Pharmacogenetic Relevance of Diazepam Metabolism in Human Liver Samples*

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= Abstract =

Diazepam 대사의 약물유전학적 규명

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Background : It has been well documented that the disposition of diazepam may differ markedly between different individuals. There is evidence to suggest a possible association between the variability of *N*-demethylation of diazepam and the *S*-mephenytoin hydroxylase polymorphism. However, the pharmacogenetic relevance of other metabolic pathways of diazepam is obscure at present.

Method : Fresh human liver samples ($n=14$) were obtained as an excess material removed during surgery on the liver. After preparing the microsomes, the kinetic parameters of *S*-mephenytoin 4-hydroxylase in human liver microsomes were assessed. Enzyme kinetic parameters were obtained for the formation of oxazepam, temazepam and demethyldiazepam. The ability of *S*-mephenytoin to inhibit the formation of the metabolites was determined by measuring the velocity of production of these metabolites from diazepam in human liver microsomes in the presence of mephenytoin.

Results : After incubating with 200 μ M diazepam, the average velocities of formation of demethyldiazepam, temazepam and oxazepam in 14 different liver preparations were 11.3 ± 5.9 , 23.7 ± 16.9 and 1.5 ± 1.1 pmole/mg protein per min, respectively. Among the individual Michaelis-Menten parameters of diazepam metabolism, only the $V_{\max}$ of *N*-demethylation gave a close correlation ($r_s=0.821$, $p<0.05$) with the $V_{\max}$ of *S*-mephenytoin hydroxylation. Three liver samples showed a 9-fold smaller mean $V_{\max}$ of diazepam demethylation than that of the remaining 11 samples.

Conclusion : This study provided further evidence that *S*-mephenytoin 4-hydroxylase is primarily responsible for the formation of demethylation but not for the C_3 -hydroxylation of diazepam

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in Korean liver samples.

KEY WORDS : Pharmacogenetics • Diazepam metabolism • Human liver • Korean.

The role and importance of genetically determined polymorphic drug oxidation have been of an increasing interest since the discovery of the polymorphic oxidation of debrisoquin or sparteine^{1,2)}. The metabolism of more than two dozen drugs has now been shown to depend on the debrisoquin 4-hydroxylase (CYP2D6) activity^{3,4)}. The more recently discovered polymorphic hydroxylation of *S*-mephenytoin (mediated by way of CYP2C subfamily)^{5,6)}, which is independent of debrisoquin/sparteine-type hydroxylation^{7,8)}, is inherited as an autosomal recessive trait^{9,10)}. The frequency distribution of metabolic capacity of mephenytoin shows a clear-cut bimodality and is expressed by two distinct phenotypes: poor metabolizers (PMs) and extensive metabolizers (EMs)^{8,11-17)}. Like the debrisoquin/sparteine-type hydroxylation pharmacogenetics⁴⁾, the hydroxylation polymorphism of *S*-mephenytoin shows a marked interethnic difference in the incidence of the PM phenotype: the frequency of occurrence of the PM phenotype of mephenytoin was much greater in Korean, Japanese and Chinese populations^{11,13,14,16)} compared with that of American^{14,16)} and Canadian white subjects¹⁷⁾ and European populations¹⁸⁾. This genetic polymorphism now concerns the oxidative metabolism of several clinically important drugs such as propranolol¹⁹⁾, imipramine²⁰⁾, hexobarbital^{21,22)}, mephobarbital²³⁾, omeprazole^{24,25)}, chlorguanide^{26,27)}, and diazepam^{13,28)}.

It has been well documented that the disposition of diazepam shows an wide interindividual difference^{29,30)}. Several possible factors have been reported to account for this variability³¹⁻³³⁾. It has recently been reported that one of the factors governing interindividual difference in the disposition of diazepam is genetically determined hydroxylation polymor-

phism of *S*-mephenytoin in Korean subject and Swedish white subject^{28,34)}. However, this was not the case with Chinese subjects³⁴⁾, suggesting the possibility that the pharmacogenetic information on a drug metabolism obtained from an ethnic group should not be easily translated to another ethnic group.

