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Determination of clopidogrel metabolite (SR26334) in human plasma by LC–MS

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Abstract

A new, sensitive, specific and reproducible method for determination of clopidogrel metabolite (SR26334) in human plasma has been developed. After liquid–liquid extraction on Chem Elut cartridges with dichloromethane, samples were quantified using reversed-phase high performance liquid chromatography with mass detection. The determination was performed on a Luna C18, 3 μm (75 mm × 4.6 mm i.d.) column with an acetonitrile-water-formic acid mixture (60:40:0.1, v/v/v) as a mobile phase. The flow rate was set at 0.2 mL/min. Repaglinide was chosen as an internal standard and the time of analysis was 12 min. For SR26334 the limits of detection and quantification were 7.5 ng/mL and 20 ng/mL, respectively, and the calibration curve was linear up to 3000 ng/mL. The extraction recovery of SR26334 from plasma was within the range of 85–90%. The method has been successfully used to study clopidogrel metabolite pharmacokinetics in healthy volunteers.

Keywords: Clopidogrel; Human plasma; Liquid chromatography; Liquid–liquid extraction on Chem Elut cartridges; Validation

1. Introduction

Clopidogrel, methyl (+)-(*S*)-2-(2-chlorophenyl)2-{6,7-dihydrothiene[3,2-*c*]pyridine-5(4*H*)}-acetate (SR25990C, Fig. 1A), inhibits platelet aggregation by selective preventing of the binding of adenosine diphosphate (ADP) to its platelet receptor [1]. The drug reduces thrombotic events in a broad range of patients (e.g. recent myocardial infarction, recent stroke, established peripheral arterial disease, or acute coronary syndrome). Clopidogrel is an inactive prodrug, and a biotransformation by the liver is necessary to induce expression of its antiaggregating activity [2]. It is rapidly absorbed and undergoes extensive metabolism after oral administration and its plasma concentration goes down very fast [3]. Moreover, the compound belongs to a family of eight stereoisomers with chemical structure of 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene}acetic acid, that is believed to be the active metabolite of clopidogrel and is highly labile [4]. The carboxylic acid derivative of clopidogrel (SR26334, Fig. 1B), which is its inactive metabolite, is the major circulating compound and information on the absorption and elimination of clopidogrel is derived from the pharmacokinetics of SR26334 [3]. It is formed by hydrolysis of the ester function by carboxylesterase [5].

In general, two methods for determination of SR26334 in plasma have been reported in literature: HPLC with UV detection and GC–MS. The former was used for multiple-dose pharmacokinetics study [3], the latter—for single-dose pharmacokinetics of SR26334 [3,6]. Recently, several LC–MS–MS methods for the study of pharmacokinetics of clopidogrel and SR26334 have been published [7–9].

The purpose of the study described below was to develop and validate a specific, simple and reproducible method for determination of clopidogrel metabolite (SR26334) in human plasma using a liquid–liquid extraction procedure on Chem Elut cartridges followed by HPLC analysis on reversed phase with single mass detection. In the study, repaglinide (Fig. 2) was used as the internal standard. The method was subsequently applied to bioavailability studies.

2. Experimental

2.1. Reagents and chemicals

The metabolite of clopidogrel was supplied by Adamed Ltd. (Pieńków, Poland). Repaglinide (internal standard, I.S.)

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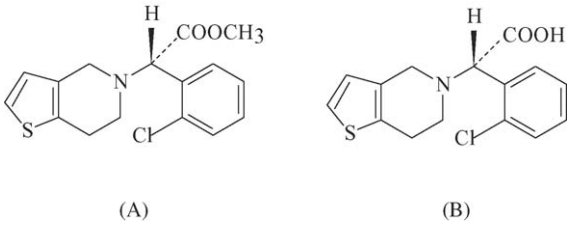

Fig. 1. Chemical structures of clopidogrel (A); and its inactive metabolite SR26334 (B).

