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HPLC–MS/MS method for the simultaneous determination of clopidogrel, its carboxylic acid metabolite and derivatized isomers of thiol metabolite in clinical samples

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ABSTRACT

A fast and reproducible HPLC–MS/MS method was developed for the simultaneous determination of clopidogrel (CLP), its carboxylic acid derivative (CLPM), derivatized thiol metabolite isomers MP-H3 and the active MP-H4 in incurred human plasma. CLP, CLPM, MP-H3 and MP-H4 isomers together with the internal standard piroxicam were extracted from plasma samples using a simple protein precipitation with acetonitrile. The analytes were separated on HPLC Zorbax Plus C18 column via gradient elution with water and acetonitrile, both containing 0.1% (v/v) formic acid. Detection of the analytes were performed on a triple-quadrupole MS with multiple-reaction-monitoring via electrospray ionization. Calibration curves of the analytes prepared in 250 μL plasma were found to be linear in ranges: 0.25–5.00 ng/mL for CLP, 0.25–50.00 ng/mL for MP-H3 and MP-H4 isomers and 50–10,000 ng/mL for CLPM. The lower limit of quantitation was 0.25 ng/mL for CLP, MP-H3, MP-H4 and 50.00 ng/mL for CLPM. Intra- and inter-assay precision, expressed as relative standard deviation, was ≤18.1% for CLP, ≤15.2% for CLPM, ≤10.1% for MP-H3 and ≤19.9% for MP-H4. Intra- and inter-day accuracy of the method, expressed as relative error, was ≤16%. The analytes were stable in samples stored for 6 h in autosampler, in plasma samples for 24 h at room temperature and for 3 months at −25 °C. Resolution of CLP, CLPM and MP-H3 and MP-H4 isomers of thiol metabolite during one analytical run was reported in patient plasma. The HPLC–MS/MS method was applied for pharmacokinetic studies of CLP and its metabolites in patients treated with daily dose of 75 mg CLP.

1. Introduction

Clopidogrel (CLP), methyl (+)-*S*-2-(2-chlorophenyl)-2-(6,7-dihydrothiene(3,2-c)pyridine-5(5H))-acetate, 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 used in prevention of ischemic events in patients with myocardial infarction or undergoing percutaneous coronary intervention (PCI) [2]. Despite obvious advantages, many clinical studies have shown that about 5–40% of patients treated with conventional doses of CLP do not display adequate antiplatelet response which may lead to serious cardiovascular complications such as stent thrombosis, myocardial infarction, stroke and death [3]. The mechanism underlying CLP resistance is multifactorial and includes genetic polymorphisms of transporters and enzymes participating in CLP absorption and metabolic transformation, and non-genetic causes (drug–drug interactions, co-morbidities, age) [4]. CLP absorption in the intestine may be diminished by active secretion via an efflux pump P-glycoprotein (P-gp) encoded by the multidrug resistance gene (MDR-1). The differences in MDR-1 genotype may contribute to the visible inter-patient variability of CLP and its metabolites levels in plasma [5]. The metabolism of CLP undergoes through two different pathways in the liver. The major metabolite is carboxylic acid derivative of CLP (CLPM), to which up to 85% of the administered parent drug might be transformed [6]. It is formed by hydrolysis of the ester function by carboxyl esterase. Although it is inactive, for many years its determination in plasma was used

Abbreviations: CLP, clopidogrel; CLPM, carboxylic acid derivative of clopidogrel; CTM, thiol metabolite of clopidogrel; CTMD, derivatized thiol metabolite of clopidogrel; IS, internal standard; MP-H1, MP-H2, MP-H3, 3′-methoxyacetophenone derivatives of isomers of clopidogrel thiol metabolite; MP-H4, derivatized active isomer of thiol metabolite of clopidogrel; MPB, 2-bromo-3′-methoxyacetophenone; PRX, piroxicam.

