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An evaluation of para toluene-sulfonamide metabolism and effect with regard to CYP isoforms, P-glycoprotein, and drug interactions

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Aim of this study was to investigate liver metabolism of with regard to para toluene-sulfonamide (PTS), CYP isoforms, P-glycoprotein (P-gp), and drug interactions. Known substrates, inducers and inhibitors of CYP and inhibitor of P-gp were employed and metabolites were determined with HPLC. Male Wistar rats were pretreated with ip phenobarbital (PB), ketoconazole (Ket), or verapamil (Ver) for 3 days and *in situ* liver perfusion of PTS was conducted in a recirculation system. Rats were also pretreated with ip PTS (33 mg · kg^{-1} · d^{-1} or PTS 99 mg · kg^{-1} · d^{-1}) for 4 days before liver perfusions with dextromethorphan (Dex) and phenacetin (Phe) preparations were conducted. Microsome incubation was used to investigate PTS effect on five CYP isoforms and PTS-drug interactions probability with phyllotoxin and 5-fluorouracil (5-FU) *in vitro*. PTS at 60 min perfusates had areas of 61.4% and 133.6% of the blank control in PB group and Ket group, respectively. The result that PTS metabolism was enhanced by PB and inhibited by Ket treatments suggested liver CYP was attributed to PTS metabolism. PTS 99 mg · kg^{-1} · d^{-1} pretreatment slowed down the metabolism of Dex and Phe while *in vitro* incubations did not show a PTS (0–160 μmol/L) effect on CYP activities. PTS metabolite formation when co-incubated with phyllotoxin was 50.7% of the negative control. The potent inhibitory ability of phyllotoxin to PTS requires further clinical investigation regarding in concomitant administration.

1. Introduction

Para toluene-sulfonamide (p-methylbenzenesulfonamide; PTS) is an anticancer reagent, which is now in Phase II clinical trials in China for the treatment of breast cancer and head and neck squamous cancer as an adjunct to chemotherapy and radiotherapy. The substance shows good lipophilicity and accords with one compartment model in clinical pharmacokinetics.

While the prominent role of CYP in the metabolism of many pharmaceutical agents and in the activation or deactivation of potential carcinogens is known, it would be important to know as early as possible in the drug development process which CYPs are likely to process a drug candidate (DC), and which CYP activities to which extend are likely to be altered by the drug. According to our previous study (Zhou 2006), CYP2C7, CYP2D1 and CYP3A2 might contribute to PTS metabolism in the rat model; CYP3A2 inhibitors had the most effective inhibitory effect on PTS metabolism; LC-MS-MS analysis revealed that the PTS metabolite could be a hydroxylated derivative.

Moreover, CYP genes have great promise for applications designed to enhance the sensitivity of tumor cells to cancer chemotherapeutic drugs (Zhou 2005a). Many anticancer agents are extensively metabolized by CYPs, mostly CYP3A, e.g. cyclophosphamide ifosfamide, the taxanes paclitaxel and docetaxel, the *Vinca* alkaloids, anthracyclines, camptothecin derived topoisomerase I inhibitors, and epipodophyllotoxins (Schellens et al. 2000). P-Glycoprotein (P-gp), a secretary transporter in the intestine, a member of the multidrug resistance (MDR) associated protein family, and breast cancer resistance protein (Schellens et al. 2000) is believed to decrease the bioavailability of many CYP3A substrates by lowering the drug concentration at intracellular target sites. Compounds transported by P-gp include important anticancer drugs like *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxanes. We hope to understand P-gp function in the metabolism of PTS and we therefore conducted a preliminary investigation.

In situ liver perfusion is an appropriate model simulating *in vivo* condition for examining drug metabolism, and drug interaction. Known substrates, inducers and inhibitors of CYP and inhibitor of P-gp were employed. We gave rats pretreatment of verapamil, a selective P-gp inhibitor, ketoconazole, a known CYP inhibitor, and phenobarbital, a stimulator of CYP. Three major CYP isoforms were examined in the perfusion experiments and five CYP isoforms were examined in microsome incubation. Isoform substrates (phenacetin for CYP1A2, dextromethorphan for CYP2D1, and CYP3A2, tolbutamide for CYP2C7, and chlorozoxazone for CYP2E1) with their specific metabolites were employed.

