Prediction of in Vivo Carbamazepine 10,11-Epoxidation from in Vitro Metabolic Studies with Human Liver Microsomes: Importance of Its Sigmoidal Kinetics

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Hepatic intrinsic clearance (CL_{int}) of drugs is often predicted based on in vitro data that are obtained from the Michaelis–Menten analysis. While most of the metabolic rate-substrate concentration kinetic curves fit to the Michaelis–Menten equation, cytochrome P450 (CYP) and uridine 5’-diphosphate (UDP)-glucuronosyltransferases exhibit sigmoidal kinetics for certain drugs. In our study, the kinetics of CYP3A4-catalyzed carbamazepine 10,11-epoxidation in human liver microsomes was sigmoidal and fitted to the Hill equation, revealing the S_{50} value of 358 µM, n of 2.0, and the V_{max} value of 463 pmol/min/mg. While the intrinsic clearance calculated from Michaelis–Menten parameters (CL_{int}) overestimated the observed in vivo intrinsic clearance (CL_{int, in vivo}), the maximum intrinsic clearance calculated based on the Hill equation (CL_{max}) exhibited better predictions of CL_{int, in vivo}. Such better prediction using the CL_{max} was also observed for other four drugs, all of which also exhibited sigmoidal metabolic rate-concentration curves, according to the literature data. However, even if we assume such Hill equation, intrinsic clearances predicted at their therapeutic concentrations from in vitro data were still much lower than their CL_{max, in vivo}, suggesting the existence of unknown factors causing discrepancy between in vitro intrinsic clearance in human liver microsomes and in vivo data. Thus, even if we assume sigmoidal kinetics, that would not be enough for accurate prediction of CL_{int, in vivo}, and it would be preferable to use CL_{max} to quantitatively extrapolate the in vitro data to in vivo clearance.

Key words carbamazepine metabolism; sigmoidal kinetics; in vitro–in vivo extrapolation; cytochrome P450 3A4

One of the ultimate goals of in vitro drug metabolism research is the prediction of in vivo clearance of drugs from in vitro data. Phase I and II drug-metabolizing enzymes such as cytochrome P450 (CYP) and uridine 5’-diphosphate (UDP)-glucuronosyltransferases (UGT) metabolize drugs to generate metabolites, which are generally pharmacologically inactive and more hydrophilic than the parent compounds, and are therefore easily excreted from the body through the kidney or the liver. Since these metabolic processes play a central role in the clearance of drugs, the metabolic clearance of drugs, which is commonly described as an intrinsic clearance (CL_{int}—often estimated as the ratio of V_{max} to K_{m}), is calculated from in vitro research to estimate the in vivo clearance of the drugs.3 Carbamazepine (5-carbamoyl-5H-dibenzo-[b,f]azepine) is a widely used anti-epileptic drug, and the first-line drug of choice in partial epilepsy. Since its increased concentration in the blood is tightly linked to the onset of adverse effects of carbamazepine including drug addiction, the blood levels of carbamazepine are monitored so as not to exceed the therapeutic range, which is normally 4–12 µg/mL.4 Carbazepine is metabolized to over 30 metabolites in humans such as carbamazepine 10,11-epoxide, 2- and 3-hydroxy carbamazepine, and carbamazepine N-glucuronide.5 Among the 30 metabolites, carbamazepine 10,11-epoxide is the major metabolite, which accounts for approximately 40% of carbamazepine metabolism.6 The metabolism of carbamazepine to its 10,11-epoxide is mediated mainly by CYP3A4, while CYP2C8 partially contributes to the epoxidation reaction.7 Due to the fact that carbamazepine is eliminated by hepatic metabolism, determination of the hepatic metabolism rate is especially important to predict its clearance from the body. Since CYPs and UGTs are enzymes expressed in the endoplasmic reticulum, human liver microsomes (HLMs) are used in the in vitro metabolic research to obtain the kinetic parameters of carbamazepine 10,11-epoxidation in the human liver. Recombinant systems, such as insect cells expressing human CYP3A4, are also useful to conduct in vitro kinetic analysis of drugs.

