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Identification of alcohol-dependent clopidogrel metabolites using conventional liquid chromatography/triple quadrupole mass spectrometry

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Abstract

RATIONALE—Clopidogrel (CLO) is a prodrug used to prevent ischemic events in patients undergoing percutaneous coronary intervention or with myocardial infarction. A previous study found ethyl clopidogrel (ECLO) is formed by transesterification of CLO when incubated with alcohol in human liver microsomes. We hypothesize that ECLO will be subject to further metabolism and developed an assay to identify its metabolites.

METHODS—A liquid chromatography/triple quadrupole mass spectrometry (LC-MS/MS) method was developed to identify metabolites of ECLO. According to the predicted metabolic pathway of ECLO, precursor–product ion pairs were used to screen the possible metabolites of ECLO in human liver S9 fractions. Subsequently, the detected metabolites were characterized by the results of product ion scan.

RESULTS—In the presence of alcohol, CLO was tranesterified to ECLO, which was further oxidized to form ethylated 2-oxo-clopidogrel and several ethylated thiol metabolites including the ethylated form of the H4 active metabolite.

CONCLUSIONS—The ECLO formed by transesterification with alcohol is subject to metabolism by CYP450 enzymes producing ethylated forms of 2-oxo-clopidogrel and the active H4 thiol metabolite.

Keywords

clopidogrel; alcohol; liquid chromatography; triple quadrupole mass spectrometer; carboxylesterase

Introduction

Clopidogrel (CLO) is an antiplatelet prodrug used in a variety of cardiovascular disorders including acute coronary syndromes and in patients undergoing percutaneous coronary intervention.^[1] The CLO metabolic pathway in humans is summarized in Figure 1.

Clopidogrel itself is pharmacologically inactive and must be metabolized by cytochrome

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P450 (CYP450) enzymes to a highly unstable thiol metabolite, which is the active moiety that wields the antiplatelet activity.^[2, 3] The majority of CLO (~85%) is hydrolyzed by carboxylesterase-1 (CES1) to form inactive clopidogrel acid (CLOA).^[4–7] This hydrolysis pathway competes with active metabolite formation catalyzed by hepatic CYP450 enzymes.^[2, 3, 6] Clopidogrel's metabolism by CYP450 enzymes forms the inactive intermediate 2-oxo-clopidogrel (OCLO), which is further metabolized by CYP450 enzymes to three thiol metabolites in humans of which only the H4 metabolite has antiplatelet activity.^[8, 9]

Because of the competition between pathways for inactive and active metabolite formation, the resulting *in vivo* plasma concentrations of the H4 active metabolite and subsequent antiplatelet activity are susceptible to changes in both CES1 and CYP450 enzyme activity. For example, reduced CYP2C19 activity decreases H4 production resulting in an attenuated antiplatelet effect while loss-of-function CES1 polymorphisms reduce hydrolysis resulting in increased substrate available for CYP450-mediated H4 formation and enhanced platelet inhibition.^[4, 10]

Alcohol (ethanol) inhibits CES1 hydrolysis and results in transesterification (e.g. exchange of the ethyl group of alcohol with a methyl ester) of some CES1 substrate drugs, most notably cocaine and methylphenidate.^[11–14] In the presence of alcohol, clopidogrel is also subject to transesterification by CES1 (Figure 1) *in vitro* resulting in the formation of ethyl clopidogrel (ELCO).^[15] Given the minor structural change resulting from transesterification (changing a methyl to an ethyl ester), we speculated that the transesterified ELCO product will be susceptible to metabolism by the same CYP450 enzymes as CLO, producing ethylated forms of OCLO and the active H4 metabolite.

Given the widespread consumption of alcohol, the high probability of co-ingestion with clopidogrel, and the potential for formation of uncharacterized metabolites with unknown pharmacological activity, it is essential to determine the effects of alcohol on clopidogrel metabolism. This report describes the identification of ECLO metabolites in hepatic S9 fractions utilizing a new liquid chromatography/mass spectrometry method, and is seen as an initial step in elucidating the interaction between alcohol and clopidogrel.

