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Chapter 3

CLOPIDOGREL PHARMACOKINETIC: REVIEW OF EARLY STUDIES AND NOVEL EXPERIMENTAL RESULTS

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ABSTRACT

In 1998 the European Commission granted the first marketing authorization for the potent anti-platelet aggregation agent clopidogrel to Sanofi Pharma Bristol-Myers Squibb SNC; the pharmacokinetic section of the Scientific Discussion which was part of the European Public Assessment Report stated that plasma levels of the parent compound beyond 2 hours from administration are very low, below the quantification limit of the existing method, and the only pharmacokinetic information available were on the inactive clopidogrel carboxylic metabolite. Clopidogrel appeared from the beginning to be a prodrug; however, detailed information on the chemical structure and pharmacology of the active moiety, the thiol metabolite responsible of the therapeutic action, were only published in 2000 by Savi et al., leaving in any case open the discussion on the pharmacokinetic aspects of this molecule. Four years later, in 2004, Lainesse et al. presented the first clopidogrel pharmacokinetic data in man and, in the same year, Taubert et al. published the first paper on the determination of clopidogrel active metabolite in plasma. It is noteworthy that for many years the analytical methods aiming to quantify clopidogrel in human plasma have encountered big problems. Until in 2010, a more clear understanding of the problem was reached, discovering that a new metabolite of clopidogrel is responsible for back-conversion to clopidogrel during sample handling (Silvestro et al., 2010). In the same years the oxidative steps involved in the synthesis of the active metabolite were studied and cytochrome P450 isoenzymes 2B6, 3A4, 1A1, 1A2 and 2C19 were identified as the most important. As a result of these findings, and of several PK/PD and genotype studies, in 2010, the FDA approved a new label for clopidogrel with a "boxed warning" about the diminished effectiveness of the drug in patients with impaired ability to convert the prodrug into its active form. Meanwhile a lot of progress was done also in the identification and quantitation of other clopidogrel metabolites like oxo-clopidogrel and clopidogrel acyl-glucuronide. Despite 15 years are passed from the introduction of clopidogrel in the market, the pharmacokinetic of this molecule still intrigues and attracts the attention of the scientific community.

1. INTRODUCTION

Clopidogrel, as already known from the available literature, is an oral thienopyridine derivative, used as anti-platelet aggregation agent to inhibit blood clots formation in coronary artery diseases, peripheral vascular diseases,

and cerebrovascular diseases (SmPC (Summary of Product Characteristics) of Plavix, revised 26 July 2013). Clopidogrel is a prodrug that is absorbed in the intestine and activated in the liver. The conversion of clopidogrel to its active metabolite requires two sequential steps: it is first oxydized to 2-2-oxo-clopidogrel (intermediate metabolite) and subsequently metabolized to the active metabolite through a reaction mediated by multiple P450 cytochromes.

The metabolite exerts its pharmacodynamic action by selectively inhibiting the binding of adenosine diphosphate (ADP) to its platelet $P2Y_{12}$ receptor and the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation. Due to the irreversible binding, platelets exposed are affected for the remainder of their lifespan (approximately 7-10 days).

Although a large number of publications provide useful information on clopidogrel, some particularities relating to its pharmacokinetic and pharmacodynamic behavior as well as the sources of the observed intra-individual variability with respect to therapeutic efficacy remain open for debate.

The purpose of this chapter is to provide a comprehensive image of what was thought to be known about clopidogrel pharmacokinetic at the time of the marketing authorization, the new information gathered so far and the aspects that still need further clarifying in the future.

The European Commission granted a marketing authorization valid throughout the European Union for Plavix (clopidogrel) to Sanofi Pharma Bristol-Myers Squibb SNC on 15 July 1998. The pharmacokinetic section of the Scientific Discussion which was part of the EPAR (European Public Assessment Report) of Plavix stated that plasma levels of the parent compound beyond 2 hours from administration are very low and below the quantification limit (0.00025 mg/L) of the existing method, and that the active metabolite, a thiol derivative, was formed by oxidation of clopidogrel to 2-oxo-clopidogrel and subsequent hydrolysis.

The oxidative step was thought to be regulated primarily by the Cytochrome P450 isoenzymes 2B6 and 3A4 and to a lesser extent by 1A1, 1A2 and 2C19 (EMA Scientific Discussion on Plavix, first published 24.10. 2006).

The active thiol metabolite, isolated in vitro, could not be detected in plasma at that time. Detailed information on the chemical structure and pharmacological activity of the active metabolite were published in 2000 by Savi et al., leaving in any case open the discussion on the pharmacokinetic behavioral aspects of this molecule.

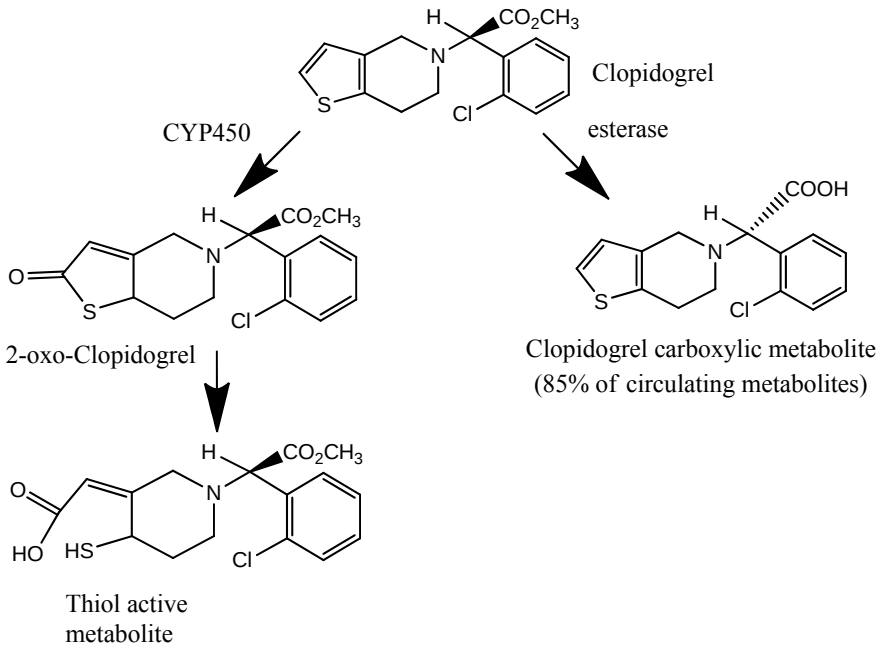


Figure 1. Clopidogrel metabolic pathway according to SmPC.

The known metabolic pathway of clopidogrel, as stated in the SmPC and presented in figure 1, is simple having two directions: on one hand, a desesterification to clopidogrel carboxylic acid, and on the other hand, an oxidation to 2-oxo-clopiopgrel, which undergoes a further oxidation leading to the active metabolite (Figure 1).

Suitable quantification assays were at the time only available for the main circulating inactive metabolite, the carboxylic acid derivative of clopidogrel, which represents about 85% of the circulating compound in plasma; though useful as a marker of absorption, it was admitted that the relevance of conventional pharmacokinetic studies of the inactive metabolite was limited. Thus, many aspects of clopidogrel behavior in special populations or potential interactions with other drugs were studied using dynamic markers such as inhibition of ADP-induced platelet aggregation and prolongation of bleeding time. In the years later, thanks to the improvement in liquid-chromatography-mass spectrometry (HPLC-MS/MS) techniques, methods were successfully developed to determine the parent molecule, clopidogrel, in biological samples of subjects treated with clopidogrel at therapeutic dosage. It is noteworthy to mention here the work performed by Lainesse et al. in 2004.

Still, it was evident that a main open issue for PK/PD studies was the fact that clopidogrel itself is inactive, and the determination of its active metabolite was expected to be much more revealing. Also in 2004, Taubert et al. published the first paper on the determination of clopidogrel active metabolite in plasma. Because a pure synthetic standard of active metabolite was unavailable, the authors based the quantitative evaluation on a surrogate calibration curve of clopidogrel.

Despite this shortcoming the work of the German group was quite a milestone and presented interesting observations also on PK/PD correlation. In 2006 Nirogi et al. published another interesting work comparing the PK of clopidogrel administered in fasting and fed conditions, showing a relevant food effect that was not observed, as reported in the Plavix SmPC, in studies employing a PD approach.

The group of Takahasi et al. described in 2008 the first quantitative method for the determination of clopidogrel active metabolite in plasma using an adequate analytical standard; the method employs a derivatization procedure just upon sampling to minimize the degradation of the metabolite, problem already discussed by Taubert group that also developed and patented (Bundesrepublik Deutschland Deutsches Patent DE 102004046159A1; 2006) a complex procedure to stabilize the samples.

Several other publications presented studies on clopidogrel PK and large variations of C_{max} and AUC_{0-t} have been observed with the same dose administered. The scientific community raised concerns regarding overestimation of the quantitative results, due to back-conversion of some clopidogrel metabolites into the parent compound.

As a consequence, regulatory authorities were forced to take position on this issue, as it can be seen in EMA's "Questions and Answers: Positions on specific questions addressed to the pharmacokinetics working party" (EMA/ 618604/2008).

According to this paper, the fact that plasma levels of clopidogrel carboxylic acid observed in patients or healthy volunteers treated with clopidogrel are 3 orders of magnitude higher than those of clopidogrel, only a minimum back-conversion of this metabolite could potentially lead to a huge overestimation of clopidogrel plasma levels and would bias the outcome of bioequivalence studies. It was therefore concluded by EMA experts, that clopidogrel bioanalytical method validations should include a demonstration that there is no back-conversion of the major metabolite to the parent drug clopidogrel under all conditions of sample handling (including extraction procedures) and storage.

However, a few publications pointed out that a clopidogrel metabolite, other than carboxylic acid, is the source of back-conversion (Yerino et al. (2006), Garofolo et al. (2009), Silvestro et al. (2010)). The studies carried out by our group showed that clopidogrel acyl glucuronide, rather than oxo-clopidogrel or the active metabolite, is responsible for the back-conversion of clopidogrel (Silvestro et al. (2011)); more information on this aspect, along with representative results are presented in the section regarding analytical issues in clopidogrel determination from plasma.

