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# Evaluation of Microdosing to Assess Pharmacokinetic Linearity in Rats using LC/MS/MS

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**Abbreviations**:

LC/MS/MS, liquid chromatography tandem mass spectrometry; AMS, accelerator mass spectrometry

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**Abstract**

The microdosing strategy allows for early assessment of human pharmacokinetics of new chemical entities using more limited safety assessment requirements than those requisite for a conventional Phase I program. The current choice for evaluating microdosing is accelerator mass spectrometry (AMS) due to its ultrasensitivity for detecting radiotracers. However, the AMS technique is still expensive to be used routinely and requires the preparation of radiolabeled compounds. This report describes a feasibility study with conventional LC/MS/MS technology for oral microdosing assessment in rats, a commonly used preclinical species. The non-labeled drugs fluconazole and tolbutamide were studied due to their similar pharmacokinetics characteristics in rats and humans. We demonstrate that pharmacokinetics can be readily characterized by LC/MS/MS at microdose of 1 μg/kg for these molecules in rats, and, hence, LC/MS/MS should be adequate in human microdosing studies. The studies also exhibit linearity in exposure between the microdose and ≥1000-fold higher doses in rats for these drugs, which are known to show linear dose-exposure relationship in the clinic, further substantiating the potential utility of LC/MS/MS in defining pharmacokinetics from the microdose of drugs. These data should increase confidence on the use of LC/MS/MS in microdose pharmacokinetics studies of new chemical entities in humans. Application of this approach is also described for an investigational compound MLNX where the pharmacokinetics in rats were determined to be nonlinear, suggesting that MLNX pharmacokinetics at microdoses in humans also might not reflect those at the therapeutic doses. These preclinical studies demonstrate the potential applicability of employing traditional LC/MS/MS for microdose pharmacokinetic assessment in humans.

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## Introduction

Microdosing has the potential for estimating human pharmacokinetics at therapeutic dose levels with much lower preclinical safety, and compound requirements (ICH, 2000; Lappin and Garner, 2003; EMEA, 2004; FDA Draft Guidance, 2005; Garner, 2005; Wilding and Bell, 2005). Microdosing studies performed before Phase I have the potential to reduce the attrition of drugs due to inadequate pharmacokinetic properties in humans and reduce the cost and time to reach this decision point. Currently, accelerator mass spectrometry (AMS) is the technology of choice for microdosing assessments, allowing ultra sensitive, femto or attomol drug level measurements. There is a growing literature on the use of AMS in preclinical and clinical research (Kaye et al., 1997; Young et al., 2001; Garner et al., 2002; Sandhu et al., 2004; Liberman et al., 2004, Choi et al, 2005, Sarapa et al, 2005). The extremely low amounts of radioactivity required for AMS permits human studies to be conducted without extensive regulatory approvals for radiation safety (Barker et al, 1999; NRC; WHO). Microdosing, as defined in the EMEA position paper (EMEA, 2004), is $1/100^{th}$ of the dose calculated to yield a pharmacological effect of the test substance in humans based on primary pharmacodynamics data obtained in vitro and in vivo. The EMEA paper also limits the maximum dose to 100 μg only, which biases towards the use of AMS only for the microdosing purpose, even if one can establish no safety concerns at $1/100^{th}$ of the projected therapeutic dose. It is prudent to demonstrate linear pharmacokinetics between microdoses and higher, therapeutically equivalent doses in an appropriate preclinical species before microdosing could be applied to humans to predict the PK at pharmacological doses. Though AMS is an excellent tool it requires the preparation of

radioactive drug and is costly for a routine use. Two orally administered drugs and an investigational compound with pharmacokinetic parameters similar in rats and humans (Table 1), were studied at microdoses in rats in order to determine if conventional LC/MS/MS was sensitive enough to quantitate drug levels following microdosing. The linearity in exposures between micro- and macrodoses was also assessed, to investigate the utility of microdosing for predicting exposures at 100 to ≥1000-fold higher doses.