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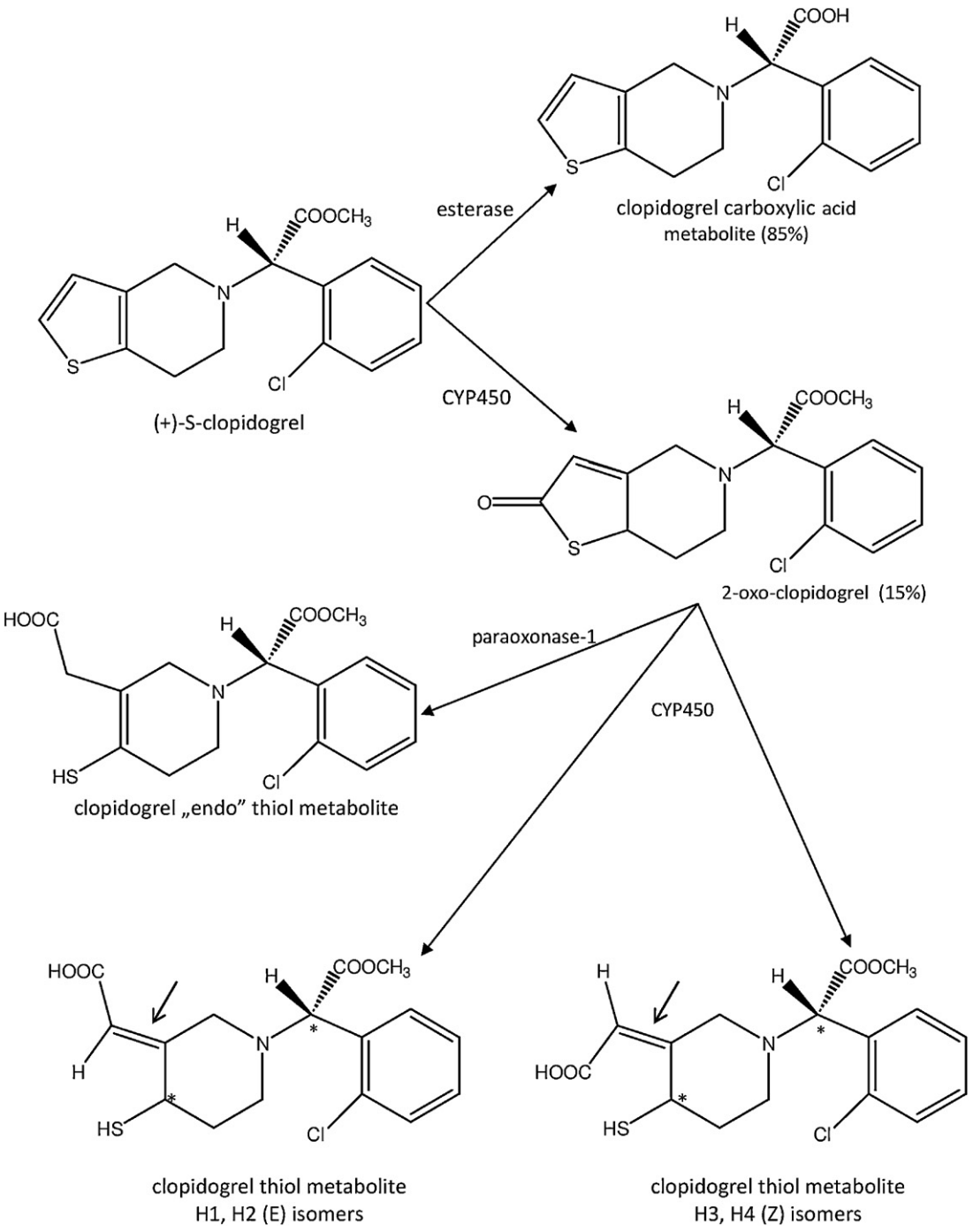

Fig. 1. Metabolic pathway of clopidogrel; *chiral center; →, geometric center.

for studying the pharmacokinetics of CLP in indirect manner, as the plasma concentrations of the parent drug are very low (pg/mL levels) due to its rapid metabolism [7]. Antithrombotic effect of CLP depends on its biotransformation to a thiol metabolite (CTM) through a two-step hepatic pathway involving cytochrome P450 isoenzymes including CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4 [8]. In the first step, a small fraction of CLP is converted to an intermediate product 2-oxo-clopidogrel, which is subsequently hydrolyzed to isomers of CTM (Fig. 1). Among various CYP450 enzymes catalyzing oxidative activation of CLP, mainly CYP2C19 polymorphic variants *2 and *3 are responsible for the reduced exposure to CTM leading to the decreased antiplatelet effect of CLP in patients [9,10]. CTM selectively inhibits ADP-induced platelet aggregation by direct inhibition of ADP binding to a $P2Y_{12}$ receptor located on a platelet surface leading to the inhibition of ADP-mediated activation of the glycoprotein GPIIb–IIIa complex [11]. CTM possesses three stereochemical sites: two chiral centers at C4 and C7 and one geometric center at C3 (ethylenic bond) [1]. However, as the result of an administration of pharmaceutical formulations containing only CLP with S configuration at C7, CTM may be present in human body as four diastereoisomers: H1 and H2, possessing the 3E configuration, and H3 and H4, which are the 3Z compounds (Fig. 1). Only 15% of the absorbed CLP dose is transformed to CTM, which was proved to be present in incurred plasma only in a form of H3 and H4 isomers [12]. In vitro studies confirmed, that H4 isomer can be considered as the only active circulating