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Structural characterization of anti-HIV drug candidate PA-457 and its acyl glucuronides in rat bile and evaluation of in vitro stability in human and animal liver microsomes and plasma

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Running title page

Running title:

Characterization and stability of PA-457 acyl glucuronides

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Abbreviations used in this paper:

PA-457, 3-O-(3',3'-dimethylsuccinyl)-betulinic acid; DSD, 3-O-(3',3'-dimethylsuccinyl)-dihydrobetulinic acid; PA-457Gs, acyl glucuronides of PA-457; UGT, UDP-glucuronosyltransferase; UDPGA, Uridine 5'-diphosphoglucuronic acid; HPLC, high-performance liquid chromatography; LC-ESI-MS, liquid

chromatography-electrospray ionization-mass spectrometry; D-SL, D-saccharic acid 1,4-lactone; PMSF, phenylmethylsulfonyl fluoride.

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ABSTRACT

PA-457 [3-O-(3',3'-dimethylsuccinyl)-betulinic acid] represents a new class of anti-HIV drug candidate termed maturation inhibitors. After oral administration to rats, PA-457 was metabolized to several glucuronide conjugates and mainly eliminated into rat bile. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis showed that the glucuronidation products of PA-457 were acyl glucuronides including one di-glucuronide, di-PA-457G, and two mono-glucuronides, referred to as mono-PA-457G (I) and mono-PA-457G (II), respectively. In-source fragmentation of MS spectra supported the conclusion that mono-PA-457G (I) was glucuronidated at the C-28 carboxyl of PA-457, while mono-PA-457G (II) was conjugated at the dimethylsuccinic acid side chain of the C-3 position. Quantification demonstrated that the predominant glucuronide of PA-457 in rat bile was mono-PA-457G (I) with lower amounts of mono-PA-457G (II) and di-PA-457G. In vitro stability indicated that the mono-acyl glucuronides of PA-457 were not degraded after incubation with 0.1 M phosphate buffer (pH 4, 7.4 and 9), plasma (human, rat and mouse) and UDP-glucuronosyltransferases (UGTs) reaction media (without uridine 5'-diphosphoglucuronic acid (UDPGA)) with microsomes (human, rat and mouse liver microsomes), respectively, whereas the minor diglucuronide was unstable in rodent liver microsomes. All glucuronides of PA-457 could be hydrolyzed both by β-glucuronidase and alkaline (1M NaOH). Minor putative acyl migration products were slowly formed at pH 9, suggesting that the acyl glucuronides of PA-457 have relatively high in vitro stability.

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Introduction

PA-457 [3-O-(3',3'-dimethylsuccinyl)-betulinic acid], a betulinic acid derivative containing double carboxylic acid groups (Fig. 1), is the first in a new class of anti-retrovirals termed maturation inhibitors that disrupt viral maturation, the last step in the virus life-cycle. PA-457 specifically inhibits processing of the HIV-1 capsid precursor, capsid-SP1, thereby preventing normal viral core condensation (Li et al., 2003). Preclinical studies have shown that PA-457 retains full activity against viral strains resistant to currently available treatments, and is effective in an animal model of HIV infection. Recently, PA-457 has demonstrated positive results in a phase I/II clinical trial in HIV-infected patients.

Glucuronidation, a major conjugation reaction catalyzed by UDP-glucuronosyltransferases (UGTs), is an important pathway for drug metabolism and detoxification in humans (Radominska-Pandya et al., 1999; Ritter, 2000; Tukey and Strassburg, 2000; Fisher, et al., 2001). Glucuronidation is sometimes the predominant metabolic pathway of carboxylic acid-containing drugs like PA-457. Acyl (ester) glucuronides have been reported to be unstable at neutral and basic environments, undergoing possible intramolecular acyl migration and direct hydrolysis (Smith et al, 1985; Spahn-Langguth and Benet, 1992, 1993; Bailey and Dickinson, 2003). The common degradation products of positional isomers of glucuronic acid formed by acyl migration are generally resistant to cleave by β-glucuronidase, a commonly used reagent employed in studies of glucuronides (Faed and McQueen, 1978; Hasegawa, et al., 1982; Spahn-Langguth and Benet, 1992; Bailey and Dickinson, 2003). Since PA-457, a triterpenne derivative, is a poor UV chromaphore, it is difficult to analyze by chromatographic assay with UV

detection. To understand the metabolite profile, we developed a method for the simultaneous analysis of PA-457 and its acyl glucuronidation products (PA-457Gs) by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and characterized the structures of PA-457 and PA-457Gs in rat bile. In vitro stability and possible acyl migration of PA-457 glucuronides were also investigated to determine if glucuronides of PA-457 were labile as reported for other acyl glucuronides.

Materials and Methods

Chemicals. PA-457 [DSB, 3-O-(3',3'-dimethylsuccinyl)-betulinic acid], DSD [3-O-(3',3'-dimethylsuccinyl)-dihydrobetulinic acid] were provided by Panacos (Gaithersburg, MD). DSD (a reduced analog of PA-457) was used as the internal standard for the quantification of PA-457 and its acyl glucuronides (PA-457Gs). Uridine 5'-diphosphoglucuronic acid (UDPGA), β-glucuronidase (EC 3.2.1.31, Type B-10 from bovine liver), magnesium chloride, D-saccharic acid 1,4-lactone (D-SL, specific β-glucuronidase inhibitor), phenylmethylsulfonyl fluoride (PMSF, carboxylesterase inhibitor), alamethicin and Tris-HCl (pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO).

