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Forensic toxicology: development of an SNP-assay for genotyping *CYP2D6* and *CYP2C19* variants

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Abstract. A procedure was developed for genotyping one *CYP2C19* and eight *CYP2D6* polymorphisms. After multiplex PCR of the exons containing the SNPs, a mini-sequencing reaction was performed with the SNapShot™ Multiplex Kit and the resultant primer-extension products were analyzed on an ABI PRISM® 3100 Genetic Analyzer. The developed procedure was validated in a population of 199 Caucasians.

Keywords: Pharmacogenetics; Single nucleotide polymorphism; Mini-sequencing; Cytochrome *P450*

1. Introduction

Forensic toxicology can contribute substantially to the interpretation and the determination of the causes and manners of death. Most fatal intoxications concern suicidal overdoses, but during recent years, chronic high dosage has attracted much attention. A high ratio between the parent drug and the metabolite could be interpreted not only as an overdose but could also be the result of a reduced metabolism of the parent drug by enzymes belonging to the cytochrome *P450* family. Several of these enzymes, such as *CYP2D6* and *CYP2C19,* are known to be polymorphic resulting in a different metabolic behaviour. The presence of the polymorphism is population dependent with about 5–10% of the Caucasian population belonging to the poor metabolisers [1]. Determination of the presence of a mutation in *CYP2D6* or *CYP2C19* may provide answers concerning the circumstances of death in cases with suspicion of overdose [2]. The present study describes the first results of the development of an Single Nucleotide Polymorphism (SNP)-assay for genotyping one *CYP2C19* and eight *CYP2D6* alleles. These assays are capable to identify at least 83% (*CYP2C19*) to 95% (*CYP2D6*) of the poor metabolizers in a Caucasian population.

2. Materials and methods

DNA was extracted by the chelex method from blood samples of 199 anonymous donors and quantified with the Quantiblot DNA Quantification Kit (Applied Biosystems).

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Two multiplex PCR-reactions were developed for the amplification of the coding sequences of *CYP2C19* and *CYP2D6*. Exons 4 (198 bp) and 5 (168 bp) of the *CYP2C19* were amplified in one reaction while exons 1 (519 bp), 3 and 4 (460 bp), and 5 and 6 (609 bp) of *CYP2D6* were amplified in a second PCR. The sequences of the PCR-primers and the SNapShot™ primers are available upon request. The amplification was done in a 25-μl reaction containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 U AmpliTaq Gold DNA-polymerase (Applied Biosystems), 1 × PCR-Gold-buffer, primers and 5 ng DNA. The PCR-conditions were as follows: denaturation of 10 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and final extension of 10 min at 72 °C in a GeneAmp® PCR System 9700.

The primers for genotyping *CYP2C19*2* and the *CYP2D6* alleles were selected with the READIT® Interrogation Probe Design software from Promega (http://www.promega.com/readit/probedesign/default.asp). The 10 detection primers (Table 1) for *CYP2D6* genotyping were combined into two SNapShot™ reactions. Free dNTPs and primers from the PCR-reaction were removed with Shrimp Alkaline Phosphatase (SAP) and Exonuclease I as described in the SNapShot™ manual. The SNapShot™ reactions and a final treatment with SAP were done according to the recommendations of the manufacturer. Sizing of the extension products was done in POP-6™ on an ABI PRISM® 3100 Genetic Analyzer with GeneScan™-120 LIZ™ as the internal size marker. The results were analyzed with GeneScan® v3.7.

3. Results and discussion

A multiplex PCR was developed for exons 4 and 5 of the *CYP2C19* gene, which contain, respectively, the *CYP2C19*2* and *CYPC19*3* alleles. The *CYP2C19*3* allele was not yet included in the SNapShot™ reaction due to its low occurrence in a Caucasian population (0.2%). Validation of the developed method in a population of 199 individuals showed in total 6 individuals (3%) homozygous for the mutation (PM). The allele frequency for *CYP2C19*2* was 13.8% which was in accordance to other studies in Caucasian populations [2].

Table 1
Detected mutations (header) for *CYP2D6* and their corresponding alleles (first column)

Allele	2549delA	1846G>A	100C>T	1661G>C	1707delT	2935A>C	124G>A	2850C>T	138-139insT	1979T>C
CYP2D6*3	■									
CYP2D6*4		■	■	■						
CYP2D6*6					■					
CYP2D6*7						■				
CYP2D6*10			■	■						
CYP2D6*12				■			■	■		
CYP2D6*15									■	
CYP2D6*20				■				■		■

Gray boxes indicate the presence of the mutated position.

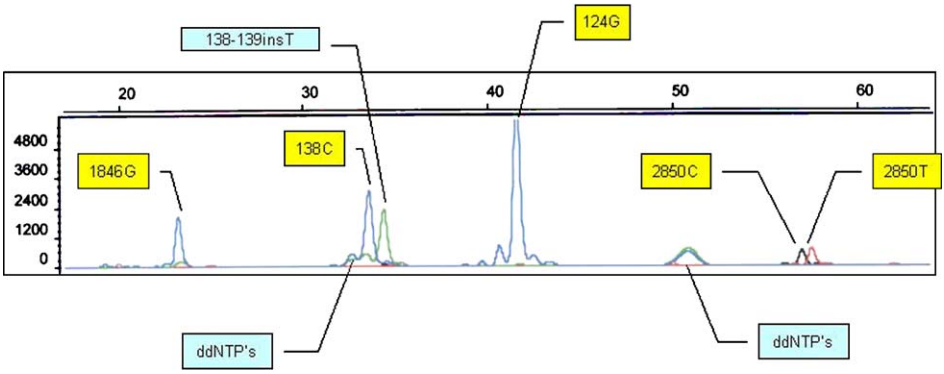

Fig. 1. SNaPshot™ result for genotyping *CYP2D6* (individual heterozygous for 2850C>T).

Exons 1, 3 and 4, and 5 and 6 of the *CYPDC6* gene were co-amplified in one PCR reaction. The primers for exon 1 co-amplified also several pseudogenes (*CYP2D7AP*, *CYP2D7BP* and *CYP2D8P*) of *CYP2D6*. Theoretically, it was possible to identify individuals homozygous for the *CYP2D6*5* allele (deletion of the complete gene; frequency of 2% in Caucasian populations). The 10 SNapShot™ primers were split into 2 SNapShot™ reactions after some initial experiments: one with 4 primers showing a high signal (Fig. 1) and one with 6 primers showing a lower signal. Validation of this assay in the population sample of 199 individuals showed a "clear" result for only 57 individuals. Several problems were encountered: (1) some SNapShot™ primers did not give any signal; (2) a background signal from free [F]-ddNTPs was seen that coincided with some of the SNapShot™ primers (Fig. 1); (3) the primer detecting mutation 138–139insT showed in most individuals a heterozygous result. The SNapShot™ primers showing a weak or no result hybridized to the "Exon 3–4" PCR-fragment indicating a not optimal amplification of this fragment. The background signal from the free [F]-ddNTPs could only be removed by replacing the SAP-treatment after the extension reaction with gel filtration through Sephadex G-50. The 138–139insT mutation is localized in exon 1 of the *CYP2D6* gene and is also present in several pseudogenes. As the multiplex PCR was set up to identify individuals with the gene deletion (*CYP2D6*5*), it was not possible to avoid initially this problem. Further experiments to overcome these problems are in progress and the results of the final *CYP2D6* assay will be reported elsewhere.

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