EXPERIMENTAL

Materials

(S)-(+)-clopidogrel (CLO), clopidogrel acid (CLOA), 2-oxo-clopidogrel (OCLO, mixture of diastereomers), 2-oxo-clopidogrel acid (OCLOA, mixture of diastereomers), 2-bromo-3'-methoxyacetophenone (MP) derivatized clopidogrel active metabolite (MP-H4), and ethyl clopidogrel (ECLO) were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). 2-bromo-3'-methoxyacetophenone (MP) and L-Glutathione reduced (GSH) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). LC-MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was prepared with an in-house Milli-Q Advantage A10 Ultrapure water purification system (Bedford, MA, USA). Human liver S9 (HLS9) fractions (150 pooled donors of mixed sex) were

obtained from BD Gentest (San Jose, CA). Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate monosodium salt (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were obtained from Sigma-Aldrich (St. Louis, MO).

LC-MS/MS analyses

An AB SCIEX 3000 triple quadrupole mass spectrometer (Redwood City, CA) interfaced via a turbo ion spray (ESI) source with an Agilent 1100 HPLC module (Santa Clara, CA) was used for metabolite profiling of clopidogrel. The LC separation for both initial targeted detection and final product ion detection was achieved on a 3.5 μ m Waters XSelect CSH C18 column (100 mm $\times$ 2.1 mm I.D.; Milford, MA) at 24°C according to previous reports with minor modification^{[16, 17][15]}. Mobile phases were acetonitrile/water, 1:99 (v/v), containing 2.5 mM formic acid for A, and acetonitrile/water, 99:1 (v/v), modified with the same electrolyte for B. Separation was optimized using a gradient method with mobile phase A/B set to 95%/5% from 0.00 to 0.10 min and 28%/72% from 0.11 to 5.00 min and then back to 95%/5% from 5.01 to 8.00 min.

Using chemical standards, ionization conditions were optimized to maximize generation of the singly protonated ions and to produce the characteristic product ions for the compounds. The parent-product ion pairs used for multiple reaction monitoring (MRM) of CLO, CLOA, OCLO, OCLOA, MP-H4, and ECLO were m/z 322 $\rightarrow$ 212, 308 $\rightarrow$ 198, 338 $\rightarrow$ 155, 324 $\rightarrow$ 169, 504 $\rightarrow$ 155, and 336 $\rightarrow$ 226, respectively. The parameters of the ESI source (DP = 26 to 40, FP = 190 to 260, Temperature = 500 °C, Curtain Gas = 8, Nebulizer Gas = 12, Ion Spray Voltage = 5000, and CAD = 4) were optimized under the above-mentioned chromatographic conditions. The LC eluent was introduced to the ESI source over the period of 2.5–5.6 min at a flow rate of 0.30 mL/min.

In vitro metabolism of CLO (with and without alcohol) and ECLO (without alcohol) in HLS9 fractions

The metabolism of CLO in incubations containing HLS9 fractions, NADPH generating system (0.5 U/mL of G-6-PDH, 1.3 mM of NADP, 3.3 mM G-6-P), and GSH (50 mM) was tested at 37°C. Assays were conducted in duplicate in 96-well cluster tubes with a total assay volume of 100 μ L in each well. The assay buffer was 0.1 M potassium phosphate, pH 7.4. Incubation times were 120 min. The HLS9 protein and substrate concentrations in the incubations were 4.0 mg/mL and 20 μ M, respectively. The alcohol concentrations in the incubations were 0 or 200 mM. The final concentration of acetonitrile (the substrate solvent) was not greater than 0.1% for all assays. Assays were initiated by adding the substrate/buffer mixture (with or without alcohol) to the HLS9/NADPH/buffer mixture (50 μ L). A negative control (buffer substituted for HLS9 mixture) was included to assess the chemical stability of CLO in buffer at 37°C. The reaction was terminated by the addition of an equal volume (100 μ L) of ice-cold acetonitrile containing 5 mM MP (used as the derivatizing agent to stabilize highly unstable thiol metabolites). After centrifugation at 16,000 $\times$ g for 5 min, 10 μ L of supernatant was injected onto the LC-MS/MS.

The conditions for the metabolism of ECLO in HLS9 fractions were similar to those for CLO. However, the ELCO concentration was 200 μ M and no alcohol was added to the incubations.

