Journal of Pharmacy and pharmacology

Research Paper

Evaluation of changes in hepatic disposition of phenolsulfonphthalein, indocyanine green and FITC-dextran at low temperatures by rat liver perfusion system

Hirotaka Miyamoto[1], Hideaki Miyake[1], Naoki Yoshikawa[1], Haruna Hirata[1], Yuichi Ohwaki[1], Shintaro Fumoto[1], Hitoshi Sasaki[2], Junzo Nakamura[1] and Koyo Nishida[1,3]

[1] Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[2] Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

[3] To whom correspondence should be addressed. (e-mail: dds.yakuzai@gmail.com)

RUNNING HEAD: Changes in hepatic disposition at low temperature

Objectives. The aim of this study is to determine the factor changing the hepatic disposition of a drug during hypothermia using rat liver perfusion system.

Methods. The liver of male Wistar rats was perfused at 37°C, 32°C, or 28°C in the single-pass mode. Venous outflow dilution patterns and biliary excretion rate patterns of phenolsulfonphthalein (PSP), indocyanine green (ICG) and fluorescein isothiocyanate (FITC)-dextran (FD-4, MW 4400) after the injection of a bolus into the perfused rat liver were analyzed based on statistical moment theory.

Key findings. The first-pass extraction ratio (Eh) of PSP was significantly decreased at 32°C and 28°C compared to 37°C. The biliary recovery of PSP and its conjugate were decreased and their biliary excretion kept high concentration and prolonged by low perfusion temperatures. ICG was almost extracted by a single-pass through the liver even at 32°C and 28°C. The biliary recovery of ICG was significantly decreased at low temperature. Although the distribution volume of FD-4 as a vascular reference was not changed by perfusion temperature, the Eh of FD-4 was decreased at 28°C although not markedly.

Conclusion. The change in hepatic disposition of a drug at low perfusion temperatures differed according to disposition processes under hypothermia.

KEY WORDS: phenolsulfonphthalein; indocyanine green; liver perfusion; therapeutic hypothermia; hepatic disposition.

INTRODUCTION

Hypothermia is a therapeutic strategy used after cerebral ischemia and cardiac arrest to protect the brain. Although several clinical studies have reported that treatment with hypothermia in patients with severe head injury, acute stroke, or hastened neurologic recovery improved the outcome (1-3), therapeutic hypothermia can have side effects such as arrhythmia, blood coagulation problems, and impaired immune function (4). Several medicines such as anti- arrhythmic or antibiotics are administered to negate these effects. Furthermore, alterations in the drug disposition of midazolam (5) and phenytoin (6,7) under hypothermia have been reported from clinical studies. However, there has been little systematic information concerning changes in the pharmacokinetics of drugs during hypothermia.

In this study, we defined 32 and 28^{o}C as hypothermia. Because therapeutic hypothermia is done at 32-34^{o}C, it is useful to know the alternation of hepatic disposition of drugs at 32^{o}C. Moreover, we examined the change of hepatic disposition at 28 °C, aiming to consider the unexpected conditions such as too much cooling and body temperature dependency on pharmacokinetic change of a drug in the patients fully by three different body temperatures.

We have already reported that the pharmacokinetics of phenolsulfonphthalein (PSP), indocyanine green (ICG) and fluorescein isothiocyanate-dextran (FD-4, Mw 4400) changed under hypothermic conditions in rats (8). However, as many factors affect drug disposition such as blood flow, transporter and drug metabolizing enzyme, it is difficult to determine individual factors in studies *in vivo*. It is necessary to determine the individual factors affecting drug disposition for prediction the pharmacokinetics during hypothermia. Generally, the hepatic disposition of drugs

consists of four steps (i) uptake into liver from blood, (ii) efflux from the liver, (iii) elimination by metabolism and (iv) excretion into bile from liver. Under the hypothermia, the activity of several transporters and enzymes could changed and this alternation affects on the hepatic disposition of drugs. In this study, we tried to evaluate the effect of hypothermia on hepatic disposition by isolated liver perfusion. The isolated liver is often used to explore hepatic physiology and pathophysiology, because it is easy to control the flow rate and temperature of the perfusate.

We chose PSP, ICG and FD-4 as model compound and these compounds eliminate by different process. PSP is conjugated by enzymes and excreted into bile via multidrug resistance associated protein2 (Mrp2), while ICG is excreted via multidrug resistance P-glycoprotein2 (Mdr2). On the other hand, FD-4 is eliminated by glomerular filtration from kidney and taken up into cell by endocytosis. In this study, we can evaluate the effect of hypothermia on these transporter activities and transport process using PSP, ICG and FD-4. In the present study, we examined the effect of temperature on hepatic disposition of three model compounds, PSP, ICG and FD-4 by isolated liver perfusion system to exclusive of another factor affecting the hepatic disposition, such as flow rate.