Along with the analytical advances made over the past years, a great effort has been also oriented to better understand the metabolic pathway of clopidogrel, with emphasys on the identification of markers that can be associated with individual pharmcodynamic variability through genotyping.

In-vitro tests carried out by Kazui et al. (2010) using cDNA-expressed human P450 isoforms have shown that clopidogrel is metabolized to 2-oxo-clopidogrel, the immediate precursor of its pharmacologically active metabolite, in a reaction catalyzed by CYP1A2, CYP2B6 and CYP2C19 with an individual contribution to the formation of 2-oxo-clopidogrel of 35.8, 19.4 and respectively 44.9%. In the same system using 2-oxo-clopidogrel as substrate, detection of the active metabolite of clopidogrel required the addition of glutathione to the system. CYP2B6, CYP2C9, CYP2C19 and CYP3A4 contributed to the production of the active metabolite and their individual contribution was found to be 32.9, 6.76, 20.6 and respectively 39.8%. The studies showed that CYP2C19 contributed substantially to both oxidative steps required in the formation of clopidogrel active metabolite and that CYP3A4 contributed significantly to the second oxidative step and helped explaining the role of genetic polymorphism of CYP2C19 and also the effect of potent CYP3A inhibitors on the pharmacokinetics and pharmacodynamics of clopidogrel. To date, the reduced-function genetic variants in the hepatic cytochrome CYP2C19 gene have been identified as the most prominent contributors to the variability of clopidogrel. As a result of these findings, as well of other studies, in March 12, 2010, FDA approved a new label for clopidogrel with a “boxed warning” about the diminished effectiveness of the drug in patients with impaired ability to convert the drug into its active form. The boxed warning is based on the concern that the anti-platelet aggregation effect of clopidogrel depends primarily on its activation by the cytochrome P450 (CYP) system. Patients with decreased CYP2C19 function because of genetic polymorphisms metabolize clopidogrel poorly and have higher rates of cardiovascular events after acute coronary syndrome (ACS) and percutaneous coronary interventions (PCIs) than patients with normal CYP2C19 function.

The warning also notes that tests are available to identify patients with genetic polymorphisms, and that alternative treatment strategies should be considered in poor metabolizers of the drug (Holmes et al. (2010)).

It is interesting to observe that despite the huge improvement in the analytics of clopidogrel and its metabolites, no work has been published on therapeutic drug monitoring of clopidogrel and only scarce PK data, beside method development, is available for the active metabolite of clopidogrel.

2. PHARMACOKINETICS OF CLOPIDOGREL CARBOXYLIC ACID AND CLOPIDOGREL

In the early phase development of clopidogrel, modest analytical sensitivity was a major issue for quantitative measurements in biological samples. As aforementioned, plasma levels of the parent drug were considered below the limit of detection of most methods; therefore bioavailability studies have been conducted first using the major inactive metabolite, clopidogrel carboxylic acid *(SR26334)*, as main parameter.

Back in 1998, Lagorce et al. published for the first time information about plasma concentrations of SR26334.The authors used GC-MS as analytical technique and the sample preparation protocol was quite complex, involving liquid-liquid extraction followed by solid-phase extraction and derivatization with *n*-ethyl diisopropyl-ethylamine and α-bromo-2,3,4,5,6-pentafluoro toluene.

Table 1 resumes the PK data measured in published papers up to date and also data available from studies of our group.

All authors describe quantification methods based on HPLC-MS/MS, except Lagorce (1998) employing GC-MS and Yousef et al. (2013) using liquid chromatography with UV detection. Von Beckenrath et al. (2005) developed and validated a method for simultaneous determination of clopidogrel, its carboxylic acid metabolite (SR26334) and its active thiol metabolite (the later on a surrogate curve of clopidogrel) on a Kromasil C8 column, using 1-methyl-4-phenylpyridinium bromide as internal standard. Ksycinska et al. (2006) validated on Luna C18 column a method only for the quantification of the carboxylic acid, repaglinide being the internal standard. Two studies performed in our laboratory (Pharma Serv1 (2007) and Pharma Serv 2 (2010) (unpublished data) focused on clopidogrel and SR26334 quantification; d3-clopidogrel was used as internal standard.

Table 1. Clopidogrel carboxylic acid main PK data

Author/source	Administered dose	Mean C_{max} (ng/mL)	Mean AUC_{0-t} (ng/mL*h)	Median T_{half} (h)
Lagorce (1998)	75 mg	2700.00	N/A	6.2
Hanna Ksycinska (2006)	75 mg	3200.00	8100.00	10.0
Pharma Serv 1 (2007)	75 mg	2807.81	8386.80	7.8
EMA/CHMP/595926/2010 – Study 07-197 (2007)	75 mg	2608.90	6674.70	N/A
El Ahmady et al. (2009)	75 mg	3501.00	9170.00	6.3
Pharma Serv 2 (2010)	75 mg	3164.84	10554.95	9.7
Marta Karazniewicz-Łada (2012)	75 mg	2821.00	9782.00	N/A
von Beckerath et al. (2005)	300mg	18000.00	35765.50	N/A
	adjusted to 75 mg*	4500.00	8941.38	
von Beckerath et al. (2005)	600mg	42000.00	97236.00	N/A
	adjusted to 75 mg*	5250.00	12154.50	
Yousef et al. (2013)	600 mg	24490.00	90400.00	4.3
	adjusted to 75 mg*	3061.25	11300.00	-
von Beckerath et al. (2005)	900mg	45000.00	129825.00	N/A
	adjusted to 75 mg*	3750.00	10818.75	

*Assuming dose-linearity.

Karazniewicz-Łada et al. (2012) describe a simultaneous determination of clopidogrel, SR26334 and 2 isomers of the active thiol metabolite. The clean-up of plasma samples was performed either by protein precipitation (Pharma Serv studies, Von Beckenrath et al., Lada et al.), by solid-phase extraction (Ksycinska et al.) or liquid-liquid extraction (Yousef et al.).

As it can be seen, quite similar results were obtained by different groups, irrespective of the analytical technique selected. An evaluation of PK dose linearity (see Table 2) was then carried out, based on mean of all studies for each dose strength reported in Table 1; according to the current Guideline on the Investigation of Bioequivalence (CPMP/EWP/QWP/1401/98 Rev. 1/ Corr**) assessment of dose linearity should consider whether differences in dose-adjusted AUC meet a criterion of ± 25%.

Table 2. Evaluation of dose linearity for clopidogrel carboxylic acid

Administered dose	AUC_{0-t} value adjusted to 75 mg	% mean AUC_{0-t} (AUC_{0-t} adjusted * 100/ AUC_{0-t} observed for 75 mg)	Dose linearity
300mg	8941.38	101.86	YES
600mg	11727.00	133.59	NO
900mg	10818.75	123.25	YES

A mean pharmacokinetic curve of clopidogrel carboxylic acid obtained in our lab after clopidogrel 75 mg administration in 12 healthy volunteers, in fasting conditions, is presented in Figure 2.

While it seems that from an analytical perspective, clopidogrel carboxylic acid is a desirable PK endpoint, due to the fact that reproducible results are obtained irrespective of the methodology employed, the question that still remains to be considered is whether there is any merit to this approach from a pharmacological point of view, especially keeping in mind that the metabolic pathways leading to the formation of the carboxylic acid and respectively the thiol-active metabolite are different.

Table 3 presents a similar PK comparison that has been carried out for clopidogrel itself. It is interesting to observe that an almost linear relationship between AUC_{0-t} and administered dose has been found from 75 mg and 900 mg. The situation is quite different from that of the previous table on clopidogrel carboxylic acid; PK parameters differ by a factor of 6-7 at the same administered dose (studies at different dosages were corrected for 75 mg assuming dose-PK linear response), and this fact can be seen also in two studies performed by our group. Although all authors used HPLC-MS/MS as analytical technique, plasma samples preparation was carried out either by liquid-liquid extraction (Robinson et al., Nirogi et al.) or protein precipitation at room temperature or in cold conditions (Pharma Serv studies, Taubert and Von Beckenrath group).

These PK differences between studies were already observed in the past and brought to the conclusion that in the case of clopidogrel, metabolites back-conversion in biological samples can play an important role and the measured concentrations can be highly influenced by storage, transport and processing methods.

These findings determined also the regulatory authorities to enforce particular stability and re-conversion studies in method validations for clopidogrel quantification, like mentioned in the previous section.

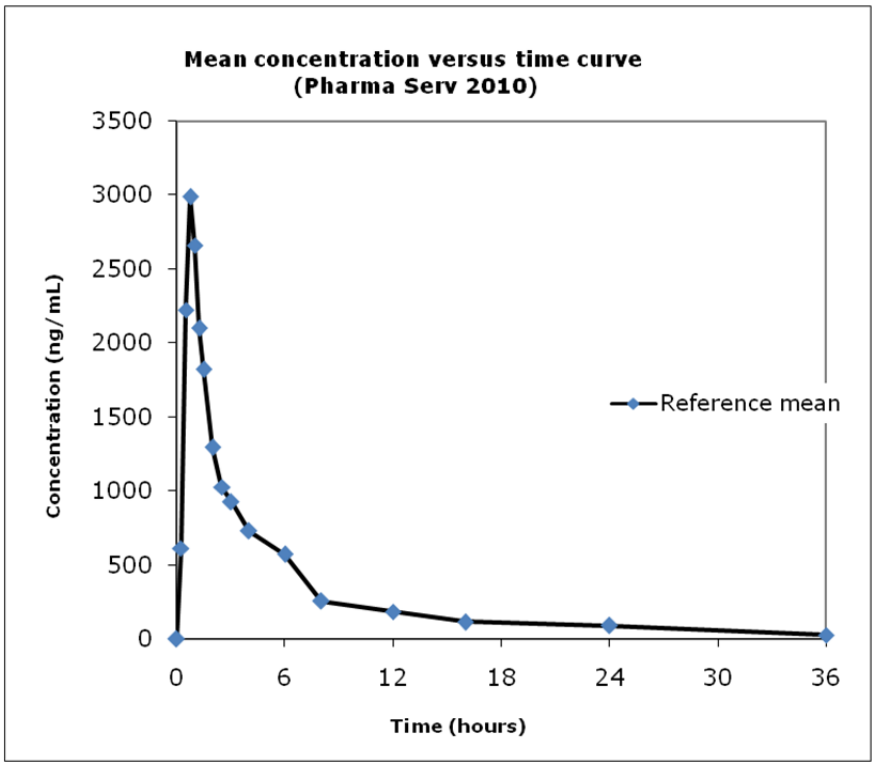


Figure 2. Mean concentration of SR26334 in 12 subjects orally treated with clopidogrel 75 mg.

