Int J Legal Med (2011) 125:265–269
DOI 10.1007/s00414-011-0550-0

ORIGINAL ARTICLE

Post-mortem ABCB1 genotyping reveals an elevated toxicity for female digoxin users

Anu M. Neuvonen · Jukka U. Palo · Antti Sajantila

Received: 8 September 2010 / Accepted: 10 January 2011 / Published online: 11 February 2011

Abstract
Background P-glycoprotein, a common transporter molecule, is known to affect metabolic functions in humans. Polymorphisms of the multidrug resistance gene (ABCB1/MDR1) coding for P-glycoprotein have been linked to changes in the processing of several commonly used medications in patients. Here, we have evaluated the impact of ABCB1 single nucleotide polymorphisms on digoxin fatality, through investigation of the relationship between post-mortem digoxin concentration and ABCB1 genotype.
Methods The effect of three ABCB1 single nucleotide polymorphisms (SNPs) (3435C>T, 1236C>T, and 2677G>T) on digoxin concentration was examined in 112 deceased Finnish subjects through RT-PCR genotyping of post-mortem blood samples. These subjects were selected on the basis of digoxin findings during the post-mortem toxicology screen, and categorized by digoxin concentration into three distinct groups. The distributions of mutant alleles and haplotypes in the deceased were compared to a random sample of 143 Finns.
Results Mutant genotype frequencies showed a positive relationship with post-mortem digoxin concentration for all SNPs. Female subjects showed a more emphatic pattern, suggesting a higher risk of digoxin intoxication.
Conclusions These findings demonstrate a link between ABCB1 polymorphisms and increased mortality, and suggest that individualized genotyping should be considered prior to digoxin treatment. This research also exemplifies the value of gender-segregated genotyping studies in helping establish drug safety parameters, while allowing more decisive determination of cause and manner of death in a medico-legal context.

Keywords ABCB1 · Gene · Single nucleotide polymorphism · Digoxin · Pharmacogenetics

A. M. Neuvonen (✉) · J. U. Palo · A. Sajantila
Department of Forensic Medicine, Hjelt-Institute,
Laboratory of Forensic Biology, University of Helsinki,
PO Box 40, (Kytösuontie 11),
Helsinki FI-00014, Finland
e-mail: anu.neuvonen@helsinki.fi

Introduction

Digoxin is a frequently used drug intended for treatment of heart conditions, e.g., atrial fibrillation and flutter, and in cases where patients fail to respond to other medication [1]. Digoxin has several concentration-dependent adverse effects, and these along with a narrow therapeutic range, slow excretion, and accumulation can contribute to its lethality. In post-mortem toxicology analyses, digoxin is a common finding [2] and can impede the decisive establishment of the cause and manner of death when the drug is present within the limits of toxicity.

General metabolic efficiency and the processing of xenobiotics such as digoxin are linked to the transporter P-glycoprotein, encoded by the ABCB1 (also known as MDR1) gene and expressed in multiple tissues [3]. In the past decade, the exact effect of single nucleotide polymorphisms of ABCB1 on digoxin processing has been a matter of controversy. Hoffmeyer et al. showed that subjects with the 3435C>T genotype displayed higher levels of digoxin in plasma after a single dose than those with wild-type alleles [4]. These results were contradicted in studies on Japanese subjects [5]; however, more recent research has confirmed the link between mutant genotype and decreased P-glycoprotein activity in Caucasian as well as Asian populations [6–8]. The cause of the discrepancy is still unknown and has been suggested to result, e.g., from

dietary differences; an alternate explanation or contributory factor may be genetic variation among populations [7, 9].

Two widely researched ABCB1 single nucleotide polymorphisms (SNPs), 3435C>T and 1236C>T, are synonymous polymorphisms on exons 26 and 21, respectively, while 2677G>T/A on exon 12 is nonsynonymous with a T-allele coding for serine instead of wild-type alanine. The 2677G>T/A has never, to our knowledge, been associated with any metabolic phenotype independently. The SNPs are strongly linked and the TTT (mutant) haplotype may play a stronger role in digoxin metabolism than any of the three singularly [6, 9, 10].