MATERIALS AND METHODS

Materials

Phenolsulfonphthalein and indocyanine green were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Daiichi Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Fluorescein isothiocyanate-dextran (FITC-dextran) with an average molecular weight of 4400 (FD-4) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Male Wistar rats (180-210 g) were housed in a cage in an air-conditioned room and maintained on a standard laboratory diet (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments in the present study conformed to the Guidelines for Animal Experimentation of Nagasaki University and approved by Committee of Animal Experimentation of Nagasaki University (Approval number: 0506280443).

Liver perfusion

Rat liver was perfused *in situ* as described by Mortimore *et al*. (9) with slight modifications. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After the middle abdomen was cut open, the common bile duct was cannulated with a polyethylene tube (i.d. 0.28 mm, o.d. 0.61 mm, Becton Dickinson & Co., Parsippany, NJ, U.S.A.). The portal vein was rapidly catheterized with a polyethylene tube (i.d. 1 mm, o.d. 2 mm, Hibiki), and infusion of the perfusate, Krebs-Ringer bicarbonate buffer with 10 mM glucose (oxygenated with 95% O_2-5% CO_2 to pH 7.4 at 37^{o}C), and was

started immediately. The inferior vena cava was catheterized through the right atrium with a polyethylene tube (i.d. 1.7 mm, o.d. 2.7 mm, Hibiki, Tokyo, Japan) and then ligated right above the renal vein. The perfusate was circulated using a peristaltic pump (SJ1211, ATTO Co., Tokyo, Japan) at a flow rate of 13.0 ± 0.2 mL/min (mean ± S.D.). The experiments at low temperature were carried out with the perfusate maintained at 32ºC or 28ºC. To avoid the effect of interaction with albumin and simplify the perfusion system, liver perfusion was carried out with albumin-free perfusate.

After a stabilization period of 30 min, the drug solution (0.1 mL) was injected into the perfusion route. After administration of the drug solution, venous outflow samples were collected into tubes at appropriate time intervals for 1 min. The sampling interval was 1 sec at first and gradually prolonged. Bile samples were collected into weighed test tubes at 5 min for 60 min. The bile sample volumes were calculated from the gain in weight in the test tube assuming the density of the bile to be 1.0. The sampling time was taken as the midpoint of the sampling period. After the perfusion experiment, the whole liver was excised and weighed. The mean weight of the liver was 9.1 ± 0.9 g.

Assay

The concentration of model compounds in the venous outflow perfusate and bile sample was determined as follows. The concentration of free PSP was determined spectrophotometrically at 560 nm after dilution with 1 M NaOH. In the case of bile sample, the total concentration of free PSP and its conjugate was measured in the same manner after the samples were subjected to acid hydrolysis (1 M HCl at

131 100°C for 30 min) (10). The concentration of PSP conjugate was estimated from the
132 difference between these values.

133 The concentration of ICG was determined spectrophotometrically at 805 nm
134 after proper dilution with saline containing 0.1% (w/v) bovine serum albumin as a
135 stabilizer.

136 The concentration of FD-4 was determined spectrophotofluorometrically at
137 excitation and emission wavelengths of 489 and 515 nm, respectively.

138

139 **Pharmacokinetic analysis of outflow patterns and biliary excretion rate-time**
140 **curves**

141 The first two (zeroth to first) moments for the outflow pattern are defined as
142 follows:

143 $$auc = \int_0^\infty \mathrm{C}\,\mathrm{dt} \quad (1)$$

144 $$\bar{t} = \int_0^\infty t \cdot \mathrm{C}\,\mathrm{dt} / auc \quad (2)$$

145 where *t* is the time and C is the concentration of substances normalized by the injection
146 dose as the percentage of the dose per milliliter, and *auc* and $\bar{t}$ are the area under the
147 concentration-time curve and mean transit time, respectively. The moments were
148 calculated by numerical integration using a linear trapezoidal formula and extrapolation
149 to infinite time based on a monoexponential equation (11). We chose two
150 representative parameters, apparent distribution volume (V) and hepatic extraction ratio
151 (E_h), to assess local drug disposition. These parameters were derived from moments
152 described previously (12) as follows:

153 $$\mathrm{V} = \mathrm{Q} \cdot \bar{t} / \mathrm{F} \quad (3)$$

154 $$\mathrm{F} = \mathrm{Q} \cdot auc \quad (4)$$

$$E_h = 1 - auc \cdot Q \quad (5)$$

Where Q is flow rate of perfusate and F is the recovery ratio.