Pooled human liver microsomes (HLM) were obtained from In Vitro Technologies (Baltimore, MD). Pooled rat and mouse liver microsomes (RLM and MLM) were purchased from BD Gentest (Woburn, MA). Pooled human plasma was obtained from Valley Biomedical (Winchester, VA). Pooled rat and mouse plasma were purchased from Harlan (Indianapolis, IN). Acetonitrile (ACN, HPLC grade) was obtained from Mallinckrodt (Phillipsburg, NJ). Bond Elut® C18 cartridges (100 mg, 1 ml) for solid-phase extraction (SPE) were obtained from Varian (Palo Alto, CA). All other chemicals and reagents were of

analytical grade.

Animals. Male Sprague-Dawley rats (chronic bile duct cannulated, body weight ~250 g, n = 2) were obtained from Charles River (Wilmington, MA). Animals were maintained under a 12-h light/dark cycle in a temperature-controlled environment, with free access to food and water. Animals were administered oral doses of PA-457 (100 mg/kg). Bile samples were collected via the cannulated bile duct after oral dosing at 1-h intervals up to 9 h. Bile volumes were measured and samples were immediately adjusted to pH 3~3.5 with glacial acetic acid (HAc) and stored at –80ºC until use. All animal studies were conducted after approval by the Institutional Animal Care and Use Committee.

Analysis of PA-457 and PA-457Gs. Aliquots (0.10 ml) of rat bile, human and animal plasma, and incubation samples (see below) were protein-precipitated by the addition of 0.20 ml of ice-cold ACN containing 0.5% formic acid and internal standard DSD (100 ng). After the removal of protein by centrifugation at 15,000g for 10 min at 4ºC, the supernatants were transferred to a clean tube. Following the addition of 1 ml of 0.1% HAc to reduce the organic solvent level, the resultant supernatants were loaded onto a SPE cartridge pre-conditioned by washing with 1 ml of MeOH and then 1 ml of 0.1% HAc. The SPE cartridge was washed with 1 ml of ACN/0.1% HAc (5:95, v/v) and then eluted with 2 ml of ACN/0.1% HAc (90:10, v/v). The eluant was dried with a stream of N_2 at 45ºC in a water bath and the residue was reconstituted in 0.10 ml of ACN/0.1% HAc (80:20, v/v) for LC-ESI-MS assay. Preliminary experiments indicated that the reconstituted samples containing PA-457, PA-457Gs and internal standard (DSD) were stable at least for 72 h at 4ºC. The stock solutions of PA-457 and DSD were stable for 1 year

with the storage at -20ºC.

HPLC separation was carried out using an Agilent HP 1050 LC system (Palo Alto, CA) with a guard column (Zorbax RX-C8, 12.5 × 4.6 mm i.d., 5 µm) and an analytical column (Zorbax RX-C8, 150 × 2.1 mm i.d., 5 µm). HPLC conditions: mobile phase, A-0.1% formic acid/50 mM ammonium formate (pH ~4.5), B-ACN containing 0.1% formic acid; gradient elution, 0-10 min, 20% B to 90% B, 10-16 min, 90% B, 16-18 min, 90% B to 20% B; column temperature, ambient; flow rate, 0.3 ml/min; injection volume, 10 µl; run time, 18 min; post time, 4 min.

MS analysis was performed by a PE Sciex API 100 LC/MS system (PE Sciex, Toronto, Canada) with a TurboIonspray interface in the negative ESI ionization mode. MS parameters for qualitative analysis: ionspray voltage (IS), -4500 V; ionspray temperature (TEM), 450ºC; orifice voltage (OR), -100 V; focusing ring voltage (RNG), -300 V; nebulizer gas (NEB), 10 L/min; curtain gas (CUR), 8 L/min; step size, 0.4 amu; dwell time, 1 ms; pause time, 5 ms; scan time, 2.2 s; scan mode, full scan at the range of 100-1,000 *m/z*. MS parameters for quantitative analysis: IS, -3100 V; TEM, 450ºC; OR, -50 V; RNG, -250 V; NEB, 10 L/min; CUR, 8 L/min; step size, 1 amu; dwell time, 300 ms; pause time, 5 ms; scan time, 1 s; scan mode, selective ion monitoring (SIM) with $[M-H]^-$ for di-PA-457G (*m/z* 935.6), mono-PA-457Gs (*m/z* 759.6), PA-457 (*m/z* 583.5) and internal standard DSD (*m/z* 585.5), respectively.

Enzyme and alkaline hydrolysis of PA-457Gs. Preliminary experiments showed that PA-457 was predominantly metabolized to its acyl glucuronides after oral administration to rats with most excretion into bile. The individual glucuronide metabolites are not available, therefore, all studies were conducted

with a mixture of high concentrations of the metabolites from rat bile (Fig. 2, TIC). Rat bile samples containing PA-457Gs were spiked in 0.1 M phosphate buffer (pH 4, 7.4, and 9) and plasma (human, rat and mouse plasma, pH 7.3-7.5). The resultant samples were hydrolyzed with β-glucuronidase (0-4,000 U/ml in the final incubation) at pH 5.0 and 37ºC for 2 h. In addition, alkaline hydrolysis was also conducted by incubation with 1 M NaOH at 60ºC for 2 h. Preliminary studies had indicated that PA-457 was stable under these conditions. Sample preparation was performed as the procedures of analysis of PA-457 and PA-457Gs and then analyzed by LC-ESI-MS assay.

In vitro stability of PA-457Gs in phosphate buffer. Rat bile samples containing PA-457Gs were spiked in 0.1 M phosphate buffer (pH 4.0, 7.4 and 9.0), and then incubated at 37ºC. Aliquots of the incubations were taken at 0, 4, 12, 24, 36, 48 and 72 h, and then treated with and without β-glucuronidase (4,000 U/ml in the final incubation) at pH 5.0 and 37ºC for 2 h. Sample preparation was conducted as described above for the analysis of PA-457 and PA-457Gs. The contents of PA-457Gs, as well as PA-457, in the incubations with and without enzyme hydrolysis were analyzed by LC-ESI-MS assay.

