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The influence of diabetic status on the pharmacokinetics of clopidogrel and its metabolites in patients suffered from cardiovascular diseases

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ABSTRACT

Aim. A significant percentage of individuals treated with an anti-platelet agent clopidogrel do not receive the expected therapeutic effect. Clopidogrel resistance is even more prevalent in patients with type 2 diabetes mellitus (DM). An extensive investigation on pharmacokinetics of clopidogrel and its metabolites in patients with type 2 DM suffering from cardiovascular diseases were performed following an administration of 75 mg of the drug.

Material and methods. Plasma concentrations of clopidogrel, its carboxylic metabolite (CLPM) and diastereoisomers of a thiol metabolite (the inactive H3 and the active H4) were determined by a validated HPLC-MS/MS method. The pharmacokinetic parameters of the analytes in diabetic (n = 16) and non-diabetic (n = 28) patients were compared and correlated with platelet aggregation.

Results. DM patients exhibited a slightly higher Cmax of clopidogrel (2.34 ± 2.29 ng/mL) compared with non-diabetic group (1.82 ± 1.86 ng/mL), whereas plasma levels of clopidogrel metabolites were lower in DM than in non-DM patients (2339 ± 989 ng/mL vs. 2662 ± 2090 ng/mL, 4.64 ± 4.79 ng/mL vs. 5.42 ± 4.55 ng/mL and 6.42 ± 4.80 ng/mL vs. 7.44 ± 7.18 ng/mL, respectively for CLPM, H3 and H4). A significant correlation was found between platelet aggregation and the Cmax of the active H4 metabolite in non-diabetic patients.

Conclusions. Pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 isomers in patients with DM did not differ significantly from those determined in non-diabetic group. Moreover, the antiplatelet response to clopidogrel therapy measured by ADP-stimulated platelet aggregation was similar in both groups of patients.

Keywords: clopidogrel active metabolite, diabetes mellitus, platelet aggregation.

Introduction

Clopidogrel is a pro-drug from the thienopyridine group with an absolute S configuration at carbon 7 [1]. The drug inhibits platelet aggregation and it is widely used in prevention of ischemic events [2]. However, variability of response to clopidogrel treatment associated with an increased risk of death or thrombotic recurrences is present in 5–40% of patients treated with conventional doses of clopidogrel [3]. The etiology behind a reduced efficacy of clopidogrel has not been completely investigated. Multiple studies attempting to characterize this phenomenon have identified genetic polymorphisms of transporters and enzymes participating in clopidogrel absorption and metabolic transformation, and non-genetic factors including co-morbidities, drug-drug interactions and age [4]. Clopidogrel resistance is even more prevalent in patients with type 2 diabetes mellitus (DM)

and leads to a 2- to 4-fold higher risk of developing cardiovascular disease compared to non-diabetic subjects [5]. Evidence suggests that platelets from patients with DM have an increased reactivity and baseline activation compared to healthy controls [6]. The mechanisms involved in platelet dysfunction include hyperglycemia, insulin deficiency and resistance, associated metabolic conditions and other cellular abnormalities, such as increased P-selectin and glycoprotein expression, oxidative stress or increased production of thromboxane [7]. An impaired response to clopidogrel treatment may be also associated with changes in the drug metabolism observed in DM patients [8]. The metabolic transformation of clopidogrel undergoes through two different pathways in the liver. Up to 85% of the absorbed drug might be transformed by carboxyl esterases to an inactive carboxylic acid derivative of clopidogrel (CLPM) [9]. Because the plasma concentrations of the parent drug are very low, the CLPM determination in plasma might be applied to study the pharmacokinetics of clopidogrel in an indirect manner [10]. Only 15% of the absorbed clopidogrel dose is transformed by isoenzymes of cytochrome P450 (CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4) to a thiol metabolite (CTM), which is responsible for the clopidogrel pharmacological effect [11]. CTM selectively and irreversibly blocks ADP binding to a P2Y12 receptor located on the platelet surface and thus inhibits ADP-induced platelet aggregation [12]. It possesses three stereochemical sites but only two isomers named H3 and H4 are present in clinical samples obtained from patients treated with clopidogrel [13]. Moreover, in vitro studies have confirmed, that only the H4 isomer possesses pharmacological activity [1]. An extensive investigation on pharmacokinetics of clopidogrel and its metabolites in patients with DM would greatly contribute to optimization of the clopidogrel therapy in this disease. There is only one paper focused on the pharmacokinetic aspects of the clopidogrel treatment in diabetic patients. Erlinge et al. compared the plasma levels of CTM in DM patients to those determined in non-diabetic group [8]. However, conditions of the applied HPLC-MS/MS method allowed to determine CTM as a mixture of isomers. Such an approach may lead to overestimation of patient exposure to the active metabolite of clopidogrel because only the H4 isomer is clinically relevant. Moreover, the levels of clopidogrel or its main metabolite CLPM were not considered in that study.