The benzodiazepines are eliminated from the body mainly by metabolic processes. Thus, diazepam is metabolized to the *N*-demethylated form, *N*-demethyldiazepam and the *C*₃-hydroxylated form, temazepam, both of which are eventually converted to the *N*-demethylated- and *C*₃-hydroxylated product, oxazepam^{29,30)}. Both Jack and Colburn³⁵⁾ and Dasberg³⁶⁾ have calculated that more than 50% of a dose of diazepam is demethylated to demethyldiazepam. Early study showed that diazepam undergoes both *N*-demethylation and *C*₃-hydroxylation *in vitro* in dog and rat liver, and that these reactions are NADPH-dependent and are inducible by phenobarbital³⁷⁾. Subsequent study using human fetal liver preparations demonstrated that these oxidative processes are mediated by the cytochrome P450 (CYP) system³⁸⁾. Recent *in vitro* investigation with liver microsomes from adult humans showed that the intrinsic clearance (V_{max}/K_m) for the formation of temazepam was higher than that for the degeneration of demethyldiazepam³⁹⁾. This implies that temazepam should be a more prominent metabolite of diazepam *in vivo* than demethyldiazepam, in contrast to what is observed in practice.

The objective of the present investigation was to examine the possible link between diazepam metabolic pathways and genetically determined *S*-mephenytoin hydroxylation polymorphism in human liver samples.

Materials and Methods

1. Drugs and chemicals

Diazepam, demethyldiazepam, temazepam and oxazepam were obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Glucose 6-phosphate dehydrogenase, NADP⁺ and glucose 6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methanol, triethylamine and other reagents of analytical or high-performance liquid chromatographic grade were obtained from either Sigma Chemical Co. or Boehringer Mannheim GmbH (Mannheim, Germany).

2. Instruments and chromatography

Analyses were performed with the following high-performance liquid chromatography system: a model L-6000 pump, a model L-4000 ultraviolet absorbance detector, a model AS-2000 autosampler, a model D-2500 integrator (Hitachi Ltd., Tokyo, Japan) and a 4.6mm×5cm Zorbax BP-ODS column (Dumont Instruments, DE, U.S.A.). The mobile phase consisted of a 70/30/0.1 (by volume) mixture of methanol, H₂O and triethylamine adjusted with phosphoric acid to pH 5.50. The flow rate was 1.0 mL/min. Each analyte was monitored at 240 nm.

3. Preparation of human liver microsomes

Fresh human liver samples (n=14) were obtained from the patients who underwent a partial hepatectomy at the Department of General Surgery (College of Medicine, Gyeongsang National University) as an excess material removed during surgery on the liver. None of the patients had been phenotyped *in vivo* with the racemic mephenytoin before undergoing the hepatectomy. All the surgeries were performed for the removal of intrahepatic stone from the liver. The use of human liver for the study had been approved by the Institutional Ethics Committee. It took less than 5 min from the removal of liver until

collection and freezing a sample in liquid nitrogen. The liver parenchyma of the part used for the study was shown later to be histopathologically normal in all cases. The liver samples obtained from the following donors were not included in the study: patients with acute or chronic hepatitis, those with cirrhosis and those who were taking medications known to induce or inhibit the hepatic monooxygenase activity.

Microsomes were prepared by differential centrifugation and the 105,000 g pellet was washed and resuspended in 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. After the determination of protein concentration⁴⁰⁾, the suspended microsomes were aliquoted, frozen and kept -80°C until used.

4. Incubation and sample preparation

The basic incubation medium contained 0.1 or 0.2 mg/mL of microsomes, 0.5 mM NADP, 2.0 mL glucose-6-phosphate dehydrogenase, 4 mM MgCl₂, 0.1 mM EDTA, 100 mM potassium-phosphate buffer (pH 7.4) and 40 to 400 µM of diazepam or 25 to 400 µM of *S*-mephenytoin in a final volume of 250 µL. The mixture was incubated at 37°C for 20 and 60 min for diazepam and *S*-mephenytoin, respectively, and the reaction was stopped by adding 2 mL diethyl ether-isopropanol (7:3 v/v). At this point 0.5 µg of the internal standard prazepam was added to each tube. Each tube was vortexed and centrifuged before the organic layer was transferred to a 3 mL tube and then dried under nitrogen. Before HPLC analysis, the residue was dissolved in acetone and a 1-2 µL of aliquot was analyzed.