In vitro stability of PA-457Gs in incubation media with human and animal liver microsomes. Rat bile samples containing PA-457Gs were incubated in glucuronidation reaction media (alamethicin, 25 μg/mg protein; $MgCl_2$, 5 mM; without UDPGA) with HLM, RLM and MLM (1 mg protein /ml) at pH 7.4 (0.1 M Tris-HCl) and 37ºC for 0, 0.25, 0.5, 1, 2 and 4 h. Aliquots of the incubations with different microsomal sources were withdrawn at different time-points and prepared as described above for the analysis of PA-457 and PA-457Gs. The contents of PA-457Gs, as well as PA-457, in the incubations with

and without inhibitors (D-SL, 5 mM and PMSF, 0.5 mM) were analyzed by LC-ESI-MS assay.

In vitro stability of PA-457Gs in human and animal plasma. Rat bile samples containing PA-457Gs were directly spiked in pooled human, rat and mouse plasma (pH 7.3-7.4), and then incubated at 37ºC for 0, 0.25, 0.5, 1, 2 and 4 h. Aliquots of the incubations with different plasma sources were withdrawn at different time-points and prepared as the procedures of analysis of PA-457 and PA-457Gs. The contents of PA-457Gs, as well as PA-457, in the incubations were analyzed by LC-ESI-MS assay.

Results and Discussion

Characterization of PA-457 and PA-457Gs. Characterization of PA-457 and its possible acyl glucuronidation conjugates was investigated by LC-ESI-MS assay. Figure 2 shows the representative LC-ESI-MS chromatograms and MS spectra of PA-457, PA-457Gs and DSD (internal standard) from rat bile. PA-457 and DSD were identified and confirmed by comparing their chromatographic and mass spectrometric characterizations of authentic standards. The retention times of PA-457 and DSD at the experimental chromatographic conditions were 14.1 and 14.6 min, respectively. Because PA-457 has a carbon-carbon double bond in comparison with its reduced analog DSD (Fig. 1), its polarity is a little higher than that of DSD resulting in earlier elution from reversed-phase column. In the negative ESI-MS mode, the main fragment ions of PA-457 and DSD were their deprotonated molecular ions ($[M-H]^-$), 583 *m/z* for PA-457 and 585 *m/z* for DSD, respectively. Characteristic fragmentation ions, 455 *m/z* for PA-457 and 457 *m/z* for DSD, were also observed corresponding to the cleavages of betulinic acid and dihydrobetulinic acid. Di-glucuronide of PA-457 (di-PA-457G; t_R = 7.9 min) was identified by the main

deprotonated molecular ion, 935 *m/z* and fragmentation ions, 759 *m/z* for the neutral loss of one glucuronide moiety (176 Da), 583 *m/z* for the neutral loss of double glucuronide moieties (2 ×176 Da), and 455 *m/z* for the following cleavage of betulinic acid. Two mono-glucuronides of PA-457, named mono-PA-457G (I) and mono-PA-457G (II), were found in rat bile characterized by the main deprotonated molecular ion, 759 *m/z* and fragmentation ion, 583 *m/z* for the neutral loss of glucuronide moiety (176 Da). Differences in retention time on HPLC chromatogram and cleavage of betulinic acid moiety suggested that, for mono-PA-457G (I) (t_R = 10.1 min), glucuronic acid was conjugated to the carboxylic group at C-28 position because it did not show the related subfragmentation ion (455 *m/z*) from the cleavage of betulinic acid moiety. In contrast, mono-PA-457G (II) (t_R = 10.5 min) clearly revealed a similar cleavage to that of the parent compound (PA-457), indicating glucuronic acid was conjugated to the carboxylic group at C-3 position (side chain of dimethylsuccinyl group). In the negative ion mode, all glucuronides of PA-457 exhibited fragment ions at *m/z* 175 (deprotonated glucuronide moiety). In addition, disappearance of all peaks of PA-457Gs and increase of the peak of PA-457 after hydrolysis by β-glucuronidase and strong base further supported the metabolites as being acyl glucuronides.

Quantification of PA-457 and PA-457Gs. Calibration curves were established using PA-457 standards spiked in blank sample matrices (rat bile, human and animal plasma, and incubation buffer) in the linear concentration ranges of 0.02-10 μg/ml with $1/x^2$ weighted least-squares regression equation driving from the peak area ratio of PA-457 to that of internal standard (DSD). The limit of detection (LOD), defined as the concentration of PA-457 giving a signal-to-noise (S/N) ratio >3:1 and the relative standard deviation

(RSD, n = 8) <15%, was 10 ng/ml. At five concentration levels of PA-457 (0.02, 0.05, 0.1, 1 and 10 μg/ml) spiked in blank sample matrices, the intra-day precisions were 3.5-9.7% and the inter-day precisions were 3.3-13.1% (RSD, n = 4), respectively. Since di-PA-457G and mono-PA-457Gs standards were not available, the ESI-MS responses and related quantitative coefficients of PA-457Gs were estimated by comparing the initial and released contents of PA-457 in rat bile (mainly containing PA-457Gs) with and without β-glucuronidase hydrolysis (4,000 U/ml at pH 5.0 and 37ºC for 4 h). The contents of PA-457Gs were then calculated using the calibration curves of PA-457 with the quantitative coefficients estimated, assuming all forms of PA-457Gs have similar ESI-MS response ratios derived from the peak area ratio to that of internal standard (DSD).