**Materials and Methods**

Fluconazole, tolbutamide were obtained from Sigma-Aldrich (St. Louis, MI). Fluconazole was obtained from MP Biomedicals (Aurora, OH). Compound MLNX was synthesized at Millennium Pharmaceuticals, Inc. All solvents and other chemicals were of analytical or HPLC grade.

**PK Studies**

Male jugular vein cannulated SD rats (n=3-9; Hilltop Labs) were fasted overnight prior to oral dosing of the compounds. The compounds were formulated with 0.5% hydroxypropylmethylcellulose + 0.2% Tween80 solution doses for oral administration. Fluconozole was administered at 5, 0.05, 0.005 and 0.001 mg/kg; tolbutamide at 1, 0.1, 0.01, 0.002, 0.001 mg/kg; and MLNX at 10, 1, 0.1 and 0.01 mg/kg. To minimize variability, a stock solution of each compound was prepared in the dosing vehicle and serial dilutions were made with the vehicle to correspond to individual doses. Serial plasma samples were collected at predose and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h postdose. At low doses of $\leq$ 0.1 mg/kg, a group of nine rats were separated into 3x3 groups and pools of plasma (0.5 mL blood draws) were prepared from within subgroup animals to generate 3 pools per time point. For fluconazole the low dose of 1 μg/kg was analyzed individually from nine rats. For MLNX, an alternate scheme also was tested for the lowest dose group of 0.01 mg/kg, where the compound was administered to 4 rats with drawn blood volume of 0.8 mL replaced by blood from donor rats. Samples were processed by solid-phase extraction. Standard curves were generated for each compound

with the lowest quantitation limit of 0.1, 1 and 0.1 nM for fluconazole, tolbutamide, and MLNX, respectively.

**LC/MS/MS Analysis**

The rat plasma concentrations of the compounds were determined using LC/MS/MS based methods. Solid phase extraction method was used for sample preparation. A 100 μL aliquot of plasma samples was mixed with 50 μL of an internal standard, carbutamide, for the drugs, whereas a stable isotope-labeled internal standard was used for MLNX. For pooled samples, from three animals, a 200 μL aliquot was used for extraction. The mixed samples were diluted with an equal volume of water before loading onto the extraction plate. The mixed samples were extracted on either a 3M Empore $C_8$ or $C_{18}$ solid phase extraction plate which was preconditioned with methanol and then water. After washing with water and then 5% methanol, the compound was eluted with acetonitrile. The eluent was evaporated to dryness under a stream of nitrogen. The residual in the plate was reconstituted in 100 μL of 10% acetonitrile in water containing 0.1% formic acid and 20 μL injected for analysis.

A reverse-phase gradient HPLC method provided sample stacking and separation for all compounds. The mobile phases were water and acetonitrile, and each was supplemented with 0.1% formic acid. A YMC ODS-AQ column (S5, 200A, 2.1x50 mm) was used for the separation at a flow rate of 0.4 mL/min under ambient temperature. An MDS SCIEX API-4000 mass spectrometer (Toronto, Canada) was used for analysis. Each compound was tuned on the mass spectrometer to establish a quantification method under the

multiple reaction monitoring mode (MRM). For high dose (≥1 mg/kg) studies, the calibration standard curve ranged from 1 to 1,000 nM. A 0.1 to 10 nM range was used for low MLNX dose studies. The accuracy of measurement was within ±20% of the nominal concentrations.

**PK and Statistical Analysis**

Noncompartmental PK parameters ($AUC_t$, $AUC_{inf}$, $C_{max}$, $T_{max}$, $t_{1/2}$) were calculated using WinNonLin V4.1 (Pharsight, CA). Dose linearity tests on $AUC_{inf}$, $C_{max}$, and $t_{1/2}$ were carried out by the regression of log-transformed data (power regression model) (Gough et al., 1995; Smith et al., 2000). Doses and PK parameters were log-transformed and correlation coefficient ($R^2$), slope, and 95% confidence intervals (CI) were calculated using Data Analysis tool in Microsoft Excel. Inferences were made based on the theoretical slope of 1, and confidence limits of 0.8 and 1.25.

