

Title page

**Endogenous plasma kynurenic acid in human: a newly discovered
biomarker for drug-drug interactions involving in OAT1 and OAT3 inhibition**

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Abbreviations: *AUC*, area under the plasma concentration-time curve; *C_{max}*, peak concentration;

OAT, organic anion transporter; *LC-MS/MS*, liquid chromatography–tandem mass spectrometry;

DDI, drug-drug interaction

Abstract

As an expansion investigation of drug-drug interaction (DDI) from previous clinical trials (Shen et al., 2019), additional plasma endogenous metabolites were quantitated in the same subjects to further identify the potential biomarkers of OAT1/3 inhibition. In the single dose, open label, three-phase with fixed order of treatments study, 14 healthy human volunteers orally received 1000 mg probenecid alone, or 40 mg furosemide alone, or 40 mg furosemide at 1 h after receiving 1000 mg probenecid on Days 1, 8, and 15, respectively. Endogenous metabolites including kynurenic acid, xanthurenic acid, indo-3-acetic acid, pantothenic acid, *p*-cresol sulfate, and bile acids in the plasma were measured by LC-MS/MS. The C_{\max} of kynurenic acids was significantly increased about 3.3- and 3.7-fold over the baseline values at pre-dose followed by the treatment of probenecid alone or in combination with furosemide respectively. In comparison with the furosemide alone group, the C_{\max} and AUC_{0-12} of kynurenic acid were significantly increased about 2.4 and 2.5-fold by probenecid alone, and 2.7 and 2.9-fold by probenecid plus furosemide, respectively. The increases in C_{\max} and AUC of plasma kynurenic acid by probenecid are comparable to the increases of furosemide C_{\max} and AUC reported previously. Additionally, the plasma concentrations of xanthurenic acid, indo-3-acetic acid, pantothenic acid, and *p*-cresol sulfate, but not bile acids, were also significantly elevated by probenecid treatments. The magnitude of effect size analysis for known potential endogenous biomarkers demonstrated that kynurenic acid in the plasma offers promise as a superior addition for early DDI assessment involving OAT1/3 inhibition.

Significance Statement

We reported that probenecid, an OAT1 and OAT3 inhibitor, significantly increased the plasma concentrations of kynurenic acid and several uremic acids in human subjects. Of those, the increases of plasma kynurenic acid exposure are comparable to the increases of furosemide by OAT1/3 inhibition. Effect size analysis for known potential endogenous biomarkers revealed that plasma kynurenic acid is a superior addition for early DDI assessment involving OAT1/3 inhibition.

INTRODUCTION

The kidneys are specialized organs to remove undesirable substances from the body and renal elimination is the most common route for many exogenous and endogenous compounds. The processes consist of glomerular filtration, tubular secretion and reabsorption. While generally glomerular filtration is a passive process, tubular secretion, and in some cases renal reabsorption, are active processes that are accomplished by a variety of renal transporters located on the basolateral and apical membranes of the renal tubular epithelial cells. Renal transporters that are involved in active renal tubular secretion or reabsorption can be disrupted by inhibitor drugs, leading to clinically significant changes of plasma and/or kidney exposure of victim drugs (Morrissey et al., 2013; Nigam et al., 2015; Ivanyuk et al., 2017). Recently it has become clear that renal transporters play important roles in drug clearance and can be the major determinants of drug exposure in the kidney and plasma. In order to elucidate the impact of transporter functions on the variations of pharmacokinetics (PK) and/or pharmacodynamics (PD) of a drug, characterizing the role of renal transporters in the drug disposition and elimination is required by regulatory agencies for new molecular entities (NMEs) to understand the potential drug-drug interactions (DDIs). The US Food and Drug Administration (FDA) recently published the guides to investigate the clinical DDIs for renally cleared drugs (USFDA-Guidance-2020, 2020).

Renal transporters on proximal tubular cells work in concert to eliminate exogenous and endogenous substrates from the systemic circulation. The basolateral transporters that take up drugs from blood into proximal tubular cells include organic anion transporter (OAT) and organic cation transporter (OCT) subfamilies. Subsequently the apical transporters such as multidrug and toxin extrusion proteins (MATEs) and ATP-binding cassette (ABC) transporters can excrete these substrates from the tubular cells into the urine. The basolateral uptake processes often act as the rate-determining step in renal tubular secretion of anionic compounds (Watanabe et al., 2011; Mathialagan et al., 2017). The uptake of positively charged organic

cations is mostly mediated by OCT2. For many negatively charged organic substances, the basolateral uptake is mainly attributed by OAT1 and OAT3, and to a minor extent also OAT2 (Sekine et al., 2006). OAT1 and OAT3 share substrate specificities with some exceptions. For example, both OAT1 and OAT3 can transport cyclic nucleotides, corticosteroids, and prostaglandins (Cha et al., 2001; Ahn and Bhatnagar, 2008), while OAT3 has a preference of more bulky and lipophilic organic anionic substrates such as glucuronide conjugates (Srimaroeng et al., 2008). Inhibition of OAT1 and OAT3 can reduce the renal clearance of drugs that are eliminated from renal tubular excretion, resulting in increase of plasma exposure (Ivanyuk et al., 2017). It is exemplified that probenecid can reduce the basolateral tubular uptake of cidofovir and protect against cidofovir-induced nephrotoxicity, via the inhibition of OAT1 and OAT3 activities (Yin and Wang, 2016). In addition, in the second third of the twentieth century, probenecid was used to prolong the half-life and reduce the expensive penicillin doses (Gibaldi and Schwartz, 1968). We now know that OAT1 and OAT3 inhibition can result in increases of plasma exposure for a number of widely prescribed anionic drugs such as furosemide, famotidine, and ciprofloxacin (Inotsume et al., 1990; Jaehde et al., 1995; Vree et al., 1995).

In order to assess the potential risk of renal transporter DDIs, *in vitro* testing is recommended by regulatory agencies for all drugs whose tubular secretion constitutes at least 25% of the total clearance. A clinical DDI study with a known inhibitor of these transporters is warranted according to the decision trees in the regulatory guidance (USFDA-Guidance-2020, 2020). Probenecid is recommended as a clinically relevant inhibitor for OATs. As a general practice, these expensive clinical DDI trials are determined by a static mathematical approach that is conservative by design with a high false positive rate in prediction. Thus, identifying a circulating endogenous biomarker to aid the DDI assessment in phase I clinical trials becomes an attractive and cost-effective means of assessing transporter-mediated DDI potential (Chu et al., 2017, Mariappan et al., 2017, Rodrigues et al., 2017, Muller et al., 2018). In fact, the latest

USFDA Guidance for Industry on Drug-Drug Interaction Studies suggests that serum/plasma creatinine levels can be an early index of OCT2 and MATE1/2K inhibition by the investigational drugs (USFDA-Guidance-2020, 2020).

One of the major functions of the kidney is to filter and excrete a sort of water-soluble metabolites yielded from many metabolic processes. Kidney failure causes accumulation of uremic toxins including creatinine, cresol sulfate, hippurate, indole acetate, and indoxyl sulfate. OAT1 and 3 transporters are responsible for tubular uptake of the uremic toxins from blood (El-Sheikh et al., 2008). Through metabolomics analysis, we previously reported that plasma pyridoxic acid (PDA) and homovanillic acid (HVA) and another 27 metabolites can be potential markers of OAT1/3 inhibition in monkeys (Shen et al., 2018). A follow-up clinical study showed that PDA is a promising plasma-based marker of OAT1 and 3 inhibition in humans (Shen et al., 2019). However, as PDA is the primary catabolic product of vitamin B6, the plasma exposure of PDA can be varied by the intake of supplements containing vitamin B6 during the study. Hence, the aim of this follow-up investigation was to further analyze the human plasma samples from the same subjects of clinical furosemide-probenecid DDI studies to assess if these endogenous uremic toxins and bile acids can be potential biomarkers of OAT1 and OAT3 inhibition.

MATERIALS AND METHODS

Probenecid (Bencid, 500 mg tablets) and Furosemide (Lasix®, 40 mg tablets) were obtained from Geno Pharmaceuticals Limited (Goa, India) and Sanofi India Limited, respectively. Kynurenic acid, xanthurenic acid, pantothenic acid, indo-3-acetic acid, bile acids, and Labetalol were purchased from Sigma Aldrich. *p*-Cresol sulfate was purchased from Apexbio Technology (Houston, TX). GS-A was synthesized by chemists at Gilead. All other reagents and solvents used for liquid chromatography–tandem mass spectrometry (LC-MS/MS) were of high-performance liquid chromatography (HPLC) grade, unless specified, and purchased from Sigma-Aldrich Corporation (Bangalore, India, and St. Louis, MO).

Clinical Study

As disclosed previously, the trial was an open-label, three-treatment, three-period, single dose crossover clinical study. The study protocol and the informed consent were reviewed and approved by an independent ethics committee. The study met the Syngene Clinical Development standard operating procedures, International Council for Harmonization “Guidance on Good Clinical Practice” (<https://www.ich.org/page/efficacy-guidelines>), Declaration of Helsinki (<https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/>), Central Drugs Standard Control Organization guidelines (<http://www.cdsc.nic.in/writereaddata/cdsc-guidanceforindustry.pdf>), Indian Council of Medical Research guidelines (https://ethics.ncdirindia.org/ICMR_Ethical_Guidelines.aspx) and other applicable regulatory requirements. The clinical part of the study was conducted at one clinical site at Human Pharmacology Unit, Syngene International Limited Clinical Development, Electronics City, Bangalore, India. Fourteen healthy, male, Indian, adult subjects aged between 18 and 45 years were enrolled and written informed consent for participation was obtained from the subjects prior to initiation of study procedures. No clinically relevant conditions were identified from the medical history, physical examination, electrocardiography, or chest X-ray. Any

clinically relevant laboratory abnormalities in clinical chemistry tests including hepatic and renal biochemistry, hematology tests, or urinalysis were criteria for exclusion. Subjects were asked to not take supplements containing vitamin B6 during the study.