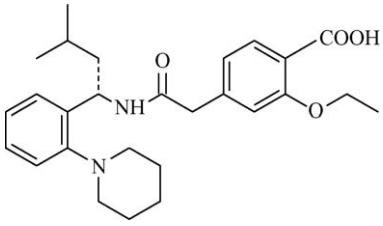

Fig. 2. Chemical structure of repaglinide (the internal standard).

was synthesized in Pharmaceutical Research Institute (Warsaw, Poland). HPLC-grade acetonitrile and dichloromethane were purchased from LabScan (Dublin, Ireland), acetic acid and HPLC-grade ammonium acetate were from T.J. Baker (Deventer, Netherlands) and formic acid was from Riedel-de Haën (Seelze, Germany). Distilled water was purified by a Millipore System Milli Q (Molsheim, France). Other reagents were of analytical grade.

2.2. Instruments and conditions

HPLC analyses were performed using a system consisting of a pump (LC-10AD*VP*), an autosampler with a controller (SIL-HTA), an oven (CTO-10A) and a quadruple mass spectrometer with an electrospray ion source (LCMS-2010) (Shimadzu Europe, Duisburg, Germany). Data acquisition and processing software LCMS solution version 2.02 Su 1 was from Shimadzu.

A Luna 3 μ C18 column (75 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) preceded by C18 guard column (4 mm × 3 mm i.d., Phenomenex, Torrance, CA, USA) was used for all chromatographic separations. The mobile phase was an acetonitrile-water-formic acid mixture (60:40:0.1, v/v/v). The flow rate was set at 0.2 mL/min, the injection volume at 10 μL and the column temperature was maintained at 35 °C. Time of an analysis was 12 min.

The electrospray ionization (ESI) was carried out using nitrogen to assist nebulisation (the flow rate was set at 4.5 L/min). Selected ion monitoring (SIM) with positive ion mode, capillary voltage at 4.5 kV and temperature of Curved Desolvation Line (CDL in which the solvent contained in electrically charged particles generated by ESI is removed by heating) and block set at 250 °C and 200 °C, respectively, were used. Target ions were monitored at *m/z* 308.00 for SR26334 $[M+H]^+$ and at *m/z* 453.55 for I.S. $[M+H]^+$. The product ion mass spectra of these protonated molecular ions are shown on Figs. 3 and 4, respectively.

2.3. Preparation of standard solutions, calibration standards and quality control samples (QC)

Stock solutions of clopidogrel metabolite (CLM) and repaglinide (I.S.) were prepared at 1 mg/mL in methanol and stored at −20 °C.

The working solution of I.S. (16 μg/mL) was obtained by diluting the stock solution with water and stored in a refrigerator.

The highest-concentration calibration standard of CLM (3000 ng/mL) was prepared by spiking the appropriate amount of the stock solution in the drug-free human plasma. The other calibration standards (20, 50, 100, 250, 1000, 1500, 2500 ng/mL) were prepared by diluting the highest CLM concentration solution (3000 ng/mL) with the drug-free human plasma.

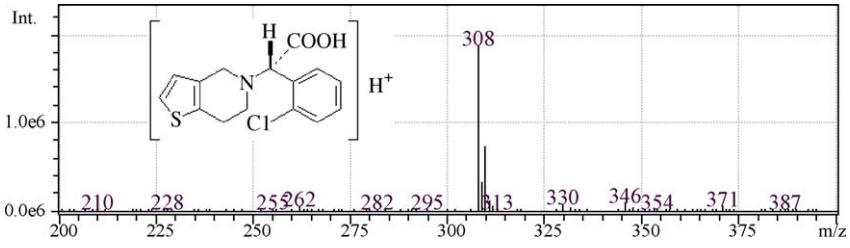

Fig. 3. Positive production mass spectra of the protonated molecular ions $[M+H]^+$ of clopidogrel metabolite.

