

DETECTION OF CYP2D6*6 ALLELE BY REAL TIME POLYMERASE CHAIN REACTION IN ROMANIAN POPULATION

ANDRIES ADRIAN*, CIOACA DANIELA, GUTIU IOAN AXENTE, OPREA IONUT, RADULESCU FLAVIAN, GEORGESCU ANA-MARIA

University of Medicine and Pharmacy "Carol Davila", Faculty of Pharmacy, 6 Traian Vuia street, 020956, Bucharest, Romania.

**corresponding author: aaandries@yahoo.co.uk*

Abstract

Cytochrome P450 2D6 isoform (CYP2D6) is one of the best-known polymorphic enzymes responsible for the metabolism of several drugs with considerable therapeutic impact. Mutation analysis and detection of CYP2D6 gene variations allow risk predictions for adverse drug responses or reduced therapeutic benefits. The distribution of CYP2D6*6 poor metabolizer allele was analyzed in a group of 37 Romanian healthy subjects by real time polymerase chain reaction (qPCR). The results indicated the presence of 4 heterozygotes (carrying a wild-type and a CYP2D6*6 allele) and 33 wild-type homozygotes subjects (carrying two functional wild-type alleles).

Rezumat

Izoforma 2D6 a citocromului P450 (CYP2D6) este una dintre cele mai cunoscute enzime polimorfe implicată în metabolismul mai multor substanțe medicamentoase cu impact terapeutic considerabil. Analiza mutațiilor și detecția variațiilor genei CYP2D6 permite analiza riscului apariției unor reacții adverse la diverse medicații sau reducerea beneficiilor terapeutice. Distribuția alelei CYP2D6*6, corespunzătoare izoformei de metabolizator lent, a fost analizată pe un grup de 37 de subiecți sănătoși români, prin reacția calitativă de polimerizare în lant (qPCR). Rezultatele au indicat prezența a 4 subiecți heterozigoți (purători a alelei normale și ai alelei CYP2D6*6) și a 33 de subiecți homozigoți (purători a două alele normale).

Keywords: CYP2D6*6, genotype, poor metabolizers.

Introduction

Cytochrome P450 isoenzymes are a group of heme-containing enzymes involved in the oxidative metabolism of an important group of xenobiotics [1]. The P450 genes are polymorphic and display a functional significance for drug metabolism as certain allelic variants exhibit either altered activity or complete absence of enzymatic activity. Cytochrome

CYP2D6 (CYP2D6) enzyme is one of the most important drug-metabolizing enzyme that metabolizes many drugs, such as antidepressants, antipsychotics, antiarrhythmics, opiates, antiemetic and beta-adrenergic receptor blocker drugs [2]. The distribution of functional, reduced function and nonfunctional CYP2D6 alleles shows racial/ethnic differences [3,4]. Genotyping techniques permit the identification of single-nucleotide polymorphisms (SNPs) and thereby a prediction of individual metabolic capacities for CYP2D6 substrates [5,6]. CYP2D6 activity ranges widely within a population comprising ultrarapid (UM), extensive (EM), intermediate (IM) and poor (PM) metabolizer phenotypes. The incidence of PM subjects carrying two alleles encoding a nonfunctional or unstable enzyme is 5–10% in Caucasians, 3.5–8% in African Americans and ~1% in Asians. Genotyping strategies comprising a relatively small number of allelic variants (*CYP2D6**3, *4, *5, *6) detect almost all poor metabolizers among the Caucasian population [7].

Several methods based on polymerase chain reaction (PCR) amplification of the CYP2D6 locus are used to genotype the PM alleles. The most widely used methods to detect PM alleles with small nucleotide mutations and deletions are restriction fragment length polymorphism (RFLP) analysis, allele-specific amplification (ASA) [8-12].

Our study focused on the detection of CYP2D6*6 allele on a group of 37 Romanian subjects by real time PCR technique using TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination CYP2D6*6 Kit (Applied Biosystems).

