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Research Article

Direct measurement of active thiol metabolite levels of clopidogrel in human plasma using tris(2-carboxyethyl)phosphine as a reducing agent by LC–MS/MS

A simple, robust, and rapid LC–MS/MS method has been developed and validated for the simultaneous quantitation of clopidogrel and its active metabolite (AM) in human plasma. Tris(2-carboxyethyl)phosphine (TCEP) was used as a reducing agent to detect the AM as a disulfide-bonded complex with plasma proteins. Mixtures of TCEP and human plasma were deproteinized with acetonitrile containing 10 ng/mL of clopidogrel-d_4 as an internal standard (IS). The mixtures were separated on a C_{18} RP column with an isocratic mobile phase consisting of 0.1% formic acid in acetonitrile and water (90:10, v/v) at a flow rate of 0.3 mL/min. Detection and quantification were performed using ESI-MS. The detector was operated in selected reaction-monitoring mode at m/z 322.0→211.9 for clopidogrel, m/z 356.1→155.2 for the AM, and m/z 326.0→216.0 for the IS. The linear dynamic range for clopidogrel and its AM were 0.05–20 and 0.5–200 ng/mL, respectively, with correlation coefficients (r) greater than 0.9976. Precision, both intra- and interday, was less than 8.26% with an accuracy of 87.6–106%. The validated method was successfully applied to simultaneously analyze clinical samples for clopidogrel and its AM.

Keywords: Clopidogrel / Clopidogrel active metabolite / Human plasma / Reducing agent / tris(2-Carboxyethyl)phosphine / LC-MS/MS
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1 Introduction

Clopidogrel (Fig. 1) is a platelet aggregation inhibitor that is commonly prescribed to reduce cardiovascular event rates for patients with acute coronary syndromes, particularly coronary interventions [1–3]. It prevents platelet aggregation by irreversibly inhibiting the platelet P2Y12 adenosine diphosphate receptor by covalent modification of cysteine residues in the P2Y12 receptor [4–7]. To exert this antiplatelet effect, clopidogrel requires biotransformation to a thiol-containing active metabolite (AM) of clopidogrel (3-(carboxymethylene)-a-(2-chlorophenyl)-4-mercapto-1-piperidineacetic acid; Fig. 1). The conversion to the AM is facilitated by hepatic cytochrome P450 enzymes, mainly CYP2C19, and to a lesser extent, CYP1A2, CYP2B6, and CYP3A4/5 [8–10]. The reactive thiol group of the AM covalently modifies the cysteine residues of the platelet P2Y12 receptor, thereby preventing platelet aggregation [4–7]. Although clopidogrel is widely used as an antiplatelet agent, it has shown significant interindividual variability with regard to drug response. Lack of response or "resistance" to clopidogrel therapy may be seen in ~45% of the patient population [11]. To date, several genetic markers for these variations, such as CYP2C19 [12–15], CYP3A5 [16], MDR1 [17], and paroxonase 1 [18], have been identified. Recent data suggest that the resistance to clopidogrel therapy may be primarily attributable to failure of the biotransformation of clopidogrel to its AM [19, 20]. To properly estimate the patient response to clopidogrel, an accurate quantification method is needed for measuring levels of the AM in plasma. However, the reactivity of thiol-containing compounds such as clopidogrel AM typically renders them unstable in biological samples [20, 21]. Specifically, the thiol group forms a disulfide bond with endogenous, low-molecular-weight compounds or with proteins, for example, dimers, cysteine conjugates, glutathione conjugates, or complexes with albumin [20, 21]. The labile nature of the AM has prevented the acquisition of its pharmacokinetic properties.

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Abbreviations: **AM**, clopidogrel active metabolite; **IS**, internal standard; **LLOQ**, lower limit of quantification; **QC**, quality control; ***r***, correlation coefficients; **TCEP**, Tris(2-carboxyethyl)phosphine