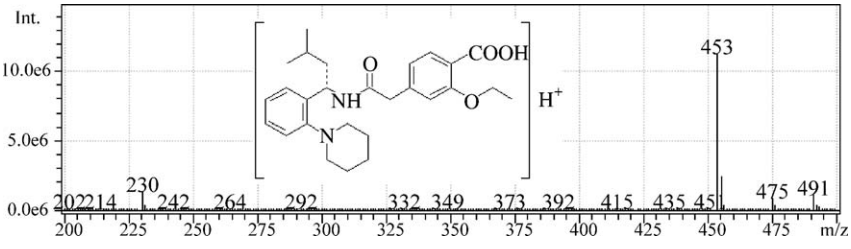

Fig. 4. Positive production mass spectra of the protonated molecular ions $[M+H]^+$ of repaglinide.

Quality control samples (of low, medium and high concentration) at 50, 1000 and 2500 ng/mL were prepared in the same way as the calibration standards. All standard solutions in plasma were stored at −20 °C.

2.4. Plasma samples

Venous blood samples were withdrawn into heparinised tubes, and then centrifuged at 1000 × *g* for 10 min at 4 °C. The plasma obtained in that way was stored at −20 °C until analysis.

2.5. Extraction procedure

To 250 μL of plasma in a glass tube 50 μL of I.S. (repaglinide at 16 μg/mL in water), 250 μL of water and 500 μL of 0.1 M ammonium acetate (pH 4.0) were added and vortex-mixed (IKA, IKA Labortechnik, Staufen, Germany) for 10 s. The sample was loaded on a dry 1 mL Chem Elut extraction cartridge (Varian Sample Preparation Products, Harbor City, CA, USA). After 10 min, liquid–liquid extraction was performed with 4 mL of dichloromethane without vacuum. The extract was evaporated to dryness at 40 °C under nitrogen stream in a Multivap evaporator (Organomation Associates Inc., Berli, MA, USA). The extraction was repeated two more times. Each portion of the extract was collected to the same tube. The dry residue was reconstituted in 200 μL of the mobile phase, transferred to an autosampler vial and injected onto the chromatographic column (10 μL).

2.6. Assay validation

2.6.1. Limits of detection and quantification

The limit of detection (LOD) – defined as a peak height equal to the value of 3 times of a baseline noise [10] – was determined as 7.5 ng/mL. The lower limit of quantification (LLOQ) is defined as the lowest concentration of a compound that can be determined with an acceptable accuracy and precision of 20% [11] with a particular method. In our method, the LLOQ value was established as 20 ng/mL. The precision, characterized by the relative standard deviation ($n = 6$), for LLOQ was determined as 7.7% and for upper limit of quantification ULOQ (3000 ng/mL) it was determined as 1.6%.

2.6.2. Linearity

Calibration standard solutions of eight CLM concentrations in the range of 20–3000 ng/mL were extracted according to the procedure described in Section 2.5. A calibration curve was constructed by plotting the height ratios of CLM to the I.S. against CLM concentrations in the plasma. It was calculated by the least-squares linear regression method: $y = a + bx$, where y is a peak height ratio of drug and internal standard, a and b are an intercept and a slope, respectively, and x is an analyte concentration.

2.6.3. Precision and accuracy

The precision of an analytical method is a measure of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogeneous sample. It is determined by assaying a number of aliquots of a homogenous sample sufficient for calculation of statistically valid estimates of a standard deviation or a relative standard deviation [11].

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value.

The precision and accuracy of the assay were determined from the low (50 ng/mL), medium (1000 ng/mL) and high (2500 ng/mL) QC plasma samples. The within-day assay variations were determined by repeated analysis of each QC sample on one day (in six replicates). The between-day assay variations were determined by analyzing QC samples in duplicates during three separate, successive days. The concentration of each QC sample was calculated using a calibration curve prepared each day.

2.6.4. Extraction recovery

The recovery of an analyte in an assay is the detector response corresponding to an amount of the analyte added to and extracted from the biological matrix, compared with the detector response obtained from the pure authentic standard that represents 100% recovery [11].

In the described method, the absolute recovery was calculated by comparing the CLM peak heights obtained from extracts of spiked plasma samples and peak heights obtained from direct injection of known amounts of the CLM standard solution. The absolute recovery of CLM through the extraction procedures was determined by analysis of each QC sample (in six replicates) at low, medium and high concentrations. Additionally, the absolute recovery of the internal standard after the extraction was determined.

