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Formation Of The Thiol Conjugates And Active Metabolite of Clopidogrel By Human Liver Microsomes

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Running title: Metabolism of clopidogrel by HLMs

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Nonstandard abbreviations: 2-oxo, 2-oxo clopidogrel; AM, active metabolite of clopidogrel; ASC, ascorbic acid; AUC, area under the curve; CYP or P450, cytochrome P450; DTT, dithiothreitol; EIC, extracted ion chromatogram; ESI-LC/MS, electrospray ionization liquid chromatography mass spectrometry; GSH, glutathione; HLM, human liver microsome; IS, internal standard; L-Cys, L-cysteine; NAC, N-acetyl-L-cysteine.

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ABSTRACT

We have previously reported the formation of a glutathionyl conjugate of the active metabolite (AM) of clopidogrel and the covalent modification of a cysteinyl residue of human cytochrome P450 2B6 in a reconstituted system (Zhang et al, *Mol. Pharmacol.* 80:839-847 (2011)). In this work, we have extended our studies of the metabolism of clopidogrel to human liver microsomes in the presence of four different reductants: GSH, L-cysteine (L-Cys), N-acetyl-L-cysteine (NAC) and ascorbic acid (ASC). Our results demonstrated that formation of the AM was greatly affected by the reductant used and the relative amount of the AM formed was increased in the order of NAC (17%) < L-Cys (53%) < ASC (61%) < GSH (100%). In addition, AM-thiol conjugates were observed in the presence of NAC, L-Cys, and GSH. In the case of GSH, the formation of both the AM and the glutathionyl conjugate were dependent on the concentrations of GSH with similar K_m values in the range of ~0.5 mM, indicating that formation of the thiol conjugates constitutes an integral part of the bioactivation processes of clopidogrel. It was observed that the AM was slowly converted to the thiol conjugate with a $t_{1/2}$ of ~10 hrs. Addition of dithiothreitol to the reaction mixture reversed the conversion resulting in a decrease in the AM-thiol conjugate with a concomitant increase in the AM, whereas addition of NAC led to formation of the AM-NAC with a concomitant decrease in AM-GSH. These results not only confirm that the AM is formed through oxidative opening of the thiolactone ring, but also suggest the existence of an equilibrium between the AM, the thiol conjugates and the reductants. These factors may potentially affect the effective concentration of the AM in vivo.

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INTRODUCTION

Clopidogrel (Plavix) is commonly prescribed to reduce cardiovascular events in patients with acute coronary syndromes, particularly among those undergoing percutaneous coronary interventions because of its antiplatelet activity. Clopidogrel prevents platelet aggregation by irreversible inhibition of the platelet $P2Y_{12}$ receptor through covalent modification of the cysteinyl residues of the $P2Y_{12}$ receptor (Algaier et al., 2008; Ding et al., 2003; Savi et al., 2006). It is well established that clopidogrel is bioactivated to its pharmacologically active metabolite (AM) by hepatic cytochromes P450 (P450s or CYPs) enzymes (Kazui et al., 2010; Savi et al., 1992). Specifically, clopidogrel is bioactivated through two sequential oxidative processes as elegantly elucidated by Dansette and coworkers (Dansette et al., 2009; Dansette et al., 2011; Dansette et al., 2012). In the first step, clopidogrel is mono-oxygenated to give 2-oxo clopidogrel which has no antiplatelet activity. 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 opening of the thiolactone ring to form a glutathionyl conjugate followed by a thiol-disulfide exchange to form the AM. The reaction scheme is illustrated in Figure 1. Recent studies by Dansette et al (Dansette et al., 2011; Dansette et al., 2012) demonstrated that the AM produced by P450s possesses a *cis* exocyclic double bond at C3-C16, while hydrolysis of 2-oxo clopidogrel by the esterase PON1 leads to formation of an *endo* isomer, an inactive form. It is generally thought that the reactive thiol group of the active metabolite covalently modifies the cysteinyl residues of the platelet $P2Y_{12}$ receptor leading to the prevention of platelet aggregation. It is currently unknown whether the glutathionyl conjugate of clopidogrel exhibits antiplatelet activity.

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Although widely used as an antiplatelet agent, clopidogrel has shown significant interindividual variability in drug response. Lack of response or "resistance" to clopidogrel therapy may be seen in as high as ~45% of the patient population (Mason et al., 2005). A large body of pharmacogenetic and pharmacogenomic studies have been carried out in the past 5-6 years in attempts to identify genetic markers for this variable drug response. Many target genes have been indicated such as CYP2C19 (Collet et al., 2009; Hulot et al., 2006; Mega et al., 2009), CYP3A5 (Suh et al., 2006), ABCB1 (Taubert et al., 2006), the $P2Y_{12}$ receptor (Fontana et al., 2003), PON1 (Bouman et al., 2011), etc. Most of the studies on the CYP2C19 polymorphisms, but not all, have established a correlation between the variable drug response and a loss-of-function mutation in the CYP2C19 gene (CYP2C19*2) (Collet et al., 2009; Hulot et al., 2010; Mega et al., 2010; Simon et al., 2011). Correlations of the variable drug response with other genetic markers have not been established. However, all these studies have failed to account for the majority of the variable drug response. Even in the case of CYP2C19, CYP2C19*2 accounts for only ~12% of the overall variation in the response to clopidogrel therapy (Shuldiner et al., 2009). Therefore, other factors are most likely involved, but not yet identified.

These factors may be associated with the bioactivation of clopidogrel. In particular, from both the mechanistic and clinical points of view, some important questions regarding the formation of the AM and the glutathionyl conjugate have not yet been addressed adequately. For example, what are the factors that determine the concentration of the AM? Does the AM undergo thiol-disulfide exchange? Does the glutathionyl conjugate of the AM have antiplatelet activity? Answers to these questions

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will undoubtedly provide crucial information to fully understand the variable drug response to clopidogrel therapy.

Recently we reported the formation of a glutathionyl conjugate of the AM in a reconstituted system of CYP2B6, one of the key P450 isoforms involved in the bioactivation of clopidogrel (Zhang et al., 2011). In addition, we also observed that Cys 475 of CYP2B6 was covalently modified to form an AM-Cys conjugate. These observations prompted us to conduct further studies to understand the general reactivity of the AM. In this work, we have extended our previous studies to human liver microsomes (HLMs) to investigate the factors that affect the formation of the AM. Our results show that GSH, L-Cys, and NAC are all capable of forming AM-thiol conjugates and that formation of the AM is greatly affected by the identity of the reductants used. In addition, we observed thiol-disulfide exchange reactions between the AM, the conjugates and various reductants. These factors may potentially affect the effective concentration of the AM in vivo.

MATERIALS AND METHODS

Chemicals. HLMs pooled from 150 donors were purchased from BD Biosciences. Racemic 2-oxo clopidogrel hydrochloride and S-(+)-clopidogrel hydrogen sulfate were purchased from Toronto Research Company (Toronto, Canada). GSH, L-Cys, NAC, dithiothreitol (DTT), ascorbic acid (ASC), $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO).

Detection of the AM and the AM-thiol conjugates of 2-oxo clopidogrel formed by HLMs. To generate the AM and AM-thiol conjugates, the metabolic reactions were

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performed in 0.2 ml of 50 mM K phosphate solution (pH 7.4) containing 1 mg/ml HLM, 80 µM 2-oxo clopidogrel, 1 mM of each of the four reductants (GSH, NAC, L-Cys, or ASC), 0.1 M KF, 1 mM glucose-6-phosphate, and 1 mM $NADP^+$. KF was used to inhibit the activities of esterases; hydrolysis of the methylester clopidogrel to the carboxylic acid metabolite is fully inhibited by 0.1 M KF, whereas the esterase activity of PON1 is only partially inhibited at this concentration (Dansette et al., 2012). The catalytic reaction was initiated by the addition of 1 unit of glucose-6-phosphate dehydrogenase and then incubated at 37 ^oC for 30 min. The reaction was then terminated by the addition of 0.1 ml of 10% acetic acid in acetonitrile. For quantitative analysis, 200 pmoles of clopidogrel was spiked into the quenched samples as the internal standard (IS). The samples were then centrifuged at 16,000xg for 10 min to precipitate the protein aggregates and the supernatants were stored on ice. Aliquots of 50 µl of the supernatants were loaded into an ESI-LC/MS to analyze the AM and AM-thiol conjugates as described below.