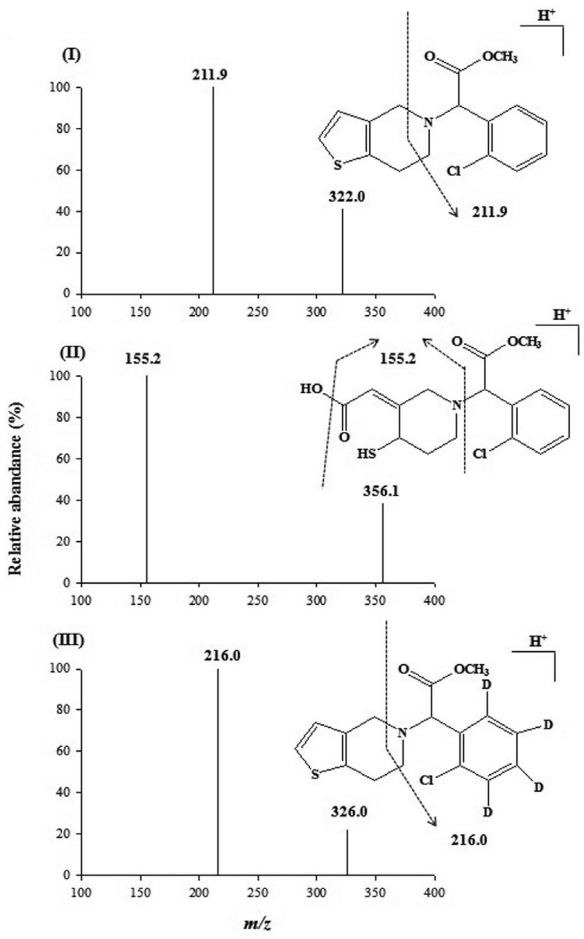

Figure 1. Product ion mass spectra of clopidogrel (I), clopidogrel active metabolite (II), and clopidogrel-d_4 (IS) (III).

Several years ago, a carboxylic acid derivative, formed by de-esterification, was identified in humans as a major metabolite of clopidogrel (85% of total metabolites) [22]. Since the AM is unstable in biological samples, levels of exposure and the pharmacokinetic parameters of clopidogrel have been indirectly determined by quantifying the inactive carboxylic acid derivative of clopidogrel [22] or by simultaneous measurements of clopidogrel [23–30] and its carboxylic acid metabolite. However, both clopidogrel and its carboxylic acid metabolite are inactive. Furthermore, the back-conversion acyl glucuronide clopidogrel, another metabolite of clopidogrel typically present in biological samples, to clopidogrel or its carboxylic acid metabolite has been recently reported [31–33]. To date, only a few analytical LC–MS/MS-based methods for determining levels of clopidogrel AM have been reported [21, 34–37]. These methods required a preparation step, consisting of reaction with an alkylating reagent such as 3′-methoxyphenacyl bromide immediately after blood collection to stabilize the free thiol group of the AM [21, 34–37]. In addition, none of these methods was applied to direct measurements of clopidogrel levels. They limit their widespread use in retrospective clinical studies if there is no immediate derivatization step during blood collecting.

In general, two methods are employed with biological samples to allow direct measurements of compounds bearing thiol groups. The first is derivatization of the thiol group into a stable moiety. The second is a directed binding between the thiol group and proteins using reducing agents such as L-cysteine, glutathione, β-mercaptoethanol, DL-dithiothreitol, and tris(2-carboxyethyl)phosphine. Of these, the two most traditionally used thiol reductants are β-mercaptoethanol and DL-dithiothreitol [38, 39]. Recent research has demonstrated the stability of tris(2-carboxyethyl)phosphine (TCEP) over DL-dithiothreitol at 4 and 25°C, from pH 6 to 8, and with different buffers and metal ions. In addition, TCEP is suitable for use with proteins [38–40]. TCEP reduces disulfide bonds just as effectively as DL-dithiothreitol. However, unlike DL-dithiothreitol and other thiol-containing reducing agents, TCEP does not have to be removed from the sample matrix prior to certain sulfhydryl-reactive, crosslinking reactions [38–40]. Additionally, four different reductants, glutathione, L-cysteine, *N*-acetyl-l-cysteine, and ascorbic acid, reportedly led to the formation of the AM from 2-oxoclopidogrel in human liver microsomes [20].

Therefore, the current report compares glutathione, DL-dithiothreitol, and TCEP for use in a new method for the quantification of clopidogrel AM in human plasma. A 50 mM solution of TCEP exhibited clear advantages over DL-dithiothreitol for the detection of clopidogrel AM in plasma *via* disulfide bond formation with plasma proteins. The current method, which employs a simple deproteinization procedure without derivatization, requires very small plasma volumes (100 μL) and only 2.0 min per run. Analyzing concentrations of the AM in human plasma was achieved from clinical samples stored long term at –80°C without immediate derivatization during blood collecting. The established method was successfully applied to the simultaneous analyses of clinical samples for clopidogrel and its AM.

2 Materials and methods

2.1 Materials and reagents

Clopidogrel hydrogen sulfate and clopidogrel-d_4 hydrogen sulfate were at least 99.0% pure and purchased from Toronto Research Chemicals (North York, ON, Canada). Clopidogrel's AM (3-(carboxymethylene)-a-(2-chlorophenyl)-4-mercapto-1-piperidineacetic acid) was a product of SynFine Research (Richmond Hill, ON, Canada). DL-Dithiothreitol, formic acid, reduced glutathione, and tris(2-carboxyethyl)phosphine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were of HPLC grade and were obtained from Burdick & Jackson (Morristown, NJ, USA). All other chemicals were of the highest quality available. Drug-free heparinized human plasma was obtained from the Clinical Trial Center of Chungnam National University Hospital (Daejon, South Korea).

