ORIGINAL ARTICLE

Genetic Polymorphism of NAT2 Metabolizing Enzymes on Phenytoin Pharmacokinetics in Indian Epileptic Patients Developing Toxicity

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Epilepsy; Pharmacokinetics; Phenytoin; NAT2 enzymes.

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SUMMARY

Objective: To investigate the effects of NAT2 metabolizing enzymes on the pharmacokinetics of antiepileptic drug phenytoin in the epileptic patients showing toxicity. **Methods:** Fifty epileptic individuals who had developed toxicity to phenytoin and 50 control epileptic subjects who had not developed toxicity to phenytoin were genotyped for NAT2 (NAT2*5A, NAT2*5C, NAT2*7, NAT2*6) polymorphisms by polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLP method). Phenytoin plasma levels were analyzed by reversed phase HPLC method and pharmacokinetic parameters such as area under the concentration curve (AUC), maximum concentration (C_{max}), time to C_{max} (t_{max}) and half-life ($t_{1/2}$) were estimated by noncompartmental analysis using PK Solutions[®] software. **Results:** The NAT2 polymorphism was seen to be in Hardy–Weinberg equilibrium and showed significant genotypic as well as allelic association with phenytoin toxicity for NAT2*5A (481C>T) and NAT2*5C (803A>G). Pharmacokinetic parameters for phenytoin in toxicity group of poor metabolizers showed a longer elimination half-life of a drug ($t_{1/2}$ = 35.3 h) and less clearance rate (CL = 468 mL/h) compared to intermediate metabolizers ($t_{1/2}$ = 33.2 h, CL = 674 mL/h) and extensive metabolizer ($t_{1/2}$ = 20.7 h, CL = 977 mL/h) in NAT2*5A polymorphism. **Conclusion:** Our findings suggest that the NAT2*5A genetic polymorphisms plays a significant role in the steady-state concentrations of phenytoin and thereby have impact on toxicity in epileptic patients.

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Introduction

Epilepsy is a chronic disorder, characterized by recurrent, unprovoked epileptic seizures. It is the commonest chronic neurological disorder after headache, with a prevalence of 5–10 per 1000 persons and an incidence of 50–120 per 100,000 per year [1]. India accounts for 0.5–1% of the world population with epilepsy. Studies to estimate prevalence and patterns of epilepsy in India found that urban men and women had a higher prevalence of epilepsy compared with rural areas [2]. The exact pathophysiology of epilepsy remains unknown, but hyperexcitability and altered synaptic transmission of brain neurons are known to be cardinal features in epileptogenesis [3]. Phenytoin, a widely used antiepileptic drug, is known to exhibit marked interindividual variation in pharmacokinetics and a narrow range of therapeutic serum concentrations (10–20 μ g/mL) [4]. The role of phase II metabolizing enzymes, which are responsible for conjugation and detoxification of reactive metabolites in antiepileptic drug

(AED) metabolism, is currently less well defined. Phase II enzymes with a role in AED metabolism includes the *N*-acetyltransferases (NAT1 and NAT2) and glutathione S-transferase (GST). A few studies have reported correlations of variants in genes encoding drug metabolizing enzymes (DMEs) and AED response, most of them relating to drug toxicity. More recently, attention has been focused on the genetic polymorphism of Phase II, NAT2 enzymes involved in phenytoin biotransformations. *N*-acetylation polymorphism causes interindividual variations in the biotransformation of various drugs, procarcinogens, and other xenobiotics with a primary aromatic or hydrazine structure [5]. The drugs sulfadimidine, hydralazine, dapsone, procainamide, sulfapyridine, nitrazepam, and caffeine are metabolized by NAT enzyme [6]. Hereditary differences in *N*-acetylation activity among individuals and in populations of diverse racio-geographic origin, have led to the phenotype classification of humans as rapid and slow acetylators [7]. There exists two functional human *N*-acetyltransferases: NAT1 [8] and NAT2 [9]. The human NAT2 gene is located

on chromosome 8p22 [8,10] and encodes a 290-amino-acid protein. The gene is polymorphic and 36 alleles have been so far described [11]. Seven missense (G191A, T341C, A434C, G590A, A803G, A845C, and G857A) and four silent (T111C, C282T, C481T, and C759T) substitutions have been identified thus far in the NAT2 coding exon. NAT2 alleles containing the G191A, T341C, A434C, G590A, and/or G857A missense substitutions are associated with slow acetylator phenotypes. This study was undertaken to investigate the possible association of NAT2 polymorphism on the pharmacokinetics of phenytoin in epileptic patients showing toxicity.

