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Assay method for the carboxylic acid metabolite of clopidogrel in human plasma by gas chromatography–mass spectrometry

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Abstract

This paper describes a GC–MS method for the analysis of the carboxylic acid metabolite (SR26334, **II**) of methyl (+)-(*S*)-α-(*o*-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate hydrogensulfate (clopidogrel, SR 25990, **I**) in plasma and serum. The analytical procedure involves a robotic liquid–liquid extraction with diethyl ether followed by a solid–liquid extraction on C_{18} cartridges. The derivatization process was performed using *n*-ethyl diisopropylethylamine and α-bromo-2,3,4,5,6-pentafluoro toluene. A structural analogue (**III**) of **II**, was used as internal standard. The $1/X^2$; weighted calibration curve obtained in the range 5–250 ng/ml was well described by a quadratic equation. The extraction efficiency was better than 48% over the range studied; for the internal standard it averaged 51% at 50 ng/ml. Precision ranged from 3.6 to 15.8%, and accuracy was between 92 and 114%. Dilution has no influence on the performance of the method which could then be used to quantitate plasma samples containing up to 25 000 ng/ml. The limit of quantification was 5 ng/ml. The method validation results indicate that the performance characteristics of the method fulfilled the requirements for assay methods for use in pharmacokinetic studies. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Clopidogrel; SR26334

1. Introduction

Clopidogrel hydrogen sulfate, methyl (+)-(*S*)-α-(*o*-chlorophenyl)-6, 7-dihydrothieno[3, 2-c]pyridine-5(4H)-acetate hydrogensulfate (SR25990, I, Fig. 1) is a thienopyridine compound structurally related to ticlopidine, that irreversibly inhibits platelet aggregation [1]. Unlike aspirin and related drugs, clopidogrel does not inhibit platelet aggregation through effects on arachidonic acid metabolism, but via selective binding to adenylate cyclase-coupled ADP receptors on the platelet surface [2–4]. This drug does not inhibit cyclo-oxygenase or prostaglandin synthesis, and does not have a direct effect on adenylate cyclase, cAMP phosphodiesterase, phospholipase-A_2 activity or thromboxane A_2 and prostacyclin synthesis. Moreover, clopidogrel does not influence coagulation, fibrinolysis or prolong bleeding time.

Clopidogrel is inactive in vitro and needs hepatic metabolism which generates an active metabolite, suggesting that its activity is dependent on hepatic biotransformation [5,6]. In humans, very low levels of the parent compound were detectable in plasma

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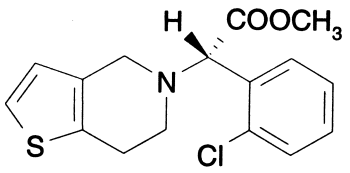
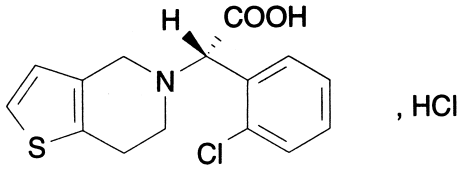


Sulphuric salt of clopidogrel

(SR 25990, I)

(Molecular mass: 419.9; salt/base ratio: 1.305)



Hydrochloride salt of the carboxylic acid

(SR 26334, II) of clopidogrel

(Molecular mass: 344.3; salt/base ratio: 1.119)

Fig. 1. Structural formulae of **I** and **II**.

samples after multiple oral doses [7]. The main identified compound in plasma and urine was the carboxylic acid derivative of **I** ((*S*)-(2-chlorophenyl)-6,7-dihydro-4H-thieno [3,2-c] pyridin-5-yl)-acetic acid hydrochloride, SR26334, **II**), formed by hydrolysis of the methyl ester moiety, in the free form or in the glucuroconjugated forms. Other minor compounds were also identified. The carboxylic acid metabolite (85% of the circulating compound in human) was devoid of any pharmacological activity. Nevertheless, as the active metabolite is not detectable in blood, it was used to document the pharmacokinetic profile of clopidogrel.

A large phase III trial (CAPRIE) has examined its use in the prevention of ischaemic stroke, myocardial infarction and vascular disease. The drug was well tolerated and demonstrated clinical efficacy superior to that of aspirin. Applications for registration are in progress in many countries [6]. Today, this drug is registered in Europe and in the USA.

