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Correlation of tramadol pharmacokinetics and *CYP2D6*10* genotype in Malaysian subjects

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Abstract

The aim of the present study is to investigate the influence of the *CYP2D6*10* allele on the disposition of tramadol hydrochloride in Malaysian subjects. A single dose of 100 mg tramadol was given intravenously to 30 healthy orthopaedic patients undergoing various elective surgeries. After having obtained written informed consents, patients were genotyped for *CYP2D6*10*: the most common *CYP2D6* allele among Asians by means of allele-specific polymerase chain reaction. The presence of other mutations (*CYP2D6*1*, **3*, **4*, **5*, **9* and **17*) was also investigated. Tramadol was extracted from 1 ml serum with an *n*-hexane: ethylacetate combination (4:1) after alkalinisation with ammonia (pH 10.6). Serum concentrations were measured by means of high-performance liquid chromatography. The pharmacokinetics of tramadol was studied during the 24 h after the dose. As among other Asians, the allele frequency for *CYP2D6*10* among Malaysians was high (0.43). Subjects who were homozygous for *CYP2D6*10* had significantly ($P = 0.046$) longer mean serum half-life of tramadol than subjects of the normal or the heterozygous group (Kruskal–Wallis test). When patients were screened for the presence of other alleles, the pharmacokinetic parameter values were better explained. CYP2D6 activity may play a main role in determining tramadol pharmacokinetics. The *CYP2D6*10* allele particularly was associated with higher serum levels of tramadol compared with the *CYP2D6*1* allele. However, genotyping for *CYP2D6*10* alone is not sufficient to explain tramadol disposition.

Keywords: *CYP2D6*; Polymerase chain reaction; High-performance liquid chromatography; Tramadol hydrochloride; Pharmacokinetics

1. Introduction

The cytochrome P450 enzyme (CYP2D6 or debrisoquine hydroxylase) is involved in the oxida-

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tive metabolism of numerous drugs. Its clinical importance is emphasised by the existence of genetic polymorphism. Since the discovery of the debrisoquine/sparteine oxidation polymorphism in the 1970s, numerous studies have investigated its status in various ethnic groups. Approximately 7–10% of Caucasians and 1% of Asians are homozygous for mutant *CYP2D6* alleles, resulting in no functional protein being formed [1]. Despite the low frequency of poor metabolisers (PMs) of CYP2D6 substrates in the Asian populations, they have been found to carry a high frequency (51%) of the C_{188} to T_{188} mutation in exon 1, which causes a $Pro_{34} \rightarrow Ser$ amino acid substitution leading to a form of an unstable enzyme with lower metabolic activity [2]. This mutation, which is a defining mutation for *CYP2D6*10* allele is relatively rare in Caucasian populations [3]. Therefore, in the Asian populations, *CYP2D6*10* allele seems to be more clinically important than other CYP2D6 mutant alleles such as *CYP2D6*3*, **4* or **5* which cause absence of enzyme activity.

Even within the extensive metaboliser (EM) group, CYP2D6 activity is known to differ widely. For example, the CYP2D6 activity represented by the metabolic ratio value of debrisoquine has been reported to show more than a 70-fold variation within the EMs of the Korean population [4]. A few reports have also suggested that the number of *CYP2D6*10* alleles may be a useful predictor for the pharmacokinetics of several CYP2D6 substrates including metoprolol [5], nortriptyline [6] and haloperidol [7].

In most studies that have attempted to correlate *CYP2D6* genotype with the pharmacokinetics of a specific probe drug, subjects have been recruited on the basis of their *CYP2D6*10* genotype without regard to the whole range of CYP2D6 activity. In this study, the pharmacokinetics of tramadol hydrochloride (another CYP2D6 substrate) was compared (1) when patients were grouped based on their *CYP2D6*10* genotype alone and (2) when other genotypes were taken into consideration, to see any real influence of the CYP2D6 genotypes on tramadol pharmacokinetics.

2. Materials and methods

2.1. Selection of subjects and sampling

All data was collected prospectively. Patients included were those going for elective surgeries such as removal of an orthopaedic implant and open reduction internal fixation of fracture. Those with head injuries or those who could not understand instructions regarding the study protocol were excluded. Patients were blinded as to both the study drug and dose. They were allowed to withdraw from the study at any time. The study was approved by the local university's Research and Ethics Committee.