Materials and Methods

Study Subjects

The epileptic patients were recruited from outpatient and wards of Neurology, All India Institute of Medical Sciences, New Delhi. The diagnoses of epilepsy were based on the classification systems for seizure types provided by the International League Against Epilepsy (ILAE). The study protocol was approved by the Institute Ethics Committee and all studies were performed with full and informed consent of the patients. Patients with normal therapeutic doses who develop toxicity in the form of nystagmus, ataxia, dysarthria, fatigue, cognitive impairments, and gingival hyperplasia were included. Patients treated with other anticonvulsant drugs, cytochrome P450 (CYP) inducers like haloperidol, diazepam, and rifampicine, and CYP inhibitors like sulphonamides, ranitidine, and antipsychotics etc., patients with severe liver/kidney dysfunctions or diabetes mellitus, and patients taking phenytoin, less than 1 month, were excluded from the study. A total number of 100 epileptic patients, in which epileptic patients showing toxicity and adverse drug reaction to phenytoin monotherapy (50 cases) and epileptic patients without showing toxicity and adverse drug reactions to phenytoin monotherapy (50 cases) were recruited from North Indian origin.

Sample Collection

Eight milliliters of venous blood were collected in 2% EDTA tubes (5 mL) and plain vial (3 mL) from each of the subjects participated in the study, and serum was isolated within 2 h by centrifugation at 2000 *g* for 20 min and stored at -20°C for drug assay. Peripheral blood leukocytes were also stored at -20°C for extracting DNA and to perform the molecular tests.

Therapeutic Drug Assay for Phenytoin

For serum phenytoin level determination, the blood was collected between 8.00 a.m. to 10 a.m. before the morning dose and the drug levels were measured by enhanced chemiluminescence's enzyme immunoassay method (Immulite[®], 1000 systems, DPC, Siemens Health Care Diagnostics Ltd., Flanders, NJ, USA). A polystyrene bead enclosed within the Immulite Test Unit is coated with a polyclonal antibody specific for phenytoin. Phenytoin in the sample competes with (ALP) enzyme labeled phenytoin for a limited number of antibody binding sites on the bead. Unbound

enzyme conjugate is then removed, after which chemiluminescent substrate (adamantyl dioxetane phosphate) is added. This undergoes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate, which results in the sustained emission of light, measured by the luminometer, intensity of which is inversely proportional to the concentration of phenytoin in the sample (reference values: 10–20 $\mu\text{g/mL}$ for serum phenytoin therapeutic range).

DNA Extraction

DNA was extracted from blood by phenol–chloroform method [12] and was purified by ethanol precipitation. DNA was used as a template for NAT2 polymorphism analysis.

NAT2 Genotyping (PCR–RFLP)

Polymorphisms in the NAT2 alleles were determined using polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) analysis. The NAT2 gene was amplified with the primers described earlier [13]. The PCR mixture was constituted in a total volume of 25 μL , containing 200 ng DNA, 0.2 mM of each of the deoxynucleotide triphosphates; 20 nM of each oligonucleotide primer each forward and reverse, reaction buffer (1 $\times$ as per commercial catalog guidelines), 1.5 mM MgCl_2 (present in the buffer itself) and 1 unit of *Taq* polymerase (Perkin-Elmer). The conditions for denaturation, annealing, polymerization, and extension were 94°C for 5 min, and 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and a final extension period of 72°C for 5 min. The amplified PCR products were digested with 5U restriction enzymes Kpn I, Dde I, *Taq* I, and Bam HI by overnight incubation at 37°C and separated by 8% PAGE and 3% agarose gel electrophoresis. The primers used in the study and the expected sizes of the alleles after restriction digestion are presented in Table 1.

Pharmacokinetics Study

Preparation of Stock and Working Standard Solutions

Stock solutions of 1 mg/mL of standard phenytoin, was prepared using methanol as a diluent. From the stock solutions, working standard solutions 5, 10, 20, 50, 100, 250, 500, and 1000 $\mu\text{g/mL}$ of phenytoin were prepared.

