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Title: In Vitro and In Vivo Determination of Piperacillin Metabolism in Humans

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Running title: metabolism of piperacillin in humans

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Non-standard abbreviations: Sandwich-cultured human hepatocytes (SCHH), Biliary Excretion Index (BEI), dexamethasone (DEX), Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA), human liver microsomes (HLM), Dulbecco's Modified Eagle's Medium (DMEM), nonessential amino acids (NEAA), internal standard (IS), multiple reaction monitoring (MRM), multidrug resistance associated protein (MRP).

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ABSTRACT

Piperacillin metabolism and biliary excretion are different between humans and preclinical species. In the present study, piperacillin metabolites were characterized in bile and urine of healthy humans and compared with metabolites formed *in vitro*. Volunteers were administered 2 g piperacillin IV; blood, urine and duodenal aspirates (obtained via a custom-made oroenteric catheter) were collected. The metabolism of piperacillin in humans also was investigated *in vitro* using pooled human liver microsomes and sandwich-cultured human hepatocytes. Piperacillin and metabolites were estimated by HPLC with MS-MS detection. Piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide were detected in bile, urine and human liver microsomal incubates. Similar to the *in vivo* results, desethylpiperacillin was formed and excreted into bile canaliculi of sandwich-cultured human hepatocytes. This is the first report of glucuronidation of desethylpiperacillin *in vitro* or *in vivo*. The clinical method employed in this study to determine biliary clearance of drugs also facilitates bile collection as soon as bile is excreted from the gallbladder, thereby minimizing the exposure of labile metabolites to the intestinal environment. This study exemplifies how a combination of *in vitro* and *in vivo* tools can aid in the identification of metabolites unique to the human species.

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The characterization of metabolic pathways that are unique to humans is very important in the drug development process. The formation of human-specific metabolites can be predicted using *in vitro* systems including pooled human microsomes, suspended human hepatocytes or recombinant enzymes; nevertheless, these tools do not allow for mechanistic studies concerning the excretory route of these metabolites from the liver (canalicular versus basolateral excretion). Often, biliary excretion of drugs and metabolites can only be characterized using animal models, primarily rodent isolated perfused livers and bile duct cannulated animals. However, significant inter-species differences in the function and regulation of transport proteins and drug metabolizing enzymes have been reported, and these could compromise the ability to extrapolate from animal data to humans (Wang and LeCluyse, 2003; Zhang et al., 2005).

Aspiration of bile from the duodenum of healthy volunteers can be used to determine biliary clearance of drugs (Ghibellini et al., 2006a). Additionally, this methodology, combined with the sensitivity of mass spectrometry, may offer a new tool to identify metabolites formed in humans that might not be predicted from preclinical studies. Similarly, sandwich-cultured human hepatocytes (SCHH), a particularly powerful tool with respect to metabolic and transport capabilities, may be a useful *in vitro* tool to predict the formation and route of excretion of metabolites (LeCluyse et al., 2000; Hoffmaster et al., 2004).

Piperacillin is a third-generation, broad-spectrum, penicillin derivative. The biliary excretion of piperacillin in animal models is extensive, ~15% of the piperacillin dose was recovered in rat bile after single-pass isolated perfused liver experiments (Calhoun et al., 1987), and 37% of the dose was recovered in recirculating rabbit isolated perfused livers (Brogard et al., 1994). In contrast, the biliary excretion of piperacillin in healthy humans is negligible (Ghibellini et al., 2006b). Very little is known about the metabolism of piperacillin in humans;

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the liver-specific metabolism of this compound to desethylpiperacillin was reported previously using human liver homogenates (Minami et al., 1991) and this metabolite was identified in human urine (Komuro et al., 1997). No other metabolites have been described to date, and the excretion of piperacillin metabolites into human bile has not been characterized.

During a clinical study designed to determine the biliary clearance of piperacillin in healthy volunteers, bile and urine samples were obtained from three subjects and analyzed by HPLC-MS-MS to estimate the contribution of the biliary route to the systemic clearance of the parent compound (Ghibellini et al., 2006b). Although desethylpiperacillin has been recovered previously in human urine and in human liver homogenates, the enzymes involved in the formation and the preferential route of excretion of desethylpiperacillin have not been demonstrated to date. Accordingly, one of the goals of this study was to use mass spectrometry to evaluate whether sampling bile immediately upon secretion into the intestine would enable identification of potentially unstable drug metabolites. In addition, piperacillin metabolism was evaluated also using hepatic microsomes and sandwich-cultured human hepatocytes to allow for the comparison of metabolites generated *in vitro* to those formed *in vivo*.

