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# ESTABLISHMENT OF RAT PRECISION-CUT FIBROTIC LIVER SLICE TECHNIQUE AND ITS APPLICATION IN VERAPAMIL METABOLISM

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## SUMMARY

**1. Liver fibrosis is the compensatory state of cirrhosis. In the long asymptomatic period, it is imperative to select a proper dosing regimen for drugs that are applicable to hepatic fibrosis. Otherwise, progressive deterioration to uncompensated cirrhosis may occur. The present study explored the characteristics of drug metabolism in fibrotic liver.**

**2. A rat precision-cut fibrotic liver slice (PCFLS) technique was established and the metabolism of verapamil was studied employing this technique. A rat hepatic fibrosis model was successfully induced integrating complex factors that included a high-fat diet, alcohol and $CCl_4$. The PCFLS were incubated under different conditions and lactate dehydrogenase leakage, glutathione S-transferase activity and 3[4,5-dimethythiazole-2-yl]-2,5-diphenyltetrazolium bromide reduction were used as indices to assess PCFLS viability. Activities of phase I and phase II metabolizing enzymes were monitored following treatment with cytochrome P450 (CYP) inducers. Normal and fibrotic liver slices were incubated individually with 10 μmol/L verapamil. The concentration of verapamil in the medium was determined by high-performance liquid chromatography and intrinsic clearance ($Cl_{int}$) was calculated on the basis of the concentration–time curve.**

**3. The results showed that the PCFLS viability remained steady throughout the 6 h of culture when the thickness of slices was 300 μm and pH of the medium was 7.0; CYP inducers (phenobarbital and ethanol) enhanced CYP2E1, CYP3A1/2 and uridine diphosphate-glucuronate transferase (UDPGT) activities, respectively, in a time-dependent manner. The $Cl_{int}$ (μL/min per mg) values differed significantly between normal ($9.7 \pm 1.8$) and fibrotic ($5.6 \pm 1.4$) liver slices ($P < 0.01$).**

**4. These results suggested that the PCFLS could remain viable for 2–6 h under appropriate conditions. The stability and inducibility of drug-metabolizing enzymes of PCFLS were also demonstrated. Furthermore, the metabolic rate of verapamil in PCFLS was decreased. These findings add further support to the use of PCFLS as a tool to study drug metabolism and to guide clinical medication.**

**Key words: inducibility, intrinsic clearance, liver fibrosis, precision-cut fibrotic liver slice, verapamil.**

## INTRODUCTION

Liver fibrosis is a common response to chronic liver injury, manifested as hepatocyte necrosis, regeneration and collagen deposition, which is at best reversible and at worst may result in potentially lethal sequelae; that is, cirrhosis. Up to 40% of patients with compensatory cirrhosis are asymptomatic and may remain so for more than a decade.[1] In the long asymptomatic period, it is imperative to select the proper dosing regimen for drugs that are applicable to patients with liver fibrosis otherwise progressive deterioration to uncompensated cirrhosis may occur. However, the pharmacokinetic information on the metabolism of drugs by a healthy individual does not tally with data obtained from patients. Thus, the research on the characteristics of drug metabolism in fibrotic liver plays an important role in the instruction to clinical medication.

Drug metabolism research is based on both *in vitro* models and experiments *in vivo* in various animal species. *In vitro* models are used to obtain early information about biotransformation pathways and to predict drug–drug interactions at the metabolic level.[2] Compared with *in vivo* experiments, *in vitro* models elucidate the biotransformation pathway of a drug and avoid a time-consuming and expensive process.[3] As liver is the predominant organ in which biotransformation of xenobiotic compounds takes place, several *in vitro* liver models have been developed in the past two decades, including microsomes, primary hepatocytes, liver slices and perfused liver.

The use of precision-cut liver slices (PCLS), which is a technique between organ and cell culture, offers the advantages of preserving the normal tissue architecture, the cell heterogeneity and cell–cell interactions, whereas some *in vitro* systems, such as cultured hepatocytes, do not.[4] Previous studies have demonstrated that the activities of certain phase I and phase II xenobiotic-metabolizing enzymes remained stable in both fresh and cryopreserved liver slices,[5–7] and that xenobiotic-metabolizing cytochrome P450 (CYP) isoforms are inducible by exposure of the slices *in vitro* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), β-naphthoflavone (β-NF), phenobarbital (PB), 16$\alpha$-carbonitrile (PCN)[8–10] and other so-called inducers. On the basis of those studies, PCLS have been used widely to study biotransformation and toxicology of xenobiotics, including metabolic pathways, metabolites, drug interaction and drug toxicity.[11,12] Several incubation systems for normal liver slices have been reported,[13,14] and our laboratory has recently established the technique of normal rat PCLS.[15] However, the PCLS technique has not been used in pathological animal models.