Preparation of Calibration Curve Standards in Human Serum

The studies were carried out using aliquots of human serum (90 μL) pipetted into microcentrifuge tubes, spiked with 10 μL of the working standard solutions of phenytoin to reach final concentration of 0.5, 1, 2, 5, 10, 25, 50, and 100 $\mu\text{g/mL}$. To this 400 μL of acetonitrile was added and vortexed for 2 min, followed by centrifuge at 10,000 rpm for 5 min in a cold centrifuge at 4°C . After centrifugation, the supernatant layer was separated and 50 μL was injected to HPLC system. An internal standard carbamazepine

Table 1 Primer and RFLP patterns of NAT2 polymorphism

Gene	Primers	Product size	Restriction enzymes and product size after digestion
NAT2*5A (481C>T)			Kpn 1 digestion 547, 433, (wild-type [wt]); 547, 433, 114 (heterozygous [ht]); 547 (homozygous mutant [mt])
NAT2*5C (803A>G)	5'- GCT GGG TCT GGA AGC TCC TC-3' 5'-TTG GGT GAT ACA TAC ACA AGG G-3'	547 bp	DdeI digestion 345, 137, 65 (wt); 345, 137, 114, 65 (ht); 345, 114, 65 (mt)
NAT2*6 (590 G>A)			TaqI digestion 222, 170, 155 (wt); 392, 222, 170, 155 (ht); 392, 155 (mt)
NAT2*7 (857 G>A)			BamHI digestion 490 and 57 bp (wt); 547, 490, and 57 (ht); 547 (mt)

(50 µg/mL), to blank plasma was prepared. The calibration curves were assessed by the coefficients of correlation (*r*) of the logarithmic equation using $\log Y = a \log X + b$ (*a*: slope, *b*: intercept, *Y*: peak height ratio, *X*: concentration).

Bio Analysis of Serum Samples

To 100 µL of serum samples, 400 µL of acetonitrile was added and vortexed for 2 min followed by centrifuge at 10,000 rpm for 5 min in a cold centrifuge at 4°C. After centrifugation, the supernatant layer was separated and 50 µL was injected to HPLC system.

Preparation of Mobile Phase

For this, 2.6 g of potassium dihydrogen phosphate was weighed and dissolved in 1000 mL of milli-Q water. The pH of the mobile phase was adjusted to 3.0 using orthophosphoric acid. Then the mobile phase was filtered through 0.45 µm and degassed.

Chromatographic Conditions

Stationary phase	Grace Vydac C ₁₈ (250 × 4.6 mm, 5µ)
Mobile phase	Solvent A: 20 mM phosphate buffer pH 3.0 Solvent B: Acetonitrile
Solvent ratio	Isocratic run for 16 min using ratio 60: 40 of A: B
Detection wavelength	210 nm
Flow rate	0.8 mL/min
Injection volume	50 µL
Column Temperature	: 25°C
Sample temperature	: 4 ± 2°C

HPLC Method

Phenytoin level was estimated in human serum by reverse phase HPLC method [14]. The standard and sample solutions were injected with the above chromatographic conditions and the chromatograms were recorded. The response factor and peak ratio of the standard and samples were calculated and the concentration

of phenytoin present in the serum samples was calculated from the calibration curves. The retention time (RT) of phenytoin (PHT) was found to be 11.3 min (Figure 3). The method was found to be specific and no interferences from the serum sample at RT.

Pharmacokinetic Evaluation

Five epileptic patients were enrolled for the pharmacokinetic study and six samples were collected from each patients showing extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) in phenytoin for NAT2*5A polymorphism toxicity groups spread over a period of 12 h who had attended the steady-state concentration. The sample collection times were 0, 2, 4, 6, 8, and 12 h, respectively. The pharmacokinetic parameters of phenytoin, was determined by noncompartmental methods with the aid of PK Solutions[®] 2.0 software (Summit Research Service, Montrose, CO, USA). Peak serum concentrations (*C*_{max}) and the time to reach peak concentrations (*t*_{max}) were determined by observation of individual concentration time data. The terminal elimination half-life (*t*_{1/2}) was calculated. The area under the concentration curve (AUC_{0–12h}) was calculated by the trapezoidal rule. The apparent oral clearance (CL) was calculated as the ratio of administered dose to AUC (0–12 h).