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METHODS

Chemicals and Reagents for *In Vitro* Studies: Insulin/transferrin/selenium (ITS+) was purchased from BD Biosciences (Bedford, MA). Dexamethasone (DEX), piperacillin, β -NADPH, Hanks' balanced salt solution (HBSS) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade and were available from commercial sources.

Phase I Metabolism of Piperacillin: Mixed gender human liver microsomes (HLM, 2.5 mg/mL from XenoTech, LLC, Lenexa, KS) were incubated in triplicate in 100 mM potassium phosphate buffer (pH 7.4), 3.3 mM MgCl_2 and a concentration of piperacillin within the range of the observed clinical C_{max} (200 μM), in a total incubation volume of 0.5 mL. After pre-incubation (5 min, 37°C), the reactions were initiated by the addition of 1 mM β -NADPH; as a control, incubations without β -NADPH also were performed. At 0, 5, 15, 30, 60 and 90 min, aliquots (50 μL) were removed and the reactions stopped with an equal volume of ice-cold acetonitrile containing cimetidine as the internal standard (IS). Proteins were precipitated by centrifugation (3500xg for 5 min) and the supernatant was analyzed immediately by HPLC-MS-MS.

Liver cytosolic incubations: Individual incubations (total volume 0.5 mL) were composed of 0.25 mg/mL, mixed gender, pooled human liver cytosol (XenoTech, LLC, Lenexa, KS) in 100 mM phosphate buffer (pH 7.4), 3.3 mM MgCl_2 , 1 mM NADH and piperacillin at three different concentrations (50, 100 and 200 μM). After 5 min pre-incubation at 37°C, the reaction was initiated by the addition of NADH. Aliquots (50 μL) were removed at 0, 2, 5, 10, 15, 30 and 60 min and quenched with 25 μL of ice-cold acetonitrile. Proteins were precipitated and 70 μL of the supernatant was analyzed immediately using HPLC-MS-MS as described below.

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Glucuronidation of Piperacillin: Incubations were performed in duplicate using HLM (2.5 mg/mL from XenoTech, LLC, Lenexa, KS), 100 mM potassium phosphate buffer (pH 7.4), 3.3 mM MgCl₂, 50 µg alamethicin/mg protein, 5 mM saccharolactone and 500 µM piperacillin in a total incubation volume of 0.5 mL. After pre-incubation (5 min, 37°C), the reactions were initiated by the addition of β-NADPH (2 mM) and UDPGA (5 mM). Reactions were terminated at 0, 5, 15, 30, 60, and 90 min by quenching 50 µL of the incubation mixture with an equal volume of ice-cold 80:20 (v/v) acetonitrile:1% acetic acid containing cimetidine (IS). After protein precipitation, the supernatant was analyzed by HPLC-MS-MS.

Isolation and Culture of Human Hepatocytes: Human liver tissue was obtained by qualified medical staff from the University of North Carolina at Chapel Hill, School of Medicine, as waste from surgical resection. Donor consent and IRB approval were obtained for all studies involving human liver tissue. Hepatocytes were isolated by modifications of the two-step collagenase digestion method (Hamilton et al., 2001). Hepatocytes were cultured for 6 days according to the methods described by Hoffmaster *et al.* (Hoffmaster et al., 2004) with modifications, and medium was replaced every 24 h. Briefly, 1.5x10⁶ hepatocytes/well were seeded on 6-well Biocoat™ plates (BD Bioscience) in 1.5 mL of Dulbecco's Modified Eagle's Medium (DMEM, without phenol red) supplemented with 5% fetal bovine serum, nonessential amino acids (NEAA), L-glutamine, penicillin/streptomycin, and 1 µM dexamethasone (DEX). After cell attachment (2–6 h at 37°C in a humidified incubator with 95% air/ 5% CO₂), medium was replaced with DMEM containing 0.1% v/v ITS+, penicillin/streptomycin, NEAA, L-glutamine, and 0.1 µM DEX; 6-12 hr later, cells were overlaid with ice-cold medium containing 0.25 mg/mL Matrigel™ (BD Bioscience).