A written informed consent was obtained from each patient after a full explanation of the study. All patients were healthy as assessed by physical examination and routine laboratory tests of peripheral blood, kidney and liver function. Thirty (23 males and seven females) unrelated healthy patients were selected based on the study's inclusion and exclusion criteria. Their ages ranged between 13 and 51 (mean $\pm$ S.D. = 24.1 ± 8.9 years) with mean body weights of 56.2 ± 12.9 kg. Twenty-eight were Malays while two were of Chinese origin.

In the operation theatre, an independent anaesthetist placed an intravenous line for subsequent blood sampling for the determination of tramadol pharmacokinetics. Patients' arms were swabbed before the veins were punctured with standardised canullas (sized 16 G) and kept patent by heparin lock (2 units/ml). The venipuncture sites were standardised to the *antecubital fossa* only, contralateral to the limb bearing the line for drug or infusion, in order to avoid the dilution effect of any infusions given concurrently. All patients were placed on a standardised general anaesthesia regimens by the anaesthetist.

Two milliliters of blood was collected from each patient into EDTA tubes (LD®, Italy) for genotyping. Each sample was shaken gently for adequate mixing with the anticoagulant before being immediately transported on ice to the laboratory. The samples were stored at -20 °C until DNA extraction.

2.2. Genotyping

Genomic DNA was isolated from peripheral lymphocytes of each subject. Five milliliters of thawed blood contained in EDTA tubes was diluted with cold lysis buffer (0.64 M sucrose, 0.02 M tris-HCl, 2% Triton-X 100) and centrifuged at 3500 rpm for 15 min. The pellet obtained was rinsed with Tris–EDTA (TE), centrifuged and resuspended in saline–EDTA to which a 20% sodium dodecyl sulphate solution was added. RNAse-A was added to the samples which were incubated at 37 °C for 1 h followed by the addition of Proteinase-K and an overnight incubation at 37 °C. The DNA was precipitated with 2 M KCl followed by cold absolute ethanol. The precipitate was then spooled up and washed with cold 70% ethanol. It was left to air dry on the bench for 15 min before reconstitution in TE buffer and storage at −20 °C until polymerase chain reaction (PCR) analysis.

PCR was performed to detect the C188/T mutation using two primer sets 9/10 and 9/10B according to the method by Johansson et al. [8] with some slight modifications. Patients were also genotyped for other alleles to check whether they have additional mutant alleles that might have caused absence or reduction of CYP2D6 activity. *CYP2D6*3* and **4* alleles were determined according to the modified method by Heim and Meyer [9]. The determination of the *CYP2D6*5*, *CYP2D6*9* and *CYP2D6*17* alleles were based on the methods described by Steen et al. [10], Tyndale et al. [11] and Masimirembwa et al. [12] respectively with some slight modifications.

All PCR reactions were performed on a Perkin-Elmer GeneAmp PCR System 2400®. Briefly, in the first PCR of *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*9*, 1.0 U of Biotool Taq DNA polymerase was used to amplify fragment A and 0.5 U to amplify fragment B. The reactions utilised 0.25 μmol/l of each primer and 200 ng of DNA. Hot start was then performed by subjecting the DNA to 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min 30 s, 72 °C for 1 min 30 s and final extension at 72 °C for 10 min. The first PCR product was analysed on a 1.2% agarose gel. The appropriately diluted product was then used as templates in three parallel allele-specific reactions. Fragment A served as templates for the second PCR of *CYP2D6*3* and **9* and fragment B for *CYP2D6*4*. The second PCR reaction was carried out using 0.25 U of Biotool Taq DNA polymerase and 0.25 μmol/l of each primer. The second PCR comprised of only 15 cycles of 45 s at 94 °C, 45 s at 50 °C and 45 s at 72 °C.

In the amplification of the first fragment for *CYP2D6*10* and **17* spanning exon 1 and 2, the PCR was performed using 200 ng genomic DNA, 0.25 μmol/l of each primer and 1 U of rTth DNA polymerase (Gene Amp XL, Perkin-Elmer). Hot start was performed at 93 °C for 2 min followed by 35 cycles at 93 °C for 45 s, 66 °C for 45 s and 72 °C for 3 min and final extension at 72 °C for 10 min. The second PCR used 1.5 μl of diluted first PCR product as the template. It consisted of 15 cycles of 1 min at 94 °C and 1 min at 54 °C and 2.5 min at 72 °C.

For the determination of *CYP2D6*5*, 1.0 U rTth DNA polymerase (Gene Amp XL, Perkin-Elmer), 0.35 μmol/l of each primer and 50 ± 100 ng of DNA were used in a two-step PCR that consisted of 12 s of denaturation at 94 °C and 5 min of annealing at 68 °C for 35 cycles followed by a final extension at 72 °C for 10 min.

