Genotyping of human cytochrome P450 2A6 (CYP2A6), a nicotine *C*-oxidase

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Abstract Cytochrome P450 2A6 (CYP2A6) is a polymorphic enzyme responsible for the oxidation of certain precarcinogens and drugs and is the major nicotine *C*-oxidase. The role of CYP2A6 for nicotine elimination was emphasised recently by the finding that smokers carrying defective *CYP2A6* alleles consumed fewer cigarettes [Pianezza et al. (1998) Nature 393, 750]. The method used for *CYP2A6* genotyping has, however, been found to give erroneous results with respect to the coumarin hydroxylase phenotype, a probe reaction for the CYP2A6 enzyme. The present study describes an allele-specific PCR genotyping method that identifies the major defective *CYP2A6* allele and accurately predicts the phenotype. An allele frequency of 1–3% was observed in Finnish, Spanish, and Swedish populations, much lower than described previously.

Key words: Cytochrome P450; Coumarin; Allele; Genotype; Phenotype; Methoxyflurane

1. Introduction

Cytochromes P450 is a superfamily of haem thiolate enzymes involved in the metabolism of numerous endogenous and exogenous compounds including steroid hormones, fatty acids, precarcinogens, and a variety of clinically used drugs. A pronounced interindividual variability in the expression of drug-metabolising P450s exists because of genetic and environmental factors. Based on the genetic variation, subjects are classified into poor (PM), extensive (EM), or ultrarapid metabolisers (UM) [1] and accordingly a pronounced interindividual difference in the metabolism of and sensitivity to drugs and other xenobiotics is seen [1,2]. PMs for CYP2D6 or CYP2C19, enzymes which metabolise many clinically used drugs, have a reduced metabolic capacity which can result in higher plasma concentrations and increased risk of adverse effects at ordinary drug dosages, whereas UMs, carrying duplicated or multiduplicated genes, do not achieve therapeutic drug levels. In addition, prodrugs such as proguanil and codeine need to be metabolically activated by these enzymes, and are therefore inactive in PMs.

Cytochrome P450 2A6 (CYP2A6) was first identified as the human coumarin 7-hydroxylase [3,4] and has been shown to activate a variety of precarcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosodiethylamine, aflatoxin B1, 4,4′-methylene-bis(2-chloroaniline), 1,3-butadiene and 2,6-dichlorobenzonitrile (see [5–7] and references therein). In addition, CYP2A6 is involved in the metabolism of certain pharmaceuticals, such as methoxyflurane [8], halothane [9], (+)-*cis*-3,5-dimethyl-2-(3-pyridyl) thiozolidin-4-one hydrochloride (SM-12502) [10], losigamone [11], letrozole [12], valproic acid [13], and disulfiram [14]. Nicotine is primarily metabolised to cotinine in humans and this reaction has been shown to be catalysed mainly by CYP2A6, as well as the subsequent 3′-hydroxylation of cotinine [15–18].

In vivo phenotyping studies using coumarin as a probe drug have shown a pronounced interindividual variability in the expression of CYP2A6 [19–21]. A similar variability has also been observed when CYP2A6 was measured in human liver microsomes, with some livers lacking the enzyme [4,22,23]. The explanation is inherent in a polymorphism and three different apparently defective allelic variants of the *CYP2A6* gene have been described. *CYP2A6*2*[1] encodes an enzyme with a $Leu^{160} \rightarrow His$ substitution, which yields an inactive enzyme in a vaccinia virus-based expression system [3]. One individual was recently reported to be homozygous for this allele and lacked coumarin 7-hydroxylation capacity in vivo [25]. The rare *CYP2A6*3* allele, generated through a gene conversion between *CYP2A6* and *CYP2A7*, was also proposed to be inactive [26]. Recently, it has been reported that some Japanese individuals had a part of the *CYP2A6* gene deleted and subjects homozygous for this allele also exhibited the PM phenotype [27].

