Relations of CYP2C19*2 genetic polymorphisms to plasma and saliva concentrations of diazepam in patients hospitalized for alcohol withdrawal

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Abstract: Diazepam is one of the most widely prescribed tranquilizers for the therapy of alcohol withdrawal syndrome (AWS). However, diazepam therapy often turns out to be ineffective, and some patients experience dose-dependent adverse drug reactions. Previous studies have shown that the metabolism of diazepam involves the CYP2C19 isoenzyme, whose activity is highly dependent on polymorphism of the encoding gene. The objective of our study was to investigate the effects of *CYP2C19*2* genetic polymorphisms on plasma and saliva concentrations of diazepam as well as its impact on the efficacy and safety rates of therapy in patients with AWS. The study was conducted on 100 Russian male patients with AWS who received diazepam in injections at a dosage of 30.0 mg/day for 5 days. Genotyping was performed by real-time polymerase chain reaction. The efficacy and safety assessment was performed using psychometric scales. We revealed differences in the efficacy and safety of therapy in patients with different *CYP2C19 681G>A* genotypes. Therapeutic drug monitoring (TDM) revealed the statistically significant differences in the levels of diazepam plasma concentration: (*GG*) 199.83 [82.92; 250.58] vs (*GA+AA*) 313.47 [288.99; 468.33], p=0.040, and diazepam saliva concentration: (*GG*) 2.80 [0.73; 3.80] vs (*GA+AA*) 5.33 [5.14; 6.00], p=0.003.

Keywords: *pharmacogenetics; benzodiazepines; diazepam; biotransformation; personalized medicine; CYP2C19; alcohol withdrawal*

Introduction

Today benzodiazepines (BZDs) are among the most widely prescribed medicinal products in the world [1]. They have the largest and the best evidence base in the treatment of alcohol withdrawal syndrome (AWS), and are considered the gold standard [2]. Currently the problem of personalized approach to the prescription of BZDs is poorly developed in the scientific community. The wide use of these medications creates a misleading impression that it does not require the personalized approach. Therefore, despite the high frequency of administration of BZDs, their dose selection is currently empirically based. According to the available scientific data, in a subset of patients, AWS worsens despite escalating doses of BZDs [3]. Such patients represent a severe alcohol withdrawal state and a serious challenge to practitioners due to the acuity and refractoriness of the disorder [4, 5]. The incidence rates of this state are unknown, but patients suffering from the resistant AWS were found to have a higher rate of intubation, longer ICU stays, and a greater risk of nosocomial infections in comparison with the patients with the AWS who

response to BZDs [5, 6]. Meanwhile, the use of the exceedingly high doses of BZDs in this cohort of patients may result in the occurrence of adverse drug reactions (ADRs).

Today it is well known that clinical responses to BZDs vary widely between individuals. [1]. The studies of the pharmacogenetics of BZDs usually focus on the genes of cytochrome P450 (CYP) enzymes, which are among the factors that contribute to the pharmacokinetic (PK) variability of drugs. Diazepam is mainly metabolized via CYP2C19 and CYP3A4 to its major active metabolite, desmethyldiazepam. Recent studies revealed the effect of *CYP2C19* and *CYP3A4* genetic polymorphisms on the pharmacokinetics of BZDs [7-10]. The differences in the activity of these enzymes are genetically determined. Currently there is a lack of data on the pharmacogenetics of BZDs in patients with AWS [11]. It creates conditions for examining this issue.

Objective

The objective of our study was to investigate the effect of *CYP2C19*2* genetic polymorphisms on both plasma and saliva concentration levels of diazepam as well as its impact on the efficacy and safety rates of therapy in patients with AWS.

Materials and Methods

Clinical characteristics of patients

The study included 100 male patients (average age — 42.66±9.8 years). Inclusion criteria were the diagnosis of "Mental and behavioral disorders due to use of alcohol. Withdrawal state, uncomplicated" (F10.30, according to ICD-10); written informed consent obtained from the patient; an initial phase of AWS (abstinence from alcohol for at least 8 hours, but no longer than 48 hours prior to the inclusion in study); presence of anxiety, fear or emotional tension in the clinical presentation of the patient; Clinical Institute Withdrawal Assessment for Alcohol scale (CIWA-Ar) score more than 10. Exclusion criteria were presence of any other mental disorders or severe somatic disorders (except alcoholic hepatitis and toxic encephalopathy); presence of any other psychotropic medications in treatment regimen except diazepam; creatinine clearance values <50 mL/min, creatinine concentration in plasma ≥1.5 mg/dL (133 mmol/L); body weight less than 60 kg or greater than 100 kg; age of 75 years or more, and presence of any contraindications for diazepam use.