**Results**

Sensitive and specific bioanalytical assay methods were developed using LC/MS/MS. Using 100 μL of plasma sample and the API-4000 mass spectrometer, a concentration range of 1-1000 nM was readily established for all the compounds. The quantification of MLNX and fluconazole was extended to a lower quantification limit of 0.1 nM. However, due to the carryover, two separate concentration ranges were established (0.1-10 and 1-1000 nM) for analyzing samples from high and low doses of MLNX in rats.

**Fluconazole**. The plasma concentration-time profiles of fluconazole in rats after single oral doses are shown in Fig. 1 and the PK parameters are summarized in Table 2. The exposure increased proportionally in the dose range of 0.001 to 5 mg/kg.

**Tolbutamide**. The plasma concentration-time profiles of tolbutamide in rats after single oral doses are shown in Fig. 2 and the PK parameters are summarized in Table 2. The exposure increased proportionally in the dosing range of 0.001 to 1 mg/kg.

**MLNX**. The plasma concentration-time profiles of MLNX in rats after single oral doses are shown in Fig. 3 and the PK parameters are summarized in Table 2. The exposure was found to increase linearly over the dose range of 0.01 to 1 mg/kg, but non-linearly between 1 and 10 mg/kg.

Results from regression analyses of the $C_{max}$, $AUC_{inf}$, and $t_{1/2}$ data of the model compounds are summarized in Table 3. Plots of log-transformed $C_{max}$ and $AUC_{inf}$ data and the regression lines are shown in Fig. 4.

**Discussion**

Microdosing allows determination of pharmacokinetic parameters in humans at sub-pharmacological doses, which may then be extrapolated to those at therapeutically relevant doses. Currently, AMS is the only technology that offers the ultrasensitivity required for the quantitation of drugs using radiotracers. The level of sensitivity is reported to be 100-1000-fold lower than that can be achieved using traditional mass spectrometry (Garner, 2000). However, AMS analysis is expensive and requires radiolabel drug and, therefore limits the broader application of microdosing studies in drug development. We have assessed the use of the traditional LC/MS/MS for microdosing in rats for two drugs, fluconazole and tolbutamide, which are known to show similar pharmacokinetic properties in humans and rats (Debruyne and Ryckelynck, 1993; Balant, 1981; Sawada et al., 1985; Yang et al., 1996: Yamao et al., 1994). The microdoses for rats were proximated on mg/kg body weight basis (Fan et al., 1995) from the clinical doses of the two drugs. Thus, oral microdose of 1 μg/kg for tolbutamide and fluconazole was studied in rats. For fluconazole and tolbutamide a lower limit of quantitation of 0.1 and 1 nM, respectively, was found to be sufficient to characterize the pharmacokinetics. Because of the low plasma clearance, low volume of distribution and high oral bioavailability recorded in literature for these compounds, the plasma concentrations in rats declined slowly and were easily quantifiable in 24 hr samples (Fig. 1 and 2). Thus, LC/MS/MS sensitivity of 0.1 - 1 nM was adequate to support microdosing studies for these non-labeled compounds in rats. By analogy microdosing should also be assessable by LC/MS/MS in humans.