Analysis of the AM and the AM-thiol conjugates of 2-oxo clopidogrel by ESI-LC/MS. The AM and AM-thiol conjugates were analyzed on a LCQ Deca XP ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The IS and metabolites were separated on a reverse phase C18 column (2x100 mm, 3 µm, 110 Å, Phenemonex, CA) using a binary mobile phase as reported by Dansette et al. (Dansette et al., 2012). The mobile phase consisted of 10 mM ammonium acetate (Solvent A) and a mixture of acetonitrile:methanol:water at a 7:2:1 ratio (Solvent B). The gradient used to elute metabolites and IS was as follows: 40% B for 4 min, linearly increasing to 100% B in 15 min and held at 100% B for 2 min. The flow rate was 0.2 ml/min. The mass

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spectrometer was tuned with 1 μM clopidogrel in 50% Solvent B in the positive electrospray ionization mode. The instrumental settings for the MS were as follows: heated capillary temperature, 200 ^{o}C; Spray voltage, +4.5 kV; sheath gas flow, 60; auxiliary gas, 20. The precursor ions were scanned from m/z 300-700, while the MS^2 spectra were obtained using the dependent scan mode where the four most abundant ions were fragmented by collision induced dissociation at 35% energy level and the fragment ions were recorded from m/z 100 to 700.

Determination of the K_m and V_{max} values for the metabolism of 2-oxo clopidogrel by HLMs. To quantitatively evaluate the formation of the AM and AM-thiol conjugates, we determined the kinetic parameters for the metabolism of 2-oxo clopidogrel. The metabolic reactions were performed as described above except that 2-oxo clopidogrel was incubated at 0, 1, 3, 5, 10, 30, and 50 μM at 37 ^{o}C for 30 min. The calibration standards were prepared under the same conditions as for the samples except that the standards were quenched before the reactions were initiated. The concentrations of 2-oxo clopidogrel remaining were quantified based on the calibration curve. The K_m and V_{max} values were obtained by fitting the specific activity obtained at various concentrations of 2-oxo clopidogrel to the Michaelis-Menten equation using GraphPad Prism5 (GraphPad Software, Inc.).

Since genuine standards of the AM and AM-thiol conjugates were not commercially available, we were able to determine only the relative amounts of the AM and AM-thiol conjugates by calculating the area ratios of the analytes to IS. The area ratio for the AM was calculated as the AUC of m/z 356.1 to that of m/z 322.1, whereas the area ratios of the AM-GSH, AM-L-Cys and AM-NAC conjugates were calculated as

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the AUCs of m/z 661.0, 475.1, and 517.1 to that of m/z 322.1, respectively. The same approach was used to determine the K_m for GSH for the metabolism of 2-oxo clopidogrel at various concentrations of GSH (0 - 5 mM).

Studies of thiol-disulfide exchange in HLMs. To examine the thiol-disulfide exchange reactions, the metabolism of 2-oxo clopidogrel by HLMs was first carried out in the presence of 1 mM GSH in 50 mM K phosphate solution (pH 7.4). The reaction mixture (2 ml) was incubated at 37 ^{o}C for 30 min in the presence of 1 mg/ml HLM, 80 µM 2-oxo clopidogrel, 1 mM GSH, 0.1 M KF and the $NADP^{+}$-regenerating system. The reaction was then terminated by aliquoting 0.4 ml of the reaction mixture into three separate vials containing 0.2 ml of ice-cold acetonitrile. Small volumes (~6 µl) of DTT or NAC were added into each vial to give final concentrations of 5 mM. An equal volume of water was added to the control sample. The samples were then incubated at room temperature for 30 min to allow thiol-exchange to occur. The samples were then acidified with acetic acid and stored on ice prior to ESI-LC/MS analyses. In a separate experiment to measure thiol-disulfide exchange between the AM and AM-GSH conjugate, aliquots of the reaction mixture were analyzed immediately after the quenching without pre-incubation at room temperature. The same samples were then analyzed under the same conditions over a period of 20 hrs.

RESULTS

Metabolism of 2-oxo clopidogrel by HLMs in the presence of GSH. Traditionally, the metabolism of clopidogrel has been carried out in the presence of 1-10 mM GSH. Therefore, we first characterized this metabolic reaction in the presence of 1 mM GSH.

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As shown in Figure 2A, the metabolism of 2-oxo clopidogrel by HLMs led to the formation of multiple metabolites. The extracted ion chromatogram (EIC) at m/z 356.1 (MH^+ of the AM) showed the formation of metabolites M1-M5 eluting at 6.7, 8.8, 9.2, 9.6 and 9.9 min, respectively. All five peaks in the MS exhibited a base peak at m/z 356.1 along with an isotope peak at m/z 358.1 whose intensity was ~40% of the base peak (data not shown). The presence of this pair of isotope peaks indicates that M1-M5 contain one chlorine atom presumably originated from 2-oxo clopidogrel. The identities of M1-M5 can be assigned on the basis of their MS^2 spectra. The MS^2 spectra of M2-M5 are very similar. A representative MS^2 spectrum for M5 is shown in Figure 2B. This spectrum shows major fragment ions at m/z 211.90, 322.07, and 172.08 and is almost identical to that of the AM as reported in the literature (Dansette et al., 2011; Dansette et al., 2012; Pereillo et al., 2002). The MS^2 of M1 differs from that of M5 as shown in Figure 2C, but is consistent with that of an *endo* isomer of the AM where the double bond has migrated from the exocyclic to the endocyclic piperdinyl ring (Dansette et al., 2011; Dansette et al., 2012). The *endo* isomer is an inactive product of 2-oxo clopidogrel not produced by P450s, but rather by PON1 through hydrolysis of 2-oxo clopidogrel.

In addition to the multiple active metabolites, we also observed the AM-GSH conjugate. The EIC at m/z 661.0, equivalent to MH^+ of the AM-GSH conjugate, exhibits a peak at 5.5 min, as shown in Figure 2A (trace b). The MS^2 spectrum of this peak shown in Figure 2D is in excellent agreement with what we reported previously for the glutathionyl conjugate of the AM observed in a reconstituted system (Zhang et al., 2011). The two major MS^2 peaks at m/z 531.83 and 353.92 can be attributed to a

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neutral loss of 129 from the precursor ion m/z 661.0 and the AM fragment cleaved from the mixed disulfide bond of the glutathionyl conjugate, respectively. Clearly metabolism of 2-oxo clopidogrel by HLMs produced both the AM and the AM-GSH conjugate. When clopidogrel was substituted for 2-oxo clopidogel as substrate, we observed similar metabolic profiles for the AM and AM-GSH conjugate, but with lower yields (data not shown).

Dependence of the metabolism of 2-oxo clopidogrel by HLMs on the concentrations of GSH. To examine the dependence of 2-oxo clopidogrel metabolism on GSH, we determined the K_m values for GSH for the formation of the AM and the AM-GSH conjugate. As shown in Figure 3, the formation of M3-M5 and the AM-GSH conjugate requires GSH, as observed before (Kazui et al., 2010), and they exhibit saturation kinetics with K_m values of 0.3 mM for AM-GSH, 0.49 mM for M3, 0.53 mM for M4 and 0.60 mM for M5. The K_m values for the formation of the AM and the AM-GSH are very similar, indicating that formation of the glutathionyl conjugate constitutes an integral part of the bioactivation processes of 2-oxo clopidogrel via CYP-catalyzed reactions.

