A Rapid and Simple CYP2D6 Genotyping Assay—Case Study With the Analgetic Tramadol

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There is substantial evidence for a causal relationship between genetic variability of the CYP2D6 gene and changes in the pharmacokinetics of drugs. Therefore, knowledge of single-nucleotide polymorphisms (SNPs) prior to drug administration is highly desired for assisting in the development of individualized pharmacotherapy. We therefore developed a robust assay that detects common CYP2D6 alleles within 60 minutes of blood withdrawal and links carriers of the variant CYP2D6*3 and *4 alleles to the pharmacokinetics of tramadol. This new genotyping assay employs fluorescence resonance energy transfer (FRET) analysis, which permits parallel identification of the CYP2D6*3 and CYP2D6*4 alleles within 60 minutes of blood withdrawal. We determined the genotypes of 100 healthy unrelated individuals and studied the pharmacokinetics of tramadol in 24 CYP2D6 genotyped healthy subjects. The total allelic frequencies of homozygote carriers were 0.015 and 0.25 for the CYP2D6*3 and *4 alleles, respectively, and the plasma area under the curve (AUC) was 84% above those of extensive metabolizers (homozygous EM group): 3,941.2 ng/mL · h (95% confidence interval [CI], 2,928.9 ng/mL · h to 4,953.5 ng/mL · h) versus 2,142.6 ng/mL · h (95% CI, 1,829.6 ng/mL · h to 2,455.7 ng/mL · h). Likewise, the AUC for the O-desmethyl-tramadol metabolite (M1) was significantly reduced in poor metabolizers (PMs): 300.2 ng/mL · h (95% CI, 260.3 ng/mL · h to 340.0 ng/mL · h) versus 842,6 ng/mL · h (95% CI, 715.1 ng/mL · h to 970.0 ng/mL · h). We observed a statistically significant correlation between plasma tramadol AUC and production of the O-desmethyl metabolite in CYP2D6 genotyped healthy volunteers. Our assay can be used reliably in clinical pharmacology studies and may be used for dose adjustment.

IDIOSYNCRATIC RESPONSES to drug therapy may be linked to genetic polymorphisms and indeed, more than 40 drugs and environmental chemicals and as many as 20% of all commonly prescribed drugs are subjected to CYP2D6-mediated metabolism.[1,2] Importantly, more than 77 variant alleles have been identified for this particular mono-oxygenase (see http://www.imm.ki.se/CYPalleles/), but only certain nucleotide changes of the CYP2D6 gene are of functional importance and result in the poor or ultrarapid metabolizer phenotype, which leads to significant changes in the pharmacokinetics of CYP2D6 substrates.[1,3]

From a practical point of view it is literally impossible to screen for all possible allelic variants of drug metabolising enzymes on a routine basis. Nonetheless, genotype information is highly desirable, particularly when the prescribed drugs are substrates for enzymes with clinically relevant genetic polymorphisms. Thus, genetic information is required to secure therapeutic efficacy and safety based on dose adjustments, especially when drugs with narrow therapeutic windows or cocktails of medicines are given.[1]

We therefore developed a robust assay that would detect within 60 minutes of blood withdrawal, 2 common CYP2D6 alleles that usually confer the poor metabolizer (PM) phenotype. We developed a new genotyping protocol employing fluorescence resonance energy transfer (FRET) analysis and applied the assay to the Roche Diagnostics (Mannheim, Germany) LightCycler to allow polymerase chain reaction (PCR) amplification and product analysis simultaneously. Our PCR assay monitors fluorescence quantification of DNA binding dyes, which hybridize to selected target sequences; using this approach, we can specifically assay nucleotide polymorphisms of the CYP2D6*3 and CYP2D6*4 allele. We report the genotypes of 100 healthy unrelated individuals from Southern Germany enrolled in clinical phase I trials, of which 24 subjects participated in a clinical trial with tramadol. This drug is a synthetic 4-phenyl-piperidine analogue of codeine and is widely prescribed as a centrally acting analgesic with a dual mechanism of action that includes low agonistic effects for the μ-opioid receptor, as well as inhibition of monoamine (serotonine, norepinephrine) reuptake.[4] Furthermore, tramadol is mainly metabolized to the N- and/or O-desmethyl-metabolite. CYP2D6 is the major isoform responsible for tramadol's oxidation; nonetheless CYP2B6 and CYP3A4 are minor contributors.[5]

We studied the relationship between carriers of the CYP2D6*3 and *4 alleles and the production of the O-desmethyl-tramadol metabolite (M1). This metabolite is exclusively produced by CYP2D6 and thus allows genotype/phenotype correlation. Overall, we report an assay that enables swift, large-scale, and cost-effective determination of major CYP2D6 polymorphisms for clinical pharmacology studies and provides guidance for dose adjustment within 60 minutes of blood sample withdrawal.