2.2 Calibration standards and quality controls (QCs)

Stock solutions of clopidogrel (as a free salt), AM, and clopidogrel-d_4 (internal standard, IS) were prepared by dissolving each material in acetonitrile at a concentration of 1 mg/mL. The IS stock solution was further diluted to 10 ng/mL in acetonitrile for routine use. The stock solutions of clopidogrel and the AM were serially diluted with acetonitrile. These diluted solutions were added to drug-free human plasma to produce final concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 20 ng/mL for clopidogrel and 0.5, 1, 5, 10, 50, 100, and 200 ng/mL for the AM. On the day of analysis, calibration curves for clopidogrel and the AM in human plasma were derived from peak area ratios of the analyte relative to the IS as a function of the respective plasma concentrations of clopidogrel and the AM, respectively, using a linear regression with $1/x^2$ as a weighting factor. QC samples were assayed along with each batch of plasma samples.

QC samples were prepared daily by spiking diluted stock solutions into drug-free human plasma to achieve final concentrations of 0.05 (lower limit of quantification, LLOQ), 0.15 (low QC), 3 (medium QC), and 15 (high QC) ng/mL for clopidogrel and 0.5 (LLOQ), 1.5 (low QC), 30 (medium QC), and 150 (high QC) ng/mL for the AM. The QC samples were used to evaluate the intra- and interday precision and accuracy of the method. All of the prepared plasma samples and stock solutions were stored at –80°C (Revco ULT 1490 D-N-S; Western Mednics, Asheville, NC, USA).

2.3 The effects of reduced glutathione, DL-dithiothreitol, and tris(2-carboxyethyl)phosphine

To detect clopidogrel AM as a disulfide-bonded complex with plasma proteins, reduced glutathione, DL-dithiothreitol, or tris(2-carboxyethyl)phosphine were used as reducing agents in human plasma; 50 μL aliquots of water containing reduced glutathione (0 to 10 mM) were added to two different QC samples (0.15 and 3 ng/mL for clopidogrel and 1.5 and 30 ng/mL for the AM) and mixed by a vortex mixer for 5 min. Then, a 300 μL aliquot of acetonitrile containing 10 ng/mL clopidogrel-d_4 (IS) was added to each sample. After the mixture was centrifuged at 13 000 × *g* for 15 min at 4°C, the supernatant was transferred to another vial, and the sample was passed through a 1.2 μm syringe filter (Acrodisc; Pall Gelman Laboratory, Ann Arbor, MI, USA). Five microliter aliquots of the filtered samples were injected into the LC–MS/MS system as described in Section 2.4.

DL-Dithiothreitol (0 to 5 M) or tris(2-carboxyethyl) phosphine (0 to 150 mM) were also used to detect the AM in human plasma. Additionally, to evaluate the effects of the reducing agents themselves on the levels of clopidogrel in human plasma, the chromatographic peak areas of clopidogrel were compared in the presence of reduced glutathione, DL-dithiothreitol, TCEP, or without any reductants. All experiments were performed in triplicate and mean values were used in data analyses.

2.4 Sample preparation by deproteinization

Fifty microliter aliquots of water containing 50 mM of TCEP were added to 100 μL aliquots of human plasma and mixed by a vortex mixer for 5 min. Then, a 300 μL aliquot of acetonitrile containing 10 ng/mL clopidogrel-d_4 (IS) was added to each sample. After the mixture was centrifuged at 13 000 × *g* for 15 min at 4°C, the supernatant was transferred to another vial, and the sample was passed through a 1.2 μm syringe filter (Acrodisc; Pall Gelman Laboratory). Five microliter aliquots of the filtered samples were injected into the LC–MS/MS system as described in Section 2.4. All prepared samples were kept in an autosampler at 4°C until injection.

Derivatization, on the other hand, should be included an immediate stabilization step with an alkylating reagent such as 3′-methoxyphenacyl bromide after drawing blood [21, 34–37]. It should be directly added into each whole blood sample. As mentioned earlier, it limits its widespread use in retrospective clinical studies if there is no immediate derivatization step during blood collecting.

2.5 LC–MS/MS conditions

The samples were analyzed using an API 5500 Q-Trap mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with a 1260 HPLC system (Agilent Technologies, Wilmington, DE, USA) in ESI mode to generate positive molecular ions $[M+H]^+$. The compounds were separated on a C_{18} RP column (YMC Pro C_{18} column, 50 × 2.0 mm id, 3.0 μm particle size; YMC) with an isocratic mobile phase consisting of 0.1% formic acid in acetonitrile and water (90:10, v/v) at a flow rate of 0.3 mL/min. The column and autosampler temperatures were maintained at 40 and 4°C, respectively. The total run time was 2.0 min for each sample.