An aspect of microdosing is the evaluation of linearity of pharmacokinetics between the microdoses and the therapeutically equivalent doses of new chemical entities, leading to predictability of pharmacokinetic parameters at high doses from the microdose data. Before this aspect is applied in the clinical setting it is prudent to demonstrate linearity in pharmacokinetic parameters in a relevant preclinical model which shows pharmacokinetic properties and metabolism broadly similar to those projected in humans. Preclinical species that can be used for allometric scaling (Boxenbaum, 1984) to project human pharmacokinetic parameters may be used as a preclinical model for microdosing. More than one preclinical species could also be used to strengthen the projection of linearity in humans, thereby increasing confidence in the utility of microdosing studies in humans. In the current study, demonstration of linear increases in exposure upon oral administrations between microdoses and higher doses in rats for tolbutamide and fluconazole, which are reported to show linear increases in exposure after oral doses in humans (PDR; Balant, 1981), would corroborate this system of microdosing for projecting pharmacokinetic parameters at high doses. These drugs were ideally suited for showing linear pharmacokinetics after administration of microdoses since both drugs are reported to show low $CL_p$, low $Vd_{ss}$ and high oral bioavailability and similar metabolism in rats and humans (Ashforth et al., 1995; Balant, 1981; Debruyne and Ryckelynck, 1993; Yamao et al., 1994; Yang et al., 1996; Sawada et al., 1985). Unlike high clearance compounds, these drugs were not expected to exhibit saturation of $1^{st}$ pass effect and non-linear kinetics. However, the microdosing assessment using LC/MS/MS is expected to be applicable to compounds with varied pharmacokinetic properties. Tolbutamide was tested in rats with higher oral doses of 0.002, 0.01, 0.1 and 1 mg/kg, and fluconazole was

tested at higher doses of 0.005, 0.05 and 5 mg/kg. Doses higher than 1 mg/kg were not tested for tolbutamide since comparison of exposure with a published report (Yamao et al, 1994) showed that in rats this compound at a dose of 13 mg/kg gave linear increase in AUC, within 2-fold margin of error. The exposures at various doses of these molecules in rats are included in Table 2. The terminal disposition phases at different doses were parallel to each other. The exposures as measured by $C_{max}$ and $AUC_{0\text{-}inf}$ were linear over the ≥1000-fold dose ranges studied for these compounds, allowing for 2-fold variability. This was also evident by the power regression analysis of the plots of exposure vs. dose (Fig. 4, Table 3). These results establish the use of LC/MS/MS for microdosing assessments.

The published plasma $C_{max}$ for fluconazole after a 400 mg oral dose to human subjects is 62-100 μM (Grant, 1990). Assuming pharmacokinetic linearity to microdose, the plasma $C_{max}$ after a 100 μg dose (current maximum microdose limit; EMEA and FDA guidelines) would be 15-25 nM. Given its monoexponential decay and a long elimination half-life of ~30 hr, it could be adequately monitored by LC/MS/MS with an LLOQ of 0.1 nM. The reported plasma $C_{max}$ for tolbutamide after 1 g oral dose to human subjects is 315 μM (Balant, 1981) and the elimination half-life of 7 hr. The extrapolated $C_{max}$ at a 100 μg dose, assuming linearity and monoexponential decay, would be 32 nM. Thus, with an LC/MS/MS sensitivity of 1 nM, tolbutamide could also be adequately monitored by LC/MS/MS for characterization of its pharmacokinetics at the microdose in humans.

We have applied the microdosing approach on an investigational compound MLNX which in rats showed $CL_p$ of 17 mL/min/kg, $Vd_{ss}$ of 1.2 L/kg and F of >95%, properties