Materials and methods

As a part of the *CYP2D6* polymorphism inclusion criteria for a clinical study, a total of 37 healthy subjects were recruited for this research. The study protocol was reviewed and approved by an independent ethics committee. Volunteers were recruited from a single site, and each volunteer signed an informed consent before screening according to specific inclusion/exclusion criteria. The medical history of each subject was recorded in order to determine alcohol consumption or drug abuse pattern, donation of blood or plasma in the last 30 days, prescription or nonprescription drug intake in the last 2 weeks, participation in any investigational drug study in the last 30 days and any other condition that could have interfered with the study protocol specific evaluations. In addition, a urine drug screen was performed at the time of recruitment in order to check the presence of any drugs of abuse. Volunteers were asked to refrain from alcohol, coffee, tea,

chocolate, and any other xanthine containing beverages for 24 h before the start of the study and through its completion.

Genomic DNA was extracted and purified from EDTA-supplemented blood samples with the Wizard SV Genomic DNA Purification Kit (PROMEGA). Purified genomic DNA was subjected to 1% agarose gel electrophoresis, stained with ethidium bromide (500mg/L in water) and visualized under an UV transilluminator.

Identification of CYP2D6*6 allele was accomplished using TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination CYP2D6*6 Kit (Applied Biosystems) according to the protocol provided by the producer. Determinations were made in the presence of two controls, AL-1 (wild-type) and AL-2 (CYP2D6*6), using a Rotor-Gene Q 2plex 6000 real-time cycler machine with two channels (Qiagen, Germany).

The TaqMan Allelic Discrimination Kit employs a probe technology that exploits the 5'-3' nuclease activity of AmpliTaq Gold DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR.

Two TaqMan probes are used in this allelic discrimination assay, one probe for each allele in a two-allele system. Each probe consists of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. TET (6-carboxy-4,7,2',7'-tetrachlorofluorescein) is covalently linked to the 5' end of the probe for the detection of Allele 1. FAM (6-carboxyfluorescein) is covalently linked to the 5' end of the probe for the detection of Allele 2. Each of the reporters is quenched by TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) attached via a linker arm located at the 3' end of each probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product.

The AmpliTaq Gold enzyme cleaves the TaqMan probe with its 5'-3' nuclease activity. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter. The 3' end of the TaqMan probe is blocked to prevent extension of the probe during PCR.

The separation of the reporter dyes from the quencher dye results in increase in fluorescence for each of the FAM and TET reporters. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR. The fluorescence signals are generated only if the target sequences for the probes are amplified during PCR.

Allele 1 and Allele 2 probes supplied in the Probe and Primer Mix with the TaqMan Allelic Discrimination Kit are used with the specific Genomic Control DNA included in the kit.

The AL1 VIC dye-labeled probe correspond to the wilde-type. The AL2 FAM dye-labeled probe correspond to the mutant type (CYP2D6*6). The table below shows between fluorescence signals and sequence in a sample:

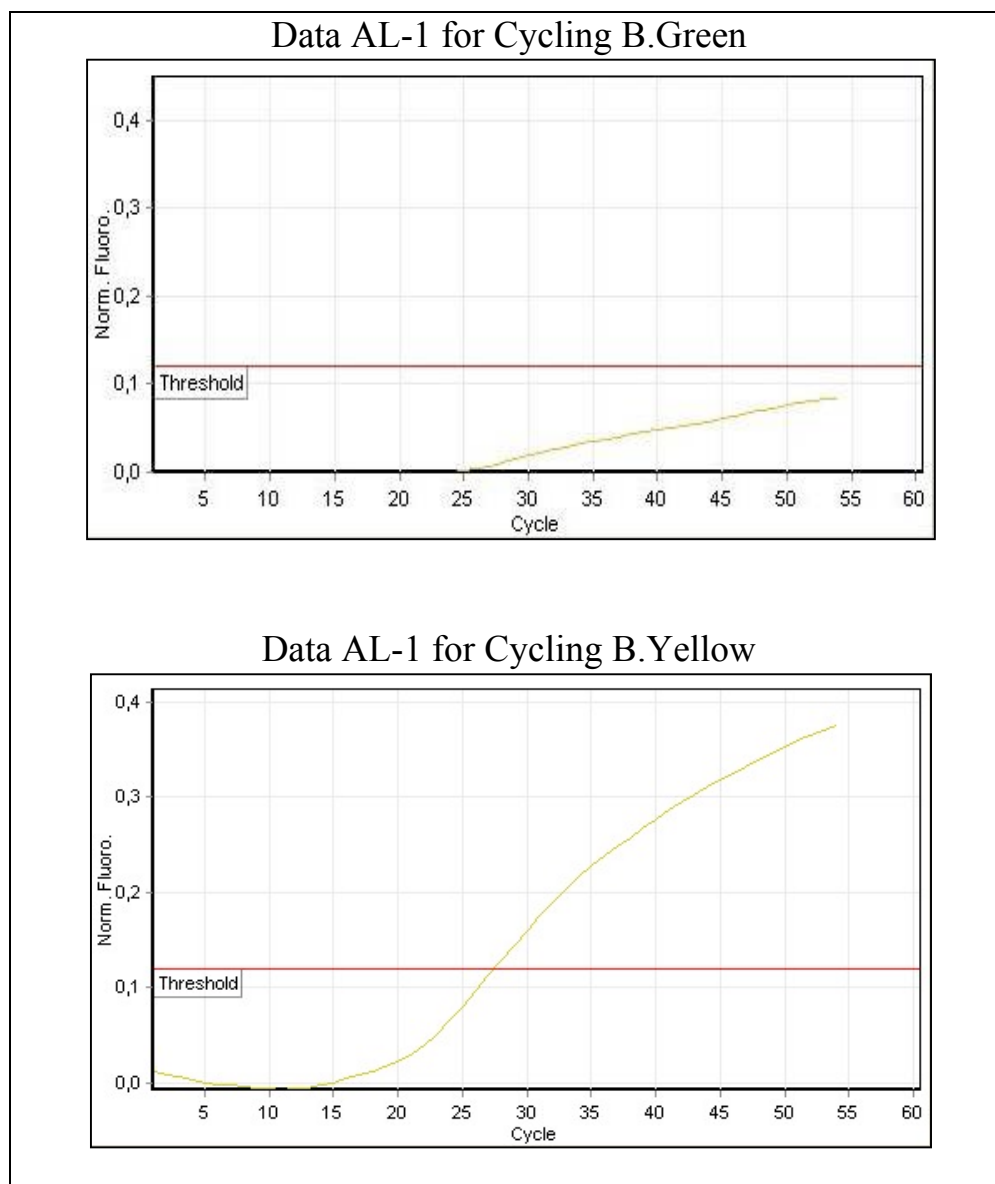
Table 1
Fluorescence signals for AL1 and AL2

	AL1 (VIC dye)	AL2 (FAM dye)	TYPE
Fluorescence signals	+	-	Homozygote wilde-type
	-	+	Homozygote CYP2D6*6
	+	+	Heterozygote wilde-type/ CYP2D6*6

Results and discussion

TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination CYP2D6*6 Kit (Applied Biosystems) were used on the purified genomic DNA, according to the protocol provided by the producer. Determinations were made in the presence of two controls, AL-1 (wild-type) and AL-2 (CYP2D6*6). See figures 1 and 2.

Our results indicated that 4 subjects were heterozygotes (carrying a wild-type allele and a CYP2D6*6 allele) and 33 subjects were wild-type homozygotes (carrying two functional wild-type alleles). The subject's phenotype, estimated by the metabolic ratio of metoprolol *versus* the inactive alpha-hydroxy metabolite, could not be predicted. For a 90-95% accuracy of prediction, it is necessary to analyze the presence of allelic variants CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6. See figures 3 and 4.