The matrix effect was evaluated by comparing the peak height ratio of neat CLM and I.S. solutions and post-extraction control plasma spiked with standards at three QC levels (50, 1000 and 2500 ng/mL) in five different lots of plasma.

2.6.5. Stability

Stability of the CLM was evaluated after sample extraction process (in an autosampler), three freeze-thaw cycles and long-term freezing at −20 °C (1 month).

Autosampler stability of the CLM and I.S. in plasma samples was performed on three sets of a low (50 ng/mL), medium (1000 ng/mL) and high (2500 ng/mL) concentration of CLM and 3200 ng/mL of I.S., each of 6 replicates. The samples were analyzed immediately after preparation and after 18 h of standing in the autosampler at room temperature (about 22 °C).

Freeze and thaw stability of CLM in plasma samples was determined after three cycles. Six aliquots of the low (50 ng/mL), medium (1000 ng/mL) and high (2500 ng/mL) concentrations were stored at −20 °C for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h. The cycle of thawing and freezing was repeated two more times, and then samples were analysed. The concentration was calculated from the daily calibration curve.

Stability of CLM in plasma samples during storage in a freezer at −20 °C was determined using two sets of low (50 ng/mL) and high (2500 ng/mL) concentration of CLM, each

Table 1
The extraction recovery of CLM and I.S. from human plasma ($n = 6$)

Nominal concentration (ng/mL)		Peak height (mean ± S.D.)		Extraction recovery (%) (mean ± S.D.)
		Without extraction	After extraction	
	50	5909 ± 139	5377 ± 387	90 ± 5
CLM	1000	126674 ± 5650	113412 ± 9839	90 ± 10
	2500	308516 ± 9266	260994 ± 24995	85 ± 9
I.S.	3200	342048 ± 12355	313208 ± 25446	92 ± 6

n, number of replicates.

set including 6 replicates. Each set was analyzed at the beginning and at the end of the study (after 1 month).

3. Results

3.1. Method validation

3.1.1. Specificity

Drug-free human plasma from six different lots was tested for endogenous interference. In Fig. 5 representative chromatograms of drug-free human plasma, spiked plasma and plasma sample from a volunteer who received clopidogrel as a single 75 mg tablet are shown. The extraction procedure and chromatographic conditions make it possible to separate both compounds. The chromatogram of drug-free human plasma shows that any interfering endogenous substances did not extract from the plasma. The retention times for CLM and I.S. during the validation were 4.2 ± 0.1 min ($n = 20$) and 5.9 ± 0.2 min ($n = 10$), respectively.

The carry-over test was performed by injection of mobile phase on the column following a high concentration of CLM and I.S. There were no peaks in the standard places on the chromatogram.

3.1.2. Extraction recovery

Chem Elut 1 mL cartridges were chosen for liquid–liquid extraction of CLM and I.S. from plasma because of their very useful features. They protect from emulsion forming, the extraction is carried out with gravity only, there is no drying step after the extraction and an adsorbed sample is stable and does not need to be extracted immediately.

The initial solvent screening for liquid–liquid extraction of clopidogrel metabolite (CLM) and repaglinide (I.S.) on Chem Elut 1 mL cartridges included: tert-butyl methyl ether, ethyl acetate, ethyl ether and dichloromethane without addition and after adding a small amount of ammonium acetate buffer or acetic acid solutions to drug-free human plasma spiked with the standards. Eventually, it was found that after buffering plasma solution with ammonium acetate (pH 4.0, 0.1 M), extraction with dichloromethane (12 mL) gave sufficient recovery of both CLM and I.S. on Chem Elut extraction cartridges. Plasma buffering weakens drug-plasma protein bonds and facilitates-or even makes it possible-to extract the drug from water environment to the organic solvent. For better recovery of CLM and I.S., the 12 mL volume of the solvent was divided into three 4 mL separate portions. Each of them was collected to the same tube (to minimize of a CLM loss) and evaporated immediately from the tube upon the partial extraction. The extraction yields of CLM and I.S. are shown in Table 1. The recovery of clopidogrel metabolite (CLM) for three QC standard concentrations (50, 1000 and 2500 ng/mL) was 85–90% ($n = 18$) and for I.S. (3200 ng/mL) 92% ($n = 6$).