Table 3. Clopidogrel main PK data

Author/Source	Administered dose	Subject selection by genotype	Mean C_{max} (ng/mL)	Mean AUC_{0-t} (ng/mL*h)	Median T_{half} (h)
Nirogi (2006)	75mg (fasting)	NO	1.019	1.764	N/A
	75mg (fed)	NO	6.273	17.105	N/A
EMA/CHMP/595926/2010 - Study 07-197 (2007)	75 mg (fasting)	NO	0.9814	1.9097	N/A
Pharma Serv Study 3 (2008)	75 mg	NO	0.8032	1.8133	7.8
Pharma Serv Study 4 (2008)	75 mg	NO	1.2395	1.9175	8.5
EMA/CHMP/595926/2010 - Study 80430 (2009)	75 mg (fed-normocaloric)	NO	3.0029	5.5469	5.39

Author/Source	Administered dose	Subject selection by genotype	Mean Cmax (ng/mL)	Mean AUC0-t (ng/mL*h)	Median Thalf (h)
Pharma Serv Study 5 (2009)	75 mg (fed-high fat; high calories)	NO	4.8901	10.4251	6.8
Pharma Serv Study 6 (2009)	75 mg	NO	1.4163	2.4808	6.2
UK/H/1662/001/DC (2010)	75 mg	NO	0.9986	2.0242	N/A
Pharma Serv Study 2 (2010)	75 mg	NO	1.3990	2.6590	2.1
Robinson et al. (2007)	150 mg	NO	2.1285	4.4090	N/A
	dose adjusted to 75 mg		1.0645	2.2045	
von Beckerath et al. (2005)	300mg	NO	12.53	28.58	N/A
	dose adjusted to 75 mg (assuming dose linearity)		3.13	7.15	
Taubert et al. (2006)	300mg (dose adjusted to 75 mg (assuming dose linearity)*	3435T/T	8.6 (2.15)	11.35 (2.84)	N/A
		3435C/T and 3435C/C	14.4 (3.60)	35.58 (8.90)	
von Beckerath et al. (2005)	600mg	NO	39.25	74.40	N/A
	dose adjusted to 75 mg (assuming dose linearity)		4.91	9.30	
Taubert et al. (2006)	600mg (dose adjusted to 75 mg (assuming dose linearity)*	3435T/T	13.3 (1.66)	25.03 (3.13)	N/A
		3435C/T and 3435C/C	49.7 (6.21)	117.62 (14.70)	
von Beckerath et al. (2005)	900mg	NO	46.25	91.88	N/A
	dose adjusted to 75 mg (assuming dose linearity)		3.85	7.66	
Taubert et al. (2006)	900mg (dose adjusted to 75 mg (assuming dose linearity)*	3435T/T	55 (4.58)	142.27 (11.86)	N/A
		3435C/T and 3435C/C	34.7 (2.89)	93.03 (7.75)	

*In this work, the authors tested the hypothesis that the intestinal efflux transporter P-glycoprotein (P-gp) limits the oral bioavailability of clopidogrel and that variance in the MDR1 gene encoding P-gp predicts absorption variability. Results were presented in correlation with the MDR1 genotype subjects pertain to.

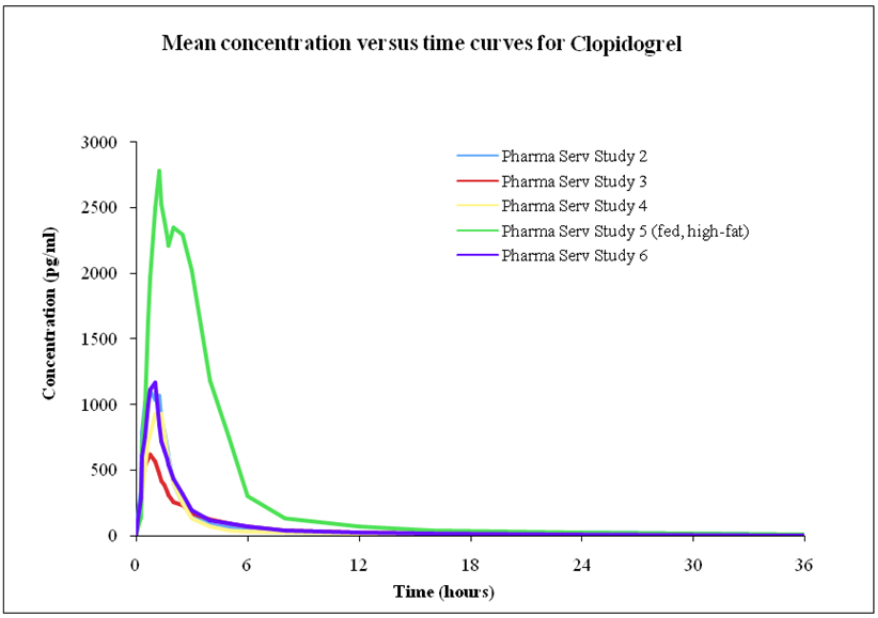


Figure 3. Mean clopidogrel concentrations obtained in 5 studies with a fixed dose of 75 mg.

Mean pharmacokinetic curves of clopidogrel obtained in the bioequivalence studies performed in our lab with a fixed dose of 75 mg per os are presented in Figure 3.

3. CLOPIDOGREL ANALYTICAL CHALLENGES

In 2007 our group started the development and validated an analytical method to quantify clopidogrel and clopidogrel carboxylic acid in plasma samples of subjects involved in bioequivalence studies; the classical FDA bioanalytical validation rules (2001) were followed. A fully validated method based on protein precipitation with trichloroacetic acid 20% in water/methanol 1/1 (v/v) was applied to a pharmacokinetic study. While analyzing the clinical samples, it was observed that reinjecting the extracts after an overnight stay in the autosampler, in the conditions validated with spiked plasma QCs (10°C), clopidogrel concentrations were definitively higher (more than double) than those measured in the first run. The measured concentrations of SR26334 metabolite on the other hand were stable.

Prompted by these results, a second method to determine only clopidogrel in plasma was validated, using incurred samples of real subjects in order to verify the plasma sample and extract stability. Setting of an unusually low autosampler temperature (-5ºC) was needed to obtain reproducible results in clinical samples, in contrast to the results achievable in blank plasma spiked with clopidogrel and/or SR26334 standards, which didn't show any issue. Here we must note that in this method the precipitation agent was changed to acetonitrile; still, the solution of internal standard (d3-clopidogrel) added to the samples was prepared in methanol. The problems that arose in these studies prompted us to perform, first, back-conversion tests of the metabolites described in literature (clopidogrel carboxylic acid, oxo-clopidogrel, clopidogrel active metabolite), diluted in plasma and stored or processed in different conditions. In all these experiments, clopidogrel was not measured; therefore it is evident that other metabolites from those of the known metabolic pathway could be responsible for back-conversion. In parallel, we have performed back-conversion tests on incurred samples and developed and validated a third analytical method, involving a clean-up step of the supernatant resulted from protein precipitation on HybridSPE-Precipitation 96-well plates (containing 50 mg bed). Although the internal standard solution was also prepared in methanol, the extracts were stable at 10°C, no back-conversion was observed and measured clopidogrel concentrations were reproducible, so it can be concluded that the metabolite (or metabolites) generating back-conversion were retained in the SPE-Precipitation plate (Silvestro et al., 2010).

Further studies of urine or plasma from healthy volunteers treated with a high oral dosage of clopidogrel (8 x 75 mg) revealed a series of other metabolites scarcely described in the literature. Among them, clopidogrel acyl glucuronide, initially identified from the mass spectrum, and subsequently confirmed with a commercially available standard, was recognized as the major source of back-conversion (Silvestro et al., 2011). The results of clopidogrel acyl glucuronide stability tests in different solvents (all dilutions at a concentration of 1µg/mL), pH and temperatures showed a very high rate of back-conversion, probably through transmethylation, in alkaline conditions in methanol (resulting clopidogrel concentrations up to 300ng/ml); acidic conditions were also favoring the back-conversion. Even at neutral pH the measured clopidogrel concentration was 11 ng/mL after 24 h at room temperature; at lower temperature (4 ºC) the back-conversion rate decreased with 85% (1.5 ng/mL).

This behavior of the acyl glucuronide metabolite is in agreement with the results described in a few papers (Yerino et al. (2006), Garofolo et al. (2009)).

The transesterification between the glucuronide group and the methanol used in sample preparation (to dilute the internal standard and/or for protein precipitation) was the cause of these unexpected results and in fact a method avoiding the use of methanol during sample preparation and chromatographic separation showed optimal validation results; a schema of the chemical reactions involved in this back-conversion process is presented in Figure 4.