Verapamil, a calcium channel blocker that is in clinical use,[16] is mainly metabolized by the major and most important CYP isoform; that is, CYP3A4 in human liver,[17] which contributes to the interaction

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of verapamil with other co-administered drugs.[18,19] In our recent work, its antifibrotic action was found to involve the inhibition of proliferation of hepatic stellate cells (data not shown). Thus, it is necessary to investigate the pharmacokinetic alteration of verapamil in the presence of fibrosis to guide its clinical application.

We have previously investigated the changes and characteristics of drug-metabolizing enzymes in a varying range status of hepatic injury and found that a decrease in enzyme activity is correlated to an increase in the degree of hepatic injury. However, there are few details on drug metabolism in the case of hepatic fibrosis, especially for verapamil. We hypothesized that, during the earlier period of hepatic fibrosis when no symptoms are presented, the alteration of drug metabolism may be due mainly to the degression of enzyme function rather than a decrease in hepatocyte amount. Thus, we are greatly interested in investigating the change in drug metabolism (rate, pathway and drug–drug interaction) resulting directly from fibrosis of the liver.

In a previous study of normal rat PCLS, we found that slice viability was influenced by many factors, such as slice thickness, the acidity or alkalinity of the medium and culture time. So as to obtain information about the effect of hepatic fibrosis on intrinsic clearance ($Cl_{int}$) *in vitro*, our aim in the present study was to optimize the incubation conditions of rat precision-cut fibrotic liver slices (PCFLS); to investigate the stability and inducibility of phase I and phase II enzymes; and to use PCFLS as a tool to study verapamil metabolism.

## METHODS

### Materials

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco BRL (Paisley, Scotland, UK). 3[4,5-dimethythiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco Chemical Co. (Solon, OH, USA). 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (BSA), β-NF, erythromycin, isocitric acid, isocitric acid dehydrogenase, 7-ethoxyresorufin, resorufin, dextromethorphan and verapamil standard were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Methanol (high-performance liquid chromatography (HPLC) grade) was obtained from Fisher (Waltham, MA, USA). Verapamil injection was obtained from Hefeng Pharmaceutical Group Inc. (Shanghai, China). All other chemicals and reagents were of AR grade.

### Establishment of liver fibrosis

Male Wistar rats (223 ± 13 g bodyweight) were obtained from the Medical Scientific Academy of Hubei Province (China). Animals were allowed free access to a high-fat diet (88% corn flour, 11.5% lard and 0.5% cholesterin) and drink 5–10% ethanol (5% ethanol for the first 3 days and 10% ethanol from the 4th day) for 3 weeks. From the second week, rats were injected subcutaneously with $CCl_4$ (dissolved in olive oil; 0.5 mL/100 g on the first occasion, then 0.3 mL/100 g) twice a week for 2 weeks.

### Preparation and incubation of rat precision-cut fibrotic liver slices

Rats were killed by decollation. Livers were removed and stored in a refrigerator (4°C) for 10 min, then placed in ice-cold Krebs'–Henseleit (KH) buffer. Slices were prepared in oxygenated ice-cold KH buffer, using a Krumdieck slicer according to a procedure described elsewhere.[6] The slices were incubated in 24-well culture plates containing 1.0 mL per well of DMEM supplemented with 10% newborn calf serum, 100 nmol/L insulin, 10 nmol/L dexamethasone, 2.5 μg/mL amphotericin, 50 μg/mL streptomycin and 100 IU/mL penicillin. The plates were shaken horizontally 120 times per min. In the experiments exploring the culture conditions, the slices were transferred to other wells containing fresh medium after 1 h of pre-incubation. Slice viability was assessed over a range of slicing thicknesses (200, 300 and 400 μm), pH of the medium (6.8, 7.0, 7.2 and 7.4) and culture time (0, 2, 4 and 6 h). Time 0 h, which represents slices incubated for 5 min after pre-incubation, is taken as the control value. After incubation, the medium and slices were analysed further.

### Treatment of rat precision-cut fibrotic liver slices

A CYP inducer (i.e. PB (final concentration 0.5 mmol/L), ethanol (final concentration 50 mmol/L) or β-NF (final concentration 0.05 mmol/L in 0.2% dimethylsulfoxide)) was added to the medium after pre-incubation. Fibrotic slices were cultured for 0, 2, 4 and 6 h under optimal culture conditions, in the presence of a CYP inducer.

### Histological study

Slices were fixed in 4% formaldehyde for histopathological analysis. Paraffin sections (5 μm thick) were stained with haematoxylin and eosin.

### Detection of slice viability

The viabilities of liver slices were estimated by measuring lactate dehydrogenase (LDH) leakage, cytosol glutathione S-transferase (GST) activity and MTT reduction. The activity of LDH was detected using a LDH biochemistry assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's protocol and expressed as IU LDH per mg of protein. To detect GST activity, liver slices were homogenized with 1 mL of 50 mmol/L Tris buffer (pH 7.4) and the activity was measured with CDNB as substrate.[20] For MTT reduction, slices were rinsed with phosphate-buffered saline (PBS) and incubated with 1.21 mmol/L MTT solution. Slices were rinsed again with PBS after 45 min of incubation and the MTT–formazan product was extracted using 1.0 mL isopropanol. The eluent was determined at 570 nm using a scanning spectrophotometer.[21] The formazan formed in each slice was expressed as absorbance (OD) per gram of slice weight.