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Human Hepatocyte Studies: Four different sandwich-cultured human hepatocyte (SCHH) preparations were used for these experiments. The biliary excretion index (BEI) was calculated according to the method described by Hoffmaster *et al.* (Hoffmaster *et al.*, 2005) using B-CLEAR[®] technology (Qualyst, Inc., Research Triangle Park, NC). The BEI indicates the fraction of the total accumulation of drug that resides in the bile compartment. Accumulation of [³H] taurocholate (1 μM, 100 nCi) in cellular and cells plus bile compartments was determined over 10 min; piperacillin (300 μM) accumulation was determined over 30 min. Only preparations with a [³H] taurocholate BEI ≥ 50% were incubated with piperacillin. Since 30 min was not long enough to allow for the formation of Phase II metabolites in SCHH of Liver 1, additional experiments were performed with hepatocytes from Livers 2, 3, and 4. Hepatocytes were incubated for 2 hr in HBSS (Livers 2, 3, and 4), and for 24 and 48 hr (Liver 4 only) in DMEM, both containing 500 μM piperacillin, in an attempt to increase intracellular concentrations of piperacillin. To measure the accumulation of piperacillin in the presence or absence of intact canalicular spaces, hepatocytes were lysed with 70/30 (v/v) methanol/water. Samples were analyzed directly, or evaporated and reconstituted in 1/10 of the original volume containing cimetidine (IS) prior to analysis by HPLC-MS-MS. Nonspecific binding was accounted for by including a blank plate (Biocoat[™] plus Matrigel[™] overlay). Protein content in cell lysate was quantified with the BCA method (Smith *et al.*, 1985) and was used to normalize accumulation. Due to the incompatibility of methanol with the protein assay, the average protein content for standard HBSS or Ca²⁺-free HBSS incubations with taurocholate was used to normalize piperacillin and metabolite content of the same liver preparation.

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Clinical Study:

The design and conduct of the clinical study has been described previously (Ghibellini et al., 2006b). In brief, three male volunteers completed the study. The Clinical Research Advisory Committee and the Committee on the Protection of the Rights of Human Subjects (IRB) at the University of North Carolina at Chapel Hill School of Medicine approved all procedures. Subjects provided written informed consent prior to participation in the study. After an overnight fast, a custom-made oroenteric tube, described in detail previously (Ghibellini et al., 2004), was passed through the mouth and positioned in the duodenum. The distal end of the tube was fitted with a polyethylene balloon, which was inflated to occlude the intestine during bile collection. Subjects were administered 2 g of piperacillin (generous gift of American Pharmaceutical Partners) as a 15-min IV infusion, and blood, bile and urine samples were collected at predetermined intervals over 6 hr (bile) and 10 hr (urine and plasma).

Analysis of Piperacillin and Metabolites: Bile, urine and samples from the *in vitro* experiments were analyzed for piperacillin and metabolites using HPLC-MS-MS. Bile and urine samples were diluted 1:7 with methanol containing cimetidine (0.5 µg/mL) as the internal standard (IS). The Agilent 1100 HPLC system (Agilent, Germany) was coupled to an Applied Biosystems API4000 mass spectrometer with a TurboIonSpray source (Foster City, CA) as described previously (Ghibellini et al., 2006b). User controlled parameters were optimized via direct infusion for the detection of the most intense parent-to-product transitions in both positive and negative ionization modes when analytical standards were available (piperacillin and cimetidine). For desethylpiperacillin and the two glucuronides, the most likely transitions were monitored under conditions that were assumed to be optimal. For example, conditions for desethylpiperacillin were the same as the structurally similar piperacillin. For the glucuronide