The biliary excretion rate-time curves of free and conjugated PSP were analyzed independently based on the statistical moment theory (11). In the case of ICG, a biliary recovery ratio ($F_{b,free}$) was determined, because the biliary excretion rate-time curve was not appropriate for the monoexponential extrapolation due to incomplete biliary excretion. Biliary moment parameters for FD-4 were not calculated because of biliary excretion.

Biliary moment parameters are defined as follows:

$$auc_{b,free} = \int_0^\infty (dX_{b,free}/dt)dt \quad (6)$$

$$auc_{b,conj} = \int_0^\infty (dX_{b,conj}/dt)dt \quad (7)$$

$$\bar{t}_{b,free} = \int_0^\infty t \cdot (dX_{b,free}/dt)dt / auc_{b,free} \quad (8)$$

$$\bar{t}_{b,conj} = \int_0^\infty t \cdot (dX_{b,conj}/dt)dt / auc_{b,conj} \quad (9)$$

$$F_{b,free} = auc_{b,free} / dose \quad (10)$$

$$F_{b,conj} = auc_{b,conj} / dose \quad (11)$$

where t is the time, and $dX_{b,free}/dt$ and $dX_{b,conj}/dt$ are the biliary excretion rates of free and conjugated PSP, respectively. The values of $dX_{b,free}/dt$ and $dX_{b,conj}/dt$ are normalized with the injected dose per mL. $F_{b,free}$ and $F_{b,conj}$ are the biliary recovery ratios of free and conjugated PSP, respectively. $\bar{t}_{b,free}$ and $\bar{t}_{b,conj}$ are the biliary mean transit times of free and conjugated PSP, respectively. The moments are calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation, from the excretion rate-time curves.

Statistical Analysis

Animal experiments were performed at least 3 times, and the mean and standard error (S.E.) were calculated. Statistical comparisons were performed with Dunnett's test after an analysis of variance (ANOVA). $p < 0.05$ was considered to be indicative of statistical significance, compared to the control condition (control group at 37ºC).

RESULTS

Hepatic disposition of PSP at low perfusion temperatures

Fig. 1 shows the outflow concentration-time curves of free PSP after a bolus was injected into the perfused rat liver at a dose of 0.1 mg under the different perfusion temperatures. Table 1 lists the moment and disposition parameters for the outflow patterns. The outflow peak concentration of free PSP increased according to the decrease in the perfusion temperature (Fig. 1), and *auc* increased to about 1.4 times that of the control condition, respectively, at perfusion temperatures of 32°C and 28°C (Table 1). The E_h of PSP was significantly decreased in the low perfusion temperature group compared to control, and V of PSP was also decreased according to the perfusion temperature.

Figs. 2A, B illustrate the biliary excretion rate-time curves of free PSP and its conjugate after the injection of PSP at a dose of 0.1 mg under the different perfusion temperatures. Similar to the change in E_h, the maximum biliary excretion rates of free PSP and its conjugate decreased according to the perfusion temperature. Table 2 lists the moment parameters for the biliary excretion rate of free PSP and its conjugate under the different perfusion temperatures. The biliary excretion rates in 60 min for free PSP ($F_{b,free}$) and its conjugate ($F_{b,conj}$) in the low perfusion temperature group were decreased to about 50% of the control (Table 2). In addition, the $\bar{t}_{b,free}$ and $\bar{t}_{b,conj}$ of PSP were significantly prolonged under the low perfusion temperatures.

Hepatic disposition of ICG at low perfusion temperatures

Table 1 lists the moment and disposition parameters for outflow patterns of ICG in the perfused rat liver at a dose of 0.1 mg under the different perfusion temperatures. The -outflow patterns are not shown because of the extremely low ICG concentration in the outflow caused by the almost complete hepatic extraction of ICG (Table 1). The *auc* values of ICG were extremely low (Table 1) compared to the other model compounds (Tables 1 and 4). It was thus clarified that hepatic extraction of ICG was almost 100% even at low perfusion temperatures.

Fig. 3A shows the biliary excretion rate-time curves of ICG after the injection of a bolus of 0.1 mg under the different perfusion temperatures. The biliary excretion rate decreased with the perfusion temperature and either plateauted or continued to rise until 60 min at low perfusion temperatures. As shown in Fig. 3B, the F_b of ICG in 60 min at 28 and 32 ^oC was significantly decreased to less than about 40% of the control value.

Hepatic disposition of FD-4 at low perfusion temperatures

Fig. 4 shows the outflow patterns of FD-4 after a bolus was injected into the perfused rat liver at a dose of 0.1 mg under the different perfusion temperatures. There were no considerable changes among the perfusion temperatures in the outflow concentration of FD-4. Moment and disposition parameters of outflow patterns of FD-4 are summarized in Table 1. V of FD-4 as a vascular reference was unchanged under the low perfusion temperatures, and well correlated to the previously obtained data (12). While the E_h of FD-4 at 32 ^oC was not changed, the E_h of FD-4 was significantly decreased at 28^oC compared to 37^oC.

