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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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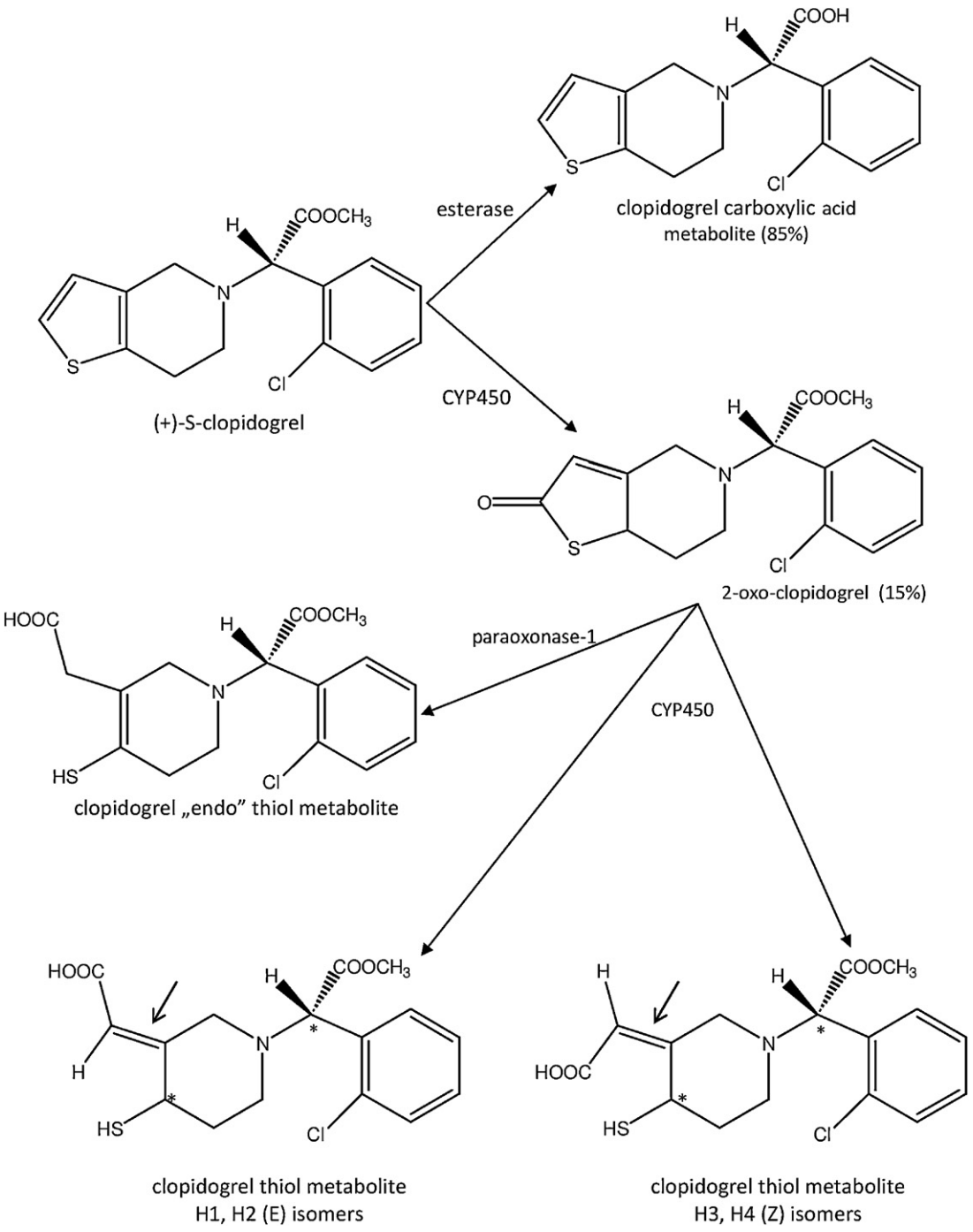


**Fig. 1.** Metabolic pathway of clopidogrel; *chiral center; →, geometric center.

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

isomer of CTM [1,12]. CTM is highly labile because of the reactivity of thiol group, which may form a disulfide bond with endogenous compounds with low molecular weight or with proteins. To prevent that reaction CTM in patients' plasma needs stabilization with an alkylating agent, such as 2-bromo-3′-methoxyacetophenone (MPB) [13]. Due to the above mentioned analytical problems only few HPLC–MS/MS assays have been published for determination of a derivatized CTM (CTMD) in human plasma [12–15]. Most of the methods do not distinguish between the four different isomers and enabled the determination of CTMD as a mixture of them which could be visible on the chromatograms as a single or partially resolved peak [12–15]. Only Tuffal et al. presented the HPLC–MS/MS method for separation of all four CTMD stereoisomers and determination of two of them with Z configuration in human plasma [12]. However, the studies were applicable only for CTMD and do not consider the levels of CLP or its main metabolite CLPM. There are few papers describing the HPLC–MS/MS methods for simultaneous quantification of either CLP with CTMD [15] or CLP with CLPM [7,16,17] but there is none reporting resolution of the parent drug and its two main metabolites including active isomer. Peer et al. developed an ultra HPLC–MS/MS method for determination of CLP and CTMD in human plasma after a simple protein precipitation but it was not utilized for CLPM analysis [15]. The HPLC–MS/MS method was also reported for the simultaneous analysis of CLP and CLPM in human plasma after SPE [7]. Silvestro et al. established the optimal conditions for HPLC–MS/MS quantification of CLP, CLPM and clopidogrel acyl glucuronide in plasma samples allowing to avoid acyl glucuronide back-conversion to CLP [17]. There is only one paper focusing on the simultaneous determination of CLP, CLPM and underivatized CTM in biological samples. However, due to a lack of the CTM standard, its concentration was approximated based on the calibration curve prepared for CLP [18].