**Figure 1**

Normal fluorescence ($\log_{10}$ – logarithmic scale for AL-1 control sample)

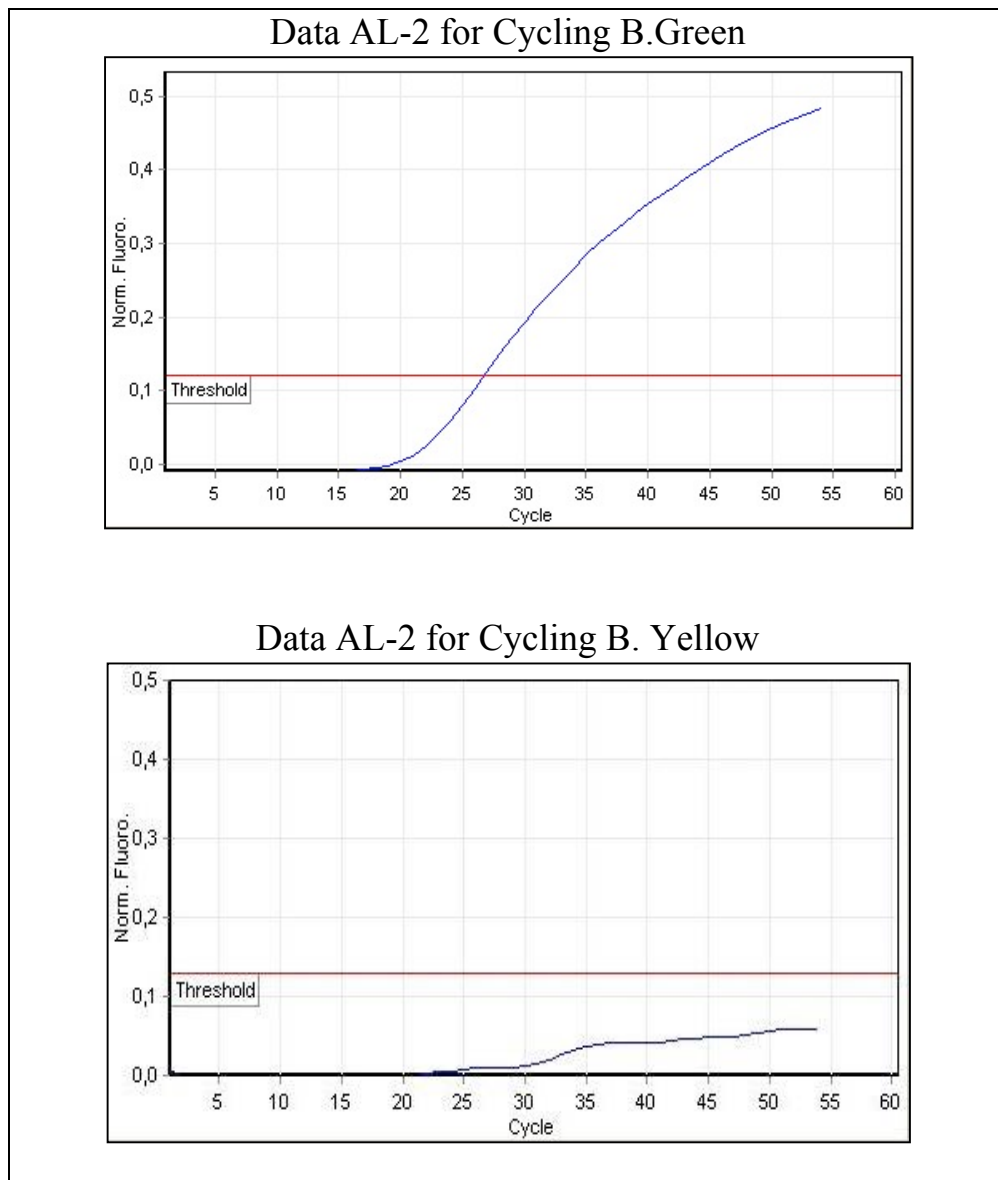


Figure 2
Data for AL-2 control