Identification of potential metabolites

A targeted screening method was used in the identification of new metabolites. Five potential metabolites of ECLO were predicted, i.e. a pair of diastereomers for ethyl 2-oxo-clopidogrel (EOCLO), ethyl MP-H endo (EMP-H endo), ethyl MP-H3 (EMP-H3), and ethyl MP-H4 (EMP-H4). The predicted parent-product ion pairs were m/z 352 $\rightarrow$ 125 for EOCLO diastereomers, and m/z 518 $\rightarrow$ 125 for EMP-H endo, EMP-H3, and EMP-H4. The predicted ion pairs were used in the MRM mode to search for all of the potential metabolites. Three different values of collision energy (CE) were tested for each ion pair (29, 34, and 39 for m/z 352 $\rightarrow$ 125; 50, 55, and 60 for m/z 518 $\rightarrow$ 125). The detected metabolites were confirmed and characterized by a product ion scan. The major parameters of the product ion scan for EOCLO diastereomers were: Parent ion = m/z 352, Scan range = m/z 50–355, and CE = 34 v. For the remaining metabolites, the parameters were: Parent ion = m/z 518, Scan range = m/z 50–520, and CE = 50 v.

RESULTS AND DISCUSSION

Detection of CLO metabolites in HLS9 fractions without alcohol

Two different chromatographic conditions were tested, a short runtime (8 min) and a long runtime (15 min). Though the long runtime showed better separation of the isomers than the short runtime, the sensitivity achieved with the long runtime was significantly lower than that achieved with the short runtime. Since separation of the isomers with the short runtime was adequate for the study purpose, this chromatographic condition was used in the study. The MRM-based chromatograms of HLS9 fractions spiked with standards of CLO, CLOA, OCLO, OCLOA, ECLO, and MP-H4 are shown in Figure 2. The chromatographic retention times and product ions of these compounds (with chemical standards) are shown in Table 1. A single peak was observed for CLO, CLOA, ECLO, and MP-H4 whereas two peaks were found for OCLO and OCLOA. This occurred because the OCLO and OCLOA standards each contained a pair of diastereomers rather than a single stereoisomer.

After incubation of CLO in HLS9 fractions (fortified with NADPH and GSH) without alcohol, CLOA, OCLO, and OCLOA were detected (Figure 3). Consistent with the published studies in humans, three thiol metabolites (MP-H endo, MP-H3, and MP-H4) were detected (Figure 3).^[8, 16, 17] In addition, OCLO and OCLOA showed double peaks that have the same chromatographic behaviors as those of OCLO/OCLOA chemical standards, indicating they probably share the same chemical structures. Considering that there are only two chiral carbons in the structure, the two diastereomers of OCLO/OCLOA are probably (7S, 4S) OCLO/OCLOA and (7S, 4R) OCLO/OCLOA, respectively (Figure 1). To our knowledge, this is the first study showing the existence of two diastereomers of OCLO/OCLOA in incubations of human liver fractions.

Identification of ethylated clopidogrel metabolites in HLS9 fractions

Incubation of CLO and alcohol in HLS9 fractions resulted in significant formation of the ethylated metabolite ECLO (Figure 3). Thus, our results are consistent with previous *in vitro* findings showing that CLO undergoes transesterification in the presence of alcohol and CES1.^[15] As discussed in the Introduction, clopidogrel and ECLO should have a similar metabolic pathway. Therefore, five potential metabolites of ECLO were predicted, including EOCLO (a pair of diastereomers), EMP-H endo, EMP-H3, and EMP-H4. According to the product ions of the chemical standards for CLO and its metabolites (Table 1), all the potential metabolites were predicted to have the same product ion of m/z 125 with high relative abundance. Therefore, the predicted parent-product ion pairs used in the MRM detection mode were 352.3→125.0 for EOCLO diastereomers, and 518.1→125.0 for EMP-H endo, EMP-H3, and EMP-H4. All the predicted metabolites were observed when CLO and 200 mM alcohol were co-incubated in HLS9 fractions (Figure 3). Alcohol concentrations of 17 mM is equal to 0.08 g/dl, which is commonly achieved in humans and the legal intoxication limit in the US. Accordingly, we also tested the alcohol concentration of 12.5 and 25 mM. The same metabolites detected in incubations with 200 mM alcohol were also found in incubations containing 12.5 and 25 mM alcohol (see Figure S1 in Supporting Information).

To further characterize the chemical structures of the detected metabolites, ECLO was directly incubated in HLS9 and the resulting samples subjected to product ion scan (Figure 4). A summary of the product ions and chromatographic behaviors of these newly identified metabolites are shown in Table 1. It is noteworthy that a very low amount of CLO was detected when high concentration of ECLO was incubated in HLS9 (Figure 4). The peak area ratio of CLO/ECLO is only about 0.2%. We speculate this minimal amount of CLO is a result of incomplete reaction of CLO during the synthesis of ECLO.

