

Simultaneous detection of *CYP2C8*2*, *CYP2C8*3*, *CYP2C8*4* and *CYP2C8*5* alleles by a nested PCR method

Wardah Yusof^{*1,2} and Siew Hua Gan¹

¹ Department of Pharmacology, ² Central Research Lab, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia.

Received 28 Feb 2009 / Accepted 20 April 2009

Abstract. Cytochrome P450 (CYP) 2C8 plays a significant role in metabolizing many clinically important drugs such as paclitaxel, amiodarone and amodiaquine. A simultaneous detection of *CYP2C8*2*, *CYP2C8*3*, *CYP2C8*4* and *CYP2C8*5* alleles by a PCR method will be useful for detecting single nucleotide polymorphisms (SNPs) on the *CYP2C8* gene that may contribute to the inter-individual variability in metabolizing these drugs. This is a blinded randomised cross over study conducted in 24 healthy normal volunteers who received the anti-malarial drug amodiaquine and artesunate both as individual agents and in a fixed combination, over two study phases to determine the safety profile of the two preparations. Genomic DNA was isolated from the volunteers using a DNA extraction kit. A nested PCR method was applied to detect all of the *CYP2C8* variants. Regions from exons 3, 5 and 8 of the gene were simultaneously amplified using a first PCR step (PCR1). Products from PCR1 were then used to run the second PCR (PCR2) for detection of the variant alleles. This method was verified in 24 healthy Malaysian volunteers where all four variant *CYP2C8* alleles were successfully amplified and confirmed by DNA sequencing. We have developed and optimized a two-step multiplex nested PCR method for genotyping all of the important *CYP2C8* variants found to date.

Keywords: *CYP2C8*, genetic polymorphism, nested PCR

INTRODUCTION

Inter-individual variation in drug metabolism is a major cause of adverse drug events worldwide. It is estimated that genetic factors can account for up to 20 to 95% of variability in drug's disposition and effects (Ingelman-Sundberg, 2001). There are many examples of cases that show interindividual differences in drug response due to sequence variations or SNPs in genes encoding drug metabolizing enzymes (DME) such as cytochrome P450.

Phenotypes resulting from these genetic changes influence the rate of drug metabolism. This can result in the drug being either eliminated too quickly to be effective or accumulated to toxic levels. The impact of SNPs in the genes will be determined by its position in the coding sequence. SNPs could result in incorrect insertion or deletion of one or more nucleotides that may result in transcription of a protein with abnormal function contributing to decreased activity of enzymes as compared to the fully functional wild type gene (Sweeney, 2005). On the other hand, multiple bases polymorphism from gene deletions and conversions causing a frame shift and premature termination of translation will result in non-functional proteins (Ingelman-Sundberg, 2001).

The CYP super family of microsomal hemoproteins

is a large, highly polymorphic family of heme-containing mono-oxygenases that catalyses the monooxygenation of a large number of endogenous and exogenous compounds (Guengerich, 2003; Taavitsainen, 2001). Some CYPs play a role in both the formation and elimination of endogenous compounds, while some other CYPs, especially those from the families 1-3, seem to be principally-involved in xenobiotic metabolism (Nelson *et al.*, 1996). The cytochrome P450 (CYP) enzyme super family is primarily located in the liver and some are distributed in other tissues such as the intestine, lung, kidney and brain (Frye, 2004). The clinical implications of genetic variability in P450 genes depend on the function of the encoded enzyme, the drug and the chemical sensitivities to cancer (McKinnon and Evans, 2000). The most important cytochrome P450s involved in drug metabolisms are from the members of *CYP1*, *CYP2* and *CYP3* families (Taavitsainen, 2001; Walsky, and Obach, 2004).