Statistical Analysis

The sample size was determined using the Power and precision software (<http://www.power-analysis.com>). Allele frequencies were derived from genotype data, and deviations from the Hardy–Weinberg equilibrium were tested using χ^2 -test. Differences in allelic, genotypic frequencies in toxicity, nontoxicity, and controls were compared using the χ^2 -test. Odds ratio (OR) and confidence interval (95% CI) were also calculated. Correlation between the metabolizers, EMs, IMs, and PMs, with median drug levels was analyzed between toxicity and nontoxicity group using Kruskal–Wallis test and Mann–Whitney U test. Correlations between Pharmacokinetic parameters were analyzed using one-way ANOVA. The *P* values of less than 0.05 were considered to be significant. The Statistical Package for Social Sciences (SPSS, version 11.5 for Windows) was used for all analyses.

Table 2 Demographic and clinical characteristics of study population

Demographic characteristics	Phenytoin toxicity, N = 50	Phenytoin nontoxicity, N = 50
Mean age $\pm$ SD, year	22.1 $\pm$ 9.6	24.6 $\pm$ 10.4
Gender		
Male	36	39
Female	14	11
Type of seizures		
GTCS ^a	36	32
SPS ^b	–	3
SPS sec. gen ^c	11	12
CPS ^d	–	–
CPS sec. gen ^e	3	3
Mean weight $\pm$ SD kgs	50.8 $\pm$ 9.93	52.8 $\pm$ 8.93
Therapeutic doses, mg	300 mg	300 mg

^aGeneralized tonic–clonic seizures.^bSimple partial seizures.^cSimple partial seizures with secondary generalization.^dComplex partial seizures.^eComplex partial seizures with secondary generalization.

Results

Demographic and clinical characteristics of patients with epilepsy enrolled in the study are shown in Table 2. Total 100 patients with epilepsy (50 patients with phenytoin toxicity and 50 patients with phenytoin nontoxicity) cases were recruited. Their mean age and standard deviation of each group were (22.1 $\pm$ 9.6, 24.6 $\pm$ 10.4), respectively. The seizure types were generalized tonic–clonic seizures (GTCS) 68%, simple partial seizures with secondary generalization (SPS sec. gen.) 33%, complex partial seizures with secondary generalization (CPS sec. gen) 6%, and CPS and SPS 3%, respectively.

PCR–RFLP was used to examine NAT2*5A (481C>T), NAT2*5C (803A>G), NAT2*6 (590G>A) and NAT2*7 (857G>A) genotype distributions in epileptic patients showing toxicity and nontoxicity to phenytoin (Figure 1). These polymorphisms were polymorphic in the study population and seen to be under the Hardy–Weinberg equilibrium ($P > 0.01$). The genotype and allele frequencies for these variants for phenytoin toxicity groups and nontoxicity groups were shown in (Tables 3 and 4). The allelic and genotypic frequency for NAT2*5A polymorphism was significantly associated with phenytoin toxicity and nontoxicity groups (allelic $\chi^2 = 7.33$, $P = 0.006$, OR = 2.29 [1.20–4.41]; genotypic $\chi^2 = 4.84$, $P = 0.02$, OR = 2.45, [1.02–5.94]). Similarly, allelic and genotypic frequency for NAT2*5C (803A>G) was significantly associated with phenytoin toxicity and nontoxicity groups (allelic $\chi^2 = 5.53$, $P = 0.01$, OR = 2.25 [1.08–4.73]; genotypic $\chi^2 = 4.34$, $P = 0.037$, OR = 2.42, [0.97–6.15]). Whereas allelic and genotypic frequency for NAT2*6 (590G>A) and NAT2*7 (857G>A) was not significantly associated with phenytoin toxicity and nontoxicity groups ($P > 0.05$).

Metabolizers were classified into EMs carrying both wild-type alleles (wt/wt), IMs carrying one normal allele and one variant allele (wt/mt), and PMs carrying both variant allele (mt/mt) according to their genotypes of NAT2 metabolizing enzymes. The serum phenytoin levels in toxic cases ranges from 17.3 μ g/mL to