broadly similar to those projected by allometric scaling for humans (Table 1), and the in vitro metabolism of MLNX also was similar in the two species (Internal communication). The clinical oral dose of MLNX is predicted to be 10 mg/kg BID. Accordingly, the doses of MLNX studies in rats were 0.01, 1 and 10 mg/kg. MLNX is ionized well on LC/MS/MS and gave a strong signal, leading to LLOQ of 0.1 nM. The two methods used for plasma collection at the lowest dose of 0.01 mg/kg, sample pooling and individual animal sampling per time point with blood transfusion from donor rats, generally gave similar results (Table 2). Thus, either method for obtaining larger volumes of plasma could be adopted for sample collections, and could possibly enhance the LLOQ. At low doses MLNX showed quantifiable concentrations only up to 8 h. Since the half-life of the compound was short, extrapolation of AUC to infinity added only a fractional area ($\leq$ 10%) to $AUC_{0\text{-}8h}$. Therefore, an $AUC_{0\text{-}inf}$ comparison across dose groups was considered appropriate for this compound. The increase in exposure in rats was linear in the dose range of 0.01 to 1 mg/kg (Fig. 4), however, nonlinearity in the exposure was observed between 1 and 10 mg/kg. Because of this nonlinearity at the high dose, it was not considered necessary to assess doses lower than 10 μg/kg. These results showed that MLNX was assessable for microdosing using LC/MS/MS, and also suggested that microdosing to determine the pharmacokinetics at therapeutic doses might not work for this investigational compound in humans.

Overall, oral microdose pharmacokinetics of tolbutamide, fluconazole and MLNX were successfully characterized in rats using conventional LC/MS/MS technique. Linearity of exposure between microdoses and ≥1000-fold higher doses in rats was demonstrated for tolbutamide and fluconazole which are known to show linear pharmacokinetics in

humans, providing a validation of the system. Application of this preclinical approach to the investigational compound MLNX showed that the exposures at therapeutic doses may not be predictable from the microdoses in humans. These preclinical assessments may help select molecules for microdosing in humans. The readily achievable LC/MS/MS sensitivity of 0.1 to 1 nM was sufficient for these microdose assessments in rats. Thus, with the current trends toward higher sensitivity LC/MS/MS systems the potential of conducting microdosing studies in the clinic without radiolabeled compounds is becoming feasible.

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**Footnotes**

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**Figure Legends**

**Fig. 1:** Plasma concentration-time profiles of fluconazole in rats (N=3 or 9) after single oral doses of 0.001 **(●)**, 0.005 (○), 0.05 (▼), and 5 (▽) mg/kg.

**Fig. 2:** Plasma concentration-time profiles of tolbutamide in rats (N=3) after single oral doses of 0.001 (●), 0.002 (○), 0.01 (▼), 0.1 (▽), and 1 (■) mg/kg.

**Fig. 3:** Plasma concentration-time profiles of MLNX in rats (N=3) after single oral doses of 0.01 (●), 0.1 (○), 1 (▼), and 10 (▽) mg/kg.

**Fig. 4**. Dose linearity test by power regression analysis for $C_{max}$ and AUC of fluconazole (A), tolbutamide (B), and MLNX (C)

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**Table 1.** Historic or projected clinical PK parameters of compounds that were assessed preclinically for microdosing

| **Compound** | **Dose regimen** (mg) | **Bioavailability** (%) | **$CL_p$** (L/h/kg) | **$Vd_{ss}$** (L/kg) | **$t_{1/2}$** (h) | **Reference** |
|---|---|---|---|---|---|---|
| Fluconazole | 400, qd | >90 | 0.016 | 0.6 | 32 | (Debruyne and Ryckelynck, 1993) |
| Tolbutamide | 500, bid | 93 | 0.014 | 0.1 | 6 | (Balant, 1981) |
| MLNX | 500, bid[1] | >90[1] | 0.42[1] | ~1[1] | 2[1] | |

[1] Predicted

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**Table 2.** PK parameters of test compounds after oral administration to rats