### Assays of drug-metabolizing enzymes

Following incubation, the slices were washed briefly and homogenized with 50 mmol/L Tris buffer (pH 7.4) containing 0.154 mol/L KCl, and fragmentated by an ultrasonic tissue destructor. The postmitochondrial fraction ($S_9$) was prepared by centrifugation and stored at −80°C before use. The following assays were carried out on $S_9$: O-de-ethylation of 7-ethoxyresorufin was used to monitor CYP1A1, with resorufin as standard;[22] CYP2E1 was monitored with aniline as the diagnostic substrate, as its *p*-hydroxylation is catalysed primarily by CYP2E1;[23] erythromycin demethylation monitored CYP3A1/2;[24] *p*-hydroxy-biphenyl and CDNB were used as substrates of uridine diphosphate-glucuronate transferase (UDPGT) and GST,[20,25] respectively. The homogenate protein was determined by the method described by Lowry and colleagues,[26] using BSA as standard.

### Verapamil metabolism and determination of intrinsic clearances in liver slices

Normal and fibrotic liver slices were incubated individually in 24-well plates in the presence of 10 μmol/L verapamil in 1 mL DMEM after pre-incubation. After 2, 5, 15, 30, 45, 60, 90, 120, 240 and 360 min of incubation, the medium was removed to determine the residual concentration of verapamil using HPLC. Each slice was homogenized and centrifuged at 9000 *g*, and the protein content was determined. The intrinsic clearance ($Cl_{int}$) of verapamil was calculated as the ratio of its initial concentration to the area under the concentration–time curve (*AUC*). The *AUC* was calculated using WinNonlin

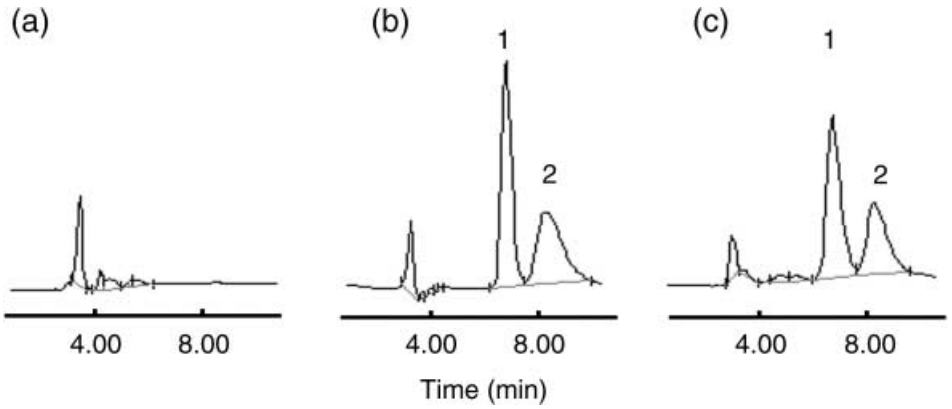


**Fig. 1** Chromatograms of verapamil (1) and dextromethmorphan (2) in medium. (a) Blank medium, (b) blank medium spiked with 5000 ng/mL verapamil and 5000 ng/mL dextromethmorphan, and (c) medium sample after 60 min of incubation with 1 μg of verapamil hydrochloride.

4.0.1 software (Pharsight Co., Mountain View, CA, USA) and the statistical analysis was performed using SPSS for Windows® 11.5 software (SPSS Inc., Chicago, IL, USA).

## High-performance liquid chromatography assay of verapamil

A 0.25 mL volume of medium was spiked with dextromethorphan (final concentration 5.4 μmol/L) as the internal standard. Following the addition of 1.25 mL organic phase (*n*-hexane : butyl alcohol = 10 : 1, v/v), the mixture was shaken vigorously for 1 min and then centrifuged at 1630 *g* for 10 min. The pellet was discarded and the organic solvent was evaporated at 45°C under nitrogen. Dry residue was re-dissolved in 50 μL of the mobile phase and a 20 μL sample was injected into the HPLC system.

Determination of verapamil in the medium was carried out by HPLC by the method described by Tan *et al.*,[27] with minor modifications. The HPLC system was composed of a pump (LC-10AT; Shimadzu Co., Tokyo, Japan) and a UV detector (SPD-10A; Shimadzu). Separations were accomplished on a Hypersil ODS-$C_{18}$ (250 × 4.6 mm I.D.) reversed-phase column. The column was eluted isocratically with a mobile phase of methanol–water–triethylamine (75 : 25 : 0.3, v/v). The pH was adjusted to 6.70 by adding acetic acid. A flow rate of 1.0 mL/min was used and the UV detector was set at 279 nm. All chromatography was performed at room temperature and the overall run-time was 20 min. The retention time of verapamil and dextromethorphan was 6.69 min and 8.76 min, respectively (Fig. 1).