In human, cytochrome P450C (CYP2C) subfamily includes *CYP2C8*, *CYP2C9*, *CYP2C18* and *CYP2C19* all of which are polymorphic. *CYP2C8*, *CYP2C9* and *CYP2C19* proteins are located in the liver where they account for 20%

* Author for correspondence:

Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia. Tel. 609-7664187. Fax. 609-7653370. Email address: wadiocomel@yahoo.com

of the total P450 (Shimada *et al.*, 1994). CYP2C18 proteins on the other hand, are primarily expressed in the skin. Among the four subfamilies, CYP2C8 genetic polymorphisms have only been described recently. The four variant alleles of CYP2C8 are CYP2C8*2, CYP2C8*3, CYP2C8*4 and CYP2C8*5. *In vitro*, CYP2C8*2 and CYP2C8*3 have been shown to encode for an enzyme with decreased activity (Dai *et al.*, 2001) while CYP2C8*4 appears to cause a decrease in CYP2C8 activity (Bahadur *et al.*, 2002). CYP2C8 plays a significant role in the metabolism of the anti-cancer drug paclitaxel (Polasek *et al.*, 2004), the anti-malarial drug amodiaquine (Walsky and Obach, 2004, Li *et al.*, 2002), amiodarone (Ohyama *et al.*, 2000), and ibuprofen (Martínez *et al.*, 2004). CYP2C8 also plays a secondary role in the metabolism of fluvastatin, perphenazine, gallopamil and omeprazole [reviewed by Martínez *et al.*, 2004].

Previously described genotyping methods for CYP2C8*2, CYP2C8*3, CYP2C8*4 and CYP2C8*5 alleles [Dai *et al.*, 2001, Nakajima *et al.*, 2003] involve multiple amplification reactions and PCR cycles. The methods were tedious, thus delaying genotyping process especially when a large number of subjects in population studies are involved. Muthiah *et al.*, (2004 & 2005) have also studied the CYP2C8*2, CYP2C8*3, CYP2C8*4 alleles among Malaysian subjects as they are commonly found among Asians. Unfortunately, the CYP2C8*5 allele which was detected among the Japanese (Nakajima *et al.*, 2003) and may be present among Malaysians was not investigated. Therefore, in this study a simple nested multiplex PCR method for the simultaneous detection of the four CYP2C8 alleles has been developed. Our simultaneous detection method is more rapid and cost effective for the detection of genetic polymorphism that may contribute to the variability in drugs metabolised by this enzyme.

MATERIALS AND METHODS

Sample collection. This is a blinded randomised cross over study conducted in 24 healthy normal volunteers who received the anti-malarial drug amodiaquine and artesunate both as individual agents and in a fixed combination, over two study phases to determine the safety profile of the two preparations. Written informed consents were obtained from all volunteers and ethical approval was obtained from the local ethics committee in Universiti Sains Malaysia, Kubang Kerian, Kelantan. Human genomic DNA samples were isolated from peripheral blood lymphocytes by using QIAamp® DNA Blood Midi Kit (QIAGEN) as proposed by the manufacturer.

Genotyping of the CYP2C8 variant alleles. Nested multiplex PCR methods were developed to detect four variants of CYP2C8 genes which are CYP2C8*2, CYP2C8*3, CYP2C8*4 and CYP2C8*5. Regions from exons 3, 5 and 8 of CYP2C8 genes were simultaneously amplified by the first PCR (PCR1). The products were then

used in the second PCR (PCR2) for concurrent detection of CYP2C8 variant alleles. Genotyping of the CYP2C8*2, CYP2C8*3, CYP2C8*4 [Dai *et al.*, 2001, Muthiah *et al.*, 2004, Muthiah *et al.*, 2005] and CYP2C8*5 (Nakajima *et al.*, 2003) alleles were initially performed as reported previously. When none of the detected mutants were detected, it is assumed to be the wild-type CYP2C8*1 gene.

To enable simultaneous genotyping of all four amplicons, primers shown in Tables 1 and 2 were used. PCR1 was optimized by gradually increasing annealing temperatures and primer concentrations. The optimization was performed under the following conditions: an initial denaturation step at 94°C for 3 mins, followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 45 secs and extension at 72°C for 1 min followed by a final extension at 72°C for 10 mins. Genomic DNA samples (200 ng) (n= 24) were then added to the PCR mixtures (25 µl) which consisted of a 1X PCR buffer [10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)], 1.5 mM MgCl₂, 0.4 mM dNTPs, primers (concentration as shown in Table 3) and 1.5U of Taq DNA polymerase (Biotoools, B&M Lab, Spain).