In the present study, a fast and reproducible HPLC–MS/MS method was developed and validated for the simultaneous analysis of CLP, CLPM and H3 and H4 isomers of CTM in clinical samples. The method was successfully applied for determination of the analytes levels in plasma of patients treated with 75 mg CLP and it may be useful for the pharmacokinetic study of CLP, CLPM and CTM isomers.

## 2. Experimental

### 2.1. Materials

The 3′-methoxyacetophenone derivatives of clopidogrel thiol metabolite H3 (MP-H3) and H4 (MP-H4) isomers were obtained from Sanofi Aventis (Montpellier, France). The 3′-methoxyacetophenone derivatized mixture of CLP thiol metabolite isomers with E configuration (MP-H1 + MP-H2) was purchased from Alsachim (Illkirch, France). (+)-*S* clopidogrel bisulphate (purity 99%) and its carboxylic acid metabolite (CLPM; purity 99.6%) were made available from Pharmaceutical Research Institute (Warsaw, Poland). Piroxicam (PRX, internal standard, IS) was obtained from Jelfa (Jelenia Góra, Poland). Acetonitrile (Merck, Darmstadt, Germany) were of HPLC gradient grade. The alkylating agent 2-bromo-3′-methoxyacetophenone (MPB) and formic acid (purity ≥ 95%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). De-ionized water was always used to prepare a mobile phase for HPLC (Simplicity UV, Millipore, USA). Drug free human plasma was obtained from Regional Centre of Blood Donation (Poznań, Poland).

### 2.2. HPLC–MS/MS equipment and conditions

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, USA). Data processing was performed using MassHunter workstation software (Agilent Technologies, USA). CLP, CLPM, MP-H1, MP-H2, MP-H3 and MP-H4 isomers of CTM and PRX were separated in the Zorbax Plus C18 column (100 mm × 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. Mobile phase flow was set to 0.35 mL/min and injected volume was 25 μL. The eluent from HPLC column was introduced directly to the MS interface using the electrospray ionization in the positive ion mode. MS parameters were as follows: capillary voltage 4000 V, nebulizer gas (nitrogen) pressure 40 psi (275.8 kPa), desolvatation gas (nitrogen) flow 10 L/min and desolvatation 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. MS collision parameters for each compound are listed in Table 1.

### 2.3. Standard solutions

Stock solutions of CLP, CLPM, MP-H1 + MP-H2, MP-H3 and MP-H4 isomers and IS were prepared by dissolving the appropriate amount of a compound in anhydrous acetonitrile. The stock solutions were of followed concentrations: 200 μg/mL for CLPM, MP-H3, MP-H4 and MP-H1 + MP-H2, 500 μg/mL for CLP and 1000 μg/mL for PRX.

For plasma samples analysis, the standard solutions of CLP, MP-H3, MP-H4, CLPM and PRX were prepared from stock solutions by diluting the appropriate volume of the stock solution

**Table 1**
MS/MS parameters.

| Compound | Parent ion (*m*/*z*) | Daughter ion (*m*/*z*) | Fragmentor voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| CLP | 322.1 | 212[a] | 96 | 13 |
| | 322.1 | 184 | 96 | 21 |
| | 322.1 | 155 | 96 | 37 |
| MP-H1, MP-H2, MP-H3, MP-H4 | 504.1 | 354.1 | 144 | 17 |
| | 504.1 | 212 | 144 | 25 |
| | 504.1 | 155[a] | 144 | 45 |
| CLPM | 308.1 | 198[a] | 100 | 13 |
| | 308.1 | 152 | 100 | 21 |
| | 308.1 | 125 | 100 | 41 |
| PRX | 332.1 | 121 | 100 | 25 |
| | 332.1 | 95[a] | 100 | 17 |

[a] Transition used for quantification.

with anhydrous acetonitrile in 10 mL glass flasks. The standard solutions were of 1.0; 2.5; 5.0; 7.5; 10.0; 25.0; 37.5 and 50 ng/mL CLP, 2.5; 5.0; 25.0; 50.0; 125.0; 250.0 and 500 ng/mL MP-H3 and MP-H4 isomers, 0.5; 1.0; 5.0; 10.0; 50.0; 75.0 and 100.0 μg/mL CLPM, and 1000 ng/mL IS.

