Hepatic Cytochrome P450 2E1 Activity in Nonalcoholic Fatty Liver Disease

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Background: *Nonalcoholic fatty liver disease (NAFLD) is a worldwide phenomenon spanning all the continents. The pathogenesis of NAFLD has not been completely elucidated. One hypothesis is that hepatic cytochrome P450 2E1 (CYP2E1) plays an important role in increasing the lipid peroxidation and oxidative stress in NAFLD.*
Objective: *The aim of the present study was to examine hepatic CYP2E1 activity in patients with NAFLD.*
Material and Method: *Healthy subjects were included. After an overnight fasting, the subjects were orally administered 400 mg chlorzoxazone (CHZ) and serial blood samples were collected at 0 (predose), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 and 8 hours after dosing. For patients with NAFLD, plasma samples were collected at 0 (predose), 1.5, 2, 2.5 and 3 hours after dosing. Plasma CHZ and 6-hydroxychlorzoxazone (6-OH-CHZ) was assayed by reversed-phase high-performance liquid chromatography (HPLC) with UV detector. Hepatic CYP2E1 activity was calculated by using concentration ratio of 6-OH-CHZ / CHZ.*
Results: *High concentration levels of CHZ and 6-OH-CHZ in healthy subjects were found between 1.5 to 3 hours after the dose. At 1.5 to 3 hours, the concentration ratio of 6-OH-CHZ /CHZ of patients with NAFLD seemed to be more than of healthy subjects. The time point which showed most different was 2.5 hours. (0.40 $\pm$ 0.27 vs. 0.25 $\pm$ 0.12 μg/ml, respectively, p = 0.10).*
Conclusion: *Although significant difference of the concentration ratio of 6-OH-CHZ / CHZ between the two groups was not exhibited, the data demonstrated the possibility of the increasing hepatic CYP2E1 activity in NAFLD. The concentration ratio of 6-OH-CHZ / CHZ at the point 2.5 hours may be the best index for measuring hepatic CYP2E1 activity in NAFLD.*

Keywords: *Nonalcoholic fatty liver disease, Chlorzoxazone, CYP2E1*

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Nonalcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome that encompasses several clinical entities. The spectrum of conditions ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which may advance to cirrhosis and end-stage liver disease[1,2].

The pathogenesis of NAFLD remains poorly understood. It is a complex, multifactorial process that involves genetic and environmental elements. In 1998, a "two-hit" hypothesis was proposed to explain the pathogenesis of NAFLD[1]. The first "hit" is thought to be an accumulation of fat, specifically fatty acids and triglycerides, within the liver[3]. Insulin resistance is believed to lead to the accumulation of triglycerides in hepatocyte as a result of more fatty acids being synthesized, more free fatty acids (FFA) being delivered to the liver, less fatty acids being degraded, and less triglycerides being released from the liver. The second "hit" is thought to be the progression from steatosis to steatohepatitis. Oxidative stress is the likely cause for progression of NAFLD. Oxidative stress occurs when more oxidant substances are produced than the antioxidant process of the liver can handle. Increased FFA

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levels, in addition to hepatic mediating insulin resistance and causing oxidative stress, can be directly hepatotoxic, leading to cellular injury[4]. Cytochrome P450 2E1 (CYP2E1) is a major microsomal source of oxidative stress, and it has been explored as a candidate in the pathogenesis of NASH[5]. Recent studies have shown that there is an increase of hepatic CYP2E1 activity in patients with NASH[5-7], findings that are supported by data from increasing hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation[7,8]. Thus, these supported the idea that CYP2E1 plays a role in the pathogenesis of NASH[5].

Chlorzoxazone (CHZ) has been used extensively as a selective *in vivo* probe of CYP2E1 activity in humans[7,9]. CHZ is a centrally acting skeletal muscle relaxant that is used in the treatment of muscle spasm[10]. CHZ is almost exclusively metabolized in the liver by CYP2E1 to a single major metabolite, 6-hydroxychlorzoxazone (6-OH-CHZ), which has been rapidly glucuronidated and eliminated by the kidney[11]. In the present study, the authors aimed to examine hepatic CYP2E1 activity in patients with NAFLD, as assessed by using concentration ratio of 6-OH-CHZ/CHZ.

Material and Method

Subject

Ten healthy Thai subjects participated in the present study. All of the subjects were considered healthy by physical examination, no medical history of allergy, no suffering from impaired hepatic function and no history of liver disease. They were non-smokers. They abstained from alcohol and medication a week before the present study and during the present study. Twelve patients with NAFLD were defined based on clinical and histological criteria to ascertain the diagnosis of NAFLD. Other courses of liver disease, including alcohol abuse, were excluded. The protocol was approved by the Institutional Ethical Review Committee of Faculty of Medicine, Chulalongkorn University.

