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Review

Quantitative determination of clopidogrel and its metabolites in biological samples: A mini-review

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ABSTRACT

Clopidogrel has been applied in antiplatelet therapy since 1998 and is the thienopyridine with the largest clinical experience. By 2011, clopidogrel (Plavix®) was the second top-selling drug in the world. Following complete patent expiry in 2012/2013 its use is expected to grow even further from generics entering the market. Prefaced by a brief description of clopidogrel metabolism, this review analyzes analytical methods addressing the quantification of clopidogrel and its metabolites in biological samples. Techniques that have been applied to analyze human plasma or serum are predominantly LC–MS and LC–MS/MS. The lowest level of clopidogrel quantification that has been achieved is 5 pg/mL, the shortest runtime is 1.5 min and almost 100% recovery has been reported using solid-phase extraction for sample preparation.

Contents

1. Introduction

Clopidogrel, (+)-(*S*)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl)acetate, along with prasugrel, ticlopidine, and ticagrelor, is part of a group of antiplatelet drugs that target $P2Y_{12}$ adenosine diphosphate (ADP) receptors [1]. As potent antiplatelet and antithrombotic drugs, members of this group have been approved and marketed globally for the reduction of atherosclerotic events in patients, especially in the presence of comorbid conditions like ischemic stroke, myocardial infarction and cardiovascular disease. Of these, the thienopyridines clopidogrel, prasugrel, and ticlopidine are prodrugs that require metabolization to pharmacologically active thiol intermediates, which lead to irreversible inhibition of $P2Y_{12}$ ADP receptors [2]. In contrast, ticagrelor, cangrelor, and elinogrel, the latter two still in development, act as reversible inhibitors of $P2Y_{12}$ ADP receptors and do not require prior metabolic activation [4].

In the first of two metabolization steps the thiophene ring of clopidogrel is subject to a monooxygenation by hepatic cytochrome P450 isozymes (mainly CYP2C19) producing the corresponding 2-oxo-clopidogrel intermediate, a thiolactone. In the second step this thiolactone is further oxidized by cytochrome P450 isozymes, then undergoes a hydrolytic ring-opening and finally is subjected to a glutathione-dependent reduction giving the active thiol metabolite (Fig. 1) [3]. The pharmacodynamic activity of clopidogrel originates solely from this thiol metabolite, which accounts for approximately 10% or less of all metabolites [2]. Besides this CYP dependent metabolic pathway recent reports have discussed the contribution of paraoxonase-1 (PON-1) to clopidogrel activation [3,5,6]. However, initial assumptions that PON-1 provides a major contribution to the bioactivation and thus clinical activity of clopidogrel have been refuted.

Inhibition of $P2Y_{12}$ ADP receptors, either reversibly or irreversibly, suppresses platelet aggregation by blocking activation of glycoprotein IIb/IIIa. Upon activation, glycoprotein IIb/IIIa is subjected to a conformational change resulting in enhanced affinity to

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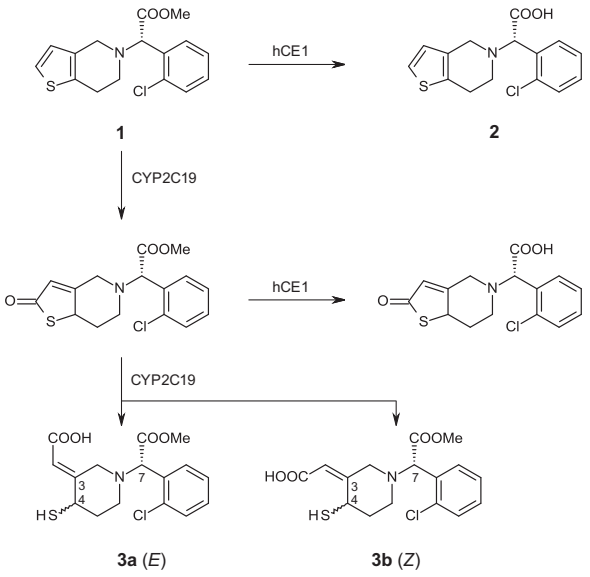

Fig. 1. Clopidogrel metabolism: structures of clopidogrel (**1**), its inactive (**2**) and active metabolite in *E*- (**3a**) or *Z*-configuration (**3b**), as well as of the 2-oxo intermediates (adapted from [2]).

fibrinogen and other adhesion molecules like von Willebrand factor, vitronectin and fibronectin [7]. Thus inhibition of glycoprotein IIb/IIIa activation reduces the risk of excess/uncontrolled platelet aggregation or thrombosis, *e.g.*, atherosclerotic events.