| **Dose** (mg/kg) | **$AUC_{inf}$** [a] (nM*h) | **$C_{max}$** (nM) | **$T_{max}$** (h) | **$t_{1/2}$** (h) |
|---|---|---|---|---|
| **Fluconazole** | | | | |
| 0.001 | 51.6 ± 20.3 | 5.20 ± 5.12 | 2.2 ± 2.0 | 5.5 ± 0.6 |
| 0.005 | 233 ± 89.9 | 33.6 ± 17.1 | 1.2 ± 0.8 | 7.3 ± 2.3 |
| 0.05 | 2031 ± 262 | 188 ± 10.5 | 1.5 ± 0.9 | 4.6 ± 0.7 |
| 5 | 158691 ± 10125 | 17667 ± 702 | 0.7 ± 0.3 | 5.6 ± 1.1 |
| **Tolbutamide** | | | | |
| 0.001 | 575 ± 31.2 | 40.2 ± 4.15 | 0.7 ± 0.3 | 7.9 ± 1.7 |
| 0.002 | 967 ± 189 | 59.5 ± 5.92 | 0.6 ± 0.4 | 9.9 ± 3.7 |
| 0.01 | 2133 ± 628 | 198 ± 46.7 | 0.8 ± 1.0 | 10.1 ± 2.4 |
| 0.1 | 37340 ± 6078 | 3420 ± 1148 | 1.1 ± 0.9 | 7.4 ± 1.0 |
| 1 | 269902 ± 39427 | 22967 ± 2977 | 1.0 ± 0 | 6.8 ± 1.2 |
| **MLNX** | | | | |
| 0.01 (Sample pooling method) | 12.8 ± 1.84 | 8.48 ± 1.69 | 0.5 ± 0 | 2.1 ± 0.6 |
| 0.01 (blood transfusion method) | 12.7 ± 1.84 | 6.31 ± 0.50 | 0.5 ± 0 | 2.6 ± 1.6 |
| 0.1 | 133 ± 45.4 | 89.3 ± 38.6 | 0.5 ± 0 | 1.6 ± 0.2 |
| 1 | 1148 ± 211 | 781 ± 252 | 0.5 ± 0 | 2.3 ± 1.0 |
| 10 | 48067 ± 5010 | 15367 ± 1914 | 0.8 ± 0.3 | 1.4 ± 0.7 |

[a] Extrapolated AUC was generally less than 15% of $AUC_{0-t}$

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**Table 3.** Dose Proportionality Test Using Power Model

| Compound | Dose Range (mg/kg) | Parameter | $R^2$ | Slope | 95% Lower CI | 95% Upper CI | Inference |
|---|---|---|---|---|---|---|---|
| **Tolbutamide** | 0.001-1 | $C_{max}$ | 0.987 | 0.96 | 0.89 | 1.02 | Linear |
| | | $AUC_{inf}$ | 0.980 | 0.92 | 0.84 | 1.00 | Linear |
| | | $t_{1/2}$ | 0.058 | 0.02 | -0.03 | 0.08 | Dose independent |
| **Fluconazole** | 0.001-5 | $C_{max}$ | 0.984 | 0.96 | 0.91 | 1.01 | Linear |
| | | $AUC_{inf}$ | 0.984 | 0.96 | 0.90 | 1.01 | Linear |
| | | $t_{1/2}$ | 0.069 | -0.02 | -0.09 | 0.04 | Dose independent |
| **MLNX** | 0.01-10 | $C_{max}$ | 0.985 | 1.09 | 1.01 | 1.17 | Nonlinear |
| | | $AUC_{inf}$ | 0.981 | 1.18 | 1.08 | 1.29 | Nonlinear |
| | | $t_{1/2}$ | 0.012 | -0.01 | -0.08 | 0.06 | Dose independent |
| | 0.01-1 | $C_{max}$ | 0.966 | 1.03 | 0.87 | 1.19 | Linear |
| | | $AUC_{inf}$ | 0.976 | 1.02 | 0.89 | 1.15 | Linear |
| | | $t_{1/2}$ | 0.009 | -0.02 | -0.15 | 0.12 | Dose independent |

System considered to be linear when $R^2 \sim 1$, $CI_{lower} \geq 0.8$, and $CI_{upper} \leq 1.25$