Chemical

CHZ tablets (Maselax®, 200 mg) were obtained from the Thai Japan Laboratories Co., LTD. Standards of CHZ, 3-aminophenyl sulfone (internal standard) and β-glucuronidase (Type G0751), were purchased from Sigma. 6-OH-CHZ was purchased from Ultrafine Chemicals (Manchester, UK). HPLC grade acetonitrile and diethyl ether were purchased from Merck.

Drug administration

After an overnight fasting, all of the healthy subjects were administered 400 mg dose of CHZ orally. Breakfast and lunch were given at 2 hours and 4 hours after the dose, respectively. The healthy subjects had serial blood samples (5 ml each) collected from an arm vein at the following intervals: 0 (predose), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 and 8 hours after dose. The samples were collected in sodium citrate tubes, centrifuged, and the separated plasma was stored at -70°C until analysis. For the patients with NAFLD overnight fasting, blood samples were obtained at 0 (predose), 1.5, 2, 2.5 and 3 hours after the 400 mg dose of CHZ orally.

Method validation

Method for CHZ and 6-OH-CHZ analysis was validated following Guidance for industry: Bioanalytical method validation (U.S. Department of Health and Human Series FDA, CDER, CVM. May 2001, BP)[12].

Sample preparation

All of the plasma samples were determined by a modification of HPLC assay as described previously by Frye et al[10] and Mishin et al[13].

0.5 ml plasma was added with 1 ml of 0.2 M sodium acetate buffer (pH 4.75) and 200 units of β-glucuronidase (1,000 units of β-glucuronidase dissolved in 0.5 ml of 0.2% sodium chloride). Sample was incubated at 37°C for overnight. After incubation, 50 µl of 170 µg/ml 3-aminophenyl sulfone (internal standard) and 2 ml of acetonitrile were added. The sample was centrifuged at 4,000 rpm for 10 min. The supernatant was added with 4.5 ml of diethyl ether, mixed and centrifuged at 4,000 rpm for 10 min. The organic layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 500 µl of mobile phase. 50 µl was injected onto the column.

Instrument and HPLC condition

The HPLC column was a phenomenex® column (C_{18} reversed-phase column, 250 x 4.6 mm I.D., particle size 5 mm). The mobile phase was composed of phosphoric acid (0.5%, pH 3.5): acetonitrile (70:30, v/v) and the flow rate was 0.8 ml/min. The absorbance of compounds was monitored by UV detector at 287 nm.

Pharmacokinetic analysis

Maximum concentration (C_{max}) and time to reach the maximal concentration (T_{max}) for CHZ and 6-OH-CHZ were evaluated for each patient. The area under the concentration-time curve $(AUC)_{CHZ(0-8)}$ and $AUC_{6\text{-}OH\text{-}CHZ(0-8)}$ were determined by trapezoidal rule. $AUC_{CHZ(0-\infty)}$ and $AUC_{6\text{-}OH\text{-}CHZ(0-\infty)}$ were calculated by the

sum of AUC_{0-8} and C_{last}/K_{el}. K_{el} is the elimination rate constant estimated from the slope of log concentration-time curve in elimination phase. An elimination half-life ($t_{1/2}$) for CHZ and 6-OH-CHZ was calculated from $0.693/K_{el}$.

Hepatic CYP2E1 activity

The concentration ratio of 6-OH-CHZ/CHZ at 1.5 to 3 hours was used to assess hepatic CYP2E1 activity by means of mean ± standard deviation (SD). Hepatic CYP2E1 activity between healthy subjects and patients with NAFLD were compared by Student-t test using SPSS for Windows. Results were considered statistically significant if the p-values were less than 0.05

Results

The present method used for determination of CHZ and 6-OH-CHZ in plasma showed high selectivity with clear separation of peak of CHZ, 6-OH-CHZ, internal standard and any endogenous substances (Fig. 1). The least-squares regression analysis gave a linear correlation coefficient of r = 0.9999 and 0.9989 for CHZ and 6-OH-CHZ, respectively. The intra-day and inter-day precision were in the acceptable range (%CV < 15%) and the accuracy was within the acceptable limit (85-115%). The recovery of extraction was within the acceptable range (60-120%).