According to our best knowledge, investigation on the pharmacokinetics of clopidogrel and its metabolites: the pharmacologically active H4, and the inactive H3 and CLPM have been performed in diabetic patients for the first time. Moreover, a Multiplate analyzer has been applied to determine the platelet reactivity in this group of subjects in order to estimate the pharmacodynamic-pharmacokinetic correlation.

Material and methods

Chemicals

(+)-S clopidogrel bisulphate (purity 99%) and its carboxylic acid metabolite (CLPM; purity 99.6%) were the generous gift of Pharmaceutical Research Institute (Warsaw, Poland). The 3'-methoxyacetophenone derivatives of clopidogrel thiol metabolite H3 (MP-H3) and H4 (MP-H4) isomers were obtained from Sanofi Aventis (Montpellier, France). Piroxicam (PRX, internal standard, IS) was obtained from Jelfa (Jelenia Góra, Poland). The alkylating agent 2-bromo-3'-methoxyacetophenone (MPB) and formic acid (purity >95%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Acetonitrile (Merck, Darmstadt, Germany) was of HPLC gradient grade. De-ionized water was always used to prepare a mobile phase for HPLC (Simplicity® water purification systems, Merck Millipore, Billerica, USA). Drug free human plasma was obtained from Regional Centre of Blood Donation (Poznań, Poland).

Study population

The study involved 44 patients of Caucasian origin from central Poland undergoing elective coronarography, carotid artery stenting or peripheral artery interventions. Patients received an oral clopidogrel formulation under fasting conditions as a 75 mg maintenance dose for at least 7 days prior to the procedure. In 16 patients taking clopidogrel the concomitant type 2 DM was observed. All diabetic patients were on hypoglycemic treatment (oral anti-diabetic agents or insulin). Exclusion criteria included acute myocardial infarction, malignancies, oral anticoagulation therapy with a coumarin derivatives, treatment with a glycoprotein IIb/IIIa antagonist or other antiplatelet drugs except for aspirin, thrombocytopenia (platelet count < 100 000/μL), chronic liver disease and impaired renal function (serum creatinine level > 2 mg/dL). A detailed characteristics of subjects was presented in **Table 1**. The study protocol was approved by the Ethical Committee at Poznan University of Medical Sciences. All patients gave written informed consent for participation in the study.

Sample collection

Blood samples for pharmacokinetic analysis were collected immediately prior to administration of clopi-

Table 1. Patients' characteristics

	Patients	
	diabetic (n = 16)	non-diabetic (n = 28)
Age [y]	65.5 ± 8.3	61.6 ± 8.6
Body weight [kg]	80.0 ± 12.9	83.0 ± 13.6
BMI [kg/m^2]	29.4 ± 3.8	26.9 ± 6.2
Sex (male/female)	9/7	21/7
Medical history: Hypertension	15	23
Hypercholesterolemia	1	6
Dyslipidemia	5	4
Medical therapy: Proton pump inhibitors	7	11
Statins	15	27
Beta blockers	13	25
ACE inhibitors	10	19

Age, body weight andbody mass index (BMI) are presented as mean ± SD. ACE – angiotensin-converting enzyme

dogrel and at 0.5, 1, 2, 3, 4, 6, 12 and 24 hours post-dosing. An aliquot of 7.5 mL of blood was drawn into collection systems containing K_2EDTA as the anticoagulant (Sarstedt AG&Co., Nűmbrecht, Germany). Due to the limited stability of CTM in human whole blood, 37.5 µL of a 500 mM acetonitrile solution of MPB was added to the systems according to the procedure reported by Takahashi et al. [14]. The blood samples were centrifuged at 1620×g for 10 min and the obtained plasma samples were stored at –25°C until analysis. Following the suggestion of Tuffal et al. [13] the samples with poor signs of the haemolysis after the addition of MPB were considered as not sufficiently stabilized, and the concentrations of both H3 and H4 were not taken into account during the calculation of pharmacokinetic parameters.