The peak height ratios of CLM and I.S. after spiking evaporated plasma samples at three QC levels (50, 1000 and 2500 ng/mL) compared to neat standard solutions ranged 98–106%. The results were obtained while testing 5 different lots of drug-free human plasma and indicate that the evaluated method is free from the matrix effect.

3.1.3. Calibration curve

The standard curve for CLM was linear within the range of 20–3000 ng/mL. A typical calibration curve for CLM had a slope of 0.000308, an intercept of 0.001727 and $r = 0.9999$ ($n = 6$). For calculation of lower CLM concentration, the range 20–1500 ng/mL was used. A typical calibration curve for lower range of CLM concentration had a slope of 0.000308, an intercept of 0.001900 and $r = 0.9996$ ($n = 6$). A calibration curve was prepared for each individual batch during validation procedure and examination of plasma samples from volunteers.

3.1.4. Precision and accuracy

The results of the precision and accuracy determination of the method within-day and between-days are shown in Table 2. In the case of the within-day assay, the accuracy for three investigated QC concentration was in the range of 95–106%. The precision expressed as relative standard deviation for each QC concentration was 3.4–5.0%. In the case of the between-day assay the accuracy for three investigated QC concentrations was in the range of 99–108%. The precision expressed as relative standard deviation for each QC concentration was in the range of 2.4–5.6%.

3.1.5. Stability

The peak height comparison of immediately analyzed and stored samples showed percent relative error in the range of −8.0–10.3% for CLM and 1.8% for I.S. The results in Table 3 indicate that the processed samples are stable at room temperature for 18 h.

Percent relative errors of QC concentration calculated after three freeze and thaw cycles amounted to 2.5–8.0%. The results

