Short communication

Capillary Zone Electrophoresis method for determination of (+)-S clopidogrel carboxylic acid metabolite in human plasma and urine designed for biopharmaceutic studies

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ARTICLE INFO

ABSTRACT

Fast and reproducible Capillary Zone Electrophoresis (CZE) method for the quantification of (+)-S clopidogrel carboxylic acid metabolite in human fluids was elaborated for the first time. Optimal buffer and CZE conditions were established to obtain the complete separation of clopidogrel, its metabolite and piroxicam (internal standard), during one analytical run. Finally, resolution of the analytes was obtained in an uncoated silica capillary filled with a phosphate buffer of pH 2.5. The analytes were isolated from plasma and urine samples using solid phase extraction (SPE). Validation of the CZE method was carried out. The calibration curve of clopidogrel was linear in the range of 0.5–10.0 mg/L in plasma and urine, whereas for (+)-S carboxylic acid metabolite linearity was confirmed in the range of 0.25–20.0 mg/L in plasma and 0.25–10.0 mg/L in urine. Intra- and inter-day precision and accuracy were repeatable. LOD and LOQ were also estimated. SPE recovery of the analytes from plasma and urine was comparable and greater than 80%. The validated method was successfully applied in pharmacokinetic investigations of (+)-S carboxylic acid metabolite of clopidogrel following the oral administration of clopidogrel to patients prior to percutaneous coronary intervention.

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1. Introduction

Clopidogrel (CLP, SR25990C), methyl (+)-S-2-(2-chlorophenyl)-2-(6,7-dihydrothiene[3,2-c]pyridine-5(5H))-acetate, is an antiplatelet and anti-thrombotic drug. It inhibits platelet aggregation by selective preventing of the binding adenosine diphosphate (ADP) to its platelet receptor and blocks the subsequent platelet aggregation. The drug is indicated for the reduction of thrombotic events including myocardial infarction, ischaemic stroke and vascular death in patients with atherosclerosis [1]. It has an absolute S configuration at carbon 7, while the corresponding R enantiomer is devoid of anti-aggregating activity [2]. CLP is inactive pro-drug and hepatic activation by cytochrome P450 isoenzymes, mainly CYP3A4, CYP3A5 and CYP2C19, is necessary to induce expression of its anti-aggregating properties [3,4]. In the biotransformation process, a small fraction of CLP is converted into the intermediate metabolite 2-oxo-clopidogrel, which is subsequently hydrolyzed to the active thiol metabolite. This compound reacts with the thiol of an amino acid of the platelet receptor and causes an irreversible blockade of ADP binding for the platelet's life span [5]. The products of CYP-mediated metabolism, 2-oxo-clopidogrel and the active thiol metabolite of CLP, are highly labile and usually undetectable in plasma. The major metabolite of CLP is carboxylic acid derivative of CLP (CLPM, SR26334), which represents 85% of circulating drug-related compounds in plasma [6]. CLPM is formed in the liver by hydrolysis of the ester function by carboxylesterase. Although it is inactive, it could be used as a pharmacokinetic marker of CLP [7–9]. Up to now, numerous HPLC assays for the determination of CLP and CLPM have been reported in the literature. Recently, a review was published comprising the methods used for the determination of CLP and its metabolites in biological fluids [10]. Mitakos and Panderi reported a non-stereospecific HPLC method with UV detection for the determination of CLP in oral dosage forms. The method was applied to degradation studies under stress conditions [11]. Moreover, an enantiospecific validated HPLC–UV method was reported for the determination of CLP and its impurities in 19 drug product tablets containing CLP [12]. This year, capillary electrophoresis method was published for the separation and determination of CLP and its impurities in commercial bulk samples [13]. CLPM was determined in human plasma using HPLC methods with UV detection. The methods were applied for bioavailability studies of CLP in

healthy volunteers [8,9]. Moreover, GC–MS, LC–MS and LC–MS–MS methods were reported for the determination of CLPM in biological fluids [7,14–17]. However, the methods required time and solvent consuming extraction from biological matrix [7,14] and derivatization of the analyte [14]. In recent years, the capillary electrophoresis methods have been widely applied for the analysis of drugs and/or their metabolites in biological matrices due to the low solvent consumption, short analytical time and a high resolving power. However, up to now capillary electrophoresis assay for the determination of CLPM in biological fluids has not been published. The aim of this study was to develop and validate a fast and reproducible CZE method for the determination of (+)-S carboxylic acid metabolite in patients' plasma and urine. The validated method was applied to pharmacokinetic studies of CLPM in patients following the oral administration of 300 mg clopidogrel loading dose prior to percutaneous coronary intervention (PCI).