### 2.4. Preparation of plasma samples

The volume of 25 μL of the standard solutions of the analytes and 25 μL of IS solution were transferred into an Eppendorf mini-centrifuge tube containing 250 μL human blank plasma. The resulting plasma samples contained 0.1; 0.25; 0.5; 0.75; 1.0; 2.5; 3.75 and 5.0 ng/mL CLP, 0.25; 0.5; 2.5; 5.0; 12.5; 25.0 and 50.0 ng/mL MP-H3 and MP-H4 isomers, 50; 100; 500; 1000; 5000; 7500 and 10,000 ng/mL CLPM, and 100 ng/mL IS. Protein precipitation was performed by adding 450 μL of acetonitrile to each sample. The mixture was vortexed and centrifuged for 10 min at 22,570 × *g* and temperature 20 °C before the supernatant was filtered using Mini Uni Prep filters (Agilent Technologies, UK). The resulting filtrate was evaporated under vacuum at 40 °C and the dry residue was reconstituted in 200 μL of a mobile phase containing A and B solution (50:50, v/v). Then, a 25 μL aliquot was injected onto the HPLC–MS/MS system.

### 2.5. Method validation

#### 2.5.1. Selectivity

Selectivity was evaluated by comparing the chromatogram of blank plasma, which was processed by protein precipitation (Section 2.4) with the chromatogram of plasma spiked with respective standards of CLP, CTMD isomers, CLPM and PRX to detect any peaks interfering the target compounds. Six different specimens of blank plasma were applied for the specificity evaluation. Moreover, drugs: amiodarone, amlodipine, atorvastatin, betahistine, doxazosin, furosemide, hydrochlorotiazide, metformin, metoprolol, omeprazole, pantoprazole, perindopril, ramipril, rosuvastatin, salicylic acid, simvastatin and telmisartan, potentially co-administered with CLP were examined for possible interference with this method.

#### 2.5.2. Linearity

Linearity of the calibration curves was estimated for the ratio of the peak area of CLP, CLPM or MP-H3 and MP-H4 isomers to that of the IS as a function of the analyte concentration covering the range of 0.25–5.0 ng/mL for CLP, 50–10,000 ng/mL for CLPM and 0.25–50.0 ng/mL for MP-H3 and MP-H4 isomers in plasma. The equations of calibration curves were used to calculate concentration of CLP, CLPM and H3 and H4 isomers in patients' plasma. Mandel's fitting test has been applied for the evaluation of the linearity of a straight line regression model, test value (TV) < $F_{crit}$ means statistically nonsignificant differences [19]. The correlation coefficients *r* were also calculated.

#### 2.5.3. LLOQ, precision and accuracy

LLOQ was defined as the lowest concentration of CLP, CLPM and MP-H3 and MP-H4 isomers determined by the method within the relative standard deviation (%RSD) and relative error not exceeding 20%. The LLOQ samples with concentration 0.25 ng/mL of CLP, MP-H3 and MP-H4 isomers and 50 ng/mL of CLPM in plasma were independently prepared and determined. Intra-day and inter-day precision of the method, expressed as %RSD, has been estimated for quality control samples (QCS) at concentrations of 0.5, 2.5 and 5.0 mg/L of CLP, 0.5, 5.0 and 50.0 ng/mL of MP-H3 and MP-H4 isomers, and 100, 1000 and 10,000 ng/mL of CLPM in plasma, prepared in five replicates analyzed over five different days. Accuracy, expressed as relative error (%RE) was estimated for the same ranges of analytes concentrations as for the evaluation of precision of the method and expressed as the percent difference between the mean determined concentration and the nominal concentration.

#### 2.5.4. Stability

Stability of CLP, CLPM and MP-H3 and MP-H4 isomers in plasma samples was evaluated at concentrations of 0.5 and 5.0 ng/mL of CLP, 100 and 10,000 ng/mL of CLPM, 0.5 and 50 ng/mL of MP-H3 and MP-H4 isomers (in three replicates for each analyte concentration) after three freeze–thaw cycles as well as short-term storage (24 h of standing at room temperature) and long-term storage (3 month at −25 °C). Moreover, stability of the analytes in samples stored for 6 h in autosampler was also analyzed. The concentrations of the analytes after each storage period were calculated using a calibration curve, obtained from freshly prepared samples in the same analytical run. The analytes are stable if the deviation from the nominal concentration is within ±15% [20]. Stability of the analytes in stock solutions was determined against a freshly prepared stock solution during storage for 3 months at −25 °C.

#### 2.5.5. Matrix effect

The influence of co-eluting matrix components on the analytes ionization was evaluated for each analyte and the internal standard. For this purpose the matrix factor (MF) was calculated, by dividing the peak measured by analyzing blank matrix spiked with analyte after protein precipitation, to the peak area in absence of matrix. The investigations were performed using samples spiked with low (0.5 ng/mL of CLP, MP-H3 and MP-H4 isomers and 100.0 ng/mL of CLPM) and high (5.0 ng/mL of CLP, 50.0 ng/mL of MP-H3 and MP-H4 isomers and 10,000.0 ng/mL of CLPM) concentrations of the analytes. Moreover, the IS normalized MF was calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalized MF should not be greater than 15% [20].