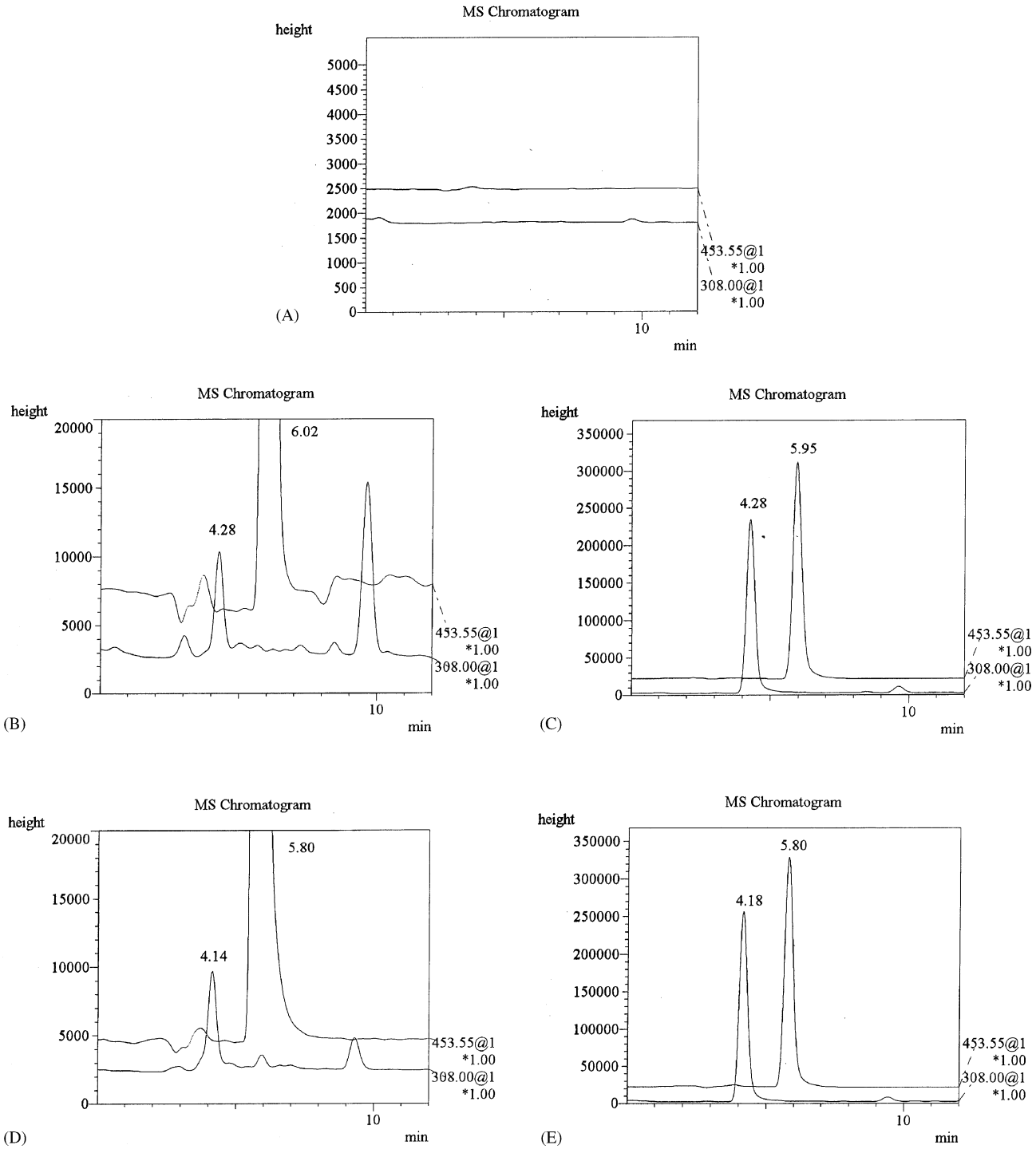

Fig. 5. Chromatograms of drug-free human plasma (A); drug-free human plasma spiked with CLM (50 ng/mL) and I.S. (B); drug-free human plasma spiked with CLM (2500 ng/mL) and I.S. (C); plasma sample from a volunteer who received clopidogrel as a single 75 mg tablet at 24 h (D); and at 0.75 h (E) after the drug administration. Chromatographic conditions: column, Luna C18 (75 mm × 4.6 mm i.d.) 3 μm (Phenomenex); guard column, C18 (4 mm × 3 mm i.d.) (Phenomenex); mobile phase, acetonitrile-water-formic acid (60:40:0.1, v/v/v); flow rate, 0.2 mL/min; temperature, 35 °C; injection volume, 10 μL; *m/z* 308.00 and 453.55 for CLM and I.S., respectively.

summarized in Table 4 confirm CLM stability in plasma after three freeze and thaw cycles.

The results obtained during the long-term stability study (Table 5) were within the range of −3.8 to 5.5%. The data indicate that 1-month storage in a freezer at −20 °C had no effect on CLM determination.

3.2. Application

The applicability of the assay procedure is illustrated in Fig. 6. It shows an average plasma concentration–time curve of clopidogrel metabolite (SR26334) after administration of a single 75 mg Plavix tablet to 32 volunteers. Plasma samples were collected

Table 2
Accuracy and precision for HPLC determination of CLM concentration in human plasma ($n = 6$)

Nominal concentration (ng/mL)	Observed concentration (ng/mL) (mean ± S.D.)	Accuracy (%) (mean ± S.D.)	Precision (%)
Within-day			
50	53 ± 3	106 ± 5	5.0
1000	946 ± 29	95 ± 3	3.4
2500	2447 ± 84	98 ± 4	3.8
Between-day			
50	54 ± 1	108 ± 3	2.4
1000	995 ± 55	99 ± 6	5.6
2500	2519 ± 141	101 ± 6	5.6

n, number of replicates.