2. Investigations and results

The present study was designed: (1) to elucidate CYP responsible for PTS metabolism; (2) to investigate the effect of PTS on CYP isoforms *in vivo* and *in vitro*; (3) to examine P-gp function on PTS metabolism under experimental conditions; (4) to examine drug interactions between PTS and two currently used anticancer drugs, phyllotoxin and 5-FU; (5) to compare liver perfusion results with incubation experiment results.

2.1. Calibration curves and HPLC method for in vitro incubation study

The HPLC methods were validated with five calibration standards and three QC samples of each substrate. Standard samples were added to the boiled microsomes and prepared as described in *Experimental*. The calibration curve was constructed by linear least-squares regression of standard concentrations against metabolite peak area. The mean recovery (accuracy) was within the range 91.1–110.6%. The relative standard deviations (RSD) of intra-day and inter-day in the concentrations studied were less than 4.99% and 6.51%, respectively.

2.2. PTS liver perfusion after treatment with PB, Ket and Ver

As shown in Fig 2, in the PB treatment group PTS concentration at 60 min perfusate was 61.4% (8.6 ± 2.8 μmol/L) of blank control (14.0 ± 3.0 μmol/L). In the Ket treatment group PTS concentration at 60 min was 133.6% (18.7 ± 3.9 μmol/L) of blank control. Pretreatment with Ver showed little effect on PTS metabolism under the experimental conditions.

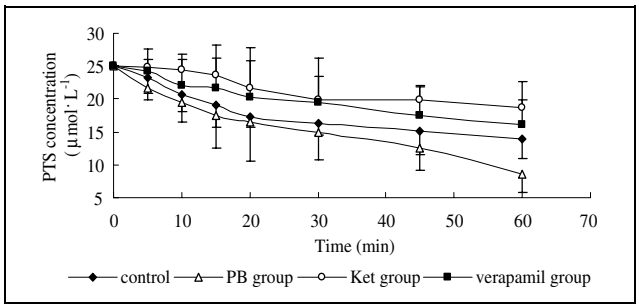

Fig. 2: PTS 25 μmol/L liver perfusion in groups of control, PB, Ket, or Ver pretreatment at 0, 5, 10, 15, 20, 30, 45, and 60 min (n = 4)

