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# STUDY OF THE PHENOTYPIC VARIATION EXPRESSION IN THE METABOLIZING STATUS OF ROMANIAN EPILEPTIC PATIENTS

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**Abstract. Aim of the study.** The present study oversees the influence of CYP polymorphisms on the metabolism of VPA and the correlation between the genotype and the plasma levels. **Materials and method.** 46 patients either with idiopathic or secondary epilepsy, with a mean age of 37,37 ± 1,87, evaluated in the Neurology Clinic of Cluj-Napoca were included. All patients were under stable VPA treatment for at least a month. Steady state plasma concentrations were determined using the GC/FID technique. We considered therapeutic level between 50-100 μg/mL. Using the PCR-RFLP method for each patient we've determined allelic variant of CYP2C19*2 and CYP2C19*3. **Results.** 65% of the patients had therapeutic level of valproic acid, 15% supra-therapeutic and 20% sub-therapeutic level of it. 23,91% of the patients were heterozygous for CYP2C9*2 and 19,57% CYP2C9*2 for CYP2C9*3. Regarding CYP2C19*2 15,22% were heterozygous and 8.70% homozygous. Allele CYP2C19*3 was absent. There were no significant correlation established between the genetic metabolizer status and it's phenotypic expression represented by the plasma concentrations. **Conclusions.** The metabolizer status defined by the different allelic expression of CYP2C9 and CYP2C19 doesn't present statistically significant influences on VPA steady-state plasma concentrations.

**Keywords:** pharmacogenetics, metabolism, valproic acid

## Introduction

Epilepsy is an invalidating chronic neurological disorder, with an unpredictable character. Despite the availability of numerous antiepileptic drugs (AEDs), there is still a significant percent of the patients without proper control of the disease (1).

Pharmacogenomics investigates the pathways behind the influences of the human genome's variations on the responses to medication and permits to modify it according to the patient's individual genetic constellation (2). Genotyping before drug administration seems to be a promising clinical approach in order to reduce adverse effects and to increase the efficacy of the treatment (3).

The polymorphisms of the genes involved in the metabolism of AEDs can potentially modify the activity of the coded enzymes. The CYP2C9 and CYP2C19 genes belong to the P450 (CYP) enzymatic system, being involved in the metabolism of the main AEDs.

Valproic acid/Sodium valproate (VPA) is an AED with a broad spectrum, and it is extensively metabolized in the liver, the enzymes of the P450 system being involved in the process (4). The polymorphism of the CYP2C9 and CYP2C19 isoenzymes represent a potentially determinant factor in

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the alteration of some drug's metabolism (5). There are relatively few studies investigating the influence of the CYP2C9 and CYP2C19 polymorphisms over VPA pharmacokinetics. The present paper oversees the influence of these polymorphisms on the metabolism of VPA and the correlation between the genotype and the plasma levels.

## Materials and method

### The patients

46, either idiopathic or secondary epilepsy patients were included, evaluated in the Neurology Clinic of Cluj-Napoca, all being under stable VPA treatment for at least a month. The average age was 37,37 ± 1,87 years. The distribution on gender was 63% women, and 37% men. The patients were assessed according to international diagnostic criteria. The study also got the approval from the Ethics Committee of the University of Medicine and Pharmacy "Iuliu Hatieganu" Cluj-Napoca, each patient was instructed and has signed an Informed Consent.

### VPA plasma concentrations

A simple capillary gas chromatographic assay with liquid-liquid extraction and flame ionization detection (GC/FID) was developed and validate for the determination of VPA in human plasma. In order to achieve this aim we use caprilic acid as internal standard.

The blood samples (5 ml) were centrifuged (5000 rpm) in the first 4 hours, followed by plasma separation and freezing at -20°C until the determination of concentrations. To do this, 200 μl of serum was mixed with an aliquot of caprilic acid as internal standard and extracted by stepwise addition of hydrochloric acid and chloroform with slight agitation between each step. After centrifugation, 1.0 μl of the bottom layer was injected into the capillary column.

Determinations were performed using an Agilent 6890N GC system, with split/splitless injector, and a 7673 autosampler. We used a HP-5 capillary column (30m, 320 μm (i.d.), 0.25 μm) and a temperature regimen. Capillary gas chromatographic conditions yielded typical retention times of 4.4 min for VPA and 5.5 min for the internal standard. The limit of detection was 0,34mg/L. Calibration was linear between 5,215 and 333,76mg/L. The simplicity of sample preparation with no derivatization, short run-time and high sensitivity sufficient to detect low concentrations of the drug makes this method suitable for research as well as routine use. The method was used to determine therapeutic levels of VPA for more then 100 patients.