Metabolism of 2-oxo clopidogrel by HLMs in the presence of alternative reductants. In addition to GSH, ascorbic acid, NAC, and L-Cys were also used to generate the AM**.** This is the first report to compare their effects on the production of the AM under the same conditions as used for GSH. As shown in Figure 4A (trace a), metabolism of 2-oxo clopidogrel in the presence of L-Cys yielded the AM similar to that observed in the presence of GSH. Namely, M2-M5 were observed at the same retention times as in the presence of GSH; however, the relative amount of the AM formed is

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44% less compared with in the presence of GSH. M2-M5 were also observed in the presence of NAC (trace b). However, their amounts were significantly decreased. Compared with GSH, only ~17% of the AM was produced in the presence of NAC. The relative amount of the AM generated in the presence of 1 mM ascorbic acid is approximately 61% compared with GSH (trace c). Interestingly the intensity of M4 was negligible (trace c, Figure 4A). It appears that ascorbic acid showed stereo-selectivity for M3 and M5.

In addition to the AM, thiol conjugates with the AM were also observed in the presence of NAC and L-Cys. As shown in Figure 4A (trace d), the AM-L-Cys conjugate eluted at 5.6 min in the EIC of m/z 475.1 (MH^+ of AM-L-Cys conjugate). The MS^2 spectrum of the AM-L-Cys conjugate, shown in Figure 4B, exhibits a major fragment ion at m/z 354.01 that is attributed to the AM fragment cleaved from the mixed disulfide bond, similar to the AM-GSH conjugate shown in Figure 2. In contrast, two major AM-NAC conjugates were observed at 6.0 and 6.3 min and a minor one at 5.8 min (trace e). The MS^2 spectra of the precursor ion at m/z 517.1 (MH^+ of the AM-NAC conjugates) of the two major AM-NAC conjugates are similar to that of the AM-L-Cys conjugate with a major peak at m/z 354, as shown in Figure 4C.

Kinetic parameters for the metabolism of 2-oxo clopidogrel by HLMs in the presence of GSH, NAC and L-Cys. To quantitatively compare the effects of the different reductants, we determined the K_m and V_{max} values for the metabolism of 2-oxo clopidogrel and the results are shown in Figure 5. As shown in Figure 5A, the rates of consumption for 2-oxo clopidogrel by HLMs do not differ significantly in the presence of any of the three reductants. For example, the K_m and V_{max} for the consumption of 2-oxo

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clopidogrel in the presence of GSH are 75 μM and 0.4 nmol 2-oxo/min/mg HLM, respectively, which gives a catalytic efficiency (V_{max}/K_m) of 0.0053 as shown in Table 1. The K_m, V_{max}, and catalytic efficiencies for the consumption of 2-oxo clopidogrel in the presence of L-Cys and NAC are similar to those for GSH (within 11%). Thus, the presence of different reductants does not appear to affect the rates of consumption for 2-oxo clopidogrel.

As shown in Table 1, the K_m values of 2-oxo clopidogrel for the production of the total amount of the AM were determined to be 13 μM for GSH, 14 μM for NAC and 18 μM for L-Cys (see in Figure 5B). These values are not significantly different. However, the V_{max} for the production of the AM in the presence of NAC differs greatly from that for the other two; the V_{max} for the formation of the AM in the presence of NAC is 7.6-fold less than that in the presence of GSH and 7.3-fold less than in the presence of L-Cys. Thus, NAC seems to decrease the formation of the AM. The K_m values for the formation of the AM-thiol conjugates are also similar, with K_m values in the range of 46-66 μM. However, it is difficult to directly compare the V_{max} values for the different thiol conjugates based solely on the area ratios because their responses to the MS may differ.

Thiol-disulfide exchange between the AM, AM-thiol conjugates, and thiol reductants. Since the reaction mixture for the metabolism of 2-oxo clopidogrel by HLMs in the presence of GSH already contained the AM, the AM-GSH conjugate and GSH in both its reduced and oxidized forms after a 30 min of incubation, one simple way to examine the thiol-disulfide exchange reaction is to monitor the time dependent changes in the levels of the AM and the AM-GSH conjugate. As shown in Figure 6, the

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relative amounts of the major AM metabolites (M3-M5) decrease slowly with identical rate constants of 1.1×10^{-3} min^{-1} ($t_{1/2} \cong 10$ hrs), while the relative amount of the AM-GSH increases slowly with a comparable rate constant of 0.4×10^{-3} min^{-1}. Simultaneous decrease in the amount of the AM and increase in the amount of the conjugate indicates that the AM is converted to the AM-GSH conjugate, at least partially.

To further examine the thiol-disulfide exchange, we added a second reductant such as DTT or NAC after a 30 min of incubation with GSH and monitor the changes in the amounts of the AM and the AM-thiol conjugates. As shown in Figure 7 (traces b), addition of 5 mM DTT led to a decrease in the amount of the AM-GSH conjugate by 86% with a concomitant increase in the amount of the AM by 40%. This observation indicates that the disulfide bond of the AM-GSH conjugate was reduced by DTT through thiol-disulfide exchange. Addition of excess NAC also led to decrease in the AM-GSH conjugate by ~80% without significantly affecting the amount of the AM. Interestingly addition of NAC led to formation of the AM-NAC conjugate as evidenced by the two peaks at 6.0 and 6.3 min in the EIC of m/z 517.1 (trace d, Figure 7B). These results clearly demonstrate that the thiol-disulfide exchange can occur between the AM, the conjugates and reductants such as DTT and NAC.

DISCUSSION

We have demonstrated in HLMs that metabolism of 2-oxo clopidogrel in the presence of four different reductants, i.e., GSH, NAC, L-Cys, and ascorbic acid, leads to the formation of the active thiol metabolites. Furthermore, in the presence of the three thiol-containing reductants, we also observed formation of the corresponding AM-thiol

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conjugates (see Figures 2 & 4). The multiplicity of the active metabolite observed is due to existence of multiple diastereomers of 2-oxo clopidogrel. In principle, eight diastereomers would be expected as a result of the combination of two chiral centers at C7 and C4 and one exocyclic double bond between C3-C16 (see Figure 1). According to Pereillo et al (Pereillo et al., 2002), the (7S, 7R) diastereomers exhibit very similar chromatographic properties and are not able to be resolved on conventional reverse phase C18 columns. Therefore, M2-M5 would in fact be expected to include the two *trans* and two *cis* isomers of the (4S, 4R) diasteromers. The MS^2 spectra of M2-M5 are in excellent agreement with that reported for the AM of clopidogrel (Dansette et al., 2011; Dansette et al., 2012; Pereillo et al., 2002). Quantitative analysis of the AM of clopidogrel in human plasma, historically referred to as H1, H2, H3 and H4, showed that H3 and H4 are the major metabolites (Tuffal et al., 2011). The double bonds of H3 and H4 are in the *cis* configuration (Dansette et al., 2012; Pereillo et al., 2002). Therefore, metabolites M3 and M5 are most likely the *cis* isomers of the active metabolite, while the minor metabolites M2 and M4 are the *trans* isomers.

As shown in Figure 3, production of the major metabolites M3-M5 is clearly dependent on the concentrations of GSH. In the absence of GSH, only negligible amounts of M3-M5 were produced, which might be due to the presence of GSH or other alternative reductants in the HLM samples. In the presence of GSH, however, formation of the active metabolites is evident and exhibits saturation kinetics with a K_m of 0.3 mM for GSH. The intracellular level of GSH in mammalian cells is in the range of 0.5-10 mM (Meister and Anderson, 1983). This high level of GSH should readily allow the microsomal P450s to produce the active metabolite. Similar K_m values for the formation

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of the AM and the AM-GSH conjugate suggests that the formation of the AM and the AM-GSH conjugate are both dependent on GSH and that the formation of the conjugate is an integral part of the metabolic bioactivation process of clopidogrel by P450s. Our results support the two-step bioactivation mechanism proposed by Dansette and coworkers (Dansette et al., 2009; Dansette et al., 2012).