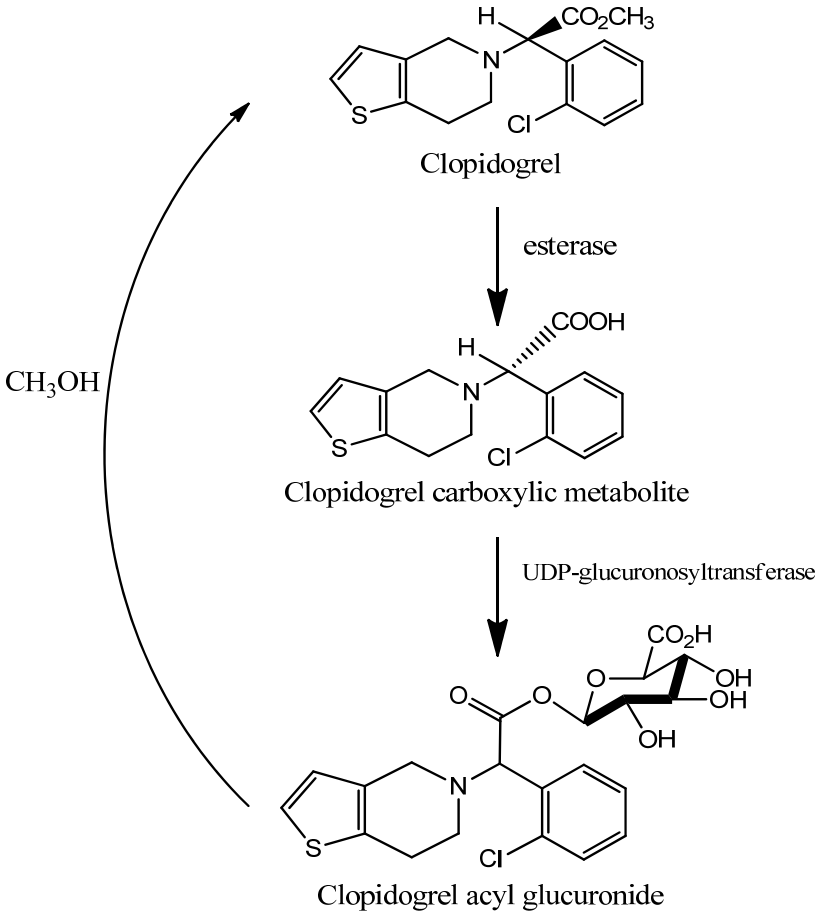


Figure 4. Metabolic reaction to form clopidogrel acyl glucuronide and back-conversion reaction.

As it can be expected, ethanol or isopropanol also produce transesterification; however the reaction products differ from clopidogrel. It is notewhorty that the reactivity decreases significantly (10 to 100 fold) with the increase of the alkyl chain length [Silvestro et al. (2011)].

In any case methanol should be avoided when analysing clopidogrel in biological fluids, keeping in mind that the concentrations of clopidogrel acyl-glucuronide in those fluids are about 500-fold higher than the concentrations of clopidogrel itself (details in the next section).

4. EXPERIMENTAL DATA ON CLOPIDOGREL ACYL GLUCURONIDE PK

Clarified that clopidogrel acyl glucuronide metabolite plays a critical role in the analytical back-conversion problems observed in clopidogrel quantification, it was interesting to evaluate its PK profile in plasma and compare it with clopidogrel and clopidogrel carboxylic acid PK.

We have then established a new method for the simultaneous quantification of all three analytes (clopidogrel acyl-glucuronide, clopidogrel carboxylic acid and clopidogrel) in plasma using the best conditions to avoid back-conversion; clopidogrel-d_3 was added as internal standard (Silvestro et al. (2011)).

Later, the method was revalidated and a second internal standard, clopidogrel carboxylic acid-$^{13}C_6$, was introduced beside d3-clopidogrel, in order to compensate properly the matrix effects for the two metabolites, eluting very early compared to clopidogrel retention time. A representative chromatogram is presented in Figure 5.

Samples from 10 subjects included in a bioequivalence study with clopidogrel 75mg, administered orally and in single dose, were analysed with this method. PK data (semilogarithmic plot) for clopidogrel acyl-glucuronide are presented in Figure 6 in comparison to the data obtained in the same study for clopidogrel and clopidogrel carboxylic acid; mean PK variables are available in Table 5.

It is interesting to observe that the exposure to this metabolite in man, based on $AUC_{0\text{-}inf}$ data, is practically 500 times higher than that observed in the same subjects for clopidogrel and 6 times lower than that of clopidogrel carboxylic acid.

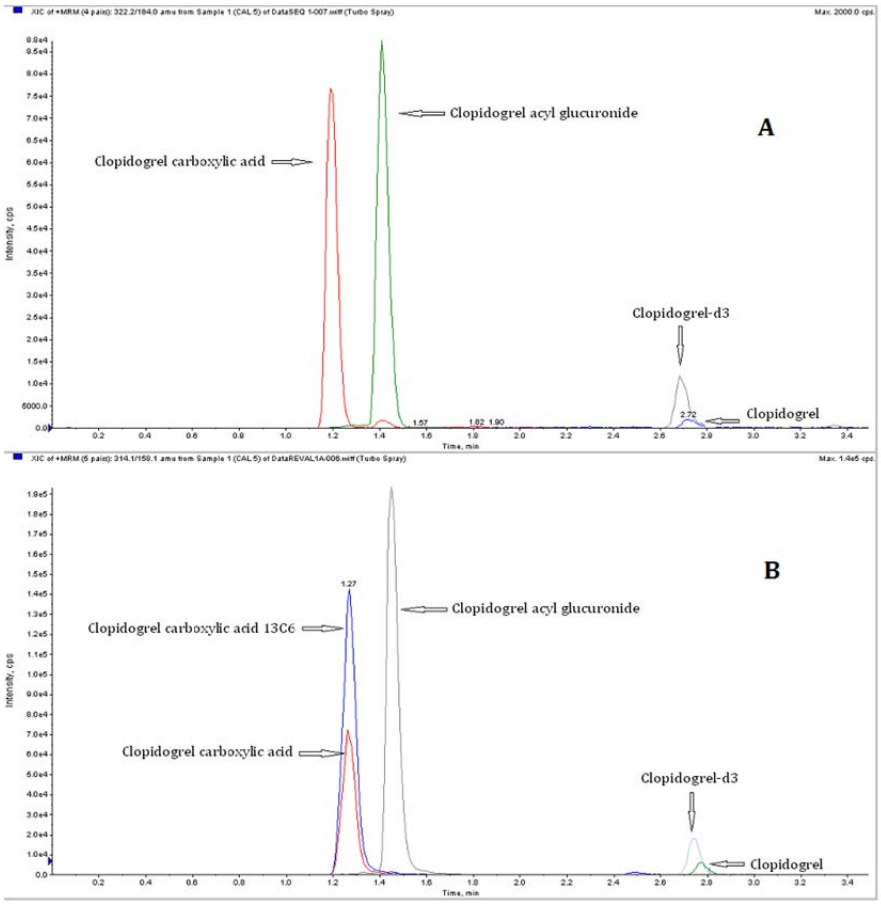


Figure 5. (A) Chromatograms recorded on the MRM transitions of clopidogrel (322.2/184.0), clopidogrel-d3 (327.2/189.2), clopidogrel carboxylic acid (308.2/95.0), and clopidogrel acyl glucuronide (484.3/198.1) after the injection of a spiked sample precipitated with acetonitrile. (B) Chromatograms of the same method, revalidated adding clopidogrel carboxylic acid $^{13}C_6$ (314.1/158.1) as internal standard for the early-eluting analytes. Mass spectrometer: AB Sciex API 4000Qtrap. Column: Ascentis Express RP-Amide (100x2.1 mm, 5µm). Mobile phase: gradient of formic acid 0.1% in water and acetonitrile, at a flow rate of 0.2 mL/min. Injection volume: 10µL.

Little other information is available on clopidogrel acyl glucuronide. A poster by Yerino et al. showed in 2006 that this metabolite has a modest activity as CYP2C8 inhibitor, while a very recent work [Inoue et al. (2013)] evaluating the inhibitory effect of this acyl glucuronide metabolite on human carboxylesterases concluded that it selectively inhibits hCES1 but not hCES2.

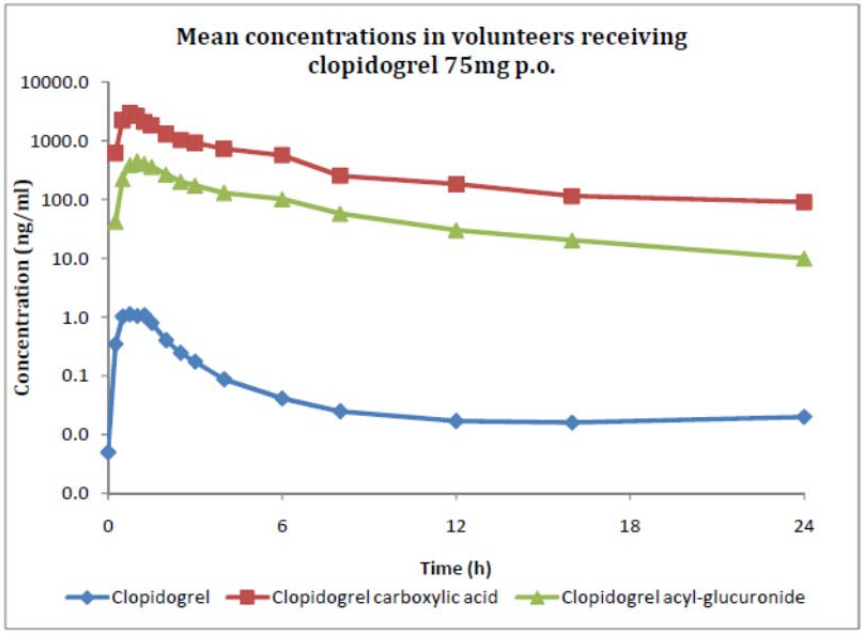


Figure 6. PK curve of clopidogrel acyl-glucuronide, SR26334 and clopidogrel.

Besides this, no other data is available and, in particular, it is unclear if this metabolite is mainly excreted in urine, bile or both elimination pathways. It is interesting to remember that, in case of a biliary excretion, an entero-hepatic circulation can be expected, bringing to a prolonged half-life of the carboxylic acid metabolite in plasma.