2. Experimental

2.1. Materials

(+)-S clopidogrel bisulphate (optical purity, o.p. 99.0%) and its carboxylic acid metabolite (o.p. 99.6%) were a generous gift of Pharmaceutical Research Institute (Warsaw, Poland). Piroxicam (PRX, internal standard, IS) was purchased from Jelfa (Jelenia Góra, Poland). Potassium dihydrogen phosphate and disodium hydrogen phosphate anhydrous were from Xenon (Łódź, Poland) and Fluka (Buchs, Switzerland), respectively. 1.0 M and 0.1 M NaOH, water for HPCE, 50 mM phosphate buffer of pH 2.5 and 7.0 for HPCE (Agilent Technologies, Waldbroon, Germany) were used. Methanol (Merck, Darmastadt, Germany) was of HPLC grade. Demineralised water was always used to prepare buffer solutions applied to SPE procedure (Simplicity UV, Millipore, USA). The cartridges for SPE, of 1 mL capacity with 100 mg of octadecyl phase chemically bound to silica gel (Bakerbond SPE^{TM}, J.T. Baker, The Netherlands), were applied for the isolation and purification of analytes from plasma and urine.

2.2. Equipment and CE conditions

Analytes were determined on an Agilent model ^{3D}CE apparatus (Agilent Technologies, Waldbronn, Germany) with UV detector set at $\lambda = 220$ nm. The samples were automatically injected using hydrodynamic injection at the anode. The temperature of the capillary was maintained by a thermostatic system at 25 °C. The separation was performed in a fused silica capillary, 35 cm × 50 μm i.d., 26.5 cm to the detector. The system was controlled by Chem-Station software. All experiments were carried at 25 kV and 50× 5 mbar s injection (12 nL injected volume). The volume of a sample loaded to capillary was calculated using Hagen–Poiseuille equation [18]. The background electrolyte (BGE) used for the study on the optimal electrophoretic resolution of CLP, CLPM and PRX was composed of commercially available phosphate buffers (pH 2.5 and 7.0) or it was prepared by mixing appropriate volumes of 0.025 M aqueous solutions of Na_2HPO_4 and KH_2PO_4 (pH 3.0 and 4.0). Finally, the analytes were determined using a 0.025 M phosphate buffer of pH 2.5. The solution was degassed by ultrasounds before injection into the capillary. A new capillary was conditioned with 1.0 M sodium hydroxide, subsequently with 0.1 M sodium hydroxide, water for HPCE and finally with BGE for 10, 10, 5 and 8 min. Before each run the capillary was rinsed out with 0.1 M sodium hydroxide, water for HPCE and BGE for 2, 5 and 5 min.

2.3. Sample preparation for calibration curves

CLP, CLPM and IS stock solutions of 1 g/L were prepared in methanol. Then, standard solutions of 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 mg/L of CLP, as well as 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 and 400.0 mg/L of CLPM and 200 mg/L IS were prepared also in methanol. The volume of 50 μL of the sample was transferred to a vial containing 1.0 mL human blank plasma. The resulting plasma samples contained 0.25, 0.50, 1.00, 2.50, 5.00 and 10.0 mg/L of CLP and 0.1, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0 and 20.0 mg/L of CLPM and 10.0 mg/L IS. Urine samples were prepared according to the same procedure but with concentration range of 0.1–10.0 mg/L. The plasma and urine samples were mixed with 1 mL phosphate buffer of pH 5.0 (prepared as a mixture of 0.95 mL of 1/15 M Na_2HPO_4 and 99.05 mL of 1/15 M KH_2PO_4) and applied on C_{18} bonded SPE cartridges activated with 2× 1 mL of methanol and 2× 1 mL of demineralised water. Subsequently, the adsorbed analytes were washed with 2 mL of water and eluted with 2× 0.5 mL of methanol. The organic liquid was evaporated under a gentle nitrogen flow at 50 °C and the dry residue was dissolved before injection in 50 μL of methanol and 50 μL of BGE.