Furthermore, simultaneous formation of both the AM and the AM-thiol conjugate under a variety of conditions seems to indicate the existence of an equilibrium between the AM, GSH and AM-GSH conjugate, as illustrated in the following equations:

$$\text{R-S-OH} + \text{GSH} \rightarrow \text{R-SSG} + \text{H}_2\text{O} \qquad \text{(Eq. 1)}$$

$$\text{R-SSG} + \text{GSH} \leftrightarrows \text{R-SH} + \text{GSSG} \qquad \text{(Eq. 2)}$$

where R-S-OH, R-SSG, and R-SH represent the sulfenic acid intermediate, the AM-GSH conjugate and the AM, respectively. It is well established that thiol-disulfide interchange occurs in a number of biochemical processes with a range of rate constants ($k \cong 10^3 - 10^7$ s^{-1}) (Houk et al., 1987). As shown in Figure 6, conversion of R-SH back to R-SSG conjugate is a much slower process than the formation of the active metabolite. As such, formation of the R-SH is favored. The concentration of R-SH in this equilibrium is ultimately governed by the redox potentials of R-S-OH, R-SSG and GSH. Thus, the use of alternative reductants with more positive redox potentials would be expected to favor formation of the conjugate R-SSG leading to a decrease in the active metabolite. This is exactly what we observed in the presence of NAC and L-Cys (see Figure 4).

The standard redox potentials (E^o) for GSH and L-Cys are -0.24 and -0.22 V (pH 7.0, 25 ^oC) for the redox pairs of GSH/GSSG and CSH/CSSC respectively, as

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determined using the thiol-disulfide exchange reaction (Jocelyn, 1967). No experimental data can be found for the absolute redox potentials of NAC, but its redox potential has been calculated to be +63 mV above E^o of GSH/GSSG (Noszal et al., 2000). Thus NAC would be expected to exhibit a higher propensity to form a disulfide bond than GSH and L-Cys. Conversely, NAC would disfavor formation of the active thiol metabolite. The level of the AM produced in the presence of GSH, L-Cys, and NAC is in the order of GSH (100%) > L-Cys (56%) > NAC (17%). Moreover, as shown in Figure 5C, more of the AM-NAC conjugate appears to be formed than the AM-GSH conjugate. It should be noted that direct comparison of the relative amount of the AM-NAC with that of the AM-GSH conjugate based on the area ratios is difficult because their responses to the MS detector may differ. Nonetheless, the large difference (~4-fold) in the relative amounts of the AM-NAC and the AM-GSH conjugates formed in the presence of 50 μM 2-oxo clopidogrel provides indirect evidence that NAC has a higher propensity for forming disulfide bond than GSH. This is consistent with the observations that the amount of the AM produced in the presence of NAC is ~17% of that produced in the presence of GSH while the overall consumption of 2-oxo clopidogrel remained almost constant. One plausible explanation is that the AM-NAC conjugate produced in Eq.1 is not converted to R-SH in Eq. 2 due to the higher E^o of NAC. This is also consistent with the observation that the addition of NAC led to a decrease in R-SH with concomitant increase in the AM-NAC conjugate (see Figure 7). It should be emphasized that the concentration of the AM is largely dependent on Eq. 2. This is because the sulfenic acid is an unstable intermediate that likely has a high redox potential. Its reduction is not likely to be the rate-limiting step.

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Metabolism of 2-oxo clopidogrel in the presence of ascorbic acid differs from the other reductants in two aspects. First, ascorbic acid favors formation of the *cis* isomers over the *trans* isomer. The *cis/trans* ratio in the presence of ascorbic acid is approximately 6-fold higher than in the presence of GSH, indicating that ascorbic acid preferably reduces the *cis* isomer of sulfenic acid. Second, the redox potential of ascorbic acid is -0.081 V (pH 7) (Fruton, 1934), which is the highest in the four reductants used. Nonetheless it is more effective than NAC in producing the active thiol metabolite. Compared with GSH, the relative amount of the active thiol metabolite is 61%, similar to L-Cys. This is likely due to direct reduction of the sulfenic acid without the aforementioned equilibrium involving the thiol-disulfide exchange.

Due to its high concentration in hepatocytes, GSH probably serves as the primary reductant for the generation of the active thiol metabolites in human livers. Inevitably, the AM-GSH conjugate will be generated as well. Presence of the equilibrium as indicated in Eq. 2 may contribute to the variations in the concentration of the active thiol metabolites of clopidogrel in blood plasma. This is particularly relevant considering that clopidogrel has a narrow therapeutic range; over-dosing may cause severe bleeding while under-dosing may not achieve the desired therapeutic effect. The C_{max} of the active isomer H4 in blood plasma is approximately ~30 ng/ml (or 0.1 μM) after a 300-mg loading dose (Takahashi et al., 2008; Tuffal et al., 2011). This low level of active metabolite may be prone to changes due to factors other than P450-catalyzed reactions such as the concentrations of GSH or other reductants that may be associated with oxidative stress, inflammation, etc. In addition, a recent study by Hagihara and coworkers demonstrated that glutaredoxin and thioredoxin accelerate the

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conversion of the glutathionyl conjugate of the active metabolite of prasugrel, another thienopyridine antiplatelet agent like clopidogrel, to the active metabolite (Hagihara et al., 2011). It is unclear to what extent glutaredoxin and thioredoxin may contribute to the metabolism of clopidogrel to its active metabolite. More studies are required to investigate the factors that determine the effective concentration of the active metabolite in vivo in order to give patients correct doses for maximal efficacy.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Zhang

Conducted experiments: Zhang

Performed data analysis: Zhang, and Hollenberg

Contributed new reagents or analytic tools: Zhang, and Hollenberg

Wrote or contributed to the writing of the manuscript: Zhang, Lau, and Hollenberg

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Bioactivation of clopidogrel by P450s. Clo, 2oxo, AM, and AM-GSH represent clopidogrel, 2-oxo clopidogrel, the active metabolite and the glutathionyl conjugate of the AM, respectively. The numeric letters shown in the AM structure represent the numbering of the two chiral centers (C4 and C7) and the exocyclic double bond (C3 and C16).

Figure 2. Extracted ion chromatograms (EICs) and MS^2 spectra of the AM and AM-GSH conjugate produced by HLMs. The AM and AM-GSH conjugate were generated by incubation at 37 ^oC for 30 min in a reaction mixture containing 1 mg/ml HLM, 80 µM 2-oxo clopidogrel and 1 mM GSH as described in Materials and Methods. (A), EICs at m/z 356.1 for the AM (Trace a) and at m/z 661.0 for the AM-GSH conjugate (Trace b). (B), MS^2 spectrum of the precursor ion m/z 356.1 at 9.9 min; (C), MS^2 spectrum of the precursor ion m/z 356.04 at 6.7 min; (D), MS^2 spectrum of the precursor ion m/z 661.0 at 5.5 min.

Figure 3. Dependence of the metabolism of 2-oxo clopidogrel by HLMs on GSH. Metabolism of 2-oxo clopidogrel was performed in 50 mM K phosphate buffer (pH 7.4) in the presence of 80 µM 2-oxo clopidogrel and varying concentrations of GSH (0-5 mM) at 37 ^oC for 30 min and the AM and AM-GSH conjugate were analyzed by ESI-LC/MS as described in Materials and Methods. The relative amount of the AM was calculated as the area ratios of m/z 356.1 to that of m/z 322.0, while the relative amount of the AM-GSH conjugate was calculated as the area ratios of m/z 661.0 to that of m/z

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322.0. Legend: (●), M3 eluting at 9.2 min; (■), M4 eluting at 9.6 min; (▲), M5 eluting at 9.9 min; (○), AM-GSH eluting at 5.5 min.