5. CLOPIDOGREL ACTIVE METABOLITE PK

Pioneering work on clopidogrel active metabolite PK was presented by the group of Taubert and Von Beckenrath in 2004 and 2005. They have used high loading dose of medication (300, 600 and 900 mg) and a tentative quantification of the active metabolite was achieved employing a surrogate calibration curve of clopidogrel (an appropriate standard was not commercially available).

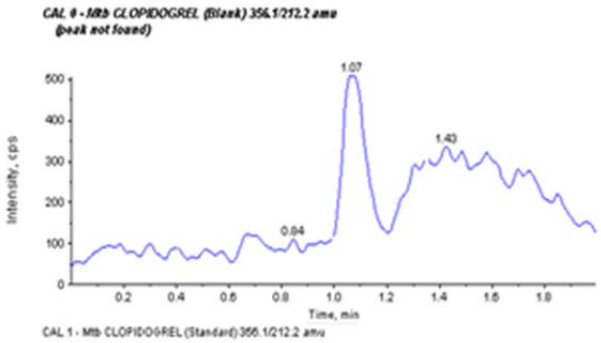
Analyses were carried out with LC-MS/MS, the sample preparation was performed by protein precipitation, and 1-methyl-4-phenylpyridinium bromide was added as internal standand.

As it can be seen in Table 4, that resumes the data available so far on clopidogrel active metabolite PK, the plasma concentrations were very low in these first studies, probably due to the thiol metabolite instability but more likely to the already mentioned analytical drawbacks of using a surrogate calibration curve and non-labeled internal standard.

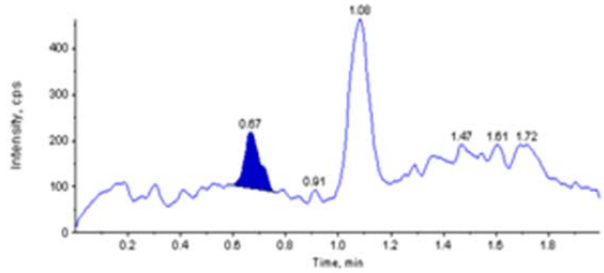
Takahasi et al. described in 2008 the first validated method using an active metabolite analytical standard. They also applied LC-MS/MS and, in order to stabilize the thiol group, a derivatization reaction with 2-bromo-3-methoxy-acetophenone was carried out just after blood sample collection. A 4-bromo-acetophenone analog of clopidogrel active metabolite was added as internal standard prior to the solid-phase extraction clean-up. It is important to observe that the derivatization approach was partially pushed by the consideration that the active metabolite containing a thiol is a highly reactive compound with critical aspects of stability. Interestingly, the group of Taubert, in a German patent application to cover a specific method of samples stabilization, showed that in adequate cold conditions the active metabolite has an acceptable stability in plasma, while it is definitively unstable at ambient temperature [DE 10 2004 046 159 A1 (2006)]. Later in 2010 three groups (Delavenne et al., Furlong et al., Lada et al.) have published new studies on clopidogrel active metabolite PK. All authors followed the same approach of Takahasi and used 2-bromo-3-methoxy-acetophenone to stabilize the thiol in blood samples. It is important to mention that 4 isomers of this active metabolite were already observed in the first study "in vitro" of Savi et al. in 2000 and only one was active; the groups of Lada et al. and Furlong et al. dedicated particular attention to this fact, thus observing that in plasma samples only form 3 and 4 were present (only form 4 is active). A quantification method developed and fully validated (unpublished data) in our laboratory in about the same period was based on a similar analytical approach, but without derivatization and with sample handling at very low temperatures. Plasma storage was at -70°C and processing (by simple protein precipitation with acetonitrile) at 2-4°C. Samples were analyzed by HPLC-MS/MS following the fragmentation of the protonated ions from clopidogrel active metabolite (MRM transition 356.1/212.2) and its deuterated analogue (d_3) used as internal standard; in the case of the later, the isotope ^{37}Cl was used (MRM transition 361.1/217.1). Both the active metabolite used for the calibration curves and quality controls, and the internal standard were obtained *in vitro* as described by Savi et al. (2000) starting from synthetic oxo-clopidogrel (d_3-oxo-clopidogrel in case of the internal standard), purified and quantified by inductive coupled plasma-

MS; the method selectively measured only the active form (4) of this metabolite.

Representative chromatograms are presented in Figure 7.



a



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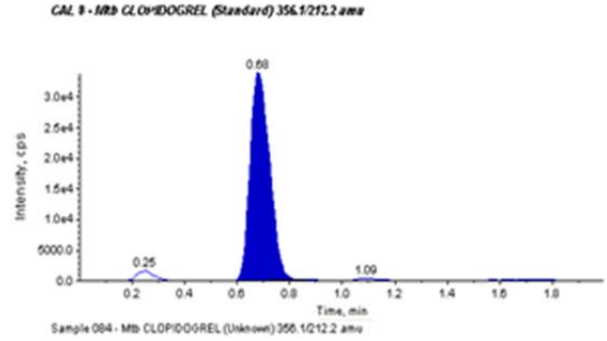
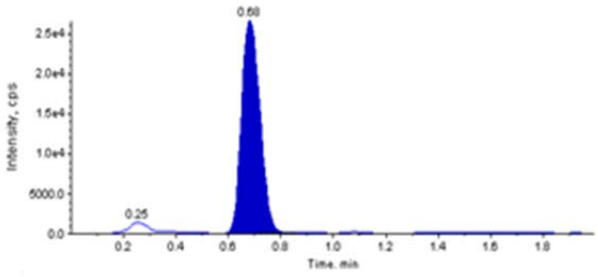


c

Figure 7. (Continued)



d

Figure 7. Chromatograms of clopidogrel active metabolite in blank plasma (A), plasma spiked at Cal 1 - 100 pg/mL (B), Cal 8- 60000 pg/mL (C) and in a sample from a subject treated with clopidogrel 75 mg (D). Mass spectrometer: AB Sciex API 5000. Column: Ascentis Express RP-Amide (100x2.1 mm, 5µm) maintained at 35°C. Mobile phase: gradient of (A) formic acid 0.1% in water/methanol 65/35 (v/v) and (B), formic acid 0.1% in water/methanol 10/90(v/v) at a flow rate of 0.25 mL/min. Injection volume: 20µL.

To help understanding the first results of Taubert et al., Figure 8 presents plasma concentrations obtained in one of our PK studies (clopidogrel dose 75 mg, fasting conditions), as first determined in 2008 using a surrogate calibration curve of clopidogrel (according to the method described by Taubert in 2004) and as determined again in 2009, using a calibration curve of clopidogrel active metabolite. As it can be observed, a substantial underestimation of active metabolite concentration occurs when using a surrogate calibration curve. It is interesting to note that results obtained in our laboratory in 2009 are in good agreement with those obtained by the authors using derivatization while the original results from 2008 closely resemble those of Taubert et al. and von Beckerath et al. (see Table 4).

Among the presented studies, the one performed by von Beckerath (2005) took into consideration the aspect of PK linearity for the active metabolite, within the dose range of 300 to 900mg clopidogrel, following the administration of a single loading dose. Due to the fact that only three distinct doses were administered, no clear statistical evaluation of linearity can be made. However, it seems easily noticeable that from 300 to 600 mg the PK is almost linear, while the dose increase from 600 to 900mg (150%) results in only modest increase in C_{max} (108.4%) and AUC_{0-t} (137.8%), suggesting a saturation of the metabolism.

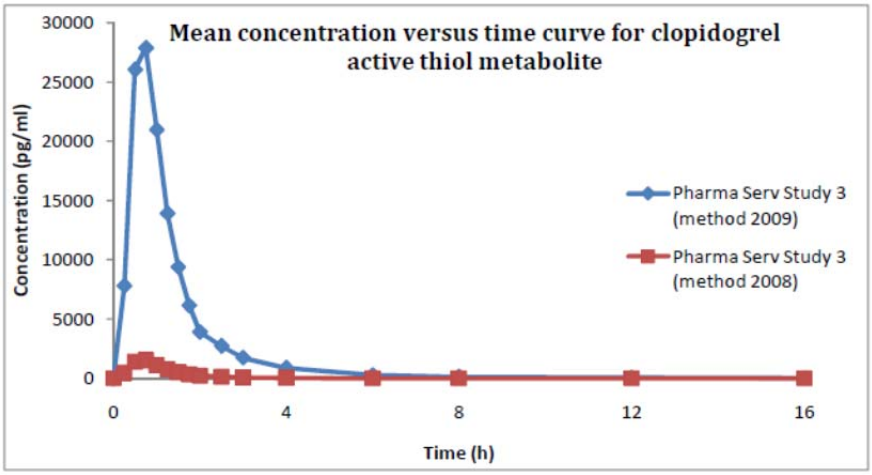


Figure 8. PK curve of clopidogrel active metabolite, same study (single dose, 75mg), quantified using two distinct analytical methods.