In the initial phase, patients were grouped into three main classes based merely on the genotyping of the *CYP2D6*10* allele: Group 1 (*CYP2D6*1/*1*), Group 2 (*CYP2D6*1/*10*) and Group 3 (*CYP2D6*10/*10*). Later, when the same patients were genotyped for other *CYP2D6* alleles, they were regrouped into five main classes which exhibit slower and slower enzyme activity from Group A to Group E: Group A (*CYP2D6*1/*1*), Group B (*CYP2D6*1/*9* and *CYP2D6*1/*10*), Group C (*CYP2D6*1/*4* and *CYP2D6*1/*5*), Group D (*CYP2D6*10/*10* and *CYP2D6*10/*17*) and Group E (*CYP2D6*4/*10*, *CYP2D6*5/*10*).

The pharmacokinetic profile was compared between the different groups and also when grouped differently followed by the determination of allele frequency [13].

2.3. Pharmacokinetic analysis method

One hundred milligrams of tramadol hydrochloride (Boehringer Mannheim, Grunenthal, Germany) was diluted with 10 ml of normal saline and was administered by intravenously over 2–3 min to all patients. It was given as the first analgesic in the recovery room (regardless of whether patients had pain or not) by the investigator to ensure that all patients received the drug. Patients complaining of nausea or vomiting received 10 mg intramuscular (IM) metoclopramide. In case of breakthrough pain, patients were also allowed standardised rescue analgesia of IM pethidine 100 mg at any time.

Three milliliters of patients' blood was sampled at various time intervals: 0, 15, 30 min, 1, 2, 4, 8, 16, 20 and 24 h. The first 1 ml of diluted blood was discarded. The samples were withdrawn into plain tubes and immediately transported to the laboratory. They were spin at $3500 \times g$ for 10 min to give about 1 ml of serum. The product was stored for a maximum duration of three months at -20 °C until HPLC analysis.

The HPLC system consisted of a 307 Gilson pump coupled to a 115 Gilson Variable UV detector set at 218 nm. The analytical column used was an RP-18 Lichrosorb® (California) (5 μm: 250 × 4.6 mm^2 I.D.) coupled to a guard column (Bellefonte) (5 μm: 4×4 mm^2 I.D.). The mobile phase was a mixture of 70% phosphate buffer (0.01 M) and 30% acetonitrile with the addition of 0.1% triethylamine (v/v). The pH of the final mixture was adjusted to 3.5.

Liquid–liquid extraction was carried out using an *n*-hexane: ethylacetate combination (4:1) mixture after alkalinisation with ammonia (pH 10.6). Mean recovery was $94.36 \pm 12.53\%$. The developed method was selective and linear for concentrations ranging from 50 to 3500 ng/ml. The limit of quantitation (LOQ) was 50 ng/ml. Intraday accuracy ranged from 95.48 to 114.64% and the interday accuracy, 97.21–103.24%. Good intraday and interday precision (coefficient of variation $< 20\%$) was obtained at LOQ.

PCNONLIN® (software for the statistical analysis of nonlinear models, version 4.0) was used to determine individual and population pharmacokinetics of tramadol.

2.4. Statistics

Data was expressed as mean value $\pm$ S.D. The pharmacokinetic parameter values among groups of different genotypes were compared with Kruskal-Wallis test. Chi-squared was used to determine the relationship between the groups and sex, race, smoking status and fracture types. When more than 20% of the cells in the chi-squared table had expected frequency less than five, Fisher's exact test was used.

A P value of less than 0.05 was considered to be statistically significant.

3. Results

There was no significant difference in terms of age, sex, race, body weight, smoking status or fracture types among the groups. No patients were PM. Table 1 show the pharmacokinetic parameter values for the different groups.

Subjects having slower CYP2D6 activity (Group 3) were found to have a significantly longer half-life ($t_{\frac{1}{2}}$) compared to the subjects in other groups. When the mean serum concentration–time profiles of tramadol were plotted, there were differences between the three groups (Fig. 1). The area under the curve from zero to infinity

Table 1
Mean pharmacokinetic parameter values of different groups when patients were grouped based on *CYP2D6*10* allele alone

Group	$t_{\frac{1}{2}}$ (h)	CL (ml/min)	$AUC_{0-\infty}$ (ng/ml h)
Group 1 ($n = 8$)	7.2 ± 1.7	19.3 ± 6.2	5078.4 $\pm$ 1961.1
Group 2 ($n = 13$)	10.0 ± 10.0	17.8 ± 7.7	5906.2 $\pm$ 2759.7
Group 3 ($n = 9$)	12.1 ± 8.8	13.2 ± 4.4	7106.4 $\pm$ 2844.9
Kruskal-Wallis test	$P = 0.046$	$P = 0.147$ (NS)	$P = 0.252$ (NS)

$t_{\frac{1}{2}}$, half-life; CL, clearance values; $AUC_{0-\infty}$, area under the curve from zero to infinity; Group 1, *CYP2D6*1/*1*; Group 2, *CYP2D6*1/*10*; Group 3, *CYP2D6*10/*10*.