The objective of this study was to replace an HPLC–UV method (not published) for the quantitation of **II** in human plasma in order to decrease its limit of quantitation, and to increase its specificity which is necessary to quantify this drug in plasma samples drawn during a Phase III study (patients undergoing polytherapy). This method was validated according to validation procedures, parameters and acceptance criteria [8–10] based on USP XXIII guidelines [8] and recommendations of Shah et al. [9]. During the validation step, the influences of the matrix (serum or plasma) and of the anticoagulant have been investigated. Moreover, stability tests under different conditions have been performed. A quadratic equation has been used to link peak area ratios (**II**/internal standard) to drug concentrations with $1/X^2$ weighting, where X is the theoretical concentration. This weighting factor has been chosen on a statistical basis. The low quantitation limit (5 ng/ml) was accomplished by a combination of a selective extraction procedure and an efficient chromatography. This method has enhanced precision due to the high selectivity of gas chromatography coupled with mass spectrometry. It was used to assay samples from a large multicentric Phase III study quoted CAPRIE [6].

2. Experimental

2.1. Materials and Reagents

Compound **II** (molecular mass, 344.3, salt/base= 1.119, Fig. 1) and the internal standard (**III,** an analogue of **II**, molecular mass, 323.8, salt/base= 1.127) were provided by Sanofi Recherche (Montpellier, France) in the form of hydrochloride salt.

Methylene chloride, *n*-hexane, diethyl ether, ethyl acetate, acetonitrile, absolute ethanol and formic acid were chromatography grade and were purchased

from Merck (Darmstadt, Germany). Methanol was Chromasol grade (SDS, Peypin, France). α-Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr) and *n*-ethyl diisopropylethylamine were obtained from Fluka (Buchs, Switzerland). Double-distilled deionised water, obtained by passing it through a Milli-Q reagent water system (Millipore, Saint Quentin/Yvelines, France), was used.

PFBBr (30%) and *n*-ethyl diisopropylethylamine (10%) solutions were prepared in acetonitrile.

Stock solutions of **II** and **III** (1 mg/ml) were prepared in methanol. Standard solutions of **II** were prepared extemporaneously in methanol by diluting stock solution to obtain concentrations ranging from 0.05 to 100 μg/ml. Working solution of **III** was prepared in methanol to obtain a final solution of 1 μg/ml.

Pooled blank (drug-free) plasma samples from healthy volunteers were used for the validation of the method. To prevent coagulation, blood was collected on CPD (citric acid, 327 mg; sodium citrate, 2.36 mg; anhydrous dextrose, 2.32 mg; sodium diphosphate, 222 mg and water 100 ml), and plasma was obtained by centrifugation at 1000 *g* for 10 min.

2.2. Apparatus and chromatographic conditions

The chromatographic separation was performed by injection in the splitless mode (valve time: 30 s), of 1 μl of the extract in a DB1 capillary column (length: 30 m; I.D. 0.25 mm; film thickness: 0.1 μm) (J&W Scientific, Folsom, CA, USA). Helium flow was adjusted to 40 cm/s. The injector temperature was 290°C and the initial oven temperature was 120°C then the temperature was programmed as follows: 30°C/min up to 290°C. This temperature was maintained for 3 min. The transfer line temperature was set to 270°C.

2.3. Detection

The detection was performed using a Finnigan MAT GCQ Ion Trap mass spectrometer (Finnigan MAT, San Jose, CA, USA), coupled to a computer (Gateway 2000), using the Finnigan MAT GCQ software (version 2.0). Analysis was performed by negative chemical ionisation with methane as gas reagent. The ion source temperature was set to 180°C. The electron energy was 70 eV. **II** (exact mass of the base: 307; exact mass of the derivatized compound: 565) and **III** (exact mass of the base: 287; exact mass of the derivatized compound: 545) were analyzed in the full scan mode. The ions m/z 262 and m/z 286 were selected to monitor **II** and **III**, respectively, due to their abundance and to their specificity. Ion m/z 262 of the derivatized **II** arises from the loss of the esterifying PFBBr group (m/z: 259) and of CO_2 group (m/z: 44), while ion m/z 286 of the derivatized internal standard arises from the loss of the esterifying PFBBr group (m/z: 259) (Fig. 2).

2.4. Analytical procedure

2.4.1. Calibration curves

Quantitation was based on the internal standard method. Blank plasma was spiked with **II** to obtain concentrations of 5, 10, 25, 50, 80, 100 and 250 ng/ml. A 100-μl volume of the adequate methanolic standard solution and 50 μl of a 1 mg/l methanolic solution of internal standard were transferred to 10-ml glass screw-capped tube and evaporated to dryness at 40°C under nitrogen stream. Then, 1 ml of blank plasma was added.