The fragmentation patterns were predicted for the compounds with chemical standards, including CLO, CLOA, OCLO, OCLOA, ECLO, and MP-H4 (Figures 5–6). Then, the chemical structures of the newly identified alcohol-dependent metabolites were characterized and confirmed by comparing their respective production ions with those of the compounds with chemical standards (Figures 5–6). The fragmentation pattern of CLO (blue and pink lines in Figure 5) suggest the formation of two different m/z 212 fragments. The structures for the m/z 184 fragments from active H4 metabolite of CLO were proposed in a published study.^[2] However, the actual m/z for the reported structure of m/z 184 fragment is 185, suggesting the reported structure for m/z 184 fragment is not correct. We suggest m/z 184 fragment is double charged and formed from two m/z 183 fragments (see Figure 5). For the formation of m/z 141 fragment from CLOA, OCLOA, ECLO, and EOCLO, rearrangement may have occurred to form the formyl group (Figures 5–6).

Thus, our results show, for the first time, the comprehensive fragmentation patterns of CLO and its various metabolites. Furthermore, we describe a novel alcohol-dependent metabolic pathway for CLO. Our results confirm a previous study demonstrating, in the presence of alcohol, CLO undergoes transesterification by CES1 to form ECLO.^[15] Similar to the parent CLO compound, ECLO is metabolized by CYP450 enzymes to EOCLO then to the three

corresponding ethylated thiol metabolites EMP-H endo, EMP-H3, and EMP-H4. This transesterification of clopidogrel in the presence of alcohol is similar to that reported with the CES1 substrate drugs cocaine and methylphenidate in which each drug's methyl ester is exchanged for the corresponding ethyl ester resulting in ethylated metabolites that are also pharmacologically active.^[11, 14, 18, 19] Whether the effects of alcohol on CES1-mediated clopidogrel hydrolysis or the formation of these newly identified ethylated metabolites affects the pharmacological actions of clopidogrel and its efficacy and safety is unknown. Our results describing a method to identify these alcohol-dependent metabolites provide an initial basis to further investigate the interaction between clopidogrel and alcohol.

Though triple quadrupole mass spectrometry (MS/MS) is a commonly used analytical tool in pharmaceutical research for quantitative purposes (MRM detection), this instrument is not commonly used in metabolite profiling due to its poor sensitivity in full-scan mode (non-targeted mode). We have addressed this question by using a targeted detection method, which is rapid and sensitive.^[20] However, a product ion scan is still required to characterize the chemical structure of the metabolites that were initially found by the targeted detection. Considering the non-selective nature of a product ion scan, a long LC runtime is commonly used in the separation of metabolites from their isomers and/or interfering components. However, use of a long runtime is not only time-consuming but also results in significantly impaired detection sensitivity (decreased ionization efficiency because of decreased concentration of organic solvent in the mobile phase when a long chromatographic condition is utilized). In this study, we used a short runtime LC method for both the initial targeted detection and the final product ion scan.

CONCLUSIONS

In the present study, a simple and sensitive LC-MS/MS-based approach was developed to identify unique clopidogrel metabolites formed in the presence of alcohol. The comprehensive fragmentation patterns of CLO and its various metabolites were also characterized. The assay presented here can be applied to further investigate the interaction between alcohol and CLO in humans. The systemic exposures to alcohol-derived metabolites should be studied in humans to evaluate the potential safety issues when CLO and alcohol are co-ingested. In addition, further in vitro and in vivo studies are required to characterize which CYP450 enzymes are involved in the metabolism of ECLO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The metabolic pathway of clopidogrel in humans (black arrows). The blue arrow shows the effect of alcohol on clopidogrel metabolism. **CLO**, clopidogrel; **CLOA**, clopidogrel acid; **OCLO**, 2-oxo-clopidogrel; **OCLOA**, 2-oxo-clopidogrel acid; **H3/H4/H endo**, clopidogrel thiol metabolites; **ECLO**, ethyl clopidogrel.

Figure 2.

