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Short communication

Quantitative determination of clopidogrel active metabolite in human plasma by LC–MS/MS

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ABSTRACT

A quantitative method for the determination of clopidogrel active metabolite (AM) in human plasma was developed and validated using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Clopidogrel AM contains a thiol group, thus requiring stabilization in biological samples. The alkylating reagent 2-bromo-3′-methoxyacetophenone was used to stabilize clopidogrel AM in blood. An analog of the derivatized clopidogrel AM was used as the internal standard (IS). The derivatized samples were subjected to solid-phase extraction with a C2 disk plate and the overall procedure exhibited good reaction (more than 90%) and recovery efficiencies (from 85% to 105%). The derivative of clopidogrel AM (MP-AM) and IS were separated on an ODS column and quantified by tandem mass spectrometry with electrospray ionization. No significant endogenous peaks corresponding to MP-AM or IS were detected in blank human plasma samples, and no significant matrix effect was observed for MP-AM and IS in human plasma samples (from 102% to 121%). The calibration curve ranged from 0.5 to 250 ng/mL with good linearity, and extended by validation of a 50-fold dilution. In the intra- and inter-assay reproducibility tests, the accuracy and precision were within 12% relative error and 6% coefficient of variation, respectively. The derivatized MP-AM was stable in human plasma for 4 months at −80 °C. The validated method was successfully used to analyze clinical samples and determine the pharmacokinetics of clopidogrel AM.

1. Introduction

Clopidogrel bisulfate, a thienopyridine antiplatelet agent, is marketed worldwide as Plavix®/Iscover® (Sanofi-Aventis, Paris, France) and is used in the prevention of myocardial infarction, stroke and death in patients with acute coronary syndromes. The drug is not active *in vitro* and hepatic biotransformation is necessary to produce the platelet antiaggregating effect [1,2]. Clopidogrel is primarily metabolized to an inactive carboxylic acid derivative, generated by hydrolysis, and to the pharmacologically active metabolite (AM) via an inactive 2-oxoclopidogrel by a two-step cytochrome P450 oxidation process, as shown in Fig. 1 [3–5]. The clopidogrel AM has a thiol group that irreversibly inhibits the binding of 2MeS-ADP to $P2Y_{12}$ by covalent binding to a cysteine residue in the receptor through a disulfide bond [6,7].

Because the inactive carboxylic acid metabolite of clopidogrel is the major circulating metabolite in human plasma, and the AM is unstable, the exposure and pharmacokinetic parameters for clopidogrel have been indirectly determined by quantifying the inactive carboxylic acid metabolite [3,8]. Consequently, several HPLC and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for the determination of clopidogrel and its carboxylic acid metabolite have been developed and published [9–15]. On the other hand, no quantitative methods for the clopidogrel AM have been reported. Recently, however, the plasma levels of the AM were reported in order to evaluate the pharmacokinetics of clopidogrel [8,16–18]. In these studies clopidogrel was used as the reference to establish a calibration curve for its AM, thus only approximating the actual concentration of the AM. Furthermore, the need to stabilize clopidogrel AM has not been investigated or evaluated, in spite of the fact that the labile AM degrades rapidly in blood after collection [19]. Hence, the plasma concentrations of clopidogrel AM

Abbreviations: AM, active metabolite; LC–MS/MS, liquid chromatography–tandem mass spectrometry; IS, internal standard; MPB, 2-bromo-3′-methoxyacetophenone (3′-methoxyphenacyl bromide); EDTA, ethylenediaminetetraacetic acid; MP-AM, 3′-methoxyacetophenone derivative of clopidogrel active metabolite; MRM, multiple reaction monitoring; RE, relative error; CV, coefficient of variation; C_{max}, maximum plasma concentration.