The mean ± SD of demographic data and clinical laboratory data of healthy subjects are presented in Table 1. They were in good health with normal liver and renal function. For patients with NAFLD, the mean ± SD of demographic data and clinical laboratory data are presented in Table 1. They had abnormal liver function with NAFLD confirmed by biopsy and all classified as NASH varying of severity.

All subjects completed the study with no adverse events observed following CHZ administra-

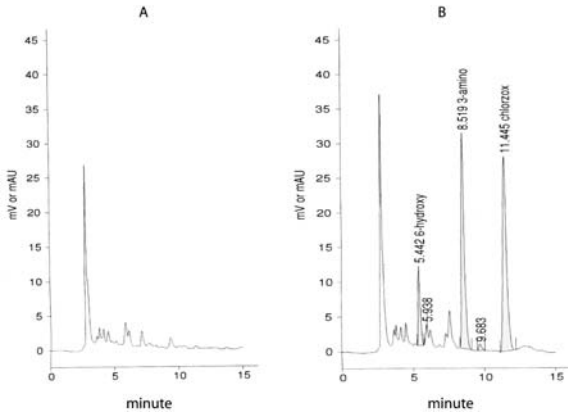

Fig. 1 Chromatograms of blank plasma (A), and plasma sample of subject after administered 400 mg chlorzoxazone (B). The retention time of 6-OH-CHZ, internal standard and CHZ were 5.4, 8.5 and 11.4 min, respectively

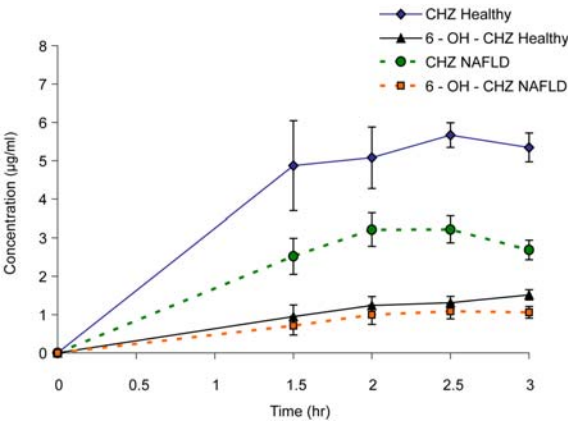

Fig. 2 CHZ and 6-OH-CHZ plasma concentration (mean ± SEM) vs. time in healthy subjects and patients with NAFLD

Table 1. Demographic and clinical laboratory data of healthy subjects and patients with NAFLD enrolled in the study

	Healthy subjects (n = 10)		Patients with NAFLD (n = 12)	
	Mean ± SD	Range	Mean ± SD	Range
Age (years)	33.80 ± 4.85	26-38	50.17 ± 12.82	29-79
Weight (kg)	57.60 ± 7.93	41-70	73.79 ± 10.21	49-89
Height (cm)	160.00 ± 0.09	150.0-175.0	165.60 ± 10.84	150.0-181.0
Body mass index (BMI) (kg/m^2)	22.56 ± 2.20	18.22-25.25	26.94 ± 3.29	23.15-32.89
SGOT (Normal, 0-38 U/L)	21.20 ± 5.51	14-28	45.08 ± 14.79	29-70
SGPT (Normal, 0-38 U/L)	17.80 ± 9.02	9-37	76.92 ± 28.05	40-121
Alkaline phosphatase (Normal, 39-117 U/L)	63.50 ± 13.17	44-93	81.18 ± 16.14	57-117

Table 2. Pharmacokinetic parameters of CHZ and 6-OH-CHZ in healthy subjects

Pharmacokinetic parameters	CHZ (n = 10)		6-OH-CHZ (n = 10)	
	Mean ± SD	Range	Mean ± SD	Range
C_{max} (μg/ml)	7.15 ± 2.09	9.89-4.91	1.77 ± 0.50	2.78-1.01
T_{max} (hr)	2.00 ± 0.82	3.00-1.00	3.05 ± 1.17	5.00-1.50
AUC_{0-8} (μg.hr/ml)	25.47 ± 7.11	34.16-15.70	7.32 ± 2.21	10.63-4.88
$AUC_{0-\infty}$ (μg.hr/ml)	27.52 ± 8.05	39.44-16.58	8.50 ± 2.78	12.14-5.25
K_{el} (hr^{-1})	0.48 ± 0.10	0.62-0.34	0.40 ± 0.13	0.59-0.21
$T_{1/2}$ (hr)	1.49 ± 0.32	2.01-1.12	1.95 ± 0.73	3.32-1.17