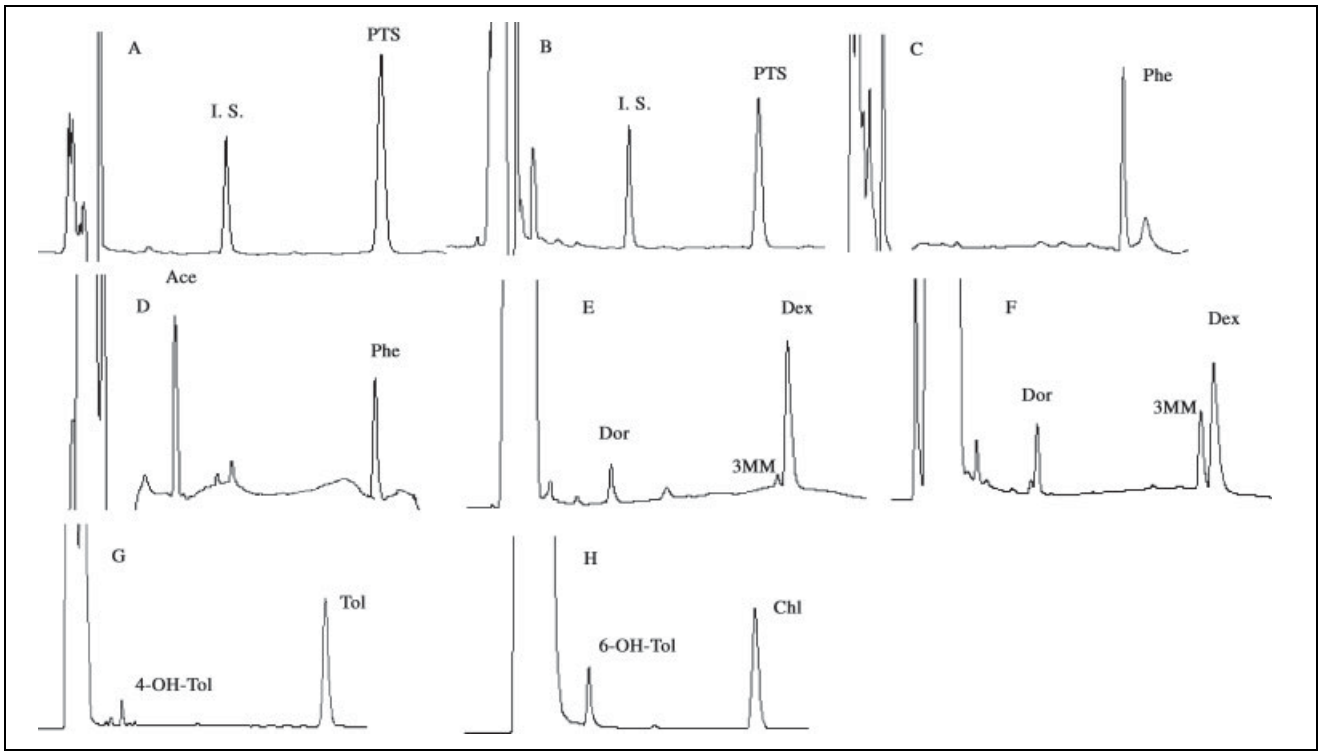

Fig. 1: HPLC chromatograms: (A): PTS perfusion at 0 min; (B) PTS perfusion at 60 min; (C) Phe perfusion at 0 min; (D) Phe perfusion at 60 min; (E) Dex perfusion 60 min; (F) Dex incubation; (G) Tol incubation; (H) Chl incubation

Table: Accuracy and precision of HPLC determination (n = 5)

Metabolite	Ace			6-OH-Chl			4-OH-Tol			Dor			3MM		
Added(μmol/L)	10	20	40	10	20	40	20	40	60	5	10	20	5	10	20
Recovery/%	98.5	105.6	96.4	98.3	92.3	94.6	96.3	99.3	93.3	96.4	110.6	107.4	101.6	91.1	94.0
RSD/% (Intra-day)	0.68	1.27	1.54	3.80	3.84	4.99	1.78	1.32	1.93	2.12	1.80	4.28	3.35	1.65	4.40
RSD/% (Inter-day)	3.69	2.55	2.49	6.51	4.08	4.0	1.09	1.45	3.40	0.9	3.4	3.9	5.88	1.45	6.39
Lineary	Y = 1.0653X + 0.0572 γ = 0.9997			Y = 3.8354X + 13.742 γ = 0.9991			Y = 35.388X − 20.575 γ = 0.9993			Y = 3.8354X + 13.742 γ = 0.9986			Y = 10.498X + 0.6615 γ = 0.9996		

2.3. Dex and Phe perfusion after treatment with PTS

After treatment of rats with PTS (33 mg · kg^{-1} · d^{-1} or 99 mg · kg^{-1} · d^{-1}), Dex 40 μmol/L and Phe 20 μmol/L liver perfusion were conducted. In PTS 99 mg · kg^{-1} · d^{-1} group Dex concentration at 60 min remained 155.7% (19 ± 2.6 μmol/L) of the blank control (12.2 ± 1.7 μmol/L); Phe at 60 min perfusate remained 138.2% (15.2 ± 1.6 μmol/L) of the blank control (11.0 ± 0.9 μmol/L). There was no significant difference between groups of PTS 33 mg · kg^{-1} · d^{-1} and blank control in both Dex and Phe perfusion. PTS ip 99 mg · kg^{-1} · d^{-1} for 4 days had inhibition impact to Dex and Phe *in vivo* metabolism (Fig. 3). The results indicated that CYP2D1, CYP3A2, and CYP1A2 activities might be influenced by of PTS pretreatment higher concentration.

