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Research Article

Impact of *CYP2C9* genetic polymorphisms on valproate dosage, plasma concentrations of valproate and clinical response to valproate

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Abstract. There is a complex genotype-phenotype relationship in epilepsy and research in the field of pharmacogenetics has shown the involvement of several genetic polymorphisms that explain some of the individual variability in the response to antiepileptic treatment. Objective: to determine the impact of *CYP2C9* genetic polymorphisms on valproate treatment. Material and methods:The study included 69 patients with epilepsy (38 women and 31 men) treated with valproate (VPA) as monotherapy for at least one month prior to their inclusion in the study. Patients were investigated in the Neurology Clinic of the Emergency Clinical County Hospital of Cluj-Napoca during 6 months, being enrolled between 2008 and 2010. Genetic analysis included the genotyping the *CYP2C9*2* and **3* polymorphisms. Gas chromatography was developed for the separation, identification and quantification of valproate plasma levels and for the accomplishment of the pharmacokinetic profile in study's patients. Results: Plasma concentrations of valproate were not correlated with patient age and gender, but were correlated with dose and dose/body surface area ratio (r=0.376, p=0.001; r=0.667, p=0.05). Valproate concentrations were higher in patients with *CYP2C9*1/*2* genotypes (85.5±30.5) compared to those with *CYP2C9*1//*1* genotype (69.4±28.9) (p=0.05). There were no differences in valproate concentrations related to the presence of *CYP2C9*3* allele. Conclusion: Patients carrying the *CYP2C9*2* allele had higher plasma concentrations of valproate, with a reduced enzymatic activity for valproate (being poor metabolizers), while patients with normal alleles had lower plasma concentrations due to the type of rapid metabolizer.

Key Words: epilepsy, CYP2C9, valproate.

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Introduction

According to current World Health Organization estimates, 8 of 1,000 people suffer from epilepsy in the world. Despite the fact that there is a wide range of antiepileptic drugs available on the market, drug resistance in epilepsy is reported in one third of patients. In these circumstances, epilepsy remains a challenge. There is a complex genotype-phenotype relationship in epilepsy and research in the field of pharmacogenetics has shown the involvement of several genetic polymorphisms that explain some of the individual variability in the response to antiepileptic treatment.

Removal of antiepileptic drugs is achieved by hepatic drug metabolism and/or renal excretion. Generally, hepatic drug metabolism is the predominant route that includes the so-called phase I reactions (oxidative reactions catalyzed by various cytochrome P450 enzymes) and phase II reactions (conjugation reactions and glucuronidation). Some polymorphic enzymes have been detected in the family of cytochrome P450 that partially contribute to the metabolism of some antiepileptic drugs (Klotz *et al* 2007; Loscher *et al* 2009).

It is known that the gene coding *CYP2C9* is polymorphic, its genetic variants being associated with differences in the enzymatic activity of CYP2C9. Studies in different ethnic groups have revealed the existence of several allelic variants of the *CYP2C9* gene (up to 30 allelic variants). Of these, the most important in terms of their frequency in the general population are *CYP2C9*2* and *CYP2C9*3* (Scordo *et al* 2001; Gaikovitch *et al* 2003; Bozina *et al* 2003; Allabi *et al* 2003; Myrand *et al* 2008). Both alleles are associated with a marked decrease in the enzymatic activity of CYP2C9, residual enzyme activity being around 12% in the case of *CYP2C9*2* and 5% in the case of *CYP2C9*3*. Subjects bearing at least one mutant allele are called "poor metabolizers" (Saruwatari *et al* 2010).

Valproate, one of the most commonly used antiepileptic agents in clinical practice, employs CYP2C9 as enzyme substrate for metabolism.

Materials and methods

This is an analytical, prospective, interventional, cohort study. The study included 69 patients with epilepsy (38 women and 31 men) treated with valproate (VPA) as monotherapy for at least one month prior to their inclusion in the study. Patients were investigated in the Neurology Clinic of the Emergency Clinical County Hospital of Cluj-Napoca during 6 months, being enrolled between 2008 and 2010.

Throughout the study, patients had 2 visits (visit 1 when enrolling in the study and visit 2 after 6 months). During the first visit, valproate doses could be modified taking into account the number of epileptic seizures in the last 6 months prior to enrollment. Valproate dosage at visit 1 refers to the baseline dosage patients benefited from. Valproate dosage at visit 2 refers to the dose prescribed after the first visit and applied throughout the 6 month follow-up.