Table 3. Comparison of the concentration (μg/ml) ratio of 6-OH-CHZ / CHZ in healthy subjects and patients with NAFLD at 1.5 to 3 hours

Time(hr)	Healthy subjects (n = 10) (Mean ± SD)	Patients with NAFLD (n = 12) (Mean ± SD)	p-value*
1.5	0.22 ± 0.10	0.33 ± 0.27	0.26
2.0	0.26 ± 0.10	0.36 ± 0.26	0.30
2.5	0.24 ± 0.11	0.40 ± 0.27	0.10
3.0	0.30 ± 0.13	0.45 ± 0.26	0.11

tion. The plasma concentration vs. time curve of CHZ and 6-OH-CHZ in healthy volunteers and in patients with NAFLD are summarized by mean ± standard error of mean (SEM) and were shown in Fig. 2. All pharmacokinetic parameters of CHZ and 6-OH-CHZ of healthy subjects are presented in Table 2. CHZ was rapidly absorbed with the maximum levels of CHZ reached within 1 to 3 hours in the healthiest subjects, presented by T_{max} of 2.00 ± 0.82 hours. CHZ was rapidly metabolized to 6-OH-CHZ with T_{max} of 3.05 ± 1.17 hours.

The concentration ratio of 6-OH-CHZ/CHZ of healthy subjects and patients with NAFLD at 1.5 to 3 hours after the dose are presented in Table 3. The concentration ratio of 6-OH-CHZ/CHZ at all time points between 1.5 and 3 hour in patients with NAFLD was more than in healthy subjects. However, significant difference was not exhibited. At time point 2.5 hours, the concentration ratio of 6-OH-CHZ/CHZ was most different in NAFLD compared to healthy subjects (0.40 ± 0.27 vs. 0.25 ± 0.12 μg/ml, respectively, p = 0.10).

Discussion

CHZ is extensively hydroxylated by CYP2E1 to 6-OH-CHZ, which is subsequently glucuronidated and renally eliminated. The involvement of enzymes other than CYP2E1, including CYP1A1, CYP1A2 and CYP3A in the hydroxylation of CHZ *in vitro* has been reported. However, recent studies confirm that the contribution of these isozymes to the metabolism of CHZ is negligible, composing less than 2% of total activity[14]. A single major metabolite of CHZ is 6-OH-CHZ[11]. CHZ has been used as a selective *in vivo* probe of CYP2E1 activity in humans[7,9].

The analysis of CHZ and 6-OH-CHZ concentration in plasma by the present method showed good selectivity and linearity. The precision, accuracy and recovery were within the acceptable range. This method could be used to determine CHZ and 6-OH-CHZ concentration in plasma samples. The maximum concentration levels of CHZ reached within 1 to 3 hours in most healthy subjects presented by T_{max} of 2.00 ± 0.82 hours and were rapidly metabolized to 6-OH-CHZ by T_{max} of 3.05 ± 1.17 hours. Consequently, time to collection of blood samples for determination the ratio of 6-OH-CHZ and CHZ in patients with NAFLD should be 1.5 to 3 hours after the dose.

A "two-hit" hypothesis was proposed to explain the pathogenesis of NAFLD. The first "hit" is thought to be an accumulation of fat within the liver[3]. The second "hit" is thought to be the progression from steatosis to steatohepatitis. Oxidative stress is the likely cause for progression of NAFLD[4]. CYP2E1 is a major microsomal source of oxidative stress[5]. Recent studies have shown that there was an increase of hepatic CYP2E1 in patients with NASH[5-7], which is the progression stage of NAFLD. The present study aimed to assess CYP2E1 activity in patients with NAFLD using the concentration ratio of 6-OH-CHZ/CHZ and evaluate the appropriate time point of this ratio.

C_{max} of CHZ in patients with NAFLD was significantly lower than in healthy subjects (4.16 ± 0.82 and 7.15 ± 2.09 μg/ml, respectively) (p < 0.05). However,

C_{max} should not be used to determine CYP2E1 activity, because of various interferences. The concentration of metabolite/drug ratio when their concentrations are at maximum, contributes to be a useful tool for CYP activity determination[15]. In the present study, the concentration ratio of 6-OH-CHZ/CHZ, when each drug levels reached highly, served as an index of CYP2E1 activity and determined its difference between healthy subjects and patients with NAFLD. The concentration ratio of 6-OH-CHZ/CHZ at 1.5 to 3 hours was higher in patients with NAFLD than in healthy subjects. At 2.5 hours, the concentration ratio of 6-OH-CHZ/CHZ was most different in NAFLD compared with healthy subjects (0.40 ± 0.27 vs. 0.25 ± 0.12 μg/ml, respectively, $p = 0.098$). Although significant difference in the concentration ratio of 6-OH-CHZ / CHZ between healthy subjects and patients with NAFLD was not exhibited, there is a trend of increased hepatic CYP2E1 activity in NAFLD. Data from the present study suggested that the concentration ratio of 6-OH-CHZ/CHZ at the point 2.5 hours may be the appropriate index for measuring hepatic CYP2E1 activity.