Determination of clopidogrel and its metabolites concentrations in plasma

A validated HPLC-MS/MS method [15] was applied for analysis of plasma concentrations of clopidogrel, CLPM and the H3 and H4 isomers in diabetic and non-diabetic patients. The HPLC analysis was performed on a chromatograph Agilent 1200, which was coupled to a triple quadrupole tandem mass spectrometer 6410 B Triple Quad (both from Agilent Technologies, Palo Alto, USA). The analytes were separated in the Zorbax Plus C18 column (100 mm x 2.1 mm, 3.5 µm) (Agilent Technologies, USA) at a column temperature of 40°C. The mobile phase was a mixture of de-ionized water (A) and acetonitrile (B), both containing 0.1% (v/v) formic acid. The gradient was as follows: 0–7 min linear from 42 to 90% B, 7–7.5 min return from 90 to 42% B and the post time of 5 min with 42%B for column equilibration. The mobile phase flow was set at 0.35 ml/min. The eluent from the HPLC column was introduced directly to the MS interface using electrospray ionization in the positive ion mode. The MS parameters were as follows: capillary voltage 4000 V, nebulizer gas (nitrogen) pressure 40 psi (275.8 kPa), desolvation gas (nitrogen) flow 10 L/min and desolvation temperature 300°C. Nitrogen was also used as collision gas. The specific transitions for the analytes were monitored using the multiple reaction monitoring (MRM) mode. Mass transition used for quantitative analysis were as follows: from m/z 322.1 to 212 for clopidogrel, from m/z 504.1 to 155 for the CTM isomers, from m/z 308.1 to 198 for CLPM and from m/z 332.1 to 95 for PRX. An aliquot of 250 µL of plasma was spiked with 25 µL of the IS solution at a concentration of 1 µg/mL. Protein precipitation was performed by adding 450 µL of acetonitrile to each sample. The mixture was vortexed and centrifuged for 10 min at 22570×g at the temperature of 20°C before the supernatant was filtered using Mini Uni Prep filters (Whatman International Ltd., Maidstone, Kent, UK). The resulting filtrate was evaporated under a vacuum at 40°C and the dry residue was reconstituted in 200 µL of a mobile phase. Then, a 25 µL aliquot was injected onto the HPLC-MS/MS system. Calibration curves of the analytes were prepared in concentration ranges of 0.25–5.00 ng/mL for clopidogrel, 50–10000 ng/mL for CLPM and 0.25–50.00 ng/mL for both MP-H3 and MP-H4. The intra- and inter-day accuracy of the method, expressed as the relative error, was ⩽ 16%. The intra- and inter-assay precision, expressed as the relative standard deviation, was ⩽ 19.9%. The analytes were stable in samples stored for 6 h in the autosampler, in plasma samples for 24 h at room temperature and for 3 months at -25°C [15].

Pharmacodynamic assay

Whole blood platelet aggregation was measured in 36 patients using an impedance aggregometer (Multiplate® analyzer, Roche Diagnostics, Mannheim, Germany). Samples for aggregation assay were collected 2–3 hours after clopidogrel administration into the S-Monovette system coated with hirudin (SARSTEDT AG&Co., Nümbrecht, Germany). Whole blood was diluted with 0.9% NaCl solution (1:1, v/v) and stirred in the test cuvettes at 37°C. After addition of 6.4 µmol/L of ADP, the increase in electrical impedance was recorded continuously for 6 min. All materials used for platelet function testing were obtained from Roche Diagnostics (Mannheim, Germany). The platelet aggregation was quantified as arbitrary units (AU) and the area under

the curve of arbitrary units (AU·minute). A cut-off point of 468 AU·min for platelet aggregation in response to ADP was used as the threshold for an increased risk of thrombotic events during clopidogrel therapy according to the recent consensus opinion of Bonello et al. [16].

Pharmacokinetic calculations

The pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 were calculated with the non-compartmental technique using WinNonlin 6.2 (Pharsight, Mountain View, USA). The maximum plasma concentration (C_{max}) and the time to reach the C_{max} (t_{max}) were directly derived from the observed plasma concentrations. The elimination half-life ($t_{0.5}$) was estimated from $\ln 2/k_{el}$, where k_{el} is the first-order elimination rate constant calculated by the terminal linear segment of the log plasma concentration–time data. The total area under the concentration–time curve $AUC_{0-\infty}$ was estimated by the trapezoidal rule with extrapolation to infinity using C_{last}/k_{el} (C_{last} – the last measurable concentration in plasma).