The second PCR step was similar to PCR1. After optimization, PCR2 products were amplified in a 25 µl PCR mixtures consisting of a 2.5 µl diluted PCR1 product (1 in 25 dilutions), 1 X PCR buffer [10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)], 2.5 mM MgCl₂, 0.6 mM dNTPs, primer concentration as shown in Table 3 and 1.5 U of Taq DNA polymerase (Biotoools, B&M Lab, Spain). Two parallel allele-specific reactions containing the wild or mutant-specific type primer respectively were subjected to the following PCR condition: an initial denaturation step at 94°C for 3 mins, followed by 15 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 45 secs and extension at 72°C for 1 min and a final extension at 72°C for 10 mins. Both products for PCR1 and PCR2 were run on SubCell®GT (BIORAD, California, USA) electrophoresis system at 100 volts for 3 hours on a 4% agarose gel (Promega, Madison, USA).

DNA sequencing. Uniplex reactions on all PCR1 and PCR2 products were sent for sequencing before the developed methods were tested on DNA samples extracted from each of the 24 healthy volunteers. Prior to that, the PCR products were purified with QIAquick® PCR Purification Kit (QIAGEN).

Table 1. Primer sequences used in the amplifications of exons 3, 5 and 8 of the *CYP2C8* gene

Exon	Primer name	Sequence	References
3	Forward	CYP2C8-FW3 (III)	5'- agg caa ttc ccc aat atc tc -3'
	Reverse	2C8Ex35R	5'- gag tca ccc acc ctt gtt tt-3'
5	Forward	CYP2C8-FW5 (II)	5'- tct gca ata att tcc ctc tac-3'
	Reverse	CYP2C8-REVEX5	5'- atc ctt agt aaa tta cag aag g-3'
8	Forward	CYP2C8-FW8 (II)	5'- ggc aca acc ata atg gca tta-3'
	Reverse	CYP2C8-REVEX8	5'- ctg ctg aga aag gca tga ag-3'

Table 2. Primer sequences used in the amplifications of the *CYP2C8**2, *3, *4 and *5 alleles

Variants	Name of primers	Primer sequence	Reference
<i>CYP2C8</i> *2	Forward	CYP2C8-FW5	5'- gtc tgc aat aat ttc cct ct-3'
	Rev wild _a	CYP2C8-REVEX5(*2)W	5'- ctt acc tgc tcc att ttg at-3'
	Rev mut _b	CYP2C8-REVEX5(*2)M	5'- ctt acc tgc tcc att ttg aa-3'
<i>CYP2C8</i> *3	Forward	CYP2C8-FWEX3	5'- gaa tca ttt cca gca atg ga-3'
	Rev wild	CYP2C8-REVEX3 (*3)W	5'- cac ggt cct caa tgc tcc-3'
	Rev mut	CYP2C8-REVEX3 (*3)M	5'- cac ggt cct caa tgc tct-3'
<i>CYP2C8</i> *3	For wild _c	CYP2C8-FWEX8 (*3)W	5'- ccg tgc tac atg atg aca a-3'
	For mut _d	CYP2C8-FWEX8 (*3)M	5'- ccg tgc tac atg atg aca g-3'
	Reverse	CYP2C8-REVEX8	5'- ctg ctg aga aag gca tga ag-3'
<i>CYP2C8</i> *4	Forward	CYP2C8-FW5	5'- gtc tgc aat aat ttc cct ct-3'
	Rev wild	CYP2C8-REVEX5 (*4)W	5'- ttt tga tca gga agc aat cg-3'
	Rev mut	CYP2C8-REVEX5 (*4)M	5'- ttt tga tca gga agc aat cc-3'
<i>CYP2C8</i> *5	Forward	CYP2C8-FWEX3	5'- gaa tca ttt cca gca atg ga-3'
	Rev wild	A475del-wt	5'- tca ccc acc ctt ggt ttt t-3'
	Rev mut	A475del-mt	5'- tca ccc acc ctt ggt ttt c-3'