When kinetics of drug metabolism is typical and follows Michaelis–Menten kinetics, the relationship between substrate concentration and velocity is described by the Michaelis–Menten equation (Eq. 1):

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]  

where V is the velocity of the metabolic reaction, and S is the substrate concentration. The V_{max} is the maximum rate of metabolism and K_m is the Michaelis constant, which is defined as the substrate concentration at 1/2 the maximum velocity. The ratio of V to S provides the clearance (Eq. 2):

\[ \frac{V}{S} = \frac{V_{\text{max}}}{K_m + S} \]  

While the clearance is substrate concentration-dependent, it is constant when the substrate concentration is much smaller than K_m providing the parameter, intrinsic clearance (CL_{int}) (Eq. 3):

\[ CL_{\text{int}} = \frac{V_{\text{max}}}{K_m} \]  

In contrast, sigmoidal kinetics can be described by the Hill equation (Eq. 4):

\[ V = \frac{V_{\text{max}} \times S}{K_m + S^n} \]  

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\[ V = \frac{V_{\text{max}} \times S^n}{S_0^n + S^n} \quad (4) \]

where \( S_0 \) is the substrate concentration showing the 1/2 \( V_{\text{max}} \) and \( n \) is the Hill coefficient. The clearance of drugs whose kinetic profiles are sigmoidal can be described by Eq.

\[ \frac{V}{S} = \frac{V_{\text{max}} \times S^{n-1}}{S_0^n + S^n} \quad (5) \]

While the clearance is substrate concentration-dependent, the maximum clearance, \( CL_{\text{max}} \), can be described by Eq. 6 as demonstrated by Houston and Kenworthy:[10]

\[ CL_{\text{max}} = \frac{V_{\text{max}}}{S_0} \times \frac{(n - 1)}{n(n - 1)\bar{n}} \quad (6) \]

In the present study, we carried out the kinetic analysis of carbamazepine 10,11-epoxidation in HLM and predicted the in vivo clearance of carbamazepine from data obtained in vitro.

MATERIALS AND METHODS

Chemicals and Reagents  Carbamazepine was purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Carbamazepine 10,11-epoxide was purchased from Tronto Research Chemicals, Inc. (Ontario, Canada). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Roche Applied Science (Indianapolis, IN, U.S.A.). HLM was purchased from BD Gentest (Woburn, MA, U.S.A.) as previously described.[10] Data are expressed as the means±S.D. of three independent determinations. Statistical significances were determined by analysis of variance followed by Dunnett’s test. A value of \( p<0.05 \) was considered statistically significant.

RESULTS

Carbamazepine 10,11-Epoxidation in HLM  To obtain the optimal condition for carbamazepine 10,11-epoxide formation in HLM, the formation rate was determined in incubation conditions using final methanol or acetonitrile concentrations of 1 to 4%. While acetonitrile was found to strongly inhibit the enzymatic activity (Supplementary Fig. 1A), the inhibitory effect of methanol on the enzyme activity was less than that of acetonitrile (Supplementary Fig. 1B). Because a wide range of the substrate concentration is required for the kinetic analysis, we determined the kinetic parameters in the incubation condition at a final concentration of 1% methanol. In the present study, the kinetic curve was better fitted to the Hill equation with an Akaike’s information criteria (AIC) value of 60.44, revealing the \( S_0 \) value of 358±8 \( \mu \)M, \( n \) of 2.0±0.1, and the \( V_{\text{max}} \) value of 463±6 pmol/min/mg (Eq. 4), while the Michaelis– Menten analysis revealed a \( K_m \) value of 808±48 \( \mu \)M and a \( V_{\text{max}} \) value of 726±28 pmol/min/mg (Eq. 1) with an Akaike’s information criteria (AIC) value of 76.79 (Fig. 1A), which are in agreement with values from previous reports (Supplementary Table 1).[9,13–15] Eadie–Hofstee plots were not linear (Fig. 1B), confirming that carbamazepine 10,11-epoxide formation in HLM was sigmoidal.