A calibration curve was obtained by adding appropriate volumes of stock solutions of verapamil to drug-free medium to give final concentrations in the range of 100–5000 ng/mL. These calibration samples then underwent the extraction procedure described earlier. The regression equation $y = 1.536 + 0.027$ ($r = 0.9992$) was obtained, and a strong linear relationship was found within the concentration range of 100–5000 ng/mL verapamil.

Recovery was calculated by comparing the peak area of standards after extraction with the peak area of standard solutions, which corresponded to a 100% recovery (Table 1). The recovery of dextromethorphan was 81.2% ± 13.0% ($n = 5$). For intraday precision, samples ($n = 5$) spiked at different concentrations (i.e. 50, 250, 1000 and 5000 ng/mL) were analysed. The precision was expressed as the relative standard deviation (% RSD), and calculated from the standard deviation divided by the mean of the detected concentration. Interday precision was determined in five replicates of the drug-free medium preparations, which were spiked with different concentrations (i.e. 50, 250, 1000 and 5000 ng/mL) of verapamil, and performed on three different days (Table 1).

## Statistical analysis

Data are expressed as the mean±SD. Difference between the control and induction groups was detected by Student's *t*-test and the difference within the same group was detected by paired Student's *t*-test.

**Table 1** Precision and recovery of the high-performance liquid chromatography method of verapamil determination ($n = 5$)

| Concentration (ng/mL) | Precision | | Recovery | |
|---|---|---|---|---|
| | Interday RSD (%) | Intraday RSD (%) | $\bar{x} \pm$ SD (%) | RSD (%) |
| 50 | 7.2 | 8.1 | 75 ± 9 | 12 |
| 250 | 7.5 | 6.3 | 79 ± 7 | 9 |
| 1000 | 6.2 | 3.4 | 85 ± 11 | 13 |
| 5000 | 6.0 | 6.5 | 83 ± 5 | 6 |

RSD, relative standard deviation.

# RESULTS

## Model of rat hepatic fibrosis

In contrast to the normal control (Fig. 2a), complex factors caused visible histopathological changes such as steatosis, macrophage infiltration and myofibroblast proliferation (Fig. 2b), which indicate an early stage of hepatic fibrosis.

## Effects of rat precision-cut fibrotic liver slices thickness on slice viability

Slice thickness (i.e. 200, 300 and 400 μm) significantly affected slice viability after it had been incubated in the medium (pH 7.0) for 4 h. Leakage of LDH was least (10.1 U/mg) (Fig. 3a) in the 300 μm-thick group, which was 83.8% of the 200 μm-thick group and 73.7% of the 400 μm-thick group. The GST activity (Fig. 3b) was highest in the 300 μm-thick group, 156.5% of GST activity in the 200 μm-thick group. The 300 μm-thick slices also showed a positive effect on MTT reduction (Fig. 3c), which was 27.5% and 72.8% higher than the 200 μm-thick and 400 μm-thick groups, respectively. These observations indicate that 300 μm is the most suitable thickness for PCFLS.

## Effects of medium pH on slice viability

Figure 4 indicates the effect of medium pH (6.8, 7.0, 7.2 and 7.4) on PCFLS viability. Slices were 300 μm thick and incubated for 4 h.

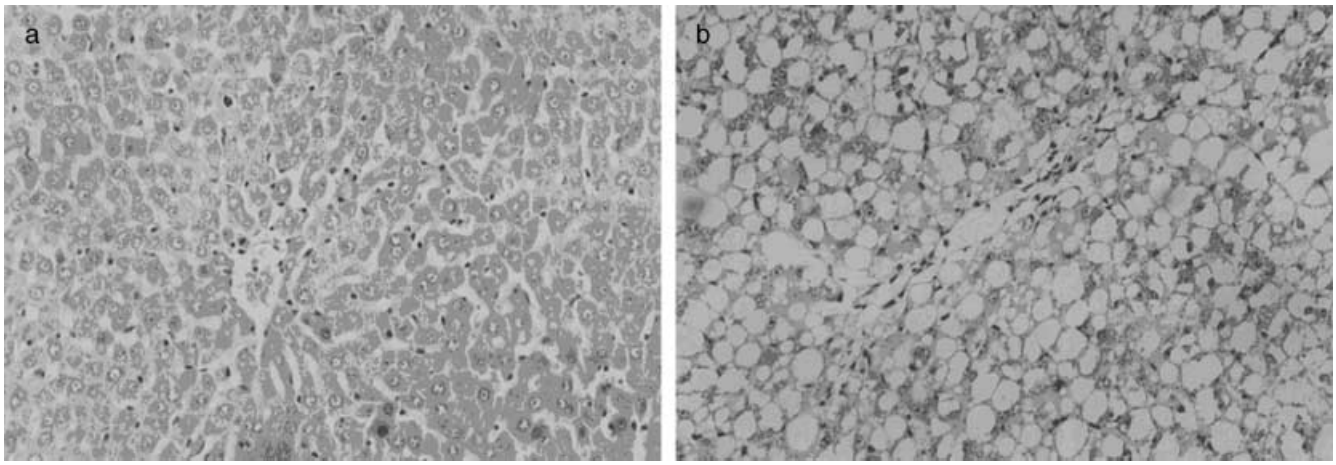


**Fig. 2** Histopathology of normal and fibrotic liver tissue in the rats (HE × 200). (a) normal control, (b) liver fibrosis is evident after hepatic complex factors (high-fat diet, consumption of alcohol and $CCl_4$ injection) were administered to rats for 3 weeks.

