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Effects of CYP3A5 and CYP2D6 genetic polymorphism on the pharmacokinetics of diltiazem and its metabolites in Chinese subjects

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Purpose: To assess the possibility of using CYP2D6*10 + CYP3A5*3 as biomarkers to predict the pharmacokinetics of diltiazem and its two metabolites among healthy Chinese subjects. *Methods*: 41 healthy Chinese were genotyped for CYP3A5*3 and CYP2D6*10, and then received a single oral dose of diltiazem hydrochloride capsules (300 mg). Multiple blood samples were collected over 48 h, and the plasma concentrations of diltiazem, N-desmethyl diltiazem and desacetyl diltiazem were determined by HPLC-MS/MS. The relationships between the genotypes and pharmacokinetics were investigated. *Results:* The pharmacokinetics of diltiazem, N-desmethyl diltiazem were not significantly affected by both CYP3A5*3 and CYP2D6*10 alleles. However, the systemic exposure of the pharmacologyically active metabolites, desacetyl diltiazem, was 2-fold higher in CYP2D6*10/*10 genotype carriers than in *1/*10 or *1/*1 ones ($AUC_{(0-inf)}$ of CYP2D6*1/*1, *1/*10 and *10/*10 are 398.2 ± 162.9, 371.0 ± 69.2 and 726.2 ± 468.1 respectively, $p < 0.05$). *Conclusions:* Two of the most frequent alleles, CYP3A5*3 and CYP2D6*10, among Chinese do not have major impacts on the disposition of diltiazem and N-desmethyl diltiazem. However, the desacetyl diltiazem showed 2-fold accumulation in individuals with CYP2D6*10/*10 genotype. Despite this, the effect of genotype of CYP2D6 on clinical outcome of diltiazem treatment is expected to be limited.

1. Introduction

Diltiazem (DTZ) is a benzothiazepine calcium channel blocker widely used in the treatment of hypertension and angina. It has three phase-I metabolic pathways: desacetylation, N-demethylation and O-demethlation (Molden, et al. 2002), mediated by esterases, CYP3A (Jones et al. 1999) and CYP2D6 separately. The pharmacological active ingredients include DTZ and three major metabolites: N-desmethyldiltiazem (MA), desacetyldiltiazem (M1) and N-demthyldesacetyl diltiazem (M2) in the aspects of vasodilatation and unti-thrombocyte aggregation, whereas the O-demethylated derivative of DTZ is almost without any vasodilating activity.

N-Demethylation of DTZ by CYP3A is quantitatively the most important metabolic route. CYP3A4 and CYP3A5 are believed to be the major isoforms responsible for CYP3A activity *in vivo* regarding to the expression level and tissue specificity of CYP3A members. CYP3A4, though predominant (Wojnowski and Kamdem 2006), is well-conserved with rare deleterious alleles, so that significant functional consequence can be avoided. CYP3A5 is highly polymorphic and is estimated to comprise on average only 7%∼8% of the total P450 hepatic expression (Wrighton, et al. 1989). CYP3A5*3 is the most common defective allele with a frequency of 90%, 75% and 20% in Caucasians, Asians and African respectively (Lee et al. 2003). Unfortunately, there is no successful marker predictive for CYP3A (Wojnowski and Kamdem 2006) due to the conservation of CYP3A4 and the low expression level of CYP3A5.

Another crucial enzyme involved in the metabolism of DTZ is CYP2D6. The variability in CYP2D6 activity is mainly genetically determined. Based on CYP2D6 alleles, individuals were mainly divided into four groups: poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM), and ultra-rapid metabolizer (UM) (Jose de Leon et al. 2005). The significant interethnic differences in the prevalence of the phenotype result from the difference of allele frequencies. The lower frequency of PM in Asian (< 1% (Qin et al. 2008), but 5-10% in Caucasians (van der Weide and Steijns 1999)) is due to the rarity of loss-of-function alleles *3, *4 and *5 (Qin et al. 2008). Whereas the high frequency of IM in Asia comes from the prevalence of the CYP2D6*10 allele (50%, but 1.5% in Caucasians (van der Weide and Steijns 1999)). CYP2D6*10 (GenBank ID: RS1065852) is a single nucleotide alteration in exon 1(100C > T) causing a Pro34→Ser amino acid substitution, and resulting a unstable enzyme with lower activity.