Difference of Predicted Clearance of Carbamazepine between Typical and Atypical Kinetic Analyses  When the metabolism of drugs shows typical Michaelis–Menten kinetics, Eq. 3 is used to predict \( CL_{\text{int, in vivo}} \). While \( CL_{\text{int}} \) is constant when the substrate concentration is much smaller than \( K_m \), the clearance is substrate concentration-dependent at higher substrate concentrations, as shown in Fig. 1C (broken line). On the other hand, Eq. 5 is used to obtain the clearance of drugs whose metabolism is sigmoidal. The clearance is substrate concentration-dependent as shown in Fig. 1C (solid line), while the maximum clearance, \( CL_{\text{max}} \), can be obtained with Eq. 6. Since it has been reported that \( CL_{\text{max}} \) correlates well to the observed hepatic intrinsic clearance, \( CL_{\text{int, in vitro}} \), we calculated the \( CL_{\text{max}} \) value of carbamazepine 10,11-epoxidation in HLM. The \( CL_{\text{max}} \) value of carbamazepine 10,11-epoxidation was 0.65±0.01 \( \mu \)L/min/mg (0.58 mL/min/kg body weight) (Eq. 6), while \( CL_{\text{int}} \) calculated based on the Michaelis–Menten analysis was 0.90±0.02 \( \mu \)L/min/mg (0.81 mL/min/kg body weight). The mobile phase was 20 \( \mu \)M phosphate buffer (pH 7.4)–acetonitrile (6:4, v/v). Quantification of carbamazepine 10,11-epoxide was carried out by comparing the HPLC peak area to that of the authentic standard.
weight) (Eq. 3). To convert from $\mu$L/min/mg HLM to mL/min/kg body weight, the following parameters were used: normal human body weight of 70kg with a 1.4-kg liver; 45 mg HLM per gram liver. Reported in vivo hepatic clearance ($CL_{int}$) and $CL_{int,in\,vivo}$ of carbamazepine is 0.40 mL/min/kg body weight and 1.32 mL/min/kg body weight, respectively. Since approximately 40% of carbamazepine is metabolized to its 10,11-epoxide to be cleared from the body, partial $CL_{int,in\,vivo}$ of carbamazepine to its 10,11-epoxide was estimated to be 0.52 mL/min/kg body weight. It was shown that calculation of $CL_{int}$ based on the Michaelis–Menten analysis resulted in a 1.5-fold overestimation of $CL_{int,in\,vivo}$ (0.81 mL/min/kg body weight to 0.52 mL/min/kg body weight). In contrast, the $CL_{max}$ value obtained by the sigmoidal analysis correlated well to the $CL_{int,in\,vivo}$ (0.58 mL/min/kg body weight to 0.52 mL/min/kg body weight).

**Prediction of Clearance at Therapeutic Concentrations**

This and other studies have demonstrated that $CL_{max}$ can be used for the prediction of $CL_{int,in\,vivo}$ of drugs whose kinetics are sigmoidal. However, it should be noted that generally, therapeutic concentrations of drugs are much lower than their $S_{50}$ and $K_{m}$ values. Although the clearance calculated from each equation (Eqs. 2, 5) is similar at the substrate concentration around the $S_{50}$ or $K_{m}$ value, the rate could be significantly different at lower substrate concentrations as shown in Fig. 1C. Alprazolam, diazepam, flunitrazepam, and nordiazepam are also drugs whose metabolism is sigmoidal. While their $S_{50}$ values range from 208 $\mu$M to 607 $\mu$M, their therapeutic concentrations are much lower than those $S_{50}$ values (Table 1). Therefore, predicted clearance rates of those drugs at the therapeutic concentrations using equation 5 significantly underestimated the observed in vivo $CL_{int}$ (Table 1). The therapeutic concentration of carbamazepine, which is 10 $\mu$M, is also much lower than its $S_{50}$ value. As with the drugs described above, the predicted clearance of carbamazepine at therapeutic concentrations using Eq. 5 also significantly underestimated the observed in vivo $CL_{int}$ (Table 1). These results suggest the possibility of the existence of factor(s) causing the discrepancy between predicted and observed in vivo clearances of drugs, whose metabolism is sigmoidal, at therapeutic concentrations, while $CL_{max}$ can be used for the prediction of $CL_{int,in\,vivo}$.

**DISCUSSION**

The kinetic curve of carbamazepine 10,11-epoxide formation has been analyzed with the Hill equation and the Michaelis–Menten equation (Supplementary Table 1). The accumulated evidence suggests that the enzymatic property is different in the recombinant systems due to different protein modification, such as glycosylation. Sigmoidal kinetics were mainly observed in human liver microsomes, while Michaelis–Menten kinetics were mainly observed in the recombinant systems. This indicates that the sigmoidal kinetics of carbamazepine 10,11-epoxidation observed in HLM is more reliable than the Michaelis–Menten kinetics. The comparison of AIC
values also supported the usage of the Hill equation for the analysis of carbamazepine 10,11-epoxidation in human liver microsomes.

To predict the in vivo clearance of carbamazepine, its hepatic clearance needs to be estimated from the in vitro data. However, $C_{L\text{int}}$ calculated from the Michaelis–Menten kinetics overestimated the actual clearance, because the metabolic rate-carbamazepine concentration curve is sigmoidal and therefore the clearance is relatively smaller than that estimated from the Michaelis–Menten analysis (Fig. 1C). In accordance with previous studies, $C_{L\text{max}}$ was in excellent agreement with $C_{L\text{int, in vivo}}$ in our study. In spite of the fact that carbamazepine 10,11-epoxidation in HLM has been reported to be sigmoidal, $K_m$ and $V_{max}$ values, which are obtained from the Michaelis–Menten analysis, were still used in a study in 2006 to predict its in vivo clearance, which can result in an overestimation. Thus, it should be re-emphasized here that $C_{L\text{max}}$ needs to be obtained for the prediction of in vivo $C_{L\text{int}}$ of drugs whose metabolic kinetics are sigmoidal.