Table 3
Autosampler stability of CLM and I.S. plasma samples ($n = 6$)

Nominal concentration (ng/mL)		Peak height (mean ± S.D.)		RE (%)
		Immediately after extraction	After 18 h in autosampler	
CLM	50	5997 ± 411	6616 ± 575	10.3
	1000	142072 ± 10605	143032 ± 12834	0.7
	2500	333007 ± 22207	306531 ± 22066	−8.0
I.S.	3200	321489 ± 23270	322059 ± 21701	1.8

n, number of replicates; RE (%), percent relative error.

Table 4
Freeze and thaw stability of CLM plasma samples ($n = 6$)

Nominal concentration (ng/mL)	After three freeze-thaw cycles	
	Observed concentration (ng/mL) (mean ± S.D.)	RE (%)
50	54 ± 4	8.0
1000	1025 ± 94	2.5
2500	2609 ± 150	4.4

n, number of replicates; RE (%), percent relative error.

up to 24 h after an oral administration of a single dose of clopidogrel in fasting conditions. The main circulating metabolite reached a maximum (t_{max}) approximately 1 h after administration (0.9 ± 0.3 h), the literature data being 0.8 ± 0.3 h [3]. The peak plasma concentration of CLM (C_{max}) was determined as 3.2 ± 0.9 μg/mL (2.8 ± 0.7 μg/mL [3]). The plasma elimination half-life ($t_{1/2}$) was determined as 10.0 ± 2.2 h (7.3 ± 1.6 h [3]). The value of area under the plasma concentration-time curve from time 0 to the last measured concentration time (AUC_t) was calculated as 8.1 ± 1.4 μg h/mL and it constituted 87% of the AUC value extrapolated from 0 to infinity (9.2 ± 1.7 μg h/mL, 7.1 ± 1.7 μg h/mL [3]) which indicates a suitability of the analytical method for pharmacokinetic investigations in humans.

Table 5
Long-term stability of CLM in plasma samples at −20 °C ($n = 6$)

Nominal concentration (ng/mL)	Observed concentration (ng/mL) (mean ± S.D.)		RE (%)
	At the beginning of the study	After 1 month storage	
50	53 ± 2	51 ± 2	−3.8
2500	2562 ± 156	2703 ± 119	+5.5

n, number of replicates; RE (%), percent relative error.

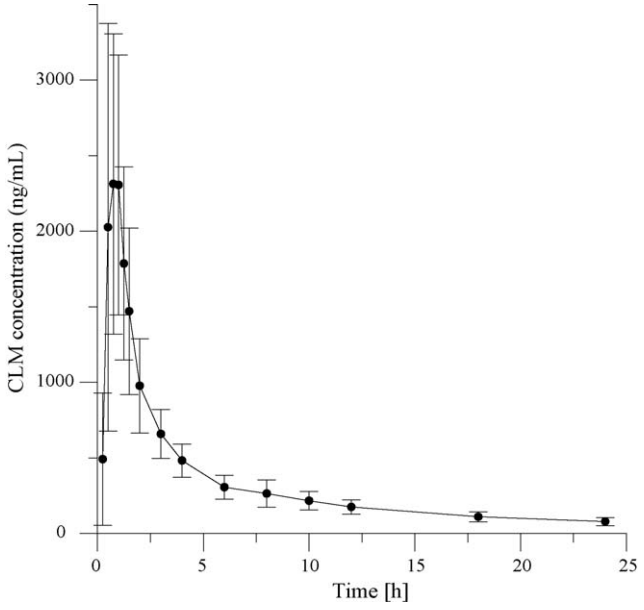

Fig. 6. Mean plasma levels of clopidogrel metabolite (SR26334) in 32 healthy volunteers following a single oral dose of 75 mg Plavix tablets.

4. Discussion and conclusion

The method of clopidogrel metabolite (SR26334) determination was developed. Repaglinide was selected as the internal standard because some of its physicochemical properties are similar to those of the clopidogrel metabolite: this compound was extracted with similar recovery to that of CLM (about 90%)

under the method conditions and retained good separation from the target compound (retention time for CLM was about 4.2 min and for I.S. about 5.9 min).

It was important that the relative short run time of the analysis (12 min) was obtained using the elution system of acetonitrile-water-formic acid (60:40:0.1, v/v/v), which allowed to separate the inactive clopidogrel metabolite and the internal standard from plasma endogenous compounds.