- a) Rev wild: reverse-wild primer;
 b) Rev mut: reverse-mutant primer;
 c) For wild: forward-wild primer;
 d) For mut: forward mutant primer

Table 3. The optimized primer concentrations and annealing temperature for PCR2

Exon	Variant	Primer	Annealing temp (°C)	Primer conc. (µM)	Product size (bp)
3	*3	CYP2C8-FWEX3	61	0.2	102
		CYP2C8-REVEX3(*3)W/M		“	
	*5	CYP2C8-FWEX3	“	“	161
		A475del-wt/mt		“	
5	*2	CYP2C8-FW5	“	“	182
		CYP2C8-REVEX5(*2)W/M		“	
	*4	CYP2C8-FW5	“	“	169
		CYP2C8-REVEX5(*4)W/M		“	
8	*3	CYP2C8-FWEX8 (*3)W/M	“	“	114
		CYP2C8-REVEX8		“	

RESULTS

In the present study, a simple nested multiplex PCR method was developed to enable simultaneous genotyping of *CYP2C8**2, *3,*4 and *5 alleles. Initially, amplifications of exons 3, 5 and 8 of the *CYP2C8* gene were performed as described by Muthiah *et al.* 2004 & 2005. Primers that amplified exons 3, 5 and 8 produced PCR products of band sizes 347 bp, 258 bp and 142 bp, respectively. Briefly, Muthiah *et al.*, (2004) performed the first multiplex PCR with primer concentration of 0.2 µM for exon 3, 1.0 µM for exon 5 and 0.15 µM for exon 8. The PCR was run at an annealing temperature of 60°C. Products from exons 3, 5 and 8 were then used as templates for allele-specific amplification of the *CYP2C8* variants. *CYP2C8**3 mutated sequences are detected in exons 3 and 8, while exon 5 carries the *CYP2C8**2 and *CYP2C8**4 variants. Detection of *CYP2C8**5 alleles was first carried out as described by Nakajima *et al.*, (2003) in which both wild and mutant sequences were amplified directly with 0.4 µM primer concentration. At an annealing temperature of 51°C, the amplifications produced PCR products with band sizes of 370 bp.

In order to simultaneously amplify the mutated sequences of *CYP2C8**5, the reverse primer of exon 3 was changed to 5'-gagtcacccaccttggtt-3' (2C8Ex35R). This is because the CYP2C8-FW3 (III) and 2C8Ex35R primers flank the mutated loci of both *CYP2C8**3 and *CYP2C8**5 variants in exon 3. This amplification produces a 363 bp PCR product. After a successful amplification of uniplex PCR1, the exons were subjected to a multiplex PCR where all of the primers needed for the amplification of all three exons were combined in a single tube.

As the first step in designing the PCR condition for multiplex PCR1, all primers were added in equal molarities. This gave an indication of how PCR parameters such as individual primer concentration, dNTPs and magnesium chloride (MgCl₂) as well as PCR cycle conditions, could further be modified. The second step in optimizing a multiplex PCR1 involved running a gradient PCR between the temperature

range of 55°C and 62°C. In a gradient PCR, aliquots of reaction mixtures were subjected to a range of annealing temperatures. This will assist the researcher in choosing the best annealing temperature that will produce the most consistent yield. From the gradient PCR performed, it can be concluded that an annealing temperature of 58°C amplified the best PCR bands for all of the three exons when compared to other temperatures [Figure 1(A)].

Determination of optimized primer concentration for the multiplex PCR were done at 58°C by gradually increasing the primers' concentrations. Primers amplifying exon 3 were maintained at 0.1 µM and 0.15µM [18] while the concentrations of primers amplifying the other two exons were increased gradually from 0.1 to 0.4 µM [Figure 1(B)]. The optimum annealing temperature was determined to be at 58°C and primers concentrations for exons 3, 5 and 8 at 0.15, 1.0 and 0.2 µM respectively as observed on lane 4 in Figure 1(B).