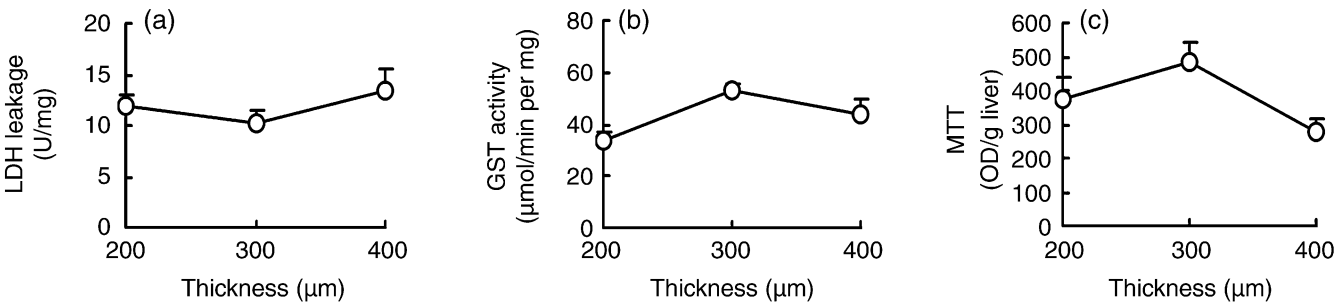


**Fig. 3** Effect of thickness of precision-cut fibrotic liver slices (PCFLS) on (a) lactate dehydrogenase (LDH) leakage, (b) glutathione S-transferase (GST) activity, and (c) 3[4,5-dimethythiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction. pH of medium was 7.0; PCFLS were incubated for 4 h. Values are the mean±SEM; $n = 4$.

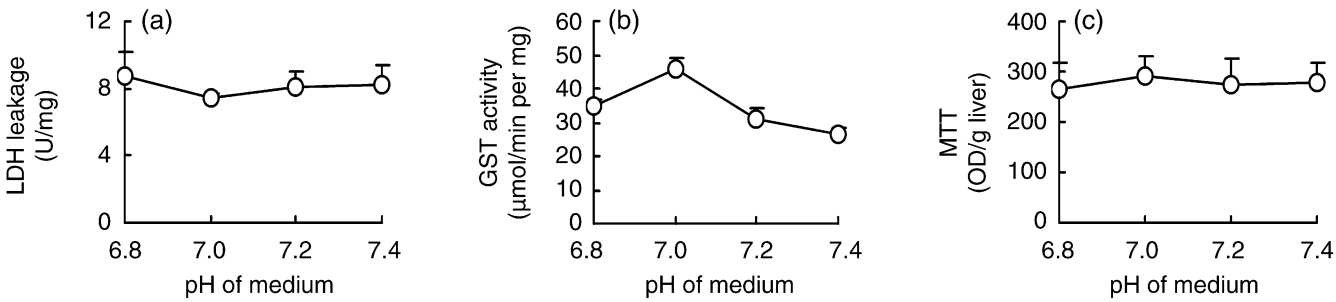


**Fig. 4** Effect of medium pH on (a) lactate dehydrogenase (LDH) leakage, (b) glutathione S-transferase (GST) activity, and (c) 3[4,5-dimethythiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction of precision-cut fibrotic liver slices (PCFLS). Thickness of slices was 300 μm; PCFLS were incubated for 4 h. Values are the mean±SEM; $n = 5–6$.

Incubation of slices in medium at pH 7.0 tended to cause the least LDH leakage (7.4 U/mg), the highest GST activity (46.0 μmol/min per mg) and the best reducing capacity of MTT (292.1 OD/g) compared with the other pH groups. However, the changes were not statistically significant.

### Effects of culture duration on slice viability

Using 300 μm-thick slices and pH 7.0 for the medium, there were slight changes in LDH leakage and GST activity, whereas MTT reduction decreased in a time-dependent manner (Fig. 5). The reduction of MTT was lowest after 6 h of incubation (293.7 OD/g), but this change was not statistically significant. These results indicate that the viability of PCFLS is maintained at a steady level within 2–6 h incubation *in vitro* when slices are 300 μm thick and medium pH is 7.0.