#### 2.5.6. In vivo application

The usefulness of the validated HPLC–MS/MS method has been demonstrated in quantitative determination of CLP, CLPM and H3 and H4 isomers of CTM in patients' plasma following oral administration of a tablet containing 75 mg CLP during their maintenance therapy with the drug. The studies were approved by the Ethical Committee at Poznan University of Medical Sciences. Three patients (two male and one female, 73 ± 1 year old) were involved in the studies. Blood samples were collected before and at 0.5; 1.0; 2.0, 3.0, 4.0, 6.0 and 12 h after administration of the drug. The aliquot 7.5 mL of blood was drawn into an EDTA tube pretreated with 37.5 μL of 500 mM of MPB to immediately derivatize the CTM isomers. The procedure and efficiency of the stabilization of CTM was previously described [13]. Plasma was separated by centrifugation for 10 min at 1620 × *g* and stored at −25 °C.

The plasma levels of the analytes were used to calculate pharmacokinetic parameters using WinNonlin 6.2 (Pharsight, USA). For the calculation non-compartmental technique has been applied. $C_{max}$ and $t_{max}$ were read from individual analyte concentration–time curve. The elimination half-life ($t_{0.5}$) was estimated from $\ln 2/k_{el}$ ($k_{el}$ – the elimination rate constant calculated by the terminal linear segment of the log plasma concentration–time data). The total area under the concentration–time curve $AUC_{0\rightarrow\infty}$ was estimated by trapezoidal rule with extrapolation to infinity using $C_{last}/k_{el}$. The apparent plasma clearance (Cl/*F*) was calculated dividing the dose (*D*) of the parent drug by $AUC_{0\rightarrow\infty}$. The apparent volume of distribution ($V_d/F$) was estimated from $D/k_{el}AUC_{0\rightarrow\infty}$.

## 3. Results and discussion

### 3.1. HPLC–MS/MS analysis

The MS conditions were optimized for obtaining a good signal and high sensitivity for CLP, CLPM, MP-H1, MP-H2, MP-H3 and MP-H4 isomers and PRX, which was used as an internal standard, in plasma samples. The transitions $m/z$ 322.1 → 212 for CLP, 308.1 → 198 for CLPM, 504.1 → 155 for MP-H1, MP-H2, MP-H3 and MP-H4 isomers and 332.1 → 95 for PRX were chosen for quantification of the analytes (Table 1). The HPLC gradient was applied in order to separate all the above mentioned analytes. The elaborated conditions allowed to analyze four isomers of CTMD in samples spiked with the analytes, but two of them, MP-H2 and MP-H3, were only partially resolved (Fig. 2). Because MP-H1 and MP-H2 were available as a mixture of isomers, while MP-H3 and MP-H4 could be used as single standards, the order of appearing peaks has been determined based on the composition of mobile phase described by Tuffal et al. The author obtained the complete separation of all four isomers of CTMD in UHPLC system using gradient elution with water and acetonitrile both containing 2 mM ammonium acetate and 2% formic acid. Using the above mentioned conditions, MP-H3 isomer was eluted between MP-H1 and MP-H2 [12]. Because of only partial separation of MP-H2 and MP-H3 isomers (Fig. 2) and the fact that in plasma samples obtained from subjects treated with CLP, only peaks originated from MP-H3 and MP-H4 were noted [12,21], for further elaboration and validation of the HPLC–MS/MS method MP-H3 and MP-H4 standards were used. Fig. 3 presents complete separation of CLP, MP-H3, MP-H4, CLPM and PRX in a plasma sample spiked with the analytes during one analytical run. In plasma sample obtained from a patient treated with CLP, one additional peak was observed, referred to as MP-H endo (Fig. 3C). The presence of MP-H endo in human plasma was also observed by other authors [12,21]. According to the new report [21], the compound is devoid of pharmacological activity and it is formed by hydrolysis of 2-oxo-clopidogrel mediated by paraoxonase-1. MP-H endo differs from H1–H4 isomers of CAM in the presence of the carbon double bond in the endocyclic position of the piperidine ring (Fig. 1) [12,21]. Specific analysis of H3 and H4 in plasma samples, apart from chromatographic separation, is ensured by the distinct MS fragmentation signatures of MP-H endo and the derivatized H1, H2, H3, H4 isomers of CTM. MP-H endo was monitored using the m/z 504.1 → 155 or 504.1 → 212 transitions, because the $m/z$ 504.1 → 354.1 transition was specific only for MP-H1, MP-H2, MP-H3 and MP-H4 isomers.