2.4. In vitro microsome incubation of PTS with CYP substrate

PTS was co-incubated with CYP substrate to investigate the effect of PTS on CYP isoforms *in vitro*. Metabolite formation was compared with the negative control (in the absence of PTS and presence of substrate in incubation). With PTS concentrations from 0–160 μmol/L,% control activity of Ace samples was within the range 98.8–106.5%; 4-OH-Tol was within the range 95.2–101.4%; 3MM was within the range 94.2–97.3%; Dor was within the range 83.6–99.6%; 6-OH-Chl was within the range 88.3–104.3%. PTS did not show a significant influence on selected substrate reactions, which indicated that CYP1A2, CYP2C7, CYP2D1,CYP3A2 and CYP2E1 activities were not affected by PTS in the concentrations of *in vitro* metabolism.

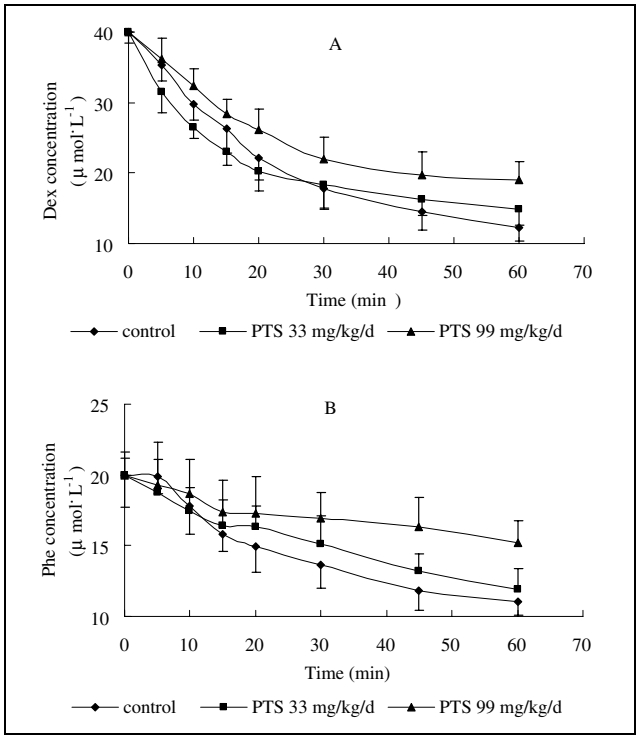

Fig. 3: *In situ* rat liver perfusion of Dex 40 μmol/L (A) and Phe 20 μmol/L (B) in groups of control, PTS 33 mg · kg^{-1} · d^{-1}, and PTS 99 mg · kg^{-1} · d^{-1} pretreatment (n = 4)

2.5. Investigation of drug-drug interaction

As shown in Fig. 5, PTS (25 μmol/L) and 5-FU or phyllotoxin at concentrations from 0–160 μmol/L were added to the reaction mixture. PTS metabolite formation when co-incubated with phyllotoxin was reduced to 50.7% of the negative control. 5-FU showed little effect on PTS metabolism. Phyllotoxin had a potent inhibitory effect on PTS metabolism which requires attention.

2.6. Conclusion

We gave a prerequisite for further toxicological risk assessment using this animal model. Evidence was not only provided that PTS was metabolized by CYP but also had relation to CYP3A activity. PTS did not show a significant effect to selected substrate reactions, which suggested that PTS clinical use with many current drugs is safe. Clinical use (and approval) of agents will depend upon a careful risk-benefit analysis. PTS administered with CYP inhibitors or CYP3A substrates still requires attention. As an anticancer drug, PTS has a simple structure which is economic and convenient in chemical synthesis and suitable for pharmaceutical development. For any given substrate, enzyme specificity, enzyme content, substrate binding affinity and sensitivity to irreversible catalytic events all play a role in determining the overall drug efficiency (Schellens et al. 2000). Further understanding of metabolism and disposition aspects of PTS are in progress which will provide a firm scientific basis of PTS usage.