### Assays of drug-metabolizing enzymes in liver slices

The activities of drug-metabolizing enzymes in the PCFLS that were treated with different CYP inducers are illustrated in Fig. 6. For the

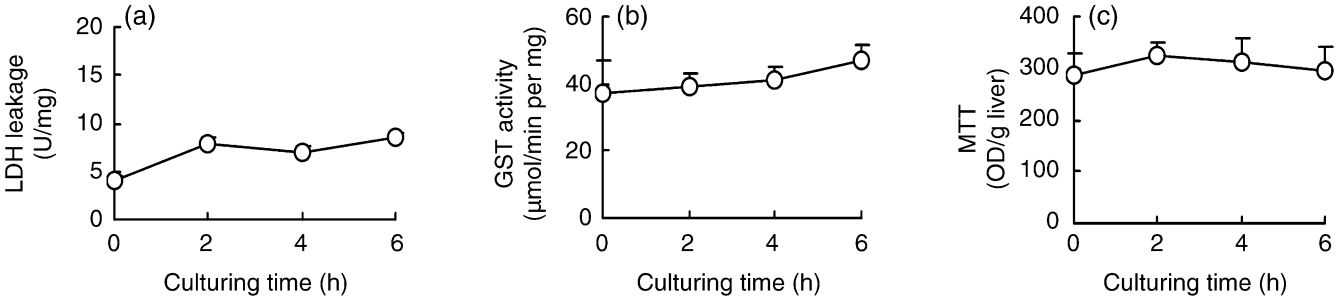


**Fig. 5** Effect of culture duration on (a) lactate dehydrogenase (LDH) leakage, (b) glutathione S-transferase (GST) activity, and (c) 3[4,5-dimethythiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction of precision-cut fibrotic liver slices. Thickness of slices was 300 μm; pH of medium was 7.0. Values are the mean±SEM; $n = 5–6$.

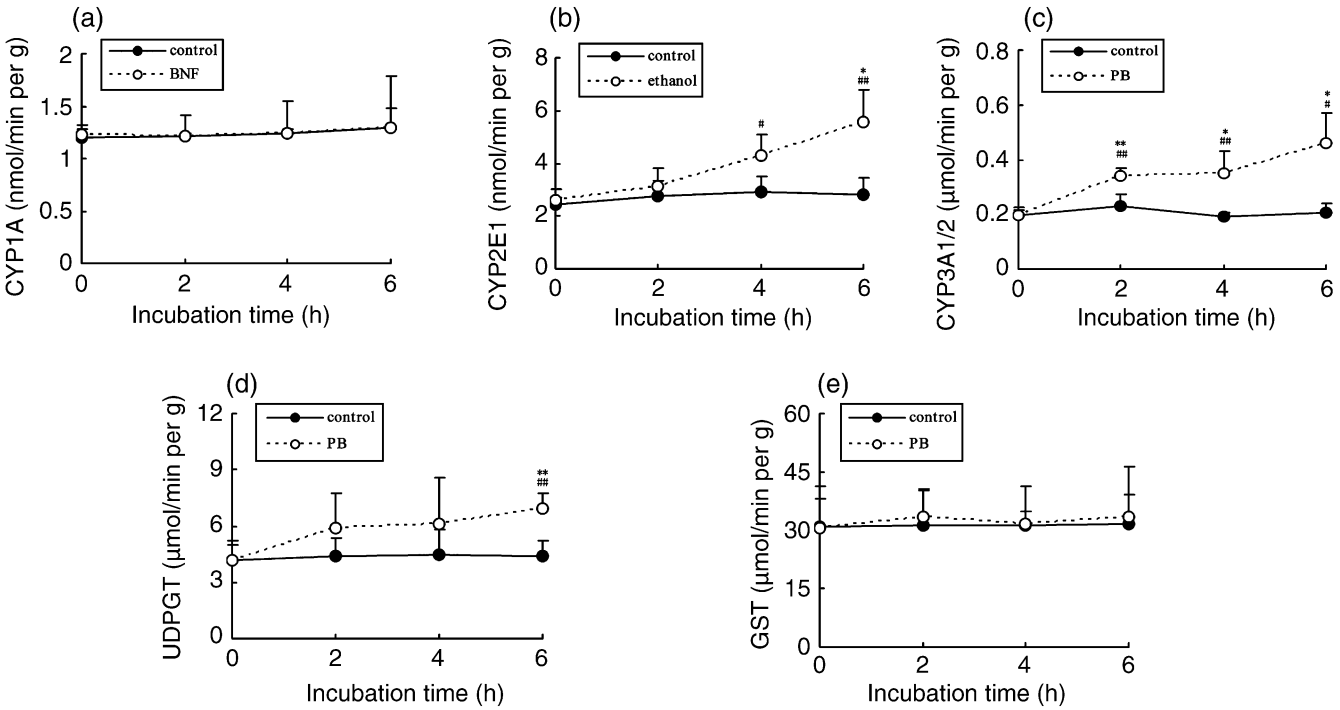


**Fig. 6** Stabilities and inducibilities of (a) CYP1A, (b) CYP2E1, (c) CYP3A1/2, (d) uridine diphosphate-glucuronate transferase (UDPGT), and (e) glutathione S-transferase (GST) activities in the presence or absence of inducers β-naphthoflavone (BNF; 0.05 mmol/L), ethanol (50 mmol/L) and phenobarbital (PB; 0.5 mmol/L), during the incubation period. Thickness of slices was 300 μm and pH of medium was 7.0. Values are the mean±SEM; $n = 4–8$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0 h; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group.

