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

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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°C as hypothermia. Because therapeutic hypothermia is done at 32-34°C, it is useful to know the alternation of hepatic disposition of drugs at 32°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% O2-5% CO2 to pH 7.4 at 37°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 32°C or 28°C. 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
The concentration of PSP conjugate was estimated from the difference between these values. The concentration of ICG was determined spectrophotometrically at 805 nm after proper dilution with saline containing 0.1% (w/v) bovine serum albumin as a stabilizer. The concentration of FD-4 was determined spectrophotofluorometrically at excitation and emission wavelengths of 489 and 515 nm, respectively.

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

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

\[
\text{auc} = \int_0^\infty C \, dt \quad (1)
\]

\[
\bar{t} = \int_0^\infty t \cdot C \, dt / \text{auc} \quad (2)
\]

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

\[
V = Q \cdot \bar{t} / F \quad (3)
\]

\[
F = Q \cdot \text{auc} \quad (4)
\]
\[ E_i = 1 - \text{auc} \cdot Q \] (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 \( (F_{b,\text{free}}) \) 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:

\[ \text{auc}_{b,\text{free}} = \int_0^\infty (dX_{b,\text{free}} / dt) dt \] (6)

\[ \text{auc}_{b,\text{conj}} = \int_0^\infty (dX_{b,\text{conj}} / dt) dt \] (7)

\[ \bar{t}_{b,\text{free}} = \int_0^\infty t \cdot (dX_{b,\text{free}} / dt) dt / \text{auc}_{b,\text{free}} \] (8)

\[ \bar{t}_{b,\text{conj}} = \int_0^\infty t \cdot (dX_{b,\text{conj}} / dt) dt / \text{auc}_{b,\text{conj}} \] (9)

\[ F_{b,\text{free}} = \text{auc}_{b,\text{free}} / \text{dose} \] (10)

\[ F_{b,\text{conj}} = \text{auc}_{b,\text{conj}} / \text{dose} \] (11)

where \( t \) is the time, and \( dX_{b,\text{free}}/dt \) and \( dX_{b,\text{conj}}/dt \) are the biliary excretion rates of free and conjugated PSP, respectively. The values of \( dX_{b,\text{free}}/dt \) and \( dX_{b,\text{conj}}/dt \) are normalized with the injected dose per mL. \( F_{b,\text{free}} \) and \( F_{b,\text{conj}} \) are the biliary recovery ratios of free and conjugated PSP, respectively. \( \bar{t}_{b,\text{free}} \) and \( \bar{t}_{b,\text{conj}} \) 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 37\(^\circ\)C).
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 $\text{auc}$ 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 plateaued 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 °C 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 °C was not changed, the $E_h$ of FD-4 was significantly decreased at 28°C compared to 37°C.
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°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 ($F_{b,\text{free}}, F_{b,\text{conj}}$) and biliary mean transit time ($\bar{t}_{b,\text{free}}, \bar{t}_{b,\text{conj}}$). 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 \textit{in vivo}, correlating with the decreasing ratio of $F_{b,\text{free}}$ and $F_{b,\text{conj}}$ in the rat liver perfusion system. In addition, $F_{b,\text{free}}/F_{b,\text{conj}}$ of PSP was not altered at 32°C while it was increased at 28°C, suggesting that the conjugation of PSP by enzymes was decreased at 28°C. 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 \textit{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 transepithelial transport of digoxin via multidrug resistant protein-1 (MDR1) was evaluated at various
temperatures *in vitro* using LLC-GA5-COL150 cells that expressed human P-glycoprotein specifically on the apical surface showed a multidrug resistant phenotype. According to this study, MDR1-mediated transport of digoxin decreased at lower temperatures. ICG was excreted into bile by Mdr2, which is one of the ABC transporters, so the decrease in biliary excretion at low perfusate temperatures would be caused by changes in Mdr2 activity. If the Mdr2 activity could decreased under hypothermia, the pharmacokinetics of digoxin and verapamil, which is substrate of Mdr2, might differ during hypothermia. Furthermore, the reduction of ICG excretion into bile under hypothermia was larger than that of PSP. This result suggests that the effect of hypothermia on Mdr2 might be greater than that on Mrp2. However, further studies are necessary for us to investigate the effect of hypothermia on ABC transporter activity by *in vitro* experiment.

