfusate remain unchanged for 2 h, even without albumin and red blood cells (Schary & Rowland 1983; Rowland et al 1984). The outflow tube was inserted into the thoracic vena cava inferior. The flow recovery was 99·8%. After the exchange of portal blood with the perfusate, the perfused liver was stabilized for 20 min. Diclofenac sodium (0·12 mL), dissolved in the perfusate buffer (200 $\mu g\ mL^{-1}$), was instantaneously injected into the liver through the portal vein cannula, using a six-position rotary valve injector (Type 50 Teflon rotary valve, Rheodyne, Cotati, CA, USA). The outflow samples were collected at intervals of 2 s for 100 s (Evans et al 1991, 1993). The bile samples were collected at various intervals for 60 min. All samples were immediately stored at −20°C until assay. The volume of the bile samples was assumed to be equal to the weight. The condition of the liver during perfusion was assessed by monitoring the bile output and the recovery of the inflowing perfusate and by visual examination of the liver (Diaz-Garcia et al 1992). All animal experiments were carried out following the guidelines of the Home Office Act 1986 (Wootton & Flecknell 1987).

Assay procedure

Diclofenac concentrations in the perfusate and bile were determined by HPLC (El-Sayed et al 1988; Ribera et al 1991). A high-performance liquid chromatograph (LC-1 OA, Shimadzu Co., Kyoto, Japan) was used with a stationary phase of Chemcosorb 5-ODS-H (150 × 4·6 mm i.d.). The mobile phases were a mixture (pH 3·3 adjusted with acetic acid) of $H_2O : CH_3CN$ (1 : 1 v/v) for analysis of the perfusate and a mixture of $CH_3OH : CH_3CN$: 1% CH_3COOH (55 : 12 : 33 v/v/v) for analysis of the bile. The detector wavelength, the flow rate of the mobile phase and the column temperature were set at 280 nm, 1·0 mL min^{-1} and 40°C, respectively. The peak area was recorded with Chromatopac C-R6A (Shimadzu, Kyoto, Japan). Calibration lines were freshly prepared by adding known amounts of drug to perfusate or bile, using four points at a range of appropriate concentrations. All correlation coefficients of the calibration lines were greater than 0·999. CH_3CN (300 μL) was added to the perfusate (100 μL) to denature the plasma protein. After precipitation of the protein by centrifugation (5 min, 2000 *g*), the supernatant (50 μL) was injected into HPLC (detection limit 0·04 $\mu g\ mL^{-1}$). To determine the concentration of unchanged diclofenac in the bile samples, the solution (90 μL) of 1% CH_3COOH/CH_3OH (33 : 67 v/v) was added to the sample (10 μL) to stabilize the conjugate. After vigorous shaking for 10 s and centrifugation for 10 min at 2000 *g*, the supernatant (50 μL) was injected into HPLC (detection limit 0·2 $\mu g\ mL^{-1}$). Total diclofenac concentration (unchanged plus conjugated) was determined by adding 10 μL 0·1 M Na_2CO_3 to a 10 μL bile sample. The mixture was incubated for 2 h at 40°C to completely hydrolyse the conjugate. The solution (80 μL) of 1% CH_3COOH/CH_3OH was then added to this incubated mixture. After shaking for 10 s and centrifuging for 10 min at 2000 *g*, the supernatant (50 μL) was injected into the HPLC. Trace amounts of diclofenac were detected in the perfusate (less than 0·1% of the dose). The moments of time courses of biliary excretion of diclofenac and its glucuronide were calculated by standard trapezoidal integration without the extrapolation to infinite time (Yamaoka et al 1978).

All experimental results were expressed as arithmetic means and standard deviations. Statistical analysis was performed by analysis of variance with the level of significance at 5%.