Rat biliary excretion profile of PA-457 and PA-457Gs. A typical chromatographic profile of PA-457 and PA-457Gs in rat bile after oral administration of 100 mg/kg PA-457 to SD rats is shown in Figure 2 (TIC). Cumulative rat biliary excretion demonstrated that PA-457 was excreted as its acyl glucuronide conjugates, mainly in the form of mono-PA-457G (I), while mono-PA-457G (II) and di-PA-457G are minor metabolites in rat bile (data not shown). The content of the parent drug, PA-457, is much lower in rat bile in comparison with its acyl glucuronide metabolites estimated by β-glucuronidase hydrolysis (data not shown).

Enzyme and alkaline hydrolysis of PA-457Gs. With the treatment of β-glucuronidase (0-4,000 U/ml) at pH 5.0 and 37º C for 2 h, PA-457Gs spiked in pooled human, rat and mouse plasma could be almost completely hydrolyzed and released back to PA-457 when the concentration of β-glucuronidase was 4,000

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U/ml (Fig. 3). After pre-incubation in 0.1 M phosphate buffer (pH 4.0, 7.4 and 9.0, respectively) for 72 h, all forms of PA-457Gs spiked in phosphate buffer demonstrated rapid hydrolysis by β-glucuronidase (4,000 U/ml at pH 5.0 and 37ºC for 2 h), though a small amount of mono-PA-457G (II) in the incubation at pH 9.0 (5.4% of the initial at start) could be observed (Table 1). When treated under alkaline hydrolysis by 1 M NaOH at 60º C for 2 h, PA-457Gs from rat bile were mostly cleaved (84, 90 and 100% for mono-PA-457G (I), mono-PA-457G (II) and di-PA-457G, respectively) (Table 1).

In vitro stability of PA-457Gs in phosphate buffer. In vitro stability of PA-457Gs in phosphate buffer showed that no significant changes of the content of PA-457Gs were observed after incubation at pH 4.0, 7.4 and 9.0 for up to 72 h in comparison with the controls at start (zero time) (Fig. 4, without β-glucuronidase). These metabolites could be rapidly cleaved back to PA-457 by β-glucuronidase (Fig. 4, with β-glucuronidase). Although some of mono-PA-457G (II) in the incubations at pH 9.0 was not cleaved by β-glucuronidase, this represented less than 6% of the total conjugates. Even lower amounts of mono-PA-457G (II) in the incubations at pH 7.4 and mono-PA-457G (I) in the incubations both at pH 7.4 and 9.0 were observed to be stable to β-glucuronidase after 72 h (<1% of the total conjugates). These data suggested that, in a high pH environment, β-glucuronidase could not completely cleave mono-glucuronides of PA-457 under the experimental conditions or some β-glucuronidase resistant isomers may be forming with time. Chromatographic conditions, such as pH, composition and content of organic solvent, composition and concentration of buffer and flow rate of mobile phase are major factors in the separation of positional isomers of acyl glucuronides (Khan et al., 1998). When these HPLC

conditions were altered, no split or new peaks were observed near the chromatographic areas of di-PA-457G and mono-PA-457Gs, indicating that putative acyl migration isomers which might be formed under the experimental incubation conditions are difficult to be resolved on HPLC. Collectively, these results suggest that the acyl glucuronides of PA-457 demonstrated relatively high in vitro stability with little putative acyl migration.

In vitro stability of PA-457Gs in incubation media with microsomes. In vitro stability of PA-457Gs in glucuronidation reaction media with pooled human, rat and mouse liver microsomes showed that, in the presence of inhibitors (D-SL and PMSF), all forms of PA-457Gs exhibited high stability in incubation media with all microsomes with no measurable hydrolysis (Fig. 5A, B and C). In the absence of the inhibitors, both mono-PA-457Gs demonstrated relatively high stability in all microsomal sources tested, however, di-PA-457G was less stable, particularly in rat and mouse liver microsomes (Fig. 5D, E and F). The degradation of di-PA-457G significantly increased the concentration of PA-457 and also slightly increased the content of mono-PA-457Gs (data not shown), suggesting that, without the protection of inhibitors, di-PA-457G is easily degraded to its parent form, PA-457, as well as to mono-glucuronides. Thus, under normal in vitro incubation conditions, the di-glucuronide formation may be underestimated in animal tissues, unless inhibitors are employed.

In vitro stability of PA-457Gs in plasma. In vitro stability of PA-457Gs in pooled human, rat and mouse plasma (without the addition of inhibitors) indicated that no significant changes of the contents of all forms of PA-457Gs were found after incubation at 37ºC for up to 4 h in comparison with the controls at

start (zero time) (Fig. 6), suggesting that PA-457Gs were stable, without hydrolysis, in plasma under the experimental conditions. Thus, inhibitors are not needed to stabilize the acyl glucuronides of PA-457 in plasma.

In this study, we demonstrated that, after oral administration, PA-457 was metabolized into its acyl glucuronides (including one di-glucuronide and two mono-glucuronides) and mainly eliminated into bile in rats. The predominant acyl glucuronide of PA-457 in rat bile was mono-PA-457G (I), with minor mono-PA-457G (II) and di-PA-457G, and very little free PA-457 was present. In vitro stability study showed that acyl glucuronides of PA-457 revealed no significant degradation after incubation 72 h in aqueous buffer at all pH conditions tested (pH 4, 7.4 and 9.0, respectively), and all forms of acyl glucuronides of PA-457 could be rapidly hydrolyzed both by β-glucuronidase and alkaline (1M NaOH) conditions. Putative acyl migration products were only observed at physiological pH and higher (pH 9.0) after many hours of incubation (72 h), suggesting that acyl glucuronides of PA-457 have relatively high in vitro stability. The relative stability of PA-457 glucuronides is possibly due to their structural characteristics with fully substituted carbon adjacent to the carboxyl group, the steric hindrance results in minimal acyl migration for glucuronides of those compounds (Benet, et al., 1993). Experiments also indicated that acyl glucuronides of PA-457 were quite stable in human and animal liver microsomes and plasma, though the apparently minor metabolite in rat bile, the di-glucuronide, is less stable.