Figure 4. Formation of the AM and the AM conjugates by HLMs in the presence of ascorbic acid, L-Cys, and NAC. The AM and the AM conjugates were formed by incubation at 37 ^{o}C for 30 min in a reaction mixture containing 1 mg/ml HLM, 80 µM 2-oxo clopidogrel, and 1 mM of the reductants as indicated in Materials and Methods. (A), Traces a, b, and c represent the EICs at m/z 356.1 in the presence of L-Cys, NAC, and ASC respectively, while Traces d and e represent the EICs at m/z 475.1 and 517.1 respectively. (B), the MS^2 spectrum of the AM-L-Cys conjugate at 5.6 min; (C) the MS^2 spectra for the AM-NAC conjugate at 6.3 min.

Figure 5. Determination of the kinetic parameters for the metabolism of 2-oxo clopidogrel by HLMs in the presence of GSH (●), L-Cys (○) and NAC (■). Metabolism of 2-oxo clopidogrel was performed in 50 mM K phosphate buffer (pH 7.4) in the presence of 1 mM reductant and varying concentrations of 2-oxo clopidogrel (0-50 µM) at 37 ^{o}C for 30 min and the amount of 2-oxo clopidogrel consumed and products formed were determined by ESI-LC/MS as described in Materials and Methods. (A), kinetics for the consumption of 2-oxo clopidogrel. The amount of 2-oxo clopidogrel remaining after 30 min of incubation was quantified by ESI-LC/MS based on a calibration curve prepared with 2-oxo clopdogrel standards in the same matrix as the samples. The net difference between substrate and the 2-oxo clopidogrel remaining gave the amount of 2-oxo clopidogrel consumed. (B), kinetics for the formation of the total amount of the AM

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(sum of M2-M5). The relative amount of the AM was calculated as the area ratios of m/z 356.1 to m/z 322.0. (C), kinetics for the formation of the AM-thiol conjugates. The relative amount of the AM-thiol was calculated as the area ratios of MH^+ of the AM-thiol to m/z 322.0. MH^+ for the AM-NAC conjugate and AM-L-Cys conjugate are m/z 517.1 and 475.1, respectively.

Figure 6. Time dependent changes in the relative amounts of the AM and AM-GSH conjugate after the metabolic reactions were terminated. The AM and AM-GSH conjugate were formed by incubation at 37 ^oC for 30 min in a reaction mixture containing 1 mg/ml HLM, 80 μM 2-oxo clopidogrel and 1 mM GSH as described in Materials and Methods. Aliquots of the reaction mixture were analyzed by ESI-LC/MS at designated times and the amount of the metabolites, M3-M5, were calculated as the area ratios of respective metabolite to that of the IS. The area of the IS was integrated from the EIC at m/z 322.0, the areas of M3-M5 were integrated from the EIC at m/z 356.1, and the area of the AM-GSH was integrated from the EIC at m/z 661.0. Legend: (●), area ratio of M3; (■), area ratio of M4; (▲), area ratio of M5; (○), area ratio of AM-GSH.

Figure 7. Thiol-disulfide exchange between the AM and the AM-GSH in the presence of excess DTT and NAC. The AM and AM-GSH conjugate were generated as described in Figure 6. For thiol-disulfide exchange, DTT or NAC were added at 5 mM as described in Materials and Methods. (A), EICs for the AM at m/z 356.1. Traces a, b, and c are EICs for the samples incubated with water, DTT or NAC, respectively. (B), EICs for AM-GSH

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at m/z 661.0. Traces a, b, and c are for the samples incubated with water, DTT, or NAC respectively. Trace d is the same as trace c except at m/z 517.1 for the AM-NAC conjugate.

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Table 1. Summary of the apparent K_m and V_{max} values for the metabolism of 2-oxo clopidogrel in the presence of GSH, NAC and L-Cys. The metabolic reactions were performed at 37 ^{o}C for 30 min as described in Materials and Methods.

Reductant	2-oxo Consumed			AM		AM-Thiol conjugate	
	K_m (μM)	V_{max} (nmol/min/mg HLM)	V_{max}/K_m	K_m (μM)	V_{max}	K_m (μM)	V_{max}
GSH	75±8	0.40±0.07	0.0053	13±2	0.92±0.02	46±5	0.41±0.05
L-CYS	64±5	0.35±0.04	0.0055	18±3	0.88±0.01	66±7	0.95±0.02
NAC	72±4	0.43±0.03	0.0059	14±1	0.12±0.01	50±4	1.6±0.09