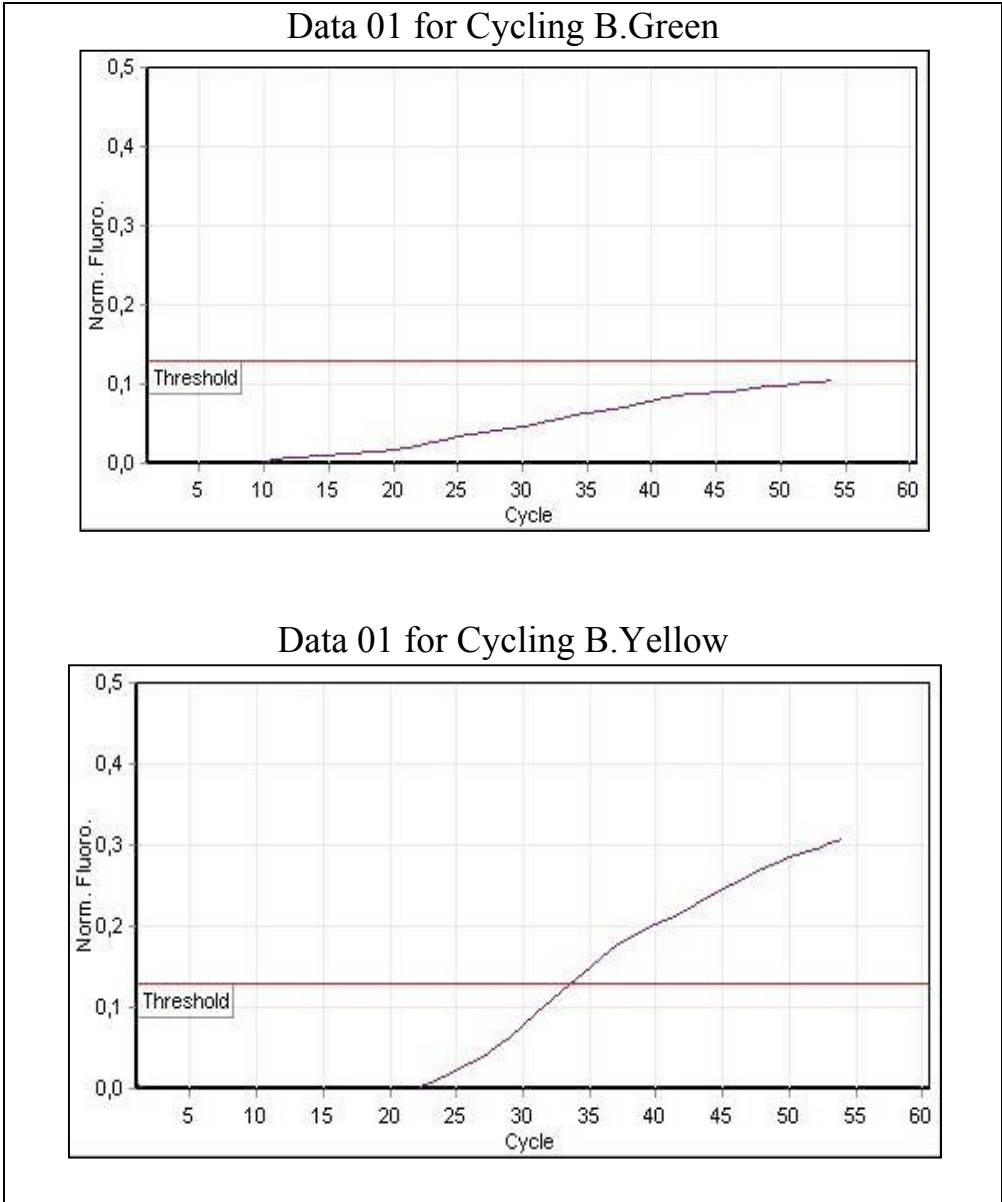
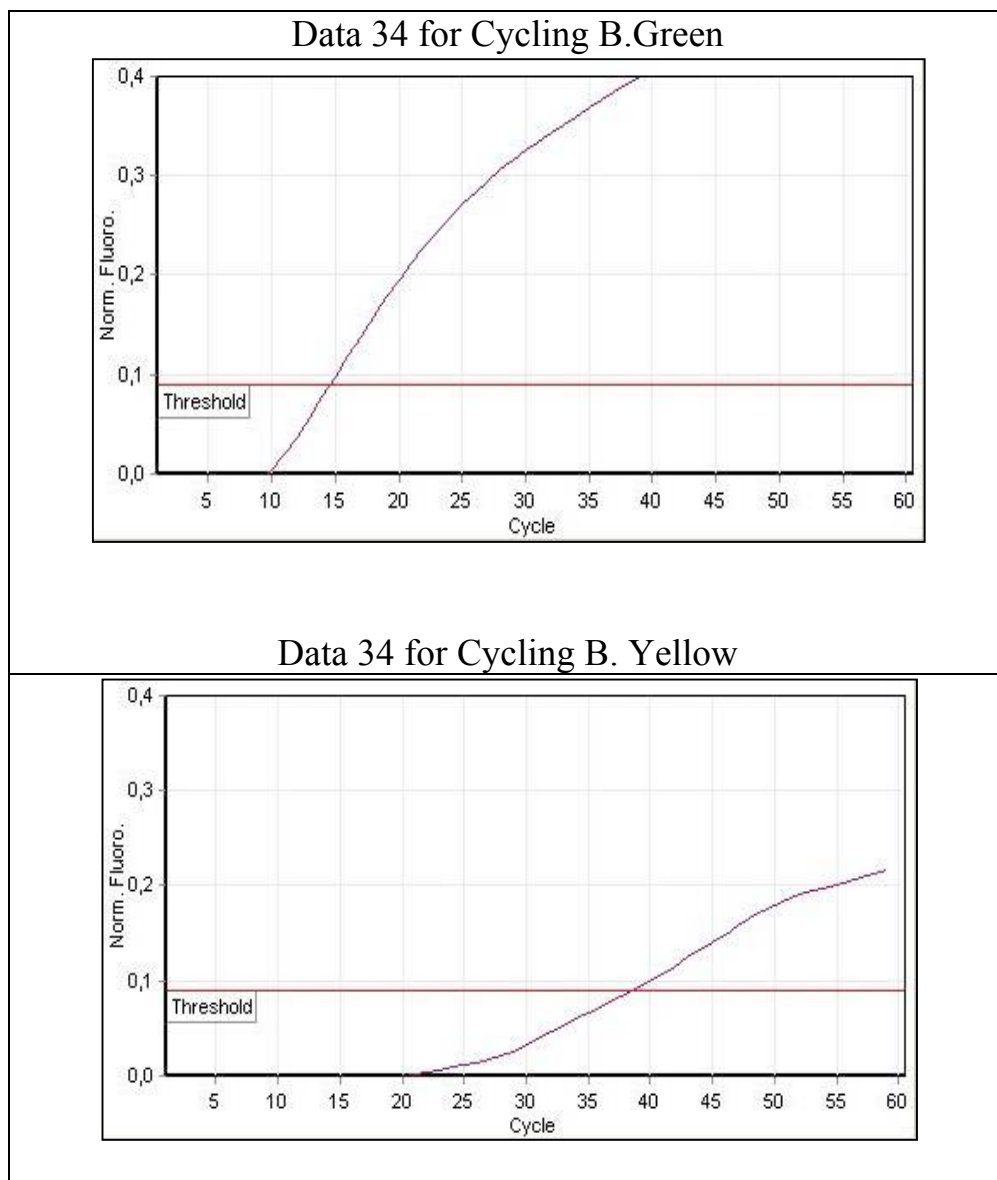