In conclusion, these studies with acyl glucuronides of PA-457 indicate that they are relatively stable compared to most other acyl glucuronides of drugs that have been reported (Spahn-Langguth and Benet,

1992, 1993; Bailey and Dickinson, 2003). The high stability suggests that these acyl glucuronides may also be less reactive in vitro and in vivo, thus there is less concern regarding possible roles that these metabolites might have as putative reactive intermediates.

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Footnotes

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Legends for figures

FIG. 1. Chemical structures of PA-457 and internal standard (DSD).

FIG. 2. Representative LC-ESI-MS chromatograms (TIC, total ion chromatogram; SIM, selective ion monitoring) and MS spectra of PA-457, PA-457Gs and internal standard (DSD) in rat bile.

FIG. 3. Enzyme hydrolysis of PA-457Gs in human and animal plasma. Aliquots of rat bile samples containing PA-457Gs were spiked in human (A), rat (B) and mouse (C) plasma and then hydrolyzed by β-glucuronidase (0-4,000 U/ml) at pH 5.0 and 37ºC for 2 h. Data (means of duplicates) were measured by LC-ESI-MS assay and expressed as remaining PA-457Gs (% of the initial at time zero) and released PA-457 (μg/ml), respectively.

FIG. 4. In vitro stability of PA-457 and PA-457Gs in phosphate buffers at different pH. Aliquots of rat bile samples containing PA-457Gs were pre-incubated with pH 4.0, 7.4 and 9.0 phosphate buffers (100 mM)

at 37ºC for 0 to 72 h, and then hydrolyzed by β-glucuronidase (4,000 U/ml) at pH 5.0 and 37ºC for 2 h. Data (means of duplicates) were measured by LC-ESI-MS assay and expressed as peak area ratios calculated from peak areas of individual metabolites divided by that of total metabolites in comparison with the controls at time zero.

FIG. 5. In vitro stability of PA-457Gs in incubation media with human and animal liver microsomes. Aliquots of rat bile samples containing PA-457Gs were incubated in glucuronidation reaction media (without UDPGA) with pooled human, rat and mouse liver microsomes (1 mg protein/ml) at 37ºC for 0, 0.25, 0.5, 1, 2 and 4 h. Data (means of duplicates) were measured by LC-ESI-MS assay and expressed as peak area ratios relative to internal standard (DSD) in comparison with the controls at time zero. A (HLM), B (RLM) and C (MLM) with D-SL (5 mM) and PMSF (0.5 mM). D (HLM), E (RLM) and F (MLM) without D-SL and PMSF.

FIG. 6. In vitro stability of PA-457Gs in human and animal plasma (without the addition of inhibitors). Aliquots of rat bile samples containing PA-457Gs were directly incubated with human (A), rat (B) and mouse (C) plasma at 37ºC for 0, 0.25, 0.5, 1, 2 and 4 h. Data (means of duplicates) were measured by LC-ESI-MS assay and expressed as peak area ratios relative to internal standard (DSD) in comparison with the controls at time zero.

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TABLE 1

Percent of Enzyme and Alkaline Hydrolysis of PA-457Gs in Rat Bile

Acyl glucuronides	β-glucuronidase[a]			NaOH[b]
	pH 4.0	pH 7.4	pH 9.0	
mono-PA-457G (I)	100	99.7	99.8	84.0
mono-PA-457G (II)	100	99.2	94.6	90.3
di-PA-457G	100	100	100	100

[a] PA-457Gs from rat bile were pre-incubated in 0.1 M phosphate buffer (pH 4.0, 7.4 and 9.0) at 37ºC for 72 h and then hydrolyzed with β-glucuronidase (4,000 U/ml) at pH 5.0 and 37ºC for 2 h.

[b] PA-457Gs from rat bile were hydrolyzed with 1 M NaOH at 60ºC for 2 h.

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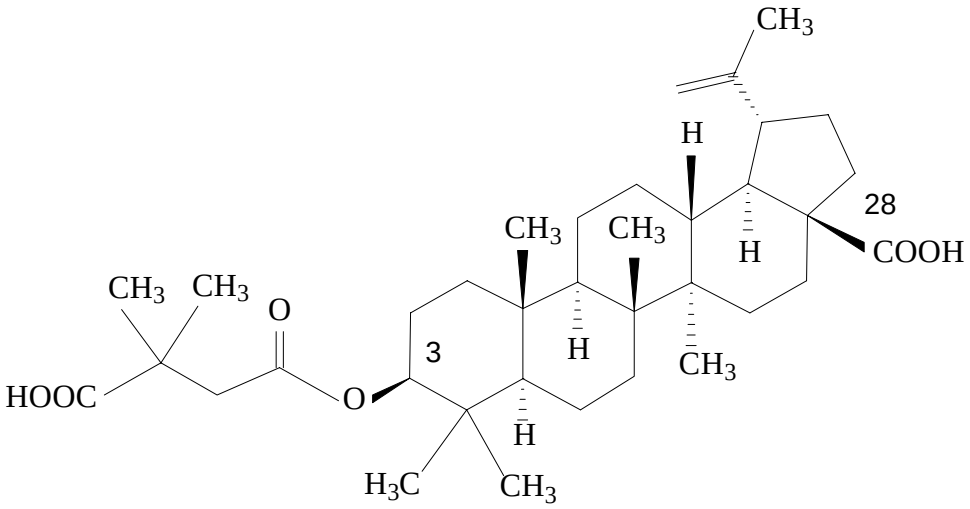
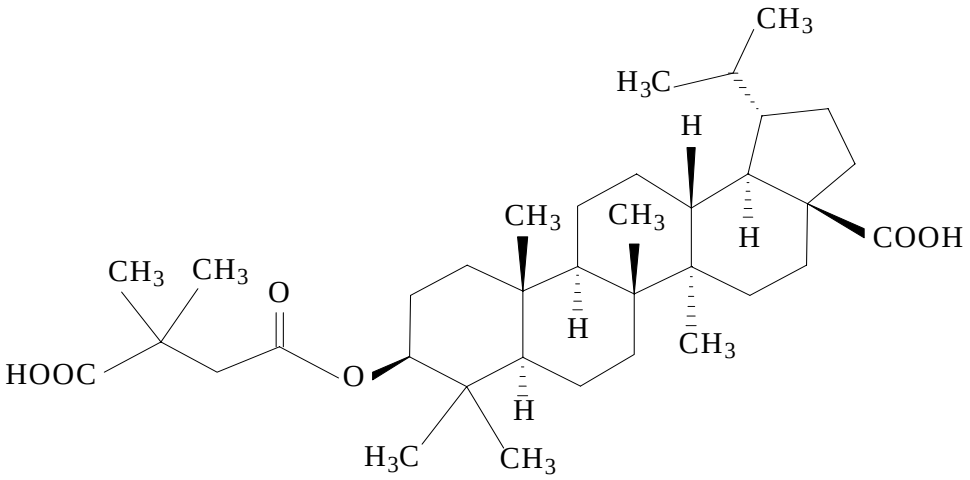
Fig. 1