Inactivation of clopidogrel results from ester cleavage by serum and intestinal esterases, especially hCE1, of either clopidogrel or 2-oxo-clopidogrel. Clopidogrel carboxylic acid is the major metabolite at approximately 90% [8], and is partially converted to the corresponding acyl glucuronide [9].

2. Methods for determination of clopidogrel and clopidogrel carboxylic acid

Analysis of human samples has been dedicated to the elucidation of possible metabolites and the investigation of clopidogrel pharmacodynamics. Most published reports describe the quantification of clopidogrel and its inactive metabolite, clopidogrel carboxylic acid. A first method addressing the latter was developed at Sanofi–Aventis and published in 1998, the same year that clopidogrel entered the market [10]. This GC–MS based assay remains the only GC method published until today, because LC–MS or LC–MS/MS techniques more suitable for clopidogrel and its metabolites were developed. In all, 24 literature reports using either LC–UV [11], LC–MS [12–14] or LC–MS/MS [9,15–33] based methods have been published. These methods differ with respect to the internal standards used, the sample preparation applied, the overall runtime required and, most importantly, the analytes of interest (Table 1).

After isotopically labeled reference compounds (*i.e.* clopidogrel-d_3/-d_4 or [$^{13}C_6$]-clopidogrel carboxylic acid/-d_4), ticlopidine is the next most common internal standard. As isotopically labeled reference compounds can only be used in MS hyphenated techniques capable of discriminating between analytes and such internal standards, other reference compounds are needed for non-mass spectrometric detection. Examples of the latter that have been applied to clopidogrel analysis belong to a diverse group of compounds more or less related to clopidogrel; diltiazem, atorvastatine, repaglinide, and gliclazide. Yet, only atorvastatine has been used in a non-mass spectrometric assay [11].

Run times of clopidogrel analysis vary depending on the applied analytical technique. Whereas LC–MS and LC–MS/MS results are less susceptible to flaws by coeluting drugs or matrix components, quantification by UV detection needs careful separation of interferences and therefore usually requires longer runtimes. Accordingly, all reports of runtimes <5 min (43%) apply either LC–MS or LC–MS/MS, followed by further MS hyphenated methods requiring 5–10 min (35%) and a smaller group including UV detection based techniques taking between 10 and 20 min (22%). Six reports require a runtime < 2 min and five of these apply simple protein precipitation for sample preparation [9,15,17,21,24]. These methods can be considered ideal for the analysis of high sample numbers.

Sample preparation has been performed three different ways. The highest recovery rates of clopidogrel and clopidogrel carboxylic acid were observed with solid-phase extraction (average: 88%, maximum: 98%), followed by liquid-liquid extraction (average: 83%, maximum: 95%), and protein precipitation (average: 73%, maximum: 81%). All methods referred to above quantify clopidogrel and/or its metabolites in plasma or serum. Until now, only one assay has been described for the analysis of clopidogrel and its inactive metabolite in urine, where capillary zone electrophoresis coupled to UV detection was applied [34].

Sensitivity of clopidogrel and clopidogrel carboxylic acid analysis has been oriented toward their physiologically relevant concentrations, which is why quantification levels of the inactive metabolite are usually three orders of magnitude higher than those of clopidogrel. Especially for pharmacodynamic investigations quantification levels of clopidogrel must be as low as possible to accurately determine its terminal elimination rate constant and half-life from the elimination phase. Three reports have reached a lower limit of clopidogrel quantification of 5 pg/mL [17,19,31].

Only one report addresses the stereochemistry of inactive metabolite formation. Chiral inversion of the inactive metabolite, *i.e.*, formation of (*R*)-clopidogrel carboxylic acid instead of the (*S*)-enantiomer, has been observed at a rate of 4–8% but was shown to not occur as a result of enzymatic cleavage [35].

Recent findings revealed that the phase II acyl glucuronide of the inactive metabolite may be reconverted (back-conversion) into clopidogrel by transesterification in the presence of methanol [9,17]. Other alcohols, such as ethanol or 2-propanol, were also found to participate in transesterification of the metabolite, but to a lesser extent. These results indicate that methanol must be avoided during workup of clopidogrel samples and acetonitrile should be preferred.

3. Methods for determination of the active metabolite

Because of its very low plasma concentrations, the thiol-derived active metabolite of clopidogrel, 2-((3*Z*)-1-((1*S*)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl)-4-sulfanylpiperidin-3-ylidene)acetic acid, has only been quantified in recent years as sufficiently sensitive instrumentation has become available [15,18,21,28]. Because of its insufficient stability determination of the unmodified active metabolite provided only semiquantitative results [32]. However, derivatization of the free thiol group by alkylation with 2-bromo-3′-methoxyacetophenone prevented disulfide conjugation to glutathione or other endogenous thiols and ensured reliable results (Fig. 2) [28]. This derivatization has been applied in combination with either solid-phase extraction or protein precipitation and is usually carried out prior to sample workup obtaining alkylation rates exceeding 90% [28].