In this study, the relationship between digoxin concentration and genotype was investigated in post-mortem subjects. The aim was to evaluate the impact of ABCB1 polymorphisms on digoxin fatality, in order to (1) help establish safety parameters for digoxin in persons of various genotypes, and (2) aid forensic pathologists in correctly determining the cause and manner of death.

Methods

The effects of ABCB1 polymorphisms were assessed in individuals who died in the years 2000–2009, and were positive for serum digoxin in the post-mortem toxicology scan at the University of Helsinki, Department of Forensic Medicine. Permission to perform genotyping studies on post-mortem samples was accorded by Finnish law and by the National Supervisory Authority for Welfare and Health. The subjects were divided into three groups according to post-mortem digoxin concentration (<2.6, ≥2.6, and ≥7 nmol/L) (Table 1). Under 2.6 nmol/L is the therapeutic concentration for digoxin, while over 7 nmol/L is considered toxic. Individuals with cause of death established as disease, undetermined, or digoxin-related were included in the study while those determined as suicide or (non-digoxin-related) accident victims in the death certificate were omitted. The case group consisted of 112 individuals. Subjects with polypharmacy were assessed with the SFINX drug-interaction database [11] to identify potential interaction with digoxin. The database categorizes interacting drugs in terms of clinical significance (A–D, D the most serious) and strength of evidence (0–4, 4 being controlled, scientifically relevant patient studies). As a control group, 143 unrelated Finnish subjects were genotyped in order to establish allele frequency at the general population level.

Table 1 Case group mutant allele frequencies (*p*T) for three ABCB1 SNPs combined

	<2.6 nmol/L		≥2.6 nmol/L		≥7 nmol/L	
	*p*T	*N*	*p*T	*N*	*p*T	*N*
Female	0.548	126	0.598	112	0.767	60
Male	0.516	192	0.583	120	0.519	54
Total	0.528	318	0.591	232	0.649	114

N denotes number of genotyped alleles

For the case group, DNA extraction was performed from femoral blood stored on FTA paper, by Chelex resin (Bio-Rad, Hercules, USA) extraction. For the control group, DNA was extracted from blood using the EZNA SE blood DNA isolation kit (Omega Bio-Tek, Norcross, USA). The subjects were genotyped for ABCB1 SNPs 3435, 1236, and 2677 using RT-PCR with Taqman Universal PCR Master Mix No AmpErase UNG, with three custom-ordered genotyping assays including sequence-specific primers from Applied Biosystems (Foster City, CA) (Table 2) Cycling conditions consisted of a 10-min activation at 95°C, denaturing at 92°C for 15 s, and extension for 1 min at 60°C for 40 cycles.

Allele frequencies were determined for each group as a whole, and established for males and females separately in the case group (Table 1). Finnish mutant allele frequency was compared with published data from 14 other populations. Haplotype frequencies were estimated using Phase v. 2.1.1 [12] using 1,000 iterations with 1,000 iteration burn-in and thinning by ten. The runs were repeated five times with different starting seeds to ensure convergence. The haplotypic genotype frequencies were tested for deviations from the Hardy–Weinberg equilibrium using Arlequin v. 3.11 [13], and haplotypic differentiation between the random sample and different post-mortem sample groups were tested for statistical significance with the method implemented in Phase v.2.1.1 (1,000 permutations).

Results

Among the case samples, there was a positive correlation between the number of mutant alleles and the amount of digoxin in the blood. This was observed for each SNP individually as well as for the TTT haplotype. In addition, differences in genotype frequencies appear to be strongly gender-specific, with the trend more conspicuous in the female group.

Background allele frequency

The frequency distribution of three SNPs of the ABCB1 gene in the Finnish population was investigated in this study. For this control group, 143 subjects were genotyped. The proportion of mutant alleles in the Finnish population is considerable when compared to populations elsewhere in Europe (Fig. 1). This holds for each individual SNP; the mutant frequencies for 3435C>T (0.661), 1236C>T (0.542), and 2677G>T (0.564) were all higher than the European

Table 2 Primer sequences. Underlined bases designate location of single nucleotide polymorphisms