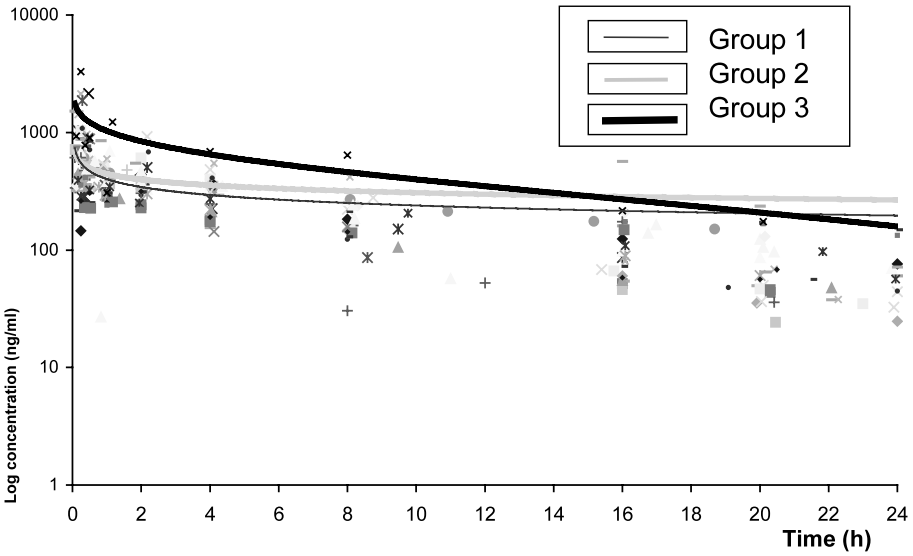

Fig. 1. The mean serum concentration–time profile of tramadol for Group 1 (*CYP2D6*1/*1*), Group 2 (*CYP2D6*1/*10*) and Group 3 (*CYP2D6*10/*10*).

($AUC_{0-\infty}$) was found to be higher and clearance values (CL) were found to be lower in this group. However, these results were not significant perhaps due to the small sample size.

Within each of the three groups there was a wide variability among the subjects as illustrated by the large S.D. When the presence of other alleles was investigated and the patients regrouped accordingly, the pharmacokinetic parameter values were better explained with a substantial improvement in the *P* values (Table 2).

Fig. 2 shows the calculated allele frequencies for the population sampled which is similar to those found by Teh et al. [14].

4. Discussion

Even though tramadol has an active metabolite known as *O*-desmethyltramadol (M1), there has been conflicting evidence on the analgesic effect of M1 in humans. It has been shown to have 2–4 times the potency of tramadol in mice and rats as assessed by tail flick response but so far no analgesic activity of M1 was reported in healthy volunteers [15]. For this reason, we have not studied the pharmacokinetics of M1.

There has been little information on the frequency of CYP2D6 mutations among South East Asian populations. The frequency of PMs has

Table 2
Mean pharmacokinetic parameter values of different groups when other alleles were taken into consideration

Group	$t_{\frac{1}{2}}$ (h)	CL (ml/min)	$AUC_{0-\infty}$ (ng/ml h)
Group A ($n=2$)	6.6 ± 0.02	25.3 ± 3.2	3501.3 ± 444.1
Group B ($n=11$)	7.4 ± 1.9	20.4 ± 7.2	4908.5 ± 1933.0
Group C ($n=5$)	7.5 ± 2.2	16.6 ± 3.7	$5471.\ 9 \pm 1003.5$
Group D ($n=6$)	8.5 ± 1.5	14.3 ± 4.5	6709.6 ± 2313.8
Group E ($n=5$)	21.6 ± 16.2	10.4 ± 3.3	8725.6 ± 3731.8
Kruskal-Wallis test	$P=0.033$	$P=0.018$	$P=0.050$ (NS)

$t_{\frac{1}{2}}$, half-life; CL, clearance values; $AUC_{0-\infty}$, area under the curve from zero to infinity; Group A, *CYP2D6*1/*1*; Group B, *CYP2D6*1/*9* and *CYP2D6*1/*10*; Group C, *CYP2D6*1/*4* and *CYP2D6*1/*5*; Group D, *CYP2D6*10/*10* and *CYP2D6*10/*17*; Group E, *CYP2D6*4/*10* and *CYP2D6*5/*10*.