MATERIALS AND METHODS

Human Subjects

General CYP2D6 screen. One hundred healthy male and female subjects from a Human Pharmacology Unit were examined for participation in various clinical research studies. These subjects were genotyped for CYP2D6*3 and CYP2D6*4 after having given written informed consent.

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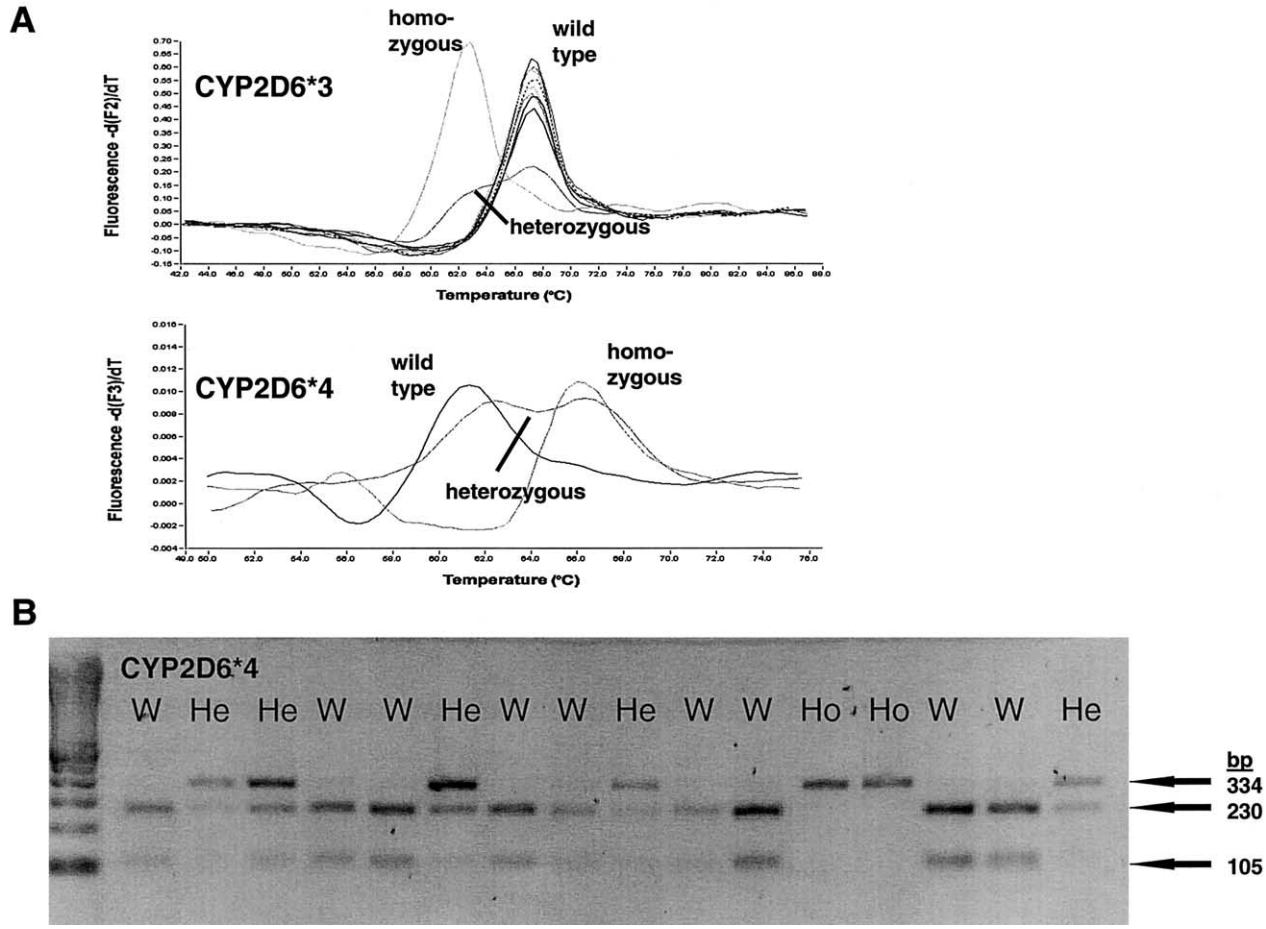