Table 4. Clopidogrel active thiol metabolite main PK data

Author/source	Administered dose	Subject selection by genotype	Mean C_{max} (ng/mL)	Mean AUC_{0-t} (ng/mL*h)	Median T_{half} (h)
Pharma Serv Study 3 (2008)	75 mg	NO	(2008[1]): 1.669	(2008[1]): 1.815	(2008[1]): 0.65
			(2009[2]): 30.915	(2009[2]): 35.383	(2009[2]): 1.14
Pharma Serv Study 5 (2009)	75 mg (fed)	NO	19.6240	34.6131	1.48
Karazniewicz-Łada et al. (2012)	75 mg	NO	H3 isomer: 14.8	H3 isomer: 18.6	0.58
			H4 isomer: 13.3	H4 isomer: 16.6	0.57
			Total: 28.1	Total: 35.2	
Furlog et al. (2013)	75 mg***	NO	H4 isomer 11.0	H4 isomer AUC_{0-12}: 11.5	0.97
von Beckerath et al. (2005)	300mg	NO	2.82	4.35	N/A
	adjusted to 75mg (assuming dose linearity)		0.71	1.09	
Taubert et al. (2006)	300mg	3435T/T carriers	1.20	1.88	N/A

Table 4. (Continued)

Author/source	Administered dose	Subject selection by genotype	Mean C_{max} (ng/mL)	Mean AUC_{0-t} (ng/mL*h)	Median T_{half} (h)
Taubert et al. (2006)	300mg	3435C/T and 3435C/C	3.20	7.85	N/A
Takahashi et al. (2008)	300mg	NO	35.9	43.8 (AUC_{0-inf})*	0.52
	adjusted to 75mg (assuming dose linearity)		8.975	10.95	N/A
von Beckerath et al. (2005)	600mg	NO	5.85	8.30	N/A
	adjusted to 75 mg (assuming dose linearity)		0.73	1.04	
Taubert et al. (2006)	600mg	3435T/T carriers	2.50	3.48	N/A
		3435C/T and 3435C/C	6.60	12.40	N/A
Delavenne et al. (2010)	600mg	NO	82.60	131.5 (AUC_{0-inf})*	0.4
	adjusted to 75mg (assuming dose linearity)		10.33	16.44	
von Beckerath et al. (2005)	900mg	NO	6.34	11.43	N/A
	adjusted to 75mg (assuming dose linearity)		0.53	0.95	
Taubert et al. (2006)	900mg	3435T/T carriers	7.30	16.90	N/A
		3435C/T and 3435C/C	5.40	10.55	N/A

*Considering the short half-life of the active metabolite and the fact that the sampling schedule was long, basically the value listed can be considered AUC0-t since no extrapolation is needed in the given study conditions.

**In this work, the authors tested the hypothesis that the intestinal efflux transporter P-glycoprotein (P-gp) limits the oral bioavailability of clopidogrel and that variance in the MDR1 gene encoding P-gp predicts absorption variability. Results are presented in correlation with the MDR1 genotype subjects pertain to.

***Treatment schedule: 600 mg clopidogrel loading dose on day 1, followed by once daily dosing of 75 mg on days 2 through 5. Pk samples collected on Day 5.

6. EXPERIMENTAL DATA ON OXO-CLOPIDOGREL PK

Despite the fact that this metabolite has been for a long time known as a key intermediate in the formation of the clopidogrel active metabolite, PK data on 2-oxo-clopidogrel are, according to our knowledge, unavailable so far. In 2013 we have developed and partially validated a HPLC-MS/MS analytical method for its determination in plasma. 2-oxo-clopidogrel and the internal standard, 2-oxo-clopidogrel-d3, were monitored on the MRM transitions 338.1/155.1 and 341.1/158.0, respectively. Sample clean-up was carried out by acetonitrile protein precipitation, in cold conditions; representative chromatograms are presented in figure 9. Preliminary experiments on 2-oxo-clopidogrel stability didn't show special problems in neat standard solutions, but degradation occured in biological samples; the optimal conditions for long term plasma storage and sample processing are still under evaluation.

Samples from a PK study with administration of 75 mg clopidogrel have been analyzed shortly after collection, in order to minimize degradation problems; the PK results are resumed in Figure 10 and PK data values are summarized in Table 5.

As it can be seen, the levels are quite similar to those obtained for the active metabolite (as presented in the same figure). We don't have at the moment any correlation to PD data or PK linearity information. In any case, this preliminary work has shown the feasibility of this determination and opens the direction for future interesting experiments of PK/PD correlations as well as the possibility to evaluate directly if the limiting metabolic rate step is from clopidogrel to 2-oxo-clopidogrel, from 2-oxo-clopidogrel to the active metabolite, or both are critical.

7. CONSIDERATIONS ON OTHER POTENTIAL METABOLITES OF CLOPIDOGREL

Other oxidative metabolites can be expected, in particular hydroxyl derivatives of clopidogrel. At the moment no data has been published showing the presence of such metabolites; these compounds can be also a starting point for additional conjugate metabolites.

Interestingly, the active thiol metabolite of clopidogrel can also participate to the formation of thiol derivatives (with glutathione, cysteine etc.) whose existence is yet unclear.

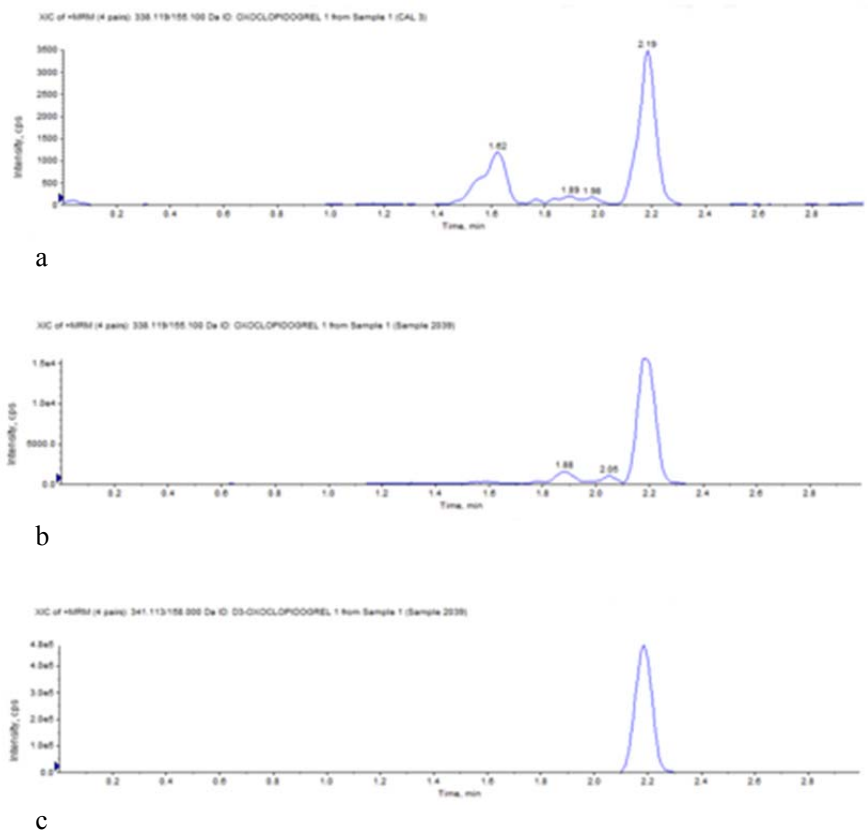


Figure 9. Chromatograms recorded on the MRM transitions of 2-oxo-clopidogrel (338.1/155.1) and 2-oxo-clopidogrel-d3 (341.1/158.0) after the injection of a spiked sample (A) and an incurred sample (B) precipitated with acetonitrile. The peak of the internal standard in the incurred sample is shown panel (C). Mass spectrometer: API 4000 Qtrap. Column: Ascentis Express RP-Amide (100x2.1 mm, 5µm) maintained at 55°C. Mobile phase: gradient of formic acid 0.1% in water and acetonitrile at a flow rate of 0.2 mL/min. Injection volume: 10µL.

A few experiments performed in our laboratory to measure the active clopidogrel metabolite after reduction of plasma samples with dithiotreitol to release the conjugates showed levels 30 to 50% higher than the ones obtained without reduction.

These few data seems to prove the existence of such conjugates; an additional aspect to be studied is also the binding to sulfhidryl compounds present in cells and tissue. All together, the clopidogrel metabolism can still offer interesting areas of investigation.

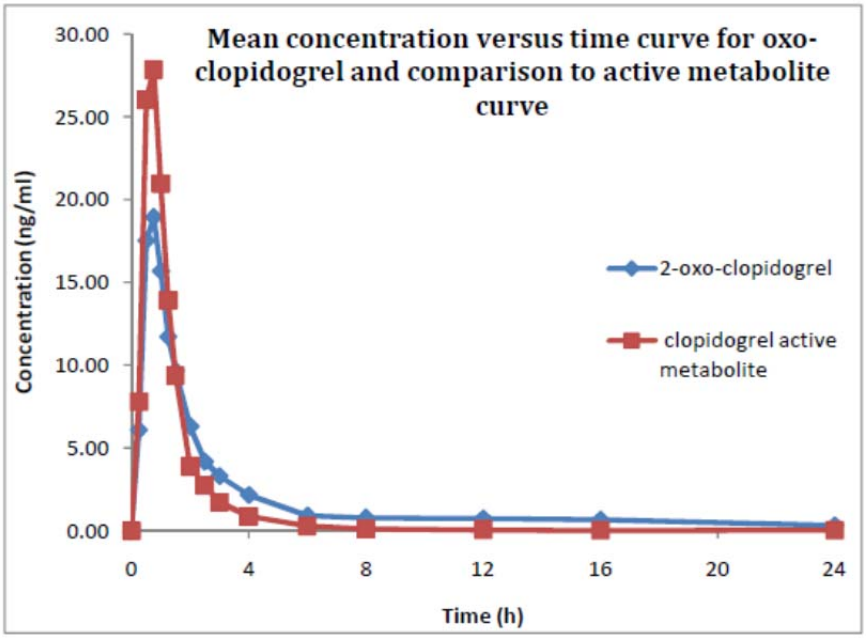


Figure 10. Pharmacokinetic curve of 2-oxo-clopidogrel (samples collected from 5 volunteers treated with clopidogrel 75 mg).

8. Overall Vision of Clopidogrel Metabolism and PK

The conversion of clopidogrel to its active metabolite is mediated by multiple P450 cytochromes, as aforementioned in the beginning of this chapter. There are 3 major CYP2C19 genetic polymorphisms. CYP2C19*1 corresponds to normal function. CYP2C19*2 and CYP2C19*3 are loss-of-function alleles and explain most of the reduced function in subjects who are poor metabolizers. CYP2C19*2 and *3 account for 85% and 99% of the nonfunctional alleles in whites and Asians, respectively. Poor metabolizers have 2 loss-of-function alleles. Intermediate metabolizers have 1 copy of a loss-of-function allele and may also have decreased active metabolite levels and reduced anti-platelet aggregation effects when treated with clopidogrel, but FDA's boxed warning only refers to poor metabolizers (Holmes et al. (2010)). It should however be noted that although the warning emphasizes the fact that there is indeed a higher risk associated with the use of clopidogrel in

poor metabolizers, it does not specifically require genetic testing nor does it state that the drug should be contraindicated in such patients. A plausible reason would be that so far it has been shown that the relation between CYP2C19*2 genotype and platelet aggregation merely accounts for 12% of the overall variation in the response to clopidogrel therapy (Shuldiner et al. (2009)).