A genotyping method for detection of the *CYP2A6*2* and *CYP2A6*3* alleles, based on PCR amplification combined with diagnostic restriction enzyme digestion, has been described previously [26]. Studies using this method have revealed pronounced interethnic differences in the frequencies of the *CYP2A6* alleles [26,28] and shown that *CYP2A6*2* is the major defective allele in Northern European populations. However, certain individuals classified as homozygous for the *CYP2A6*2* allele actually exhibited CYP2A6 activity in vivo

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Abbreviations: CYP or P450, cytochrome P450; PM, poor metaboliser; PCR, polymerase chain reaction

[1] In this report we have used the nomenclature system for *CYP2A6* alleles proposed by Daly et al. [24]: *CYP2A6*1*, 2A6wt; *CYP2A6*2*, 2A6v1; *CYP2A6*3*, 2A6v2.

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[26]. This could indicate either that variants of the *CYP2A6*2* allele exist which exhibit coumarin 7-hydroxylation activity, that coumarin is 7-hydroxylated by (an)other enzyme(s) in these individuals, or that the genotyping method gives an erroneous classification with respect to the genotype. We have investigated the origin of this problem and describe a new method for detection of the *CYP2A6*2* allele which accurately predicts the CYP2A6 phenotype. Using this new method we found a surprisingly low frequency of this allele in a random sample of 300 individuals from three different European populations.

2. Materials and methods

2.1. Subjects

Phenotype analysis using coumarin combined with genotype analysis was carried out using a selected cohort of 14 individuals in Finland. Previous phenotype analysis of 100 Finnish patients with newly diagnosed non-insulin-dependent diabetes mellitus (NIDDM) using antipyrine, caffeine, lidocaine, and coumarin as probe drugs (Rautio et al., unpublished) revealed two female siblings that were totally deficient in 7-OH-coumarin excretion after oral ingestion of 5 mg coumarin, who were included in the present study. Phenotype analysis was also carried out on a group of 11 healthy individuals (eight women, three men, age range 30–56 years), selected from the staff at the Department of Pharmacology and Toxicology, University of Oulu. Furthermore, an individual that was shown previously to display a discrepancy between genotype and phenotype [26], LV 19, was included. Blood samples were collected into EDTA tubes and genomic DNA was isolated from Ficoll-enriched leukocytes with a standard phenol extraction method.

In addition, three different Caucasian populations of Finnish, Swedish, and Spanish origin were genotyped to determine the frequency of the *CYP2A6*2* allele. Genomic DNA was obtained from 90 randomly sampled volunteers living in the Stockholm area of Sweden and 100 volunteers from the Zaragoza area in northern Spain. These individuals were used previously in *CYP2D6* genotype-phenotype correlation studies [29,30]. One hundred and forty-four DNA samples from Finnish volunteers were sampled from the FINRISK'92 study material [31]. The study was approved by the ethical committees at Karolinska Institutet and the University of Oulu.

2.2. CYP2A6 phenotyping

Following an overnight fast, the subjects voided a urine sample and ingested a capsule containing 5 mg coumarin and 25 mg rutosides (Venalot, Schaper&Brummer, Ringelheim, Germany) together with 200 ml of water. Urine was voided 2 h later and 10 ml aliquots of the urine samples were centrifuged and stored at −20°C until analysis. The amount of 7-OH-coumarin in the urine samples was measured by the HPLC method described by Rautio et al. [19] with minor modifications [32], including treatment of the urine samples with β-glucuronidase and sulphatase to liberate 7-OH-coumarin from conjugates. The coumarin index refers to the percentage of 7-OH-coumarin excreted from the total 5 mg dose during the 2 h time period.

2.3. Genotyping of the CYP2A6*2 allele

A two-step PCR method was used to detect the *CYP2A6*2* allele. In the first PCR reaction (PCR I) a part of the *CYP2A6* gene was specifically amplified and the product was subsequently used as a template in the second mutation-specific PCR reaction (PCR II). PCR I was done using primers 2A6ex1 and 2A6ex4R (see Table 1). The reaction mixture contained approximately 50 ng genomic DNA, 0.25 μM of each primer, 0.2 mM of each dNTP, 1.2 mM $MgCl_2$, 0.6 U of Taq polymerase (Advanced Biotechnologies, Epsom, UK) and was carried out in 1×Reaction Buffer IV (Advanced Biotechnologies) in a total volume of 25 μl using a Perkin Elmer GeneAmp PCR System 2400/9600. Initial denaturation was performed at 95°C for 1 min, followed by 35 cycles each consisting of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 3 min, followed by a final extension at 72°C for 7 min.