2.4.2. Quality control (QC) samples or unknown samples

QC samples were prepared at the concentrations of 5, 10, 50 and 250 ng/ml in human plasma, then aliquoted and stored at −18°C until assay. In a 10-ml glass screw-capped tube, 50 μl of a 1 mg/l methanolic solution of internal standard was added and evaporated at 40°C to dryness under a nitrogen stream. Then, 1 ml of QC sample or unknown sample was added.

2.4.3. Extraction procedure

The sample pretreatment included a liquid–liquid extraction followed by a solid-phase extraction before the derivatization process.

2.4.3.1. Liquid–liquid extraction. Liquid–liquid extraction was performed on a Zymate robot (Zymark, Hopkinton, MA, USA). To plasma (1 ml) containing the internal standard, 0.15 ml of formic acid was added and the sample was shaken by manual agitation for 20 s. Then 8 ml of diethyl ether was added to the tube and the contents were mixed for 10 min.

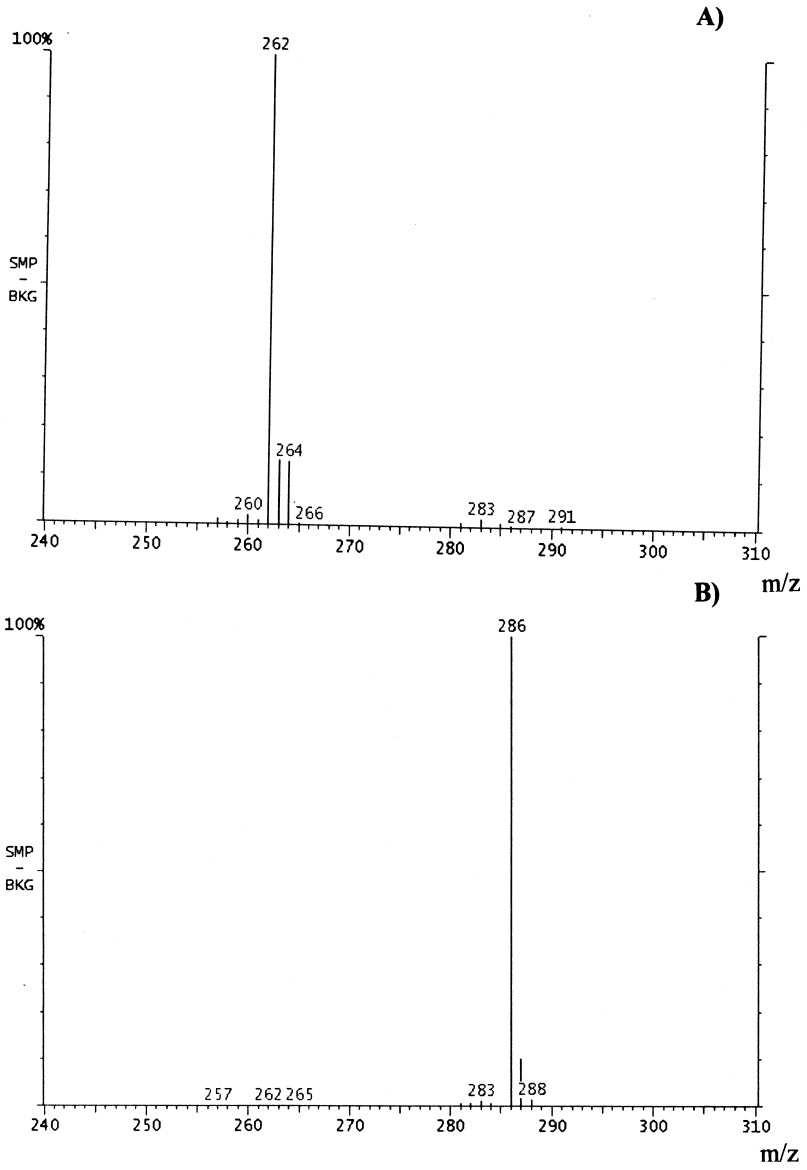


Fig. 2. Mass spectra (SIM mode) for the carboxylic acid metabolite of clopidogrel (**II**) (A) and the internal standard (**III**) (B).

After which, tubes were centrifuged at 1500 *g* for 10 min. The organic phase was withdrawn and transferred into another 10-ml glass tube, and evaporated at 40°C during 60 min at 140 kPa of nitrogen stream.