DISCUSSION

We performed single-pass rat liver perfusion experiments under different perfusion temperatures to examine the changing factors *in vivo* during therapeutic hypothermia. The perfusion can be done independently of the influence of other organ systems, plasma constituents and neural-hormonal effects. Compared with other *in vitro* models, however, the hepatic architecture, cell polarity and bile-forming capacity are preserved in the liver perfusion system.

PSP, a hydrophilic dye (organic anion), has been clinically used to test renal function in humans, and is excreted into the bile and urine as a free form or conjugative metabolite in rats (10). PSP is known to be taken up by organic anion transporter (OAT) (13) and excreted into bile via multidrug resistance associated protein2 (Mrp2) (14). In the rat liver perfusion of PSP, E_h and V were significantly decreased by 40% at 28^{o}C compared to the control condition. The decrease of V was caused by the alternation of *auc* because the V was calculated by *auc* and $\bar{t}$ (Eq. 3, 4) and then the *auc* of PSP was increased under hypothermia while the $\bar{t}$ did not change (Table 1). In this study analyzed based on moment theory, we cannot evaluate the effect of hypothermia on influx and efflux process individually. However, the increase of *auc* under hypothermia might be influenced by alternation of influx process because the peak concentration of PSP was increased (Fig 1).

Moreover, we analyzed the biliary excretion of free and conjugated PSP in terms of metabolism in the hepatocytes and secretion from the hepatocytes into the bile. These processes were characterized by the biliary recovery ratio ($F_{b,free}$, $F_{b,conj}$) and biliary mean transit time ($\bar{t}_{b,free}, \bar{t}_{b,conj}$). The biliary mean transit times of free and conjugated PSP were calculated to be 13.3 and 18.8 min and significantly prolonged

under hypothermia, respectively (Table 2). In a previous study (8), the biliary and metabolic clearance of PSP were reduced under hypothermic conditions *in vivo*, correlating with the decreasing ratio of $F_{b,free}$ and $F_{b,conj}$ in the rat liver perfusion system. In addition, $F_{b,free}/F_{b,conj}$ of PSP was not altered at 32ºC while it was increased at 28ºC, suggesting that the conjugation of PSP by enzymes was decreased at 28ºC. Because the drugs conjugated to glucronic acid is excreted into bile via Mrp2, the biliary excretion of these drugs could decreased during hypothermia in clinical.

ICG has been widely used as a diagnostic drug to evaluate liver function, especially hepatic blood flow. The characteristics of ICG are an intravascular distribution, a good capacity to bind blood protein, and excretion into the bile without biotransformation (15,16) via multidrug resistance P-glycoprotein2 (Mdr2) (17). As listed in Table 1, the E_h of ICG was not affected by the perfusion temperature. Elimination of drugs such as ICG with a high intrinsic hepatic clearance depends largely upon hepatic blood flow, whereas the clearance of drugs such as PSP with an intermediate or low hepatic extraction ratio is much less dependent on alterations in the hepatic blood flow. In a previous study (8), we clarified that total body clearance (CL_{tot}) of ICG was markedly decreased under hypothermic condition in the rat *in vivo*, according to body temperature. These results suggest that the decrease of hepatic blood flow was the changing factor of drugs with a high hepatic extraction ratio under hypothermic conditions.

The cumulative biliary excretion of ICG in 60 min decreased considerably with the decrease in perfusion temperature (Fig. 3B). The reduction in biliary excretion was likely another factor causing the decrease in CL_{tot}. The transepitherial transport of digoxin via multidrug resistant protein-1 (MDR1) was evaluated at various

277 temperatures *in vitro* using LLC-GA5-COL150 cells that expressed human
278 P-glycoprotein specifically on the apical surface showed a multidrug resistant
279 phenotype. (18). According to this study, MDR1-mediated transport of digoxin
280 decreased at lower temperatures. ICG was excreted into bile by Mdr2, which is one of
281 the ABC transporters, so the decrease in biliary excretion at low perfusate temperatures
282 would be caused by changes in Mdr2 activity. If the Mdr2 activity could decreased
283 under hypothermia, the pharmacokinetics of digoxin and verapamil, which is substrate
284 of Mdr2, might differ during hypothermia. Furthermore, the reduction of ICG
285 excretion into bile under hypothermia was larger than that of PSP. This result
286 suggests that the effect of hypothermia on Mdr2 might be greater than that on Mrp2.
287 However, further studies are necessary for us to investigate the effect of hypothermia on
288 ABC transporter activity by *in vitro* experiment.