The mixture for the mutation-specific reaction (PCR II) contained 0.8 μl PCR I product, 0.25 μM of primer 2A6wt or primer 2A6mut, 0.25 μM of primer E3R (see Table 1), 0.1 mM of each dNTP, 1.0 mM $MgCl_2$, 0.3 U of Taq polymerase, and was done in buffer consisting of 10 mM Tris-HCl pH 8.3 and 50 mM KCl in a total volume of 25 μl. The amplification was performed by initial denaturation at 95°C for 1 min, followed by 16 cycles each involving denaturation at 95°C for 15 s, annealing at 50°C for 20 s, and extension at 72°C for 45 s. PCR products (12 μl) were subsequently analysed on a 3% agarose gel (1.5% agarose, Life Technologies, Rockville, MD, USA plus 1.5% NuSieve agarose, FMC BioProducts, Vallensbaek Strand, Denmark) stained with ethidium bromide.

Sample LV 19 was analysed with all four possible combinations of the forward and reverse primers used for PCR I in the two different protocols. The PCR I reaction was carried out using the long PCR protocol [33] essentially as described by Fernandez-Salguero et al. [26].

3. Results

3.1. Phenotype analysis

HPLC profiles of the urine samples of three individuals are shown in Fig. 1. The distinct 7-OH-coumarin peak present in the sample from individual A (LV 31) is lacking in samples from individuals B (LV 29) and C (LV 26). The 2 h coumarin test was reproduced several times yielding the same results. Urine samples were also collected for up to 8 h in these individuals with no traces of 7-OH-coumarin being detected during HPLC analysis. The summarised results of the phenotypes of the 14 Finnish subjects analysed in this study are presented in Table 2.

3.2. Genotype analysis

The *CYP2A6* gene is located on chromosome 19 between 19q12 and 19q13.2 [34], which is adjacent to the *CYP2A7* gene that encodes an inactive enzyme product [3,35] and close to the *CYP2A13* gene as well as the two *CYP2A7* pseudogenes [36]. In our hands, genotype analysis using the original method described by Fernandez-Salguero et al. [26] (cf. Fig. 2A) often yielded unreliable results usually because of coamplification of the *CYP2A7* gene. Furthermore, the previously phenotyped subject LV 19 had a discrepancy between phenotype and apparent genotype, since this subject was classified as homozygous for the *CYP2A6*2* allele, but clearly exhibited coumarin 7-hydroxylation activity in vivo [26]. This prompted us to develop an alternative method for genotype determination. We designed primers which could specifically amplify a part of the *CYP2A6* gene while avoiding coamplification of *CYP2A13*, *CYP2A7* and the *CYP2A7* pseudogenes. This PCR fragment was subsequently used as a template for a mutation-specific PCR reaction as outlined in Fig. 2B.

Table 2 shows the correlation between *CYP2A6* genotype and the coumarin test in the 14 Finnish individuals. The mean coumarin index of the nine individuals with a *CYP2A6*1/*1*

Table 1
Primers used for genotyping of the *CYP2A6* gene

Primer	Sequence
2A6-ex1	5′-GCT GAA CAC AGA GCA GAT GTA CA-3′
2A6-ex4R	5′-GGA GGT TGA CGT GAA CTG GAA GA-3′
F4[a]	5′-CCT CCC TTG CTG GCT GTG TCC CAA GCT AGG C-3′
R4[a]	5′-CGC CCC TTC CTT TCC GCC ATC CTG CCC CCA G-3′
2A6wt	5′-CTC ATC GAC GCC CT-3′
2A6mut	5′-CTC ATC GAC GCC CA-3′
E3R[a]	5′-TCG TCC TGG GTG TTT TCC TTC-3′

[a]From Fernandez-Salguero et al. [26].