Figure 3
Data for subject 01 (homozygote wild-type)

**Figure 4**

Data for subject 34 (heterozygote)

Conclusions

CYP2D6 is the most widely studied member of cytochrome P450 family in order to identify various genetic mutations that could affect the biological activity of the enzyme and the metabolism process of many drugs. This is the first study focused on the detection of CYP2D6*6 allele

on a group of Romanian subjects by real time qPCR technique. Our data showed that 10% of the subjects were heterozygotes (carrying a wild-type allele and a CYP2D6*6 allele) and 90% of the subjects were wild-type homozygotes (carrying two functional wild-type alleles). We didn't identify poor metabolisers in the chosen group. Future extended studies on larger subject groups will be performed in order to obtain information about CYP2D6*6 allele distribution in the Romanian population. This data will be correlated with the distribution of the other most important poor metaboliser alleles.

Acknowledgements

This work was supported by the Romanian National Plan for Research Development and Innovation 2007 - 2013 grant 41-049/2007.

References

1. Gueguen Y., Mouzat K., Ferrari L., Tissandie E., Lobaccaro J.M., Batt A.M. *et al.*, Cytochromes P450: xenobiotic metabolism, regulation and clinical importance, *Ann Biol Clin (Paris)*, 2006, 64, 535-548.
2. Radulescu F., Miron D.S., Mircioiu C., Voicu V.A., Distribution Profiles of Drugs with Active Metabolites by Compartmental Analysis. *Farmacia*, 2007, LV, 390-395.
3. Kirchheiner J., Brockmoller J., Clinical consequences of cytochrome P450 2C9 polymorphisms, *Clin Pharmacol Ther*, 2005, 77, 1-16.
4. Lynch T., Price A., The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects, *Am Fam Physician*, 2007, 76, 391-396.
5. McElroy S., Sachse C., Brockmoller J., Richmond J., Lira M., Friedman D. *et al.*: CYP2D6 genotyping as an alternative to phenotyping for determination of metabolic status in a clinical trial setting, *AAPS PharmSci*, 2000, 2, E33.
6. Meyer U.A., Gut J., Genomics and the prediction of xenobiotic toxicity, *Toxicology*, 2002, 181-182, 463-466.
7. Marez D., Legrand M., Sabbagh N., Lo Guidice J.M., Spire C., Lafitte J.J. *et al.*: Polymorphism of the cytochrome P450 CYP2D6 gene in an European population: characterization of 48 mutations and 53 alleles, their frequencies and evolution, *Pharmacogenetics*, 1997, 7, 193-202.
8. Sandulovici R., Prasacu I., Mircioiu C., Voicu V., Medvedovici A., Anuța V., Mathematical and phenomenological criteria in selection of pharmacokinetic model for M1 metabolite of pentoxifylline, *Farmacia*, 2009, 57(2), 235-246.
9. McGuigan FE, Ralston SH: Single nucleotide polymorphism detection: allelic discrimination using TaqMan. *Psychiatr Genet* 2002, 12: 133-136.
10. Pop A., Vlase L., Leucuța S.E., Pharmacokinetic study of felodipine after single oral dose of slow release formulations in healthy volunteers, *Farmacia*, 2008, 56(5), 474-482.
11. Stamer UM, Bayerer B, Wolf S, Hoeft A, Stuber F: Rapid and reliable method for cytochrome P450 2D6 genotyping. *Clin Chem* 2002, 48: 1412-1417.
12. Syvanen AC: Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet* 2001, 2: 930-942.

Manuscript received: March 18th 2010