Assay	Primer/probe	Sequence
3435C→T		
	Forward primer	GCCGGGTGGTGTCACA
	Reverse primer	ATGTATGTTGGCCTCCTTTGCT
	VIC probe	CCCTCAC<u>G</u>ATCTCTT
	FAM probe	CCCTCAC<u>A</u>ATCTCTT
1236C→T		
	Forward primer	TCTCACTCGTCCTGGTAGATCTTG
	Reverse primer	CACCGTCTGCCCACTCT
	VIC probe	TCAGGTTCAG<u>G</u>CCCTT
	FAM probe	TCAGGTTCAG<u>A</u>CCCTT
2677G→T		
	Forward primer	GAAATGAAAATGTTGTCTGGACAAGCA
	Reverse primer	CTTAGAGCATAGTAAGCAGTAGGGAGT
	VIC probe	TTCCCAG<u>C</u>ACCTTC
	FAM probe	TTCCCAG<u>A</u>ACCTTC

average. The average frequency of mutant (T) alleles in the control group for all three SNPs combined was 0.589, with some contrast between individuals of eastern (0.615) and western (0.549) Finnish ancestry (Fig. 2). The estimated frequency of the mutant TTT haplotype was 0.512 for the Finnish population. The haplotypic genotypes were in Hardy–Weinberg equilibrium (P=0.565), despite the fact that the haplotype frequency differences between eastern and western Finland appear statistically significant (P=0.037).

ABCB1 alleles in case groups

On the haplotypic level, the case samples were in Hardy–Weinberg equilibrium (P=0.783), and the haplotype frequencies did not differ significantly from the control group (P=0.414). The case group can thus be considered to represent a random sample of the population. Contrasted with the mutant allele frequency of the control group (0.589), the frequency was found to be lower (0.528) in the <2.6 nmol/L case group but higher in the >7 nmol/L group (0.649). The middle group of 2.6–6.9 nmol/L (0.591) reflected the control in terms of allele frequencies (Fig. 2; Table 1). This pattern was also observed for each SNP individually. Analysis of 112 case subjects produced some unsuccessful allele calls in a small percentage of the cases. Some unobtained calls may be due to the presence of the untyped low-frequency allele SNP2677G>A. Ultimately, 664 alleles were genotyped for the case group.

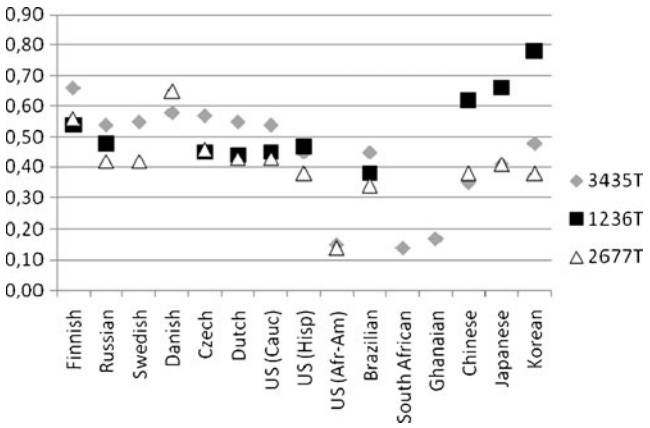

Fig. 1 Frequency of mutant alleles for three single nucleotide polymorphisms in populations worldwide [23–35]. In the Finnish population, the frequencies were: 3435C>T=0.661, 1236C>T=0.542, and 2677G>T=0.564. The sample size for the Finnish population was 143 individuals. For worldwide populations, sample size ranged from 26 to 500 individuals

Segregation of the post-mortem samples by sex revealed conspicuous differences. The upward trend observed in case samples is strongly accentuated for female subjects taken separately, both for combined alleles (Fig. 2; Table 1) as well as for each SNP individually. Genotype frequency patterns also consistently reflected those of the allele frequencies. Female subjects showed 0.238, 0.278, and 0.400 TTT/TTT frequencies in <2.6, >2.6, and >7 nmol/L concentration groups, respectively. These trends were not observed in male subjects (males=0.194, 0.200, and 0.111). On the haplotypic level, significant deviation from the control could be observed in the >7 nmol/L group, males and females combined (P=0.008) and the >7 nmol/L females (P=0.025).