A recent clinical study revealed that pharmacokinetics of DTZ were not affected by the CYP3A5*3 allele (n = 15) (Yamamoto et al. 2005), although CYP3A5 shows a higher activity level than CYP3A4's in DTZ N-demethylation *in vitro* (Yamaori et al. 2004a). However, the effect of the CYP3A5*3 allele on the clearance of M1 and MA is unclear. Based on the DTZ metabolic pathway networks, we postulated that, if CYP3A5*3 works in

the metabolism of DTZ, MA's distribution might be more sensitive than DTZ's, since MA is quantitatively predominant among DTZ's metabolites (Molden et al. 2002), and CYP3A is the most important metabolic enzyme to create it.

Another clinical study (Molden et al. 2002) suggested that M1 and M2 are extensively accumulated among CYP2D6 PM ($AUC_{(0\text{-inf})} \geq 5$ times in PM than EM). Greater reductions in blood pressure in PM were also observed. However, this study did not include the IM group which is rare in Caucasian but prevalent in Asian people.

In view of the importance of CYP3A and CYP2D6 in the metabolism of DTZ, and the prevalence of their activity related alleles among Chinese population, we expect more findings in the pharmacogenetics and therapeutic response when both CYP3A5*3 and CYP2D6*10 are considered. We carried out this study to 1) assess the influence of CYP3A5*3 allele on pharmacokinetics of DTZ, MA and M1; 2) investigate the difference in DTZ metabolism between IM group (CYP2D6*10/*10) and EM group (*1/*10 or *1/*1); 3) evaluate whether the two alleles could serve as predictive biomarkers of drug plasma levels and therapeutic efficacy in the DTZ treatment.

2. Investigations and results

There was no clinically important adverse event observed in this study.

There was no significant difference in height, weight, BMI, age and female/male ratio among the groups investigated in this study (data not shown).

The pharmacokinetic parameters of DTZ, MA and M1 in each genotype group are summarized in the Table. Figure 1 shows the plasma concentration-time profiles for DTZ, MA and M1 in each subgroup.

The pharmacokinetic parameters of DTZ, MA and M1 showed minor differences between the subgroup of CYP3A5*1/*3 and ~*1/*1 (data not shown), and between CYP2D6*1/*1 and ~*1/*10. And none of these were close to being statistically significant. Meanwhile, there is no significant difference between the subgroups of CYP3A5 in terms of the five investigated parameters of MA and M1 (except $AUC_{(0\text{-}48h)}$ in MA). There is also no significant difference between the three subgroups of CYP2D6 as to parameters of DTZ and MA.

However compared with CYP3A5 *1/*1 + *1/*3 group, $AUC_{(0\text{-}48h)}$, $AUC_{(0\text{-inf})}$ and $t_{1/2}$ of DTZ are lower in amount for approximately 26%, 28% and 18% respectively in the CYP3A5*3/*3 subgroup ($P < 0.05$) (Fig. 1-A, Table). And among CYP2D6 subgroups, the $AUC_{(0\text{-}48)}$, $AUC_{(0\text{-inf})}$ and T_{max} of the M1 were approximately 1.7 times, 1.9 times higher, and 1.6 times longer respectively in IM versus EM ($P < 0.05$) (Fig. 1-F, Table).

3. Discussion

To our knowledge, this is the first study to demonstrate the association of the CYP2D6*10 allele with pharmacokinetics of DTZ and its metabolites.

In this study, we distinguished CYP3A5*3 and CYP2D6*10 alleles in Chinese. These two alleles were abundant among Chinese and involved in the metabolism of DTZ. According to tests, the allelic frequencies of CYP3A5*3, and CYP2D6*10 were 74.48% and 51.42% respectively. This result was consistent with previous studies (Qin et al. 2008; Shih and Huang 2002).

There were no significant differences in the five investigated pharmacokinetic parameters between the CYP3A5*1/*3 and ~*1/*1 in this study. This result is in agreement with a previous *in vitro* study (Yamaori et al. 2004a) which demonstrated a similar CYP3A5 expression level (46 ± 26 and 49 ± 25 pmol/mg) in liver microsome of CYP3A5 *1/*1 and *1/*3 genotype compared with low expression (5 pmol/mg) among *3/*3 ones. Thus, in this study, the pharmacokinetic measurements of these