Ring opening as a result of metabolization gives rise to four diastereomers; 3*Z*,4*S*,7*S*, 3*Z*,4*R*,7*S*, 3*E*,4*S*,7*S* and 3*E*,4*R*,7*S* configurations (see Fig. 1 for atom numbering). Only one of these diastereomers shows physiological activity and was determined to possess *Z*-configuration, although its absolute configuration, 3*Z*,4*S*,7*S* or

Table 1
Reported methods for the quantification of clopidogrel (1), its inactive (2) and active metabolite (3); grouped by analytical techniques and descending years of publication.

Method[a]	Matrix	Analyte[b]	Linearity range [ng/mL]	Approx. run time [min]	Sample volume [mL]	Sample preparation[c]	Inter-day precision [%]	Inter-day accuracy [%]	Recovery [%]	Correlation coefficient	Internal standard	Additional validation experiments[d]
Peer et al. [15] LC–MS/MS	Human plasma	1 3	0.01–50 0.1–150	1.5	0.05	PP	n.r.	88.3–97.6 93.2–97.8	n.r.	0.9945 0.9978	Ticlopidine	1,2a,d
El-Sadek et al. [16] LC–MS/MS	Human plasma	2	25–3000	5	n.r.	LLEx	1.4–5.5	93.1–96.9	67.6–73.5	>0.999	Clopidogrel-d_4 carboxylic acid	1,2a,3
Silvestro et al. [9] LC–MS/MS	Human plasma	1 2	0.02–3.6 20–3600	2	0.2	PP	9.2–11.6 7.2–10.6	95.8–100.6 91.6–104.7	n.r.	>0.99	Clopidogrel-d_3 [$^{13}C_6$]-clopidogrel carboxylic acid	1,2a,b,d
Tuffal et al. [18] LC–MS/MS	Human plasma	3	0.5–250	8	0.2	SPE	2.8–6.4	101.1–107.4	97.4–129	n.r.	4′-Bromoaceto-phenone derivative	1,2a-e,3,4,5
Zou et al. [23] LC–MS/MS	Rat plasma	1 2	0.01–5 20–4000	n.r.	0.2	LLEx	6.18–8.44 5.50–6.30	99.88–106.66 93.14–109.99	89.8–96.0 89.4–93.5	>0.99	Ticlopidine Atorvastatin	1,2a-e
Reddy et al. [20] LC–MS/MS	Human plasma	1 2	0.1–8 70–6000	7.5	0.5	BD	1.0–7.6 0.6–12.2	95.5–103.3 91.5–101.8	94 88	>0.995	Ticlopidine	1,2a-e
Silvestro et al. [17] LC–MS/MS	Human plasma	1	0.005–2	2	n.r.	PP, SPE	2.7–12.2	96.5–102.4	73.6–80.5	>0.99	Clopidogrel-d_3	1,2a,b,d,e
Delavenne et al. [21] LC–MS/MS	Human plasma	3	1–150	1.5	0.2	PP	5.7–10.2	101.7–107-5	n.r.	0.998	Clopidogrel-d_4	1
Di Girolamo et al. [22] LC–MS/MS	Human serum	1	0.01–10	2	10	LLEx	3.8–6.9	95.2–102.3	81.7–89.4	>0.999	Ticlopidine	1,2
Zou et al. [19] LC–MS/MS	Human plasma	1 2	0.005–5 20–2500	5.6	0.5	LLEx	6.5–7.3 5.3–7.0	96.7–110.0 104.1–108.7	72.2–89.3 72.2–88.9	0.9947 0.9975	Nateglinide Pioglitazone	1,2a,b,d,e
Yang et al. [24] LC–MS/MS	Dog plasma	1	0.035–0.285	2	0.1	PP	3.01–6.32	85–115	64.0–73.7	>0.9955	Gliclazide	1,2a-d
El Ahmady et al. [25] LC–MS/MS	Human plasma	1 2	0.012–12 33–11000	n.r.	0.1	PP	n.r.	n.r.	n.r.	0.9973 0.9959	Clopidogrel-d_4 Atorvastatine	1,2a,b
Kim et al. [26] LC–MS/MS	Human plasma	1 2	0.025–20 50–25000	n.r.	0.1	LLEx, PP	4.4–9.7 5.5–8.6	n.r.	n.r.	0.9998 0.9992	Methaqualone	n.r.
Mani et al. [12] LC–MS	Human plasma	1 2	0.1–50 10–3000	12	0.5	SPE	3.6–9.2 8.3–16.1	n.r.	n.r.	n.r.	n.r.	n.r.
Patel et al. [27] LC–MS/MS	Human plasma	1 2	0.25–25 50–6000	5	0.3	SPE	2.12–10.18 2.80–6.10	100.0–102.4 106.1–110.0	97.5–99.0 83.5–89.1	>0.9989 >0.9984	Glimepiride	1,2a,b,d,e,3
Takahashi et al. [28] LC–MS/MS	Human plasma	3	0.5–250	5	0.4	SPE	0.4–3.8	92.1–99.0	85.1–93.7	0.9998	4′-Bromoaceto-phenone derivative	1,2b,3
Robinson et al. [29] LC–MS/MS	Human plasma	1	0.01–12	3	0.3	LLEx	2.2–7.2	99.3–103.0	54.9–76.0	0.9993	Clopidogrel-d_3	1,2a–d,3
Shin & Yoo [30] LC–MS/MS	Human plasma	1	0.01–10	3	0.5	LLEx	4.4–8.1	98.4–103.5	83.9–90.6	>0.999	Ticlopidine	1,2a–e
Ksycinska et al. [13] LC–MS	Human plasma	2	20–3000	12	0.25	LLEx	2.4–5.6	99–108	85–90	0.9999	Repaglinide	1,2a,b,d,e
Nirogi et al. [31] LC–MS/MS	Human plasma	1	0.005–6	2.5	0.5	LLEx	1.9–11.1	89.8–102.4	74.9–78.7	>0.9993	Ticlopidine	1,2a,b,d