Figure 1

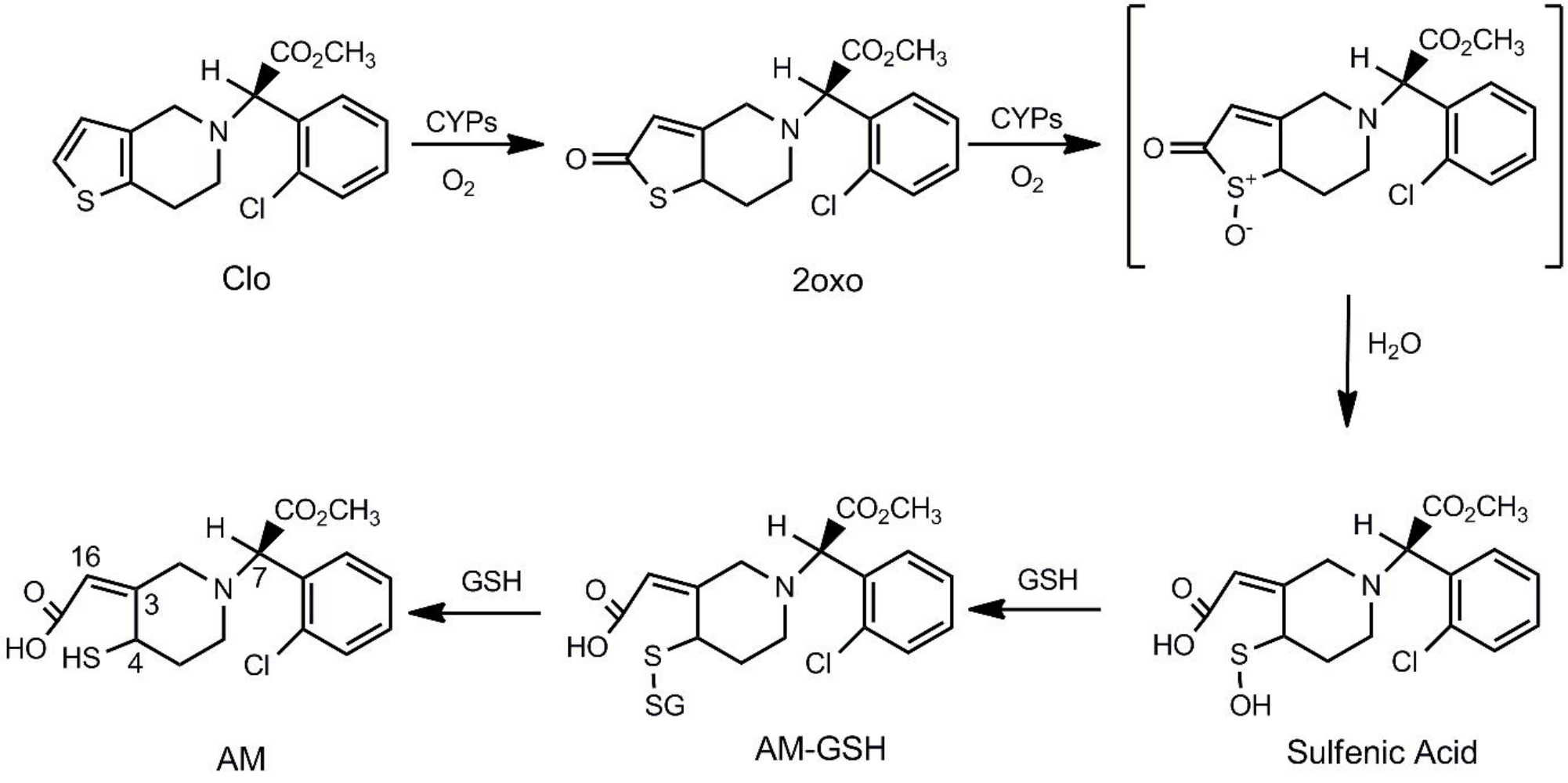


Figure 2

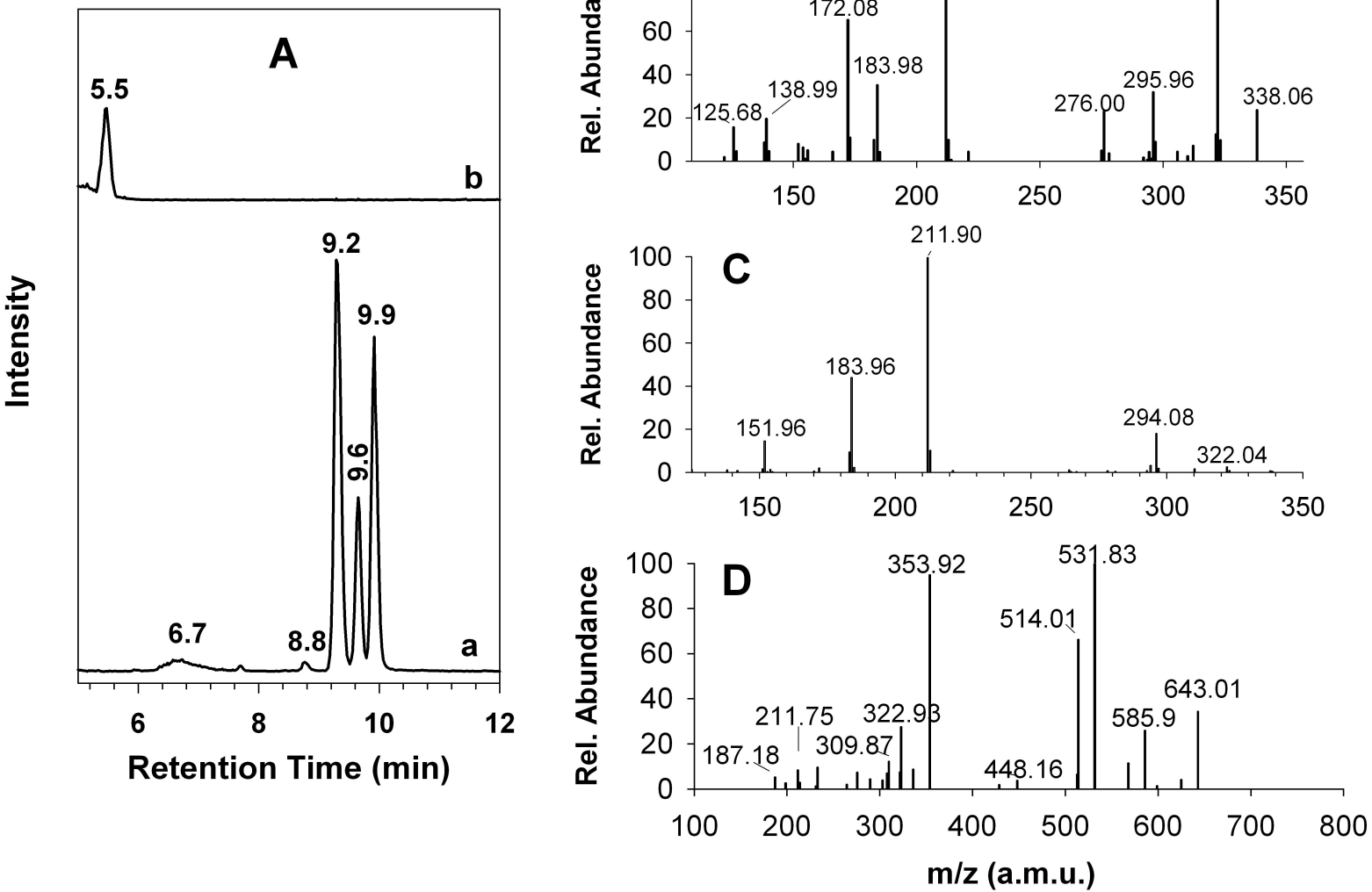


Figure 3

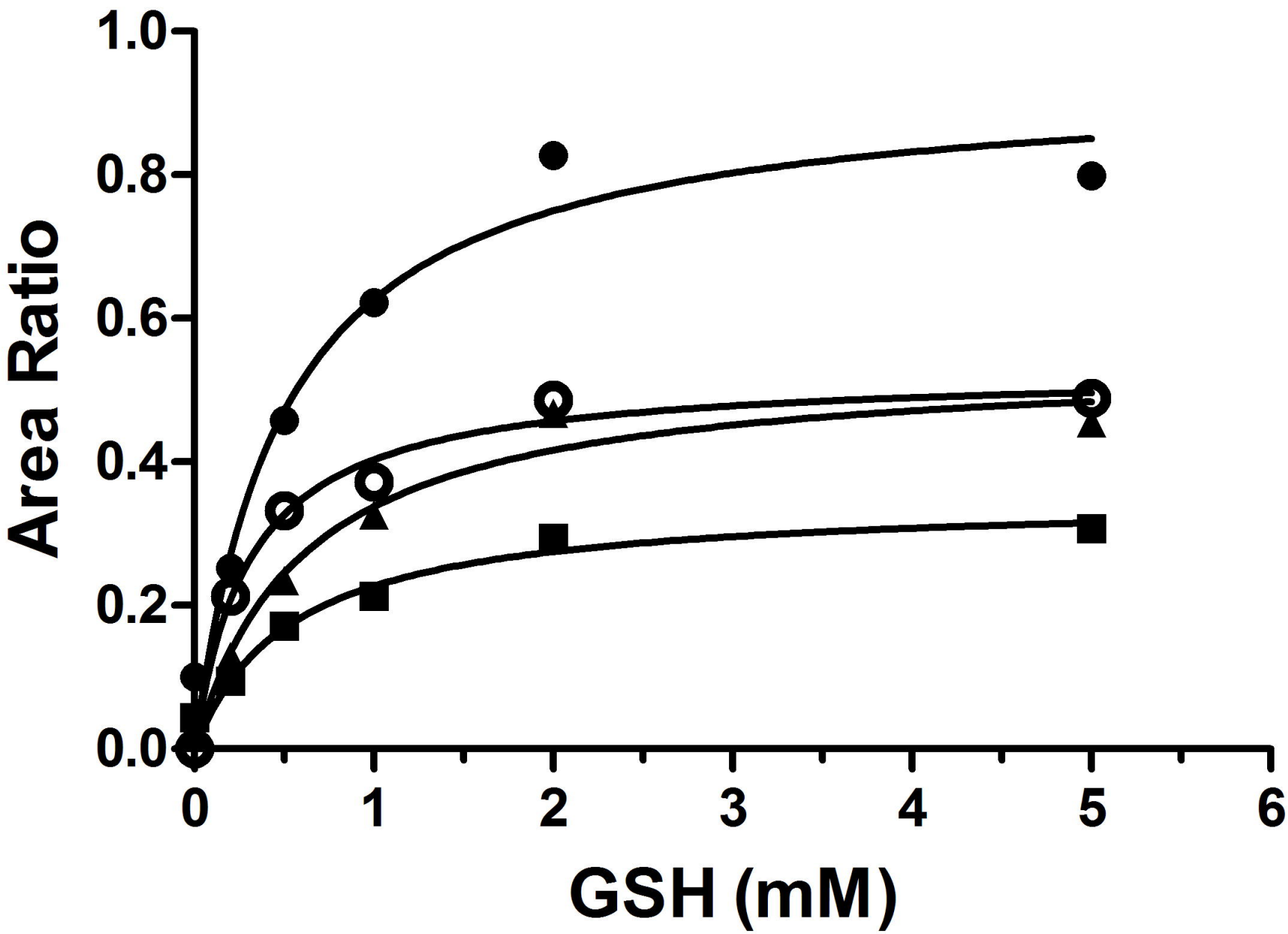
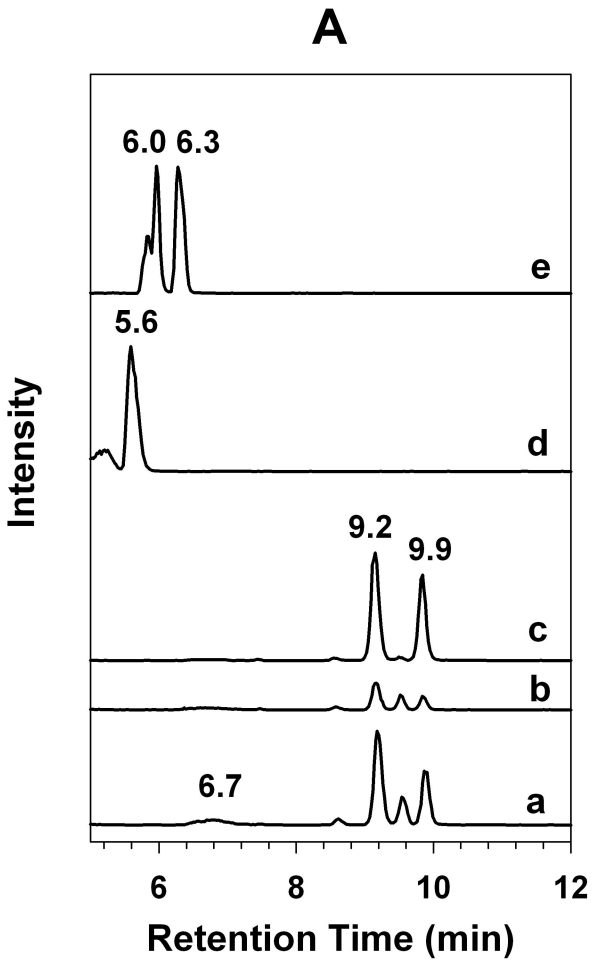
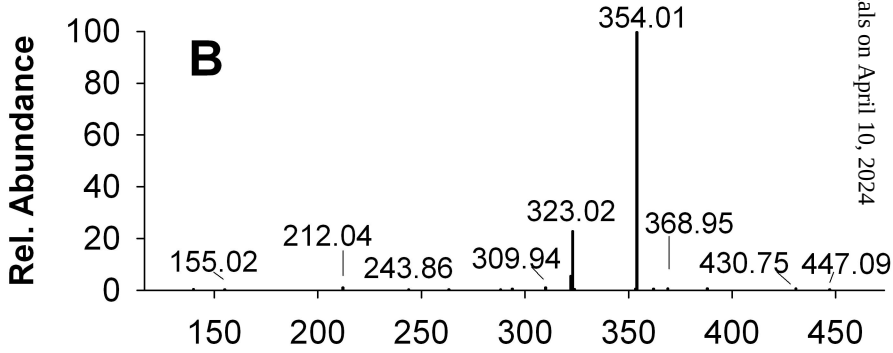


Figure 4





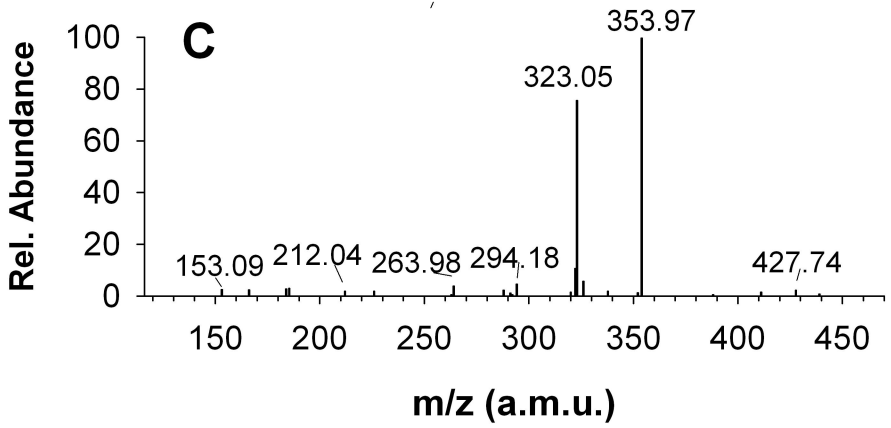
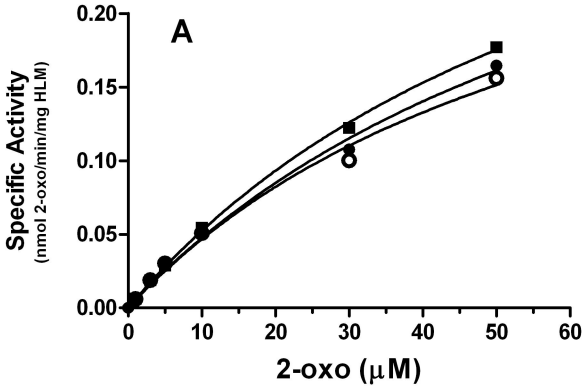


Figure 5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Specific Activity
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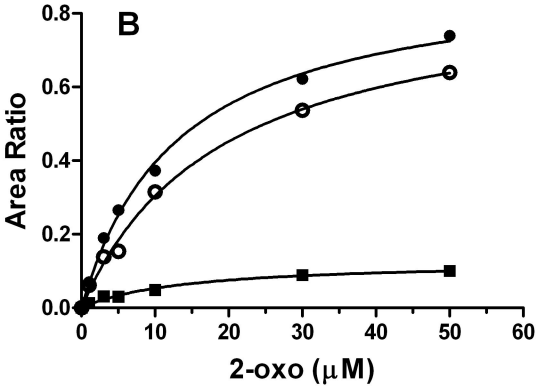
0.10
0.05
0.00
0
10
20
30
40
50
60
2-oxo (μM)


B
Area Ratio
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10
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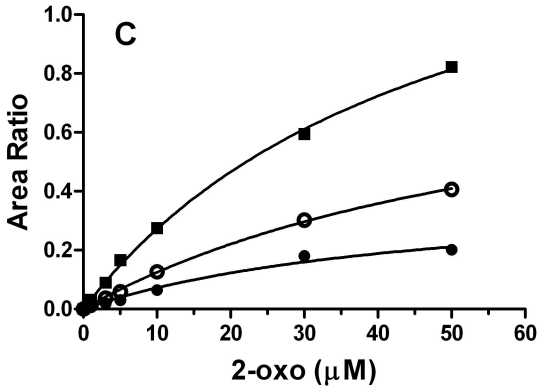
60
2-oxo (μM)


C
Area Ratio
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0.2
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10
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50
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2-oxo (μM)