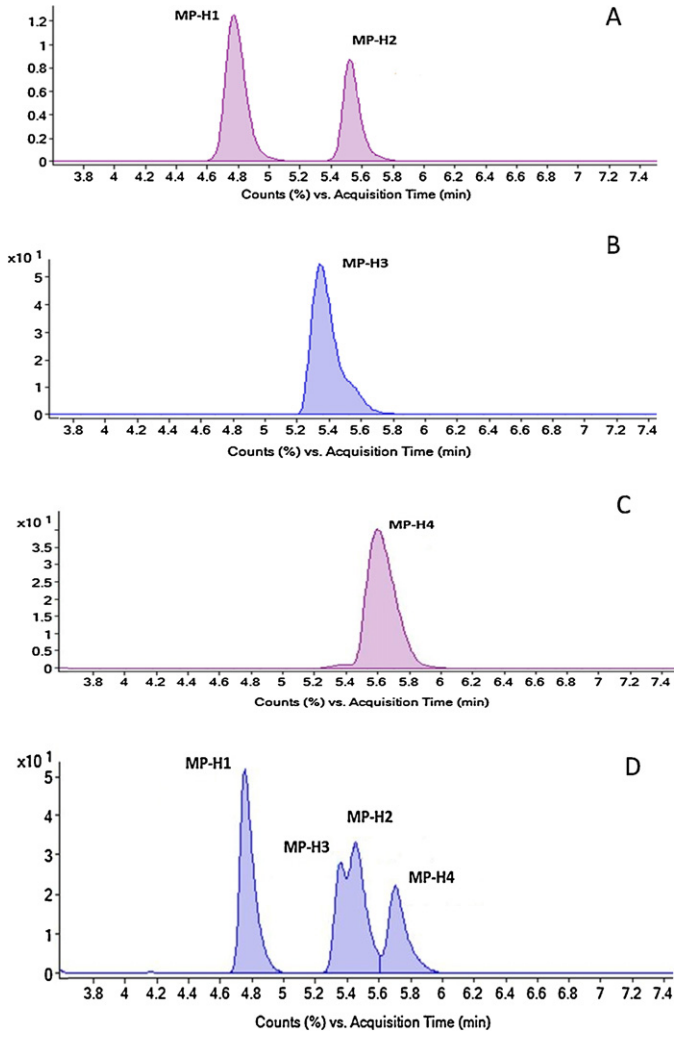


**Fig. 2.** Chromatograms of the 3′-methoxyacetophenone-derivatized isomers of CTM. (A) mixture of MP-H1 and MP-H2; (B) MP-H3; (C) MP-H4; (D) mixture of all four derivatized isomers of CTM.

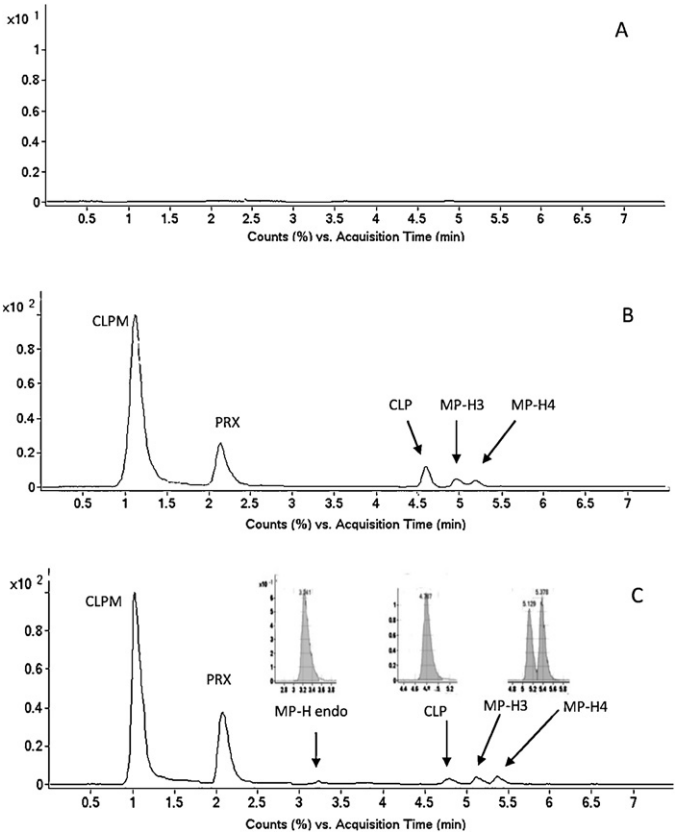


**Fig. 3.** Chromatograms of CLP and its metabolites following protein precipitation of human plasma samples. (A) Blank plasma; (B) blank plasma spiked with 5 ng/mL of CLP, 50 ng/mL of MP-H3 and MP-H4, 10,000 ng/mL of CLPM and 100 ng/mL of PRX (IS); (C) plasma sample collected from a patient at 0.5 h elapsed from administration of a tablet with 75 mg CLP.

### 3.2. Method validation

The detection of CLP, CLPM and MP-H3 and MP-H4 isomers using MRM mode was highly selective with no interferences from endogenous compounds of plasma samples obtained from six individual donors. None of the medications mentioned in Section 2.5.1, which were added to the plasma sample spiked also with CLP, CLPM, PRX, MP-H3 and MP-H4, produced the same $m/z$ ions as the