The optimized ion spray voltage and temperature were set at 4500 V and 400°C, respectively. The operating conditions, which were optimized by the infusion of 5 ng/mL standard solutions of the analytes at 20 μL/min were as follows: declustering potential, entrance potential, and collision energy were 40, 10, and 23 V for clopidogrel, 41, 9, and 41 V for the AM, and 166, 10, and 23 V for the IS, respectively. The collision cell exit potentials were all 10 V. Nitrogen gas was used as the nebulizer gas, curtain gas, and collision-activated dissociation gas, which were set at pressures of 10, 50, and 50 psi, respectively. The mass transitions used for clopidogrel, the AM, and the IS were m/z 322.0 → 211.9, 356.1 → 155.2, and 326.0 → 216.0, respectively. Data acquisition was performed with dwell times of 150 ms, and quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed using

Analyst software (version 1.5.2; Applied Biosystems, Foster City, CA, USA).

2.6 LC–MS/MS method validation

Method validation assays were performed in accordance with currently accepted United States Food and Drug Administration (US FDA) bioanalytical method validation procedures [41]. The validation parameters were selectivity, linearity, sensitivity, accuracy, precision, extraction recovery, matrix effect, and stability of clopidogrel and the AM in human plasma.

2.6.1 Selectivity

Selectivity was evaluated by comparing chromatograms of six different batches of drug-free plasma obtained from six different sources to ensure that no interfering peaks were present at the retention times of the analytes at the LLOQ.

2.6.2 Linearity and sensitivity

Calibration curves in human plasma were obtained by plotting the chromatographic peak ratios of clopidogrel and the AM to the IS against the nominal concentrations of the calibration standards at 0.05, 0.1, 0.5, 1, 5, 10, and 20 ng/mL for clopidogrel and 0.5, 1, 5, 10, 50, 100, and 200 ng/mL for the AM. The calibration curves were fitted using a linear least-squares regression with a weighing factor of $1/x^2$. The LLOQ for clopidogrel and the AM in human plasma was defined as the lowest concentration giving an S/N of at least 5, an acceptable accuracy (80–120%), and sufficient precision (within 20%). This was verified by six replicate analyses.

2.6.3 Precision and accuracy

The intraday precision and accuracy were determined by analyzing six replicates of the LLOQ sample and three different QC samples (0.05, 0.15, 3, and 15 ng/mL for clopidogrel and 0.5, 1.5, 30, and 150 ng/mL for the AM) on the same day. The interday precision and accuracy were also evaluated by analyzing ten replicates of the LLOQ sample and three different QC samples on five different days (two replicates per day). Precision was expressed as the RSD (%) and the accuracy was expressed as [(mean observed concentration)/(nominal concentration) × 100%]. The concentrations of QC samples, including the LLOQ sample, were determined from the standard calibration curve and analyzed on the same day.

2.6.4 Matrix effect and extraction recovery

Two different QC samples (0.15 and 3 ng/mL for clopidogrel and 1.5 and 30 ng/mL for the AM) and drug-free plasma were used to evaluate matrix effects and the extraction recovery of clopidogrel and the AM. All assays were performed in triplicate. Taking the analyte peak areas obtained by direct injection of diluted (or neat) standard solutions as *A*, the corresponding peak areas of diluted (or neat) standard solutions spiked into plasma extracts after extraction as *B*, and the peak areas of diluted (or neat) standard solutions spiked into plasma prior to extraction as *C*, the matrix effects and extraction recovery were calculated as [42]

$$\text{Matrix effect (\%)} = B/A \times 100 \quad (1)$$

$$\text{Extraction recovery (\%)} = C/B \times 100 \quad (2)$$

The matrix effects and extraction recovery of the IS were evaluated using the same method.

2.6.5 Stability

The stability of clopidogrel and the AM in human plasma was assessed by analyzing three replicate samples spiked with 0.15 and 3 ng/mL of clopidogrel and 1.5 and 30 ng/mL of the AM, under six conditions: (1) short-term storage in human plasma (2 h at room temperature), (2) short-term storage in human plasma (2 h on ice), (3) long-term storage in human plasma (90 days at –80°C), (4) three freeze–thaw cycles, (5) post-treatment storage (8 h at room temperature), and (6) post-treatment storage (24 h at 4°C) in human plasma. The concentrations obtained were compared with the nominal values of the QC samples. The stabilities of stock solutions of clopidogrel, the AM, and the IS, evaluated after one and two weeks at 4°C and after three months at –80°C, were evaluated by comparison with freshly prepared solutions of the same concentrations.