Statistical analysis

All statistical analyses were performed using Statistica software (version 8.0, StatSoft Inc., Tulsa, USA). Normality was estimated with the Shapiro-Wilk test. The differences between the normally distributed variables were determined with t-Student's test; in the other cases the Mann-Whitney test was applied. Correlations between the parameters were calculated with the Spearman rank correlation coefficient for all the non-normally distributed values. The differences were considered to be significant when $p \leqslant 0.05$.

Results

Pharmacokinetic study

The mean plasma concentration-time profiles of clopidogrel, CLPM, the H3 and H4 isomers of CTM obtained in diabetic and non-diabetic patients following administration of 75 mg clopidogrel were presented in **Figure 1**.

Clopidogrel was rapidly absorbed from the gastrointestinal tract yielding C_{max} values of 2.34 ng/mL and 1.82 ng/mL in plasma of diabetic and non-diabetic patients, respectively. The main clopidogrel metabolite, which is biologically inactive CLPM, reached a C_{max} in plasma thousand-fold greater compared to that of the parent drug (**Figure 1B, Table 2**). The pharmacokinetic profiles of the H3 and H4 isomers of CTM were slightly different in diabetic patients as compared to patients without DM (**Figure 1C, D**) pointing to a higher exposure to H3 and H4 in patients without diabetes. Lower plasma concentrations of the active H4 isomer were noticed in diabetic patients (**Figure 1D**), resulting in lower AUC_{0-t} values (8.7 ng·h/mL vs. 13.5 ng·h/mL in non-diabetic patients) (**Table 2**). Both the H3 and H4 isomers were eliminated rapidly and their concentrations were below limit of quantification at 6 h after clopidogrel administration in most subjects (**Figure 1C, D**). However, in DM patients values of $t_{0.5}$ determined for CTM isomers were higher (0.91 h and 1.15 h, for H3 and H4, respectively) than in non-diabetic group (0.66 h for H3 and 0.77 h for H4 isomer). Lower elimination rate was also observed for CLPM with $t_{0.5}$ of 7.50 h as compared to 7.01 h in non-diabetic patients (**Table 2**). Differences between pharmacokinetic profiles of clopidogrel and its metabolites determined for diabetic and non-diabetic patients were not statistically significant.

Pharmacodynamic study

Platelet aggregation measured in patients treated with 75 mg of clopidogrel ranged between 63–246 AU·min (mean ± SD = 120 ± 66 AU·min, n = 15) in diabetic patients and 43–747 AU·min (180 ± 151 AU·min, n = 21) in patients without DM. Statistical analysis revealed that ADP-induced platelet aggregation was strongly associated with the C_{max} of the active H4 isomer with r = -0.439, p = 0.025 for the overall patient group, and with r = -0.536, p = 0.027 observed in non-diabetic patients. The correlation was less pronounced in patients with DM (p > 0.05).

Table 2. Pharmacokinetic parameters (mean ± SD) of clopidogrel and its metabolites in diabetic and non-diabetic patients

	clopidogrel		H3		H4 (active)		CLPM	
	diabetic (n = 16)	non-diabetic (n = 24)	diabetic (n = 10)	non-diabetic (n = 20)	diabetic (n = 10)	non-diabetic (n = 20)	diabetic (n = 16)	non-diabetic (n = 28)
C_{max} [ng/mL]	2.34 ± 2.29	1.82 ± 1.86	4.64 ± 4.79	5.42 ± 4.55	6.42 ± 4.80	7.44 ± 7.18	2339 ± 989	2662 ± 2090
t_{max} [h]	1.40 ± 0.79	1.39 ± 1.26	1.25 ± 0.84	1.04 ± 0.54	0.94 ± 0.45	1.04 ± 0.54	1.38 ± 0.70	1.35 ± 0.79
$t_{0.5}$ [h]	1.33 ± 0.81	2.05 ± 1.54	0.91 ± 1.06	0.66 ± 0.62	1.15 ± 1.18	0.77 ± 0.65	7.50 ± 3.44	7.01 ± 3.34
AUC_{0-t} [ng·h/mL]	5.01 ± 4.11	4.95 ± 3.70	6.27 ± 4.86	8.61 ± 7.85	8.74 ± 7.03	13.49 ± 13.78	11061 ± 6078	11100 ± 8821
$AUC_{0-\infty}$ [ng·h/mL]	6.07 ± 4.12	6.33 ± 4.32	7.09 ± 4.68	9.11 ± 7.99	9.45 ± 6.73	14.17 ± 13.98	12695 ± 7251	12252 ± 9478