2.4.3.2. Solid–liquid extraction. Solid–phase extraction was performed with a C_{18} column (Bakerbond spe C18, Baker, Deventer, The Netherlands) using a sample processing station (Vac Elut, Varian, Harbor City, CA, USA). Preconditioning of the column was done with 1 ml of methanol and 1 ml of distilled water. The residue from the liquid–liquid extraction was dissolved in 100 μl of methanol and Vortex-mixed for 10 min, then 900 μl of water were added. This solution was transferred to the column. The column was then rinsed with 1 ml of a mixture methanol–water (25:75, v/v). Afterwards, the column was dried by suction for 10 min. Elution was

done with 1.5 ml of a mixture of methanol–water (75:25, v/v). The solvent was evaporated at 40°C to dryness under nitrogen stream.

2.4.3.3. Derivatization process. The residue was dissolved in 50 μl of ethanol, then Vortex-mixed for 10 s. A 25-μl volume of a 10% *n*-ethyl diisopropylethylamine solution and 25 μl of a PFBBr solution were added. The reaction mixture was heated for 10 min at 40°C and cooled at room temperature. The reaction solvents were evaporated at 40°C to dryness under nitrogen stream. A 2.5-ml volume of a mixture of hexane–dichloromethane (50:50, v/v) and 1 ml of distilled water were added. Then the mixture was Vortex-mixed for 10 s. The organic phase was transferred to a new 10-ml glass tube and evaporated at 40°C to dryness under a nitrogen stream.

The residue was dissolved in 200 μl of ethyl acetate. One microliter aliquot was injected into the GC–MS system.

2.5. Data analysis

From recorded peak areas the ratios of the drug to internal standard were calculated. The peak area ratios were linked to the concentrations of **II** in plasma according to a quadratic process as $Y=aX^2+bX+c$ weighting each standard in $1/X^2$. The regression curve was not forced through zero. The determination of the weighting factor has been performed by plotting ln (variance of ratio) against ln (concentration). The slope of the curve was approximately 2 which allows to choose $1/X^2$ as a weighting factor.

2.6. Specificity

To evaluate the specificity of the method, three different blank human plasma pools were tested. The retention times of endogenous compounds in the matrix were compared with that **II** and **III**.

2.7. Extraction efficiency

The extraction efficiency (recovery) was determined six times at three concentration levels for **II** (5, 50, 250 ng/ml) and at the concentration used during the assay for the internal standard (50 ng/ml). The peak areas obtained after extraction were compared to those obtained using standard solutions prepared at the same concentrations, evaporated to dryness, derivatized and injected into the GC–MS system.

2.8. Precision and accuracy

Inter-day and intra-day reproducibilities of the assay were assessed by performing replicate analyses of QC samples (5, 10, 50, 250 ng/ml) in plasma against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate the same day.

The accuracy was evaluated as [mean found concentration/theoretical concentration] $\times 100$, while the precision was given by the coefficients of variation.

2.9. Effect of dilution

In order to test whether it is possible to apply the described method to samples whose concentrations are higher than the last calibration point, spiked samples at 2 and 20 μg/ml were prepared. They were diluted 10- and 100-fold with blank human plasma in order to bring concentration within the range of standard curve, respectively. Each analysis was performed six times for each concentration, using calibration curves and QC samples. The found concentrations were reported and compared to the nominal one.

2.10. Determination of the limit of quantitation (LOQ)

The LOQ estimated on spiked samples was defined as the lowest drug concentration which can be determined with a precision $\leq 20\%$ and an accuracy between $100\pm 20\%$ on a day-to-day basis [8–10].

2.11. Stability study

The long term stability of **II** in frozen plasma (−18°C) was performed at the concentration of 5

ng/ml during a six-month period, and at the concentrations of 100, 1000 and 5000 ng/ml during 1.5 years.

Each determination was performed in replicate ($n=6$).

2.12. Influence of the matrix and the anticoagulant

Two series of QC samples (5, 250 ng/ml) were prepared, one in serum and the other in plasma. Concentrations were determined against a calibration curve prepared in plasma. The results obtained for the QC samples were compared.

In order to test the influence of the anticoagulant used (CPD versus sodium heparinate), QC samples at concentrations of 5 and 250 ng/ml were prepared. Concentrations were determined using a calibration curve prepared with CPD, while tested samples were prepared with sodium heparinate. The results obtained were compared with the theoretical values.

Each determination was performed in replicate ($n=6$).