Table: Pharmacokinetic parameters of DTZ, MA and M1 in each genotype group

	n	C_{max} (ng/mL)	T_{max} (h)	$AUC_{(0\text{-}48)}$(ng·h/mL)	$AUC_{(0\text{-inf})}$(ng·h/mL)	$t_{1/2}$ (h)
DTZ						
CYP3A5 *1/*1 + *1/*3	19	155.1 ± 60.4	13.7 ± 7.8	3403.9 ± 1568.8	3621.8 ± 1698.1	8.35 ± 2.22
CYP3A5 *3/*3	22	118.4 ± 35.4	14.7 ± 8.5	2523.0 ± 710.6[*a]	2620.3 ± 748.9[*b]	6.88 ± 1.02[*c]
MA						
CYP3A5 *1/*1 + *1/*3	19	47.6 ± 10.1	16.5 ± 7.2	1298.9 ± 304.5	1985.9 ± 1760.5	12.5 ± 4.0
CYP3A5 *3/*3	22	42.8 ± 9.0	12.3 ± 7.2	1102.5 ± 218.6[*d]	1499.2 ± 851.4	10.6 ± 2.1
M1						
CYP3A5 *1/*1 + *1/*3	19	15.8 ± 8.4	23.8 ± 0.9	414.7 ± 238.5	538.7 ± 341.9	8.38 ± 4.78
CYP3A5 *3/*3	22	14.9 ± 6.6	24.2 ± 3.1	373.8 ± 187.5	478.7 ± 327.5	9.36 ± 3.84
DTZ						
CYP2D6 *1/*1	13	147.0 ± 60.2	13.9 ± 8.4	3112.4 ± 1434.0	3280.6 ± 1537.6	7.71 ± 2.45
CYP2D6 *1/*10	14	127.3 ± 36.1	14.9 ± 8.2	2692.2 ± 584.8	2795.5 ± 592.7	7.00 ± 1.16
CYP2D6 *10/*10	14	132.7 ± 57.3	13.9 ± 8.4	3002.2 ± 1578.8	3191.1 ± 1735.3	7.98 ± 1.67
MA						
CYP2D6 *1/*1	13	47.7 ± 9.7	13.5 ± 7.3	1211.5 ± 325.3	1699.1 ± 997.0	11.53 ± 3.12
CYP2D6 *1/*10	14	42.6 ± 8.3	13.9 ± 7.4	1139.5 ± 167.2	1634.5 ± 1124.9	10.61 ± 2.21
CYP2D6 *10/*10	14	45.1 ± 11.0	15.3 ± 8.0	1230.8 ± 324.5	1827.5 ± 1862.4	12.33 ± 4.11
M1						
CYP2D6 *1/*1	13	13.1 ± 5.3	24.0 ± 0.0	326.8 ± 127.3	398.2 ± 162.9	9.95 ± 4.45
CYP2D6 *1/*10	14	13.1 ± 3.0	23.1 ± 2.3	315.5 ± 61.7	371.0 ± 69.2	7.99 ± 3.81
CYP2D6 *10/*10	14	19.9 ± 10.0[*e]	24.9 ± 3.2	531.2 ± 294.4[*f]	726.2 ± 468.1[*g]	8.97 ± 4.60

Data are shown as mean ± SD. *$P < 0.05$

[a] *3/*3 vs *1/*1 + *1/*2: pa = 0.039. if excluding two subjects whose individual AUC(0-inf) exceed mean ± 2SD, p = 0.110.

[b] *3/*3 vs *1/*1 + *1/*2: pa = 0.028. if excluding two subjects whose individual AUC(0-inf) exceed mean ± 2SD, p = 0.081.

[c] *3/*3 vs *1/*1 + *1/*2: pa = 0.009. if excluding two subjects whose individual AUC(0-inf) exceed mean ± 2SD, p = 0.023.

[d] *3/*3 vs *1/*1 + *1/*2: pa = 0.030. if excluding two subjects whose individual AUC(0-inf) exceed mean ± 2SD, p = 0.652.