CLPM – carboxylic acid metabolite of clopidogrel; H3, H4 – isomers of clopidogrel thiol metabolite; C_{max} – maximum plasma concentration; t_{max} – time to reach C_{max}; $t_{0.5}$ – elimination half-life; AUC_{0-t} – area under the plasma concentration-time curve from zero to time t; $AUC_{0-\infty}$ – area under the plasma concentration-time curve from zero to infinity

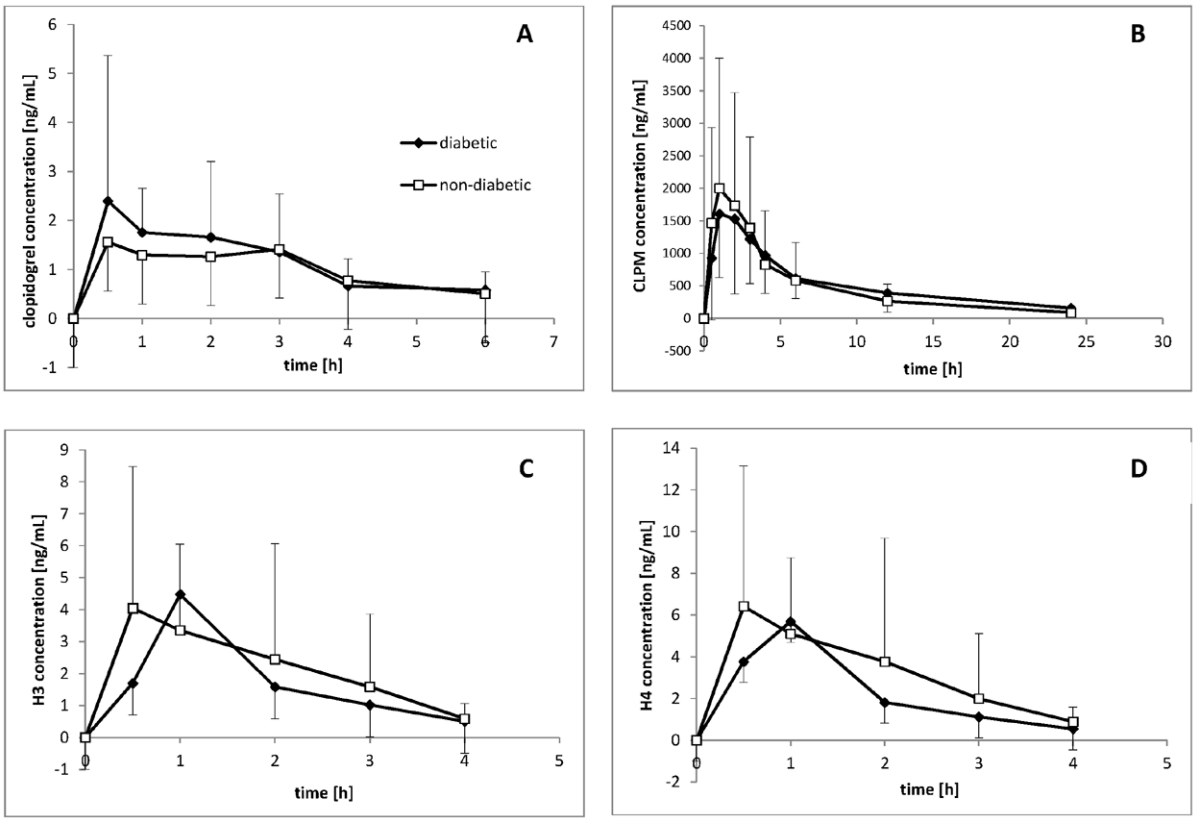

Figure 1. Mean plasma concentrations of clopidogrel (A), its carboxylic metabolite (CLPM) (B) and diastereoisomers of a thiol metabolite, the inactive H3 (C) and the active H4 (D), versus time following administration of 75 mg of clopidogrel to diabetic and non-diabetic patients