5. Kinetics of the formation of metabolites

The time-activity curves for the generation of demethyldiazepam, temazepam and oxazepam by the human liver microsome were determined at 40 and 400 µM diazepam. At the lower substrate concentration the dependence of metabolite formation with

time is linear up to 20 min for all three metabolites monitored. At the higher substrate concentration this relationship is linear past 30 min. The formation of demethyldiazepam, temazepam and oxazepam as a function of protein concentration was measured with diazepam concentrations of 40 and 400 μM , incubating samples for 15 min. At 40 μM rates of formation for all three metabolites are linear with respect to protein concentration up to 5 mg protein/mL incubation mixture and at 400 μM up to 7.5 mL incubation mixture.

The formation rate of 4-hydroxymephenytoin was linear at 37°C for up to 90 min when 200 μM of S-mephenytoin and 0.1 mg/mL of microsomal protein were present. A linear relationship was also observed between the rate of metabolite production in 60 min and protein concentration for up to 1.0 mg/mL. Accordingly, the kinetic studies were performed at 37°C with a 60-min incubation time and at a protein concentration of 0.1 to 1.0 mg/mL.

6. Analysis of data

Estimation of Michaelis-Menten parameters was achieved with the aid of the graphical technique of Hofstee⁴¹⁾. By using the method of Dixon and Webb⁴²⁾, in which $1/V$ was plotted against for two of more concentrations of substrate, K_i values were estimated. Because this method cannot distinguish competitive mixed-type inhibitions, the method of Cornish-Bowden⁴³⁾ was also used to evaluate the type of inhibition.

Results

Representative chromatograms of diazepam and its metabolites are shown in Fig. 1. The retention times for oxazepam, temazepam, demethyldiazepam, diazepam and prazepam were 8.5, 9.7, 11.5, 13.9 and 23.8 min, respectively. There were no interference peaks in the chromatograms of drug-free microsomal preparation. The conversion for all three metabolites

Fig. 1. Representative chromatogram of a extracted diazepam and its metabolites after incubation in human liver samples. Arabic numerals indicate the peaks of : 1=oxazepam ; 2=temazepam ; 3=demethyldiazepam ; 4=diazepam ; 5=prazepam(internal standard).

Fig. 2. Eadie-Hofstee plots for the formation of demethyldiazepam (■), temazepam (●) and oxazepam (▲) in human liver samples.

never exceed 16% of total substrate at the lowest concentration.

The kinetic studies of the formation of demethyldiazepam, temazepam, and oxazepam were per-

Table 1. Michaelis-Menten parameters for the *N*-demethylation and *C*₃-hydroxylation of diazepam in human liver samples

Metabolite	K_m (μM)	V_{max} (pmole/mg protein per 15min)	V_{max}/K_m (ml/gm per min)
Oxazepam	12 ± 3.5	1.08 ± 0.09	5.2
Demethyldiazepam	40 ± 7.2	2.02 ± 0.34	3.0
Temazepam	59 ± 9.0	4.52 ± 0.71	4.1

med. Fig. 2 shows representative Eadie-Hofstee plots ; linearity for each metabolite indicates a single binding site. Table 1 list the K_m , V_{max} and clearance (V_{max}/K_m) values for diazepam metabolism indicating that the formation of oxazepam has the highest affinity compared to demethyldiazepam and temazepam formation. The clearance value for oxazepam, however, was the highest among the three metabolic pathways. Due to the relatively slow production of oxazepam in human livers compared to the other pathways, the kinetic constants could not be reliably obtained. After incubation with 200 μM diazepam, the average velocities of formation of demethyldiazepam, temazepam and oxazepam in 14 different liver preparations were 11.3 ± 5.9 , 23.7 ± 16.9 and 1.5 ± 1.1

Fig. 3. Relationship between Michaelis-Menten kinetic parameter for the demethylation of diazepam, V_{max} and the value of *S*-mephenytoin 4-hydroxylation in 14 different human liver microsomes.