2.7 Clinical application

This study was approved by the Institutional Review Board of Chungnam National University Hospital (Daejon, South Korea) and was performed according to the guidelines of Korean Good Clinical Practice. Six healthy Korean male volunteers (22–31 years old; body weight 65–79 kg; height 167–183 cm) who gave written informed consent were enrolled in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values comprised the exclusion criteria. After an overnight fast, all subjects were given two oral tablets containing 75 mg of clopidogrel (Plavix®; Sanofi-Aventis Korea, Seoul, South Korea) in 240 mL of tap water. Blood samples of approximately 4 mL were collected *via* the median cubital vein prior to dosing and at 0.25, 0.5, 0.75, 1, 1.33, 1.67, 2, 3, 5, 8, 12, and 24 h after administration. The blood samples were centrifuged immediately (2000 × *g*, 10 min) at 4°C and the plasma samples were stored at –80°C until the LC–MS/MS analysis.

Pharmacokinetic parameters were calculated by a noncompartmental analysis with the WinNonlin Professional software (ver. 5.2, Pharsight, Mountain View, CA, USA) using the total area under the plasma concentration–time curve

from time zero to infinity ($AUC_{0-\infty}$) or the last measured time (AUC_{0-t}) following the linear trapezoidal rule. The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were taken directly from the experimental data.

The correlation coefficients (Pearson *r*) between the AUC_{0-t} or C_{max} of clopidogrel and those of the AM were calculated by parametric regression analysis (version 8.01; SAS Institute, Cary, NC, USA). A *p*-value less than 0.05 was considered statistically significant.

3 Results and discussion

3.1 LC–MS/MS optimization

In positive ion mode, clopidogrel, the AM, and IS yielded protonated molecular ions, $[M+H]^+$, at *m/z* 322.0, 356.1, and 326.0, respectively, as the major species. The fragmentation patterns of the protonated molecular ions were evaluated by increasing the collision energy. The product ion spectra and fragmentation patterns for clopidogrel, the AM, and the IS are shown in Fig. 1. The most intense peaks were observed at *m/z* 211.9, 155.2, and 216.0, respectively. The mass parameters were optimized by observing the maximal response of the product ions.

The relative ability of reduced glutathione, DL-dithiothreitol, and TCEP to facilitate the formation of disulfide-bonded complexes between the AM and proteins in human plasma was evaluated. Plots of the AM-to-IS peak area ratios as a function of reductant concentration reached plateaus at 5 mM reduced glutathione, 1 M of DL-dithiothreitol, and 50 mM TCEP, indicating that these concentrations were the lowest capable of yielding the highest peak ratios. The effects of TCEP concentrations on the AM-to-IS peak area ratios at 1 and 100 ng/mL of the AM are shown in Supporting Information Fig. S1. After the addition of reduced glutathione, DL-dithiothreitol, or TCEP to two different QC samples (0.15 and 3 ng/mL for clopidogrel and 1.5 and 30 ng/mL for the AM), the peak areas of clopidogrel were unchanged relative to those in the absence of each respective reducing agent (Table 1). This indicated that reducing agents used in this study for the detection of free AM in human plasma had no influence on the concentration of clopidogrel. The samples containing 50 mM TCEP showed the highest peak intensity and AM-to-IS ratios (Table 1). The tested samples using reduced glutathione showed significant back-conversion into the disulfide form of AM and precipitation under post-treatment storage conditions (8 h at room temperature; Table 1). Unlike DL-dithiothreitol, TCEP is odor-free and was required in smaller amounts (50 mM *versus* 1 M), allowing TCEP reactions to be performed conveniently on the benchtop. Therefore, 50 mM TCEP solutions were used in the sample preparation procedures for LC–MS/MS.

Since a stable isotopically labeled IS would have been ideal [43], clopidogrel-d_4, which showed no obvious matrix effects and exhibited a high extraction ratio, was used as an IS in this study.

3.2 LC–MS/MS method validation

3.2.1 Selectivity

No interfering peaks were observed at the elution times of clopidogrel (0.93 min), the AM (0.72 min), and the IS (0.93 min). Representative chromatograms of drug-free human plasma, a plasma sample at LLOQ (0.05 ng/mL for clopidogrel and 0.5 ng/mL for the AM), and a plasma sample from a volunteer at 1.67 h (0.767 ng/mL for clopidogrel and 13.3 ng/mL for the AM) after oral administration of 150 mg clopidogrel (75 mg two tablets) are shown in Fig. 2. The total run time per sample was 2.0 min.