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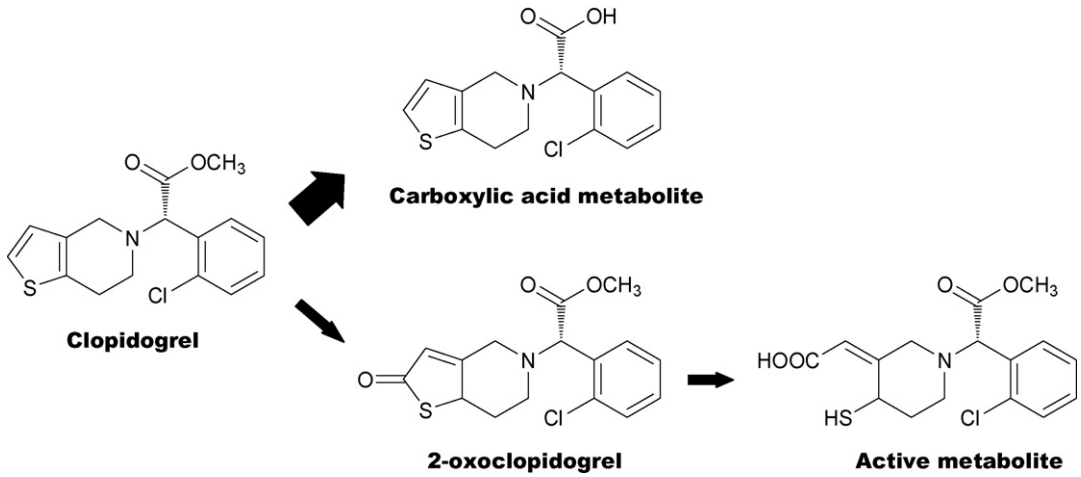


Fig. 1. Metabolic pathways of clopidogrel.

determined by the previous method probably do not accurately represent its concentrations in human plasma.

Thiol-containing compounds can generally be predicted to be unstable in blood or plasma due to the reactivity of that group. Specifically, the thiol group in a molecule forms a disulfide bond either with itself, with endogenous low-molecular-weight compounds or with proteins, e.g. dimer, cysteine conjugate, glutathione conjugate, complex with albumin [20–22]. To prevent these reactions and to stabilize the thiol compound in biological matrices, various alkylating agents, such as *N*-ethylmaleimide, 1-benzyl-2-chloropyridinium bromide, methyl acrylate, 4-bromophenacyl bromide and 2-bromo-3′-methoxyacetophenone (3′-methoxyphenacyl bromide: MPB) have been used [23–28].

In the present study, an authentic standard was used for the determination of the plasma levels of clopidogrel AM. For rapid reaction and easy handling, MPB was selected as an alkylating reagent to block and stabilize the free thiol group of the clopidogrel AM (Fig. 2). An assay method to accurately quantify the clopidogrel AM after a derivatization of the AM with MPB, was developed and validated. This method has been applied to clinical studies to accurately characterize the pharmacokinetic parameters of clopidogrel AM.

2. Experimental

2.1. Materials and reagents

Clopidogrel AM (98.2% purity by HPLC), the MPB derivative of clopidogrel AM (96.4% purity by HPLC, only used as a reference for the derivatization efficiency) and the 4′-bromoacetophenone derivative of their analog used as an internal standard (IS, Fig. 2), were synthesized in Daiichi Sankyo (Tokyo, Japan) and Ube Industries (Ube, Japan). The alkylating agent, MPB, was purchased from Tokyo Chemical Industry (Tokyo). Methanol, acetonitrile, formic acid, ammonium acetate, ammonia water and ammonium chloride, all reagent grade, were purchased from Kanto Chemical (Tokyo). Purified water was produced by a Milli-Q Academic System (Millipore Corporate, Billerica, MA, USA). Blank human ethylenediaminetetraacetic acid (EDTA)-plasma was purchased from Nippon