Fig. 1

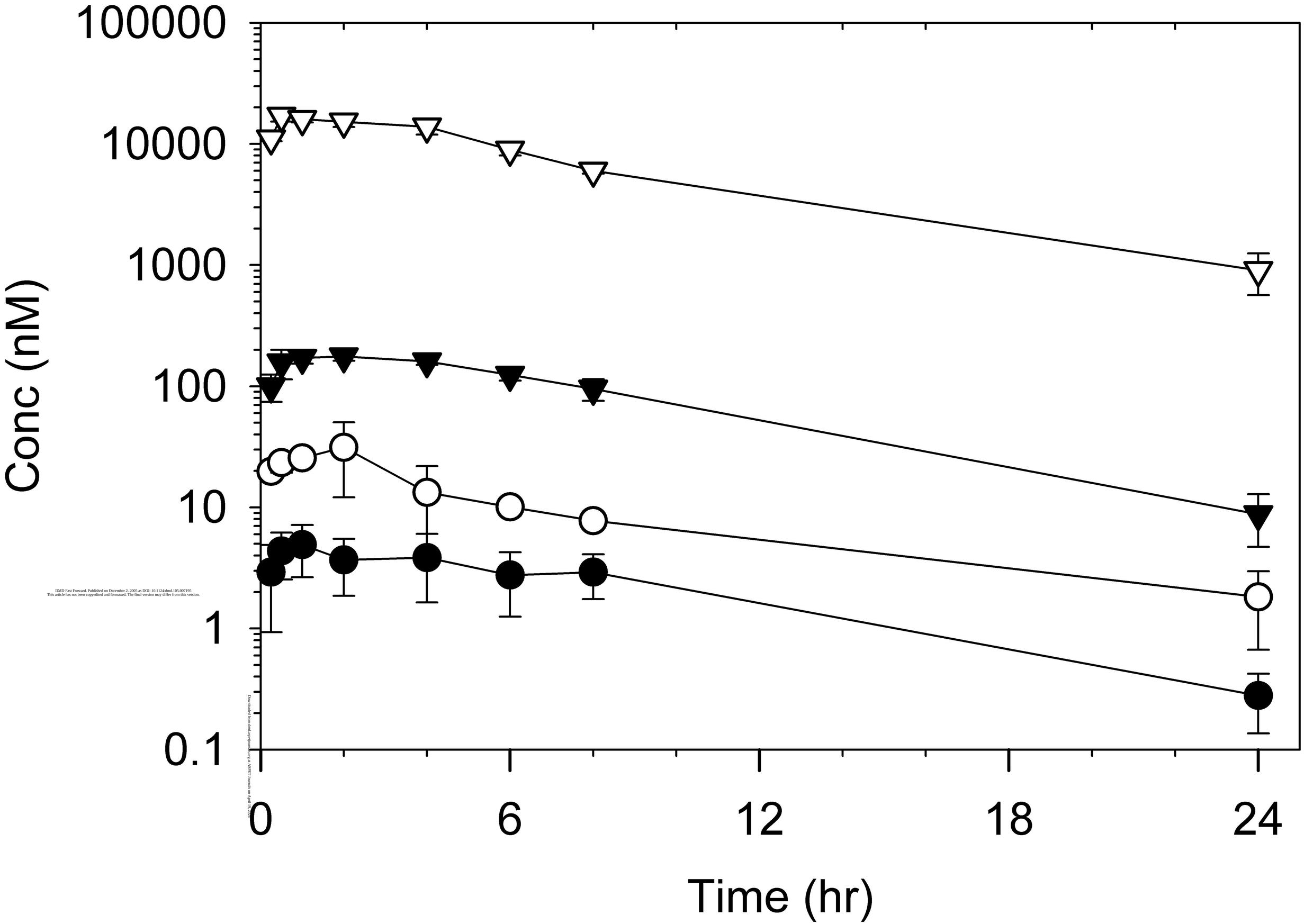