PA-457 (MW 584)



DSD (MW 586, Internal Standard)

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Fig. 2

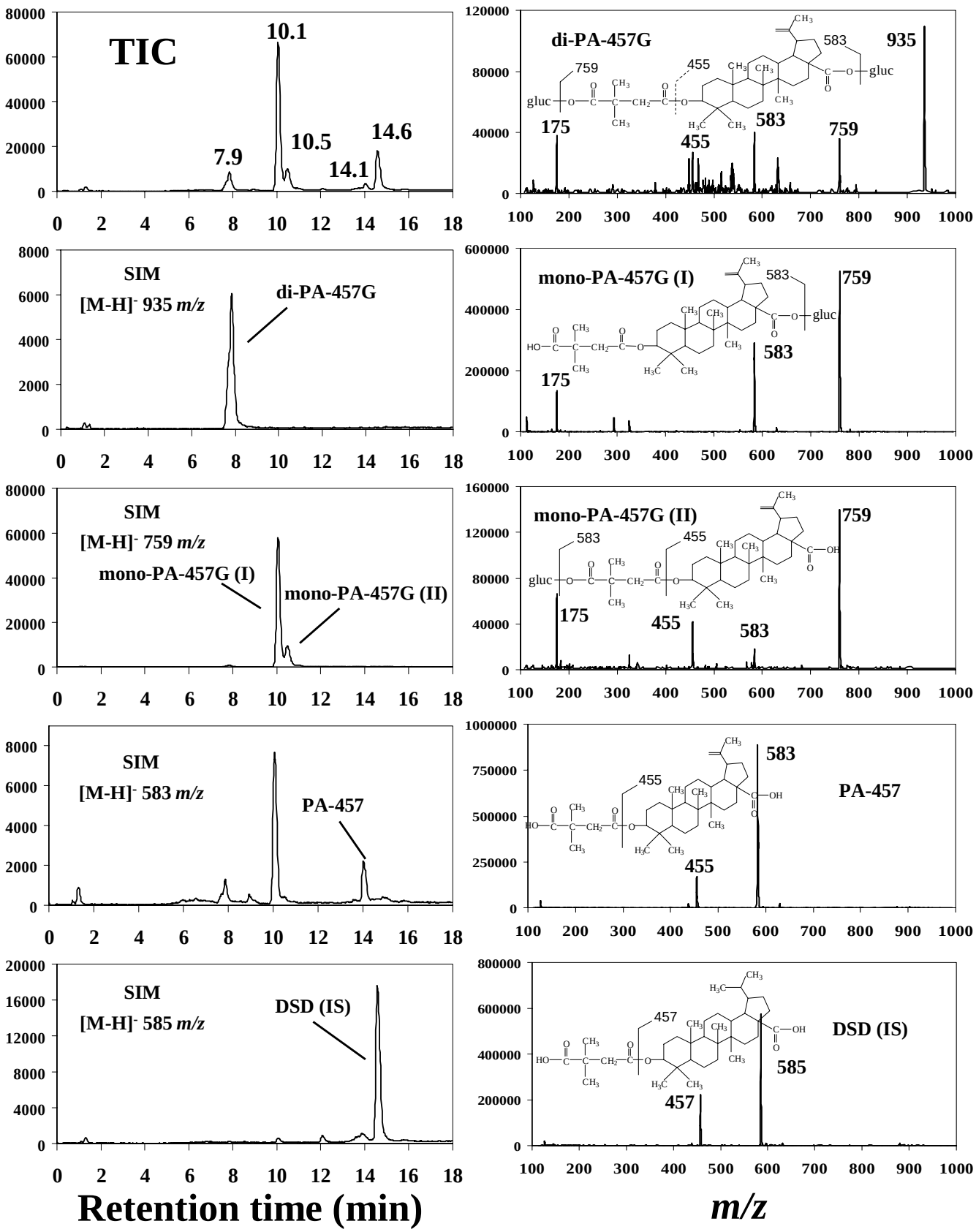