39 μ g / mL, although the normal therapeutic range was found to be 10–20 μ g/mL and in nontoxicity cases, phenytoin serum levels ranged from 10.2 μ g /mL to 20.5 μ g /mL. The median range of phenytoin drug levels in EM, IM and PM of drug metabolizing enzymes in phenytoin toxicity groups were NAT2*5A : 20.5 (EM), 22.3 (IM), 30.5 μ g/mL (PM), NAT2*6 : 20.5 (EM), 27.1 μ g/mL (IM), NAT2*5C : 20.9 (EM), 27 (IM), 32 μ g/mL (PM). NAT2*7 : 20.8 (EM), 27.2 μ g/mL (IM), respectively. Similarly for phenytoin nontoxicity groups, the median drug levels of EM, IM and PM were NAT2*5A : 12.2 (EM), 18 (IM), 21 μ g/mL (PM), NAT2*6 : 12.6 (EM), 18.9 (IM) μ g/mL, NAT2*5C : 12.6 (EM), 18.9 (IM), 21 (PM) μ g/mL, NAT2*7 : 12.3 (EM), 18.4 (IM) μ g/mL, respectively. Significant consistent rise in the genotypes (EM, IM, and PM) of median drug levels between toxicity and nontoxicity of phenytoin in epileptic patients was observed, and Kruskal–Wallis test showed a significant difference in the median drug levels between the genotypes of toxicity and nontoxicity across all NAT2 polymorphism ($P < 0.05$). Mann–Whitney's nonparametric test showed a significant difference in EM, IM, and PM of drug levels between toxicity and nontoxicity groups of phenytoin ($P < 0.05$) and are represented in Figure 2.

The peaks were well resolved and RT for phenytoin was 11.3 min (Figure 3). No interfering peaks were observed at RT. The calibration curve for the determination of phenytoin in human serum, obtained by plotting the phenytoin concentration and mean peak area ratios, was linear ($R^2 = 0.9993$) over the range of 0.5–100 μ g/mL. The corresponding regression equation was $Y = 64748X - 16008$ where, Y is the peak area ratio of phenytoin and X is the phenytoin concentration. The drug concentration in the serum was estimated using the calibration graph. The plasma concentrations of phenytoin against time of each metabolizers (EM, IM, and PM) for NAT2*5A are shown in (Figure 4, Table 5). The pharmacokinetic parameters were calculated from the plasma concentration–time curves using PK Solutions[®] software and recorded in Table 6. The C_{max} after oral administration of phenytoin was 20.4 $\pm$ 0.29 μ g/mL (EM), 24 $\pm$ 0.10 μ g/mL (IM), and 32 $\pm$ 0.16 μ g/mL (PM). The t_{max} was 6 $\pm$ 0.12 h (EM), 6 $\pm$ 0.08 h (IM) and 6 $\pm$ 0.08 h (PM). AUC (0–12 h) was 307 $\pm$ 4.45 μ g h/mL, 445 $\pm$ 3.71 μ g h/mL, and 641 $\pm$ 5.48 μ g h/mL between EM, IM, and PM. The elimination half-life ($t_{1/2}$) of phenytoin was 20.7 $\pm$ 0.46 h, 33.2 $\pm$ 0.12 h, 35.3 $\pm$ 0.13 h and CL was 977 $\pm$ 3.13 mL/h, 674 $\pm$ 2.24 mL/h, and 468 $\pm$ 1.24 mL/h. The C_{max} , AUC, $t_{1/2}$, and CL were statistically significant with the metabolizer groups ($P < 0.001$).

Discussion

Hepatic metabolism is the principal route of elimination for most AEDs. Genetic polymorphisms can alter drug metabolism at various sites. There is potential for alteration in absorption, distribution, transport, metabolism, clearance, and sites of action. AEDs of the first generation phenytoin, carbamazepine and valproate, are metabolized by biotransformation in the liver. Biotransformation of antiepileptic drugs is diverse and involves a host of different enzyme proteins [15]. Although there are known environmental influences on the biotransformation of antiepileptic drugs, genetic effects are also well-documented by virtue of the many drug metabolizing enzymes that are products of polymorphic genes [16].

Table 3 Allelic frequency of NAT2 enzymes on phenytoin

S.No	Gene name	Polymorphism	Allele frequency	Allele frequency	Allelic χ^2 value, <i>P</i> value, odds ratio, and 95 % CI
			Phenytoin toxicity N = 50	Phenytoin nontoxic N = 50	
1	NAT2*5A	481C>T	C = 0.58 T = 0.42	C = 0.76 T = 0.24	Tox versus nontox : 7.33, 0.006, 2.29 (1.20–4.41)*
2	NAT2*5C	803A>G	A = 0.70 G = 0.30	A = 0.84 G = 0.16	Tox versus nontox : 5.53, 0.01, 2.25 (1.08–4.73)*
3	NAT2*6	590 G>A	G = 0.82 A = 0.18	G = 0.89 A = 0.11	Tox versus nontox : 1.98, 0.156, 1.78 (0.74–4.30)
4	NAT2*7	857 G>A	G = 0.81 A = 0.19	G = 0.78 A = 0.22	Tox versus nontox : 0.28, 0.599, 0.83 (0.40–1.75)