Visit 1 at baseline assumed:

- Informing patients about the study and their written consent
- Age, gender, weight, height
- Patient history
- Use of valproate as the only antiepileptic drug
- Concomitant medication besides AEDs
- General and neurological examination
- Blood tests (biochemistry, hematology, ionogram)
- Blood sample for determination of plasma concentrations of valproate
- Blood sample for determination of *CYP2C9* genotype
- Counting the number of epileptic seizures (for the last 6 months prior to the study, based on patient diary if any or on patient or caregiver statements)
- Distribution of patient study diary (diary noting patient number and type of seizures, adverse effects, concomitant medication).

Visit 2 (6 months after inclusion) included the following parameters:

- Weight
- Personal history and adverse effects reported from the previous visit
- Valproate dosage and concomitant medication
- General and neurological examination
- Counting the number of epileptic seizures from the previous visit, as well as the type of seizure, based on study diary

Genotyping for *CYP2C9*2* and *CYP2C9*3* alleles

Genotyping for *CYP2C9*2* and *CYP2C9*3* alleles was performed using the PCR-RFLP method (polymerase chain reaction - restriction fragment length polymorphism) as described in 1999 by Aynacioglu *et al*. Basically, an amplicon of 372 base pairs was obtained by means of PCR in order to study the allele frequency of *CYP2C9*2*. The amplicon was subjected to overnight digestion by means of Sau96I restriction enzyme (MBI Fermentas, Vilnius, Lithuania), resulting, in the case of the wild-type allele, to 3 parts, namely 179, 119 and 74 base parts. *CYP2C9*2* allele lacks a restriction site for Sau96I enzyme, resulting in only two fragments of 253 and 119 base pairs respectively.

To analyze the variant allele *CYP2C9*3*, an amplicon of 130 base pairs was obtained by means of PCR. This amplicon was subjected to overnight digestion using the StyI restriction enzyme (MBI Fermentas, Vilnius, Lithuania). The wild-type allele was resistant to StyI digestion. However, *CYP2C9*3* allele created a restriction site for StyI enzyme, cutting the amplicon of 130 base pairs into two fragments of 104 and 26 base pairs.

Determination of plasma concentrations of valproate

Gas chromatography (GC-FID) was developed for the separation, identification and quantification of valproate plasma levels and for the accomplishment of the pharmacokinetic profile in study's patients. This method proved to be linear over the concentration range comparative to therapeutic drug levels of valproate. An Agilent 6890N network gas chromatograph with split/splitless capillary injection, flame ionization detector and 7673 autosampler was used.

Analytical reagents were provided by Sigma Aldrich, standard solutions were prepared by weighing on an analytical balance and solubilization by sonication for 2 minutes in methanol (sodium valproate solution was prepared in 0.05 M HCl in methanol).

Working concentrations were obtained from stock solutions by dilution, comparable with reference ranges for valproate, referred to in the literature:

- VPA: 33.3 to 77.7 μg/mL (reference range in the literature: 50 to 100 μg/mL).

Statistical analysis

MedCalc software version 12.7 was used for statistical analysis. Data were collected as categorical and quantitative variables. Categorical variables were described usign frequencies and percentages. Kolmogorov-Smirnov test was used to determine the normality of the distribution of quantitative variables. Continuous variables with normal distribution were described using mean and standard deviation, and the median and the 25th and 75th percentiles were calculated for those non normally distributed. Analysis of the differences in continuoas variables between two different groups was performed using the t-test for independent variables or Mann-Whitney test, when appropriate. Spearman correlation was used for the analysis of two continuous variables with a non-normal distribution. For the analysis of the same quantitative variable measured at two different moments we used the t-test for conjugate variables or Wilcoxon test, depending on the situation.

Analysis of the impact of a parameter on the change of a variable measured at two different times was performed using the General Linear Model (GLM).

Hardy-Weinberg equilibrium was tested using the chi-squared test (χ^2).

A p value of 0.05 was set as statistical significance threshold.

Results

Patients in the study were aged over 18, maximum 70 years, with a median age of 36.8 ± 13.1 years.

The study included 38 (55.1%) female patients and 31 (44.9%) male patients. There were no significant differences in patient age related to gender (p=0.5).

Valproate (VPA) dose range had a non-normal distribution. Age, body surface area, body mass index (BMI), VPA concentrations were normally distributed.