Fig 1. (A) Melting curve and (B) RFLP analysis for the wild-type (W), heterozygous (He), and homozygous (Ho) genotypes of the CYP2D6*3 and CYP2D6*4 alleles. RFLP analysis for the CYP2D6*4 allele was performed as described previously.[7]

Tramadol study. This study was a randomized, open, sex-balanced, 2-period, 2-sequence cross-over study in 24 healthy Caucasian subjects (12 female/12 male). Subjects were 18 to 42 years of age (mean, 30.7 years), and weighed 52 to 93 kg (mean, 69.3 kg). Each subject gave prior to participation written informed consent to the study, which was approved by the institutional review board of the "Landesärztekammer" Hessen. The study was conducted in accordance with the declaration of Helsinki (Somerset West Amendment, 1996) and the International Conference on Harmonisation (ICH) Guideline on Good Clinical Practice (CPMP/ICH/135/95). All subjects were healthy according to medical history, physical examination, and clinical laboratory data. Subject no. 9 dropped out after randomization but before administration of study treatment; he was replaced by subject no. 109 maintaining the same treatment sequence. Each subject received on 2 separate occasions 100 mg tramadol separated by a wash-out interval of at least 5 days.

Blood sampling and determination of tramadol and M1 metabolite. Blood was collected in 7.5 mL NH_4-heparin Monovettes (Sarstedt, Nümbrecht, Germany) pre-dose, and 0.33, 0.67, 1, 1.33, 1.67, 2, 2.5, 3, 4, 6, 8, 12, 20, 24, 30, 36, and 48 hours after dosing. The samples were centrifuged at 2,300 $\times$ *g* within 30 minutes and the plasma stored frozen at −20°C until analyzed. Concentrations of tramadol and M1 metabolite were determined by a validated gas-chromatography method with lower limits of quantification of 2.0 μg/L for tramadol enantiomers and 1.0 μg/L for M1.[6]

DNA Isolation of Human Blood Samples

DNA from whole blood was isolated using the DNA Extraction Kit NucleoSpin Blood (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

Genotyping Using FRET Analysis

The fluorogenic adjacent hybridization probes were designed and produced by TIB-MOLBIOL (Berlin, Germany). Sequences for PCR oligonucleotides were 5′-ACCCCGTTCTGTCTGGTGT (sense) and 5′-CCGAGAGCATACTCGGGAC (anti-sense) for the CYP2D6*3 allele and 5′-GCCTTCGCCAACCACTCCG (sense) and 5′-AAATCCTGCTCTTCCGAGGC (antisense) for the CYP2D6*4 allele. Probes were designed that their melting temperatures (Tm) were marginally higher than the Tm of the primers. The sensor probe of CYP2D6*3 was labeled with fluorescein (X) at the 3′ end (5′-CTAACTGAGCACAGGATGACCTG X3′) and the anchor probe was labeled with LightCycler Red 640 (LC-Red 640) at the 5′ end (5′ LC Red640-ACCCAGCCCAGCCCCCCCGAGAC phosphorylated). In the case of the CYP2D6*4 allele, the anchor probe was labeled with flourescein (X) at the 3′ end (5′-GAAGGCGACCCCTTACCCGCATCT X3′) and the sensor was labeled with LightCycler Red 705 (LC-Red 705) at the 5′ end (5′ LC-Red 705-ACCCCCAAGACGCCCCT phosphorylated). Each of the corresponding probes recognized adjacent sequences with

Table 1. Allelic Frequencies in CYP2D6 Genotyped Subjects

	No. of Patients	
	CYP2D6*3 allele	CYP2D6*4 allele
Homozygous	0	8
Heterozygous	3	34
Wild type	97	58
Total	100	100
Allelic frequencies	0.015	0.250

the shorter probe lying over the mutation site and the probes were separated by 2 (CYP2D6*3) or 3 bases (CYP2D6*4). Fluorescein was used as the donor fluorophore and blocked extension from the probe during PCR. LC-Red 640 and LC-Red 705 were used as acceptors of the FRET process with its 3′ end phosphorylated to block extension. The greater stability of the longer anchor probe meant that loss of fluorescence occurred as the shorter probe melted off the template. The probes were designed in a way that the 2 different mutation sites could be detected simultaneously (duplex-PCR).