For the therapy of anxiety, fear and emotional tension in the clinical presentation of AWS, patients received diazepam in intramuscular injections at a dose of 30.0 mg per day.

Therapy efficacy and safety evaluation

To evaluate the diazepam efficacy, an international well-validated psychometric scale was used: Clinical Institute Withdrawal Assessment for Alcohol scale (CIWA-Ar) [12]. The safety profile was evaluated using The UKU Side-Effect Rating Scale (UKU) [13]. Patients were examined on days 1 and 5 of diazepam therapy.

Genotyping

Venous blood samples collected in vacuum tubes VACUETTE® (Greiner Bio-One, Austria) on the fifth day of diazepam therapy were used for genotyping. The real-time polymerase chain reaction was performed using DNA amplifiers "Dtlite" of DNA Technology (Moscow, Russia), CFX96 Touch Real Time System with CFX Manager software of Bio-Rad Laboratories Inc. (Hercules, CA, USA) and sets "SNP-screen" of "Syntol" (Moscow, Russia). It was used to determine the single nucleotide polymorphisms

(SNP's) *681G>A* of the gene *CYP2C19*2* (*rs4244285*). In every "SNP-screen" set, two allele-specific hybridizations were used, which allowed determining two alleles of studied polymorphism separately on two fluorescence channels.

Therapeutic drug monitoring

For the therapeutic drug monitoring (TDM), venous blood samples were collected on the day 5 of diazepam therapy. The plasma calibration standards (St) and quality control samples (QC) were made from a stock solution prepared by consistent dissolving of substantial amounts in methanol with subsequent dilution to the relevant concentrations. Calibration curve was created using 5, 10, 20, 50, 100, 200, 500, 1000, 2000 ng/mL calibration standards along with 5 ng/mL (LLOQ), 15 ng/mL (Low QC), 1000 ng/mL (Medium QC), and 1500 ng/mL (High QC) quality control samples (QC). Diazepam (250 ng/mL in acetonitrile) was used as the internal standard.

Sample preparation

Samples were prepared using a protein precipitation method. A 1.5 mL tube was filled with 200mcL of analyzed plasma sample and 600mcL of acetonitrile containing the internal standard. The mixture was shaken on Vortex for 10 minutes, and then samples were centrifuged at 14,500 g for 10 minutes at 4 °C. Then the supernatant was transferred to an autosampler vial. Samples were analyzed using the HPLC system Agilent 1260 (Agilent Technologies, California, USA) and tandem mass selective detector Agilent 6460 (Agilent Technologies, California, USA) with Jet Stream Electrospray Ionization Source.

Conditions of chromatographic analysis

Stationary phase: column Agilent Poroshell 120 EC-C18 (2.7 μm, 3.0 mm × 50 mm) with the precolumn InfinityLab Poroshell 120 EC-C18 (2.7 μm, 3.0 mm × 5.0 mm) (Agilent Technologies, California, USA). The column temperature was 50°C. The mobile phase consisted of the A eluent (10 mM ammonium formate in 0.1% formic acid) and B eluent (methanol in 0.1% formic acid). The flow rate was 0.4 mL/min. The gradient elution process was performed; the gradient of the mobile phase is presented in Table 1.

The analysis time was 9.0 minutes for every sample. The volume of the inserted sample was 2 mcL. Retention time under the given conditions was 4.75 min for diazepam and 4.84 min for the internal standard.

Conditions of mass-spectrometry determination

We used positive mode electrospray ionization for mass-selective detection. Detector registered following MRM-transitions: from 349.0 m/z [M+H+] to 206.1 m/z (collision cell energy 40 V) and from 349.0 m/z [M+H+] to 184.0 m/z (collision cell energy 32 V) for diazepam; from 285.1 m/z [M+H+] to 193.1 m/z (collision cell energy 32 V) and from 285.1 m/z [M+H+] to 154.1 m/z (collision cell energy 24 V) for the internal standard. The voltage on phragmentor for diazepam and internal standard was 156V and 166V, respectively. The voltage on capillary was 3.5 kV, the temperature of desiccant gas was 350°C, nitrogen flow was 6 L/min. Nebulizers pressure was 45 psi, sheath gas temperature was 375°C, sheath gas flow was 11 L/min.

Table 1. Gradient of the mobile phase.