While $C_{L\text{max}}$ values correlate well to in vivo $C_{L\text{int}}$, the predicted clearance of drugs at the therapeutic concentrations using Eq. 5 was significantly lower than the observed in vivo $C_{L\text{int}}$ values (Table 1). This discrepancy between predicted and observed in vivo clearances of drugs might be caused by the following:

1. Presence of factor(s) that can activate the CYP3A4-catalyzed carbamazepine 10,11-epoxidation in vivo. CYPs have been known to possess an activator-binding site along with a substrate-binding site. Testosterone is one of the endogenous compounds that can activate CYP3A4-catalyzed metabolism of drugs. It was reported that 150 μM testosterone fully activated CYP3A4-catalyzed metabolism of diazepam, whose metabolic kinetics is sigmoidal, resulting in a dramatically increased maximum clearance. However, the effect of testosterone on CYP3A4 activity was moderate at the physiological concentration of testosterone in human plasma. It was also reported that other endogenous compounds such as androstenedione and dehydroepiandrosterone 3-sulfate can activate CYP3A4 activities. Taking these findings together, it can be speculated that endogenous compounds might be activating the sigmoidal metabolism of drugs in vivo, causing the discrepancy of clearance parameters between in vitro and in vivo.

2. Accumulation of carbamazepine in hepatocytes. While the predicted plasma concentration of free carbamazepine is 10 μM, there is a possibility that the drug further accumulates in hepatocytes, possibly by active drug transporters expressing in the plasma membrane, causing an even higher clearance in the hepatocytes. Although it was not in the liver, the presence of multiple transporters that can transport carbamazepine was reported. Indeed, a two-fold higher concentration of carbamazepine was observed in the liver of rats, which peritoneally received 2.5 mg/kg carbamazepine, compared to concentration in the blood.

It was also reported that liver concentrations of drugs, such as diazepam and alprazolam, greatly exceed total plasma concentrations.

We have simply extrapolated the in vitro data to in vivo $C_{L\text{int}}$ based on the assumption of 45 mg microsomal protein per g liver, in the current study. However, nowadays, it is widely accepted that such extrapolation of the absolute values for intrinsic clearance may have large bias, leading miscalculation of predicted values probably because of the quality of microsomes and other factors. An extrapolation using a scaling factor estimated from in vivo and in vitro data for reference compounds might be an alternatively way to predict in vivo clearance accurately. In addition, because approximately 40% of carbamazepine is metabolized to its 10,11-epoxide to be cleared from the body, we compared 40% of the total clearance of carbamazepine in vivo with the clearance of carbamazepine 10,11-epoxide formation in total carbamazepine metabolism between in vivo and in vitro. This discrepancy might partially contribute to the inaccurate prediction of in vivo clearance of carbamazepine at the therapeutic concentration.

In conclusion, while the intrinsic clearance calculated from Michaelis–Menten parameters ($C_{L\text{int}}$) overestimated the observed in vivo intrinsic clearance ($C_{L\text{int, in vivo}}$), the intrinsic clearance calculated based on the Hill equation ($C_{L\text{max}}$) exhibited better predictions of $C_{L\text{int, in vivo}}$. Predicted clearances of not only carbamazepine, but also of other drugs whose metabolic rate–concentration curves are sigmoidal, are significantly lower at their therapeutic concentrations than $C_{L\text{int, in vivo}}$, while intrinsic clearance at a therapeutic concentration should be close to that observed in humans in vivo. This suggests the existence of factors, such as endogenous CYP3A4 activators and drug transporters, causing the discrepancy between predicted and observed in vivo clearances at therapeutic concentrations. Even if we assume sigmoidal kinetics, that would not be enough for accurate prediction of $C_{L\text{int, in vivo}}$. Therefore, we should use $C_{L\text{max}}$ to quantitatively extrapolate the in vitro data to in vivo data instead of using intrinsic clearance at therapeutic concentration. Further studies are required to fully understand the mechanism causing discrepancies between predicted and observed in vivo clearances of drugs whose metabolism is sigmoidal.

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