Patients had an average body surface area of 1.78±0.21 m^2, with a minimum of 1.3 m^2 and a maximum of 2.28 m^2. Body mass index (BMI) had an average value of 24.9±4.5, a minimum of 16.5 and a maximum of 41.

The average number of epileptic seizures for the last six months before the first visit was of 3 (1; 4), with a minimum of 0 and a maximum of 60.
The distribution of *CYP2C9* polymorphism can be seen in Table I.

Table I. Frequency of *CYP2C9* polymorphism

Variable	Number (%)
CYP2C9*1/*1	42 (60.8%)
CYP2C9*1/*2	14 (20.2%)
CYP2C9*1/*3	10 (14.4%)
CYP2C9*2/*3	2 (2.8%)
CYP2C9*2/*2	1 (1.4%)

Median VPA dose at the first visit was of 1000 (1000; 1000) mg/day, with a minimum of 150 mg and a maximum of 2000 mg. Dose/ size average was 552.9±214.1 mg/m^2, with a minimum of 81.6 mg/m2 and a maximum of 1032.2 mg/m^2. VPA dose did not correlate with patient age, number of seizures in the last six months before the first visit, BMI ($p>0.05$) or gender ($p>0.05$). Dose/body surface area (BSA) did not correlate with patient age or gender, but was strongly correlated with the number of seizures in the last six months before the first visit ($r=0.762$, $p=0.02$). Neither VPA dose nor VPA dose reported to body surface area was dependent on *CYP2C9* polymorphisms ($p>0.05$). VPA concentration was 73.3±29.9 µg/mL, with a minimum of 2 and a maximum of 149.2 µg/mL.
Plasma concentrations of valproate were not correlated with patient age and gender, but were correlated with dose and dose/body surface area ratio ($r=0.376$, $p=0.001$; $r=0.667$, $p=0.05$). Valproate concentrations were higher in patients with *CYP2C9*1/*2* genotypes (85.5±30.5) compared to those with *CYP2C9*1/*1* genotype (69.4±28.9) ($p=0.05$).
There were no differences in valproate concentrations related to the presence of *CYP2C9*3* allele ($p>0.05$).
Six months after the first visit, 68 (of 69) patients were present at the second visit. Of these, 35 (51.4%) patients had no epileptic seizures from the previous visit. The median number of seizures at the second visit was 0 (0; 5).
Wilcoxon test was used to investigate the differences in the number of seizures between the two visits, indicating a statistically significant decrease in epileptic seizures for the second visit ($p=0.001$).
The median dose of VPA for the second visit was of 1000 (1000, 1500) mg, with a minimum dose of 150 mg and a maximum dose of 2000 mg. Average dose/body surface area ratio was 633.2±237.4 mg/m^2, with a minimum of 81.6 mg/m2 and a maximum of 1065.6 mg/m^2. We determined a statistically significant difference between dose/body surface area ratio at the first visit and at the second visit ($p=0.001$).
We applied the GLM test to study the factors that influenced the reduction in the number of seizures between the first and the second visit. The presence of *CYP2C9*2* allele and the dose/body surface area ratio for the second visit were parameters associated with the reduction in the number of seizures ($p=0.02$; $p=0.05$ respectively). We showed that patients with *CYP2C9*1/*1* genotype had significantly greater increases in VPA dosage related to body surface area for the second visit, compared to subjects with *CYP2C9*1/*2* genotype ($p=0.05$). *CYP2C9*3* allele had no influence on the change in the number of seizures or dosage related to body surface area ($p>0.05$). There was a 13% increase in average dose/body surface area ratio between the two patient groups. In these circumstances, there was a 36% decrease in the number of epileptic seizures after 6 months.