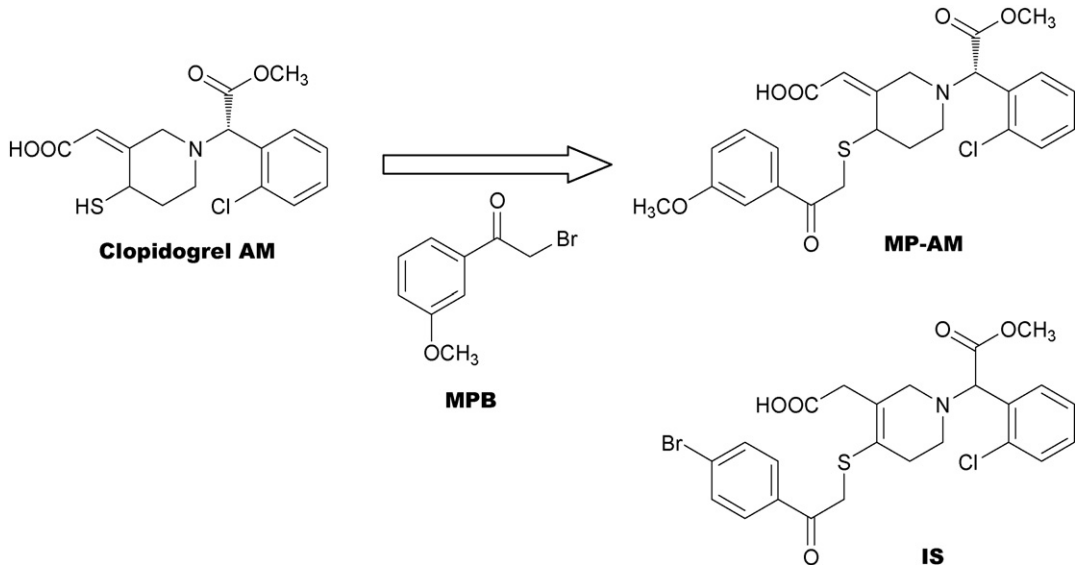


Fig. 2. Derivatization of clopidogrel active metabolite (AM) with 2-bromo-3′-methoxyacetophenone (MPB) and the chemical structures of the derivatized AM of clopidogrel (MP-AM) and its analog internal standard (IS) compound.

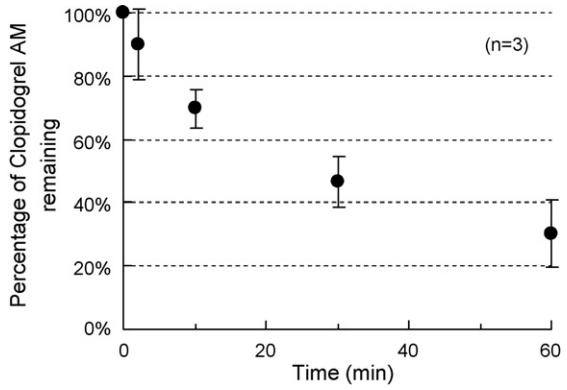


Fig. 3. Stability of clopidogrel active metabolite (AM) in human plasma at 37 °C.

Bio-Supp. Center (Tokyo). Blank rat blood was collected from male Sprague–Dawley rats purchased from Charles River Laboratories Japan (Yokohama, Japan).

2.2. Standard solutions

Clopidogrel AM was derivatized with MPB to form the 3′-methoxyacetophenone derivative of clopidogrel AM (MP-AM). Into a 25 mL volumetric flask, about 1.25 mg of clopidogrel AM was weighed and dissolved with 0.5 mL of acetonitrile. Then, 4 mL of ammonium chloride buffer (0.1 M ammonium chloride aqueous solution/0.1 M ammonia water, 4:1, v/v, pH 9) and 0.5 mL of 100 mM MPB in acetonitrile were added and mixed. The mixture was left standing at room temperature for 10 min to complete the reaction. Finally, acetonitrile was added into the flask up to 25 mL to prepare a standard stock solution of MP-AM at the concentration of 50 μg/mL. The IS stock solution was prepared in acetonitrile at the concentration of 100 μg/mL. These stock solutions were stored at −20 °C. The stock solution of the MP-AM was diluted with acetonitrile to obtain working solutions for the calibration curve and QC samples. The IS was diluted with 50% methanol aqueous solution to make an IS working solution of 100 ng/mL.

2.3. Sample preparation

To prepare plasma samples for the calibration curve and QC, each working solution of MP-AM was diluted with a 20-fold volume of blank human plasma. To 400 μL of the plasma sample, 550 μL of 1% formic acid and 40 μL of the IS working solution were added and vortexed. The mixture was applied onto an Empore Disk Plate (C2 SD, 3M Company, St. Paul, MN, USA), which had been preconditioned by 300 μL of methanol, followed by 300 μL of purified water. After the disk plate had been washed with 500 μL of 1% formic acid and 500 μL of 50 mM aqueous ammonium acetate solution, the analytes were eluted with 150 μL of methanol, followed by 100 μL of 50 mM aqueous ammonium acetate solution into a 96-well collection plate. The eluate was mixed and analyzed by LC–MS/MS as described below.