289 FD-4 is excreted mainly by glomerular filtration, and the contribution of
290 hepatic extraction is very low. (19-21) We used FD-4 as a vascular reference in the
291 rat liver perfusion study. It was reported that distribution volume of ^{131}I-human
292 serum albumin, another vascular reference substance, was not changed at 27^{o}C,
293 compared to 37^{o}C. (12) The *auc* and V were not changed under the low perfusion
294 temperatures. It was thus indicated that the distribution of FD-4 in the liver was not
295 affected by perfusion temperature.

296 The E_h of FD-4 was significantly decreased at 28^{o}C compared to 37^{o}C, while
297 there was no change at 32^{o}C, suggesting that the elimination of FD-4 by hepatic uptake
298 leading to endocytosis was slightly decreased by the perfusion temperatures. In case
299 of FD-4, the prolongation of outflow pattern might be caused by slightly release of the
300 FD-4 associated with the liver. Moreover, the $\bar{t}$ of FD-4 was larger than that of PSP,

it probably because of the difference in hepatic disposition between FD-4 and PSP. In the *in vivo* study (8), CL_{tot} of FD-4 was significantly decreased at 28^{o}C, probably because of decreased glomerular filtration as well as hepatic uptake leading to endocytosis.

CONCLUSION

We have demonstrated that the change in hepatic disposition of three model compounds under constant flow rate in the hypothermic group could differ with the disposition route and intrinsic clearance characteristics of the drug, probably due to decrease of transporter activity such as Mdr2 and Mrp2. These results might be helpful for prediction of a pharmacokinetics during hypothermia.

ACKNOWLEDGMENTS

We wish to thank Risa Ogata and Masakazu Kawamura for their skilled technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research from Nagasaki University, Japan.

REFERENCES

1. Marion D.W., Penrod L.E., Kelsey S.F., Obrist W.D., Kochanek P.M., Palmer A.M. *et al.* Treatment of traumatic brain injury with moderate hypothermia. *N Engl J Med* 1997;**336**(8),540-546.

2. Zhi D., Zhang S.,Lin X. Study on therapeutic mechanism and clinical effect of mild hypothermia in patients with severe head injury. *Surg Neurol* 2003;**59**(5),381-385.

3. Olsen T.S., Weber U.J.,Kammersgaard L.P. Therapeutic hypothermia for acute stroke. *Lancet Neurol* 2003;**2**(7),410-416.

4. Schubert A. Side effects of mild hypothermia. *J Neurosurg Anesthesiol* 1995;**7**(2),139-147.

5. Fukuoka N., Aibiki M., Tsukamoto T., Seki K.,Morita S. Biphasic concentration change during continuous midazolam administration in brain-injured patients undergoing therapeutic moderate hypothermia. *Resuscitation* 2004;**60**(2),225-230.

6. Iida Y., Nishi S.,Asada A. Effect of mild therapeutic hypothermia on phenytoin pharmacokinetics. *Ther Drug Monit* 2001;**23**(3),192-197.

7. Leslie K., Sessler D.I., Bjorksten A.R.,Moayeri A. Mild hypothermia alters propofol pharmacokinetics and increases the duration of action of atracurium. *Anesth Analg* 1995;**80**(5),1007-1014.

8. Nishida K., Okazaki M., Sakamoto R., Inaoka N., Miyake H., Fumoto S. *et al.* Change in pharmacokinetics of model compounds with different elimination processes in rats during hypothermia. *Biol Pharm Bull* 2007;**30**(9),1763-1767.

9. Mortimore G.E. Effect of insulin on potassium transfer in isolated rat liver. *Am J Physiol* 1961;**200**,1315-1319.

10. Hart L.G.,Schanker L.S. The chemical forms in which phenol red is secreted into the

bile of rats. *Proc Soc Exp Biol Med* 1966;**123**(2),433-435.

11. Yamaoka K., Nakagawa T.,Uno T. Statistical moments in pharmacokinetics. *J Pharmacokinet Biopharm* 1978;**6**(6),547-558.

12. Nishida K., Tonegawa C., Kakutani T., Hashida M.,Sezaki H. Statistical moment analysis of hepatobiliary transport of phenol red in the perfused rat liver. *Pharm Res* 1989;**6**(2),140-146.

13. Itagaki S., Shimamoto S., Hirano T., Iseki K., Sugawara M., Comparison of urinary excretion of phenolsulfonphthalein in an animal model for Wilson's disease (Long-Evans Cinnamon rats) with that in normal Wistar rats: involvement of primary active organic anion transporter. *J Pharm Pharm Sci.* 2004;**7**(2),227-34.