Another potential source of variability was recently proposed by Bouman et al. (2011) who identified paraoxonase-1 (PON1) as a major determinant of the bioconversion of clopidogrel with its common Q192R polymorphism determining the rate of active thiol metabolite formation. The paper concluded that PON1 Q192R polymorphism explained 72.5% of the variability in ADP-stimulated platelet aggregation after clopidogrel administration. However, after having genotyped PON1 (Q192R and L55M) and CYP2C19 variants in 106 patients enrolled in the PK/PD CLOVIS-2 trial, Hulot et al. (2011) have concluded that the results do not support an important contribution of PON1 Q192R and L55M as major determinants of clopidogrel PK and PD responsiveness. Nevertheless, because CYP2C19 seems to explain only a minor part of clopidogrel bioactivation, future studies to unravel the pharmacogenetics of clopidogrel are highly warranted [Jaapjan (2011)].

Furthermore, another recently proposed key contributor to the inter-individual variability in response to clopidogrel is hepatic carboxylesterase 1 (CES1) whose single nucleotide polymorphisms (SNPs) G143E (rs71647871) and D260fs (rs71647872) have been shown to exhibit markedly decreased enzymatic activity towards the hydrolysis of some CES1 substrates (eg. methylphenidate, oseltamivir, trandolapril) [Zhu et al. (2012)]. It was therefore hypothesised that clopidogrel metabolism may also be affected by variability in the expresion of the enzyme, given that hydrolysis of clopidogrel prodrug to the inactive metabolite clopidogrel carboxylic acid is catalized by CES1 and this reaction accounts for the fate of 85% of an administered dose of clopidogrel. Subsequently the ~15% of a clopidogrel dose that remains available undergoes further oxidative metabolism catalyzed by cytochrome P450 (CYP) 1A2, 2B6, and 2C19, with formation of 2-oxo-clopidogrel which is again subject to concurring reactions; a part of it is hydrolyzed by CES1 to form 2-oxo-clopidogrel carboxylate, an inactive metabolite, and the balance of 2-oxo-clopidogrel is further hydrolyzed to the unstable but active 5-thiol metabolite. Hao-Jie Zhu et al. wanted to determine whether CES1 inhibition and *CES1* genetic polymorphisms would significantly influence the biotransformation of clopidogrel and alter formation of the active metabolite, as could be asumed when considering its involvment in the biotransformation

of clopidogrel. Their conclusion was that deficient CES1 catalytic activity resulting from CES1 inhibition or *CES1* genetic variation may be associated with higher plasma concentrations of clopidogrel active metabolite and hence, enhanced antiplatelet activity.

Following upon the research of Kuzui et al. (2010) and more to the point, on the obervation that in-vitro formation of the active metabolite of clopidogrel from 2-oxo-clopidogrel required the addition of reduced glutathione (GSH) as substrate, Zhang et al. tried to establish whether a correlation can be found between varying glutathione concentrations and the amount of thiol metabolite formed. Based on the group's previous work, where the formation of a glutathionyl conjugate of the active metabolite of clopidogrel was reported, it was established that a relevant correlation would have to account for both the active metabolite formation as well as its glutathionyl conjugate. It has already been established that clopidogrel is bioactivated through two sequential oxidative processes, the first one resulting in the formation of 2-2-oxo-clopidogrel which has no antiplatelet activity. Subsequently, in the second step, 2-oxo clopidogrel is further oxidized to give an unstable sulfenic acid intermediate. Reduction of the sulfenic acid by GSH leads to thiolactone ring opening to form a glutathionyl conjugate followed by a thiol-disulfide exchange to form the thiol active metabolite. It is currently unknown whether the glutathionyl conjugate of clopidogrel exhibits antiplatelet activity, but the results of the study do indicate that the formation of both the active metabolite and the glutathionyl conjugate exhibit similar saturation kinetics, indicating that formation of the glutathionyl conjugate constitutes an integral part of the bioactivation processes of 2-oxo clopidogrel via CYP-catalyzed reactions [Zhang et al. (2012)].

It is therefore suggested that the levels of active metabolite may be prone to changes due to factors other than P450-catalyzed reactions, such as the concentrations of GSH or other reduction agents that may be associated with oxidative stress, inflammation etc., thus potentially affecting the effective concentration of active metabolite also in vivo.

Since the premises for a discussion on potential step-limiting reactions involved in the biotransformation of clopidogrel to its active metabolite have been laid out, it is important also to do a review of the information provided by food effect studies.

In Figure 11, pharmacokinetic curves obtained for clopidogrel and the active metabolite, following the administration of a single 75mg dose in fasting and fed conditions (after intake of a high fat, high calories breakfast) are shown.

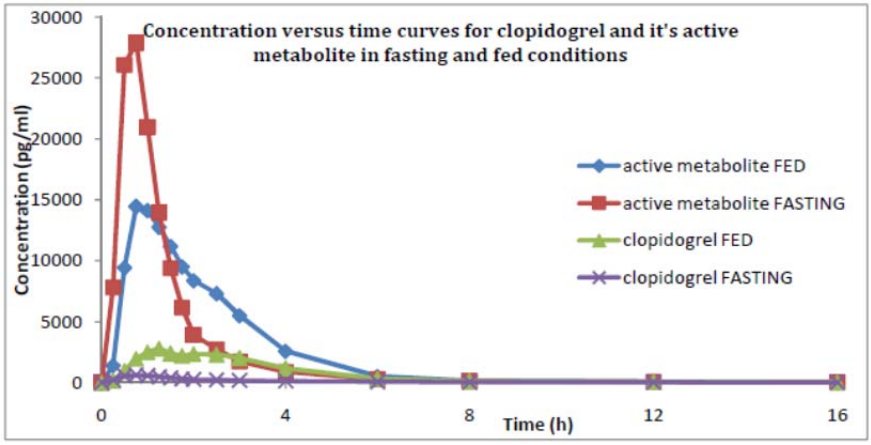


Figure 11. Concentration versus time curves for clopidogrel and its active metabolite in fasting and fed conditions.

For clopidogrel itself a significant increase is apparent with regard to both peak exposure (the ratio $C_{max\ FED}/C_{max\ FASTING}$ is 6.08) and extent (the ratio $AUC_{0\text{-}t\ FED}$ to $AUC_{0\text{-}t\ FASTING}$ is 5.75) of absorption in fed conditions, as already reported by Nirogi et al. in 2006.

For clopidogrel active metabolite, while administration in fasting conditions resulted in a peak exposure 60% higher than in fed conditions, the extent of exposure was almost identical (the ratio $AUC_{0\text{-}t\ FASTING}$ to $AUC_{0\text{-}t\ FED}$ is 1.022); in any case it is noteworthy that the increase of peak exposure and extent of exposure observed for clopidogrel didn't appear. The fact that food intake does not have a significant effect on total exposure of the active metabolite is in agreement with the pharmacodynamic findings that prompted the label claim stating that clopidogrel's effectiveness is not affected by food intake.

In conclusion, an overall summary of the proposed metabolic pathway for clopidogrel is shown in Figure 12. As it can be seen, new pathways have appeared (here represented in red color) compared to previous information available in 1998, at the time when clopidogrel was approved in the European Union. The following figures (13 and 14) and Table 5 resume the exposure parameters calculated for each metabolite (AUC and C_{max}) and the parent compound in a group of subjects receiving a standard clopidogrel dose (75 mg).

From these data it can be noticed that clopidogrel itself shows the lowest exposure, while the two oxidative metabolites, 2-oxo-clopidogrel and the

active metabolite, show 25-fold and respectively 38-fold higher values with respect to peak exposure and 26-fold and respectively 20-fold higher values for extent of exposure as compared to clopidogrel.