2.4. Validation parameters

To confirm selectivity of the elaborated CZE method, blank plasma and urine samples obtained from healthy volunteers and patients with atherosclerosis were analyzed. Moreover, resolution of CLPM and PRX was performed in patients' samples in the presence of other drugs co-administered with CLP. For this purpose the biological samples were spiked with atorvastatin, indapamide, metoprolol, omeprazole, ramipril and salicylic acid and extracted according to the procedure described in Section 2.3.

Linearity of the calibration curve was estimated for the ratio of the peak area of CLP or CLPM to IS as a function of the analyte concentration covering the range of 0.25–20.0 mg/L in plasma and 0.25–10.0 mg/L in urine. The correlation coefficient r was calculated. 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].

Limit of detection (LOD) of the analytes was determined as an S/N baseline ratio of 4:1 for plasma and urine samples. Limit of quantitation (LOQ) was defined as the lowest concentration of CLP or CLPM determined by the method within the percent RSD (100 × SD/$\bar{x}$) ≤ 15% of its nominal value. Quality control samples (QCS) at concentrations of 1.0, 2.5 and 5.0 mg/L of CLP and CLPM in plasma and urine were independently prepared and determined.

Intra-day precision of the method, expressed as %RSD, has been estimated for QCS at concentrations of 1.0, 2.5 and 5.0 mg/L of CLP and CLPM in plasma and urine, prepared in five replicates. Inter-day precision was estimated for all concentrations within the calibration curve range. Accuracy was estimated for the same ranges of analytes concentrations as for the evaluation of precision of the method.

Absolute recovery of CLP and CLPM from plasma and urine was evaluated by the analysis of each QCS (in five replicates) at concentrations of 1.0, 2.5 and 5.0 mg/L. To evaluate the recovery, I series consisted of five blank plasma or urine samples, spiked with the analytes and IS, was extracted according to the procedure presented above (Section 2.3). Subsequently, five samples of plasma or urine were supplemented with IS only (II series). The analytes was added to dry residue of the extracted samples of II series. The recovery was calculated as the area ratio of extracted to nonextracted analytes, with reference to IS.

Stability of CLP and CLPM in plasma and urine samples was evaluated at concentrations of 1.0, 2.5 and 5.0 mg/L (in three replicates) after three freeze–thaw cycles as well as short-term storage (6 h of standing in the autosampler at room temperature) and long-term storage (1 month at −20 °C). The samples were prepared according to the extraction procedure described above.

2.5. In vivo application

The usefulness of the elaborated method has been demonstrated in quantitative determination of CLPM in patients' plasma and urine. The studies were approved by the Ethical Committee at Poznan University of Medical Sciences. Three male patients (64 ± 7 years old, 87 ± 6 kg body weight) with atherosclerosis were involved in the studies. The patients, who took also other medicines (described in Section 2.4.), were administered a 300 mg clopidogrel loading dose prior to PCI. Blood and urine samples were collected for up to 24 h after administration of the drug and stored at $-20\,°C$. The plasma levels of CLPM were used to calculate pharmacokinetic parameters using Topfit 2.0 software package (Gustav Fischer, Stuttgart, 1993). 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} (k_{el}—the elimination rate constant calculated by the terminal linear segment of the log plasma concentration–time data). The elimination half-life ($t_{0.5}$) was estimated from $\ln 2/k_{el}$. C_{max} and t_{max} were read from individual CLPM concentration–time curve. The amounts of CLPM in urine were used to assess percent of eliminated dose of the unchanged drug.

3. Results and discussion

3.1. CE conditions for resolution of the analytes

CLP is a weak base with pK_a of ca. 4.5. At low buffer's pH it is dissociated and migrates as cations. The increase in pH resulted in the worsening of CLP dissociation and caused precipitation of the drug. The chemical structure of CLPM suggests more acidic nature of the compound in comparison with CLP. However, its pK_a has not been established yet. Increase of buffer's pH involved improvement of dissociation (α) and in consequence augmented electrophoretic mobility of CLPM ($\mu_{ep} = \mu^0 \alpha$). To estimate the influence of buffer's pH on migration time of CLP, CLPM and IS, series of BGE with pH of 2.5, 3.0, 4.0 and 7.0 were prepared. Although, the analysis time was extensively shortened by increasing pH, resolution of peaks was negatively affected. The most optimum conditions for the separation of CLP, CLPM and IS in one analytical run in a relatively short time of 12 min were established at buffer's pH of 2.5 and temperature 25 °C (Fig. 1).