14. Itagaki S., Sugawara M., Kobayashi M., Miyazaki K.,Iseki K. Mechanism of active secretion of phenolsulfonphthalein in the liver via Mrp2 (abcc2), an organic anion transporter. *Drug Metab Pharmacokinet* 2003;**18**(4),238-244.

15. Cherrick G.R., Stein S.W., Leevy C.M.,Davidson C.S. Indocyanine green: observations on its physical properties, plasma decay, and hepatic extraction. *J Clin Invest* 1960;**39**,592-600.

16. Wheeler H.O., Cranston W.I.,Meltzer J.I. Hepatic uptake and biliary excretion of indocyanine green in the dog. *Proc Soc Exp Biol Med* 1958;**99**(1),11-14.

17. Huang L.,Vore M. Multidrug resistance p-glycoprotein 2 is essential for the biliary excretion of indocyanine green. *Drug Metab Dispos* 2001;**29**(5),634-637.

18. Jin J.S., Sakaeda T., Kakumoto M., Nishiguchi K., Nakamura T., Okamura N. *et al.* Effect of therapeutic moderate hypothermia on multi-drug resistance protein 1-mediated transepithelial transport of drugs. *Neurol Med Chir (Tokyo)* 2006;**46**(7),321-7.

19. Mehvar R. Kinetics of hepatic accumulation of dextrans in isolated perfused rat livers. *Drug Metab Dispos* 1997;**25**(5),552-556.

20. Mehvar R., Robinson M.A.,Reynolds J.M. Dose dependency of the kinetics of dextrans in rats: effects of molecular weight. *J Pharm Sci* 1995;**84**(7),815-818.

21. Mehvar R.,Shepard T.L. Molecular-weight-dependent pharmacokinetics of fluorescein-labeled dextrans in rats. *J Pharm Sci* 1992;**81**(9),908-912.

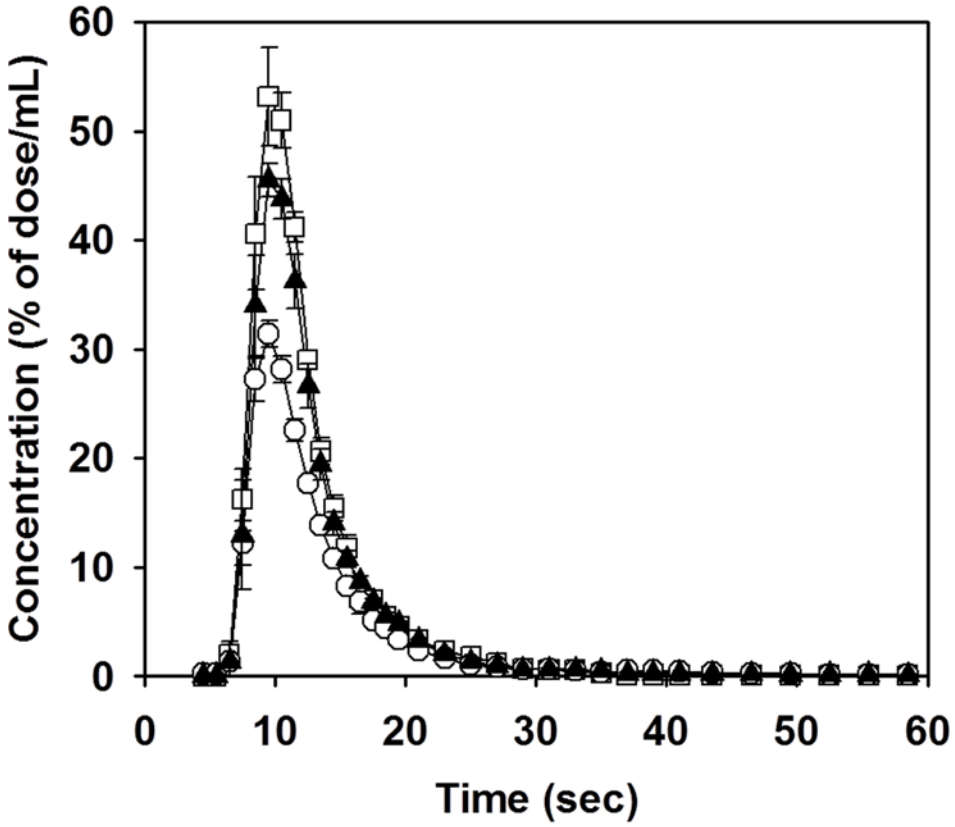
374 **Legend to Figures**

375

376 **Fig. 1** Typical outflow patterns of PSP at a dose of 0.1 mg/liver after a bolus was

377 injected in the single-pass rat liver perfusion system at 37ºC (○), 32ºC (▲) or 28ºC