Figure 6

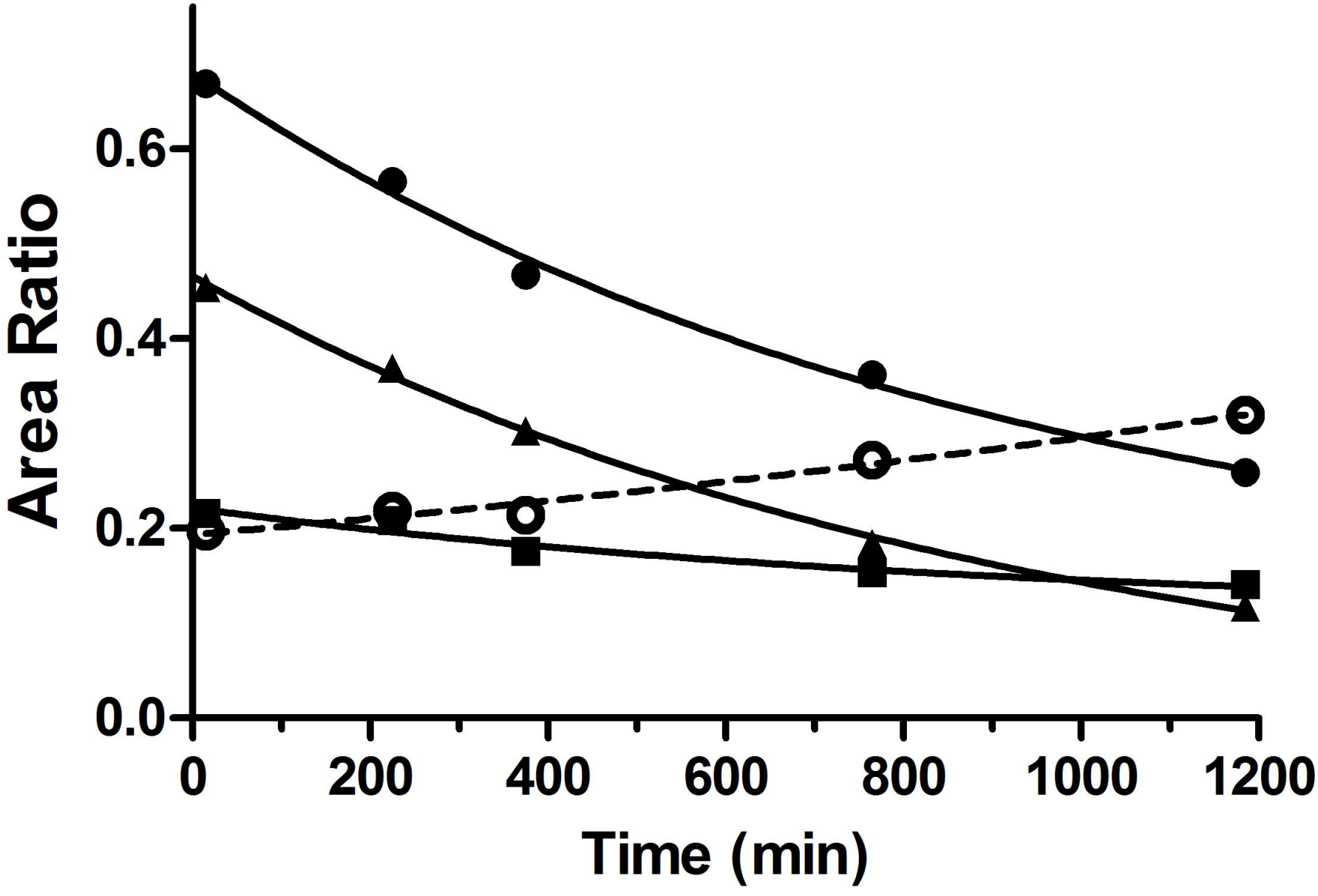
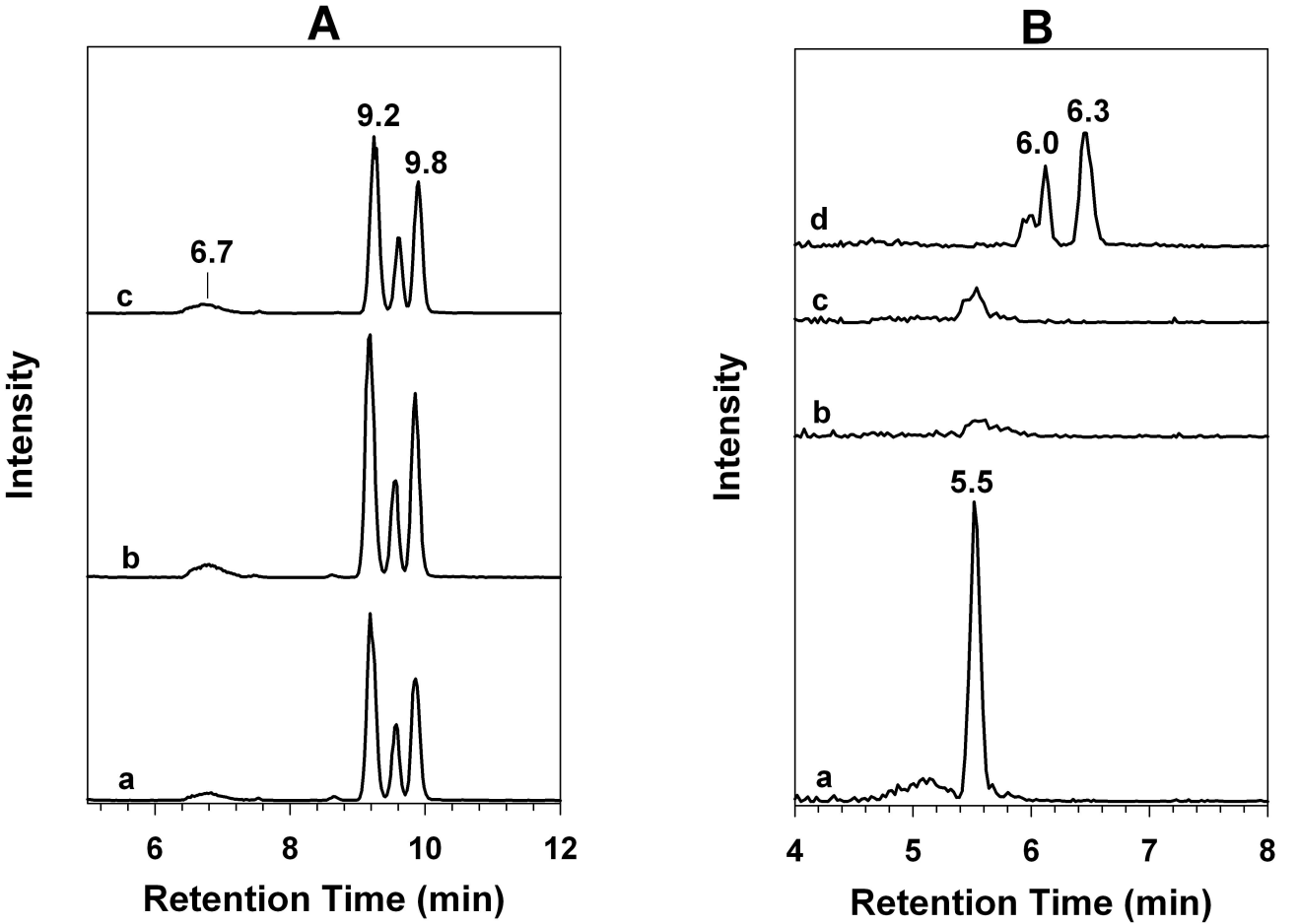


Figure 7


A
9.2
9.8
6.7
c
b
a
Intensity
6
8
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12
Retention Time (min)
B
6.3
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d
c
b
5.5
a
Intensity
4
5
6
7
8
Retention Time (min)