3.2.2 Linearity and sensitivity

Calibration curves were established using double blank (blank plasma with neither clopidogrel and the AM nor IS), zero blank (blank plasma with the IS only), and seven calibration standards with concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 20 ng/mL clopidogrel and 0.5, 1, 5, 10, 50, 100, and 200 ng/mL of the AM. During validation in human plasma, the mean correlation coefficient (*r*) of the calibration curves was 0.9987 (range, 0.9949–0.9993; $n = 5$) for clopidogrel

Table 1. Comparison peak areas of clopidogrel or the AM to the IS using different reductants of QC1 and QC2 samples

Condition ($n = 3$)	Clopidogrel		AM	
	0.15 ng/mL	3 ng/mL	1.5 ng/mL	30 ng/mL
A	0.00791 ± 0.00181	0.157 ± 0.00896	0.000770 ± 0.000234	0.0665 ± 0.0128
B	0.00808 ± 0.000380	0.166 ± 0.00896	0.00801 ± 0.000354	0.213 ± 0.0108
C	0.00847 ± 0.000120	0.166 ± 0.00278	0.00964 ± 0.00145	0.295 ± 0.0355
D	0.00799 ± 0.000371	0.155 ± 0.00286	0.0109 ± 0.000590	0.363 ± 0.0393
E	0.00825 ± 0.000254	0.168 ± 0.0307	0.00571 ± 0.000531	0.113 ± 0.00563

A, no reductants (distilled water); B, 5 mM reduced glutathione; C, 1 M DL-dithiothreitol; D, 50 mM TCEP; E, 8 h storage at room temperature under posttreatments.

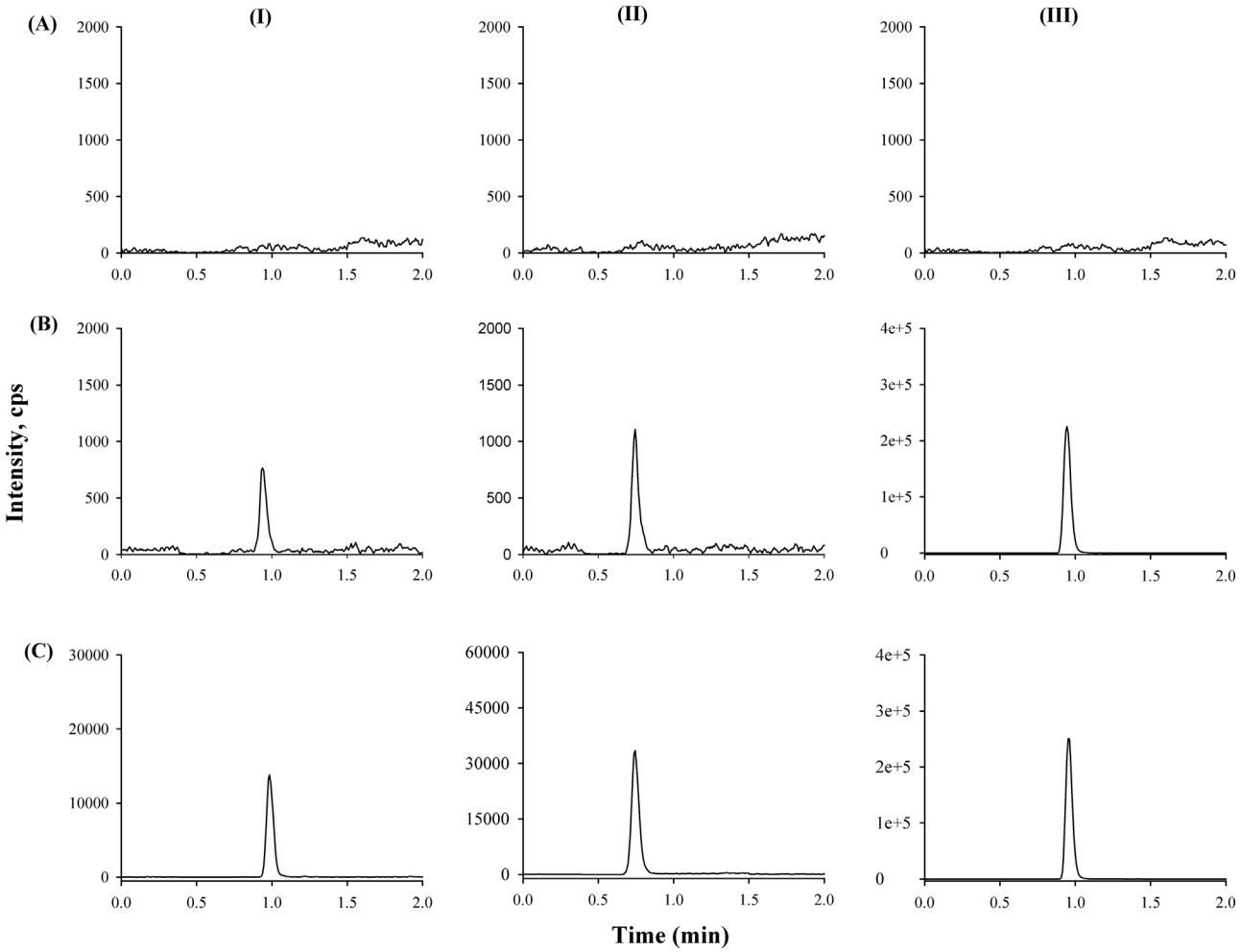

Figure 2. Representative chromatograms of clopidogrel (I), clopidogrel active metabolite (AM) (II), and clopidogrel-d_4 (IS) (III): (A) blank plasma, (B) blank plasma spiked with clopidogrel and AM at LLOQ (0.05 and 0.5 ng/mL) and the IS (10 ng/mL), and (C) a plasma sample from a volunteer at 1.67 h after oral administration of 150 mg clopidogrel (clopidogrel concentration: 0.767 ng/mL; AM: 13.3 ng/mL).