Discussion

In the present study, the selective HPLC-MS/MS method [15] permitted to perform the investigation on pharmacokinetics of the clinically relevant H4 isomer along with the parent drug and its non-active metabolites: H3 and CLPM in patients with type 2 DM accompanied by cardiovascular diseases. The values of C_{max} of clopidogrel obtained in the diabetic and non-diabetic patients (**Table 2**) refer to the published data of the C_{max} in healthy volunteers which varied from 0.9 ng/mL [17] to 4.4 ng/mL [18] following administration of 75 mg clopidogrel. There is no available numeric data regarding pharmacokinetics of the parent drug in diabetic patients. However, Park et al. [19] mentioned that they did not observe any differences in clopidogrel levels measured in plasma of Korean patients with and without DM. Some authors speculated on an increased activity of esterases in diabetic patients, which would convert more of the clopidogrel dose into the inactive CLPM [8, 20]. This has been confirmed for aspirin resistance, in which increased activity of plasma esterases hydrolyzed acetylsalicylic acid to a higher extent in patients with type 2 diabetes [21]. In the present study, plasma levels of CLPM were similar in both groups of patients (**Figure 2B**) and did not confirm the above mentioned speculation. Lower plasma concentrations of the active H4 isomer noticed in diabetic patients, resulting in lower AUC_{0-t} values (**Figure 1, Table 2**), may lead to serious clinical consequences. A similar conclusion was drawn by Erlinge et al. [8] who reported lower AUC values of CTM measured as a mixture of the H3 and H4 isomers in patients with DM compared with non-diabetic patients following administration of a loading dose of 600 mg and at day 29 of maintenance therapy with 75 mg of clopidogrel. The authors claimed that one possible reason for lower levels of CTM was reduced gastric motility in diabetic patients leading to slower absorption of the pro-drug [8].

The high inter-subject variability of pharmacokinetic parameters was demonstrated in the studied groups of patients. It requires further explanation with reference to genetic polymorphism of P-glycoprotein, which affects clopidogrel bioavailability, and CYP isoenzymes, responsible for clopidogrel metabolism. Moreover, concomitant use of certain CYP-metabolized drugs may also attenuate pharmacokinetics of clopidogrel and its metabolites, especially in a group of diabetic patients.

Harmsze et al. [22] reported an impaired response to clopidogrel therapy in diabetic patients treated with sulfonylureas as compared to patients without concomitant sulfonylurea treatment. Sulfonylureas are mainly metabolized by the CYP2C9 enzyme, which also plays an important role in the metabolic activation of clopidogrel. Because CYP2C19 is regarded as the main enzyme involved in the clopidogrel metabolism [11], a drug/drug interaction with CYP2C19 inhibitors, such as proton pump inhibitors (PPIs), would be anticipated. However, we did not observe any statistically significant influence of co-administration of PPIs (omeprazole and pantoprazole) on exposure to clopidogrel and its metabolites in the studied groups of patients.

According to the literature data, pharmacodynamic effect of the drug, expressed by inhibition of platelet aggregation was lower in diabetic compared to non-diabetic patients [23, 24]. In our study all diabetic patients had a relatively low platelet aggregation, whereas in non-diabetic group one patient could be characterized as a non-responder with AUC = 747 AU·min. This high value of platelet aggregation coexisting with the low C_{max} of the H4 isomer of 2.5 ng/mL determined in the patient plasma suggests a poor response to clopidogrel treatment associated with higher risk of cardiovascular events. According to the criteria set by Bonello et al., platelet aggregation > 468 AU·min in response to ADP may be used for identification of patients at high risk of thrombotic events. However, patients with DM may have different high on-treatment platelet reactivity cut points as compared to patients without DM [16].

Some studies have demonstrated that estimation of the platelet function may be especially useful in patients with decreased clopidogrel metabolism [25]. Therefore, the routine monitoring of platelet aggregation and the H4 plasma concentrations during clopidogrel treatment may improve the clinical outcomes in patients with cardiovascular diseases accompanied by DM.

Limitations of the study include the limited sample size to evaluate the effect of diabetes on clopidogrel pharmacokinetics in clopidogrel-treated patients. Moreover, the study did not include any genetic polymorphisms that could affect the pharmacokinetics and pharmacodynamics of the drug.

Conclusion

According to our knowledge, this is the first study of the pharmacokinetics of clopidogrel and its main metabolites, the active H4 and inactive H3 and CLPM, in patients with type 2 DM accompanied by cardiovascular diseases. Our results revealed that pharmacokinetic parameters of clopidogrel, CLPM, H3 and H4 in patients with DM did not vary significantly from those determined in non-diabetic group. Moreover, the antiplatelet response to clopidogrel therapy measured by ADP-stimulated platelet aggregation was similar in both groups of patients.

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