The trial consisted of three periods separated by a 7-day washout period between phases and the healthy volunteers were housed in the clinical facility 36 hours prior to dosing in phase 1. Volunteers orally received probenecid (1000 mg), furosemide (40 mg) and probenecid (1000 mg) plus furosemide (40 mg) with 240 mL of water in phase 1, 2 and 3, respectively. In phase 3, probenecid was dosed 1 hour prior to furosemide administration. Blood samples were collected into tubes containing dipotassium EDTA (K₂EDTA) anticoagulant and centrifuged (2600 g at 4 °C for 10 minutes) at predose and post-dose at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 hour for each phase. In period 3, post-dose time points started after furosemide administration. Subjects were housed in the clinical facility 36 hours prior to dosing in phase 1 (probenecid alone). Safety and tolerability were assessed by clinical evaluations during the study. Plasma was separated into two aliquots that were stored at -70 ± 10 °C until analysis.

Quantification of kynurenic acid, xanthurenic acid, pantothenic acid, p-cresol sulfate, indole-3-acetic acid and bile acids by LC-MS/MS

Stock solutions (10 mM) of test compounds and internal standards were prepared in dimethyl sulfoxide (DMSO) (EMD Millipore Corporation, MA, USA). Further dilutions were prepared in 50% acetonitrile in water as needed. Plasma calibration standards were prepared in 4X charcoal stripped pooled human plasma with K₂ EDTA (BioIVT, New York, USA) to obtain 13-point standard curves at concentrations ranging from 1 to 50000 nM. Similarly, quality control (QC) samples were prepared in plasma at three levels: 20 nM (low QC, LQC), 200 nM or 1000 nM (middle QC, MQC), 2500 nM or 10000 nM (high QC, HQC), respectively.

Sample extractions were conducted in 96-well plates using protein precipitation with acetonitrile. In brief, 50 µL of study/standard/QC plasma samples were mixed with 200 µL ice-cold acetonitrile containing 200 nM of Labetalol or GS-A in 96-well plate (Greiner Bio-One, Frickenhausen, Germany). Samples were vortex-mixed and centrifuged at 4°C, 3700 rpm for 15 minutes. An aliquot of 100 µL of the supernatant from each well was transferred to a clean 96-well plate and diluted with 200 µL of water. An aliquot of 10 µL was injected to the LC-MS/MS system for analysis.

A CTC PAL autosampler was used as part of a Cohesive (Thermo Scientific San Jose, CA) LX-2 multiplexed system and Dionex UltiMate 3000 UHPLC RS pump was used for elution and separation. Chromatographic separation was achieved by gradient elution on Acquity C18 BEH, 1.7µm, 2.1*100mm column (Waters Corporation, Milford, USA) maintained at room temperature. The mobile phase was a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B).

Mass spectrometric detection were performed on an AB Sciex 5500 QTRAP (Applied Biosystems, Foster City, USA), equipped with an electrospray ionization source. The detection of

p-cresol sulfate, indo-3-acetic acid, and bile acids was operated in negative ion mode and multiple reaction monitoring (MRM) transitions in the mass spectrometer, while positive ion mode was used for the detection of kynurenic acid, xanthurenic acid, and pantothenic acid. The mass spectrometer settings were as follows: ion spray voltage: 4.5 kV (negative mode) and 5.5 kV (positive mode); temperature: 550 °C; MRM transitions and collision energy (CE) are listed in Table 1 for all the analytes. All peak integration and data processing were performed using AnalystTM version 1.6.2. (Applied Biosystems, Foster City, USA). Concentrations of kynurenic acid, xanthurenic acid, pantothenic acid, cresol sulfate, indo-3-acetic acid, and bile acids in study samples were determined from the weighted (1/x) linear calibration curve obtained by plotting area ratios (analyte-to-internal standard signal) against known concentrations of analyte in calibration standards.

Data Analysis:

The pharmacokinetics was analyzed by the non-compartmental analysis in Phoenix[®] WinNonlin[®] software (Version 8.2, Certara USA, Inc., Princeton, NJ). The peak concentration (C_{max}) were recorded directly from experimental observations. The area under the plasma concentration–time curve (AUC_{0-t}) was calculated using the mixed log-linear trapezoidal rule up to 12 hr and the last detectable concentration measured (24 hr) (AUC_{0-12} and AUC_{0-24}). The relative magnitude of treatment effects of different biomarkers was ranked by standardized effect size (d) by calculating the mean difference of AUC_{0-12} between furosemide group and the mean of probenecid or probenecid plus furosemide groups, and then dividing the result by the pooled standard deviation (equation 1).

Equation 1

$$d = \frac{(M1-M2)}{SD_{pooled}}$$

$$SD_{pooled} = \frac{\sqrt{(SD1^2-SD2^2)}}{2}$$

where M1 and SD1 is the mean of AUC₀₋₁₂ and SD of probenecid or probenecid plus furosemide groups, M2 and SD2 is the mean and SD of furosemide treatment.

Statistical differences between treatments were determined using a paired two-tailed Student t-test. (GraphPad Prism version 7; GraphPad Software, Inc.; San Diego, CA). A P < 0.05 is set as statistically significant.

RESULTS

Drug-Drug Interaction of probenecid and furosemide

A total of 14 healthy volunteers were enrolled in this study and all subjects completed 3 phases of the study. As reported previously (Shen et al., 2019), the C_{max} of probenecid in plasma was 436 ± 157 and 325 ± 41.0 μM in phase 1 (probenecid alone) and phase 3 (probenecid plus furosemide), respectively. The plasma free concentration-time profiles of probenecid were re-plotted from the concentration-time profiles that are reported previously (Shen et al., 2019), normalized by the unbound fraction value of probenecid. As shown in Figure 1A, plasma free concentrations of probenecid were above the IC₅₀'s of OAT1 and OAT3, for 12 hrs (based on the highest reported IC₅₀ value) or 24 hrs (based on the reported geometric mean IC₅₀ value) (Shen et al., 2018). As a result, plasma concentrations of furosemide were significantly increased (Shen et al., 2019), confirming the inhibition of OAT1 and 3 functional activities in human.

Changes in plasma concentrations of kynurenic acid, xanthurenic acid, pantothenic acid, p-Cresol sulfate, and indole-3-acetic acid by the treatment of probenecid alone or probenecid plus furosemide

The plasma concentrations of kynurenic acid, xanthurenic acid, pantothenic acid, p-cresol sulfate, and indole-3-acetic acid were determined before or after receiving probenecid. As shown in Figure 1B, probenecid alone and in combination with furosemide significantly increased plasma concentrations of kynurenic acid. Although both AUC₀₋₂₄ and AUC₀₋₁₂ of kynurenic acid and other tested uremic acids were significantly increased by probenecid (Table 2), the plasma

free concentration of probenecid was greater than the IC₅₀ values of OAT1 and OAT3 inhibition for about 12 hrs, when the highest IC₅₀ values reported are used (Figure 1A). Therefore, the use of AUC₀₋₁₂ values is thought to be more appropriate for comparison. Accordingly, the AUC₀₋₁₂ of kynurenic acid was increased about 2.5- or 2.9-fold by probenecid or probenecid plus furosemide respectively, compared to the treatment of furosemide alone. In order to determine if the increase of plasma exposure of kynurenic acid is associated with the reduction of renal clearance, the concentrations of kynurenic acid in the urine samples were determined. As shown in supplementary Figure 1, the renal clearance of kynurenic acid in the subjects received probenecid plus furosemide (phase 3) and furosemide alone (phase 2) were 5.4±1.56 and 10.4±3.02 L/hr (mean ± SD). The renal clearance of kynurenic acid was reduced about 2-fold by the treatment of probenecid, which confirmed the inhibition of probenecid on the renal excretion of kynurenic acid.

A greater than 2-fold AUC₀₋₁₂ increase was also observed in plasma levels of *p*-cresol sulfate (Figure 2A) and indole-3-acetic acid (Figure 2B), following the treatment of probenecid alone or probenecid plus furosemide. The increases of the AUC₀₋₁₂ by probenecid was small but statistically significant for xanthurenic acid (1.4-fold) (Figure 2C) and pantothenic acid (1.2-fold) (Figure 2D) (Table 2). Similar results were obtained by the treatment of probenecid plus furosemide (Figure 2 and Table 2).

Effect of probenecid alone or probenecid plus furosemide on plasma concentration of bile acids

To elucidate the effect of administration of probenecid alone or the coadministration with furosemide on plasma bile acid levels, non-conjugated and glycine- or taurine-conjugated bile acids in the plasma including DCA, TCDCA, GCDCA, GDCA, and TDCA were also monitored. Plasma bile acid levels in all treatment groups tended to decline following the administrations up to 4 hours, and then elevate after a meal and onwards (Figure 3). Administration of either

probenecid alone or coadministration with furosemide did not significantly alter plasma concentrations of the tested bile acids compared to the pre-dose levels or levels observed in the treatment of furosemide alone (Figure 3).

The standardized effect size of known OAT inhibition biomarkers

In order to select the most sensitive biomarker to predict OAT inhibition DDI, the magnitude of effect size was estimated from the significant changes from the untreated values normalized by the interindividual variation for known potential endogenous biomarkers, including those previously reported in literature. Although the AUC₀₋₂₄ showed comparable changes, the effect size of AUC₀₋₁₂ was slightly bigger than that of AUC₀₋₂₄ (Data not shown). Therefore, the changes of AUC₀₋₁₂ values were included in the effect size analysis. As shown in Figure 4, the standardized effect size of kynurenic acid was about 3-fold higher than xanthurenic and other markers, including HVA, PDA, and 6 β -hydroxycortisol that were reported previously (Imamura et al., 2014; Shen et al., 2019). Since the effect size is a quantitative measure of the magnitude of the experimental effect, the largest effect size attributed by kynurenic acid represents the strongest relationship between kynurenic acid exposure and probenecid inhibition. As a result, changes of kynurenic acid AUC₀₋₁₂ offer promise as a superior addition for early prediction of DDIs associated with OAT1/3 inhibition.