*Statistically significant (*P* < 0.05).**Table 4** Genotypic frequency of NAT2 enzymes on phenytoin

S.No	Gene name	Polymorphism	Genotype frequency	Genotype frequency	Genotypic χ^2 value, <i>P</i> value, odds ratio, and 95 % CI
			Phenytoin toxicity N = 50	Phenytoin nontoxic N = 50	
1	NAT2*5A	481C>T	CC = 19 CT + TT = 31	CC = 30 CT + TT = 20	Tox versus nontox : 4.84, 0.02, 2.45, (1.02–5.94)*
2	NAT2*5C	803A>G	AA = 27 AG + GG = 23	AA = 37 AG + GG = 13	Tox versus nontox : 4.34, 0.037, 2.42, (0.97–6.15)*
3	NAT2*6	590G>A	GG = 32 GA + AA = 18	GG = 39 GA + AA = 11	Tox versus nontox : 2.38, 0.12, 1.99, (0.76–5.31)
4	NAT2*7	857G>A	GG = 28 GA + AA = 22	GG = 33 GA + AA = 17	Tox versus nontox : 1.05, 0.30, 1.53, (0.63–3.71)

*Statistically significant (*P* < 0.05).

This may result in the occurrence of toxic, adverse, or otherwise unintended effects. On the other hand, variants with very high enzymatic activity may be associated with a need for higher drug dosages than usually prescribed. In the case of antiepileptic drugs that are metabolically activated, low or absent biotransformation capacity may result in treatment failure due to inadequate drug levels [17]. Thus, there are a variety of potential mechanisms by which polymorphic drug metabolism can affect antiepileptic drug responsiveness. It is likely that these functional variants may contribute significantly to true drug resistance, as their effects are expected to be reflected in drug plasma levels, which can be readily monitored for most AEDs using therapeutic drug monitoring. The allele and genotypic frequency of genes encoding phase II enzymes have been reported for a number of populations and revealed a wide ethnical variation. However, there are no data available about the polymorphisms of phase II drug metabolizing enzymes in the North Indian population. Our study to the best of our knowledge is the first of its kind to be done in a North Indian population. We investigated the genotypes of some of the genes, encoding phase II enzymes NAT2 and compared our results with the corresponding data from other populations. The distribution of epileptic seizures in our study population was quite unique with generalized seizures being slightly more frequent than partial seizures. Similar findings were reported in epidemiological surveys from other developing countries [18]. Phenytoin (5, 5-diphenylhydantoin), a widely used anticonvulsant drug,

exhibits nonlinear pharmacokinetics in humans. After oral administration, four oxidative metabolites, S-5-(4-hydroxyphenyl)-5-phenylhydantoin (4-HPPH), 3-HPPH, 3,4-diHPPH, and 3, 4-dihydrodiol, have been reported in humans [19]. Phenytoin is mainly oxidized to 4-HPPH by CYP2C9 and to a minor extent by CYP2C19 [20]. *N*-acetyltransferases catalyzes the metabolism of various aromatic amine drugs and carcinogens. Sequence variations in the human *NAT1* and *NAT2* have been associated with drug-induced toxicities [21]. The NAT2 acetylating polymorphism is very important in clinical pharmacology and toxicology because of its primary role in the activation and deactivation of a large and diverse number of aromatic amine and hydrazine drugs used in clinical medicine [22,23]. *NAT2* alleles containing G191A, T341C, A434C, G590A, and G857A missense substitutions are reported to be slow acetylator phenotypes [24]. Striking ethnic differences in the frequencies of these missense substitutions are responsible for the corresponding ethnic differences in frequency of slow acetylator alleles and phenotypes. In Caucasians and Africans, the frequencies of alleles NAT2*5A (C481T) are high and the frequency of NAT2*7 (G857A) allele are reported to be low, whereas it is just opposite in the Japanese, Chinese and other Far Eastern populations [25]. The frequency of NAT2*6 in the Russian samples (31.8%) was similar to that in other Caucasians [26] whereas, in Oriental populations, the frequencies were lower (20–30%). Higher frequency of the wild-type allele NAT2*4 (44–79%) is reported in Asian population than in other ethnic groups (6–24%).