PCR was performed with 400 nmol/L CYP2D6 primers in a standard PCR reaction including a 200 nmol/L anchor and 200 nmol/L sensor hybridization probe, 100 ng DNA, 4.0 mmol/L $MgCl_2$, and 2 μL LightCycler DNA master hybridization mix in a total of 20 μL. The reaction started at 95°C for 30 seconds and amplification was done for 50 cycles of denaturation (95°C, 0 seconds, ramp rate 20°C/s), annealing (60°C, 7 seconds, ramp rate 20°C/s), and extension (72°C, 20 seconds, ramp rate 20°C/s).

PCR product identification was done by analysis of DNA melting curves in the glass capillary. DNA was denaturated at 95°C for 30 seconds and maximal fluorescence was acquired by holding the reaction at 45°C for 30 seconds. Data for the melting curves were generated by heating slowly to 80°C with a ramp rate of 0.1°C/s and were collected continuously during that time. When the shorter probe melted off the template, FRET no longer took place and fluorescence was converted to melting peaks using software that plotted the negative derivative of fluorescence with respect to temperature ($-dF/dT$ *v* T). The sequence-specific hybridization probes melt off the target sequence at a characteristic temperature: 62°C (mutation) and 68°C (wild type) in the case of CYP2D6*3 (channel F2 in the LightCycler) and 61°C (wild type) and 67°C (mutation) for CYP2D6*4 (channel F3 in the LightCycler). This shift was also observed when synthetic oligonucleotides were used as positive controls, eg, 5′-GTCCGGGGGGGCTGGGCTGGGTCCCAGGTCATCCTGTGCTCAGTTAG-3′ as a positive control for the wild-type CYP2D6*3 allele, and a similar oligonucleotide with base at position 35 missing (A deletion) was used to detect the variant CYP2D6*3 allele. In the case of the CYP2D6*4 allele, 5′-AGGGGCGTCCTGGGGGTGGGAGATGCGGGTAAGGGGTCGCCTTC-3′ was used as a positive control for the wild-type allele, and in the case of the variant CYP2D6*4 allele the base "C" was replaced by the base "T" in position 10 (G to A transition). The variant alleles resulted in a melting temperature shift of about 6°C, allowing the easy detection of a wild-type allele from the variant allele (a typical example of channels F2 and F3 is given in Fig 1A).

Restriction Fragment Length Polymorphism Analysis

Additionally, restriction fragment length polymorphism (RFLP) analysis was performed as described earlier.[7,8] A typical result after RFLP with NI is shown in Fig 1B.

Statistical Analysis

All data are given as the mean ± SD. Statistical significance was tested with the Student's *t* test for independent samples ($P < .001$). In addition, the 95% confidence intervals (CIs) are given.

RESULTS AND DISCUSSION

Figure 1A depicts a representative melting curve for the CYP2D6*3 and CYP2D6*4 alleles. The differences in Tm among individual genotypes were sufficient to permit secure discrimination of single alleles (dT +6°C). There was no difference between the theoretically predicted and sequenced PCR products (data not shown), and our RFLP analysis provided further confirmation of the correct assignment for individual CYP2D6 alleles (see Fig 1B). We thus show corroborative and conclusive evidence for the accurate DNA amplification of individual alleles.

In our cohort of 100 unrelated individuals of Southern Germany, the total allelic frequencies of homozygote carriers were 0.015 for CYP2D6*3 (A deletion) and 0.25 for CYP2D6*4 (G to A transition) (Table 1). From this pool of volunteers, we chose 24 individuals, who received on 2 separate occasions single doses of 100 mg tramadol. Blood samples were taken at regular intervals and analyzed as described in the Methods.

Table 2. AUC Values of Tramadol and M1

	PM	EM Heterozygous	EM Homozygous
Results			
AUC(tramadol) [ng/ml · h]	3,941.2	2,461.3	2,142.6
AUC(M1) [ng/mL · h]	300.2	875.0	842.5
AUC(tramadol)/AUC(M1)	13.1	2.8	2.5
Patients (n)	6	7	11
Standard deviations			
AUC(tramadol) [ng/mL · h]	1,265.1	563.4	529.7
AUC(M1) [ng/mL · h]	49.8	85.0	215.6
AUC(tramadol)/AUC(M1)	4.0	0.5	0.7
Patients (n)	6	7	11
95% C/I			
AUC(tramadol) [ng/mL · h]	2,928.9-4,953.5	2,043.9-2,878.7	1,829.6-2,455.7
AUC(M1) [ng/mL · h]	260.3-340.0	812.0-938.0	715.1-970.0
AUC(tramadol)/AUC(M1)	9.9-16.4	2.4-3.2	2.2-2.9
Patients (n)	6	7	11