In the second multiplex PCR (PCR2), primers used for allele-specific (AS) amplifications of all the alleles were designed by Muthiah *et al.*, (2004) and Nakajima *et al.*, (2003) with only 1 to 2 nucleotide bases difference between the wild and mutant type primers for each allele. The primer structure was designed as such, to ensure that both wild and mutant alleles will be amplified under the same PCR condition.

Simultaneous detection of the *CYP2C8**3 and *CYP2C8**5 alleles located on exon 3 was performed by using the same forward primer, CYP2C8-FWEX3. This eliminates the need for primer CYP2C8-FW (III) as used by Nakajima *et al.*, (2003) for the amplification of *CYP2C8**5. Allele specific amplifications of *CYP2C8**3 and *CYP2C8**5 yielded PCR products of band sizes 102 bp and 161 bp respectively. Duplex detection of *CYP2C8**3 and *CYP2C8**5 was done beforehand to determine the best ratio of the three primers to be involved in the amplification of both alleles before proceeding to the multiplex PCR. Amplification of *CYP2C8**2 and *CYP2C8**4 alleles from exon 5 were also carried out by using a common forward primer, CYP2C8-

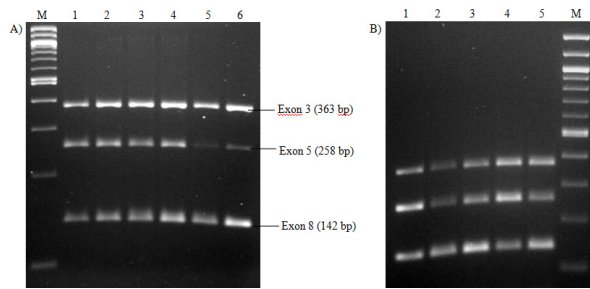


Figure 1. Optimization of PCR1 multiplex cycle and reaction. A) Gradient PCR of annealing temperatures from 55°C to 62°C. Lane 1; 56.2°C, Lane 2; 57°C, Lane 3; 58°C, Lane 4; 59°C, Lane 5; 60.2°C, Lane 6; 61°C, Lane M; 100 bp DNA Ladder (New England BioLabs). B) Optimization of primer concentration for multiplex PCR1. Primer concentration Lane 1; 0.1 μM (exon 3), 1.0 μM (exon 5), 0.2 μM (exon 8); Lane 2; 0.1 μM (exon 3), 1.0 μM (exon 5), 0.3 μM (exon 8); Lane 3; 0.1 μM (exon 3), 1.0 μM (exon 5), 0.4 μM (exon 8); Lane 4; 0.15 μM (exon 3), 1.0 μM (exon 5), 0.2 μM (exon 8); Lane 5; 0.15 μM (exon 3), 1.0 μM (exon 5), 0.3 μM (exon 8); Lane M; 100 bp DNA Ladder (New England BioLabs).