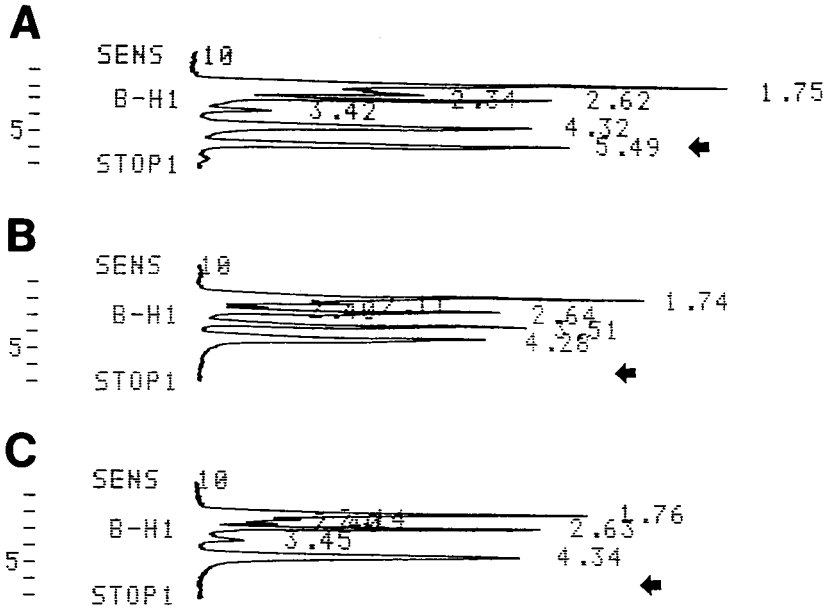

Fig. 1. HPLC chromatograms of the 2 h urine samples from poor and extensive metabolisers of coumarin. The arrows show the retention time of 7-OH-coumarin. A: LV 31; B: LV 29; C: LV 26.

genotype was 68% and a lower mean index (54%) was observed for the *CYP2A6*1/*2* individuals. The two subjects described above with no detectable coumarin 7-hydroxylation activity were both homozygous for the *CYP2A6*2* allele (see Fig. 3). Furthermore, individual LV 19 was clearly determined to be heterozygous (*CYP2A6*1/*2*; see Fig. 3) which correlates very well with the coumarin index of 40%.

In order to study further the basis for the discrepancy between the two different genotyping methods, we amplified genomic DNA from individual LV 19 using all of the four possible combinations of forward primers F4 and ex1 and reverse primers R4 and ex4R. All four PCR I products were then subjected to mutation-specific PCR (Fig. 3C). The results showed that only the *CYP2A6*2* and not the *CYP2A6*1* allele was amplified using primer R4 with genomic DNA from this individual. It is therefore reasonable to assume that many individuals have mutations at the primer binding site in the 3′ flanking region of the *CYP2A6* gene, which would result in no amplification of one of the alleles using the primers described by Fernandez-Salguero et al. [26]. This likely explains the discrepancy observed previously between phenotype and genotype using this method.

Table 2
Correlation between the CYP2A6 phenotype and genotype

CYP2A6 genotype	Subject	Coumarin index[a]
*CYP2A6*1/*1*	LV 1	81
	LV 7	70
	LV 8	44
	LV 11	70
	LV 12	84
	LV 13	66
	LV 15	61
	LV 25	72
	LV 27	64
		Mean: 68
*CYP2A6*1/*2*	LV 6	73
	LV 19	40
	LV 37	50
		Mean: 54
*CYP2A6*2/*2*	LV 26	0
	LV 29	0
		Mean: 0

[a]Percentage of 7-OH-coumarin excreted from the total 5 mg coumarin dose during a 2 h period.

3.3. Allele frequencies of the CYP2A6*2 allele

In order to estimate the prevalence of the *CYP2A6*2* allele, we genotyped more than 300 individuals belonging to three different ethnic groups from Europe, namely 90 Swedes, 144 Finns, and 100 individuals from northern Spain (Table 3). No subject was found to be homozygous for the *CYP2A6*2* allele, and the allele frequency was low in all of the populations, 1.1, 1.4 and 3.0% respectively. The observed genotype frequencies did not deviate significantly from the ones expected by the Hardy-Weinberg law.