DISCUSSION

To eliminate unpredictable clinical effects, characterization of DDIs with co-administration drugs is an important task for the development of safe drugs for complex diseases that are often treated by multidrug regimens. Conducting clinical DDI studies with investigational new drugs can be time-consuming, expensive, and sometimes cause ethical concerns. Plasma levels of endogenous or nutrient-derived metabolites that can reflect the inhibition of drug metabolizing enzymes or drug transporters *in vivo* are advantageous for early assessment of DDI

potentials in conjunction with the first-in-man tolerability and safety trials without the need to administer probe drugs to healthy volunteers (Imamura et al., 2011; Chu et al., 2018). For example, Tsuruya et al reported that probenecid can significantly reduce the urinary excretion of taurine and glycochenodeoxycholate sulfate in a dose-dependent manner (Tsuruya et al., 2016). Imamura et al observed that the plasma exposure of endogenous 6 β -hydroxycortisol, an OAT3 substrate, is increased about 2-fold in AUC by probenecid treatment (Imamura et al., 2014). Most recently, a clinical DDI trial was conducted to confirm that administration of probenecid significantly caused the AUC increase of plasma PDA and HVA (up to 3.2 and 2.1-fold, respectively) (Shen et al., 2019). The AUC increase of PDA is similar to furosemide AUC changes, demonstrating that PDA can be a clinical plasma-based biomarker for DDIs involving OAT1/3 inhibition (Shen et al., 2019). Collectively, DDIs involving OAT inhibition in humans can potentially be assessed by monitoring the plasma concentration of PDA and 6 β -hydroxycortisol (Imamura et al., 2014) or renal clearance of taurine and GCDCA-S (Tsuruya et al., 2016). Nevertheless, there are concerns regarding the sensitivity and specificity of these OAT1 and 3 inhibition biomarkers. For example, GCDCA-S is also a substrate of organic anion transporting polypeptides 1B (OATP1B) and the treatment of a single dose of rifampicin, a prototypic OATP1B inhibitor, caused a greater than 20-fold increase of GCDCA-S AUC (Takehara et al., 2019). Additionally, 6 β -hydroxycortisol is also a substrate for the multidrug and toxin extrusion protein (MATE1 and MATE2k) and its exposure is complicated by the cytochrome P450 (CYP) 3A4 inhibition and induction (Imamura et al., 2013; Imamura et al., 2014). Although PDA is relatively specific for OAT1/3 and the increases of PDA levels by probenecid treatments are more pronounced than HVA (Shen et al., 2018), the plasma PDA concentration can be affected by diet intake of vitamin B6 and metabolic interconversion (Shen et al., 2018). As such, continuous efforts are justified for better DDI biomarkers of OAT1/3 inhibition.

Previously we reported that increased plasma concentrations of 29 metabolites including PDA, HVA, and several uremic acids were detected in the plasma of monkeys receiving probenecid by untargeted metabolomics analysis (Shen et al., 2018). Similar changes of indoxyl sulfate, kynurenine, and xanthurenic acid were observed in the plasma of Oat1-gene knockout mice (Wikoff et al., 2011). These results suggested that plasma exposure of these uremic acids and metabolites may be associated with *in vivo* OAT functional actives. Therefore, the current investigation re-analyzed the plasma samples from the human trials of probenecid-furosemide DDIs for additional OAT inhibition biomarkers. As shown in Table 2, the changes of C_{max} over the baseline concentrations or the AUC₀₋₁₂ values with and without probenecid treatments demonstrated the potential use of kynurenic acid as a DDI biomarker of OAT inhibition. It is worth noting that the use of AUC₀₋₁₂ values is thought to be more appropriate, as the plasma free concentration of probenecid was greater than the IC₅₀ values of OAT1 and OAT3 inhibition for about 12 hrs, when the highest IC₅₀ values reported are used (Figure 1A).

To date, several endogenous metabolites including PDA and HVA (Shen et al., 2019), 6 β -hydroxycortisol (Imamura et al., 2014), taurine and GCDCA-S (Tsuruya et al., 2016) have been identified as potential DDI biomarkers to inform OAT1 and/or OAT3 inhibition. Although the above biomarkers, including the current additions, show statistically significant differences with the fold changes that are comparable to that in the interacting drugs, statistical significance testing does not reflect the magnitude of the changes or the strength associated with the OAT1 and 3 inhibition. Effect size is a quantitative measurement of the magnitude of the experimental effect. The larger the effect size observed, the stronger the relationship between two variables. Based on the magnitude of effect size estimated from the significant changes of the probenecid untreated group normalized by the interindividual variation for known potential endogenous biomarkers, kynurenic acid appeared to be a promising superior addition for early prediction of DDIs associated with OAT1 and 3 inhibition.

Tryptophan is an essential amino acid and acts as a precursor to redox cofactor nicotinamide adenine dinucleotide (NAD⁺) and many biologically active compounds, which play important roles in neuronal and immune function. Tryptophan is enzymatically converted to kynurenine and triggers the kynurenine metabolic pathway. Xanthurenic and Kynurenic acid are the key intermediates formed in the tryptophan catabolic pathway, and are endogenously produced by several types of cells and tissues (Walczak et al., 2020). Kynurenic acid is a substrate for both human OAT1 and OAT3 (supplementary figure 2), with the estimated K_m values of 5.06 and 4.86 μ M, respectively (Uwai et al., 2012). Indole-3-acetic acid is a breakdown product by tryptamine deamination associated minor pathways of tryptophan degradation to maintain host-microbe homeostasis (Zhang and Davies, 2016; Anesi et al., 2019). Indole-3-acetic acid is also known as a naturally occurring auxin controlling many important physiological processes of plants including cell enlargement and division, tissue differentiation, and responses to light and gravity. *p*-Cresol sulfate, a main circulating uremic toxin, is a sulfate conjugate metabolite of *p*-cresol that is biosynthesized from dietary tyrosine and phenylalanine by sulfotransferase in the gut (Cao et al., 2000). It is highly protein bound and often elevated in the urine of individuals with progressive multiple sclerosis (Cao et al., 2000). The uptake of *p*-cresol sulfate in human proximal tubule cells and rat renal cortical slices is saturable and inhibited by probenecid and other OAT inhibitors (Miyamoto et al., 2011). The data suggest that OAT transporters, preferentially OAT3, play a key role in active renal tubular excretion of *p*-cresol sulfate (Miyamoto et al., 2011). Pantothenic acid, also called vitamin B5, is one of the important water-soluble vitamins for the synthesis of coenzyme A (CoA) and acyl carrier protein, which is essential for fatty acid metabolism and synthesis. Furthermore, recent research suggests that OAT transporter-centered pathways involve essential metabolites including TCA cycle and tryptophan metabolites, vitamins and signaling molecules (Liu et al., 2016); The functional activities of OATs can regulate the plasma levels of these metabolites. In other words, the plasma level of the metabolites can reflect the OAT functional activities. On the other hand, multiple transporters that are expressed both in the kidney

and liver can be involved in the elimination of these endogenous metabolites including kynurenic acid. Inhibition of these transporters can have a greater impact on the plasma exposure of these endogenous metabolites (Niemi et al., 2003). The transporters that are involved in the transport of uremic acids or the metabolites found in the metabolic networks are not fully understood. For example, it has been reported that kynurenic acid is reportedly also a substrate for both the OATP1B1 and OATP1B3, while the transport of indoxyl sulfate involves OATP1B3 (Sato et al., 2014). Probenecid can increase the plasma exposure of OATP inhibition biomarker coproporphyrin I (Zhang et al., 2020), likely due to the inhibition of MRP2 on the canalicular membrane (Davenport et al., 2015). In addition, diet intake or the gut microbe can potentially impact the plasma levels of nutrient metabolites e.g vitamin B5 and B6 (Liu et al., 2016). As such, although these small acids are not likely eliminated from the liver, further investigation is warranted in understanding the specificity and predictive values of the biomarkers involving OAT transporter inhibition.

In summary, our results demonstrated that probenecid treatment significantly increased the plasma exposure of several uremic acids. The increases in C_{max} and AUC of plasma kynurenic acid by probenecid are comparable to the increases of furosemide C_{max} and AUC reported previously. The magnitude of effect sizes for known potential endogenous biomarkers demonstrated that the changes of kynurenic acid AUC₀₋₁₂ offer promise as a superior addition for early prediction of DDIs associated with OAT1/3 inhibition.

Authorship Contributions

Participated in research design: HS, TTM, VKH, WGH, YL

Conducted experiments: JT, XZ, YZ, EP

Contributed new reagents or analytic tools: JT, XZ, JZ

Performed data analysis: HS, JT, XZ, JZ, YL

Wrote or contributed to the writing of the manuscript: JT, HS, XZ, EP, JZ, WGH, YL

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Footnote:

Statement of Conflict Interest.

The authors were employees of Bristol-Myers Squibb (HS, TTM, VKH, WGH, YZ, EP and YL) or Gilead Sciences Inc. (JT, JZ, XZ, and YL) during this research and declare no conflicts of interest.