untreated fibrotic slices, there were no significant changes in CYP1A, CYP2E1, CYP3A, UDPGT and GST activities during an incubation period of 6 h. All enzyme activities were relatively low during pre-incubation, but they started to increase slightly in the course of incubation, except for aniline *p*-hydroxylase and UDPGT, which showed a slight decline at 6 h. After 6 h incubation, the related enzyme activities were maintained at 107.7% (CYP1A), 116.2% (CYP2E1), 105.1% (CYP3A1/2), 104.5% (UDPGT) and 102.3% (GST) of their controls (0 h).

After 6 h exposure to β-NF (final concentration 0.05 mmol/L in 0.2% DMSO), the activity of CYP1A was 105.2% of the control (0 h) and no significant induction was observed. Ethanol (final concentration 50 mmol/L) treatment enhanced the activity of CYP2E1 by 2.1-fold within 6 h ($P < 0.05$). Phenobarbital (final concentration 0.5 mmol/L) increased the activities of CYP3A and UDPGT in a time-dependent manner within 6 h, by 2.3- and 1.7-fold, respectively, compared with the control (0 h) ($P < 0.05$), but had no significant effect on GST activity in PCFLS.

## Verapamil metabolism in liver slices

For normal and fibrotic liver slices, the decline in verapamil concentrations over the time-course of incubation was mono-exponential, as shown in Fig. 7. Verapamil was metabolized at a higher rate by normal slices compared with PCFLS. After incubation with normal slices for 360 min, only 1.8% of the original drug remained. However, in the case of PCFLS, approximately 12.8% of verapamil remained unmetabolized under the same conditions.

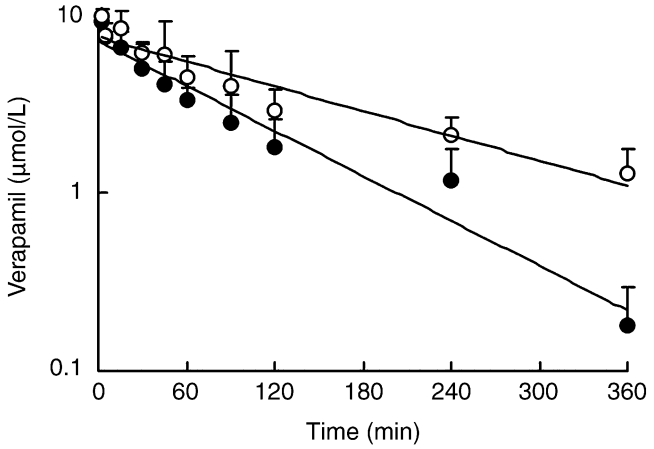


**Fig. 7** Concentration–time curves for disappearance of verapamil in incubations with both normal (●) and fibrotic (○) rat liver slices. Liver slices were incubated with 10 μmol/L verapamil for 2–360 min. Values are the mean±SEM; $n = 4$.

A significant difference between the $Cl_{int}$ (μL/min per mg) obtained with normal ($9.7 \pm 1.8$) and fibrotic ($5.6 \pm 1.4$) rat liver slices was observed ($P < 0.01$).

## DISCUSSION

Precision-cut liver slices are used widely to study pharmacotoxicology and drug metabolism under physiological status, but have been rarely used to study drug metabolism under pathological status, such as in hepatic fibrosis. In the present study, the technique of PCFLS was established and a multi-well plate incubation system was used to define the optimal incubation conditions that produced slices of the highest quality and allowed the broadest use of this technique. The results of the study herein and those of Olinga *et al*.[28] showed that PCLS viability is highly dependent on slice thickness both in normal fresh slices and in those that are fibrotic. When slice thickness is 200 μm, the slice is liable to be damaged in slicing; at 400 μm thickness, centrocytes in the slice are isolated from the nutritive matter in the medium by fibrous tissue. However, when slice thickness is 300 μm, excessive damage is avoided while still facilitating matter exchange between tissue and medium. Although medium at pH 7.0 was the optimal culturing condition for both normal[15] and fibrotic slices, we found that fibrotic slices were less sensitive than normal ones to medium pH, perhaps because of the abnormal status of the hepatocytes.