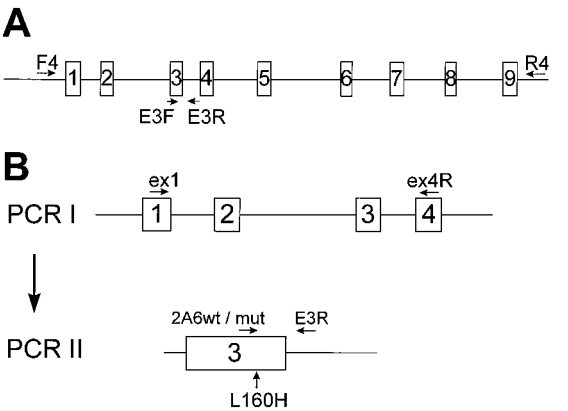

Fig. 2. Strategies for *CYP2A6* genotyping. A: Schematic representation of the genotyping method developed by Fernandez-Salguero et al. [26]. B: Schematic representation of the genotyping method used in the present study.

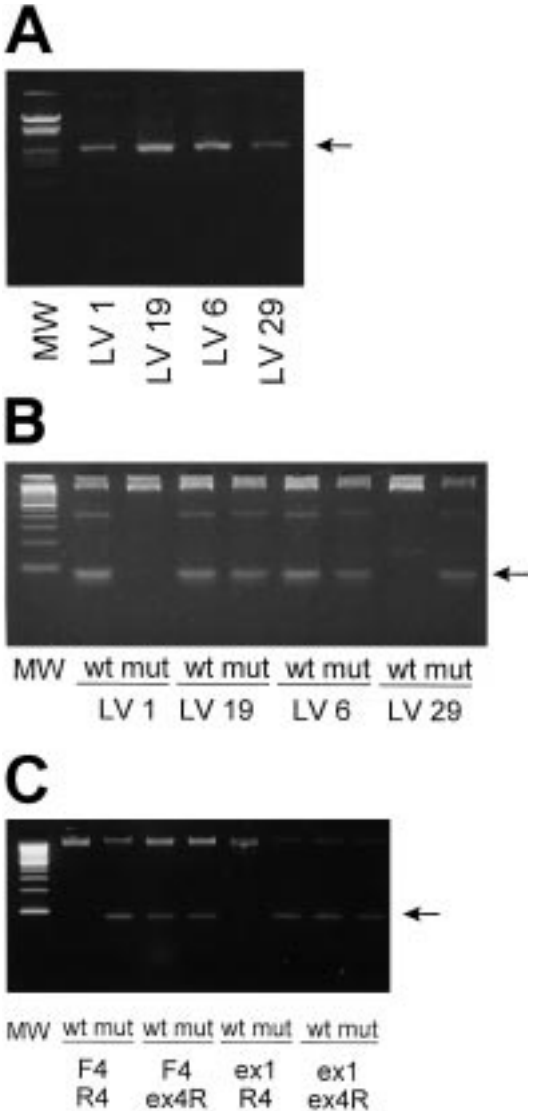

Fig. 3. *CYP2A6* genotyping based on mutation-specific PCR. A: Amplification of a part of the *CYP2A6* gene from genomic DNA ('PCR I'). MW, molecular weight marker λ digested with *Eco*RI/*Hin*dIII (Eastman Kodak Company, New Haven, CT). B: Mutation-specific PCR for detection of the *CYP2A6*2* allele ('PCR II'). Designation of the samples is the same as for Table 2. MW, 100 bp DNA ladder (Life Technologies, Rockville, MD). C: PCR II results obtained from individual LV 19 using four different combinations of primers in PCR I.

4. Discussion

Using the genotyping method presented in this report, a good correlation was obtained between the *CYP2A6* genotype and phenotype and we correctly predicted the phenotype of the individuals which had previously been misclassified as PMs. The mean coumarin index was lower in the three *CYP2A6*1/*2* heterozygotes compared with the *CYP2A6*1/*1* individuals, thereby indicating a gene-dosage effect. Furthermore, we showed that the two individuals homozygous for the *CYP2A6*2* allele displayed no coumarin 7-hydroxylation activity.