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Figure legends

Figure 1. The mean plasma concentration-time profiles of unbound probenecid (A) and kynurenic acid (B) after oral administration of probenecid alone (phase 1, open circles), furosemide alone (phase 2, open square) and the coadministration of probenecid plus furosemide (phase 3, open diamonds). The plasma free concentration-time profiles of probenecid were re-plotted from the concentration-time profiles that are reported previously (Shen et al., 2019), normalized by the unbound fraction value of probenecid (20%, the mid-point of 75-95% reported) (<https://go.drugbank.com/drugs/DB01032>). The data are expressed as the mean \pm SD of fourteen healthy volunteers. The dotted lines and hard lines in 1A represent the reported IC₅₀ boundaries of OAT1 and OAT3, respectively (Shen et al., 2018). Each OAT inhibition IC₅₀ value is depicted twice: one is the geomean of the IC₅₀ values and the other is the highest IC₅₀ value reported in literature.

Figure 2. The mean plasma concentration-time profiles of *p*-cresol sulfate (A), indole-3-acetic acid (B), xanthurenic acid (C), and pantothenic acid (D) after oral administration of probenecid alone (phase 1, open circles), furosemide alone (phase 2, open square) and the coadministration of probenecid plus furosemide (phase 3, open diamonds). The data are expressed as the mean \pm SD of fourteen healthy volunteers.

Figure 3. The mean plasma concentration-time profiles of DCA (A), TCDCA (B), GCDCA (C) GDCA (D) and TDCA (E) after oral administration of probenecid alone (phase 1, open circles), furosemide alone (phase 2, open square) and the coadministration of probenecid plus furosemide (phase 3, open diamonds). The data are expressed as the mean \pm SD of fourteen healthy volunteers.

Figure 4. Standardized effect size of known potential OAT inhibition biomarkers. The magnitude of effect size was estimated from the significant changes from the untreated values normalized by the interindividual variation of potential plasma endogenous biomarkers 6 β -hydroxycortisol (Imamura et al., 2014) (1, study 1; 2, study 2), homovanillic acid (HVA) and pyridoxic acid (PDA) (Shen et al., 2019). The effect size showed is the mean value of the effect size between probenecid alone and furosemide alone and the effect size between probenecid plus furosemide and furosemide alone.

Table 1. MRM transitions and collision energy.

Analyte	Compound ID	Q1 mass (m/z)	Q3 mass (m/z)	CE (V)
Test Compound	Kynurenic acid	190.1	144	26
Test Compound	Xanthurenic acid	206	160	26
Test Compound	Pantothenic acid	220.1	90	20
Test Compound	p-Cresol sulfate	187	107	-28
Test Compound	Indole-3-acetic acid	174	130	-20
Test Compound	TCDCA	498.3	79.9	-95
Test Compound	GDCA	448.3	74	-76
Test Compound	TDCA	498.3	79.9	-76
Test Compound	GCDCA	448.3	74	-76
Test Compound	DCA	391.2	345.4	-46
Internal standard	Labetalol	329.1	294	27
Internal standard	Labetalol	327	309.1	-25
Internal standard	GS-A	431.1	172	-44

Table 2

	Predose ^a (at time zero; drug free)	Phase 1 (probenecid alone)				Phase 2 (furosemide alone)				Phase 3 (probenecid plus furosemide)				AUC Fold difference ^b	
	C ₀ (nM)	C _{max} (nM)	Ratio of C _{max} /C ₀	AUC ₀₋₂₄ (nM*hr)	AUC ₀₋₁₂ (nM*hr)	C _{max} (nM)	Ratio of C _{max} /C ₀	AUC ₀₋₂₄ (nM*hr)	AUC ₀₋₁₂ (nM*hr)	C _{max} (nM)	Ratio of C _{max} /C ₀	AUC ₀₋₂₄ (nM*hr)	AUC ₀₋₁₂ (nM*hr)	AUC ₀₋₂₄	AUC ₀₋₁₂
Kynurenic acid	39.7 ± 16.5	130 ± 66.3*	3.3	2054 ± 342.9*	1130 ± 166.9*	55.1±22	1.4	997.8 ± 178.4	451.5 ± 61.9	149±48.9*	3.8	2424 ± 392.9*	1310 ± 170.3*	2.1/2.4	2.5/2.9
Pantothenic acid	107 ±37.8	209±98.6*	2.0	2845 ± 407.8	1591 ± 165.2*	146±46	1.4	2568 ± 343.1	1321 ± 130.3	220±55.8*	2.0	2869 ± 370.1*	1612 ± 126.1*	NS/1.1	1.2/1.2
p-cresol sulfate	13481 ±12642	30800 ±29300	2.3	559340 ± 202509*	278561 ± 91786*	23000 ±24900	1.7	357652 ± 175701	148066 ± 49605	45700 ±51800*	3.4	822134 ± 430930*	382201 ± 151317*	1.6/2.3	1.9/2.6
Xanthurenic acid	8.3±4.2	19.1±9.18*	2.3	291.9 ± 47.5*	158.8 ± 20.41*	12.7±6.42	1.5	234.5 ± 51.04	110.3 ± 14.2	17.5±7.72*	2.1	282.3 ± 50.4	152.3 ± 22.8	1.2/NS	1.4/NS
Indo-3-acetic acid	2476±2994	5300+7370	2.1	106929 ± 56027*	52381 ± 23404*	3300±3590	1.3	51141 ± 20796	24173 ± 7789	6010±7830	2.4	113804 ± 58591*	54014 ± 22524*	2.1/2.2	2.2/2.2

a, Predose represents the time zero before administration of probenecid (C₀). The values of C₀ were averaged from the values at time zero 14 subjects across three phases.

b, fold changes between phase 1 (probenecid alone) and phase 2 (furosemide alone)/fold changes between phase 3 (probenecid plus furosemide) and phase 2 (furosemide alone). NS, statistically not significant. *, statistically significant (P<0.05)

Figure 1

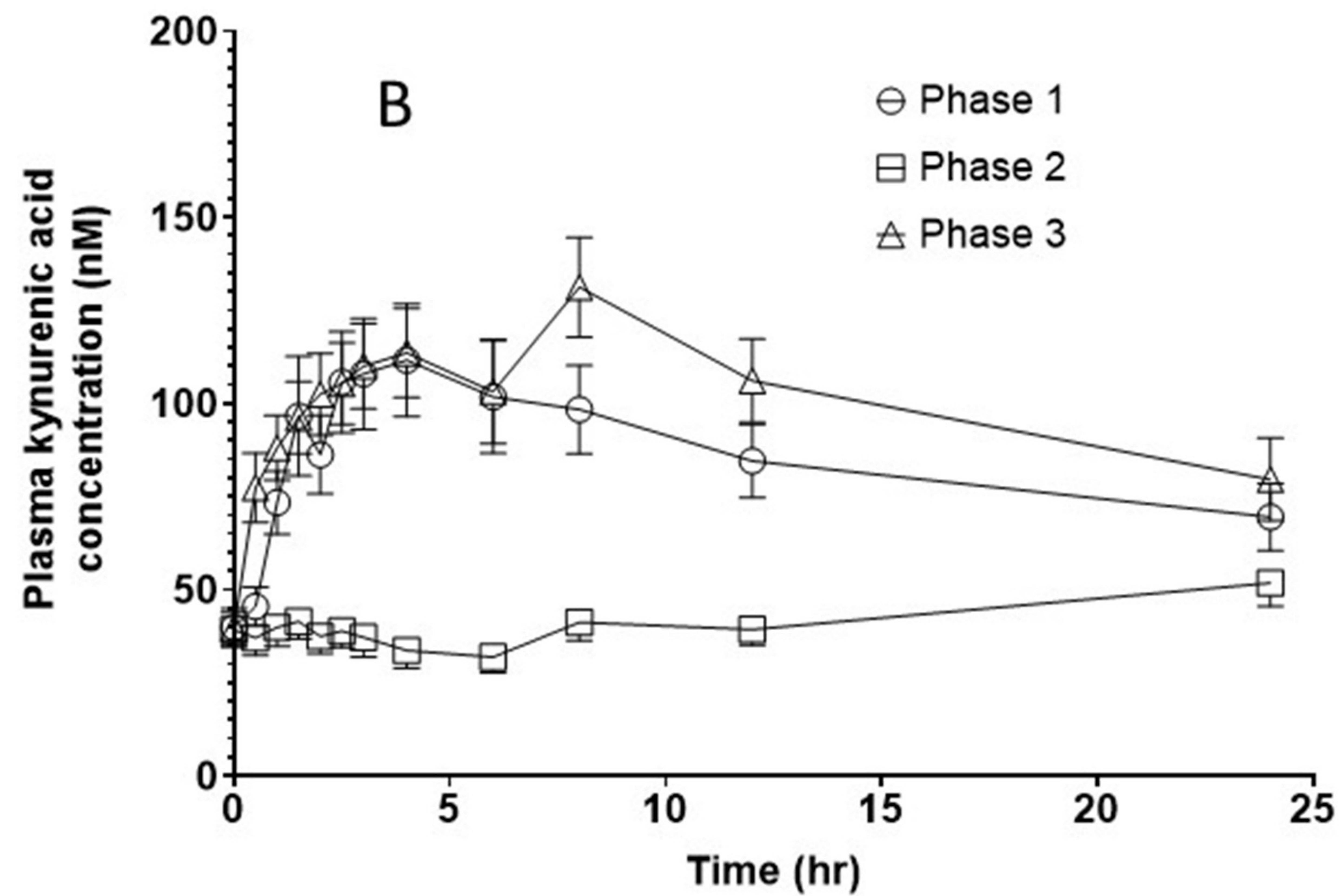
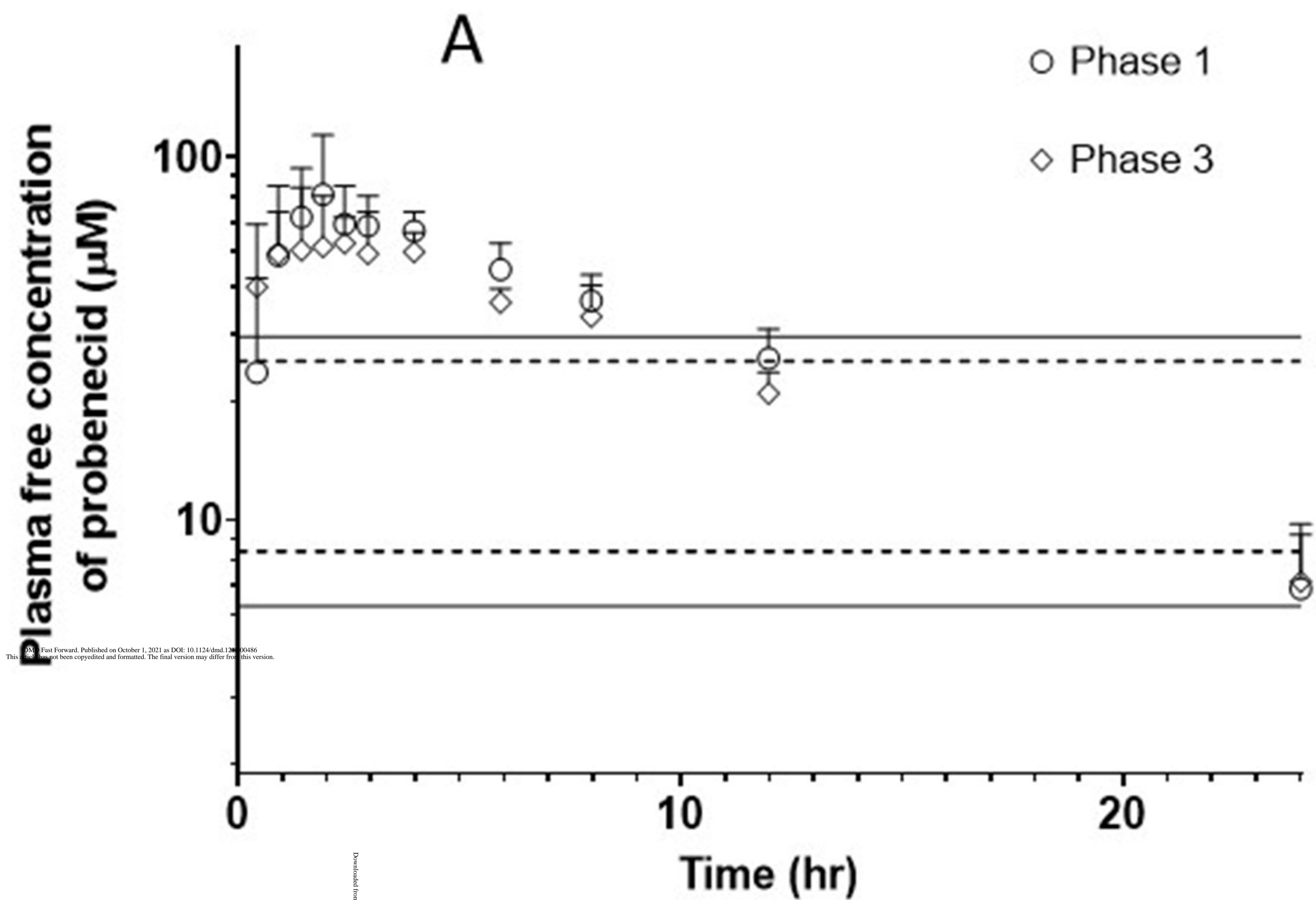