### Determining the polymorphisms of CYP2C9 and CYP2C19

Genotyping was conducted using DNA extracted from lymphocytes of peripheral blood, after venous puncturing. Probes were kept at 2-8$^0$C. DNA extraction was effected using commercial kits for genomic DNA extraction (Wizzard Genomic DNA Purification Kit, Promega, USA). Polymorphisms were determined with PCR-RFLP (Polymerase Chain Reaction - Restriction-Fragment Length Polymorphism) technique. Allelic variant detection of CYP2C19*2 and CYP2C19*3 used a modified protocol described initially by Zand et al (6). Variants of CYP2C9*2 and CYP2C9*3 were identified using the PCR-RFLP method, modified after Oijen et al (7). Fragments resulted after digestions were analyzed in a 2% agarose gel in 1x TBE tampon. Gels were colored with ethidium bromide, and visualized using a photo-documentation and gel analysis system (Vilber Lourmat Imaging System®, Franța).

## Statistical analysis

The data proposed for analysis: the placement in a plasma concentration interval, respectively the normal or polymorphic metabolizer status represents nominal variables. Contingency tables were constructed, and the independence was evaluated through the Chi squared test. The strength of the former relationship was revealed with the Phi post-test. The significance threshold was $p<0.05$. Statistical calculations were made with the help of the SPSS version 17.

## Results

After genotyping, the frequency of mutant alleles for the CYP2C9 and CYP2C19 isoenzymes was established, the values being reproduced in table 1.

| | **1*/2*** | **1/3*** | **2*/2*** | **3*/3*** |
|---|---|---|---|---|
| **CYP2C9** | 23,91% | 19,57% | 0,00% | 0,00% |
| **CYP2C19** | 15,22% | 0,00% | 8,70% | 0,00% |

**Table 1.** The frequency of polymorphic alleles (homo-, or heterozygous) for CYP2C9 and CYP2C19 in the investigated population

After the steady-state plasma concentrations were obtained, the patients were divided in three subgroups according to the VPA plasma level: patients with sub- (<50 µg/mL), supra- (>100 µg/mL) or normal (50-100 µg/mL) therapeutic levels, their distribution being reproduced below.

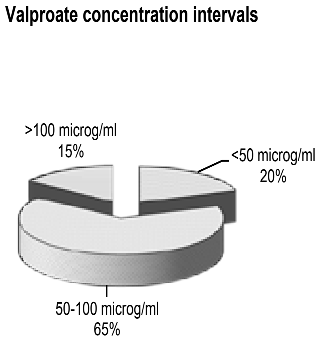

**Figure 1.** Patient distribution according to the realized steady-state plasma concentration of valproate

To test the existence of correlation between the polymorphisms of the metabolizer enzymes and individual plasma concentration (genotype-phenotype correlation), the data were categorized into contingency tables using the two nominal variables, the placement in one of the established concentration intervals and the normal or polymorphic status, the mutant being either hetero- or homozygous.

In the followings we reproduce in tables the distribution of the patients included in the study according to the steady-state plasma concentration of VPA and the characteristic of being normal homozygous (1*/1) or carrier – homo- or heterozygous – of the polymorphic alleles (2*/2*, 3*/3*, 1*/2*, 1*/3*) of the CYP2C9 and CYP2C19 enzymes, together with the statistical testing of the existence of association and the strength of it, the values of the Chi squared test and the Phi post-test for 2x2 contingency tables. We mention that lack of significance on the Chi squared test produces the Phi test to be irrelevant, overtaking the initial probability, because one cannot test the strength of an association without the existence of it.

For CYP2C19_3 the testing was impossible to be realized, the metabolizer status being a constant, every patient was normal homozygous (1*/1*).

| **Valproate microg/ml** | **CYP2C9_2** | | **Total** | **Chi-Square** | | **Phi** | |
|---|---|---|---|---|---|---|---|
| | **1*/1*** | **1*/2*** | | **Value** | **p** | **Value** | **P** |
| **<50** | 7 | 2 | 9 | 0,005 | 0,945 | 0,011 | 0,945 |
| **50-100** | 23 | 7 | 30 | | | | |
| **Total** | 30 | 9 | 39 | | | | |
| **50-100** | 23 | 7 | 30 | 0,085 | 0,048 | 0,771 | 0,771 |
| **>100** | 5 | 2 | 7 | | | | |
| **Total** | 28 | 9 | 37 | | | | |

**Table II.** Contingency tables regarding the association between the metabolizer status defined by the allelic expression of CYP2C9_2, with the sub-, supra- or normal therapeutic interval of the VPA steady-state concentration, together with the statistical tests of the existence of association and the strength of it.