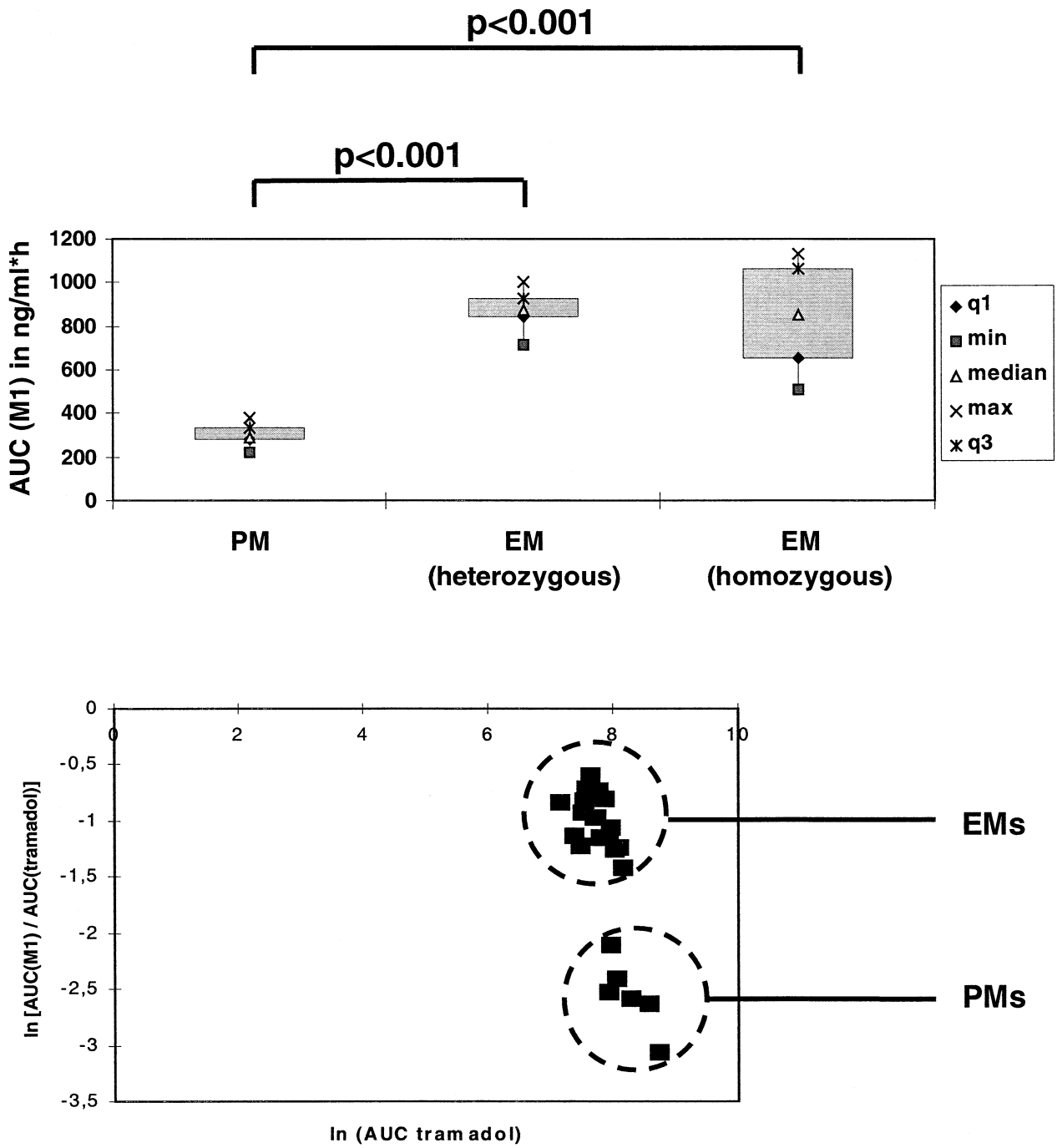

Fig 2. Box plots of $AUC_{0\rightarrow\infty}$(M1) for individual genotypes in cohort of 24 unrelated individuals from Southern Germany. (PM, poor metabolizer; EM, extensive metabolizer; q1, lower quartile [25th percentile]; q3, upper quartile [75th percentile]; min, minimum value; max, maximum value). ***P < .001). (B) Logarithmic (ln) ratio of [AUC(M1/tramadol)/AUC(tramadol)].