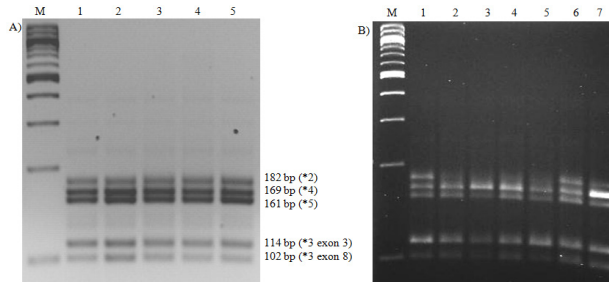


Figure 2. Optimization of multiplex PCR2. A) Optimization of PCR mixture reagents' concentration. Lane 1; 6.0 mM MgCl₂, 0.4 mM dNTPs, 1.5U Taq DNA Polymerase, Lane 2; 5.0 mM MgCl₂, 0.6 mM dNTPs, 1.5U Taq DNA Polymerase, Lane 3; 5.0 mM MgCl₂, 0.4 mM dNTPs, 2.0U Taq DNA Polymerase, Lane 4; 6.0 mM MgCl₂, 0.6 mM dNTPs, 1.5U Taq DNA Polymerase, Lane 5; 6.0 mM MgCl₂, 0.6 mM dNTPs, 2.0U Taq DNA Polymerase, Lane M, 100 bp DNA Ladder (New England BioLabs). B) Optimization of primers' concentration. Lane 1; 0.3 μM (*2), 0.2 μM (*3 exon 3), 0.3 μM (*3 exon 8), 0.15 μM (*4), 0.2 μM (*5), Lane 2; 0.2 μM (*2), 0.2 μM (*3 exon 3), 0.3 μM (*3 exon 8), 0.2 μM (*4), 0.2 μM (*5), Lane 3; 0.15 μM (*2), 0.2 μM (*3 exon 3), 0.3 μM (*3 exon 8), 0.3 μM (*4), 0.2 μM (*5), Lane 4; 0.15 μM (*2), 0.2 μM (*3 exon 3), 0.2 μM (*3 exon 8), 0.2 μM (*4), 0.2 μM (*5), Lane 5; 0.15 μM (*2), 0.2 μM (*3 exon 3), 0.3 μM (*3 exon 8), 0.2 μM (*4), 0.2 μM (*5), Lane 6; 0.2 μM (*2), 0.2 μM (*3 exon 3), 0.2 μM (*3 exon 8), 0.2 μM (*4), 0.2 μM (*5), Lane 7; 0.2 μM (*2), 0.2 μM (*3 exon 3), 0.3 μM (*3 exon 8), 0.3 μM (*4), 0.2 μM (*5), Lane M, 100 bp DNA Ladder (New England BioLabs).

FW5 with a 2:1 ratio of common forward to reverse primers of *CYP2C8*2* and *CYP2C8*4*.

Multiplex reaction on all five loci was optimized in two stages. The first stage focused on optimizing basic PCR mixture reagents; MgCl₂, dNTPs and Taq DNA polymerase. Concentration of each component was optimized before proceeding to optimizing the primers' concentration. For PCR2, concentrations of 5.0 mM MgCl₂, 0.6 mM dNTPs and 1.5U Taq DNA polymerase [Lane 2, Figure 2(A)] were found to produce the brightest bands while optimized primer concentrations could be observed in [Lane 6, Figure

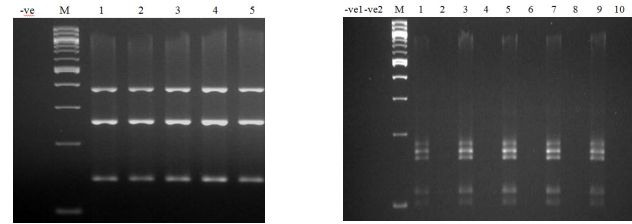


Figure 3. Genotyping of healthy volunteers by using the developed PCR methods. A) Amplification of *CYP2C8* exons 1, 3 and 5 using a multiplex PCR1. Lane 1; subject 1, Lane 2; subject 2, Lane 3; subject 3, Lane 4; subject 4, Lane 5; subject 5, Lane M, 100bp DNA Ladder (New England Biolabs). B) Amplification of *CYP2C8*2*, *3, *4 and *5 variants. Lane 1; subject 1 wild type, Lane 2; subject 1 mutant type, Lane 3; subject 2 wild type, Lane 4; subject 2 mutant type, Lane 5; subject 3 wild type, Lane 6; subject 3 mutant type, Lane 7; subject 4 wild type, Lane 8; subject 4 mutant type, Lane 9; subject 5 wild type, Lane 10; subject 5 mutant type, Lane M, 100bp DNA Ladder (New England Biolabs).

2(B)]. The optimized multiplex reaction and conditions are as shown in Table 3.

Since no positive controls were used in this study, DNA sequencing on all products for both PCR1 and PCR2 was done to confirm the sequences of the variant loci. Results from the bidirectional sequencing of wild type allele sequences were analyzed and the sequencing results were then verified against the published sequence of homo sapiens cytochrome P450, family 2, subfamily C, polypeptide 8 (*CYP2C8*) gene (GenBank accession no. AY514490). Although there was no positive control, the sequencing result of the wild-type allele sequences confirmed the specificity of the amplified fragments at all five loci.