Results and Discussion

Table 1 shows the body weights, liver weights, moment values (F_b and t_b) and ratios of biliary-excreted diclofenac (Au/A) and its glucuronide (Ag/A) to total diclofenac versus pentobarbital concentrations. Fig. 1 shows the effect of pentobarbital concentration in the perfusate on the biliary excretion of diclofenac at 60 min. The excreted percentage (F_b) of total diclofenac in the bile decreased from 23·8 to 16·3% as the pentobarbital concentration increased from 0 to 2·5 $\mu g\ mL^{-1}$. At more than 2·5 $\mu g\ mL^{-1}$, F_b of total diclofenac was approximately 14%, almost independent of pentobarbital concentration. Au/A and Ag/A were 22 and 78%, respectively, also almost independent of the pentobarbital con-

Table 1. Moment characteristics calculated by direct integration according to the trapezoidal rule.

	Pentobarbital ($\mu g\ mL^{-1}$)					
	0	1	1·5	2·5	25	250
Body weight (g)	239 ± 22	241 ± 4	237 ± 5	241 ± 4	241 ± 13	232 ± 23
Liver weight (g)	11·1 ± 1·4	10·2 ± 1·1	9·70 ± 0·64	10·8 ± 0·2	11·2 ± 1·5	8·85 ± 0·37
Total						
F_b (%)	23·8 ± 1·7	20·4 ± 2·8	16·5 ± 0·6	16·3 ± 3·0	11·9 ± 1·4	13·3 ± 4·5
t_b (min)	14·0 ± 0·5	15·1 ± 3·2	18·6 ± 3·4	14·8 ± 1·1	17·3 ± 0·3	20·6 ± 2·1
Unchanged						
F_b (%)	6·01 ± 0·72	3·65 ± 0·33	4·01 ± 0·18	5·09 ± 2·28	1·96 ± 0·25	2·40 ± 0·69
t_b (min)	14·2 ± 0·70	14·3 ± 1·8	18·0 ± 2·1	14·5 ± 0·7	17·1 ± 0·5	21·8 ± 2·7
Glucuronide						
F_b (%)	17·8 ± 2·4	16·7 ± 2·8	12·5 ± 0·6	11·2 ± 1·7	9·94 ± 1·34	10·9 ± 3·9
t_b (min)	13·9 ± 0·5	15·1 ± 3·2	18·9 ± 3·7	15·1 ± 1·3	17·4 ± 0·3	20·4 ± 2·0
Au/A (%)	25·6 ± 5·1	17·8 ± 1·8	24·0 ± 1·3	29·8 ± 10·5	16·6 ± 2·1	18·4 ± 1·3
Ag/A (%)	74·4 ± 5·1	82·2 ± 1·8	76·0 ± 1·3	70·2 ± 10·5	83·4 ± 2·1	81·6 ± 1·3

Values are shown as mean ± s.d. (n = 3).

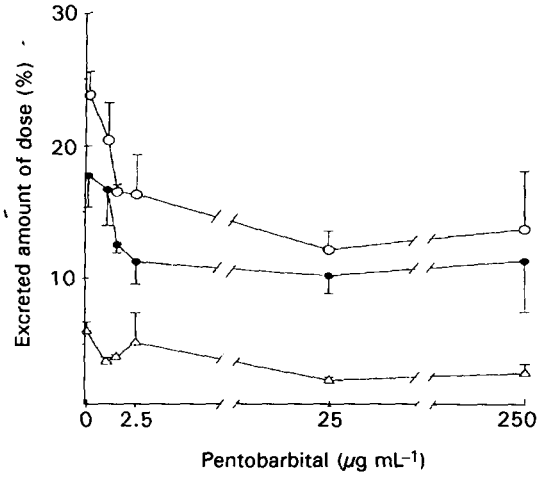

FIG. 1. Effect of pentobarbital on the biliary excretion of diclofenac, total (○), unchanged drug (△) and glucuronide (●). Each point represents the mean ± s.d. of three experiments.