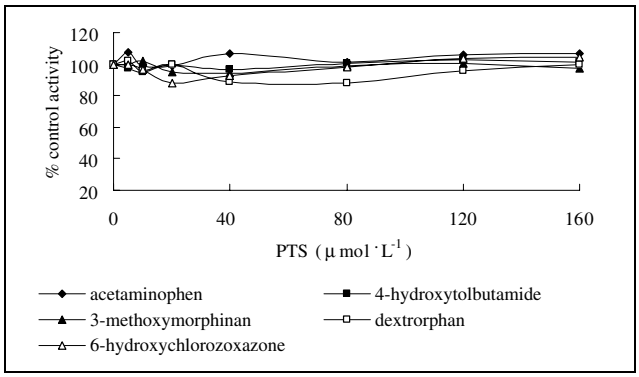

Fig. 4: PTS incubation with specific substrate. The areas of Ace, 4-OH-Tol, Dor, 3MM and 6-OH-Chl in HPLC detection were used to analysis. Each data point represents the mean of triplicate determinations

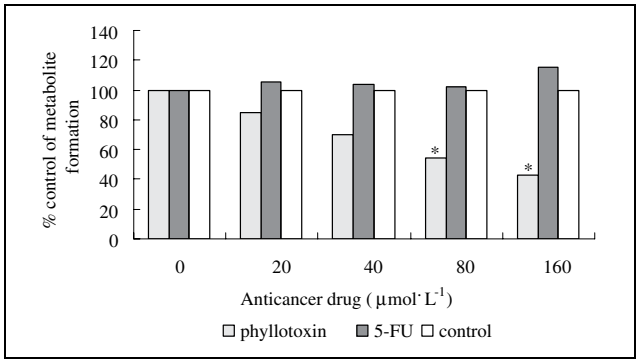

Fig. 5: PTS co-incubated with phyllotoxin and 5-FU. PTS metabolite formation was compared with the negative control. Each data point represents the mean of triplicate determinations. $^{*}P < 0.05$

3. Discussion

First of all, HPLC methods had been validated as having good linearity, recovery and RSD. The incubation contains more than one drug (PTS and substrate, or PTS and anticancer drugs) and their metabolite formation. The mobile phase, UV monitor and the flow rate should be considered to avoid inference peaks in the HPLC chromatograms.

This research investigated PTS metabolism in male Wistar rats. The experiments included liver perfusion and microsome incubation. Liver perfusion experiments provided evidence that liver CYP was responsible for PTS metabolism. Concentrations of substrates/inhibitors at target sites such as intestine and liver are varied. Theoretically, P-gp could decrease the efficient of CYP3A substrates and Ver may increase the anticancer drug efficiency in the target organ. In liver perfusion, intestinal enzyme function could not overlap with liver CYP450 regarding drug metabolism. Intestinal perfusion and more pharmacokinetic experiments are needed to verify the results.

In the perfusion model, PTS 99 $mg \cdot kg^{-1} \cdot d^{-1}$ pretreatment has shown an inhibitory effect on Dex and Phe metabolism, while CYP isoforms activities were unaffected by PTS in incubation experiments. The superfamily CYP has a complex structure and many active sites might be responsible for the metabolism of different substrates. PTS in selected concentrations and appropriate substrates representative of each isoform activity could be used in drug specificity and selectivity tests.

Many perfusion drugs could affect active molecules in the liver. Researches indicated that ochratoxin A in perfusate will influence TNF-α expression and inhibit metabolism of other drugs (AL-Anati et al. 2005). BSA added to perfusion will inhibit the activities of CYP (Vuppugalla et al. 2003). In our experiment liver perfusion and *in vitro* microsome incubation has differences in many aspects. (1) Liver perfusion lasts longer than an incubation experiment. The parent substances could transfer thoroughly and be derived in different metabolism pathways in liver perfusion. There was a significant difference between Dex perfusion and its incubation chromatograms. At the same concentration of Dex, the area of 3MM was higher in the incubation chromatogram than it was in perfusion experiments. With continuous time of perfusion, the areas of Dor and 3MM first began to increase and then decreased. Dex metabolized simultaneous by CYP2D1 and CYP3A2 to the finial metabolite hydroxymorphinan (Asha et al. 2004). After Dor and 3MM arrived to required concentrations, they began to transfer to hydroxymorphinan in a quicker process. At the same time, Dor and hydroxymorphinan would advance to glucuronidation derives (Axelsson et al. 2003). During 30 min incubation *in vitro*, Dor and 3MM concentrations increased all the time.

(2) PTS pretreatment might influence factors in the metabolism process and may lead to different results of perfusion and incubation experiments. We used the area of Dex for data analysis in perfusion while the areas of Dor and 3MM, and not Dex nor hydroxymorphinan, were used in incubation for data analysis. This leads to representative differences between them.