The optimized multiplex nested PCR developed was then tested on 24 healthy volunteers for reproducibility as shown in Figure 3. None of the reported *CYP2C8* alleles were detected among the 24 healthy volunteers recruited perhaps due to the small sample size.

DISCUSSION

In the present study, a nested multiplex allele-specific PCR was performed to enable a more rapid and sensitive detection of polymorphism in the *CYP2C8* gene. Multiplex PCR is a type of PCR in which two or more loci are simultaneously amplified in a single reaction (Henegariu *et al.*, 1997). However, establishing multiplex PCR1 and PCR2 methods is both tedious and time-consuming as it involves the usage of multiple pairs of primers for amplifications at different loci. Multiplex PCR design and optimization usually focus on primers and experimental conditions such as MgCl₂, dNTPs and reaction buffer concentrations, annealing temperature and DNA polymerase concentration (Henegariu *et al.*, 1997, Chamberlain and Chamberlain, 1994). The primers used need to have similar melting temperatures (T_m) and should not exhibit significant interactions with each other,

or among themselves, or with unwanted regions of the template (Schoske *et al.*, 2003).

In this study, the primers incorporated for all three exons are able to differentiate fragments of *CYP2C8* genes from *CYP2C9* even though they share a very close structural homology. The amplicons were then used as templates for allele-specific amplification of all four of the *CYP2C8* variants found to date for ease of use. All amplicons from PCR1 and PCR2 were then sent for sequencing to confirm the specificity of the methods developed.

In our study, we did not detect any mutations among the four investigated alleles perhaps because of our small sample size coupled with the fact that most of our subjects were of Malay in origin. This is supported by the study done by Muthiah *et al.*, 2004, who investigated three variant alleles (*CYP2C8*2*, **3* and **4*) among 548 volunteers among the Malay, Chinese and Indian origins and managed to detect only the *CYP2C8*2* and *CYP2C8*3* variants among the Indians (with allele frequencies of 0.8 and 1.2 respectively) but no mutations were detected among the Malays and Chinese volunteers. The prevalence of *CYP2C8*5* in the Malaysian population has so far, not been reported and our study did not detect any variants for this allele.

CONCLUSION

In this study, a nested PCR method for the simultaneous detection of four *CYP2C8* variants (**2*, **3*, **4* and **5*) were successfully developed. The use of nested PCR in this allele-specific PCR increases the specificity of the amplification reaction with exons 3, 5 and 8 being simultaneously amplified in the first PCR before its product was concurrently used as templates for the second PCR reaction. The developed second multiplex PCR method is able to amplify *CYP2C8*2*, *CYP2C8*3*, *CYP2C8*4* and *CYP2C8*5* alleles simultaneously. No mutations were detected among the volunteers studied.

ACKNOWLEDGEMENT

We would like to thank the Ministry of Science and Technology Malaysia for their financial support under the ScienceFund grant (305/PPSP/6113206) and Universiti Sains Malaysia for the scholarship awarded to the researcher under the USM Fellowship Programme.