**Table 2**
Mean calibration curve equations for CLP, CLPM and MP-H3 and MP-H4 isomers of CTM in plasma.

| Compound (ng/mL) | Calibration curve equations | Correlation coefficient ($r$) |
|---|---|---|
| CLP (0.25–5.00) | $P_{CLP}/P_{PRX} = 2.304 \times 10^{-2} \times C_{CLP} + 0.015$ | 0.9998 |
| CLPM (50–10,000) | $P_{CLPM}/P_{PRX} = 5.920 \times 10^{-4} \times C_{CLPM}$ | 0.9997 |
| MP-H3 (0.25–50.00) | $P_{MP\text{-}H3}/P_{PRX} = 5.398 \times 10^{-3} \times C_{MP\text{-}H3}$ | 0.9960 |
| MP-H4 (0.25–50.00) | $P_{MP\text{-}H4}/P_{PRX} = 5.101 \times 10^{-3} \times C_{MP\text{-}H4}$ | 0.9988 |

$n = 4$ curves for each analyte.

analyzed compounds. Moreover, no peaks originated from drugs co-administered with CLP were present on chromatogram within the analyte retention time. No carryover was observed for either analytes or IS by injecting a blank sample following the highest concentration of the analytes calibration standards.

Standard curves estimated for the analytes were linear in the range of concentrations: 0.25–5.0 ng/mL for CLP, 50–10,000 ng/mL for CLPM and 0.25–50.0 ng/mL for MP-H3 and MP-H4 isomers in plasma. Statistical analysis using Mandel's test with results $TV < F_{crit}$ confirmed linearity of the calibration curves. The equations of standard curves and correlation coefficients are presented in Table 2. They were prepared as the means of four calibration curves for each analyte. They were applied for the quantification of the analytes in patients' plasma following the oral administration of CLP in tablets. In the worked out conditions, LLOQ was 0.25 ng/mL for CLP, MP-H3 and MP-H4 isomers and 50 ng/mL for CLPM (Table 3). The value of LLOQ for MP-H3 and MP-H4 isomers proved to be lower than the LLOQ of 0.5 ng/mL for H4 in 200 μL of plasma sample reported by Tuffal et al. [12]. Moreover, Takahashi et al. [13] and Delavenne et al. [14] reported higher values of LLOQ of 0.5 and 0.8 ng/mL, respectively, but they regarded CTMD as a mixture of isomers. Intra-day and inter-day precision of the method, expressed as %RSD, has fitted the range required for testing drug and/or metabolites content in body fluids and it was <15.0% for QCS and <20% for LLOQ of the all above mentioned analytes. Intra-day and inter-day accuracy of the method, expressed as %RE, was ≤16% for CLP, ≤6.2% for CLPM and ≤12.0% for MP-H3 and MP-H4, pointing to relatively high accuracy in the estimation of investigated analytes concentrations in plasma samples (Table 3). No significant matrix effect was observed. The %RSD for IS-normalized MF was in the ranges of 13.8–14.2% for CLP, 2.89–12.2% for MP-H3 and MP-H4 isomers and 9.4–12.4% for CLPM.

Stability data showed that CLP, CLPM and MP-H3 and MP-H4 isomers were stable for at least three months in stock solutions in acetonitrile stored at −25 °C, as demonstrated by %RE of estimates in the range of 5.1–14.5%. The results of the stability test during storage of the analytes in samples for 6 h in autosampler were within the %RE range of 5.1–15.0%. The stability during storage of the analytes in plasma samples at room temperature for 24 h was expressed by %RE of 1.2–14.8%. The results obtained during the long term stability study were within the range of 2.0–12.0%. The data indicate that the analytes were stable in plasma samples during three months storage in a freezer at −25 °C. The analytes proved to be stable in plasma samples after three freeze–thaw cycles as demonstrated by %RE of estimates in the range of 0.1–10.0% (Table 4). The stability data obtained for CLP, CLPM and CAMD isomers are similar to those reported previously for MP-H4. According to Tuffal et al. MP-H4 was stable for at least three months in acetonitrile solutions stored at 4 °C, 24 h in plasma stored at 37 °C, four months in plasma stored frozen at −20 °C and eight months in plasma stored frozen at −80 °C [12].

### 3.3. In vivo studies

The utility of the assay for pharmacokinetic studies has been demonstrated following the oral administration of a tablet containing 75 mg CLP to patients undergoing antiplatelet treatment. The chromatogram obtained in the course of analysis of patient plasma sample collected 0.5 h following administration of a tablet with CLP presents peaks originated from CLP, CLPM, MP-H3 and MP-H4 isomers of CTM and MP-H endo (Fig. 3C). MP-H1 and MP-H2 were probably below limit of detection and were not noted. The results obtained in patients samples are in line with those reported by other authors in healthy volunteers, where out of four CTM diastereoisomers, only H3 and H4 were quantifiable and endo-CTM was also detected [12,21]. According to Gong et al., concentration of endo-CTM in plasma following administration of a tablet with 75 mg CLP to healthy volunteers was 20-fold lower compared to H4 isomer. The levels of CLP and CLPM were not analyzed in this paper [21].