and 0.9951 (range, 0.9886–0.9990) for the AM. The accuracies and precisions of all calibrations were within 88.9–105% and below 7.91%, respectively. The LLOQ for clopidogrel and the AM were 0.05 and 0.5 ng/mL, respectively, which was sufficient for pharmacokinetic studies of clopidogrel and the AM after oral administration of clopidogrel in humans.

3.2.3 Precision and accuracy

Intra- and interday precision and accuracy of the method were measured by analyzing LLOQ and QC samples (0.05, 0.15, 3, and 15 ng/mL for clopidogrel and 0.5, 1.5, 30, and 150 ng/mL for the AM) on five different days. The results are summarized in Table 2. Both precision and accuracy were well within the 15% acceptance range. The coefficients of variation for intra- and interday precision were <7.39% and <8.26%, respectively. The intra- and interday accuracies were 87.6–106 and 89.5–106%, respectively.

3.2.4 Matrix effect and extraction recovery

Two different QC samples (0.15 and 3 ng/mL clopidogrel and 1.5 and 30 ng/mL of the AM) and drug-free plasma were used to evaluate the effects of the sample matrix on the relative degree of ion suppression or enhancement in the ionization of clopidogrel and the AM. As defined in Section 2.6.4, the percentages of matrix effect were between 85 and 125%, indicating no significant matrix effects with any of the analytes (data not shown). In addition, the relative matrix effects in six different lots of plasma were within 20%, indicating that the impact of the extracted plasma matrix was negligible and consistent.

The extraction recoveries in human plasma were 70.9 ± 9.67 and 71.8 ± 8.61% at 0.15 and 3 ng/mL for clopidogrel, and 75.3 ± 6.68 and 72.3 ± 7.79% at 1.5 and 30 ng/mL for the AM, respectively.

For the IS, the extraction recovery at an initial concentration of 10 ng/mL was 77.9 ± 7.97% (data not shown). Low matrix effects and highly reproducible recovery results

Table 2. Intra- and interday precision and accuracy of QC samples for clopidogrel and the AM in human plasma

Compound	Added (ng/mL)	Precision		Accuracy (%)
		Measured (ng/mL)	RSD (%)	
Intraday ($n = 6$)				
Clopidogrel	0.05	0.0448 ± 0.00245	5.48	89.5
	0.15	0.142 ± 0.00695	4.88	94.8
	3	2.77 ± 0.118	4.26	92.3
	15	13.4 ± 0.216	1.62	89.0
AM	0.5	0.451 ± 0.0333	7.39	90.0
	1.5	1.31 ± 0.0234	1.78	87.6
	30	26.9 ± 1.66	6.19	90.6
	150	160 ± 4.28	2.68	106
Interday ($n = 5$)				
Clopidogrel	0.05	0.0487 ± 0.00402	8.26	97.3
	0.15	0.1470 ± 0.00901	6.13	98.0
	3	3.01 ± 0.0691	2.30	100
	15	14.9 ± 0.420	2.83	98.9
AM	0.5	0.514 ± 0.0405	7.89	103
	1.5	1.34 ± 0.101	7.50	89.5
	30	30.7 ± 2.07	6.74	102
	150	160 ± 13.2	8.25	106

demonstrate the reliability of the current method for bioanalyses.

3.2.5 Stability

Stock solutions of clopidogrel and the AM in acetonitrile were stable for at least three weeks at 4°C and for three months at –80°C. More than 92.6% of the initial amount of clopidogrel and the AM were recovered from samples spiked with stock solutions stored under those respective conditions.

In human plasma, no significant degradation (within ±15% deviation between the predicted and nominal concentrations) of clopidogrel and the AM occurred under the following conditions: short-term storage for 2 h at room temperature or in an ice bath, long-term storage for three months at –80°C, three freeze–thaw cycles, and post-treatment storage for 8 h at room temperature and 24 h at 4°C.