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metabolites, lower temperature and ionization energy were used to prevent in-source fragmentation and lower collision energy was used to prevent fragmentation beyond the loss of the glucuronide sugar. For positive ion multiple reaction monitoring (MRM), the following transitions were used: piperacillin, 518.2→143.3; cimetidine, 253.1→117.0; desethylpiperacillin, 490.0→115.0. For negative ion MRM, the following transitions were used: piperacillin, 516.0→141.0; desethylpiperacillin, 488.0→113.0; piperacillin glucuronide, 692.0→516.0; desethylpiperacillin glucuronide, 664.0→488.0 and cimetidine 250.9→165.9. Desethylpiperacillin and desethylpiperacillin glucuronide concentrations were estimated qualitatively and expressed as analyte:IS peak area ratios and relative amounts were expressed as the product of the area ratio and the volume collected. To confirm the presence of piperacillin glucuronide and desethylpiperacillin glucuronide, samples were injected onto an Agilent 1100MSD ion trap mass spectrometer (Agilent, Germany). Analysis was conducted in negative mode using autoMSⁿ in which parent ions of sufficient intensity were subjected to fragmentation. All product ions were scanned; since both parent and glucuronide conjugates contained similar structural elements, they were expected to form similar fragments.

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RESULTS AND DISCUSSION

Desethylpiperacillin and the glucuronide conjugate of desethylpiperacillin were identified in the urine and bile of all subjects from the clinical study described by Ghibellini *et al.* (Ghibellini *et al.*, 2006b). While dealkylation of piperacillin has been observed previously (Komuro *et al.*, 1997), the glucuronide-conjugated metabolite is a novel finding of the present study. Quantification of desethylpiperacillin and desethylpiperacillin glucuronide was not possible because analytical standards of these metabolites were not available. The relative amounts of piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide, expressed as analyte:IS peak area ratios corrected for the volume of aspirate collected in the duodenal aspirates and in urine fractions analyzed by HPLC-MS-MS in negative ionization mode, are plotted in Figure 1, (panel A). Piperacillin was excreted mostly into urine as the parent drug, while biliary excretion was more predominant for the metabolites (Figure 1A). This observation could be explained by differential affinities for transport proteins in the liver [e.g. the apical and basolateral members of the MRP (multidrug resistance associated protein) family], which may determine the major route of hepatic elimination of each species. The structure of piperacillin is reported in Fig 1 (panel B) and chromatograms of piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide in duodenal aspirates from subject number 1 are shown in Fig 2 A.

The presence of desethylpiperacillin glucuronide was investigated further. Bile samples were incubated with bovine β -glucuronidase (Sigma-Aldrich 4000 units/mL, pH 5, 37°C, over 24 hr). As expected, the desethylpiperacillin glucuronide peak was sensitive to enzymatic degradation (results not shown). Secondly, the structure of the phase II metabolite was confirmed by ion trap mass spectrometry, as shown in Figure 2, (Panel B). The fragmentation pattern of the proposed desethylpiperacillin glucuronide peak (pseudomolecular ions of 664 amu,

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176 amu greater than desethylpiperacillin) was the same as that of the desethylpiperacillin peak. Unfortunately, it was not possible to determine the location of glucuronidation based on the fragmentation pattern. The first fragment produced was desethylpiperacillin, through the loss of the glucuronide sugar. This would happen regardless of whether an O- or N- glucuronide was formed.

The products of Phase I and II metabolism subsequently were examined *in vitro*. There was no turnover of piperacillin in the cytosolic fractions of human livers (data not shown), but metabolites were formed in microsomal incubations. Figure 3, (Panel A) depicts the *in vitro* formation of desethylpiperacillin by incubation of piperacillin with pooled HLM in the presence of β -NADPH. The lack of desethylpiperacillin formation in the absence of β -NADPH suggests that this oxidation may be mediated by cytochrome P450. The determination of the extent of metabolism *in vitro* through substrate depletion was not possible due to the low turnover of piperacillin. Nevertheless, it was feasible to demonstrate that glucuronidation also took place *in vitro*, once the microsomal membrane was made permeable with alamethicin. Figure 3 (Panel B) illustrates that both piperacillin glucuronide and desethylpiperacillin glucuronide were formed over time upon incubation of the HLM with piperacillin in the presence of β -NADPH and UDPGA. Since desethylpiperacillin glucuronide formation was dependent upon the initial turnover of the parent drug into the Phase I metabolite, the amount of this glucuronide formed *in vitro* was extremely small, yet detectable after a 15-min incubation (Figure 3, Panel B insert). Although piperacillin glucuronide was detected in the *in vitro* incubations, it was not detected in the clinical samples, perhaps due to degradation in bile and urine.