3.2. Validation of the method

Comparison of electropherograms of extracted blank samples of plasma and urine (Fig. 2A1 and B1) with electropherograms of the samples spiked with 10 mg/L of the analytes (Fig. 2A2 and B2) or received from a patient following the administration of 300 mg CLP and spiked with PRX (Fig. 2A3 and B3) shows complete separation of the analytes from each other and from the peaks originated from endogenous compounds. Moreover, the peaks of CLP, IS and CLPM with migration times of: 2.7, 7.5 and 10.8 min, respectively, did not interfere with peaks from any drugs co-administered with CLP: atorvastatin (12.1 min), indapamide (12.6 min), metoprolol (2.9 min), omeprazole (2.9 min) and ramipril (3.4 min). Salicylic acid did not appear on the electropherogram during the time of the analysis. Standard curves estimated for the analytes were linear in the range of concentrations 0.5–10.0 mg/L in plasma and urine for CLP and 0.25–20.0 mg/L in plasma and 0.25–10.0 mg/L in urine for CLPM. 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 1. They were applied for the quantification of the analytes in patients' plasma and urine following the oral administration of
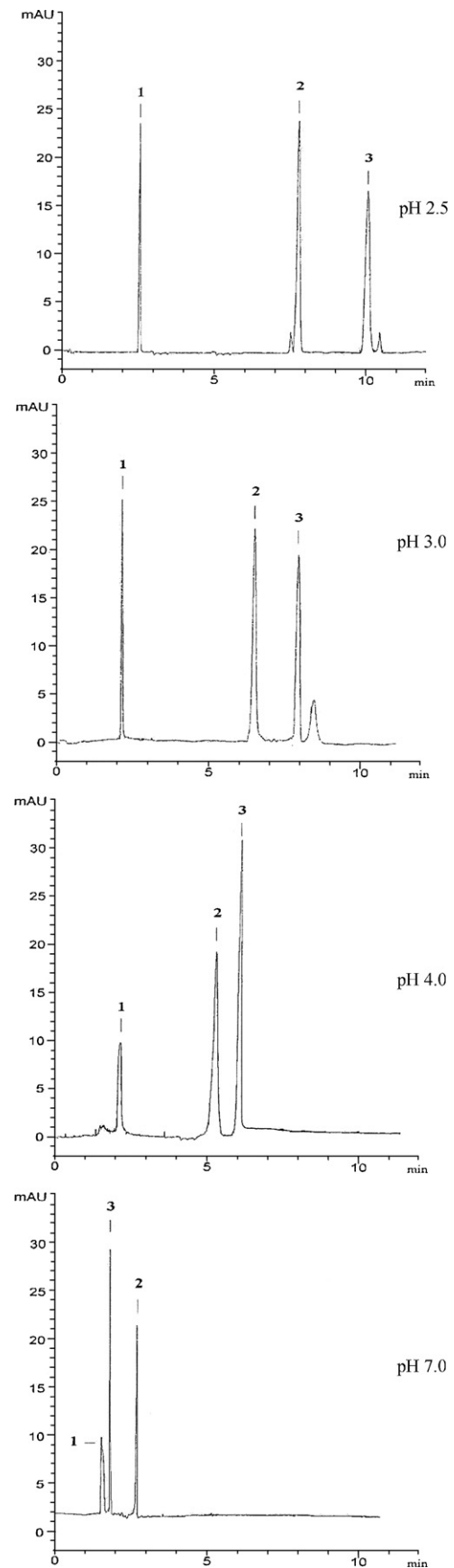