Fig. 4 shows the pharmacokinetic profiles obtained for CLP, CLPM and H3 and H4 isomers of CTM following administration of 75 mg CLP to studied patients. CLP was rapidly absorbed from GI

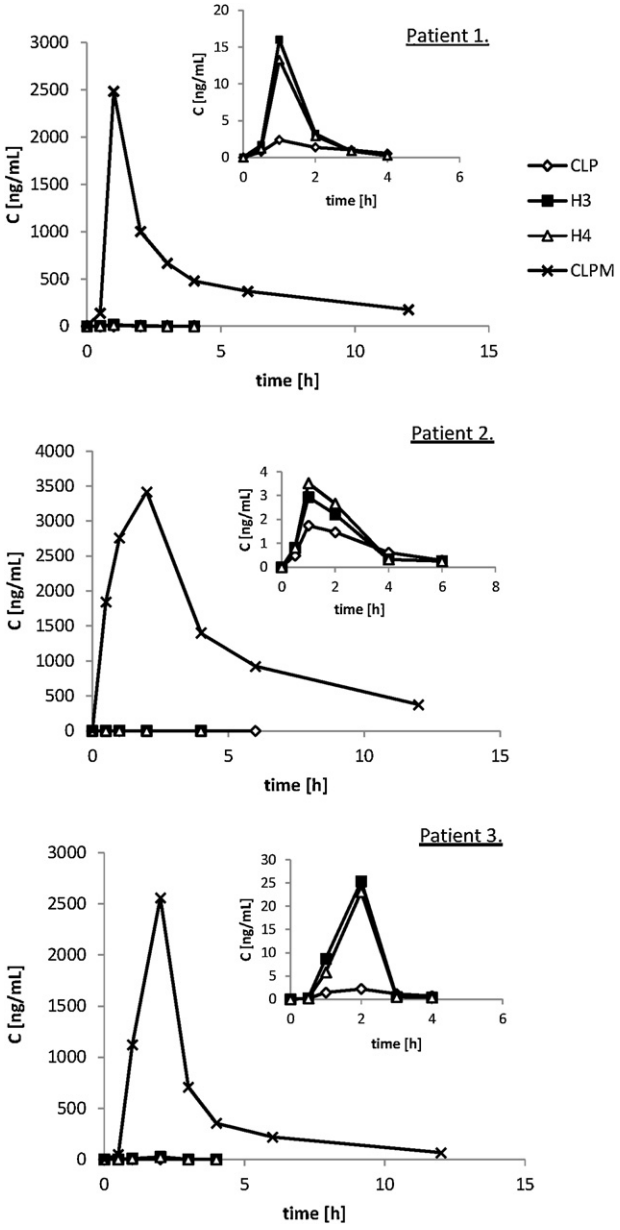


**Fig. 4.** Plasma concentrations of CLP, CLPM ad H3 and H4 isomers of CTM versus time following administration of a tablet with 75 mg CLP to three patients. Inset graphs illustrate changes in CLP and H3 and H4 concentrations.

**Table 3**
Intra-day and inter-day precision and accuracy for CLP, CLPM, MP-H3 and MP-H4.

| Nominal concentration [ng/mL] | | Intra-day ($n = 5$) | | | Inter-day ($n = 5$) | | |
|---|---|---|---|---|---|---|---|
| | | Mean assayed value [ng/mL] | Precision [%RSD] | Accuracy [%RE] | Mean assayed value [ng/mL] | Precision [%RSD] | Accuracy [%RE] |
| CLP | | | | | | | |
| LLOQ | 0.25 | 0.206 | 18.1 | 17.6 | 0.229 | 4.8 | 8.4 |
| | 0.5 | 0.556 | 9.2 | 11.2 | 0.512 | 10.2 | 2.4 |
| | 2.5 | 2.38 | 6.9 | 4.8 | 2.54 | 8.8 | 1.6 |
| | 5.0 | 4.84 | 6.5 | 3.2 | 4.97 | 6.0 | 0.6 |
| CLPM | | | | | | | |
| LLOQ | 50.0 | 50.4 | 15.2 | 0.8 | 48.2 | 9.5 | 3.6 |
| | 100.0 | 106 | 5.1 | 6.0 | 94.4 | 10.9 | 5.6 |
| | 1000.0 | 1019 | 11.3 | 1.9 | 1062 | 6.3 | 6.2 |
| | 10000.0 | 9732 | 3.1 | 2.7 | 9933 | 2.4 | 0.7 |
| MP-H3 | | | | | | | |
| LLOQ | 0.25 | 0.228 | 5.8 | 8.8 | 0.215 | 8.0 | 14.0 |
| | 0.5 | 0.522 | 8.3 | 4.4 | 0.459 | 9.0 | 8.2 |
| | 5.0 | 4.68 | 5.8 | 6.4 | 4.73 | 8.8 | 5.4 |
| | 50.0 | 50.0 | 7.8 | 0.0 | 48.0 | 10.1 | 4.0 |
| MP-H4 | | | | | | | |
| LLOQ | 0.25 | 0.245 | 19.5 | 2.0 | 0.220 | 19.9 | 12.0 |
| | 0.5 | 0.501 | 5.8 | 0.2 | 0.468 | 7.2 | 6.4 |
| | 5.0 | 5.03 | 14.4 | 0.6 | 5.37 | 7.6 | 7.4 |
| | 50.0 | 50.0 | 7.2 | 0.0 | 48.9 | 7.4 | 2.2 |