Time, min	Part of eluent A, %	Part of eluent B, %
0.00	95	5
0.50	95	5
1.00	50	50
1.50	5	95
3.00	5	95
3.01	95	5
5.00	95	5

Method validation

The methodology used in the study met FDA Guidance for Industry: Bioanalytical method validation. Calibration dependence was linear for diapason at 0.5-200 ng/mL. Correlation coefficients were normal (at least 0.99). We evaluated the intra- and inter-cycles precision and accuracy rates. Precision and accuracy rates were normal (no more than 20 % at LLOQ, no more than 15 % for other points). The matrix effect had no influence.

Local ethical committee

The research was approved by the local ethical committee of the Moscow Research and Practical Centre on Addictions of the Moscow Department of Healthcare (The protocol No. 07-17 from 11/28/2017).

Statistical analysis

Statistical analysis of the results was performed with non-parametric methods using the «Statsoft Statistica v. 10.0» (Dell Statistica, Tulsa, OK, USA). The normality of samples distribution was evaluated using W-Shapiro-Wilk test and taken into account when choosing a method. The differences were considered statistically significant at $p < 0.05$ (power in excess of 80 %). Multiple samples of continuous data were compared using the analysis of variance (ANOVA/MANOVA). To determine the correlation between quantitive characteristics Spearman rank correlation coefficient (r_s) was calculated. Research data are presented as the median and interquartile range (Me [Q1; Q3]) or, in case of a normal distribution, as the arithmetic mean and standard deviation (Mean±SD).

Results

The *CYP2C19* genotyping by polymorphic marker *681G>A* (*rs4244285*) performed in 100 patients have revealed the following:
- The number of patients with *GG* genotype accounted for 79 (79%);
- The number of patients with *GA* or *AA* genotype accounted for 21 (21%).

The distribution of genotypes corresponded to Hardy-Weinberg equilibrium for the European population ($\chi^2 = 2.18$, $p < 0.001$).

To investigate the efficacy of therapy in patients with different genotypes, the two-way analysis of variance (ANOVA) was performed including the *CYP2C19* genotype and the day of the study as covariates. The results of data analysis performed for the CIWA-Ar scale in patients with different genotypes are presented in Table 2.

Dynamics of changes in CIWA-Ar scale scores across patients with different genotypes by polymorphic marker *681G>A* (*rs4244285*) are shown in Figure 1.

Table 2. CIWA-Ar scale scores in patients with different genotypes from Day 1 to Day 6 of the study.

Genotypes	CIWA-Ar scale scores					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
GG	21 [18; 24]	14 [12; 16]	14 [11; 16]	13 [10; 14.75]	12 [10; 14]	13 [10; 15]
GA + AA	24 [22; 26]	19 [16; 22]	17 [14; 19]	14 [11; 16]	12 [9; 14]	12 [9; 14]
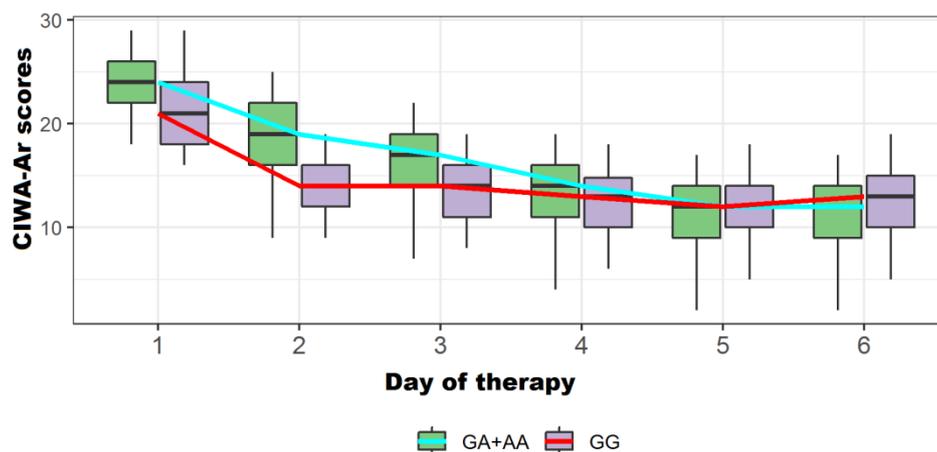
Figure 1. Dynamics of changes in CIWA-Ar scale scores across patients with different genotypes from Day 1 to Day 6 of therapy.

Table 3. Comparison of CIWA-Ar scale scores in patients with different genotypes from Day 1 to Day 6 of the study.

Variables	F	p-value
Day	114.93	<0.001
Genotype by polymorphic marker *681G>A* (*rs4244285*)	10.60	<0.001
Day + Genotype by polymorphic marker *681G>A* (*rs4244285*)	7.79	<0.001

The results of the two-way analysis of variance are presented in Table 3.