Table 1 (*Continued*)

Method[a]	Matrix	Analyte[b]	Linearity range [ng/mL]	Approx. run time [min]	Sample volume [mL]	Sample preparation[c]	Inter-day precision [%]	Inter-day accuracy [%]	Recovery [%]	Correlation coefficient	Internal standard	Additional validation experiments[d]
Taubert et al. [32] LC–MS/MS	Human plasma	1	0.5–100	13.5	n.r.	PP	n.r.	n.r.	n.r.	>0.993	Diltiazem	n.r.
		2	500–150000									
Mitakos & Panderi [14] LC–MS	Human plasma	2	100–1000	6.5	0.8	SPE	0.9–3.1	90.4–101.2	66.5–83.2	0.9998	Sulphafurazole	3
Lainesse et al. [33] LC–MS/MS	Human plasma	1	0.02–10	3.5	n.r.	LLEx	< 4.8	103.1–109.3	>83.3	0.9980	n.r.	n.r.
Singh et al. [11] LC–UV	Rat plasma	2	125–32000	20	0.18	LLEx	3.8–5.5	99.5–100.12	85.8–88.5	>0.9983	Atorvastatin	1,2a–e,3
Karaźniewicz-Łada et al. [34] CZE–UV	Human plasma	1	500–10000	12	1	SPE	4.52–11.8	99.1–110.0	81–89	0.9998	Piroxicam	1,2a,b,d,5
	Human urine	2	250–20000				1.15–10.32	88.0–104.0	85–96	0.9996		
Lagorce et al. [10] GC–MS	Human plasma Human serum	2	5–250	8	1	LLEx, SPE	4.7–15.8	94.1–112	48.2–55.6	0.996	n.r.	1,2b,3,5

n.a.: not applicable, n.r.: not reported.

[a] CZE: capillary zone electrophoresis.

[b] Clopidogrel: 1, inactive metabolite (clopidogrel carboxylic acid): 2, active metabolite (2-((3*Z*)-1-((1*S*)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl)-4-sulfanylpiperidin-3-ylidene)acetic acid): 3.

[c] BD: buffer dilution, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid-phase extraction.

[d] 1: specificity; 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), room temperature (e)); 3: dilution; 4: influence of hemolyzed plasma; 5: interference of drugs.

Fig. 2. Alkylation of the clopidogrel active metabolite with 2-bromo-3′-methoxyacetophenone protects the thiol function from conjugation to endogenous compounds like glutathione or cysteine side chains.

3*Z*,4*R*,7*S*, remained unresolved [36]. However, only one method has been reported that is capable of separating all diastereomers after solid-phase extraction from human plasma [18]. Of all methods quantifying the active metabolite, only one recent report allows simultaneous quantification of clopidogrel and its active metabolite. Unfortunately, this method lacks separation of the metabolite diastereomers [15].

4. Conclusion

Analysis of clopidogrel and its metabolites in biological samples is already supported by a broad array of analytical techniques, from more simple LC–UV approaches to very sophisticated LC–MS/MS methods. Depending on the investigation topic, a suitable method is available. Future developments should include the identification of further back-conversion phase II metabolites as well as stereoselective assays quantifying all relevant active metabolites in the presence of the parent compound.

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