FD-4 is excreted mainly by glomerular filtration, and the contribution of hepatic extraction is very low. We used FD-4 as a vascular reference in the rat liver perfusion study. It was reported that distribution volume of 131I-human serum albumin, another vascular reference substance, was not changed at 27°C, compared to 37°C. The *auc* and V were not changed under the low perfusion temperatures. It was thus indicated that the distribution of FD-4 in the liver was not affected by perfusion temperature.

The *Eh* of FD-4 was significantly decreased at 28°C compared to 37°C, while there was no change at 32°C, suggesting that the elimination of FD-4 by hepatic uptake leading to endocytosis was slightly decreased by the perfusion temperatures. In case of FD-4, the prolongation of outflow pattern might be caused by slightly release of the FD-4 associated with the liver. Moreover, the *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 \textit{in vivo} study (8), \( CL_{\text{tot}} \) of FD-4 was significantly decreased at 28\(^\circ\)C, probably because of decreased glomerular filtration as well as hepatic uptake leading to endocytosis.

\textbf{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.

\textbf{ACKNOWLEDGMENTS}

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Legend to Figures

Fig. 1  Typical outflow patterns of PSP at a dose of 0.1 mg/liver after a bolus was injected 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.
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.
Fig. 3  Biliary excretion rate-time curves of ICG (A) and F_b,free of ICG for 60 min (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. and each column represents the mean ± S.E. for at least five experiments.
Fig. 4 Typical outflow patterns of FD-4 at a dose of 0.1 mg/liver after a bolus was injected 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 three experiments.
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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Temperature (°C)</th>
<th>auc (% of dose • sec/mL)</th>
<th>( \bar{t} ) (sec)</th>
<th>( E_h ) (%)</th>
<th>V (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37</td>
<td>214 ±8</td>
<td>7.36 ±0.50</td>
<td>53.7 ±1.9</td>
<td>0.421</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>287** ±8</td>
<td>6.93 ±0.36</td>
<td>37.7** ±1.7</td>
<td>0.267**</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>310** ±8</td>
<td>7.00 ±0.38</td>
<td>32.6** ±2.0</td>
<td>0.236**</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>7.27 ±0.92</td>
<td>27.7 ±0.7</td>
<td>98.4 ±0.2</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>8.77 ±0.92</td>
<td>24.7 ±3.0</td>
<td>98.1 ±0.2</td>
<td>32.3</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>10.16 ±1.21</td>
<td>30.8 ±2.7</td>
<td>97.8 ±0.3</td>
<td>31.7*</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>431 ±11</td>
<td>7.62 ±0.59</td>
<td>8.37 ±1.86</td>
<td>0.206</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>431 ±8</td>
<td>8.33 ±0.28</td>
<td>7.57 ±1.39</td>
<td>0.207</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>454 ±10</td>
<td>9.63 ±0.95</td>
<td>1.32* ±0.97</td>
<td>0.209</td>
</tr>
</tbody>
</table>

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.
Table 2  Moment parameters for biliary excretion of free PSP and its conjugate in the single-pass rat liver perfusion system under different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( F_{b,\text{free}} ) (% of dose)</th>
<th>( t_{b,\text{free}} ) (min)</th>
<th>( F_{b,\text{conj}} ) (% of dose)</th>
<th>( t_{b,\text{conj}} ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>6.15 ± 0.67</td>
<td>13.3 ± 0.7</td>
<td>6.85 ± 0.43</td>
<td>18.8 ± 0.6</td>
</tr>
<tr>
<td>32</td>
<td>3.89* ± 0.41</td>
<td>19.0** ± 0.9</td>
<td>4.63 ± 1.01</td>
<td>23.8* ± 1.5</td>
</tr>
<tr>
<td>28</td>
<td>3.48** ± 0.31</td>
<td>20.9** ± 1.0</td>
<td>3.10** ± 0.45</td>
<td>25.0* ± 1.7</td>
</tr>
</tbody>
</table>

\( F_{b,\text{free}} \) and \( F_{b,\text{conj}} \) are the biliary recovery ratios of free and conjugated PSP, respectively.

\( t_{b,\text{free}} \) and \( t_{b,\text{conj}} \) are the biliary mean transit times of free and conjugated PSP, respectively.

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

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