Figure 2

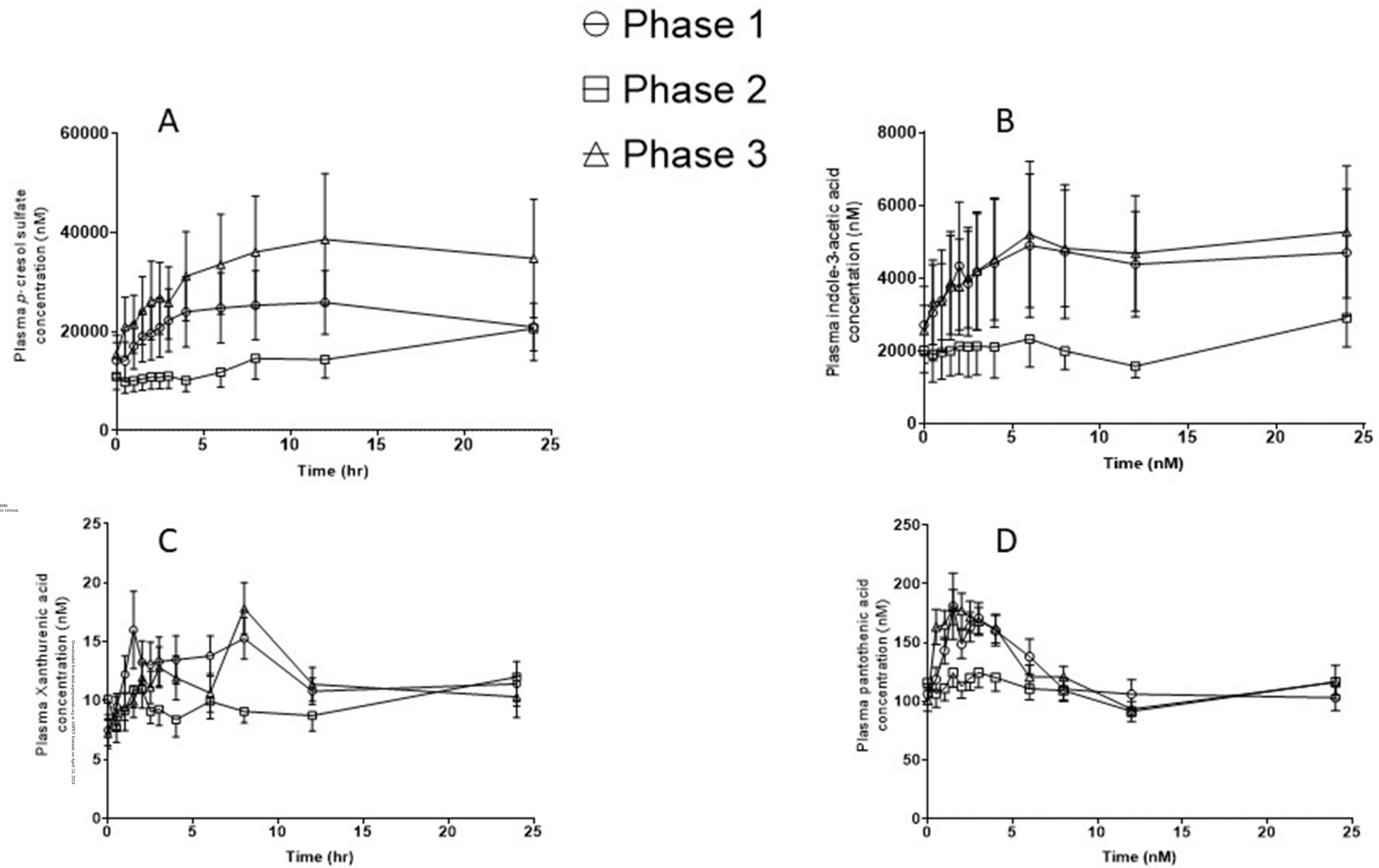
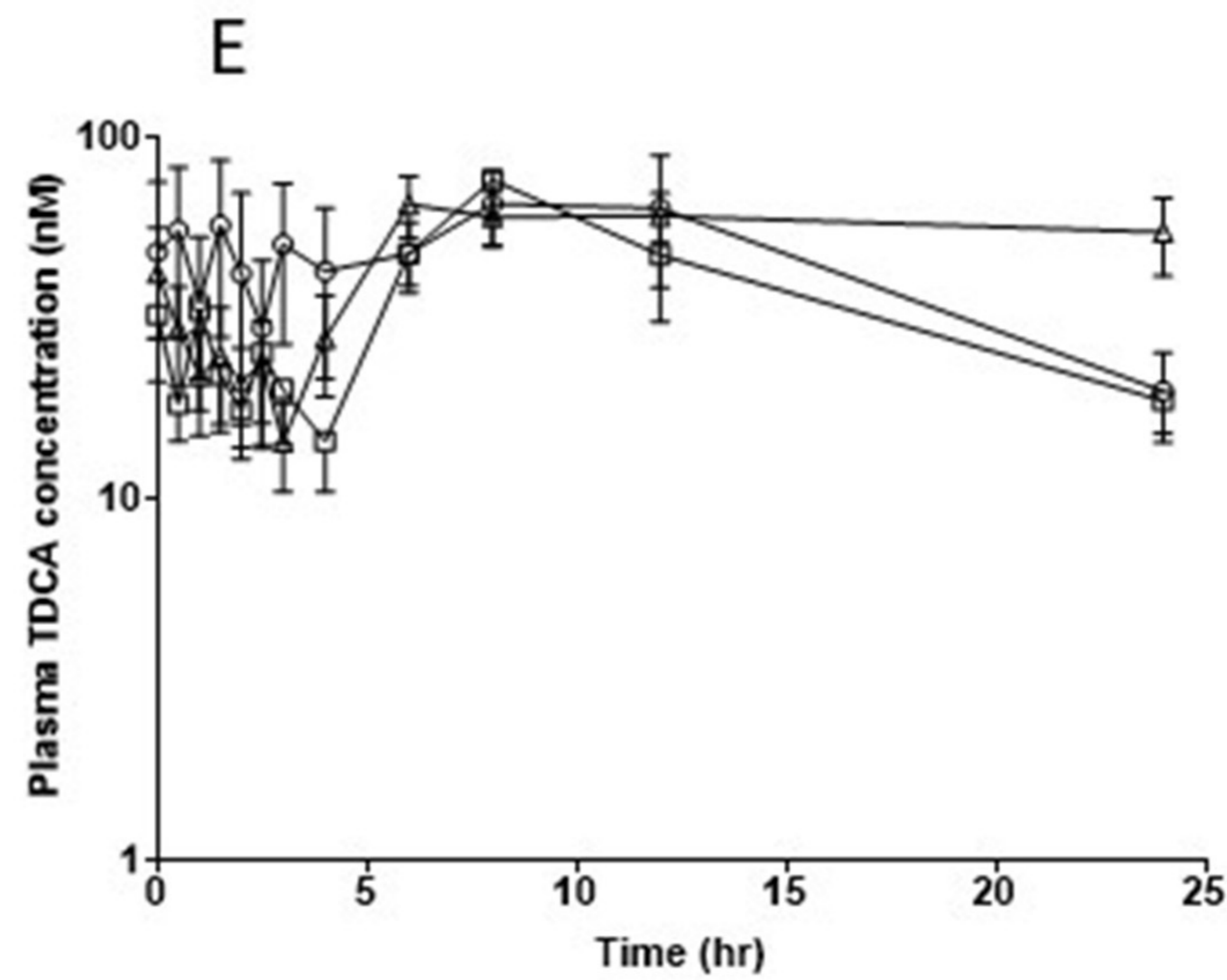
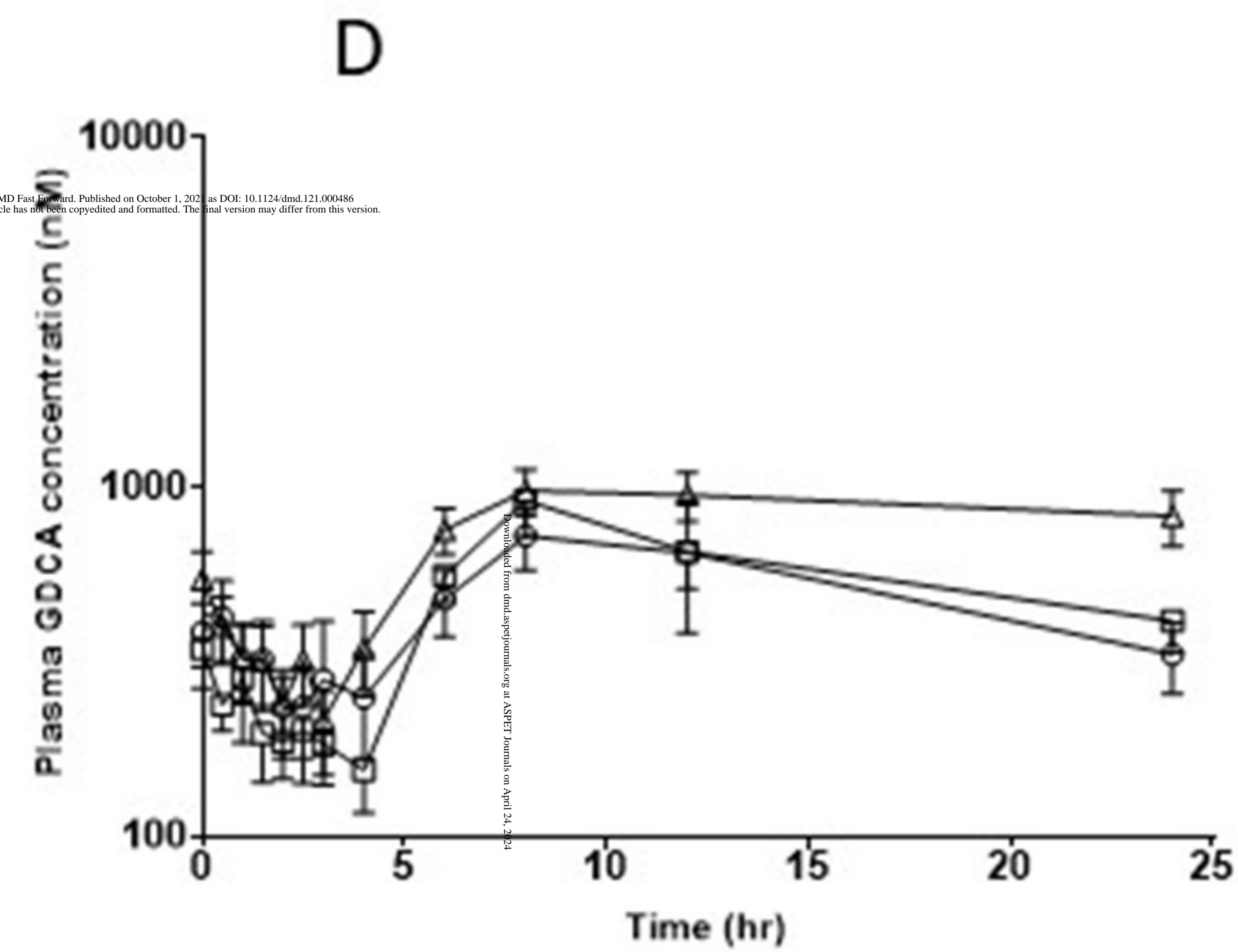