Fig. 4. The distribution of the R/S mephenytoin ratio in 14 human liver microsomes.

pmole/mg protein per min, respectively.

To assess which of the components of diazepam metabolism would be related to the activity of *S*-mephenytoin 4-hydroxylase, the correlation analyses were performed between the individual Michaelis-Menten parameters of diazepam metabolism and the corresponding individual values of *S*-mephenytoin 4-hydroxylation. Among the parameter examined, only the V_{max} of *N*-demethylation gave a close correlation ($r_s=0.821$, $p<0.05$) with the corresponding parameter (V_{max}) of *S*-mephenytoin 4-hydroxylation (Fig. 3).

Although any of the patients did not be phenotyped before their hepatectomy, the three liver microsomes might have been obtained from the patients with a PM phenotype of *S*-mephenytoin. This is because they showed a lower V_{max} and higher K_m and consequently a low V_{max}/K_m as compared with those of the remaining 11 samples. These three samples

showed a 9-fold smaller mean $V_{\max}$ of diazepam demethylation than that of the remaining 11 samples (Fig. 4).

Discussion

Recently, it was suggested on the basis of results from an *in vivo* human panel study that the *N*-demethylation of diazepam is mediated by the cytochrome P450 isozyme responsible for the polymorphic 4-hydroxylation of *S*-mephenytoin¹³. The current data derived from the *in vitro* experiments with human liver microsomes were consistent with this suggestion and provide further evidence to support this contention. The most prominent finding observed was a close correlation between the $V_{\max}$ of the *N*-demethylation of diazepam and that of *S*-mephenytoin 4-hydroxylation with the linear regression line of the relationship passing through the near-origin. In addition, the human liver microsomes which might have been obtained from the patients with a PM phenotype of *S*-mephenytoin showed a 9-fold smaller mean $V_{\max}$ of diazepam *N*-demethylation than that of the others.

These observation strongly suggest that CYP2C_{MP} is a principal enzyme responsible for the diazepam *N*-demethylation in human liver microsomes. However, the K_m values of diazepam *N*-demethylation gave no significant correlation with the K_m values of *S*-mephenytoin 4-hydroxylation. Although the reason(s) for this remains unclear, the possibility is not totally negated that a minor, but enzyme other than CYP2C_{MP} would exist in human liver microsomes. Nevertheless, it is difficult to separate such a minor enzymatic component from other with the kinetic analysis as used in the present study. This appears to be a limitation of the experiments conducted by using human liver microsomes in which the multiple forms of CYP are present. Another supportive evidence for the suggestions as mentioned above is

a mutual competitive inhibition of diazepam and *S*-mephenytoin between each other's microsomal hydroxylation.

What enzyme(s) would be involved in the *C*₃-hydroxylation of diazepam in humans cannot be assessed by the present study. However, CYP2C_{MP} does not appear to take part in this metabolic process of diazepam, because the kinetic parameters for the formation of temazepam and oxazepam did not show any significant correlation with those of 4-hydroxymephenytoin. These findings are consistent with the observation that the mean AUC ratio of temazepam to diazepam is significantly greater in the PMs than in the EMs of *S*-mephenytoin^{29,33}, implying that the formation of these metabolites from diazepam is apparently not impaired in the PMs. Therefore, other CYP(s) and/or predictably flavincontaining monooxygenase would be involved in the *C*₃-hydroxylation and *p*-hydroxylation of diazepam in humans.