PCR for NAT2 **(a) RFLP for NAT2*5A (481C>T) Polymorphism**

M - 100 bp Marker

Lane 1,3,6,7 & 9 : CC genotype (Wild type)

Lane 2 & 4 : C T genotype (Heterozygous)

Lane 5 & 8 : T T genotype (Homozygous mutant)

(b) RFLP for NAT2 *5C (803A>G) Polymorphism

M - 50 bp Marker

Lane 1,3 ,4 : AA genotype (Wild type)

Lane 6 & 7 : AG genotype (Heterozygous)

Lane 2,5 & 8 : GG genotype (Homozygous mutant)

(c) RFLP for NAT2*6(590G>A) Polymorphism

M - 100 bp Marker

Lane 1,3, 4 : GG genotype (Wild type)

Lane 2, 5-7 : GA genotype (Heterozygous)

(d) RFLP for NAT2*7 (857 G >A) Polymorphism

M - 100 bp Marker

Lane 1,2, 4-8 : GG genotype (Wild type)

Lane 3 : GA genotype (Heterozygous)

Figure 1 PCR and RFLP patterns for NAT2 polymorphism.

PT- Phenytoin toxicity, NT- Phenytoin Non Toxic

EM – Extensive Metabolizers (wt/wt), IM – Intermediate Metabolizers (wt/mt)

PM- Poor Metabolizers (mt/mt), Median Drug levels in mcg/ml

* - Kruskal-Wallis test between PT and NT, $p < 0.05$ (significant)

* * - Man Whitney U test between EM, IM & PM, $p < 0.05$ (significant)

Figure 2 Correlations between median drug levels and metabolizers with phenytoin toxicity and nontoxicity.

Figure 3 Standard chromatogram for phenytoin (retention time, RT = 11.3 min). Internal standard (IS), carbamazepine.

Figure 4 Plasma concentration profile for phenytoin.

The frequencies of point mutations, alleles, and genotypes of NAT2 in the Russian samples were reported to be similar to those in Middle and South European ethnicities [27]. According to the reported ethnic variations in the distribution of defective NAT2 alleles all over the world, two major groups could clearly be distinguished according to the incidence of the NAT2*5 and NAT2*7 variants. Caucasians, Indians, and Africans are very similar (>0.290 and <0.050 , respectively) [28,29]. Asiatic and Pacific groups also appear to make up a homogeneous group (<0.070 and >0.090), respectively. Higher variant allele frequency in NAT2*5A (0.42) was observed in phenytoin toxicity cases in our study.

Approximately 50% of people in the United States are slow acetylators and 40% intermediate acetylators [28]. The enzyme activity is expressed at highly variable levels. Three phenotypes are reported: rapid acetylators, intermediate Acetylators and extensive acetylators. Individuals who possess reduced NAT2 expression variants may exhibit different pharmacokinetics (drug levels) than normal individuals. In our study, the frequency of slow acetylators (22%) was observed in NAT2*5A phenytoin toxicity cases and intermediate acetylators (44%) was observed for NAT2*7 in