We have studied the stability and inducibility of major biotransformation enzymes of phase I and phase II in cultured PCFLS. Activities of all phase I and phase II enzymes remained relatively steady throughout a 6 h incubation period, but the extent of induction by CYP isoform inducers on the incubated PCFLS appears to be isoform-specific. After 6 h exposure to inducers, CYP2E1, CYP3A1/2 and UDPGT activities increased by 2.1-, 2.3- and 1.7-fold, respectively. However, the effect of CYP inducers on CYP1A and GST was not significant after 6 h of exposure to β-NF and PB, respectively. The indistinct effect of β-NF and PB might be because the inducibility of β-NF was not distinct to CYP1A in fibrotic slices and that PB was not a specific inducer for GST. It may also be possible that the incubation time wasn't long enough. We attempted to prolong the culture time by optimizing incubation conditions; that is, aerating the medium with $O_2 : CO_2$ (95% : 5%) accompanied by horizontal vibration.

The key parameter that enables the incorporation of *in vitro* drug metabolism data into a physiologically-based pharmacokinetic model is $Cl_{int}$, which is a pure measure of enzyme activity towards a substrate and is independent of other physiological determinants of clearance, such as blood flow or drug binding within the blood matrix.[29] The *in vitro* $Cl_{int}$ value is generally determined as the ratio of the Michaelis-Menten parameters $V_{max}$ and $K_m$ ($V_{max}/K_m$).[30] However, there are several CYP isoforms involved in verapamil metabolism and the difference in verapamil substrate concentration may lead to a disparate metabolism pathway.[31] In addition, there are 25 metabolites of verapamil transformed *via* CYP in the rat liver.[32] Hence, in the present study, the $Cl_{int}$ value was estimated from the time curve for the disappearance of the test compound. This approach requires that the $K_m$ value for the compound be higher than its initial concentration in the incubation medium.[33,34] Compared with the generally used method, this measurement method can be considered to be a simple and useful one, having the following advantages: (i) it is simple to conduct; (ii) it can be used for many compounds; (iii) metabolites do not need to be known; (iv) it can be performed easily, without requiring radiolabelling; and (v) it can yield a gross profile of an enzyme.[35,36]

In the present study, we compared the $CL_{int}$ of verapamil between normal and fibrotic rat liver slices. The results demonstrated that the rate of verapamil metabolism with rat liver slices was significantly affected by the status of fibrosis, being about half that of the normal status. This may have resulted from the decline of enzyme function in fibrotic liver rather than the loss of cells. The reasons for this suggestion are given below.

1. To remove fibrous tissue, which may interfere with protein determination, PCFLS were homogenized and centrifuged at 9000 *g*. The study of the alteration in drug metabolism was based on the equal quantity of tissue protein.
2. Histological findings (Fig. 2) showed that there was insignificant change in the number of hepatocytes in the early stage of hepatic fibrosis.
3. In addition, a comparison with the values found in a previous study we have conducted[25] showed that activities of CYP2E1, 3A1/2, GST and UDPGT in PCFLS were lower than in normal slices, after 2 h incubation.
4. Furthermore, Kleinbloesem *et al*. demonstrated low enzymatic activities and functional depression of nifedipine metabolism at the status of liver cirrhosis.[37] In a previous study, we have also observed that hepatic fibrosis reduces the activity of CYP3A in rat liver.[38]

Moreover, after intravenous or oral administration of verapamil, the plasma clearance (Cl) of verapamil in rats with hepatic fibrosis is significantly lower than that in the control group (data not shown). The results of the present study correlate well with the change of enzyme at the status of fibrosis and verapamil metabolism *in vivo*. Thus, PCFLS is useful for obtaining information concerning hepatic drug-metabolizing enzymes and liver clearance, thereby estimating the rate of drug biotransformation *in vivo*.

The present study represents the first published information regarding verapamil metabolism at the status of fibrosis. The pharmacokinetics data acquired in the present study, in combination

with the change of enzyme activity, is helpful for clarifying the entire metabolism of verapamil in patients with liver fibrosis. Furthermore, the $Cl_{int}$ values of verapamil *in vitro* can be scaled to the *in vivo* situation by employing a scaling factor,[29] as shown below.

$$Cl_{int}\ (in\ vivo) = Cl_{int}\ (in\ vitro)\ \text{Scaling factor} \qquad [1]$$

Interferon (IFN) is also an effective antifibrotic agent[39] and is the substrate of CYP3A. Thus, it is possible that IFN could be synergistic with the pharmacodynamic effect of verapamil. However, it is important to ascertain whether each affects the pharmacokinetics of the other. The present study paves the way for further investigations of the possible pharmacodynamic and pharmacokinetic interactions of verapamil and IFN at the status of fibrosis.

In summary, the technique of PCFLS has been established and slice viability can be maintained for 2–6 h under appropriate conditions. In addition, the activities of major phase I and phase II biotransformation enzymes can be maintained at a steady level in fibrotic slices within the incubation time-frame, and some of those enzymes also show good inducibility. The rate of metabolism of verapamil by rat PCLS is affected by the status of fibrosis. Thus, PCFLS proved to be a useful *in vitro* tool for studying drug metabolism and instructing clinical medication.

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