2.4. Instruments and conditions

The LC–MS/MS system consisted of an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA, USA) with an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland), coupled

Table 1
Derivatization reaction efficiency of clopidogrel active metabolite in rat blood (a), matrix effect (b) and extraction efficiency (c) of the derivatized active metabolite of clopidogrel (MP-AM) and the internal standard (IS) from human plasma

Blood concentration (ng/mL)	1	2	3	Mean	S.D.
(a) Reaction efficiency (%)[a]					
50					
MP-AM	90.3	93.5	92.4	92.1	1.6

Plasma concentration (ng/mL)	1	2	3	Mean	S.D.
(b) Matrix effect (%)[b]					
1					
MP-AM	129.7	121.7	109.7	120.4	10.1
IS	116.5	119.8	107.4	114.6	6.4
10					
MP-AM	120.7	105.9	109.2	111.9	7.8
IS	110.0	103.3	102.1	105.1	4.3
250					
MP-AM	105.2	97.2	106.1	102.8	4.9
IS	109.2	105.7	115.8	110.2	5.1

Plasma concentration (ng/mL)	1	2	3	4	5	Mean	S.D.
(c) Extraction efficiency (%)[c]							
1							
MP-AM	96.3	94.1	74.4	101.3	96.3	92.5	10.4
IS	117.2	119.9	72.9	104.4	106.1	104.1	18.7
10							
MP-AM	84.4	86.8	69.4	91.7	93.3	85.1	9.5
IS	101.9	104.2	66.8	97.0	100.8	94.1	15.5
250							
MP-AM	101.7	102.4	68.7	98.1	97.5	93.7	14.1
IS	118.4	121.7	66.9	103.3	102.9	102.6	21.7

[a] Reaction efficiency (%) = [sample reacted in blood]/[reference sample spiked with reacted MP-AM] × 100.
[b] Matrix effect (%) = [sample spiked in blank matrix extract]/[reference solution without matrix] × 100.
[c] Extraction efficiency (%) = [sample of calibration curve]/[sample spiked in blank matrix extract] × 100.

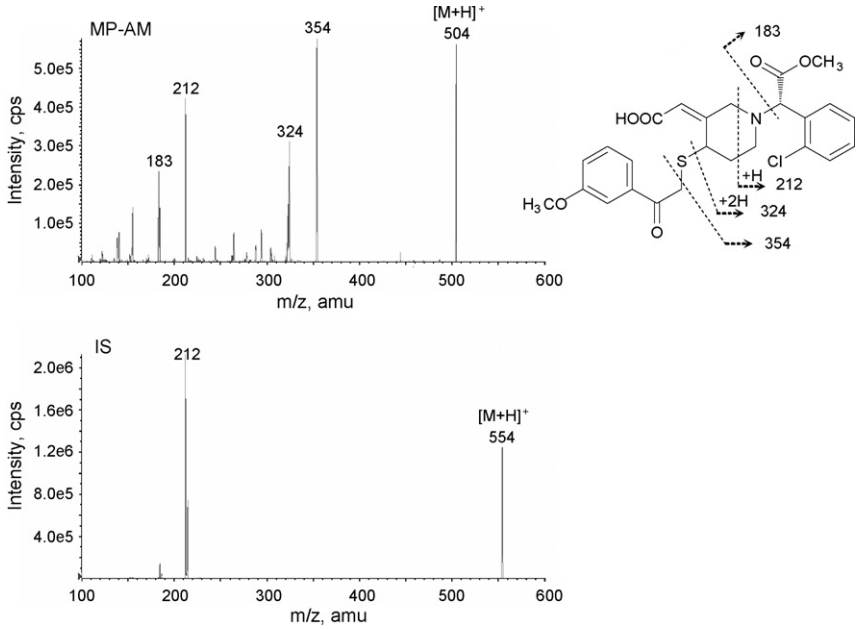


Fig. 4. Product ion spectra of the derivatized active metabolite of clopidogrel (MP-AM) and of the internal standard (IS).

to a triple quadrupole tandem mass spectrometer API4000 (Applied Biosystems/MDS SCIEX; Foster City, CA, USA/Concord, Ont., Canada). Data acquisition and processing were performed using Analyst software (version 1.1) from Applied Biosystems. The calibration curve was generated by plotting the nominal concentrations of clopidogrel AM against the peak area ratio of MP-AM to IS, and by the least-squares regression method with a weighting factor of $1/x^2$.