When SCHH preparations were used to investigate the metabolism of piperacillin and the excretion of metabolites into bile, desethylpiperacillin was formed within 30 min in all of the

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preparations (n=4). A detectable BEI value (7-20%) was determined in three out of four preparations where the accumulation of desethylpiperacillin in cells was lower than in cells plus bile, indicating excretion of this metabolite into bile canaliculi (results not shown), in accordance with data obtained in the clinical study. The parent compound (piperacillin) also was excreted into bile canaliculi in the SCHH system as detailed in Ghibellini *et al.* (2006c). Concentrations of desethylpiperacillin glucuronide and piperacillin glucuronide were undetectable after 30 min, 2, 24 or 48 hr incubation in the presence of piperacillin. This discrepancy between hepatocytes and HLM incubations could be attributed to lower intracellular concentrations of piperacillin in the SCHH compared to the concentrations available in the HLM. Although the piperacillin concentration in the medium was similar to the C_{max} obtained in healthy volunteers in the clinical study (Ghibellini *et al.*, 2006b), the intracellular concentration in SCHH was very low (less than 0.4% of the dose was taken up into the hepatocytes at 30 min). Additionally, the high concentration of microsomal protein (2.5 mg/mL) and of piperacillin (500 μ M) in the incubation mixture may have contributed to the discrepancy observed between the two *in vitro* systems, however these conditions were necessary and optimal for the formation and detection of the Phase II metabolites, given the low metabolic turnover of piperacillin. From the results obtained with this probe it can be concluded that SCHH are capable of forming desethylpiperacillin, demonstrating the maintenance of Phase I metabolizing enzymes; excretion of desethylpiperacillin into the canalicular networks of SCHH was detectable. Future studies should focus on probe substrates with higher metabolic turnover rates to assess maintenance of Phase I and II enzyme activity in SCHH.

Piperacillin was not extensively metabolized in SCHH consistent with the predominantly renal elimination of the parent compound observed *in vivo* in humans (Ghibellini *et al.*, 2006b).

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Human-specific hepatic metabolism of piperacillin into desethylpiperacillin has been reported previously (Minami et al., 1991) and piperacillin has been reported to inhibit azidothymidine glucuronidation (Rajaonarison et al., 1992). Nevertheless, no literature reports document phase II metabolism of this antibiotic *in vitro* or *in vivo*. This study demonstrated that the N-dealkylation of piperacillin is mediated by hepatic microsomes and is NADPH dependent, suggesting the involvement of a cytochrome P450 enzyme. Additional studies in the presence of UPDGA and β -NADPH demonstrated that piperacillin and its metabolite, desethylpiperacillin, undergo glucuronidation *in vitro*. Although this novel observation represents a previously unreported pathway of metabolism, the clinical relevance is not expected to be significant due to the fact that metabolism accounts for a very small percentage of piperacillin clearance.

In conclusion, *in vivo* and *in vitro* tools were used to identify novel piperacillin metabolites and clarify the routes of hepatic excretion. This type of information may be very desirable for new drugs in development, and could be obtained using SCHH and human liver microsomes during the preclinical development process, as well as by collecting duodenal aspirates in the initial clinical trials. It is possible that the acquisition and analysis of bile as soon as it appears in the duodenum could be the only way to recover metabolites that are unstable in the intestinal environment, or that undergo reabsorption along the intestine. This would allow for the full profiling of metabolites that cannot be recovered in urine or feces, and that are not predicted from preclinical studies. In the future, more extensive use of SCHH, where metabolic profiling and characterization of the routes of excretion is possible, may reduce the need for clinical studies.

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FOOTNOTES:

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FIGURE LEGENDS

Fig 1: Piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide in clinical samples.

Panel A: Relative amounts of piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide in urine (left ordinate axis) and duodenal aspirates (right ordinate axis). Relative amounts are expressed as the mean \pm SD of the analyte:internal standard (IS) peak area ratio multiplied by the collected volume.

Panel B: Structure of piperacillin.

Fig 2: Chromatograms of piperacillin, desethylpiperacillin and desethylpiperacillin glucuronide in duodenal aspirates and identification of the metabolites via HPLC-MS-MS.