Fig. 1. Effect of pH on the resolution of CLP, CLPM and PRX. CE conditions: 0.025 M phosphate buffer, 35 cm total length of fused silica capillary, 50 μm i.d., temperature 25 °C, voltage 25 kV, current 35–40 μA. Peaks denoted: 1—CLP, 2—PRX, 3—CLPM.
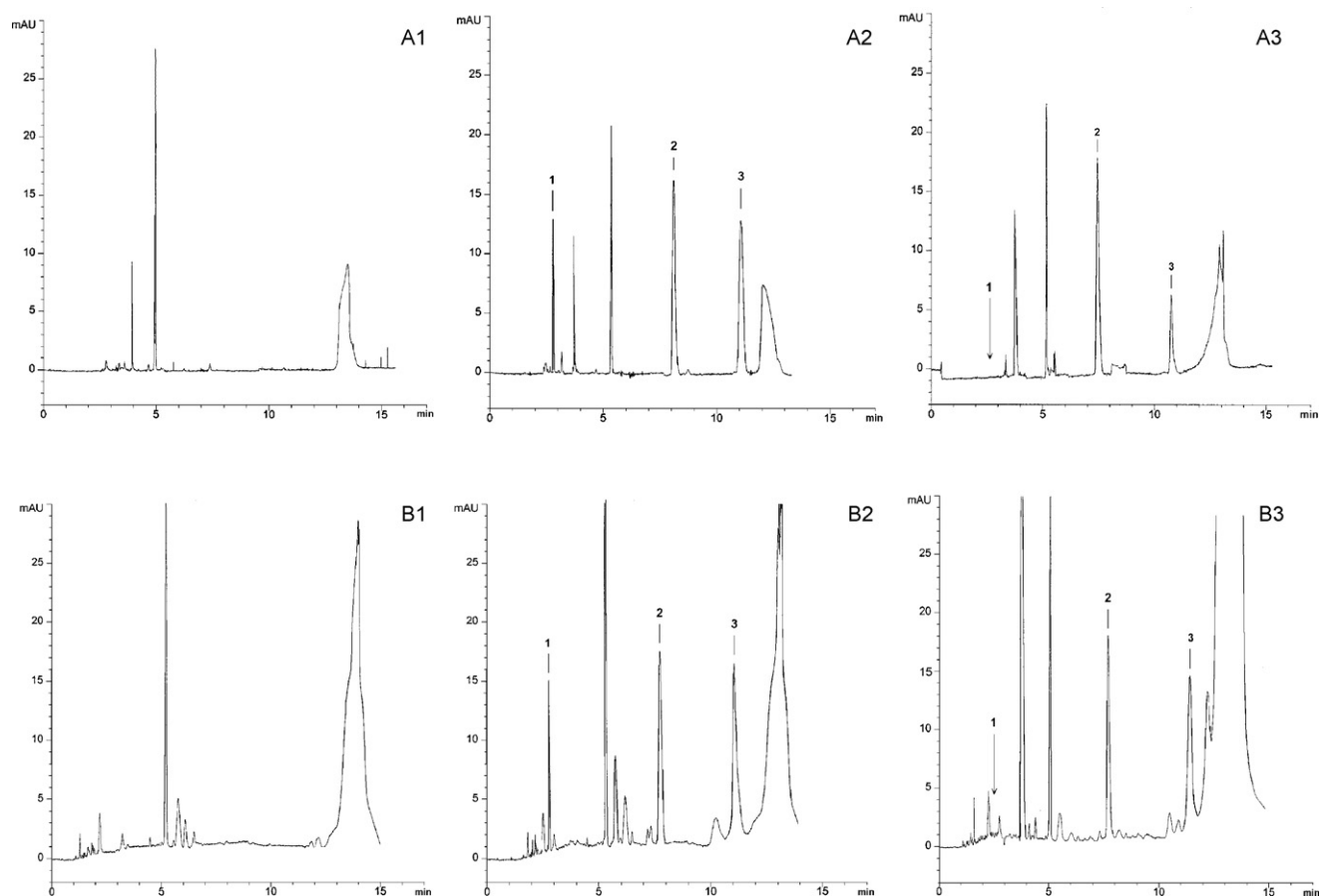
Fig. 2. Electropherograms of CLP and CLPM following SPE of human plasma and urine samples. A1—blank plasma; A2—blank plasma spiked with 10.0 mg/L of CLP, PRX and CLPM; A3—plasma sample collected from a patient at 2 h elapsed from administration of a single dose 300 mg CLP (metabolite concentration was 3.1 mg/L); B1—blank urine; B2—blank urine spiked with 10.0 mg/L of CLP, PRX and CLPM; B3—urine sample collected from a patient following the administration of a single dose 300 mg CLP (metabolite concentration was 7.4 mg/L). Peaks denoted: 1—CLP, 2—PRX, 3—CLPM.