Chromatographic separation was achieved on an Inertsil ODS-3 column (particle size 5 μm, 2.1 mm × 50 mm, GL Sciences, Tokyo) at a column temperature of 40 °C using a mobile phase of methanol and 1% formic acid (70:30, v/v) at a flow rate of 0.2 mL/min. The sample injection volume was 10 μL and the analytical run time was 6 min. The eluent from the HPLC column was introduced directly to the API4000 using the electrospray ionization interface in the positive ion mode. The ion spray voltage and turbo heater temperature were set at 5500 V and 700 °C, respectively. The transitions m/z 504 → 354 for MP-AM, m/z 554 → 212 for IS and m/z 356 → 155 for clopidogrel AM itself were monitored using the multiple reaction monitoring (MRM) mode. The declustering potential and collision energy were 60 and 29 V, respectively, for both MP-AM and IS. Those for clopidogrel AM itself were 90 and 37 V, respectively.

2.5. Validation

To validate the analytical method, tests of the specificity, calibration curve, intra- and inter-assay reproducibilities, sample dilution, extraction recovery, matrix effect, long-term stability in plasma sample and stock solution stability were conducted in compliance with the Food and Drug Administration guidelines [29].

The specificity was verified using six individual blank human plasma samples. Whether any endogenous peaks were interfering with the peak of MP-AM or IS in the MRM chromatograms was investigated using blank plasma samples without spiking the MP-AM and IS working solutions. A calibration curve was constructed with plasma samples of 0, 0.5, 1, 2, 10, 50, 125 and 250 ng of clopidogrel AM/mL. The correlation coefficient of the calibration curve and the relative error (RE, %) for each concentration were determined.

The intra- and inter-assay reproducibilities were evaluated using five replicates of QC samples at the concentrations of 0.5, 1, 10, 200 and 500 ng of clopidogrel AM/mL. The clopidogrel AM QC sample of 500 ng/mL was diluted 50-fold with blank human plasma and then analyzed. A long-term stability test for the derivatized clopidogrel AM in human plasma samples was performed with the QC samples of 1, 200 and 500 ng/mL under storage at −80 °C. The concentrations of these QC samples were determined from a cali-

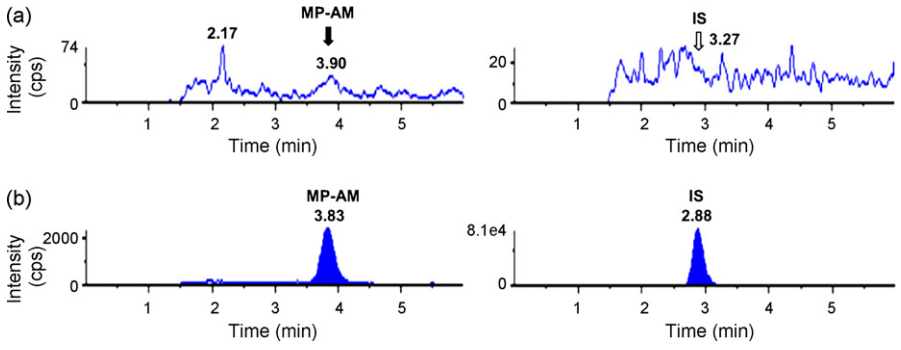


Fig. 5. Chromatograms of the derivatized active metabolite of clopidogrel (MP-AM) and of the internal standard (IS) in typical blank human plasma sample (a) and in typical lower LoQ plasma sample at 0.5 ng/mL (b).