3. Results

3.1. Retention times and specificity

Representative chromatograms of drug-free human plasma and of a serum sample from a patient before drug administration are shown in Figs. 3A and 4A, respectively. The extraction procedure and the chromatographic conditions allow separation of **II** and **III**. Observed retention times were 6.5 min for **II** and 6.2 min for **III**. Chromatograms of a human plasma spiked with **II** (5 ng/ml) and of a real sample from a patient to whom clopidogrel was administered are shown in Figs 3B and 4B, respectively.

3.2. Calibration curves

The peak area ratios were linked to concentrations (range 5–250 ng/ml) according to a quadratic equation. The regression curve was not forced through zero. The quadratic regression has been applied and gave a mean coefficient of determination of $0.993 \pm 4.82 \times 10^{-3}$.

As the variances of ratio values obtained were not homogenous over the calibration range, the regression model has been weighted in $1/X^n$. The exponent n has been defined as the slope of the curve obtained by plotting ln (variance of ratio) against ln (theoretical concentration) which gave a linear relationship with a slope of 2 (Fig. 5).

For calibration curves prepared on different days ($n=6$), mean results were as follows: $a=-1.24\times 10^{-6} \pm 2.12\times 10^{-6}$, $b=6.52\times 10^{-3} \pm 7.30\times 10^{-4}$ (C.V.=11.2%) and $c=0.0116 \pm 0.0032$.

Table 1 reports back-calculated concentrations of **II**.

3.3. Extraction efficiency, precision and accuracy

Extraction efficiency (Table 2) of **II** was better than 48% ($n=18$) over the range studied. For the internal standard it averaged 51% at 50 ng/ml ($n=18$).

For concentrations of calibration standards ranging from 5 to 250 ng/ml, the precision around the mean value not exceeded 11% (Table 1).

Inter-day and intra-day precision and accuracy of the method were assessed by analysing QC samples prepared in human plasma at different concentrations, in replicate, on the same day and on different days. The results are presented in Table 3. Precision ranged from 3.6 to 15.8%, and accuracy was between 92 and 114% over the range studied.

3.4. Influence of the dilution

The dilution has no influence on the performance of the method which could then be used up to 25 000 ng/ml which corresponded to 100-fold the last calibration point. The back-calculated concentrations averaged 2148 ng/ml (precision, 3.6%) and 20 890 ng/ml (precision, 6.3%) for the two concentrations studied (2000 and 20 000 ng/ml), respectively. The corresponding accuracy values were 107 and 104%, respectively.