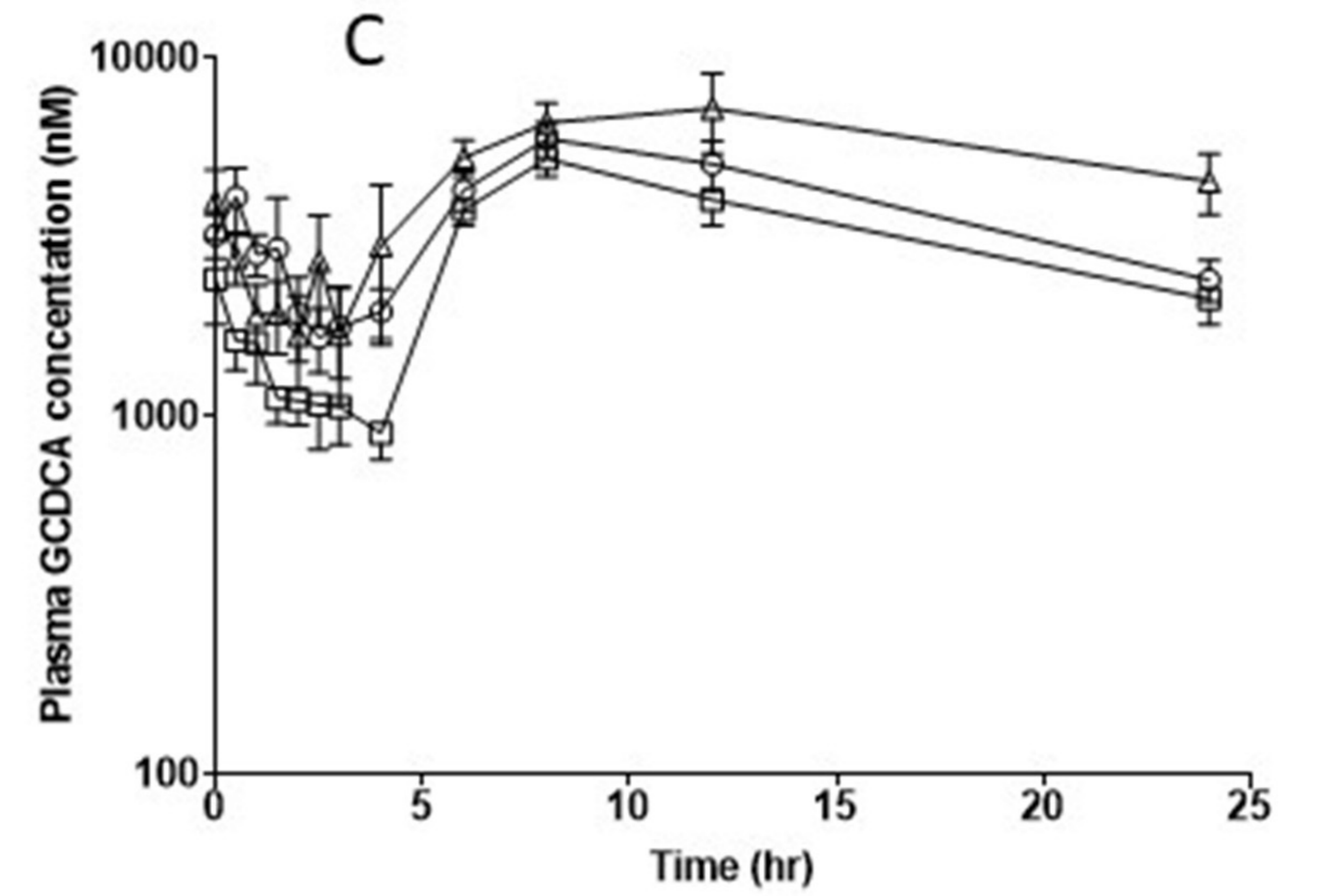
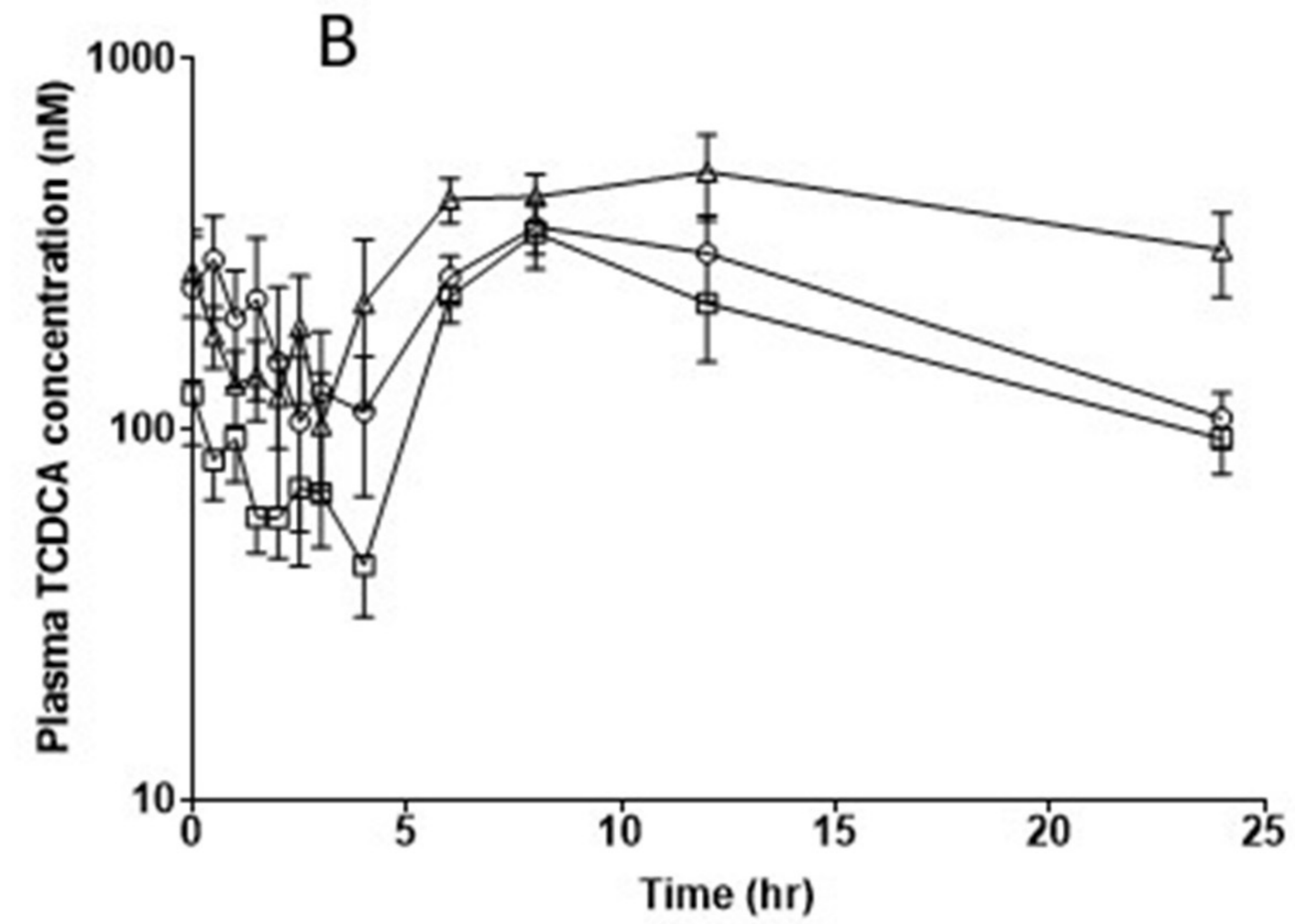
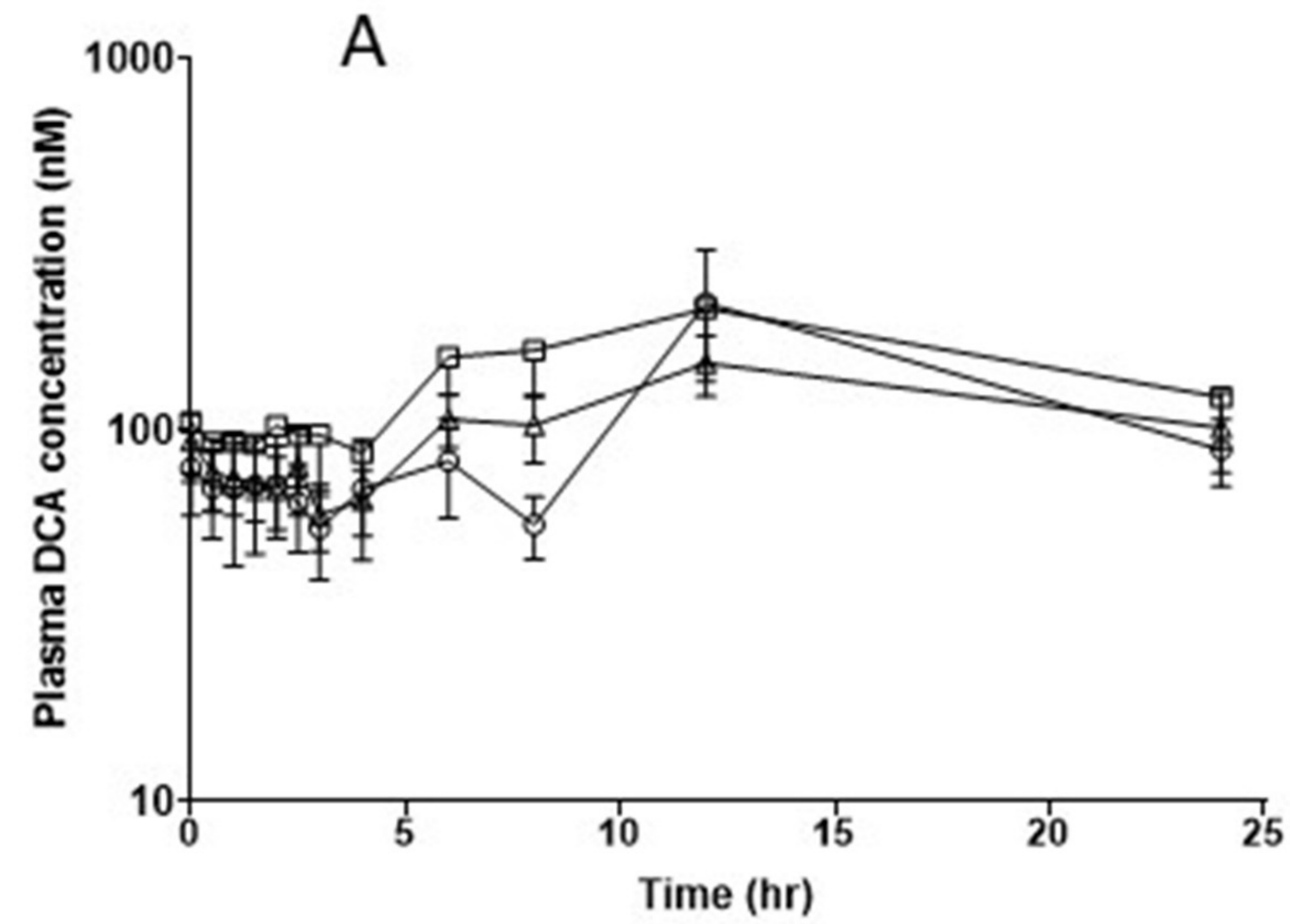