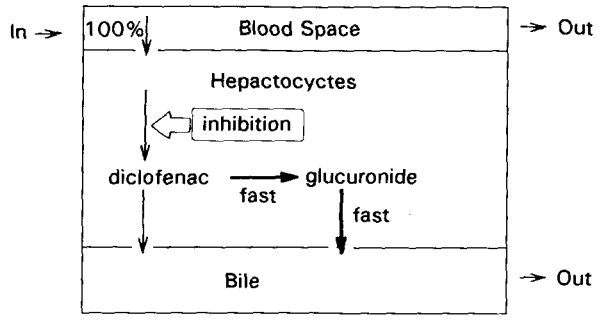

FIG. 2. Schematic representation of the processes involved in the biliary excretion of diclofenac after administration by the method single-pass through the rat liver.

centration. The mean local excretion times (t_b) of both diclofenac and its glucuronide were almost independent of pentobarbital concentration. Further, t_b of diclofenac was very close to that of its glucuronide, with a value of approximately 17 min. The biliary excretion of the glucuronide was considerably greater than that of free diclofenac. Consequently, it can be assumed that both the process of the glucuronidation and the process of biliary excretion after the glucuronidation are rapid. The inhibitory effect of pentobarbital on the biliary excretion of diclofenac was noted at a very low concentration of pentobarbital (1 μg mL^{-1}) and was saturated at a low concentration (2·5 μg mL^{-1}). The usual intraperitoneal dose of pentobarbital in rats is 30–50 mg kg^{-1} and the plasma peak concentration after intravenous administration of 15 mg kg^{-1} is reported to be approximately 25 μg mL^{-1} (Goldstein & Aronow 1960). This indicates that the inhibitory effect of pentobarbital on biliary excretion is not negligible in the usual animal experiment. The biliary excretion of diclofenac and its giucuronide were not completely inhibited even at high pentobarbital concentrations (25 and 250 μg mL^{-1}), demonstrating that three-fifths of the biliary excretion path-way is not inhibited by pentobarbital.

It has been reported that ether decreased the biliary flow rate (Cooper et al 1976) and lowered the hepatic concentration of uridine diphosphate glucuronic acid (UDPGA) (Watkins & Klaassen 1983). In the present investigation, however, the biliary flow rate was normal and the concentration of UDPGA rapidly recovered even in rat isolated hepatocytes (Shipley & Weiner 1985). It has been reported that the biliary excretion of lidocaine glucuronide was unaffected by ether (Ngo et al 1995). Accordingly, we assume that the effect of ether on diclofenac disposition would have been small in the present perfusion experiment.

Fig. 2 shows the processes through which diclofenac is transferred from the sinusoid to the bile duct across the hepatocytes in the liver. Since diclofenac was not detected in the perfusate (less than 0·1% of the dose), it can be concluded that diclofenac administered is almost completely taken up by the hepatic tissues in the albumin-free perfusion system. The major part of the diclofenac in the hepatocytes is converted to its glucuronide in the microsomes and excreted into the bile duct (Riess et al 1978; Stierlin et al 1979; Fukuyama et al 1994). Consequently, the rate of glucuronidation can be assumed to be rapid. We found here that mean local excretion time (t_b) of unchanged drug was very close to that of its glucuronide at all pentobarbital concentrations tested, indicating that the process after glucuronidation to excretion into the bile is also rapid. Further, the ratios of unchanged diclofenac and its glucuronide to the total amount excreted into the bile were approximately constant at all pentobarbital concentrations, suggesting that pentobarbital inhibits a step before glucuronidation.

In conclusion, using the perfused rat liver in-situ, we have confirmed the inhibitory effect of pentobarbital on the biliary excretion of diclofenac quantitatively. This inhibitory effect was noted at a relatively low pentobarbital concentration (1 pg mL^{-1}). Conventional attention must therefore be paid to the effect of pentobarbital anaesthesia on drug disposition in the standard animal experiment.

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