Discussion

High mutant frequency in ABCB1 SNPs among Finns

Within Europe, these polymorphisms have shown high uniformity of frequencies with few exceptions. In contrast,

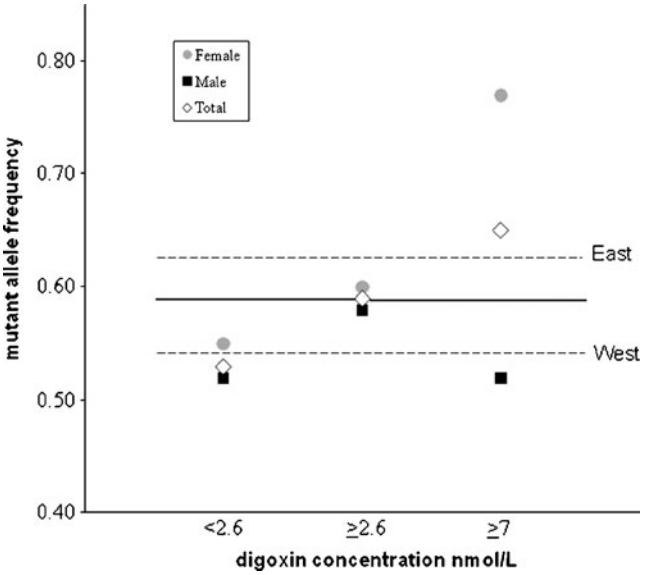

Fig. 2 Mutant allele frequencies in total for three SNPs (3435C>T, 1236C>T, 2677G>T), grouped by digoxin concentration in case subjects. For each group <2.6, ≥2.6, ≥7 nmol/L: men (*N*=192, 120, 54) and women (*N*=126, 112, 60). Horizontal lines indicate mutant allele frequency for the Finnish control population (0.589; continuous), separated further into eastern (0.615) and western (0.549) Finland (hatched lines)

we found that the proportion of mutant alleles in the Finnish population is considerably higher than elsewhere in Europe. This finding is in agreement with previous studies establishing Finns as genetic outliers among Europeans [14]. Mutant frequencies observed in this study were also higher than those found among Finns previously [15] which may partly stem from high population subdivision, as evidenced in other genetic markers in the Finnish population [16, 17] and also observed in the ACBC1 haplotypes in this study. The rare A allele of SNP 2677 was not analysed in this study; however, its estimated frequency of 3.3% [15] is low and does not cause significant deviation from Hardy–Weinberg equilibrium in the current data. The strong occurrence of the mutant TTT/TTT genotype (0.315) and TTT haplotype (0.512) in Finns may indicate a greater-than-average susceptibility to digoxin intoxication in the population as a whole. Distressingly, due to the versatile substrate affinity of P-glycoprotein, this vulnerability may not be limited to digoxin alone, but in all probability extends also to other drugs.

High mutant frequency in females suggests a toxicity risk

Remarkably, when the sexes in the case group were separated, a clear trend could be observed. The frequency of mutant alleles in the female Finnish population is notably high, especially in subjects with high post-mortem digoxin concentration (Fig. 2; Table 1). Indeed, the pattern manifests so prominently in the female subjects as to completely shift the perspective for the case group when analyzed in total, with both sexes included. This pattern remains statistically significant even in the smallest sample groups. It appears then that in comparison to wild-type alleles, mutant genotypes do not allow for efficient digoxin processing and therefore may contribute to greater lethality. This supports the clinical notion that females with mutant genotypes are more vulnerable to lethal digoxin intoxication compared to males [18, 19].

Women have a lower body mass than males, but several studies have indicated that this alone does not account for the difference in metabolic effects between the sexes. These may result instead from effects of a hormonal nature [20] such as, perhaps, the steroid-dependence of P-glycoprotein development, and in consequence the presence of less P-glycoprotein in the liver to expel toxic substances [21]. Other contributory factors could be a slower rate of renal clearance [22] and the interaction of digoxin with hormone replacement therapy in postmenopausal age groups [18].

In light of our findings, it is recommended that future pharmacokinetic studies include male–female segregation, as the genders separately can give a vastly different, more accurate perspective of metabolic effects than the two combined. Further studies on this topic are required to ensure that drugs prescribed are within the limits of safety.