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Fig. 3

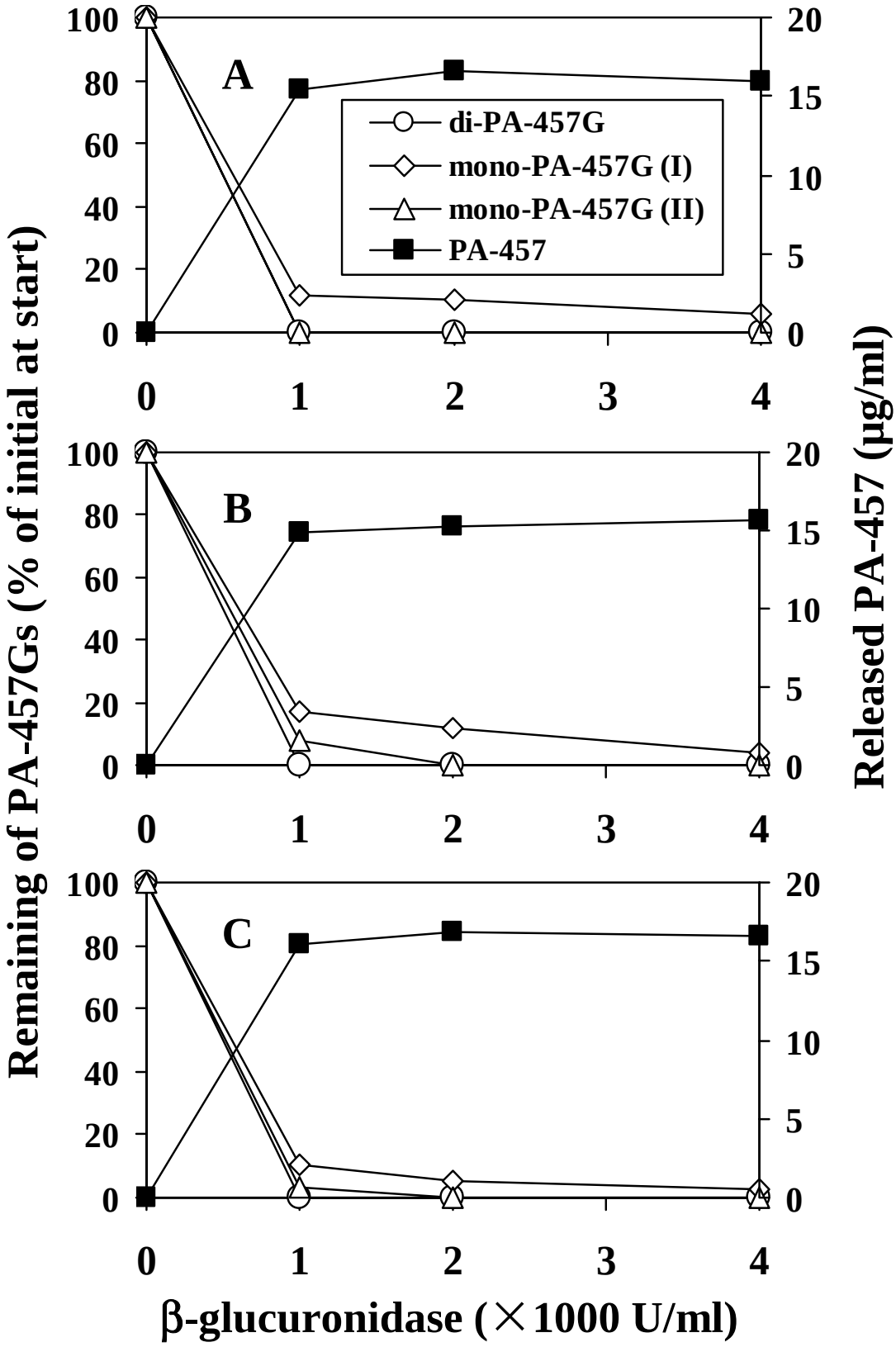

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Fig. 4

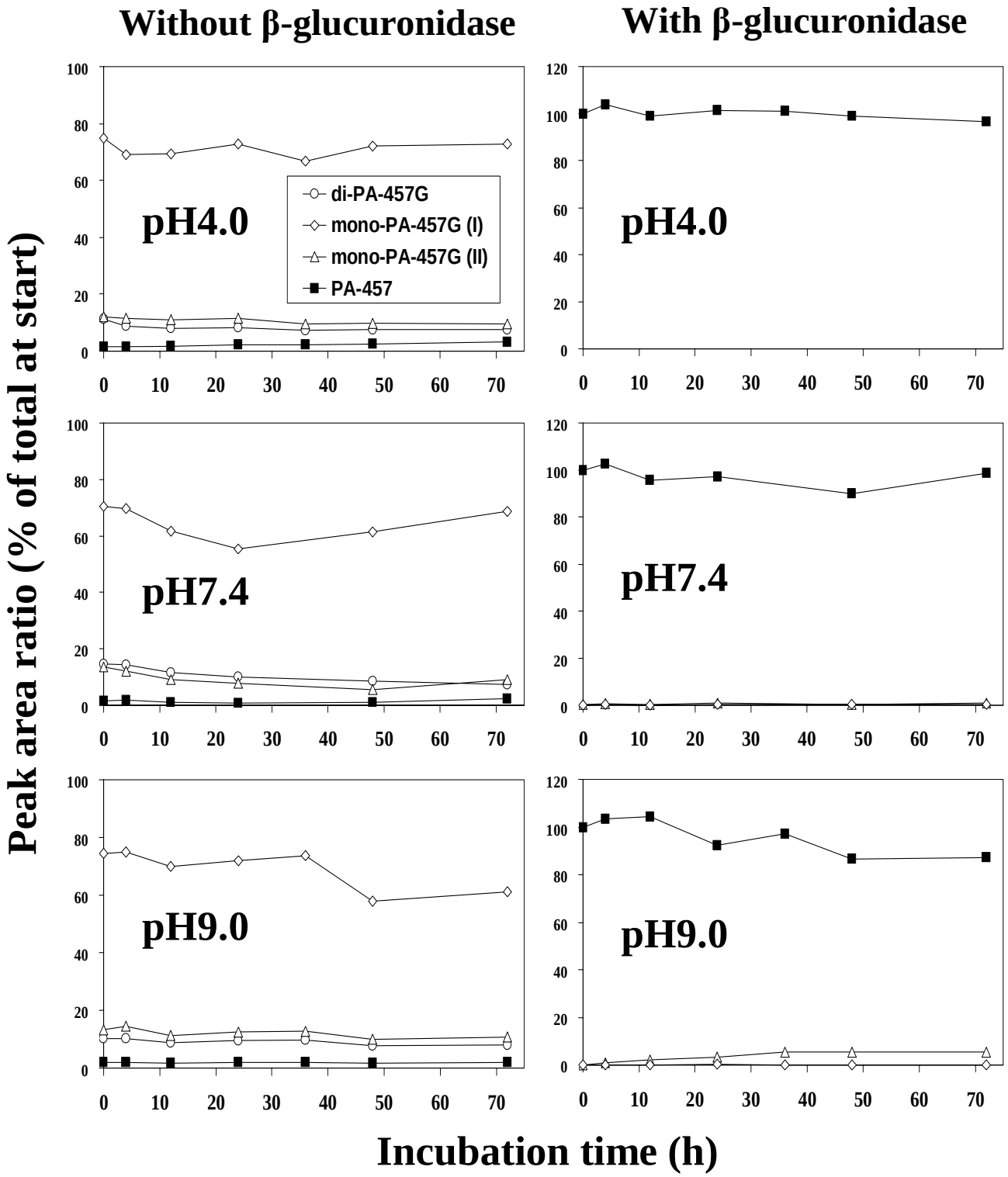