(3) Liver perfusion is a good model for metabolite analysis because the perfusate contains higher concentrations of metabolites. Metabolites could be separated from the aqueous and organic phase in microsome preparation after liver perfusion was conducted.

(4) Metabolites from both phase I and phase II (sulfotransferase, glucuronyl transferase, glutathione S-transferase) are derived from the perfusion model (Teyssier and Siess 2000).

In the treatment of cancer, drug co-administration and various individual differences in curative effectiveness are common. Inhibitors of CYP could slow down PTS metabolism and potentiate its activity or toxicity. In addition, phyllotoxin showed significant PTS metabolism inhibitory activity. We know phyllotoxin is one of the CYP3A substrates but we have no idea whether it is a CYP3A inhibitor inhibiting most of the CYP3A substrates in competitive, noncompetitive, mixed mechanism or it might take effects to PTS metabolism through any other mechanism. 5-FU belongs to the pyrimidine antagonists which is metabolized by thymidylate synthase and dihydropyrimidine dehydrogenase (Hakamada 2005) did not show any interaction with PTS.

4. Experimental

4.1. Chemicals

PTS standard was manufactured by Aldrich Company, phenacetin (Phe), acetaminophen (Ace), dextromethorphan hydrobromide monohydrate (Dex), dextrorphan (Dor), 3-methoxymorphinan (3MM), tolbutamide (Tol), 4-hydroxytolbutamide (4-OH-Tol), chlorozoxazone (Chl), 6-hydroxychlorozoxazone (6-OH-Chl), ketoconazole (Ket), verapamil (Ver), phyllotoxin, 5-FU, antipyrine (I.S.), β-NADP, G-6-P, and G-6-PDH were obtained from Sigma-Aldrich Chemical Co.(St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other supplies were of the highest grades available from standard commercial sources.

4.2. Solutions

Stock solutions of the analytes were prepared separately by dissolving each compound in water with acetonitrile at concentrations less than 1% (v/v) (Testino and Patonay 2003). G-6-PDH was dissolved in 5 mmol/L sodium citrate and kept in −80 °C until use.

4.3. Methods

4.3.1. Apparatus and chromatographic conditions (Zhou 2005b)

The samples were analyzed on Agilent 1100 series HPLC equipment at room temperature. An aliquot (50 μl) from each sample was injected onto Agilent XDB C_{18} column (4.6 mm × 250 mm, 5 μm). Peristaltic pump (Masterflex, Cole & Parmer, Illinois, USA). Compounds were quantified using their peak area.

For PTS: mobile phase A: acetonitrile/mobile phase B: water with 0.1% acetic acid (pH 3.5) was 20 : 80 at 230 nm. The retention times of metabolite, antipyrine (I.S.) and PTS were 3.0 min, 5.8 min and 9.8 min respectively. The flow rate was 1.2 ml/min.

For CYP1A2 phenacetin O-deethylase: mobile phase consisted of a gradient of A: acetonitrile and B: water with 0.1% acetic acid (pH 3.5) monitored at 230 nm. The retention time of Ace and Phe was respectively 4.7 min and 9.9 min. The flow rate was 1.2 ml/min.

For CYP2D1 dextro O-demethylation and CYP3A dextro N-demethylation: mobile phase consisted of a gradient of A: acetonitrile and B: water with 0.2% acetic acid and 0.1% triethylamine (pH 4.5) monitored at 277 nm. The retention time of Dor, 3MM and Dex was respectively 5.6, 10.6 min and 11.1 min. The flow rate was 1.0 ml/min.

For CYP2C7 tolbutamide methylhydroxylation: mobile phase A: acetonitrile/Mobile phase B: water with 0.1% acetic acid (pH 3.5) was 40 : 60 at 230 nm. The retention time of 4-OH-Tol and Tol respectively was 3.7 min and 11.2 min. The flow rate was 1.2 ml/min.

For CYP2E1 chlorzoxazone 6-hydroxylation: mobile phase A: acetonitrile/ mobile phase B: water with 0.1% acetic acid (pH 3.5) was 40 : 60 at 278 nm. The retention time of 6-OH-Chl and Chl was respectively 3.1 min and 6.2 min. The flow rate was 1.2 ml/min.