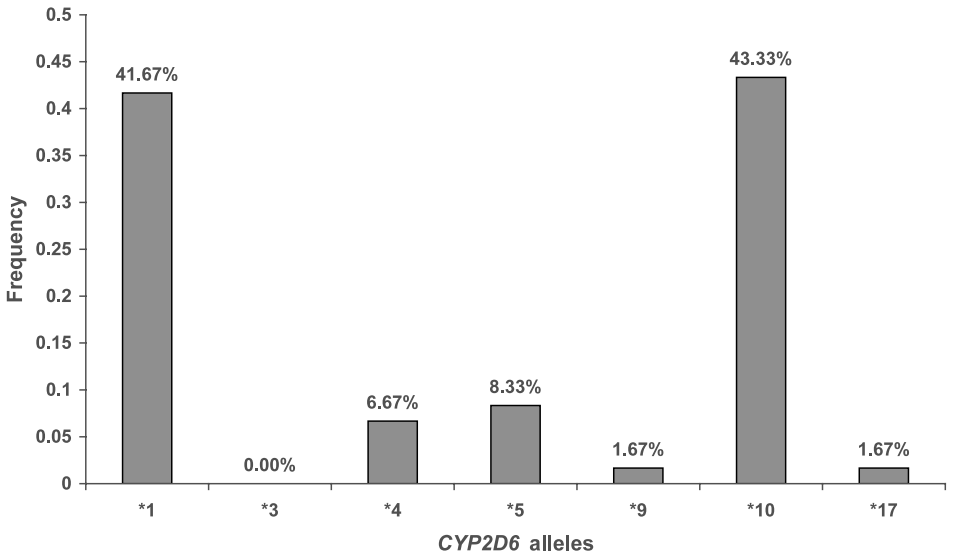

Fig. 2. Calculated allele frequency among the patients ($n = 30$).

been reported to be as low as 1% in these populations [16]. Among the 30 patients, none were PMs. Therefore a comparison between the pharmacokinetic parameter values of EMs and PMs could not be carried out in this study. Nevertheless, it was discovered that some of tramadol pharmacokinetic parameters were significantly different even within the subgroups of CYP2D6 EMs. One limitation of our study however, involved the calculation of $AUC_{0-\infty}$ for group E which could not be done properly in view of the 24 h sampling interval because of the long $t_{\frac{1}{2}}$ (21.6 ± 16.2 h) demonstrated.

Patients have been recruited rather than volunteer in order to reduce the probability of having genetic bias in the study population [17]. Genotyping is also a preferred test as compared to phenotyping due to the fact that the latter has inherent limitations such as interference by drug–drug interactions, a need for strict procedural controls and in some cases, intolerance of PMs for the test drug [18]. Furthermore, phenotyping by a probe drug has been shown not to reliably discriminate between homozygous EMs and heterozygous EMs [19]. Thus, a higher accuracy is expected using genotyping methods.

Although *CYP2D6*4* is the most common allele occurring in Caucasians [20], *CYP2D6*10* has been found to be more common among the Asians such as the Japanese and Chinese [21] as was also supported by this study (allele frequency for **10* = 0.43) and in other studies. Therefore, it is crucial to study the influence of this allele on the pharmacokinetics of CYP2D6 substrates such as tramadol. Due to the fact that most (93.3%) of the subjects were Malays, it is possible that Malay subjects have more similarity to the Chinese and Japanese populations as compared to the Caucasians in terms of debrisoquine hydroxylation capacity. It would however, be useful to conduct a similar study in a bigger sample of subjects.

5. Conclusions

It can be concluded that CYP2D6 activity may play a main role in influencing the pharmacokinetics of tramadol. The *CYP2D6*10* allele particularly was associated with higher serum levels and longer serum tramadol half-lives compared with the *CYP2D6*1* allele. Because this allele is common among Asians, lower mean doses of tramadol may be considered in these groups of

people compared to the Caucasians in order to achieve similar steady-state serum concentrations. However, individualisation of drug therapy is also of great importance considering the wide intersubject variability within both ethnic populations and genotyping for *CYP2D6*10* alone is not sufficient to explain tramadol disposition and other alleles should be taken into consideration when explaining the differences.

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