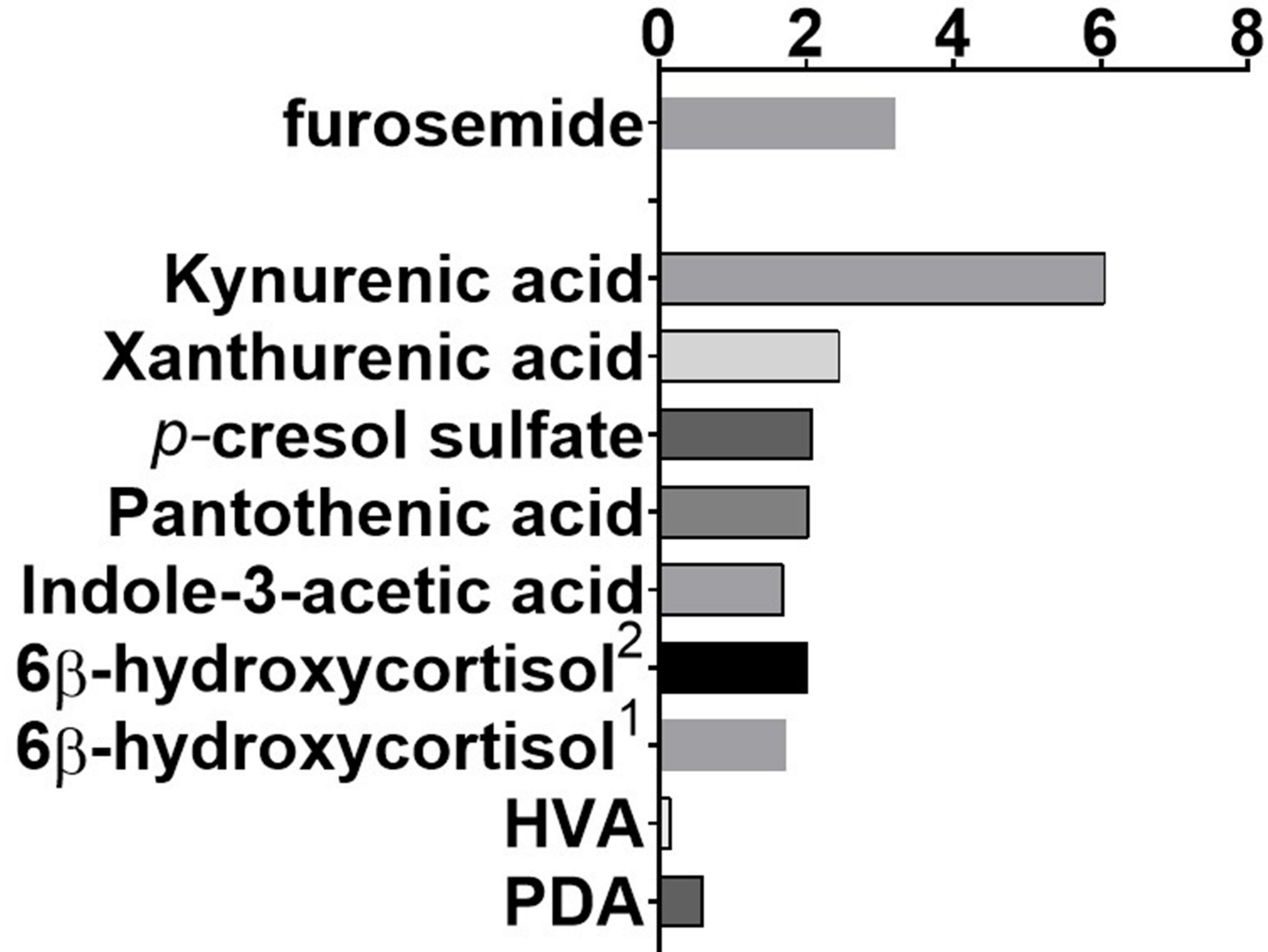
Figure 3



○ Phase 1
□ Phase 2
△ Phase 3

Figure 4

Standardized Effect Size



Supplementary Material

Endogenous plasma kynurenic acid in human: a newly discovered biomarker for drug-drug interactions involving in OAT1 and OAT3 inhibition