phenytoin toxicity cases. Significant consistent rise in the genotypes (EM, IM, and PM) of median drug levels between toxicity and nontoxicity of phenytoin in epileptic patients was observed using Kruskal–Wallis test ($P < 0.05$) and Mann–Whitney's non-parametric test showed a significant difference in EM, IM, PM of drug levels between toxicity and nontoxicity groups ($P < 0.05$). Phenytoin follows nonlinear pharmacokinetics. At a concentration of $<10 \mu\text{g/mL}$, it follows zero-order kinetics [30]. At therapeutic levels, elimination follows first-order kinetics. Patients often comply poorly with the drug regimen and phenytoin pharmacokinetics exhibits large intersubject differences implying the existence of subpopulations of patients with vastly different dose requirements. The clinical implication of Michaelis–Menten pharmacokinetics is that the clearance of phenytoin is not a constant as it is with linear pharmacokinetics, but is concentration- or dose-dependent. As the dose or concentration of phenytoin increases, the CL decreases as the enzyme approaches saturated conditions: $\text{CL} = V_{\text{max}} / (K_m + C)$. Half-life also changes with phenytoin dosage or concentration. As the dosage or concentration increases for a drug that follows Michaelis–Menten pharmacokinetics, CL decreases and half-life becomes longer for the drug. Due to nonlinear pharmacokinetic behavior, phenytoin represents a dose- or concentration-dependent CL, which means that the limited metabolic capacity may lead to a disproportionate increase in serum concentration after a small increment in dosage. Aberrations in the isoenzymes can cause clinical consequences, resulting in toxicity or altered efficacy of drugs. The apparent plasma half-life of phenytoin may vary from 7 to 60 h with most of the cases falling between 15 and 20 h. Such variability in the apparent half-life is supported by the fact that the elimination of the drug is subjected to dose-dependent kinetics and its disappearance is not always a simple monoexponential process [31,32]. The reported values for plasma CLs vary from 0.0081 to 0.0551 per h kg. Ethnic differences in phenytoin CLs have been reported [33]. The pharmacokinetic parameters for PM, IM, and EM for NAT2*5A phenytoin toxicity cases were observed, in which PM group showed a longer elimination half-life of a drug ($t_{1/2} = 35.3$ h) and less CL ($\text{CL} = 468 \text{ mL/h}$) compared to IM group ($t_{1/2} = 33.2$ h, $\text{CL} = 674 \text{ mL/h}$) and EM ($t_{1/2} = 20.7$ h, $\text{CL} = 977 \text{ mL/h}$). In our study, a significant association was found between the metabolizers of phenytoin pharmacokinetics. Our study showed clear evidence that the genetic polymorphisms in NAT2 enzymes contribute to variable pharmacokinetics that has an impact on the toxicity. This is in agreement with the findings reported from other world populations [34]. The identification of PMs and IMs based on NAT2 polymorphism will be useful in deciding appropriate dosage regimen in these groups of patients for optimal outcome. The study has implications on research and clinical aspects. It provides tools for future studies to investigate the polymorphism effect on the cellular level by conducting expression studies to verify the precise role and effect of particular polymorphisms on the metabolism and clearance, which ultimately produces unwanted adverse events. However, the clinical implications will help to design proper dosage of the drug according to the genetic make up of the patient to avoid the adverse events. The clinician on the other hand can reduce the dose in presence of certain polymorphisms or switch the patient to another mode

Table 5 Mean plasma concentration profile for phenytoin

Time, h	Plasma concentration for phenytoin (EM), n = 5, mean $\pm$ S.E.M	Plasma concentration for phenytoin (IM), n = 5, mean $\pm$ S.E.M	Plasma concentration for phenytoin (PM), n = 5, mean $\pm$ S.E.M
0	9 $\pm$ 0.252	10 $\pm$ 0.16	16 $\pm$ 0.94
2	12 $\pm$ 0.20	14 $\pm$ 0.123	19 $\pm$ 0.76
4	16 $\pm$ 0.23	20 $\pm$ 0.20	26 $\pm$ 0.40
6	20.4 $\pm$ 0.358	24 $\pm$ 0.32	32 $\pm$ 0.34
8	16 $\pm$ 0.52	21 $\pm$ 0.35	29 $\pm$ 0.358
10	11 $\pm$ 0.72	17 $\pm$ 0.52	27 $\pm$ 0.38
12	9 $\pm$ 0.312	15 $\pm$ 0.12	24 $\pm$ 0.23

EM, extensive metabolizers (wt/wt); IM, intermediate metabolizers (wt/mt); PM, poor metabolizers (mt/mt).

Table 6 Pharmacokinetics parameters for phenytoin between metabolizers

S.No	Pharmacokinetic parameters	EM, mean $\pm$ S.E.M	IM, mean $\pm$ S.E.M	PM, mean $\pm$ S.E.M	P value, one-way ANOVA
1.	C _{max} (μ g/mL)	20.4 $\pm$ 0.29	24 $\pm$ 0.10	32 $\pm$ 0.16	$P < 0.01$
2	t _{max}	6 $\pm$ 0.12	6 $\pm$ 0.08	6 $\pm$ 0.08	$P > 0.05$
3	AUC (0–12 h) μ g-h/mL	307 $\pm$ 4.45	445 $\pm$ 3.71	641 $\pm$ 5.48	$P < 0.01$
4	Half-life (t _{1/2})	20.7 $\pm$ 0.46	33.2 $\pm$ 0.12	35.3 $\pm$ 0.13	$P < 0.01$
5	Clearance (mL/h)	977 $\pm$ 3.13	674 $\pm$ 2.24	468 $\pm$ 1.24	$P < 0.01$

EM, extensive metabolizers (wt/wt); IM, intermediate metabolizers (wt/mt); PM, poor metabolizers (mt/mt).

or regimen of treatment that can be more efficacious and less toxic.

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Conflict of Interest

The authors has no conflict of interest.

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