Drug interactions

The Swedish–Finnish drug-interaction database [11] describes several drugs shown to interact with digoxin. A notable fraction of subjects (20%) had cross-reacting drugs in their system, ranging from level D4 (verapamil) to B3 (carvedilol) in the SFINX database. The cases were also examined separately by removing the subjects with verapamil interactions [2]; this did not significantly alter the results.

Cross-prescribing of medications is a common occurrence in subjects over 65 years of age. This matter is complicated by the lack of national healthcare databases to monitor the prescribing of drugs. Clearly, the current situation introduces a significant health risk and our study underlines the need for an extensive prescription database available to both the public and private sectors of medicine.

In post-mortem examinations, a drug present within the limits of toxicity can be a confounding factor for pathologists attempting to establish cause of death. This research highlights the importance of gender-segregating genotyping studies: as few as three SNPs and a limited number of subjects can reveal conspicuous patterns of broad significance. Further research on metabolic genes such as ABCB1 will help define parameters of safety for a wide range of medications and patient genotypes, with the added benefit of ultimately allowing more definitive determination of the cause and manner of death.

The original data are available upon request (anu.neuvonen@helsinki.fi or antti.sajantila@helsinki.fi).

Acknowledgments We would like to thank Bruce Budowle, Ilkka Ojanperä, and Erkki Vuori for their valuable academic input, Mikko Putkonen for assistance with RT-PCR protocols, and two anonymous reviewers for their critical comments.