Statistical significance was obtained for the study day (F = 114.93, p < 0.001) and genotype parameters (F = 10.60, p < 0.001).

To investigate the safety of therapy in patients with different genotypes, the two-way analysis of variance was performed including the *CYP2C19* genotype and the day of the study as covariates. The results of data analysis performed for the UKU scale in patients with different genotypes are presented in Table 4.

Table 4. The UKU scale scores in patients with different genotypes from Day 1 to Day 6 of the study.

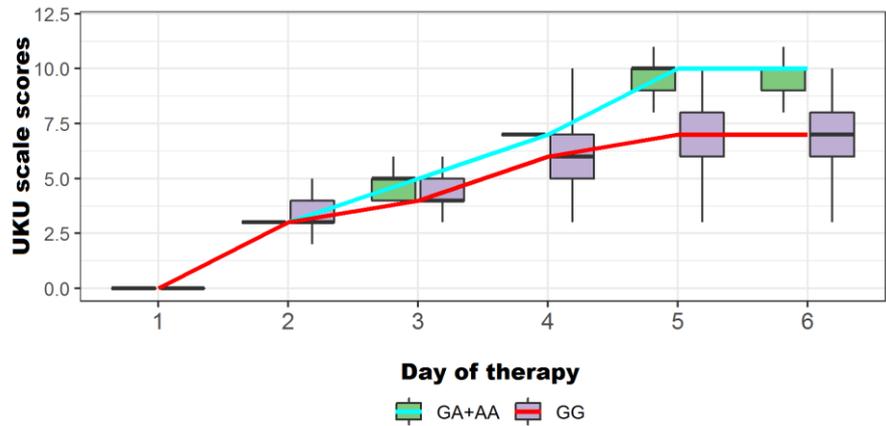| Genotypes	The UKU scale scores					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
GG	0 [0; 0]	3 [3; 4]	4 [4; 5]	6 [5; 7]	7 [6; 8]	7 [6; 8]
GA + AA	0 [0; 0]	3 [3; 3]	5 [4; 5]	7 [7; 7]	10 [9; 10]	10 [9; 10]

Figure 2. Dynamics of changes in the UKU scale scores across patients with different genotypes from Day 1 to Day 6 of therapy.

Table 5. Comparison of the UKU scale scores in patients with different genotypes from Day 1 to Day 6 of the study.

Parameter	GG	GA + AA	p-value
Diazepam plasma concentration, ng/ml	199.83 [82.92; 250.58]	313,47 [288,99; 468,33]	0,040
Diazepam saliva concentration, ng/ml	2.80 [0.73; 3.80]	5.33 [5.14; 6.00]	0,003

Dynamics of changes in the UKU scale scores across patients with different genotypes by polymorphic marker *681G>A* (*rs4244285*) are shown in Figure 2.

The results of the two-way analysis of variance are presented in Table 5.

We revealed a statistically significant difference in plasma concentration levels of diazepam across patients with different genotypes: (*GG*) 199.83 [82.92; 250.58] vs (*GA+AA*) 313.47 [288.99; 468.33], p=0.040 (Figure 3).
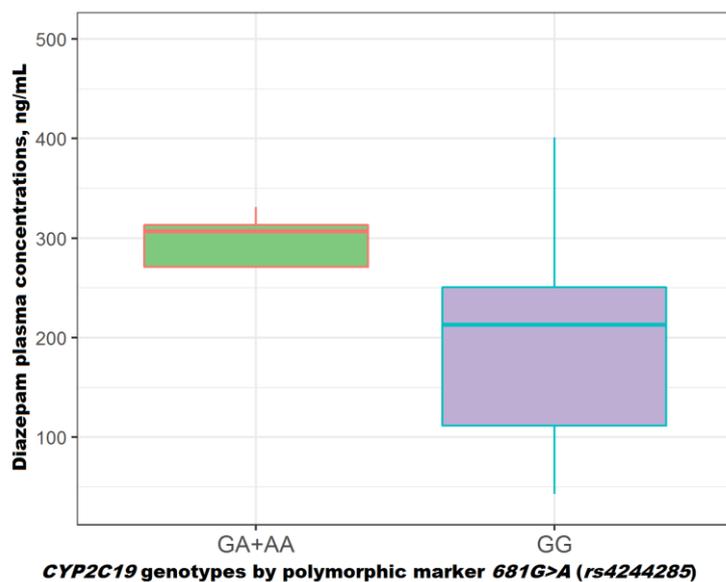