Panel A: Chromatograms of piperacillin and desethylpiperacillin, acquired in positive ionization mode, and desethylpiperacillin glucuronide, acquired in negative ionization mode, in duodenal aspirates of subject number 1.

Panel B: MS of $[M-H] = 664.0$, the predicted pseudomolecular ion of desethylpiperacillin glucuronide, and Auto MS² of $[M-H] = 488.0$, the pseudomolecular ion of desethylpiperacillin. A glucuronide-specific neutral loss of 176 amu was observed.

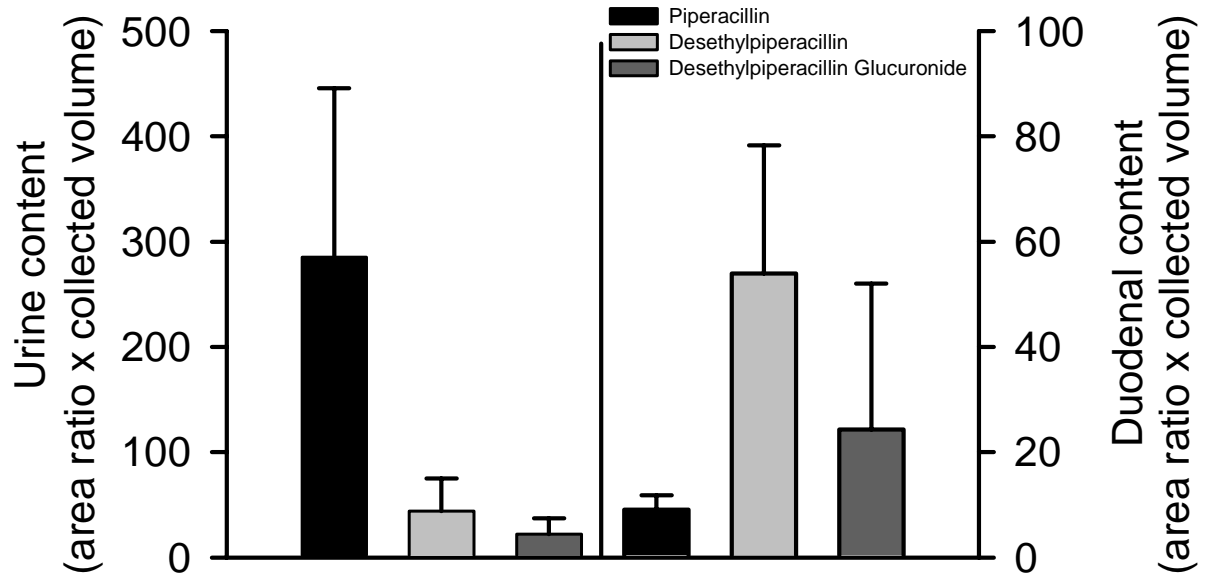
Fig 3: Piperacillin metabolism in human liver microsomal incubations.

Panel A: Desethylpiperacillin formation (mean \pm SD) over time in the presence (●) and absence (□) of NADPH. Relative amounts are expressed as the analyte:internal standard (IS) peak area ratio. NADPH incubations were performed in triplicate, and in duplicate for control incubations.

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Panel B: Desethylpiperacillin (●), desethylpiperacillin glucuronide (○) and piperacillin glucuronide (▲) formation over time in the presence of NADPH and UDPGA. All incubations were performed in duplicate and the mean value is reported. Insert shows the formation of desethylpiperacillin glucuronide over time on an enlarged scale.