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Figure 1

The renal clearance (CL_r) (A), renal secretion clearance (CL_{sec}) (B) and renal extraction ratio (ER_r) (C) of kynurenic acid in the subjects after oral administration of furosemide alone (phase 2) or coadministration of probenecid plus furosemide (phase 3). Fifty μL of urine samples were mixed with 200 μL ice-cold acetonitrile containing 200 nM of kynurenic acid-d5 as internal standard in 96-well plate. The samples were vortex-mixed and centrifuged at 4°C, 3700 rpm for 15 minutes. An aliquot of 50 μL of the supernatant from each well was transferred to a clean 96-well plate and diluted with 450 μL of 25% acetonitrile in water. An aliquot of 10 μL was injected to the LC-MS/MS system for analysis. The CL_r, ER_r and CL_{sec} were estimated by the equations as follow:

$$CL_r = \frac{X_{0-24}}{AUC_{0-24}}$$

$$ER_r = \frac{CL_r}{f_u * GFR}$$

$$CL_{sec} = CL_r - f_u * GFR$$

where the X_{0-24} is the cumulative amount excreted in the urine; AUC_{0-24} is the area under the plasma concentration-time curve from 0 to 24 hrs; GFR is the glomerular filtration rate in human (125 mL/min) (Davies and Morris, 1993); f_u is the plasma unbound fraction of kynurenic acid (0.05) (van Gelder et al., 2020). The data are expressed as mean ± standard deviation (SD). Statistical differences between two group were determined using a paired two-tailed Student t-test in GraphPad Prism (version 7, GraphPad Software, Inc.; San Diego, CA). $P < 0.05$: statistically significant.

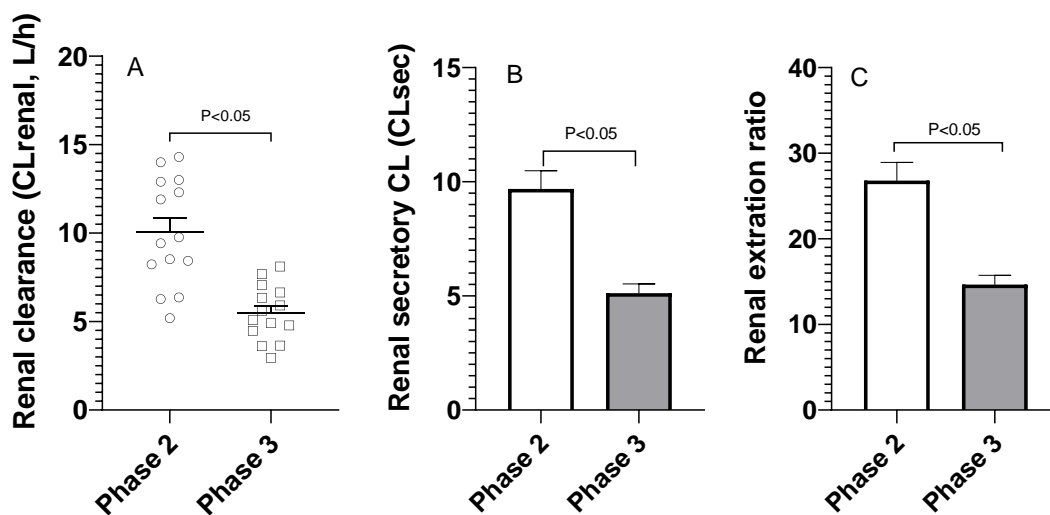
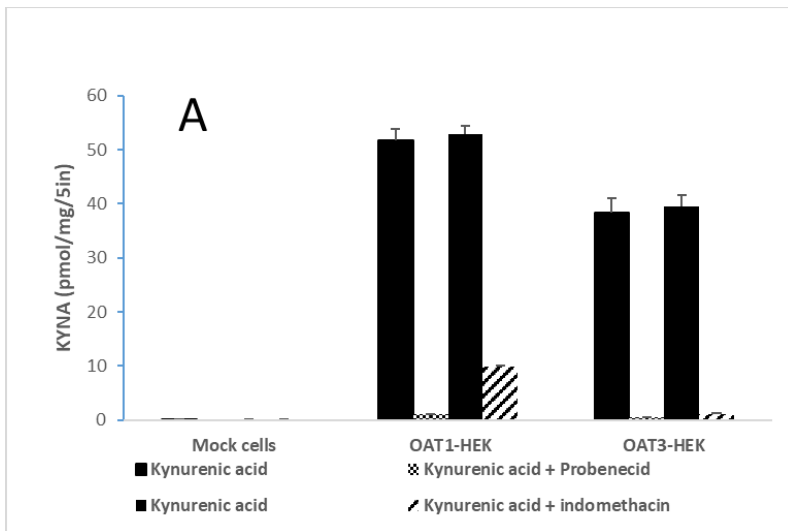
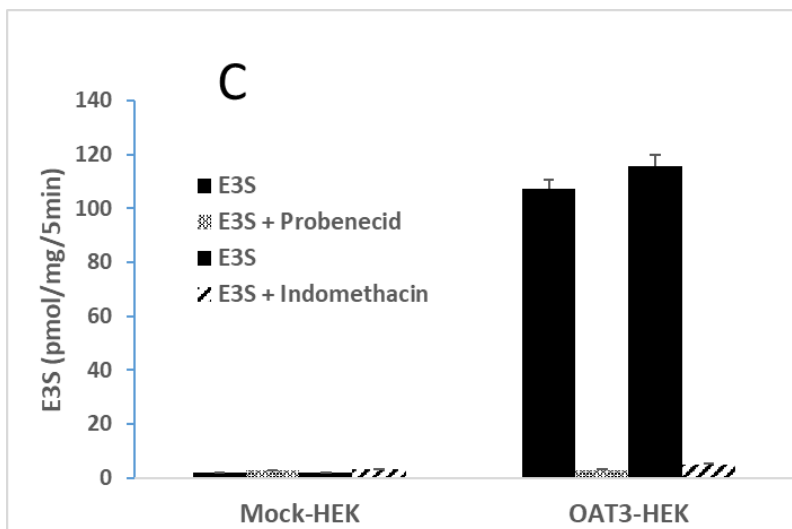
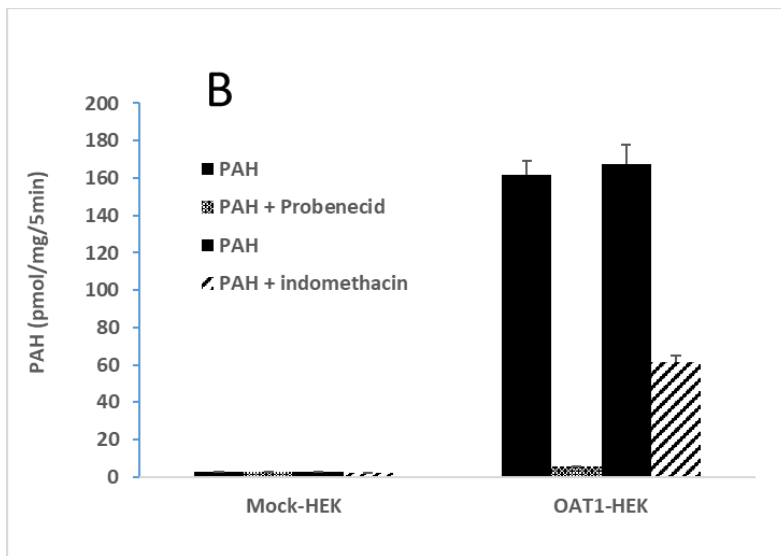


Figure 2. Measurement of kynurenic acid uptake in HEK cells lines constitutively expressing OAT1 or OAT3 transporter (OAT1-HEK and OAT3-HEK). The cellular uptake of kynurenic acid was tested at 0.5uM based on the physiological plasma concentrations and ensured analytical sensitivities. Probenecid (1mM) and indomethacin (100uM) was used as OAT inhibitors in the studies. The uptake of kynurenic acid in OAT1- and OAT3-HEK cells was approximately 727-and 541-fold higher than that in the control cells (51.6 ± 2.26 and 38.4 ± 2.57 vs 0.071 ± 0.012 pmol/mg after 5 minutes, respectively) (A). In addition, the kynurenic acid uptake in OAT1- and OAT3-HEK cells is significantly inhibited by probenecid or indomethacin, OAT1 and OAT3 inhibitors (A). The positive controls, p-aminohippurate (PAH, 1 μ M) (B) and extro-3-sulfate (E3S, 1 μ M) (C) demonstrated OAT1 and OAT3 activities, respectively.





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