REFERENCES

- Badhur, N., Leathart, J.B.S., Mutch, E., Crespi, D.S., Dunn, S.A., Gilisen, R., Van Houdt, J., Hendrickx, J., Mannens, G., Bohets, H., Williams, F.M., Armstrong, M., Crespi, C.L. and Daly, A.K. 2002. *CYP2C8* polymorphism in Caucasians and their relationship with paclitaxel 6 α -hydroxylase activity in human liver chromosomes. *Biochemical Pharmacology* 64: 1579-89.
- Chamberlain, J.S. and Chamberlain, J.R. 1994. Optimization of multiplex PCRs. In *The Polymerase Chain Reaction*, eds. Eds Mullis, K.B., Ferré, F., Gibbs, R.A., pp. 38-46. Boston: Birkhäuser.
- Dai, D., Zeldin, D.C., Blaisdell, J.A., Chanas, B., Coulter, S.J., Ghanayem, B.I. and Goldstein, J.A. 2001. Polymorphisms in human *CYP2C8* decrease metabolism of the anticancer drug paclitaxel and arachidonic acid. *Pharmacogenetics* 11: 597-607.
- Frye, R.F., 2004. Probing the world of cytochrome P450 enzymes. *Molecular Intervention* 4: 157-162.
- Guengerich, F.P., 2003. Cytochromes P450, drugs, and diseases. *Molecular Interventions* 3: 194 - 204.
- Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. 1997. Multiplex PCR: critical parameters and step-by-step protocol. *BioTechniques* 23: 504-511.
- Ingelman-Sundberg, M., 2001. Implications of polymorphic cytochrome P450-dependent drug metabolism for drug development. *Drug Metabolism and Disposition* 29: 570-573.
- Li, X.-Q., Björkman, A., Andersson, T.B., Ridderström, M. and Masimirembwa, C.M. 2002. Amodiaquine clearance and its metabolism to N-Desethylamodiaquine is mediated by *CYP2C8*: A new high affinity and turnover enzyme-specific probe substrate. *The Journal of Pharmacology and Experimental Therapeutics* 300: 399-407.
- Martínez, C., García-Martín, E., Blanco, G., Gamito, F.J.G., Ladero, J.M. and Agúndez, J.A.G. 2004. The effect of the cytochrome P450 *CYP2C8* polymorphism on the disposition of (R)-ibuprofen enantiomer in healthy subjects. *British Journal of Clinical Pharmacology* 59: 62-68.
- McKinnon, R.A. and Evans, A. M., 2000. Cytochrome P450; Pharmacogenetics. *Australian Journal of Hospital Pharmacy* 30: 102-5.
- Muthiah, Y.D., Lee, W.L., Teh, L.K., Ong, C.E., Salleh, M.Z. and Ismail, R. 2004. A simple multiplex PCR method for the concurrent detection of three *CYP2C8* variants. *Clinica Chimica Acta* 349: 191-198.
- Muthiah, Y.D., Lee, W.L., Teh, L.K., Ong, C.E. and Ismail, R. 2005. Genetic polymorphism of *CYP2C8* in three Malaysian ethnics: *CYP2C8*2* and *CYP2C8*3* are found

- in Malaysian Indians. *Journal of Clinical Pharmacy and Therapeutics* 30: 487–490.
- Nakajima, M., Fujuki, Y., Noda, K., Ohtsuka, H., Ohkuni, H., Kyo, S., Inoue, M., Kuroiwa, Y. and Yokoi, T. 2003. Genetic polymorphisms of *CYP2C8* in Japanese population. *Drug Metabolism Disposition* 31: 687-90.
- Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J, Estabrook, R., Gunsalus, I.C. & Nebert, D.W., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6: 1-42.
- Ohyama, K., Nakajima, M., Nakamura, S., Shimada, N., Yamazaki, H. and Yokoi, I. 2000. A significant role of human cytochrome P450C8 in amiodarone N-deethylation: an approach to predict the contribution with relative factor. *Drug Metabolism and Disposition* 28: 1303-1310.
- Polasek, T.M., Elliot, D.J., Lewis, B.C. and Miners, J.O. 2004. Mechanism-based inactivation of human cytochrome P450C8 by drugs in vitro. *The Journal of Pharmacology and Experimental Therapeutics* 311: 996–1007.
- Schoske, R., Vallone, P.M., Ruitberg, C.M., Butler, J.M. 2003. Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci. *Analytical and Bioanalytical Chemistry* 375: 333–343.
- Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and Guengerich, F.P. 1994. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics* 270: 414-423.
- Sweeney, B.P., 2005. *In: Pharmacogenomics: the genetic basis for variability in drug response* In: Cashman,, J. N. and Grounds, R. M. (Eds), *Recent Advances in Anaesthesia and Intensive Care*. Excerpt. Cambridge University Press, pp. 1-34.
- Taavitsainen, P., 2001. *In: Cytochrome P450 isoform-specific in vitro methods to predict drug metabolism and interactions*. Academic Dissertation, University of Oulu.
- Walsky, R.L. and Obach R. S. 2004. Validated assays for human cytochrome P450 activities. *Drug Metabolism and Disposition*. 32: 647-660.