Fig 1:
Panel A



Panel B

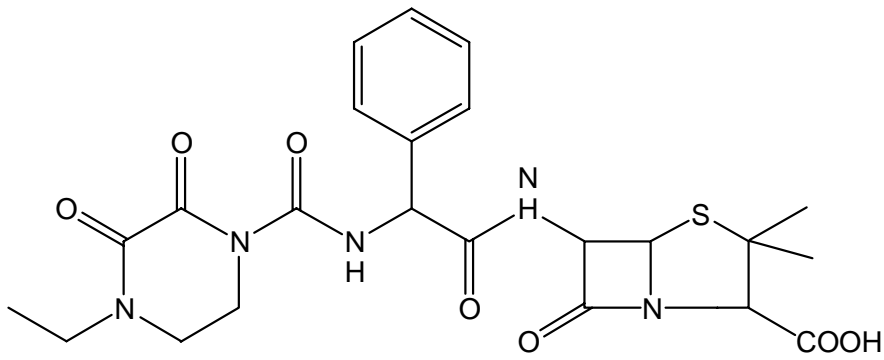
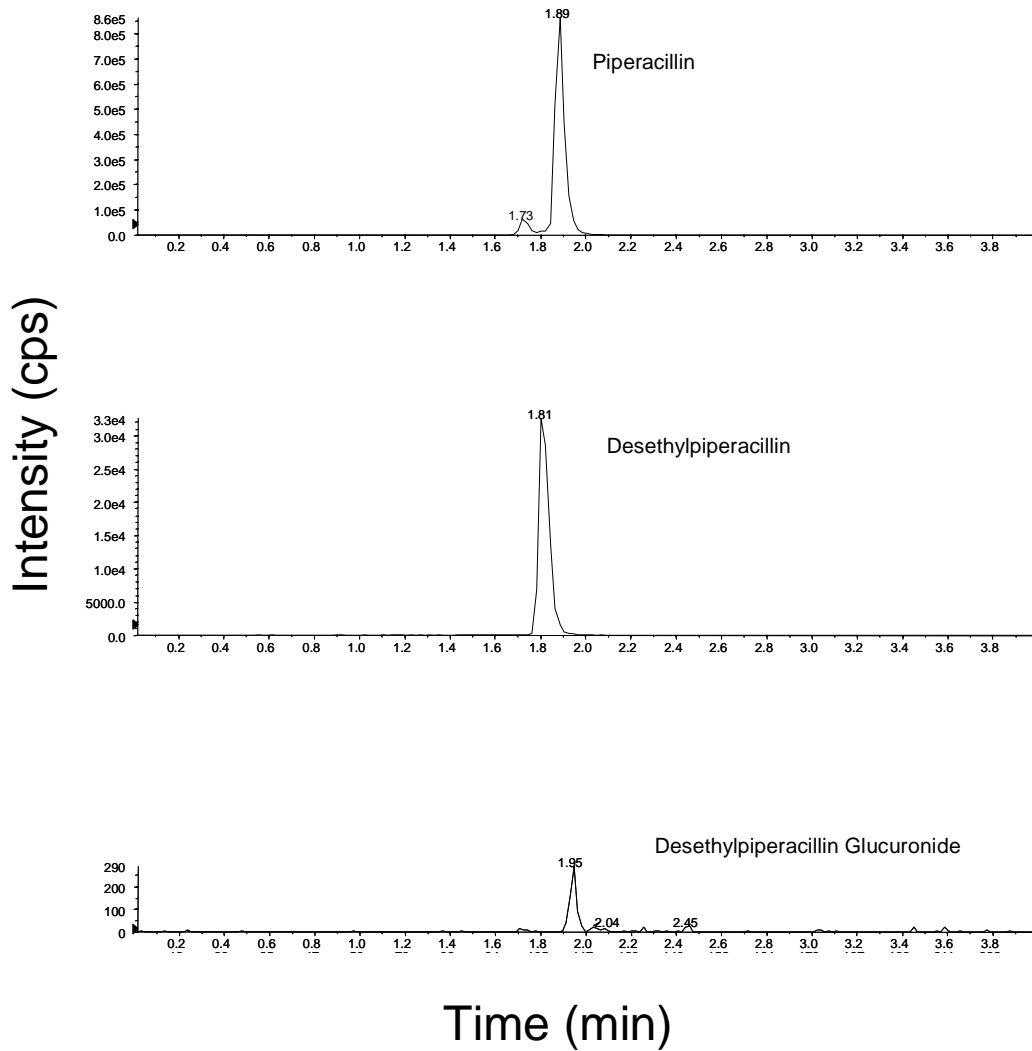