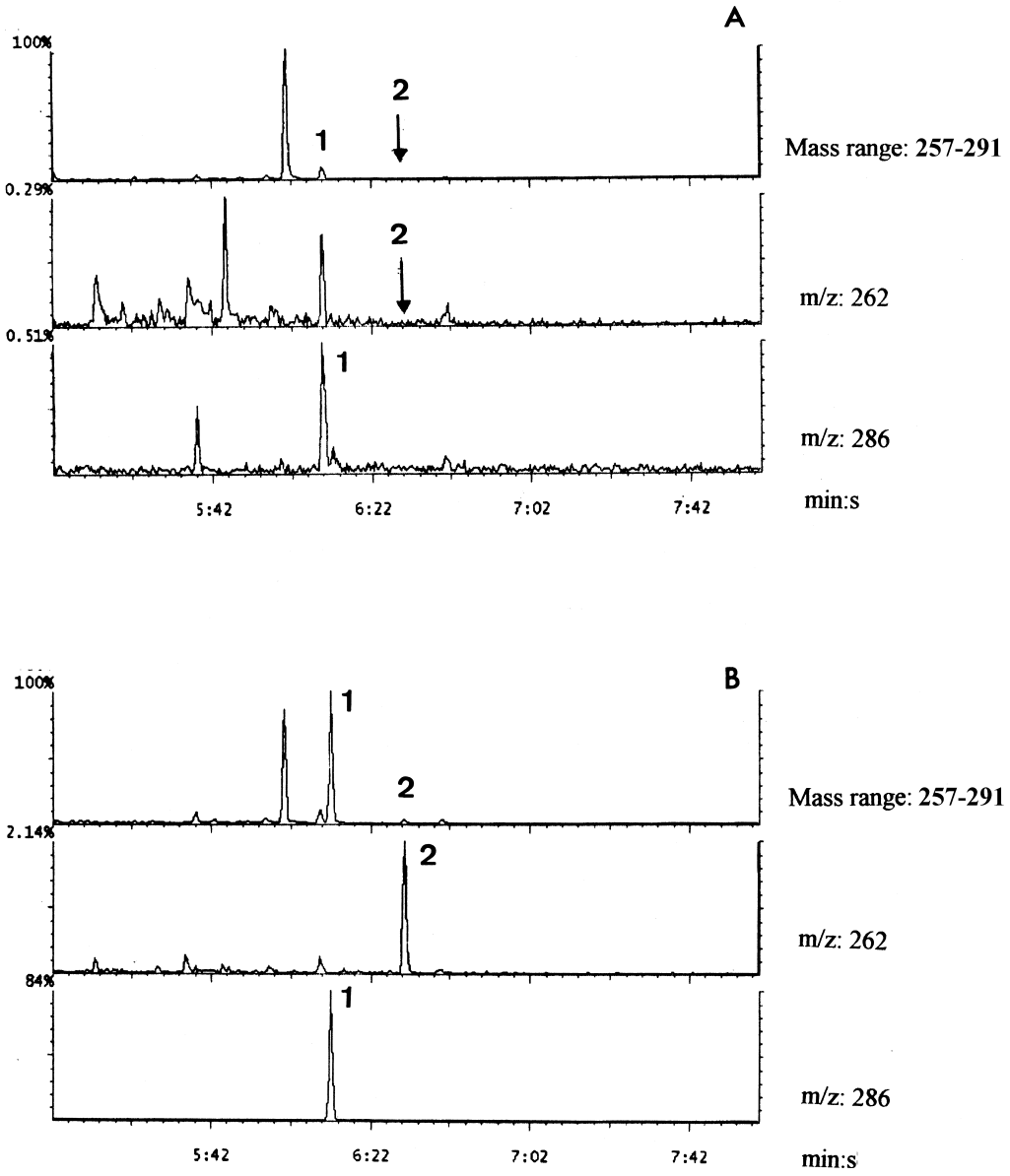


Fig. 3. Typical chromatograms of blank human plasma (A) and of plasma spiked with 5 ng/ml of **II** (B). Peak 1 is the internal standard, **III**; peak 2 is **II;** other peaks are endogenous compounds. For chromatographic conditions see sections 2.2 and 2.3.

3.5. Limit of quantitation

The limit of quantitation was 5 ng/ml**.** At this level, the analytical error was less than 11%.

3.6. Stability studies

II was stable in frozen plasma samples at the LOQ for at least six months. At the highest concentrations,