Figure 3. Differences in plasma concentration levels of diazepam in patients with different genotypes.
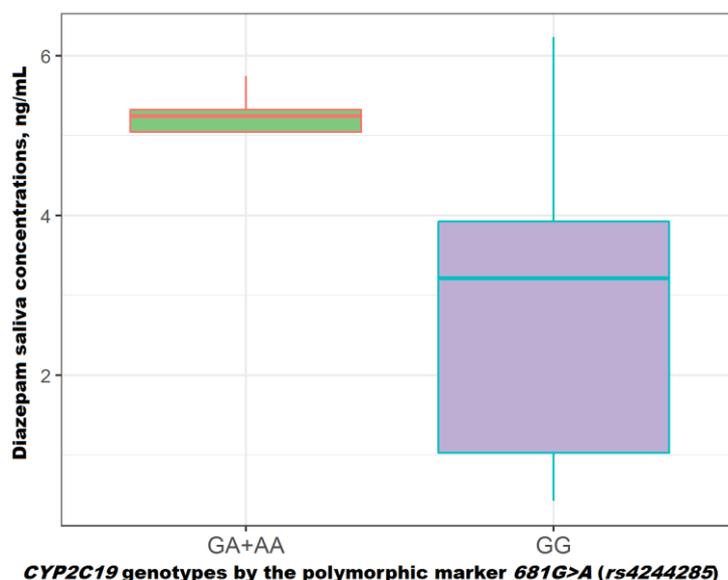
Figure 4. Differences in saliva concentration levels of diazepam in patients with different genotypes.

Pharmacokinetic study revealed that saliva concentrations of diazepam in patients with different genotypes by polymorphic marker *681G>A* (*rs4244285*) also has the statistically significant differences: (*GG*) 2.80 [0.73; 3.80] vs (*GA+AA*) 5.33 [5.14; 6.00], p=0.003). A comparison of saliva concentration levels of diazepam in patients with different genotypes is presented in Figure 4.

Discussion

The results of our study revealed the difference between the efficacy and safety profiles of diazepam in patients with AWS carrying different genotypes of the *CYP2C19* gene by polymorphic marker *681G>A* (*rs4244285*).

The efficacy of diazepam therapy (as evaluated by the psychometric scales) was different across the patients with AWS carrying different genotypes: the difference in CIWA-Ar scores before the therapy and after it was lower in patients with the *GG* genotype in comparison with those who carried the *GA* and *AA* genotypes. This is presumably related to the decreased CYP2C19 isoenzyme activity in patients carrying the *GA* and *AA* genotypes. This, in turn, leads to the reduced biotransformation rates of diazepam, an increase in concentration rates of the drug in plasma and more pronounced effect of the medication.

Patients carrying the *GA* and *AA* genotypes showed a higher increase in the UKU scale scores, which demonstrate that such patients have a higher risk of ADR occurrence than the *GG* genotype carriers do. This appears to be due to the decreased activity of the CYP2C19 isoenzyme in patients carrying the minor allele *A* by polymorphic marker *681G>A* (*rs4244285*) of the *CYP2C19* gene. The decreased activity of the CYP2C19 isoenzyme leads to the reduced biotransformation rates of diazepam, which in turn leads to an increase in plasma concentration of the drug and to an enhanced risk of undesirable side effects.

Pharmacokinetic study revealed that carriers of the wild-type genotype have a lower level of both plasma and saliva diazepam concentrations, which is probably due to the reduced diazepam biotransformation in the carriers of the minor allele *A*.

Thus, based on the study results, one would assume that patients who carry the *GG* genotype have a higher risk of absence of the intended therapeutic effect of diazepam,

which leads to persistence of the anxiety, fear and emotional tension in the clinical presentation of patients. To reduce this risk, such cohort of patients requires the prescription of medications, which are not metabolized by CYP2C19, or administration of higher doses of diazepam. Furthermore, it is possible to suppose that the carriers of the minor nonmutant genotype by polymorphic marker *681G>A* (*rs4244285*) of *CYP2C19* gene have a higher risk of ADR occurrence during the diazepam administration.

These results are consistent with the findings of our previous study focused on investigation of the *CYP2C19*2* genetic polymorphism, which had a 2-times lower sample size and enrolled 50 patients [7]. Furthermore, previous studies which were conducted by our research group [14, 15], confirm the importance, relevance and possibility to investigate the personalized approach to the prescription of BZDs (and specifically diazepam) in such cohort of patients.

Conclusions

The study conducted in 100 patients with AWS revealed the correlation between the *CYP2C19*2* genetic polymorphisms and the efficacy and safety of diazepam. Furthermore, a statistically significant difference in both plasma and saliva concentration levels of diazepam across patients with different genotypes was revealed.

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