Table 2
Intra-, inter-assay and dilution reproducibilities of the derivatized active metabolite of clopidogrel in human plasma

Plasma concentration (ng/mL)	Intra-assay ($n = 5$)		Inter-assay ($n = 5$)	
	RE (%)	CV (%)	RE (%)	CV (%)
0.5	−1.4	3.7	−5.2	3.8
1	−11.9	3.6	−7.9	1.7
10	−8.3	4.5	−3.1	0.4
200	−5.5	3.7	−1.0	1.0
500[a]	−10.2	6.0	−3.0	1.6

[a] 50-fold dilution reproducibility.

bration curve. In the reproducibility and stability tests, the accuracy expressed as RE (%) and the precision expressed as coefficient of variation (CV, %) were calculated from each concentration of the replicated samples.

The extraction efficiency was evaluated using plasma samples of the calibration curve at concentrations of 1, 10 and 250 ng/mL. The reference samples of extraction efficiency were prepared by adding MP-AM and IS working solutions to blank plasma extracts. To investigate the matrix effect, the reference samples of extraction efficiency were compared to reference solutions without matrix. The stock solution stabilities of MP-AM and IS were demonstrated following storage at −20 °C.

2.6. Application to clinical study

This method was applied to the analysis of clinical samples obtained after administration of a single oral dose of clopidogrel 300 mg to 66 healthy male and female subjects between 18 and 65 years of age [30]. The subjects signed an informed consent and the study protocol was approved by the local ethical review board. Blood samples (3–4 mL) were collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12 and 24 h after the dosing in EDTA tubes. To each of the blood samples, 25 μL of 500 mM MPB in acetonitrile was added immediately after collection. The blood samples were gently mixed and centrifuged within 30 min to separate the plasma. These plasma samples were stored at −80 °C until assay.

3. Results and discussion

3.1. Stability and derivatization of clopidogrel AM

The stability of clopidogrel AM in human plasma was initially investigated and the AM was found to rapidly degrade to less than 80% of the initial concentration within 10 min after spiking the AM solution into human plasma (Fig. 3). The clopidogrel AM was derivatized with MPB in ammonium chloride buffer for 10 min at room temperature and the unreacted AM was monitored (retention time: 2.1 min). Almost no peak of the unreacted clopidogrel AM and a peak of MP-AM nearly equal to the authentic standard were observed in the MRM chromatogram of the reacted MP-AM stock solution (data not shown). This indicated that the clopidogrel AM was fully derivatized to the MP-AM in the buffer. In addition, the reaction efficiency of clopidogrel AM in rat blood (as a substitute for human blood) was evaluated by determining the ratio of the sample reacted in blood to the reference sample spiked with the MP-AM working solution. The reaction efficiencies in the blood were more than 90% at the concentration of 50 ng/mL (Table 1(a)).

3.2. LC–MS/MS analysis

The MP-AM and analog IS were retained on the column with good peak shape at the retention times of 3.8 and 2.9 min, respectively. These analytes whose product ion spectra are shown in Fig. 4 were detected in the MRM mode with high sensitivity. By contrast, no significant endogenous peaks interfering with the peak of MP-AM or IS were observed in the MRM chromatograms of six blank human plasma samples. A typical MRM chromatogram of blank human plasma sample is shown in Fig. 5(a). The calibration curve for clopidogrel AM was established in the range of 0.5–250 ng/mL and exhibited good linearity with the correlation coefficient of 0.9998.

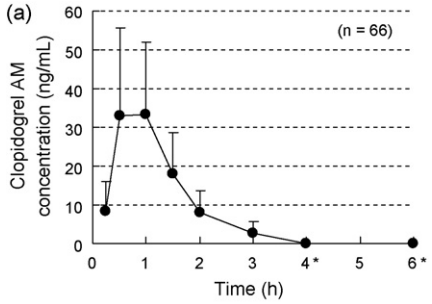
3.3. Validation parameters

The intra- and inter-assay reproducibilities of the assay were investigated in terms of accuracy and precision. As shown in Table 2, the RE (%) and CV (%) values at the concentrations of 0.5, 1, 10, 200 ng/mL were within 12% and 5%, respectively. The lower LoQ was determined to be 0.5 ng/mL and its typical MRM chromatogram is shown in Fig. 5(b). Similarly, the RE (%) and CV (%) values of 50-fold dilution samples at the concentration of 500 ng/mL were within 11% and 6% (Table 2), so that the 50-fold dilution with blank human plasma was considered to have no influence on the assay results. When the plasma samples, spiked with the MP-AM at the concentrations of 1, 200 and 500 ng/mL, were stored at −80 °C for 1, 2 and 4 months, the RE (%) of MP-AM from 0 month were within 14%, as shown in Table 3. The storage of human plasma samples for 4 months at −80 °C did not affect the assay results of clopidogrel AM.