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Fig. 5

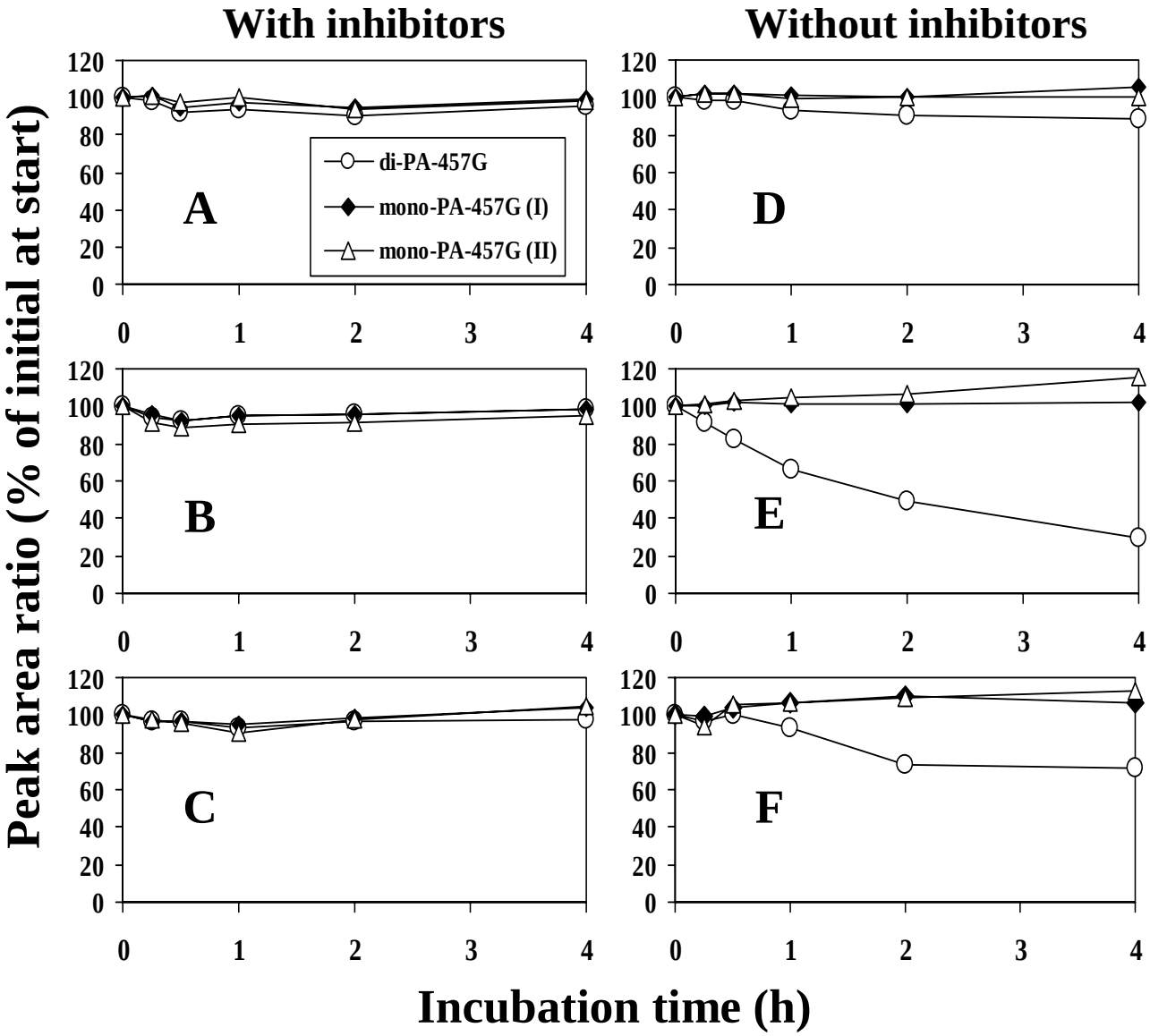

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Fig. 6