[e] *1/*1 vs *10/*10 = 0.048; f: p = 0.040 by Krus Kal-Wallis test. *1/*1 vs *10/*10: p = 0.029; *1/*10: *10/*10:p = 0.031

[g] p = 0.030 by Krus Kal-Wallis test. *1/*1 vs *10/*10: p = 0.029; *1/*10: *10/*10:p = 0.019

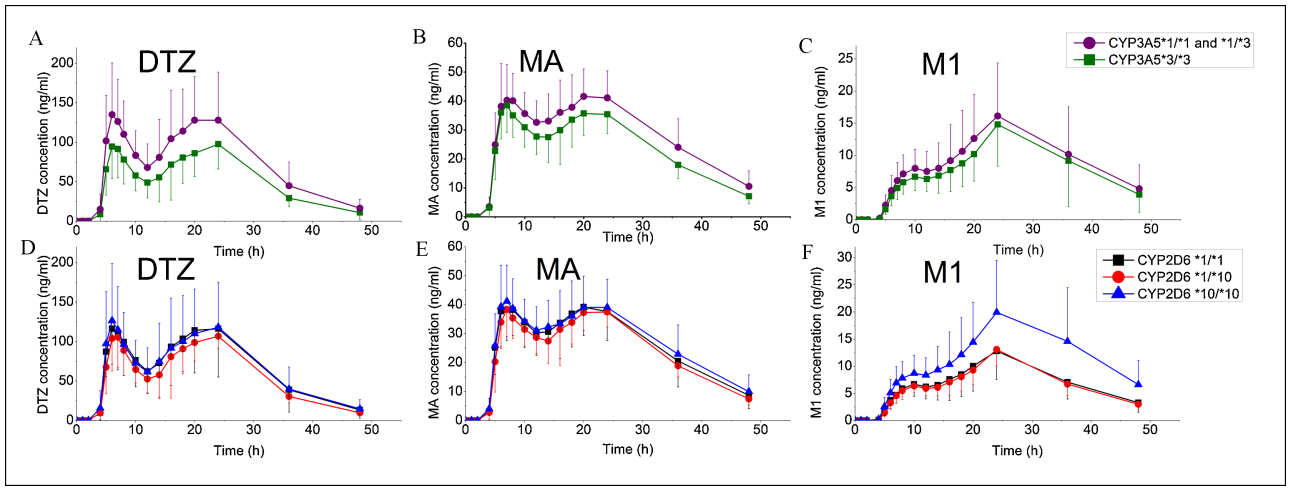

Fig. 1: Plasma concentration-time curves following oral administration of 300 mg diltiazem hydrochloride capsules. Values are given as mean ±SD.

two subgroups were combined and compared with the ~*3/*3 group.

Although the metabolic capabilities of CYP3A4 and CYP3A5 were reported to be comparable in the DTZ N-demethylation (intrinsic clearance ratio of CYP3A4/CYP3A5 from 0.4 to 3.45) (Jones et al. 1999; Williams et al. 2002; Yamaori et al. 2004b), it was not expected that CYP3A5 genotype alone could be a significant factor to affect the pharmacokinetics of DTZ, considering the limited expression level of CYP3A5 and other pathways in the DTZ's metabolism (Williams et al. 2003). However, in this study, individuals who carried at least one wild type allele (*1) show 28.0% higher AUC $_{(0\text{-inf})}$ of DTZ as compared with homozygous CYP3A5*3($P = 0.039 < 0.05$). This is inconsistent with a previous *in vivo* study (Yamamoto et al. 2005) ($n = 15$, dose = 60 mg). Such a discrepancy might be explained by a higher level of CYP3A5 activity toward DTZ N-desmethylation (Yamaori et al. 2004a), higher dosage or larger sample size. Moreover, it is reported that CYP3A5 and CYP3A4 are the main metabolic enzymes of DTZ N-demethylation (Jones et al. 1999; Yamaori et al. 2004a). But the pharmacokinetics of MA did not show more significant differences in different CYP3A5 subgroups compared with its parent drug as we postulated. This makes the result suspicious.

Thus, a repeated comparison was conducted after excluding two subjects whose individual $AUC_{(0\text{-}48)}$ exceeded mean ± 2SD (Fig. 2). Statistically non-significant differences were observed