4.3.2. Animals

Male Wistar rats (2 months old, weight 200–250 g) were purchased from the Department of Laboratory Animal Sciences, Capital University of Medical Sciences. Animals were divided into 4 groups, $n = 4$ in every group. Groups were treated as: blank control, ip PB (60 $mg \cdot kg^{-1} \cdot d^{-1}$), Ket (20 $mg \cdot kg^{-1} \cdot d^{-1}$), Ver (5 $mg \cdot kg^{-1} \cdot d^{-1}$) for 3 consecutive days before rat liver perfusion of PTS preparation was conducted in a recirculation system; the second groups of pretreatment, $n = 8$ in every group and they were treated as: blank control, PTS ip 33 $mg \cdot kg^{-1} \cdot d^{-1}$, and PTS 99 $mg \cdot kg^{-1} \cdot d^{-1}$ for 4 consecutive days before liver perfusions were conducted of Dex and Phe preparations.

4.3.3. Liver perfusion and sample preparation

Rats were anaesthetized, 1 ml of saline containing heparin (20 IU) was injected into the portal vein, the liver was then perfused at 8 ml/min through the portal vein and caval vein in a recirculating system (50 ml) for 60 min. The perfusate contains Krebs–Heinseleit buffer (Jourdan 2004) composed of NaCl 120 mmol/L, KCl 5 mmol/L, $MgSO_4$ 0.65 mmol/L, KH_2PO_4 1.17 mmol/L, $CaCl_2$ 1.29 mmol/L, $NaHCO_3$ 25 mmol/L, glucose 8.5 mmol/L (pH 7.35–7.45), saturated with a mixture of oxygen and carbon dioxide (95 : 5) with the final concentration of perfusion drug (PTS 25 μmol/L, Dex 40 μmol/L, or Phe 20 μmol/L). The perfused rat liver preparation was maintained in a humidified environment in a thermostatically-controlled cabinet (37–38 °C). All experiments were carried out after a 10 min equilibration period in order to reach the biochemical steady state. At the time of 0, 5, 10, 15, 20, 30, 45, 60 min, 0.5 ml perfusate was withdrawn and 0.5 ml blank buffer was added back to perfusion system. The perfusates were centrifuged at 6000 rpm for 5 min, then 100 μl 7% perchloric acid were added to precipitate protein. Samples were vortexed and centrifuged at 12000 rpm for 10 min. The supernatant was transferred for HPLC analysis.

4.3.4. In vitro incubation and sample preparation

Microsome preparation was prepared by differential centrifugation and stored at −80 °C until use. The incubation volume is 0.5 ml containing 1.0 mg protein, 0.1 mol/L potassium phosphate buffer (pH 7.4) and NADPH-generating system (0.5 mmol/Lβ-NADP, 2.0 mmol/L G-6-P, 5 mmol/L $MgCl_2$ and 0.1 mmol/L EDTA). The samples were preincubated for 5 min prior to the addition of 2U G-6-PDH, following 30 min incubation at 37 °C. Negative control samples (in the presence of substrate and absence of PTS) were included in each essay to ensure the integrity of the microsomal incubation system.

% control activity = (metabolite remaining/metabolite formation in negative control)×100 (Sean 2003).

To investigate PTS effect on CYP isoforms, the incubation sample contains microsome protein, phosphate buffer, NADPH, substrates and varying concentrations of PTS (from 0–160 μmol/L). Specific substrates at concentrations of around apparent K_m values (20 μmol/L Phe, 100 μmol/L Tol, 20 μmol/L Dex, 40 μmol/L Chl) were employed. The areas Ace, 4-OH-Tol, Dor, 3MM and 6-OH-Chl were used to analysis.

To examine the drug-drug interactions between PTS and 5-FU or phyllotoxin, incubation containing microsome protein, phosphate buffer, NADPH, PTS 20 μmol/L, and varying concentrations of 5-FU or poyllixoin at 0, 20, 40, 80, 160 μmol/L were constructed. PTS metabolite area was used to analyze the influential effect. Since PTS metabolite standards were not available, the analyte to internal standard peak area ratio was used instead of using absolute quantitation (Yu 2003). SPSS pharmaceutical was used for statistical comparisons. The significance level was set at $P < 0.05$.

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