3.3 Clinical application

The LC–MS/MS method described herein was successfully applied to a pharmacokinetic study on clopidogrel. The mean plasma concentration–time profiles for clopidogrel and the AM in six healthy male Korean volunteers after oral administration of two 75-mg clopidogrel tablets are shown in Fig. 3. Relevant pharmacokinetic parameters are summarized in Table 3. The sensitivity and specificity of the method were sufficient for characterizing the pharmacokinetics of clopidogrel and the AM. The QC samples ranged within 15% of their nominal concentrations, meeting the acceptance criteria of the U.S. FDA for the validation of bioanalytical methods [41]. The mean C_{max} of the AM was 54.1 ± 39.3 ng/mL occurring at

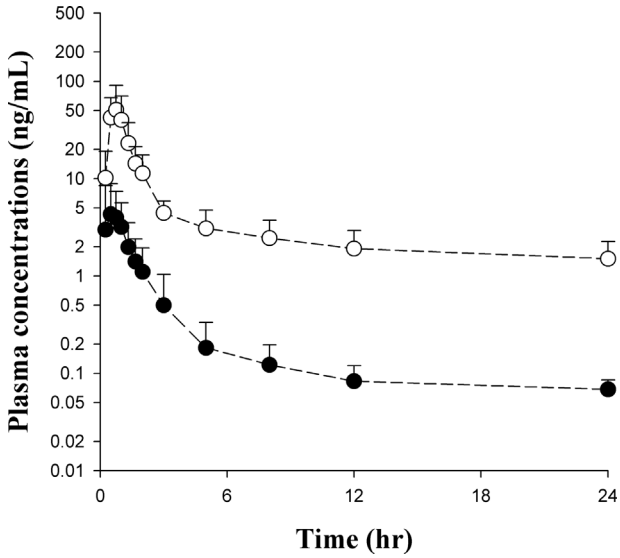

Figure 3. Mean plasma concentration–time profiles of clopidogrel (●) and its active metabolite (○) after single oral administration of clopidogrel 150 mg in six Korean healthy male volunteers. Vertical bars represent SD.

T_{max} (0.5 h; range, 0.5–1 h). The terminal half-life and AUC_{0-t} values of the AM were 21.9 ± 6.38 h and 103 ± 55.8 ng h/mL, respectively. The mean C_{max} of clopidogrel was 4.95 ± 4.51 ng/mL at T_{max} (0.693 h; range, 0.25–1 h), and the AUC_{0-t} value was 6.54 ± 7.17 ng h/mL. The plasma concentrations of the AM were significantly higher than those of clopidogrel (Fig. 3). We observed a slight increase in AUC_{0-t} and C_{max} of the AM compared with previously published data [21, 35, 38]. Although the reasons for these discrepancies are not clear,

Table 3. Pharmacokinetic parameters of clopidogrel and its AM after single oral administration of clopidogrel 150 mg (75 mg, two tablets) in six healthy Korean male volunteers.

Parameters[a)]	Clopidogrel	AM
$AUC_{0-\infty}$ (ng h/mL)	7.30 ± 7.51	150 ± 74.0
AUC_{0-t} (ng h/mL)	6.54 ± 7.17	103 ± 55.8
Terminal half-life (h)	8.29 ± 6.46	21.9 ± 6.38
C_{max} (ng/mL)	4.95 ± 4.51	54.1 ± 39.3
T_{max} (h)[b)]	0.693 (0.25–1)	0.5 (0.5–1)

a) Values are mean ± SD.
b) Median (ranges).

they may be due to such factors as the inherent variability of pharmacokinetics, different sampling times (our study was up to 24 h postdose versus previous reports that went up to 10 or 4 h postdose), or differences in populations.

The AUC_{0-t} and C_{max} of clopidogrel were also compared against those of the AM. The AUC_{0-t} and C_{max} of clopidogrel were not correlated ($r = -0.257$, $p = 0.623$ and $r = -0.207$, $p = 0.704$, respectively) with those of the AM. These indicate the necessity for direct quantification of AM and not only clopidogrel in clopidogrel therapy. As described earlier, clopidogrel, which is inactive, has shown significant interindividual variability with regard to drug response. Plasma concentrations of the AM, not clopidogrel, may be critically influenced by variability introduced by genetic polymorphisms CYP2C19, CYP3A5, MDR1, and paroxonase 1 and environmental factors such as drug and food interactions.

4 Concluding remarks

Since clopidogrel AM is highly unstable in biological samples, to date, only a few analytical LC–MS/MS-based methods for determining levels of the AM have been reported. In this study, we optimized, evaluated, and compared the effectiveness of the reducing agents reduced glutathione, DL-dithiothreitol, and tris(2-carboxyethyl)phosphine (TCEP) for the detection of clopidogrel AM in plasma via disulfide bond formation with plasma proteins. A 50 mM solution of TCEP provided greater reducing effects than the others.

The current report details the first validated LC–MS/MS method using TCEP as a reducing agent for the simultaneous quantitation of clopidogrel and its AM in human plasma. The method employs a simple deproteinization procedure without the need for derivatization and requires relatively low volumes of plasma (100 μL) and only 2.0 min per run. Furthermore, the method was applicable to clinical samples that had been stored long-term at –80°C without any steps during blood collection and was successfully applied in a pharmacokinetic study of clopidogrel and its AM.

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