| **Valproate microg/ml** | **CYP2C9_3** | | **Total** | **Chi-Square** | | **Phi** | |
|---|---|---|---|---|---|---|---|
| | **1*/1*** | **1*/2*** | | **Value** | **p** | **Value** | **P** |
| **<50** | 6 | 3 | 9 | 1,179 | 0,277 | -0,174 | 0,277 |
| **50-100** | 25 | 5 | 30 | | | | |
| **Total** | 31 | 8 | 39 | | | | |
| **50-100** | 25 | 5 | 30 | 0,024 | 0,878 | -0,025 | 0,878 |
| **>100** | 6 | 1 | 7 | | | | |
| **Total** | 31 | 6 | 37 | | | | |

**Table III.** Contingency tables regarding the association between the metabolizer status defined by the allelic expression of CYP2C9_3, with the sub-, supra- or normal therapeutic interval of the VPA steady-state concentration, together with the statistical tests of the existence of association and the strength of it.

| Valproate microg/ml | CYP2C19_2 | | Total | Chi-Square | | Phi | |
|---|---|---|---|---|---|---|---|
| | 1*/1* | 1*/2* | | Value | p | Value | p |
| **<50** | 7 | 2 | 9 | 0,005 | 0,945 | 0,011 | 0,945 |
| **50-100** | 23 | 7 | 30 | | | | |
| **Total** | 30 | 9 | 39 | | | | |
| **50-100** | 23 | 7 | 30 | 0,085 | 0,771 | 0,048 | 0,771 |
| **>100** | 5 | 2 | 7 | | | | |
| **Total** | 28 | 9 | 37 | | | | |

**Table IV.** Contingency tables regarding the association between the metabolizer status defined by the allelic expression of CYP2C19_2, with the sub-, supra- or normal therapeutic interval of the VPA steady-state concentration, together with the statistical tests of the existence of association and the strength of it.

## Discussion

Genetic polymorphism represents a factor that can influence drug metabolism, determining inter- and intraindividual variation of the therapeutic response. CYP2C9 is involved in the metabolism of more than 100 drugs, among these oral anticoagulants, antidiabetic sulphonylureas, angiotensin receptor inhibitors and some of the NSAIDs (8). In vitro studies showed that the CYP2C9*3 is associated in a more pronounced manner than CYP2C9*2 with the intrinsic clearance of drugs, their polymorphisms being responsible for the poor metabolizer status (9). At least one of the CYP2C9*2 or *3 variants are found at 8-16% and respectively 5-9% of the Caucasian population, ~2,5 % being homozygous for these alleles or heterozygous for *2/*3 (10,11). The frequency of alleles for CYP2C9 revealed in the present study is similar with the data obtained among the Romanian epileptics, the frequency for the heterozygous type being higher than in the general population (12).

Another polymorphic CYP isoform is CYP2C19, responsible for the metabolization of S-mephenytoin, diazepam, some antidepressants, omeprazole (13). In At this point it has seven variants (*2 - *8) of CYP2C19, associated with in vivo activity reduction and a new variant (CYP2C19*17), which can produce an ultra rapid metabolizer phenotype (14). CYP2C19*2 variant is relatively common in the Caucasian population, being approximately 15%, in opposition with the CYP2C19*3 variant, present in a proportion of 0,04%. The latter is more frequent in the Asian populations and at African-americans (0,4% and 5 %) (15). The present study reveals similar data, the CYP2C19*3 variant being absent both in epileptic patients and in the general population (12).

VPA is metabolized in the liver, but the pathway of P450 isoenzymes is not the main metabolic branch. The present study shows that the presence of hetero- or homozygous alleles for CYP2C9 and CYP2C19 doesn't influence the steady-state concentration of VPA. Lan Tan et al showed in a 2010 study that the presence of CYP2A6*4, CYP2B6*6 and CYP2C9*3 alleles among Chinese patients is associated with a higher steady-state concentration than the one observed in patients without these mutations (4). In Lan et al's study, the evaluations were conducted on another population, with a different allelic frequency than the Caucasian population, this possible being a reason for the different influences on VPA metabolism. These isoenzymes are probably responsible for only a reduced percent of the metabolism of VPA, it's plasma concentration being influenced by numerous other factors, among those we can mention alimentation and associated medication. In rare cases, VPA is metabolized into a product with increased liver toxicity, 4-ene-VPA (16), CYP2C9 being responsible for 70-80% of this metabolite.

## Conclusion

The metabolizer status defined by the different allelic expression of CYP2C9 and CYP2C19 doesn't present statistically significant influences on VPA steady-state plasma concentrations, for the investigated polymorphisms. There were no significant correlations established between the genetic metabolizer status and it's phenotypic expression represented by the plasma concentrations. The first line limit of the study is the dimension of the group, which does not permit sufficient statistical relevance.

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