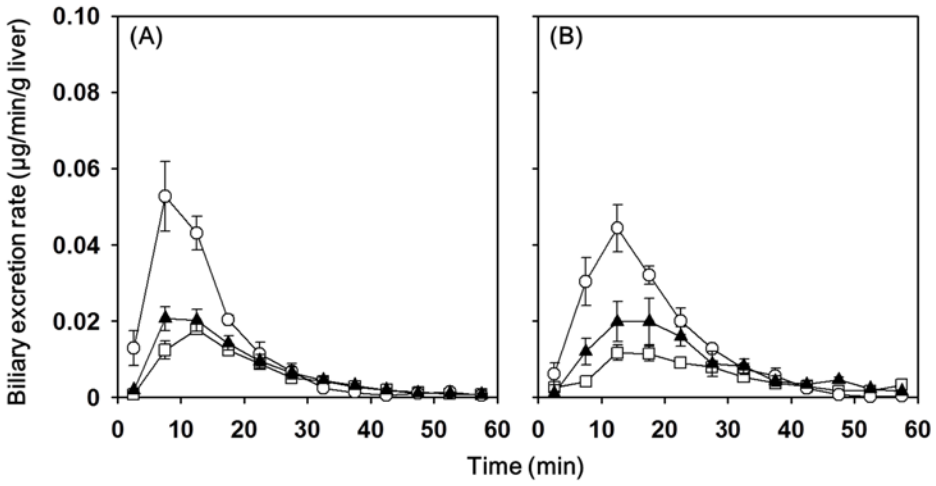
378 (□). Each point represents the mean ± S.E. for at least four experiments.



379

380

Fig. 2 Biliary excretion rate - time curves of free PSP (A) and PSP conjugate (B) at a dose of 0.1 mg/liver in the single-pass rat liver perfusion system at 37°C (○), 32°C (▲) or 28°C (□). Each point represents the mean ± S.E. for at least four experiments.

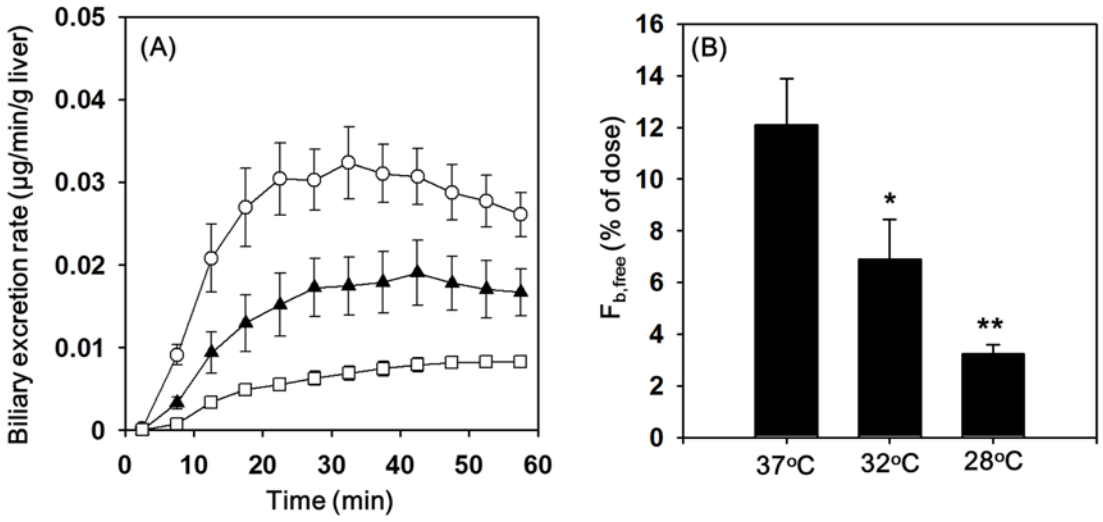


386 **Fig.3** Biliary excretion rate-time curves of ICG (A) and $F_{b,free}$ of ICG for 60 min (B)

387 at a dose of 0.1 mg/liver in the single-pass rat liver perfusion system at 37°C (○), 32°C

388 (▲) or 28°C (□). Each point represents the mean ± S.E. and each column represents

389 the mean + S.E. for at least five experiments.