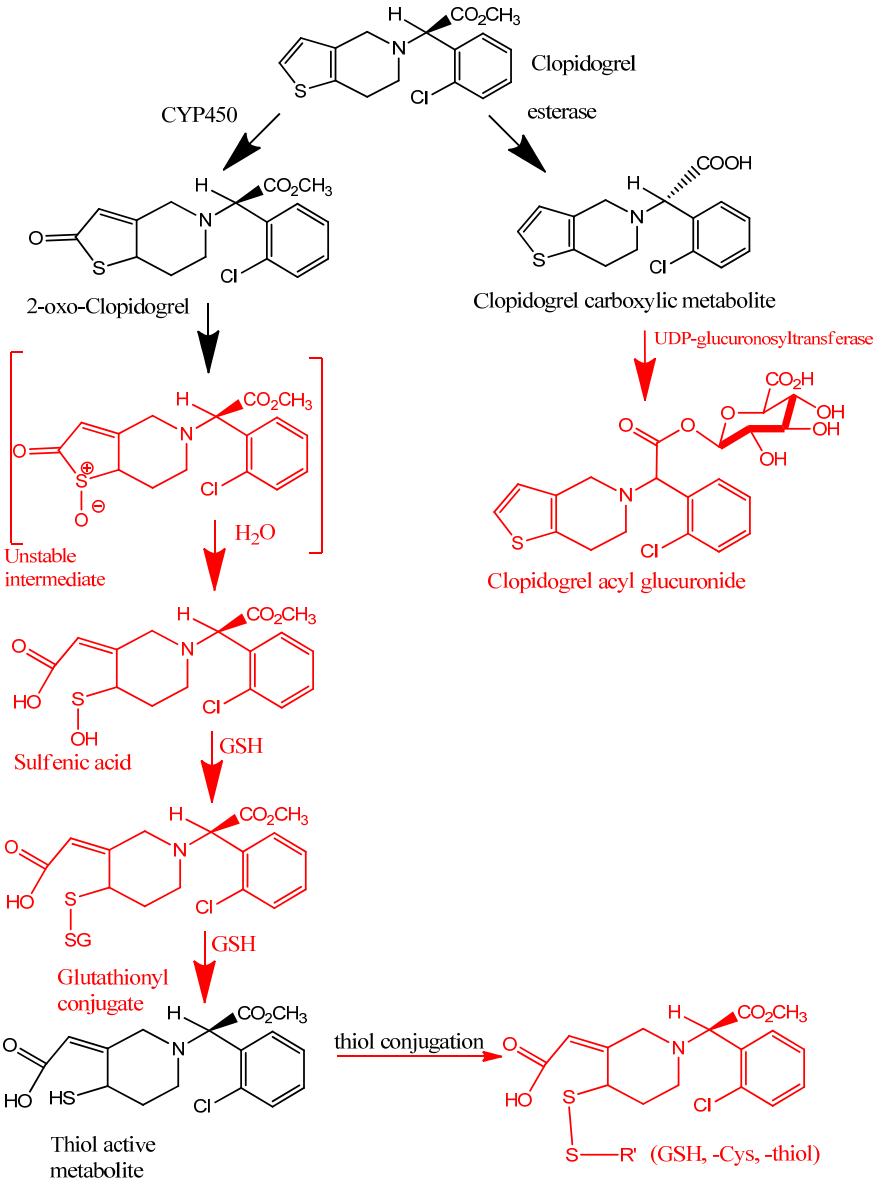


Figure 12. Clopidogrel metabolic pathway.

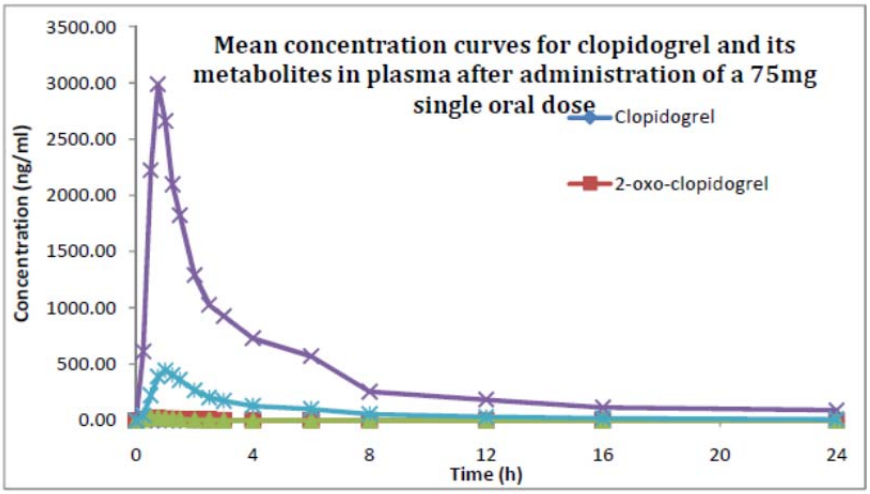


Figure 13. Linear presentation of mean PK curves for clopidogrel and metabolites.

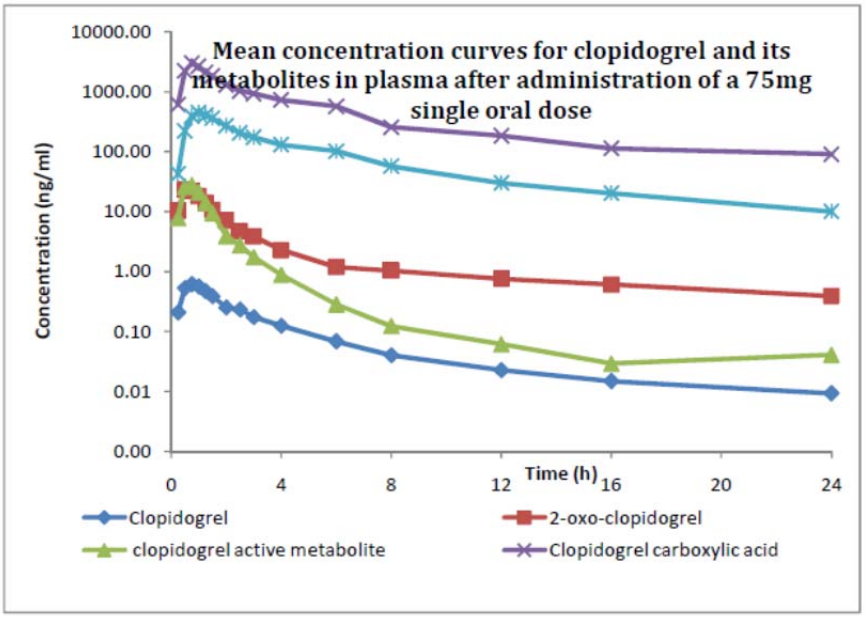


Figure 14. Semi-logarithmic presentation of mean PK curves for clopidogrel and metabolites.

Table 5. Mean PK parameters for clopidogrel and its metabolites after administration of a single 75mg oral dose in fasting conditions

Substance	Mean C_{max} (ng/ml)	Mean AUC_{0-t} (ng/ml *h)	Median T_{max} (h)	Median T_{half}
Clopidogrel (2008)	0.803	1.813	0.750	7.828
Clopidogrel active metabolite (2009)	30.915	35.383	0.750	1.143
Clopidogrel carboxylic acid (2010)	3164.835	10554.947	0.75	9.704
Clopidogrel acyl glucuronide (2010)	471.428	1706.764	1.000	6.845
2-Oxo-clopidogrel (2012)	20.390	47.353	0.750	1.800

The exposure of clopidogrel carboxylic acid and clopidogrel acyl glucuronide is definitely more significant, with C_{max} values 3940 and respectively 586 times higher than those of the prodrug and AUC_{0-t} values 5821 and respectively 941 times higher than those registered for clopidogrel itself. These data show that obviously the desesterification is a much faster and extensive metabolic reaction in comparison to the oxidative one.

9. POSSIBLE PD IMPACT OF CLOPIDOGREL PK

According to EMA's Q and A Paper of the Pharmacokinetics Working Party (EMA/618604/2008 Rev. 7), citing the work of Nirogi et al. (2006) as well as other unspecified studies reviewed, there are indications that in the fed state the bioavailability of a single oral dose of clopidogrel increases dramatically (500 - 600 %) while the systemic exposure to the major but inactive carboxylic acid metabolite increases only by approximately 10-20 %. The EWP-PK subgroup reviewed the solubility properties of clopidogrel salts and these indicate that when administration of clopidogrel occurs under fasting conditions, the dissolution in the gastric media with a subsequent hydrolysis and formation of the inactive carboxy-acid metabolite is maximal. As a consequence, the extent of unchanged drug that still is available for absorption (at the intestine level) is reduced. Conversely, the dissolution of clopidogrel is limited in the gastric media under fed conditions, the acidic hydrolysis in the stomach is reduced and the bioavailability of clopidogrel is improved (EMA/ 618604/2008).

As evidence would suggest, hepatic hydrolysis is saturable for clopidogrel while absorption from dosage form is not a limiting factor. Naturally, from a pharmacodynamic perspective, more important is the fate of the amount of circulating unchanged prodrug that remains available for oxidation to 2-oxo-clopidogrel and subsequent conversion to the thiol active metabolite. Based on the observations made by our group on yet unpublished data from fasting and fed bioequivalence studies on clopidogrel as well as on data published by Hurbin et. al (2012), it was concluded that, although exposure to clopidogrel increases dramatically in the fed state, extent of exposure (AUC) to the active thiol metabolite varies insignificantly. This important PK finding correlates well with the fact that clopidogrel active metabolite formation exhibits a plateau behavior (as apparent in the work of von Beckerath et al. 2005) that results also in a pharmacodynamic dose ceiling effect, as tripling the dosing from a regular loading dose of 300 mg to 900 mg produces only 60% inhibition of ADP induced platelet aggregation (Patent application EP 2540729 A1).

In conclusion, highly increased exposure to clopidogrel, either be it by increased bioavailability due to administration in fed state or by overdosing, does not result in significantly higher rate of metabolite formation, nor does it linearly correlate with increased effectiveness. Could the discrepancies between the levels of prodrug available and the thiol metabolite formed be explained by the fact that the first oxidative step involved in the metabolism of clopidogrel is saturable? Studies to provide evidence of that effect would be welcomed in the future and it might also be interesting to follow-up on the clinical developments in the case of 2-oxo-clopidogrel, which has been proposed as an anti-thrombotic medication itself in a recent patent application (EP 2540729 A1).

A work of Bouman et al. in 2009 is one of the best investigations on the PK/PD interaction for clopidogrel. In this paper, aimed in reality to evaluate the appropriateness of different platelet aggregation assessment methods, the authors have correlated the PK of clopidogrel active metabolite with a series of these tests and found a good correlation for some of them.

It is so far clear that PK data of the active metabolite can be a good predictor of therapeutic activity, but more work is needed to identify an adequate threshold for plasma concentrations that could serve for therapeutic drug monitoring. Despite the wider availability of mass spectrometers in clinical chemistry laboratories in the last years, the determination of clopidogrel active metabolite remains quite complex, especially due to the problems of sample stability, limiting its application.

CONCLUSION

As shown in the previous paragraphs a lot of progress has been done in the clarification of clopidogrel PK and metabolism; the situation is now adequate to perform in each subject a suitable therapeutic drug monitoring that should be most probably focused on the active metabolite. It seems interesting to evaluate this approach of therapeutic optimization, beside genotyping, due to the fact that patients often receive clopidogrel as part of complex therapies with several drugs, each influencing differently the metabolism of clopidogrel. In these conditions it is not always possible to replace all treatments with molecules without metabolic interactions and the genotype on the other side can only predict a risk, without assessing the real impact of such complex metabolic interactions.

Beside this aspect of individualized therapy, it is clear that high-tech analytical capabilities will be of help in the development of new clopidogrel derivatives and also in the optimized use of new drug combinations.

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