**Table 4**
Stability of CLP and its metabolites in plasma samples.

| Analyte | CLP | | MP-H3 | | MP-H4 | | CLPM | |
|---|---|---|---|---|---|---|---|---|
| Nominal concentration (ng/mL) | 0.5 | 5.0 | 0.5 | 50.0 | 0.5 | 50.0 | 100.0 | 10000.0 |
| Stability in autosampler (6 h) | | | | | | | | |
| Mean assayed value (ng/mL) | 0.472 | 5.53 | 0.455 | 52.8 | 0.426 | 42.9 | 115 | 10,510 |
| Accuracy (%RE) | 5.6 | 10.6 | 9.0 | 5.6 | 14.8 | 14.2 | 15.0 | 5.1 |
| Short-term stability (24 h at room temperature) | | | | | | | | |
| Mean assayed value (ng/mL) | 0.436 | 4.94 | 0.455 | 54.8 | 0.426 | 43.1 | 113 | 8818 |
| Accuracy (%RE) | 12.8 | 1.2 | 9.0 | 9.6 | 14.8 | 13.8 | 13.0 | 11.8 |
| Long-term stability (3 months −25 °C) | | | | | | | | |
| Mean assayed value (ng/mL) | 0.509 | 5.56 | 0.445 | 47.4 | 0.458 | 47.8 | 107.2 | 10,456 |
| Accuracy (%RE) | 1.8 | 11.2 | 11.0 | 5.2 | 8.4 | 4.4 | 7.2 | 4.6 |
| Freeze/thaw stability | | | | | | | | |
| Mean assayed value (ng/mL) | 0.475 | 4.89 | 0.447 | 50.1 | 0.522 | 54.4 | 91.2 | 10,914 |
| Accuracy (%RE) | 5.0 | 2.2 | 10.6 | 0.2 | 4.4 | 8.8 | 8.8 | 9.1 |

$n = 3$ samples for each concentration of the analytes.

tract obtaining $C_{max}$ of $2.10 \pm 0.33$ ng/mL after $t_{max} = 1.33 \pm 0.58$ h (Table 5), which is similar to $t_{max}$ values of about 1 h obtained in previous studies [15,22]. Low plasma levels of CLP resulted from its rapid metabolism. The main CLP metabolite, which is biologically inactive CLPM, reached $C_{max}$ in plasma of thousand-fold greater compared to the parent drug (Fig. 4 and Table 5). The second metabolite, which is a thiol derivative of CLP, was quantifiable in plasma as H3 and H4 isomers exhibiting very similar pharmacokinetic profiles (Fig. 4). Plasma levels of an biologically active H4 isomer was slightly lower than its antipode-H3 and reached $C_{max} = 13.3$ ng/mL (Table 5) similar to $C_{max} = 16.4$ ng/mL reported by Tuffal et al. following the administration of 75 mg CLP to healthy volunteers [12]. Higher values of CTM concentration over 20 ng/mL in plasma obtained by Peer et al. [15] in healthy volunteers following administration of the same dose 75 mg CLP resulted from application of the validated HPLC–MS/MS method which did not distinguish between H3 and H4 isomers. As only H4 isomer is clinically relevant, the HPLC–MS/MS methods developed

**Table 5**
Pharmacokinetic parameters (mean ± SD) of CLP and its metabolites in patients.

| | CLP | H3 | H4 (active) | CLPM |
|---|---|---|---|---|
| $C_{max}$ [ng/mL] | 2.10 ± 0.33 | 14.8 ± 11.3 | 13.3 ± 9.8 | 2821 ± 519 |
| $t_{max}$ [h] | 1.33 ± 0.58 | 1.33 ± 0.58 | 1.33 ± 0.58 | 1.67 ± 0.58 |
| $t_{0.5}$ [h] | 1.82 ± 0.29 | 0.58 ± 0.29 | 0.57 ± 0.25 | 4.05 ± 1.32 |
| $AUC_{0-t}$ [ng h/mL] | 6.02 ± 0.02 | 18.6 ± 13.3 | 16.6 ± 10.6 | 9782 ± 6518 |
| $AUC_{0-\infty}$ [ng h/mL] | 7.04 ± 0.64 | 18.9 ± 13.2 | 16.9 ± 10.5 | 10,945 ± 7075 |
| $Cl/F$ [L/h] | 11,540 ± 510 | – | – | – |
| $V_d/F$ [L] | 27,933 ± 1882 | – | – | – |

for mixture of CTM isomers [13–15] may lead to an overestimation of the patient exposure to active metabolite of CLP. Therefore, a selective method for determination of the active CTM isomer should be applied instead of measuring the mixture of isomers.

## 4. Conclusion

The HPLC–MS/MS method, according to our best knowledge, is the first analytical assay allowing to determine simultaneously CLP and its main metabolites: CLPM and H3 and H4 isomers of CTM in clinical samples. The method fulfills the validation requirements for quantitative analysis of drugs and their metabolites in biological samples. It is specific, repeatable, reproducible, adequately accurate and precise, and offers improved LLOQ of CTM isomers as compared to those previously described. The method was successfully applied for pharmacokinetic studies of CLP and its metabolites in patients undergoing anti-platelet therapy with a daily dose of 75 mg CLP.

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