CLP in tablets. In the worked out conditions, LOD at an S/N baseline ratio = 4:1 was found to be 0.25 and 0.1 mg/L, respectively, for CLP and CLPM. LOQ was 0.51–0.55 mg/L for CLP and 0.22–0.24 mg/L for CLPM (Table 1). It should be emphasized that the results were achieved using normal fused capillary of 50 μm i.d. with small volume of injected sample (only 12 nL). The value of LOQ in plasma for CLPM has proven to be similar to LOQ of 0.2 mg/L obtained by HPLC–UV method and reported by Souri et al. [8]. Very low values of LOQ of 0.05 mg/L can be obtained by a sensitive LC–MS–MS method [17]. However, the elaborated CZE method proved to be sensitive enough to determine the concentrations of CLPM in patients' plasma up to 24 h (Fig. 3).

Precision of the CE method expressed as %RSD was in the range of 1.15–11.80%, pointing to relatively high precision in the estimation of investigated analytes concentrations in both biological media. Also, accuracy of the estimations has fitted the range required for testing drug and/or metabolites content in body fluids. It has ranged from 0.30 to 12.00% (Table 1). Recovery of the analytes from plasma and urine samples following SPE procedure was comparable and amounted to 81–89% and 85–96% for CLP and CLPM, respectively (Table 2). The obtained yield corresponds to the literature data which showed 80% and higher recovery of CLPM [7–9]. However, in the reported methods time consuming procedure using multiple liquid–liquid extraction (LLE) [7] or both LLE and SPE have been used [14] or a large volume of organic solvent was applied [8,9].

CLP and CLPM proved to be stable in plasma and urine samples after three freeze–thaw cycles as demonstrated by percent relative

error of estimates in the range of 0.8–7.6% for CLP and 1.2–9.6% for CLPM. The stability during storage at room temperature for 6 h was expressed by high accuracy with percent relative error of 1.0–10.4% for CLP and 3.1–9.8% for CLPM. The results obtained during the long-term stability study were within the range of 1.2–13.6%. The data indicate that the analytes were stable during 1-month storage in a freezer at −20 °C.
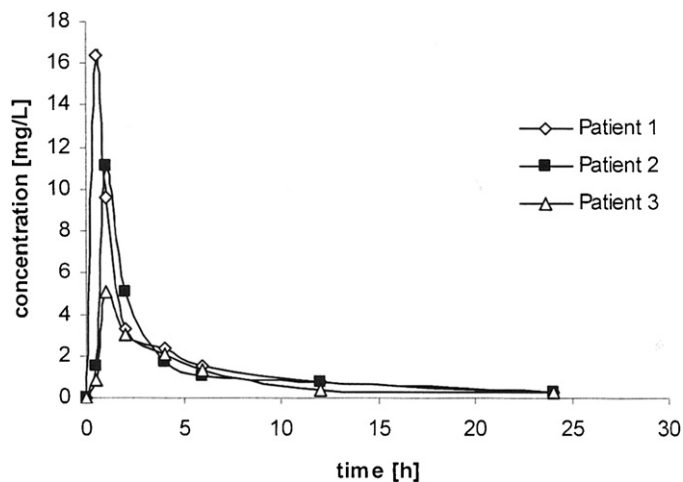

Fig. 3. Plasma concentration of CLPM versus time following the administration of a single dose 300 mg CLP to 3 patients.

Table 1
Validation parameters of calibration curves for analysis of CLP and CLPM in plasma and urine.