Ultimately, it was observed that diazepam is the potent inhibitor of *S*-mephenytoin 4-hydroxylase. Therefore, it follows that diazepam should reduce the clearance of drugs of which the metabolism is mediated *via* CYP2C_{MP}. In fact, Gugler and Jensen⁴⁴ reported that the mean elimination half-life of diazepam was increased by 130% and the mean total body clearance was reduced by 55% after a 9-day administration of omeprazole (40mg daily). The metabolism of diazepam has been reported to cosegregate with *S*-mephenytoin 4-hydroxylation polymorphism *in vivo*^{13,28}. Thus, a caution should be exercised when diazepam is administered with such drugs (e.g., imipramine, amitriptyline or propranolol) that their metabolic disposition has been demonstrated to cosegregate with the 4-hydroxylation of *S*-mephenytoin^{15,20,45}.

The relative velocities of formation of demethyl-diazepam, temzepam and oxazepam from diazepam indicate that demethyldiazepam and temazepam are

major metabolites while oxazepam is relatively minor (usually less than 7%) in humans. Even if the formation of oxidation is mediated by the same polymorphic enzyme that controls *S*-mephenytoin 4-hydroxylation, then it would be unlikely to be responsible for the considerable variation of diazepam elimination seen between subjects, since it represents such a small proportion of diazepam metabolism. Previous studies on the metabolites of diazepam in human liver microsomes indicated that *p*-hydroxylation may have been overlooked as a metabolic route^{46,47}. However, this study confirms the recent work of a British group that demonstrated *p*-hydroxylation of diazepam was minor in humans⁴⁸, any *p*-hydroxylated metabolites of diazepam were below the detection limit of their assay methods. The present study demonstrates that the formation of oxazepam is probably not the line with the *S*-mephenytoin polymorphism, or if it is of little clinical consequence.

In conclusion, the present investigation is the first report describing the metabolism of diazepam on an *in vitro* experiment by using Korean human liver microsomes, has provided further evidence that CYP 2C_{MP} is primarily responsible for the formation of demethyldiazepam but not for the C₃-hydroxylation of diazepam in humans. Thus both diazepam and demethyldiazepam may interact with several therapeutic agents of which the metabolism of a drug *in vitro* experiment by using human liver microsomes as described herein may be useful approach to understand further and scrutinize an *in vivo* drug disposition in humans.

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= 국문초록 =

배 경 :

개체간에 다양한 반응을 보이는 diazepam의 대사는 N-demethylation, C₃-hydroxylation등의 경로에 의해 이루어지며, 대사정도에 의해 각 개체의 약물효과가 결정된다. 이들의 대사경로 중 N-demethylation 과정은 *in vivo* 연구에서 약물유전학적으로 결정된 S-mephenytoin hydroxylase에 의해 이루어진다고 하나 그외의 과정은 아직 규명되어 있지 않다.

방 법 :

한국인의 간조직을 이용하여 이들의 S-mephenytoin hydroxylation 능력을 검정한 후 S-mephenytoin hydroxylase의 대사능력에 따르는 diazepam의 대사산물인 demethyldiazepam, temazepam 및 oxazepam의 형성되는 정도를 HPLC로 측정하였다.

결 과 :

14례의 한국인 간조직에서 200 μ M의 diazepam을 incubation시켰을 때 demethyldiazepam, temazepam 및 oxazepam의 평균 형성속도는 각각 11.3 ± 5.9 , 23.7 ± 16.9 , 1.5 ± 1.1 pmole/mg protein/min이었다. 각종 Michaelis-Menten 지수 중 N-demethylation의 V_{max} 와 S-mephenytoin hydroxylation의 V_{max} 만이 통계적으로 유의한 상관관계 ($r_s=0.821$, $p<0.05$)가 있었다. 14례의 간조직 중 3례는 상대적으로 낮은 V_{max} 및 높은 K_m 을 보였고 이 조직들은 나머지 11례의 간조직 보다 diazepam의 N-demethylation의 V_{max} 가 1/9에 불과하였다.

결 론 :

Diazepam의 대사물질인 demethyldiazepam의 형성에 S-mephenytoin hydroxylase가 밀접한 관계가 있음이 분명하였으나 한국인 간조직에서 diazepam의 C₃-hydroxylation에는 다른 효소가 관여할 것으로 사료된다.