The frequency of the *CYP2A6*2* allele determined by this method was between 1 and 3% in the three European populations studied. These values are considerably lower than those previously reported for European populations, namely 4, 15, and 17% in a Spanish, Finnish and English population respectively [26,28]. An explanation for this difference could be that individuals were misclassified in the earlier studies due to the erroneous method used.

Most genotyping methods using allele-specific PCR amplification are based on the assumption that the regions where the PCR primers for the first PCR reaction bind are conserved in all of the individuals studied, always resulting in reliable amplification. This may of course not always be the case, especially when the primers bind to sequences in introns and flanking regions of the genes. We therefore designed the PCR I primers in exons which are much more conserved between individuals. The results in Fig. 3C show that amplification of the *CYP2A6* gene product was also achieved for those alleles which were not amplified by the primers used by Fernandez-Salguero et al. [26]. Furthermore, we shortened the primary amplification product to 1.8 kb instead of 7.8 kb, resulting in an easier amplification and avoiding the smear problems often seen during prolonged usage of the long-PCR protocol ([33,37]; I. Johansson, M. Oscarson and M. Ingelman-Sundberg, unpublished observations). In the published protocol, the first PCR product is subjected to an additional 31 cycles in the exon-specific amplification [26]. This could be enough for coamplification of *CYP2A7* from genomic DNA carried over from the first PCR reaction if the primers used in the second PCR reaction are not specific for *CYP2A6*. This would result in erroneous evaluation of the subsequent diagnostic restriction enzyme digestion, both with respect to the *CYP2A6*2* and *CYP2A6*3* alleles.

A reliable genotyping method which accurately predicts the CYP2A6 phenotype is of interest from a clinical pharmacological point of view. It is likely that there is a pronounced variability in the disposition of pharmaceuticals which are mainly metabolised by CYP2A6, including SM-12502. This is in analogy with the PM phenotype for CYP2D6 and CYP2C19, which has been shown to be important for the interindividual disposition of a number of pharmaceuticals [38,39]. Furthermore, poor metabolisers may partially be protected from hepatotoxicity which can result from drugs metabolically activated by CYP2A6 such as valproic acid [13], and

Table 3
CYP2A6 genotypes and allele frequencies in Swedish, Finnish and Spanish populations

	Genotypes (frequency, %)			Alleles (frequency, %)	
	*CYP2A6*1/*1*	*CYP2A6*1/*2*	*CYP2A6*2/*2*	*CYP2A6*1*	*CYP2A6*2*
Population					
Swedes ($n = 90$)	97.8	2.2	0.0	98.9	1.1
Finns ($n = 144$)	97.2	2.8	0.0	98.6	1.4
Spaniards ($n = 100$)	94.0	6.0	0.0	97.0	3.0

halothane [9], and possibly from the carcinogenic effects of the precarcinogens activated by CYP2A6.

As CYP2A6 is responsible for *C*-oxidation of nicotine, which is the major metabolic pathway of nicotine [15,40], Pianezza and co-workers [41] hypothesised that lack of CYP2A6 would affect smoking behaviour. It was shown that fewer subjects carrying defective *CYP2A6* alleles were found among smokers as compared to non-smokers and they also provided evidence that smokers carrying a defective *CYP2A6* allele smoked in general six fewer cigarettes per day. Because this study used the old genotyping method, it cannot be excluded that some individuals were misclassified and that the conclusions from the study might actually be different using a correct protocol.

In conclusion, we have shown the existence of two individuals with a true PM phenotype for CYP2A6 and developed a new genotyping method for the *CYP2A6*2* allele, which well predicted the CYP2A6 phenotype. These data stress the importance that genotyping methods are validated in large population studies on phenotyped individuals. The present method can be used in future studies aimed at determining the prevalence of subjects genetically deficient for one *CYP2A6* allele among those with chemically induced cancer, experiencing side effects of drugs which are CYP2A6 substrates, or having various degrees of nicotine dependence.

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