Multiple reaction monitoring (MRM)-based chromatograms of human liver S9 fractions spiked with standards of clopidogrel and its metabolites (EOLCO and EMP-H standards are not available). **CLO**, clopidogrel; **CLOA**, clopidogrel acid; **OCLO**, 2-oxo-clopidogrel (mixture of diastereomers); **OCLOA**, 2-oxo-clopidogrel acid (mixture of diastereomers); **MP-H4**, MP derivatized clopidogrel active metabolite; **ECLO**, ethyl clopidogrel; **EOCLO**, ethyl 2-oxo-clopidogrel; **EMP-H**, ethyl MP derivatized thiol metabolites.

Figure 3.

Multiple reaction monitoring (MRM)-based chromatograms of clopidogrel and its metabolites in human liver S9 fractions (fortified with NADPH and GSH) with or without alcohol. **CLO**, clopidogrel; **CLOA**, clopidogrel acid; **OCLO**, 2-oxo-clopidogrel (mixture of diastereomers); **OCLOA**, 2-oxo-clopidogrel acid (mixture of diastereomers); **MP-H endo/-H3/-H4**, MP derivatized clopidogrel thiol metabolites; **ECLO**, ethyl clopidogrel; **EOCLO**, ethyl 2-oxo-clopidogrel; **EMP-H endo/-H3/-H4**, ethyl MP derivatized thiol metabolites.

Figure 4.

Multiple reaction monitoring (MRM)-based chromatograms of ethyl clopidogrel and its metabolites in human liver S9 fractions (fortified with NADPH and GSH). **CLO**, clopidogrel; **CLOA**, clopidogrel acid; **OCLO**, 2-oxo-clopidogrel (mixture of diastereomers); **OCLOA**, 2-oxo-clopidogrel acid (mixture of diastereomers); **MP-H**, MP derivatized clopidogrel thiol metabolites; **ECLO**, ethyl clopidogrel; **EOCLO**, ethyl 2-oxo-clopidogrel; **EMP-H endo/-H3/-H4**, ethyl MP derivatized thiol metabolites.

Figure 5.

Proposed chemical structures for the product ions of clopidogrel and its metabolites. The duplicate chemical structures are not listed (only the structures that appear at the first time are listed)

Figure 6.

Proposed chemical structures for the product ions of alcohol-dependent clopidogrel metabolites. The duplicate chemical structures are not listed (only the structures that appear at the first time are listed)

Table 1

Summary of the product ions and chromatographic retention of Clopidogrel and its metabolites

Compound ^a	<i>t_R</i> (min)	Precursor ion ^b (<i>m/z</i>)	Product ions ^c (<i>m/z</i>)
CLO	4.71	322	125 , 139, 152, 155 , 183, 184 , 212
CLOA	2.80	308	125 , 141 , 152 , 169, 198
OCLO	4.17/4.22 ^d	338	125 , 139, 155 , 183 , 184, 212 , 278
OCLOA	2.77/2.82 ^d	324	125, 141 , 156 , 169 , 198 , 278
ECLO	5.14	336	125 , 141 , 152, 154, 169, 197, 198 , 226
MP-H endo	4.05	504	125, 155, 184, 212 ^e
MP-H3/H4	4.44/4.51 ^d	504	125 , 155 , 183 , 184, 212, 324, 354 , 444
EOCLO	4.49/4.56 ^d	352	125 , 141, 154, 169 , 197 , 198, 226, 278
EMP-H endo	4.27	518	125 , 154 , 169, 197, 198 , 226
EMP-H3/H4	4.77/4.86 ^d	518	125 , 154, 169, 197 , 198 , 226 , 338, 368, 458

^a **CLO**, clopidogrel; **CLOA**, clopidogrel acid; **OCLO**, 2-oxo-clopidogrel (mixture of diastereomers); **OCLOA**, 2-oxo-clopidogrel acid (mixture of diastereomers); **MP-H endo/-H3/-H4**, MP derivatized clopidogrel thiol metabolites; **ECLO**, ethyl clopidogrel; **EOCLO**, ethyl 2-oxo-clopidogrel; **EMP-H endo/-H3/-H4**, ethyl MP derivatized thiol metabolites.

^b [M+H]⁺ (bearing ³⁵Cl isotope) generated in the positive ion ESI mode.

^c Produced from the [M+H]⁺ by collision induced dissociation (CID). The product ions of relative abundance <5% and lower than *m/z* 125 are not shown. The product ions in bold are the four most abundant ions.

^d Two retention times are shown for a pair of diastereomers.

^e Due to the low concentration of MP-H endo formed in the incubation, the product ions of MP-H endo are based on the reported product ions^[3] and MRM detection results in Figure 3.