Fig 2:
Panel A



Panel B

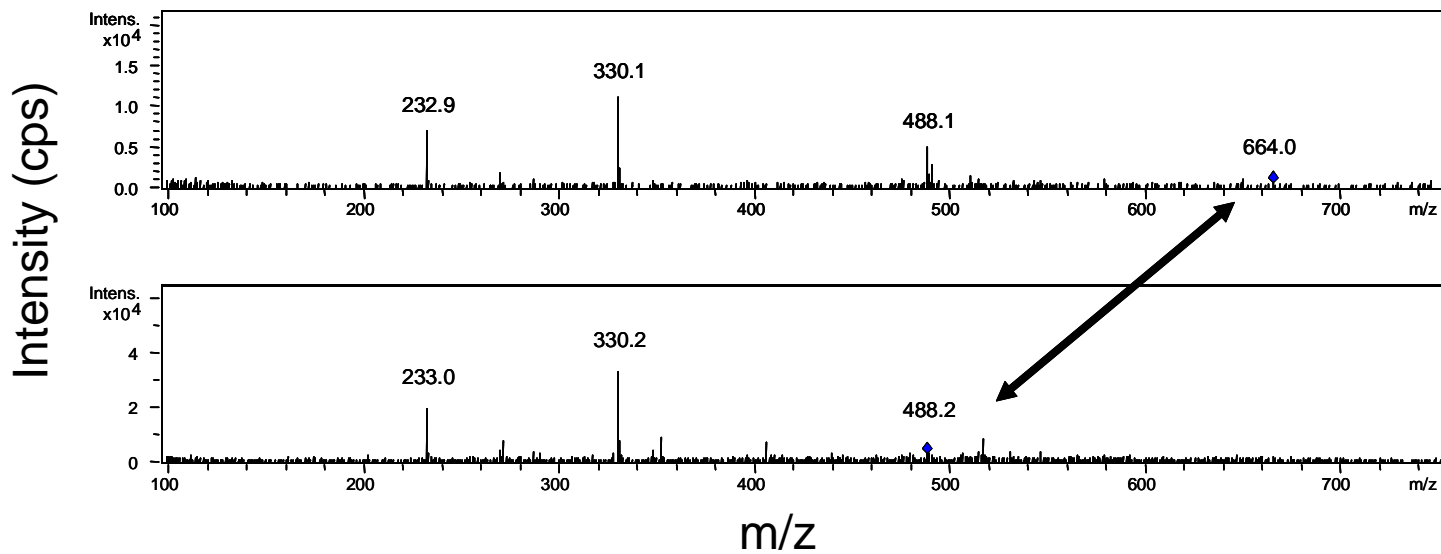
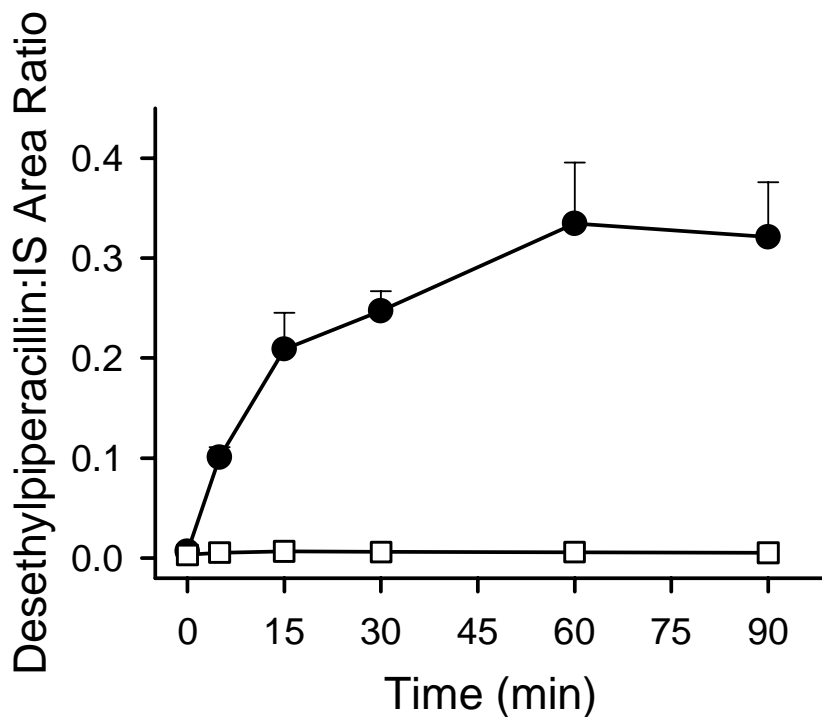


Fig 3:
Panel A



Panel B