Discussions

Recent advances in pharmacogenetics have made the hypothesis according to which inter-individual genetic variations are underlying the variability in response to treatment more attractive. This study aims to perform an analysis that would determine the relationship between *CYP2C9* genetic polymorphisms involved in the metabolism of valproate, valproate dosage, total plasma concentrations of valproate and the patient's clinical response expressed by the number of epileptic seizures/time unit (6 months in the current study).
The study population showed no significant differences in patient age according to gender.
The study of *CYP2C9* allele frequencies showed a *CYP2C9*2* allele frequency of 13%, result that is consistent with data in the literature on the frequency of this allele in the Caucasian population (8-20%) (Kirchheiner *et al* 2005).
When we calculated *CYP2C9*3* allele frequency in the study population, we found a frequency of 8.7%, which is also in agreement with the average frequency reported for this allele in the Caucasian population (6-10%) (Azarpira *et al* 2010).
Within the study population, 1 patient was homozygous for the *CYP2C9*2* allele and 2 were heterozygous for both *CYP2C9*2* and **3* alleles. We found no patients homozygous for the *CYP2C9*3* allele.
There is data in the literature reporting that the correlation between valproate dosage and the plasma concentration of valproate may be influenced by demographic factors such as age, gender or weight (Aghebati *et al* 2012). In our study, we tried to determine the influence of age, gender or weight on the relationship between valproate dosage and the plasma concentration of valproate.
Average valproate dosage at the first visit was of 1000 (1000; 1000) mg. Statistical analysis performed in the current study indicated that the dose of valproate was not correlated with patient age, number of seizures in the last 6 months or body mass index. In addition, VPA dosage did not correlate with patient gender as well. Dose/body surface area ratio did not correlate with patient age or gender, but was strongly correlated with the number of seizures in the last six months before the first visit. Both the dose of VPA and the dose/body surface area ratio were independent of *CYP2C9* genetic polymorphisms ($p>0.05$).
In the current study, plasma concentrations of valproate were not correlated with patient age and gender, but were correlated with dose and dose/body surface area ratio. Significant correlations between VPA dosage and total plasma concentrations were also found in other studies (Aghebati *et al* 2012). However, other authors have reported poor correlations between plasma levels and the dose of valproate (these correlations can be attributed to inter-individual differences in terms of drug clearance, in turn influenced by *CYP2C9* genetic polymorphisms) (Panomvana NaAyudhya *et al* 2006).

Plasma concentrations of valproate were higher in patients with *CYP2C9*1/*2* genotype (85.5±30.5) than in those with *CYP2C9*1/*1* genotype (69.4±28.9). This indicates that patients whose genotype contains *CYP2C9*1/*2* mutant allele have a reduced enzyme activity for valproate compared to wild-type genotype, so far proved in other studies as well (Rosemary *et al* 2007). There have been studies reporting the correlation between heterozygous *CYP2C9*3* genotype and higher plasma concentrations than the wild-type genotype (Tan *et al* 2010). We have not determined differences in valproate concentrations depending on the presence of *CYP2C9*3* allele.

At the first visit, a correction of valproate dosage/day was applied depending on clinical parameters (number of epileptic seizures in the past 6 months), so that the doses recorded for the second visit were the daily doses that patients received throughout the 6 month follow-up.

Six months after the first visit, 68 (of 69) patients were present at the second visit. Of these, 35 (51.4%) patients had no epileptic seizure from the previous visit. The second visit (after 6 months) revealed a statistically significant reduction in the number of epileptic seizures in comparison with the previous visit (seizure frequency was monitored over a period of 6 months in both cases).

Average dose/body surface area ratio was of 552.9±214.1 mg/m^2 for the first visit. The values recorded for the second visit were of 633.2±237.4 mg/m^2. There was a statistically significant difference between the dose/body surface area ratio for the first visit and that for the second visit.

Statistical data analysis of the two visits indicated that the presence of *CYP2C9*2* allele and the dose/body surface area ratio for the second visit were two parameters associated with a reduction in the number of epileptic seizures ($p=0.02$, $p=0.05$ respectively). We showed that patients with *CYP2C9*1/*1* genotype had significantly higher increases in VPA dosage related to body surface area from visit 1 to visit 2, compared to subjects with *CYP2C9*1/*2* genotype.

*CYP2C9*3* allele had no influence on the change in the number of epileptic seizures or valproate dosage related to body surface area.

Conclusion

Patients carrying the *CYP2C9*2* allele had higher plasma concentrations of valproate, with a reduced enzymatic activity for valproate (being poor metabolizers), while patients with normal alleles had lower plasma concentrations due to the type of rapid metabolizer.

To reduce the number of epileptic seizures, patients with *CYP2C9*1/*1* genotype needed significantly higher doses of valproate reported to body surface area compared to subjects with *CYP2C9*1/*2* genotype.

The presence of *CYP2C9*2* allele and the dose/body surface area ratio recorded during the second visit were two parameters associated with the reduced number of epileptic seizures.

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