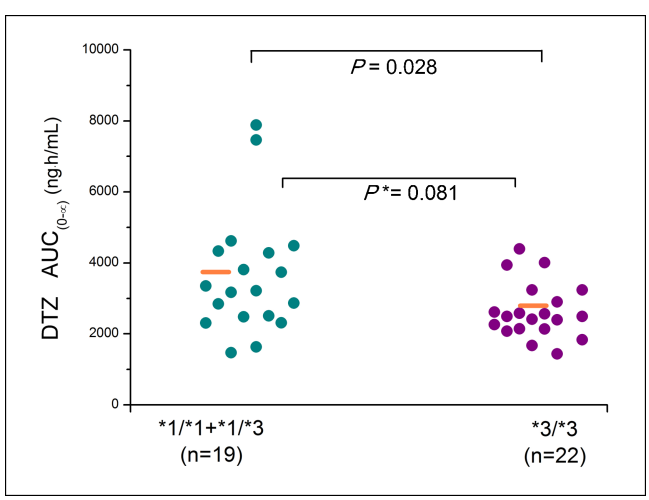

Fig. 2: ($AUC_{(0-inf)}$ of DTZ illustrated according to the CYP3A5 genotype. Thick bars indicate the mean values of individual group. P indicates the P value of Wilcoxon rank. P* indicates the P value after excluding two subjects whose individual ($AUC_{(0-inf)}$exceed mean±2SD.

in the $AUC_{(0\text{-}48)}$ ($P = 0.110$) and $AUC_{(0\text{-inf})}$ ($P = 0.081$). Various factors probably contribute to these two 'outliers': genetic factors, undetected induction, health status or environmental stimuli (Wilkinson 1996). Thus, it tends to cautiously refuse that CYP3A5*3 mutation has impact on the pharmacokinetics of DTZ as well as the 5.6-fold interindividual systemic exposure variability showed in this study.

In this study, we did not determine other mutants of CYP2D6 besides the *10 allele, due to their relatively low prevalence in Chinese populations or the uncertain function *in vivo* (Qin et al. 2008). This study showed an almost two-fold systemic exposure of M1 in the IM compared with the EM group, suggesting the disposition of M1 might be genetically determined. This result also confirms Molden's speculation (Molden et al. 2002) that IM expressers might show intermediate systemic exposure of M1 between EM and PM.

One aim of this study was to assess if the two allele markers could serve as a "switch" in DTZ's pathway networks (Molden et al. 2002). If the 'drain switch' turns down, the metabolite accumulates. If two 'switches' turn down, the accumulation effect enhanced. There are two eliminative routes for M1 with CYP2D6 and CYP3A involved separately. This study reveals that, CYP2D6*10 is responsible for the 2-fold accumulation of M1, whereas the CYP3A5*3 effect was undetectable either in IM or EM (data not shown). This result suggests that O-demethylation mediated by CYP2D6 might be the primary elimination route for M1. Similarly, CYP2D6*10 did not effect MA's pharmacokinetic profile indicating its primary elimination route might not be O-demethylation, but desacetylation mediated by esterases. If so, it could be postulated that, individuals with increased CYP3A activity and decreased CYP2D6 activity could result in the accumulation of M2, another DTZ vasodilating metabolite. However, this needs to be outlined in further studies with effective markers to evaluate the CYP3A levels *in vivo* (Luo, et al. 2009).

The pharmacodynamic outcomes were evaluated since an approximate two-fold higher systemic exposure of vasodilating active M1 was observed. The value of 'baseline minus average' and 'baseline minus minimums' of the diastolic blood pressure, systolic blood pressure and heart rate during drug expose (1 h, 3 h, 6 h, 8 h and 12 h) was compared according to Molden et al. (2002). Unlike Molden's study which revealed a significant reduction in heart rate associated with approximate 5-fold higher M1 $AUC_{(0\text{-inf})}$ in PM versus EM, there was no significant difference observed between the CYP2D6*10/*10 and *1/*10 + *1/*1 group in the present study (data not shown). Thus, it could be predicted that the effect of the CYP2D6 genotype on the clinical DTZ treatment is limited. However, this

finding requires a further study with larger sample size and a different dosing regimen, especially considering that the M1 and MA level reach a higher level in multiple dosing treatment (Höglund and Nilsson 1989).

This study suggests that, CYP2D6*10 + CYP3A4*5 are not promising predictive markers for DTZ therapy, despite CYP2D6 and CYP3A are the most important metabolic enzymes for this drug. There is also no significant evidence to show any interaction between CYP2D6*10 and CYP3A5*3 in their influence to the pharmacokinetics of DTZ, MA and M1 (data not shown). This result is probably due to the inefficiency of CYP3A5*5 as a CYP3A indicator.

In conclusion, this study suggests that CYP3A5 genotype does not play an important role in DTZ metabolism, while the CYP2D6 genotypes have impact on pharmacokinetics of M1 among healthy Chinese Han ethnic subjects. Nevertheless the clinical outcome of this impact is unclear and remains to be further examined.

4. Experimental

4.1. Subjects

This study has been approved by the Ethical committee of Sino-American Health Management Association. And written informed consent has been obtained from each of the subjects before participation.