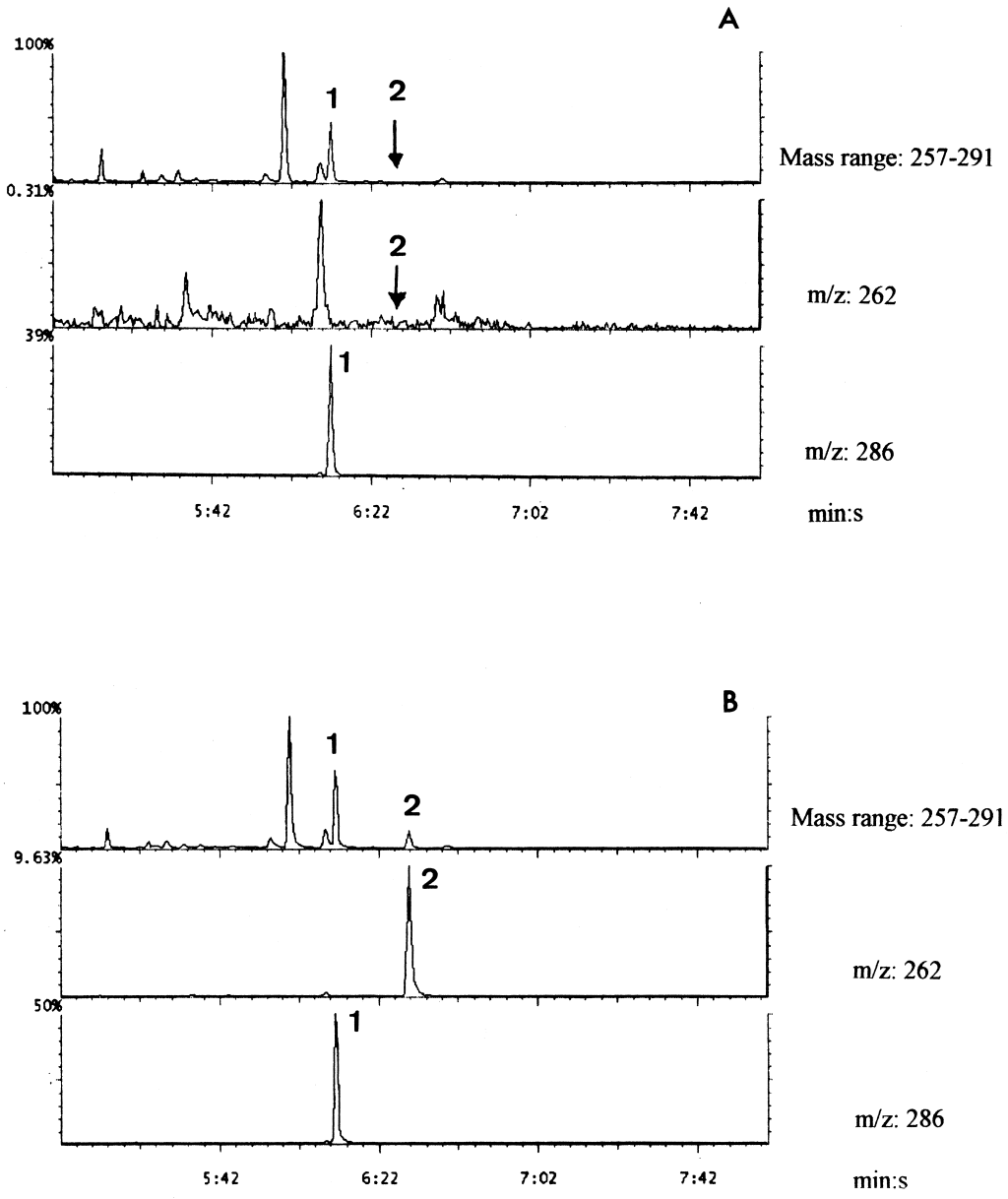


Fig. 4. Chromatograms of serum samples drawn before drug administration (A) and obtained from the same patient (B) 5.7 h after administration of clopidogrel (concentration of **II**: 364.5 ng/ml). Peak 1 is the internal standard, **III**; peak 2 is **II;** other peaks are endogenous compounds. For chromatographic conditions see sections 2.2 and 2.3.

using the old HPLC–UV method, the stability of compound **II** has been proven for 1.5 years; the percent recovery averaged 108% (C.V.=3%) at 100 ng/ml, 101% (C.V.=1%) at 1000 ng/ml and 106% (C.V.=0.9%) at 5000 ng/ml.

3.7. Influence of the matrix and of the anticoagulant

The anticoagulant used does not influence the performance of the method. Results obtained are

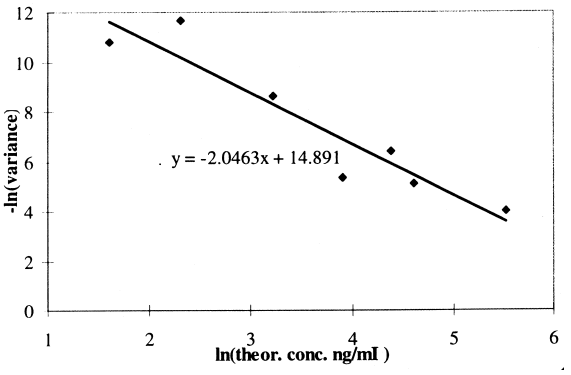


Fig. 5. Determination of the weighting factor of the calibration.

Table 1
Inter-day precision and accuracy from calibration curves ($n=6$)[a]

Theoretical concentration (ng/ml)	Back-calculated concentration (mean±S.D.) (ng/ml)	C.V. (%)	Recovery (%)
5	4.88 ± 0.21	4.3	97.5
10	10.5 ± 0.90	8.6	105
25	25.4 ± 1.76	6.9	101
50	49.1 ± 5.32	10.8	98.2
80	78.2 ± 4.06	5.2	97.7
100	96.9 ± 3.02	3.1	96.8
250	249.1 ± 12.4	5.0	99.6

[a] *n*, number of replicates

given in Table 4. Moreover, human serum samples can be assayed using a calibration curve prepared with human plasma as presented in Table 5.

4. Discussion and conclusion

In this manuscript we described a gas chromatographic method to quantify the principal circulating metabolite (85% of the circulating compound) of a new antiplatelet agent, clopidogrel. This metabolite is devoid of any pharmacological activity. However, as the active metabolite is not detectable in blood, **II** was selected to monitor the pharmacokinetic profile of the parent drug. The present GC–MS method validation results indicate that the performance characteristics of the method fulfilled the requirements for a sufficiently accurate and precise assay method to carry out pharmacokinetic studies in human plasma and serum samples. The analytical procedure involves a liquid–liquid extraction followed by a solid-phase extraction. This sample pretreatment procedure minimizes the risk of the matrix effect (especially the presence of nitrogen compounds) that has serious effects on quantitative and qualitative analysis, particularly in SIM mode. So, to avoid a reduced sensitivity of the method for the detection of target compounds, one must be aware of the presence of coeluting substances [11]. The calibration curve obtained in the range 5–250 ng/ml was well described by a quadratic equation. No interference was detected between the compounds of interest (**II** and **III**) and endogenous compounds.