The mean values of matrix effect for MP-AM and IS in human plasma samples at the concentrations of 1, 10, 250 ng/mL were 102% to 121% and 105% to 115%, respectively (Table 1(b)). The mean

Table 3
Long-term stability of the derivatized active metabolite of clopidogrel in human plasma at −80 °C

Plasma concentration (ng/mL)	Period (month)	Observed concentration (ng/mL)		RE (%)	CV (%)	RE (%) from 0 month
		Mean	S.D.			
1	0	0.924	0.045	−7.6	4.9	–
	1	1.04	0.04	4.0	3.8	12.6
	2	1.05	0.05	5.0	4.8	13.6
	4	1.03	0.05	3.0	4.9	11.5
200	0	192	4	−4.0	2.1	–
	1	193	11	−3.5	5.7	0.5
	2	218	3	9.0	1.4	13.5
	4	207	6	3.5	2.9	7.8
500	0	489	11	−2.2	2.2	–
	1	534	6	6.8	1.1	9.2
	2	522	22	4.4	4.2	6.7
	4	504	11	0.8	2.2	3.1



(b)

Pharmacokinetic parameters	Geometric mean (Geometric CV (%))
$AUC_{0-\infty}$ (ng·h/mL)	43.8 (45)
C_{max} (ng/mL)	35.9 (54)
T_{max} (h)	1.00 **
$T_{1/2}$ (h)	0.520 (50)

(n = 66)

* below the lower LOQ

** median value

Fig. 6. Mean plasma concentrations (a) and pharmacokinetic parameter estimates (b) of clopidogrel active metabolite (AM) in humans after a single oral dose of clopidogrel 300 mg.

extraction efficiencies of MP-AM and IS from human plasma samples at the three concentrations were 85% to 94% and 94% to 105%, respectively (Table 1(c)). The matrix effect and recovery for MP-AM and IS were consistent when examined repeatedly at the same concentration or at varying concentrations. The stock solutions of MP-AM and IS in acetonitrile were confirmed to be stable for 1 month at −20 °C (data not shown).

3.4. Pharmacokinetic profile of clopidogrel AM in humans

The validated method was successfully applied to determine the plasma concentrations of the clopidogrel AM after a single 300 mg clopidogrel oral dose. The principal pharmacokinetic parameters of the clopidogrel AM are shown in Fig. 6. In the previously published reports, the maximum plasma concentration (C_{max}) of the AM was estimated to be around 7 ng/mL after administration of a 600 mg clopidogrel dose [8,16–18]. Taking the dose difference into account, the C_{max} of 35.9 ng/mL determined here was approximately 10 times higher than those previously reported when quantified without an appropriate reference standard and stabilization process. These data clearly demonstrate the importance of stabilizing the clopidogrel AM upon sample collection and also of the use of an authentic reference standard for the quantitation of the AM.

4. Conclusions

This is the first report that describes a validated and robust method for the determination of clopidogrel AM in human plasma samples. Clopidogrel AM was found to be unstable in plasma and must be stabilized immediately upon blood collection. The alkylating reagent MPB used in this assay proved to be a suitable stabilizing reagent for the thiol-containing AM. Using this method, the LC–MS/MS assay for the determination of clopidogrel AM was fully validated and has been used to analyze many samples from various clinical trials [30,31]. The results obtained when using the present method in the analysis of clopidogrel AM in human plasma demonstrate that the plasma concentrations previously reported [8,16–18] were clearly underestimated by the use of a calibration curve established with clopidogrel itself rather than its active metabolite as a standard, and by not stabilizing clopidogrel AM upon sample collection. With stabilization of the active metabolite in blood, and the use of an authentic standard, the plasma concentrations of clopidogrel AM were accurately measured and used for the evaluation of the pharmacokinetic parameters of clopidogrel AM in humans.

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