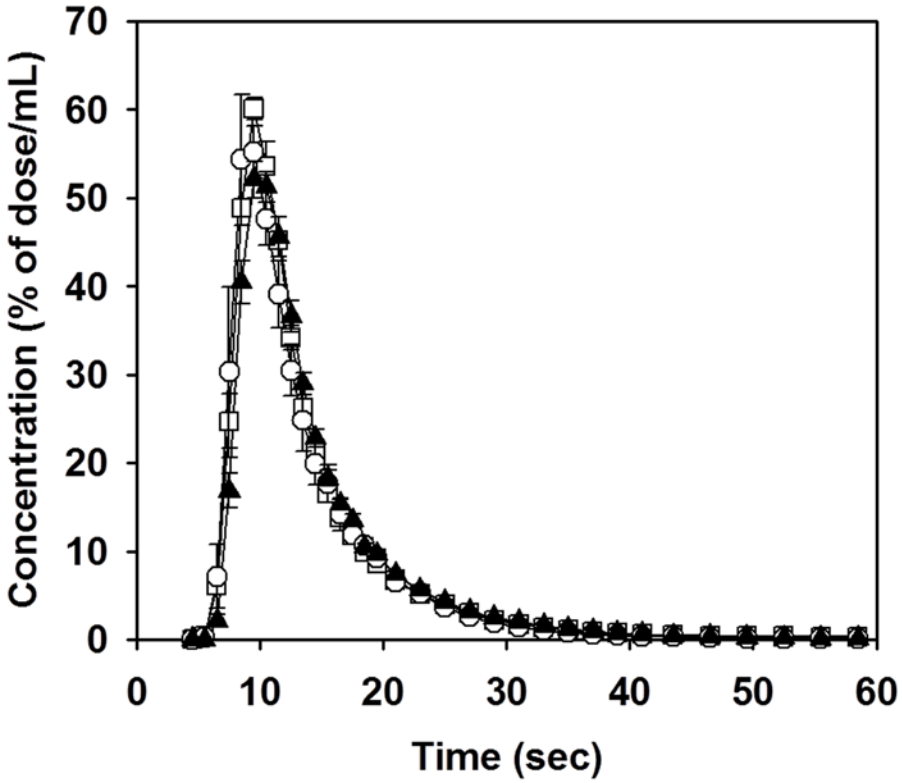
390

391

392 **Fig.4** Typical outflow patterns of FD-4 at a dose of 0.1 mg/liver after a bolus was

393 injected in the single-pass rat liver perfusion system at 37°C (○), 32°C (▲) or 28°C

394 (□). Each point represents the mean ± S.E. for at least three experiments.



395

396

Table 1 Moments and representative disposition parameters for outflow patterns of free PSP, ICG and FD-4 after a bolus was injected in the single-pass rat liver perfusion system under different temperatures.

Compounds	Temperature (°C)	auc (% of dose・sec/mL)	$\bar{t}$ (sec)	E_h (%)	V (mL/g)
PSP	37	214 ±8	7.36 ±0.50	53.7 ±1.9	0.421 ±0.031
	32	287** ±8	6.93 ±0.36	37.7** ±1.7	0.267** ±0.023
	28	310** ±8	7.00 ±0.38	32.6** ±2.0	0.236** ±0.020
ICG	37	7.27 ±0.92	27.7 ±0.7	98.4 ±0.2	47.5 ±6.6
	32	8.77 ±0.92	24.7 ±3.0	98.1 ±0.2	32.3 ±2.7
	28	10.16 ±1.21	30.8 ±2.7	97.8 ±0.3	31.7* ±3.0
FD-4	37	431 ±11	7.62 ±0.59	8.37 ±1.86	0.206 ±0.010
	32	431 ±8	8.33 ±0.28	7.57 ±1.39	0.207 ±0.005
	28	454 ±10	9.63 ±0.95	1.32* ±0.97	0.209 ±0.014

Each value represents the mean ± S.E. for at least four experiments.

*p<0.05, **p<0.01: significantly different from the results at 37°C.

405 **Table 2** Moment parameters for biliary excretion of free PSP and its conjugate in the

406 single-pass rat liver perfusion system under different temperatures.

Temperature (°C)	$F_{b,free}$ (% of dose)	$\bar{t}_{b,free}$ (min)	$F_{b,conj}$ (% of dose)	$\bar{t}_{b,conj}$ (min)
37	6.15 ±0.67	13.3 ±0.7	6.85 ±0.43	18.8 ±0.6
32	3.89* ±0.41	19.0** ±0.9	4.63 ±1.01	23.8* ±1.5
28	3.48** ±0.31	20.9** ±1.0	3.10** ±0.45	25.0* ±1.7

407

408 $F_{b,free}$ and $F_{b,conj}$ are the biliary recovery ratios of free and conjugated PSP, respectively.

409 $\bar{t}_{b,free}$ and $\bar{t}_{b,conj}$ are the biliary mean transit times of free and conjugated PSP,

410 respectively.

411 Each value represents the mean ± S.E. for at least four experiments.

412 **p<0.01; *p<0.05: significantly different from the results at 37°C.