In Table 2 we report the relationship between homozygous and heterozygous carriers of the variant alleles of the CYP2D6 gene, as well as the areasu under the curve (AUC): $AUC_{0\rightarrow\infty}$ and the ratio of AUC(tramadol total) versus AUC(M1). Noticeably, plasma AUC values for poor metabolizers (PMs) were 84% above those of extensive metabolizers (EM): 3,941.2 ng/mL · h (95% CI, 2,928.9 ng/mL · h to 4,953.5 ng/mL · h) for PMs versus 2,142.6 ng/mL · h (95% CI, 1,829.6 ng/mL · h to 2,455.7 ng/mL · h) for EMs; for further details see Table 2. Figure 2A depicts a box plot analysis, which illustrates further the relationship between EMs and PMs in the production of M1, eg, $AUC_{0\rightarrow\infty}$(M1) and Fig 2B shows good dissection of

PMs and EMs, when the ln ratio of AUC(M1/tramadol total) against AUC(tramadol total) is plotted.

We report a new genotyping assay for the determination of clinically relevant CYP2D6 alleles, which contribute significantly to a change in the metabolic clearance of CYP2D6 substrates.[7-9] Importantly, production of the O-desmethyl metabolite is dependent on CYP2D6 activity and, thus, we link the pharmacokinetics of tramadol and M1 production to carriers of the *3 and *4 variant alleles. Estimates of the ratios of AUC(tramadol)/AUC(M1) are particularly useful to phenotype CYP2D6 PMs (Table 2) and are therefore true accounts for CYP2D6 activity. We propose M1 production of tramadol to be an interesting probe for this particular mono-oxygenase; nonetheless, we acknowledge the contribution of CYP2B6 and CYP3A4 in the production of M2.[5] The latter CYP mono-oxygenases are also subject to enzyme induction. As a consequence, tramadol's metabolism of M2 may change, and this could be a confounding factor when plasma AUCs are considered indiscriminately, eg, the AUC of M1 versus M2 were not studied separately. Furthermore, Abdel-Rahman et al[10] examined the concordance between tramadol and dextromethorphan parent/metabolite ratios in CYP2D6 genotyped children (age 7 to 16 years) using standard PCR techniques (extra long PCR and PCR/RFLP). This interesting study enabled comparison of product/metabolite ratios for 2 different CYP2D6 substrates. Initially, the genotype correlation of urinary-determined metabolites was poor; however, when subjects were segregated based on number of functional CYP2D6 alleles, a much stronger relationship was observed.

The pharmacodynamics of tramadol may differ between PMs and EMs, as tramadol and the O-desmethyl metabolite are agonists for the 5-hydroxytryptamine (5-HT) or opioid receptor pathways.[4] Thus, differences in the molar concentrations of parent compound and M1 may shift the balance between these 2 receptor pathways and, thus, ultrarapid metabolizers will have significantly higher M1 levels, potentially leading to enhanced receptor signaling via the μ-opioid receptor. This shift in metabolite balance may be a cause for drug addiction, as occasionally reported for this tramadol.[11]

More than 77 variant CYP2D6 alleles are known, but as mentioned above only a few are of clinical importance. Indeed, the allelic frequencies of the *3 and *4 alleles account for the majority of the CYP2D6 PMs. Nonetheless, others such as the *5 and *6 alleles are also of functional importance, even though their contribution to the overall frequencies of variant alleles may only be on the order of 3%.[1] An array of molecular biology methods can be used to detect genetic polymorphisms, and these include the use of multiplex long PCR,[12] RFLP,[13] or single-strand conformation polymorphism analysis.[14] However, these methods are laborious and cumbersome, a major drawback for their routine use in clinical practice. Very recently, a real-time PCR-based method for additional determination of *6, *7, and *8 was reported, but the authors failed to corroborate their findings by investigating genotype/phenotype correlations using appropriate substrates.[15] Thus, it remains to be determined whether this method is of practical use for clinical pharmacology studies.

In conclusion, we report a robust and swift genotyping assay to permit analysis of the CYP2D6*3 and CYP2D6*4 alleles within 60 minutes of blood withdrawal. This assay can be used in routine clinical practice and may provide guidance for dose adjustments of CYP2D6 drugs.

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