44 healthy subjects participated and 41 (male:female = 19:22, age = 23.6 ± 2.5 years; height = 166.5 ± 9.7 cm, weight = 60.3 ± 10.1 kg, BMI = 21.9 ± 2.2 kg/cm^2) completed this study. No subject took concomitant medication within the 14 days before or during the study. Following an overnight fasting for at least 10 h, the 41 healthy Chinese Han ethnic subjects consumed a standard high-fat breakfast and then received single dose of diltiazem hydrochloride capsules (300 mg, Cardizem® CD, Sanofi-aventis U.S.LLC). Venous blood samples were collected at time 0 (40 min pre-dose) and 1.0, 2.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 13.0, 14.0, 14.0, 15.0, 16.0, 18.0, 20.0, 24.0, 36.0, 48.0 h post dose. After centrifugation, the plasma obtained was stored at approximately –70 °C for further DTZ/MA/M1 plasma concentrations determination, and blood cells were obtained for DNA extraction.

4.2. Genotyping procedure for CYP3A5 and CYP2D6

Genotyping for CYP3A5*3 allele with an A22893→G point mutation in the intron 3 of the CYP3A5 gene was done by PCR-RFLP described previously (Shih and Huang 2002). Primers pri-IN3(S) (5'-CTT CAA TTT TTC ACT GAC CTA ATA TTC-3') and IN3 (R) (5'-ACG TCC TGT TTT CTC GAG AAA TTT C-3') was used to obtain a 310-bp PCR product. And a mismatched primer IN3(S) (5'- CAC AGC ATG TTG ATC CCC ATA CCT A-3') to create the Pst I restriction site was designed. Using IN3(S) and IN3 (R), a nested PCR product of 166-bp was obtained and the resulting PCR product could be digested when there is g_{22893} mutation.

DNA fragment containing the CYP2D6*10 mutation region was amplified by PCR as described previously (Xu et al. 2008). Primers (forward: 5'-CCA TTT GGT AGT GAG GCA GGT AT-3'; reverse: 5'-CAC CAT CCA TGT TTG CTT CTG GT-3') was used to get a 272-bp fragment. This DNA fragment was subjected to direct sequencing using DNA sequencer ABI 3730xl (Applied Biosystems, ABI, CA, USA) to determine the CYP2D6*10 genotype.

4.3. Determination of DTZ, MA and M1 concentration in plasma

Plasma concentrations of DTZ, MA and M1 were determined by a validated LC-MS/MS method developed in Frontage Laboratories (Shanghai, China). The LC-MS/MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and API4000 (Applied Biosystem, Milford, USA). The calibration ranges were: 2∼500 ng/mL for DTZ (LLOQ: 2 ng/mL, CV% = 1.6 and %Nominal = 100, correlation coefficient: $r^2 \geq 0.9956$); 0.5∼125 ng/mL for MA (LLOQ: 0.5 ng/mL, CV% = 3.6 and %Nominal = 99.6, correlation coefficient: $r^2 \geq 0.9932$); 0.5∼125 ng/mL for M1 (LLOQ: 0.5ng/mL, CV% = 3.4 and %Nominal = 100, correlation coefficient: $r^2 \geq 0.9949$) respectively, with diltiazem-d4, desmethyl-d4, desacetyl diltiazem-d4 (TLC PharmaChem) as the internal standards respectively.

4.4. Pharmacokinetic analysis

The plasma concentration-time curve for time 0 to the last tested point ($AUC_{0\text{-}48h}$) was calculated using the trapezoidal rule. The AUC from time 0 to infinity ($AUC_{0\text{-}inf}$) was calculated as $AUC_{0\text{-}48h} + C_{48h}/\lambda_z$. The terminal elimination rate constant (λ_z) was estimated by linear regression of the terminal portion of the concentration-time curve. The elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$. Peak plasma concentrations (C_{max}) and time to C_{max} (T_{max}) were estimated directly from the observed data.

4.5. Statistical analysis

All data from the study were expressed as mean ± S.D. Data were analyzed with the statistical program SPSS software (version 16.0; SPSS Inc., Chicago, IL). The Wilcoxon rank sum test or Kruskal-Wallis test were used to compare the non-normally distributed data between the different genotype groups. The two-factor ANOVA was applied to detect the interaction effect of alleles. A P-value < 0.05 was considered statistically significant.

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