Stability studies carried out directly in plasma indicated that samples were stable for at least six months when stored at −18°C.

The GC–MS method has been compared to an HPLC–UV method (results not published) that has been validated for concentrations ranging from 50 to 5000 ng/ml. Its limit of quantitation was 50 ng/ml. HPLC–UV and GC–MS methods produced equivalent results in human serum for concentrations higher than 50 ng/ml.

This analytical procedure has been used during the CAPRIE trial (19185 patients), to determine concentrations of **II** in serum samples from patients with recent ischemic stroke, myocardial infarction and symptomatic atherosclerotic peripheral arterial dis-

Table 2
Extraction efficiency[a]

	II ($n=6$)			Internal standard ($n=18$)
Theoretical concentrations, ng/ml	5	50	250	50
Mean extraction efficiency, %	54.2	55.6	48.2	50.8
S.D.	5.0	5.7	6.6	6.3

[a] *n*, number of replicates.

Table 3
Accuracy and precision of the method[a]

Theoretical concentration (ng/ml)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Deviation from theoretical value (%)	Recovery (%)
Within-day (n=6)				
5	5.28 ± 0.41	7.7	5.6	106
10	11.4 ± 0.78	6.9	14.0	114
50	46.7 ± 2.08	4.5	6.6	93.4
250	230.1 ± 10.6	4.6	8.0	92.0
Between-day (n=7)				
5	5.13 ± 0.6	11.7	2.6	103
10	11.2 ± 1.77	15.8	12.0	112
50	47.8 ± 3.74	7.8	4.4	95.6
250	235.2 ± 11.1	4.7	5.9	94.1

[a] n, number of replicates.

Table 4
Influence of the anticoagulant[a]

Theoretical concentration (ng/ml)	Experimental concentration (ng/ml) (mean±S.D.) n=6	C.V. (%)	Deviation from theoretical value (%)	Recovery (%)
5	4.35±0.19	4.3	13.0	87
250	239±11.0	4.6	4.4	96

[a] n, number of replicates.

ease. The study protocol was reviewed and approved by the institutional review board or ethics committee of each of the participating centres. Each patient received repeated oral doses of clopidogrel (75 mg once daily) for 36 months. Except in the early stages of the study, follow-up visits took place monthly for the first four months and every four months thereafter. At these visits, blood samples were taken for drug assessment. Fig. 6 illustrates the serum concentrations versus time plots for **II** obtained from a patient. The individual pharmacokinetic parameters were calculated using a Bayesian approach with population parameters as a prior information together with the individual observations of the patient. The data were compatible with a two-compartment model and first-order absorption rate. For this patient, the apparent clearance, uncorrected for bioavailability and for the fraction of the administered dose con-

Table 5
Assay in serum with a calibration curve prepared in plasma[a]

Theoretical concentration (ng/ml)	Experimental concentration (ng/ml) (mean±S.D.) n=6	C.V. (%)	Deviation from theoretical value (%)	Recovery (%)
5	5.31 ± 0.44	8.2	6.2	106
250	263.0 ± 9.95	3.8	5.2	105

[a] n, number of replicates.

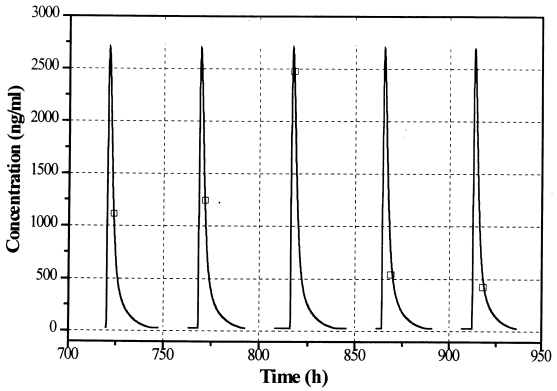


Fig. 6. Serum concentration–time profile of **II** after repeated oral administration (75 mg once daily) of clopidogrel to a patient with ischaemic stroke.

verted to **II**, and the elimination half-life were 6.2 l/h and 4.3 h, respectively.

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