References

1. The Digitalis Investigation Group (1997) The effect of digoxin on mortality and morbidity in patients with heart failure. New Engl J Med 336(8):525–533
2. Launiainen T, Vuori E, Ojanperä I (2009) Prevalence of adverse drug combinations in a large post-mortem toxicology database. Int J Legal Med 123(2):109–115
3. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC (1987) Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 84(21):7735–7738
4. Hoffmeyer S, Burk O, von Richter O et al (2000) Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci USA 97(7):3473–3478
5. Sakaeda T, Nakamura T, Horinouchi M et al (2001) MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects. Pharm Res 18 (10):1400–1404
6. Johne A, Köpke K, Gerloff T et al (2002) Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein ABCB1 gene. Clin Pharmacol Ther 72(5):584–594
7. Verstuyft C, Schwab M, Schaeffeler E et al (2003) Digoxin pharmacokinetics and MDR1 genetic polymorphisms. Eur J Clin Pharmacol 58(12):809–812
8. Morita Y, Sakaeda T, Horinouchi M (2003) MDR1 genotype-related duodenal absorption rate of digoxin in healthy Japanese subjects. Pharm Res 20(4):552–556
9. Chowbay B, Li H, David M, Cheung YB, Lee EJD (2005) Meta-analysis of the influence of MDR1 C3435T polymorphism on digoxin pharmacokinetics and MDR1 gene expression. Brit J Clin Pharmacol 60(2):159–171
10. Aarnoudse AJ, Dieleman JP, Visser LE et al (2008) Common ATP-binding cassette B1 variants are associated with increased digoxin serum concentration. Pharmacogenet Genomics 18(4):299–305
11. Böttiger Y, Laine K, Andersson ML et al (2009) SFINX–a drug-drug interaction database designed for clinical decision support systems. Eur J Clin Pharmacol 65(6):627–633
12. PHASE software for haplotype estimation. Accessed April 2010, at http://stephenlab.uchicago.edu/software.html
13. Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50
14. Lao O, Lu TT, Nothnagel M et al (2008) Correlation between genetic and geographic structure in Europe. Curr Biol 18 (16):1241–1248
15. Keskitalo JE, Kurkinen KJ, Neuvonen PJ, Niemi M (2008) ABCB1 haplotypes differentially affect the pharmacokinetics of the acid and lactone forms of simvastatin and atorvastatin. Clin Pharmacol Ther 84(4):457–461
16. Palo JU, Hedman M, Ulmanen I, Lukka M, Sajantila A (2007) High degree of Y-chromosomal divergence within Finland—forensic aspects. Forensic Sci Int-Gen 1:120–124
17. Salmela E, Lappalainen T, Fransson I et al (2008) Genome-wide analysis of single nucleotide polymorphisms uncovers population structure in Northern Europe. PLoS ONE 3(10):e3519
18. Rathore SS, Wang Y, Krumholz HM (2002) Sex-based differences in the effect of digoxin for the treatment of heart failure. N Engl J Med 347(18):1403–1411
19. Aarnoudse AL, Dieleman JP, Stricker BH (2007) Age- and gender-specific incidence of hospitalisation for digoxin intoxication. Drug Saf 30(5):431–436
20. Nicolson TJ, Mellor HR, Roberts RRA (2009) Gender differences in drug toxicity. Trends Pharmacol Sci 31(3):108–114
21. Schuetz EG, Furuya KN, Schuetz JD (1995) Interindividual variation in expression of P-glycoprotein in normal human liver and secondary hepatic neoplasms. J Pharmacol Exp Ther 275(2):1011–1018
22. Schwartz JB (2007) The current state of knowledge on age, sex, and their interactions on clinical pharmacology. Clin Pharmacol Ther 82(1):87–96
23. Gaikovitch EA, Cascorbi I, Mrozikiewicz PM et al (2003) Polymorphisms of drug-metabolizing enzymes CYP2C9, CYP2C19, CYP2D6, CYP1A1, NAT2 and of P-glycoprotein in a Russian population. Eur J Clin Pharmacol 59(4):303–312
24. Goreva OB, Grishanova AY, Domnikova NP, Mukhin OV, Lyakhovich VV (2004) MDR1 gene C1236T and C6+139T polymorphisms in the Russian population: associations with predisposition to lymphoproliferative diseases and drug resistance. Bull Exp Biol Med 138(4):404–406
25. Green H, Söderkvist P, Rosenberg P, Horvath G, Peterson C (2006) MDR1 single nucleotide polymorphisms in ovarian cancer tissue: G2677T/A correlates with response to paclitaxel chemotherapy. Clin Cancer Res 12(3):854–859
26. Larsen UL, Hyldahl Olesen L, Guldborg-Nyvold C et al (2007) Human intestinal P-glycoprotein activity estimated by the model substrate digoxin. Scand J Clin Lab Invest 67(2):123–134
27. Pechandova K, Buzkova H, Slanar O, Perlik F (2006) Polymorphisms of the MDR1 gene in the Czech population. Folia Biol (Praha) 52(6):184–189
28. Urayama KY, Wiencke JK, Buffler PA, Chokkalingam AP, Metayer C, Wiemels JL (2007) MDR1 gene variants, indoor insecticide exposure, and the risk of childhood acute lymphoblastic leukemia. Cancer Epidemiol Biomark Prev 16(6):1172–1177
29. Song P, Lamba JK, Zhang L et al (2006) G2677T and C3435T genotype and haplotype are associated with hepatic ABCB1 (MDR1) expression. J Clin Pharmacol 46(3):373–379
30. Gonzalez TP, Mucenic T, Brenol JCT, Xavier RM, Scheingold M, Chies JAB (2008) ABCB1 C1236T, G2677T/A, and C3435T polymorphisms in systemic lupus erythematosus patients. Braz J Med Biol Res 41(9):769–772
31. Chelule PK, Gordon M, Palanee T, Mosam A, Coovadia HM, Cassol S (2003) MDR1 and CYP3A4 polymorphisms among African, Indian, and white populations in KwaZulu-Natal, South Africa. Clin Pharmacol Ther 74(2):195–196
32. Ameyaw MM, Regateiro F, Li T et al (2001) MDR1 pharmacogenetics: frequency of the C3435T mutation in exon 26 is significantly influenced by ethnicity. Pharmacogenetics 11(3):217–221
33. Chen B, Fang J, Zhang W, Jin Z, Yu Z, Cai W (2009) Detection of C1236T, G2677T, and C3435T polymorphism of MDR1 by amplification refractory mutation system PCR. J Clin Lab Anal 23(2):110–116
34. Komoto C, Nakamura T, Sakaeda T et al (2006) MDR1 haplotype frequencies in Japanese and Caucasian, and in Japanese patients with colorectal cancer and esophageal cancer. Drug Metab Pharmacokinet 21(2):126–132
35. Ryu HC, Kwon HY, Choi IK, Rhee DK (2006) Analyses of single nucleotide polymorphisms and haplotype linkage of the human ABCB1 (MDR1) gene in Korean. Arch Pharm Res 29(12):1132–1139