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การทำงานของไซโตโครม พี450 2อี1 ในผู้ป่วยโรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์

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ภูมิหลัง: *โรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์เป็นภาวะที่พบได้ทั่วไป แต่พยาธิกำเนิดของโรคนั้นยังไม่ทราบแน่ชัด แต่ได้มีสมมติฐานว่าการทำงานของไซโตโครม พี450 2อี1 ในตับน่าจะมีบทบาทสำคัญในการทำให้ lipid peroxidation และ oxidative stress เพิ่มขึ้นในผู้ป่วย*

วัตถุประสงค์: *เพื่อทดสอบการทำงานของไซโตโครม พี450 2อี1 ในตับของผู้ป่วยโรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์*

วัสดุและวิธีการ: *ศึกษาในอาสาสมัครสุขภาพดีจำนวน 10 ราย หลังจากงดรับประทานอาหารหลังเที่ยงคืน ให้อาสาสมัครรับประทานยาคลอซอกซาโซนปริมาณ 400 มิลลิกรัม เจาะเลือดอาสาสมัครก่อนรับประทานยา และหลังจากรับประทานยาที่เวลา 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 และ 8 ชั่วโมง ตามลำดับ วิเคราะห์หาระดับคลอซอกซาโซนและ 6-ไฮดรอกซีคลอซอกซาโซนในพลาสมาด้วยวิธีเอชพีแอลซี โดยใช้เครื่องตรวจวัดยูวี และศึกษาในผู้ป่วยโรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์โดยให้รับประทานยาในแบบเดียวกัน และเจาะเลือดก่อนรับประทานยาและหลังจากรับประทานยาที่เวลา1.5, 2, 2.5 และ 3 ชั่วโมง ตามลำดับ แล้ววิเคราะห์หาระดับคลอซอกซาโซนและ 6-ไฮดรอกซีคลอซอกซาโซนด้วยวิธีเดียวกัน ศึกษาการทำงานของไซโตโครม พี450 2อี1 ในตับโดยใช้อัตราส่วนของ 6-ไฮดรอกซีคลอซอกซาโซนต่อคลอซอกซาโซน*

ผลการศึกษา: *ในอาสาสมัครสุขภาพดีพบระดับความเข้มข้นของคลอซอกซาโซนและ 6-ไฮดรอกซีคลอซอกซาโซนในพลาสมาสูงในช่วงเวลา 1.5 ถึง 3 ชั่วโมงหลังจากรับประทานยา อัตราส่วนของ 6-ไฮดรอกซีคลอซอกซาโซนต่อคลอซอกซาโซน ที่เวลา 1.5 ถึง 3 ชั่วโมง ของผู้ป่วยโรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์ มีค่าสูงกว่าอาสาสมัครสุขภาพดี โดยที่เวลา 2.5 ชั่วโมงมีความแตกต่างกันมากที่สุด (0.40 $\pm$ 0.27 ต่อ 0.25 $\pm$ 0.12 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ที่ $p = 0.10$)*

สรุป: *ถึงแม้ว่าอัตราส่วนของ 6-ไฮดรอกซีคลอซอกซาโซนต่อคลอซอกซาโซนระหว่างคนสองกลุ่มจะไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ แต่ข้อมูลที่ได้จากการทดลองแสดงให้เห็นความเป็นไปได้ว่า ผู้ป่วยโรคไขมันในตับที่ ไม่ได้มีสาเหตุจากแอลกอฮอล์มีการทำงานของไซโตโครม พี450 2อี1 ในตับเพิ่มขึ้น และอัตราส่วนของ 6-ไฮดรอกซีคลอซอกซาโซนต่อคลอซอกซาโซนที่เวลา 2.5 ชั่วโมง น่าจะใช้เป็นดัชนีชี้วัดการทำงานของไซโตโครม พี450 2อี1 ในผู้ป่วยโรคไขมันในตับที่ไม่ได้มีสาเหตุจากแอลกอฮอล์ได้ดีที่สุด*