Run time of the analysis was based on investigation of different lots of drug-free human plasma for 120 min. An unknown extracted endogenous compound had retention time of 12.5 min.

The LLOQ of the described method was determined as 20 ng/mL and was 2.5 times lower than that obtained with UV detection [3] and 4 times higher than that for the GC–MS method [3,6]. Chem Elut cartridges were used for sample preparation. This technique made liquid–liquid extraction simpler, faster and more reproducible procedure than a traditional liquid–liquid extraction. The recovery of CLM obtained during the extraction on Chem Elut cartridges with dichloromethane was about 90% whereas in case of a liquid–liquid extraction with diethyl ether followed by a solid–liquid extraction on C18 and derivatization for GC–MS method [6] it was about 48%. This significant improvement of the extraction recovery may be considered as one of the main achievements of the assay.

Obviously, in the light of the newest analytical methods using tandem mass spectrometry, which allow to determine clopidogrel concentration in plasma [7,9], and even to detect active metabolite of clopidogrel [8], our LC–MS method may be considered as a method of low sensitivity. Nevertheless, the present method allows to determine changes in clopidogrel metabolite (SR26334) concentration in human plasma after administration of 75 mg of clopidogrel, with the LC–MS technique being significantly cheaper than LC–MS–MS.

In the LC–MS–MS method developed by Taubert et al. [8], concentration of inactive clopidogrel metabolite (SR26334) was determined in a range of 0.5–150 μg/mL. The lower level of the range is 25 times higher than in our method. This may result from the fact that the method includes simultaneous registration of three compounds during the study performed using a high dose of clopidogrel (600 mg). It could be attended by diminishing sensitivity in relation to clopidogrel and its inactive metabolite.

In conclusion, our assay achieved a simple, sensitive, specific and reproducible method for relatively low-cost determination of clopidogrel metabolite (SR26334) in human plasma. The method was validated according to regulatory authorities requirements [11,12] and allows pharmacokinetic studies of clopidogrel in humans.

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References

[1] T.L. Lenz, A.F. Wilson, Clin. Pharmacokinet. 42 (2003) 909–920.
[2] P. Savi, J.M. Herbert, A.M. Pflieger, F. Dol, D. Delebassée, J. Combalbert, G. Defreyn, J.P. Maffrand, Biochem. Pharmacol. 44 (1992) 527–532.
[3] H. Caplain, F. Donat, C. Gaud, J. Necciari, Semin. Thromb. Hemost. 25 (1999) 25–28.
[4] J.-M. Pereillo, M. Maftouh, A. Andrieu, M.F. Uzabiaga, O. Fedeli, P. Savi, M. Pascal, J.M. Herbert, J.P. Maffrand, C. Picard, Drug Metab. Disp. 30 (2002) 1288–1295.
[5] J.M. Herbert, D. Frehel, E. Vallee, G. Kieffer, D. Gouy, Y. Berger, J. Necciati, G. Defreyn, I.P. Maffrand, Cardiovasc. Drug Rev. 11 (1993) 180–198.
[6] P. Lagorce, Y. Perez, J. Ortiz, J. Necciari, F. Bressolle, J. Chromatogr. B 720 (1998) 107–117.
[7] J. Grenier, S. Ciric, J. Curry, G. Morelli, A. Arseneault, M. Tanguay, Clin. Pharmacol. Ther. 73 (2003) P47.
[8] D. Taubert, A. Kastrati, S. Harlfinger, O. Gorchakova, A. Lazar, N. von Beckerath, A. Schomig, E. Schomig, Thromb. Haemost. 92 (2004) 311–316.
[9] A. Lainesse, Y. Ozalp, H. Wong, R. Alpan, Arzneim.-Forsch./Drug Res. 54 (2004) 600–604.
[10] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.
[11] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration. Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001.
[12] Note for Guidance on the Investigation of Bioavailability and Bioequivalence, Committee for Proprietary Medicinal Products (CPMP), London, 26 July 2001.