Nominal concentration [mg/L]	CLP			CLPM		
	Mean assayed value [mg/L]	Accuracy [% error]	Precision [% RSD]	Mean assayed value [mg/L]	Accuracy [% error]	Precision [% RSD]
Plasma						
Intra-day repeatability ($n = 5$)						
1.00	1.11	11.00	4.06	1.11	11.00	2.29
2.50	2.77	10.80	8.99	2.59	3.60	10.74
5.00	4.51	9.80	6.38	5.35	7.00	8.17
Inter-day reproducibility ($n = 5$)						
0.25	<LOQ	–	–	0.22	12.00	10.32
0.50	0.55	10.00	11.80	0.47	6.00	9.36
1.00	1.09	9.00	4.52	1.04	4.00	8.49
2.50	2.70	8.00	5.32	2.24	10.40	1.15
5.00	5.03	0.60	8.74	5.07	1.40	8.71
10.00	9.91	0.90	6.81	10.03	0.30	4.37
20.00	–	–	–	19.80	1.00	5.50
Urine						
Intra-day repeatability ($n = 5$)						
1.00	1.08	8.00	8.57	1.05	5.00	9.11
2.50	2.26	9.60	5.88	2.25	10.00	11.19
5.00	5.15	3.00	7.87	4.82	3.60	8.61
Inter-day reproducibility ($n = 5$)						
0.25	<LOQ	–	–	0.24	4.00	7.40
0.50	0.51	2.00	10.66	0.54	8.00	11.10
1.00	1.10	10.00	9.16	1.03	3.00	10.49
2.50	2.42	3.20	8.00	2.37	5.20	6.89
5.00	4.72	5.60	6.77	4.55	9.00	5.61
10.00	10.12	1.20	7.98	10.23	2.30	5.94

Equations of calibration curves for CLP and CLPM:
in plasma: $y = 0.0386C_{CLP} + 0.0051$ ($r = 0.9998$); $y = 0.1349C_{CLPM} + 0.0068$ ($r = 0.9996$).
in urine: $y = 0.0431C_{CLP} + 0.0112$ ($r = 0.9993$); $y = 0.1873C_{CLPM} + 0.0112$ ($r = 0.9984$).

3.3. In vivo application

The utility of the assay for pharmacokinetic studies has been demonstrated following the oral administration of 300 mg of CLP to each of the three patients. Due to very low blood levels of the parent drug achieved following single dose administration, the presented CZE method failed to detect peak of CLP in patients' samples (Fig. 2A3 and B3). Unchanged CLP could only occasionally be detected in studies using HPLC–MS–MS methods [16,17]. Rapid absorption of CLP from tablets in GI tract is best demonstrated by the early appearance in plasma of CLPM (Fig. 3). In the studied patients, plasma levels reached maximum value of 10.9 ± 5.6 mg/L after the time of 0.8 ± 0.3 h, which is consistent with the value of t_{max} obtained in previous studies [7,20]. The value of $AUC_{0 \to t}$ was calculated as 33.8 ± 8.3 mg h/L, which constituted 93% of the $AUC_{0 \to \infty}$ value. This fact indicates a suitability of the analytical method for bioavailability studies. The plasma elimination half-life, $t_{0.5}$ was found to be 6.1 ± 0.4 h and is similar to $t_{0.5} = 5.4$ and 7.6 h reported previously [17,20]. However, literature data reported

a wide range of $t_{0.5}$ values of CLPM, from 1.9 ± 0.9 h after a dose of 600 mg CLP [16] to 10.0 ± 2.2 h following the administration of 75 mg CLP [7]. In the present studies the mean urinary excretion of CLPM was about 1% of the dose administered. Caplain et al. also reported very low value of urinary excretion of CLPM, which was found to be about 2% following administration 50–150 mg CLP to healthy volunteers [20].

4. Conclusions

The elaborated CZE method fulfilled validation parameters required for the determination of analytes in biological samples and it can be applied for pharmacokinetic investigations of carboxylic acid metabolite of CLP in patients with atherosclerosis. Determination of CLPM in the presence of many other drugs co-administered with CLP was feasible due to a unique selectivity of the method.

Table 2
Recovery of CLP and CLPM after SPE from plasma and urine.

Concentration of the analyte [mg/L]	Recovery (%) (mean ± SD)	
	CLP	CLPM
In plasma		
1.0	82.0 ± 3.3	85.2 ± 1.9
2.5	87.9 ± 8.0	91.1 ± 8.7
5.0	86.4 ± 6.8	95.6 ± 7.8
In urine		
1.0	83.9 ± 6.8	91.2 ± 6.6
2.5	82.0 ± 5.6	85.4 ± 5.9
5.0	88.6 ± 7.0	88.01 ± 7.6

$n = 5$.

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