Fig. 2

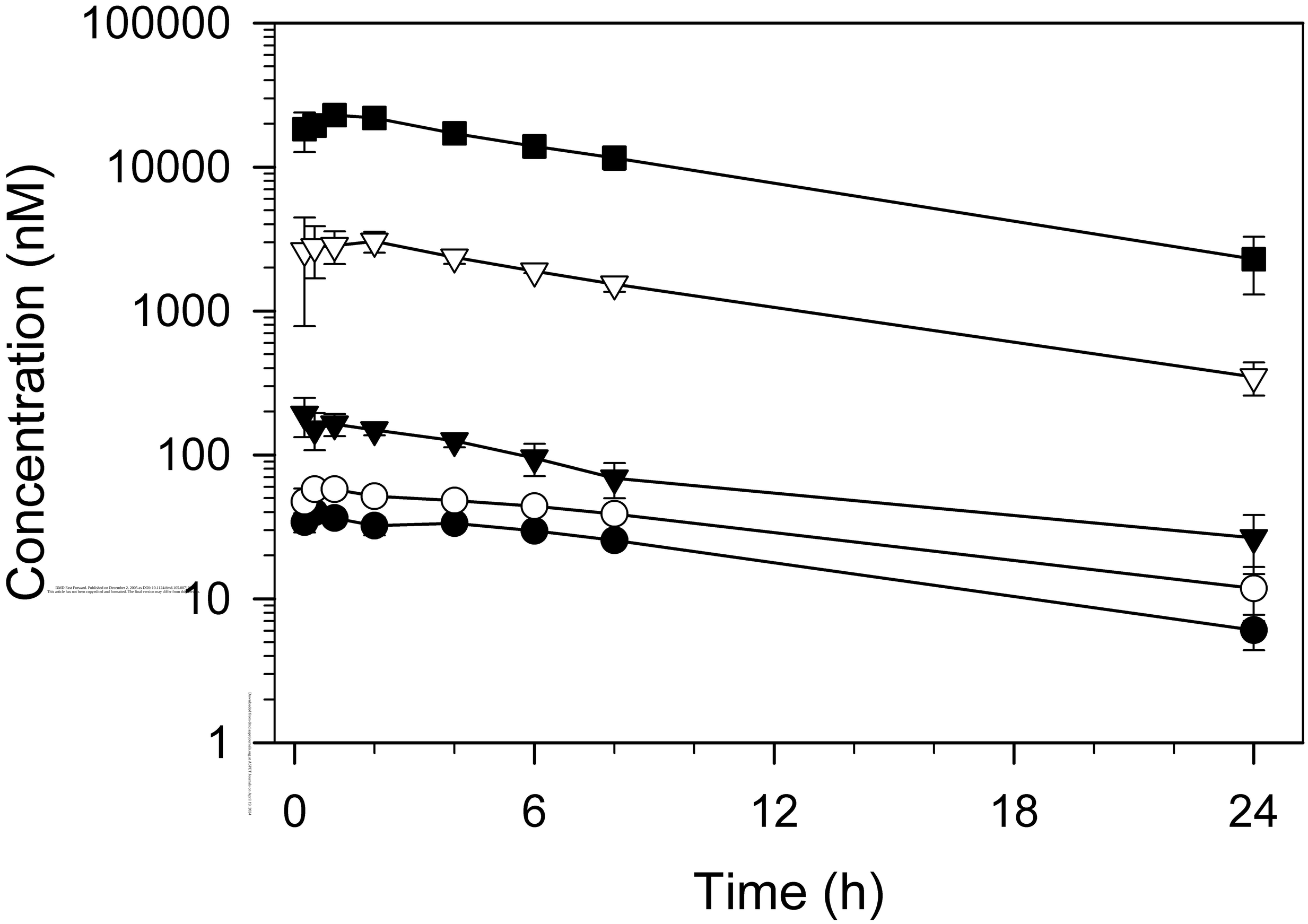

Fig. 3

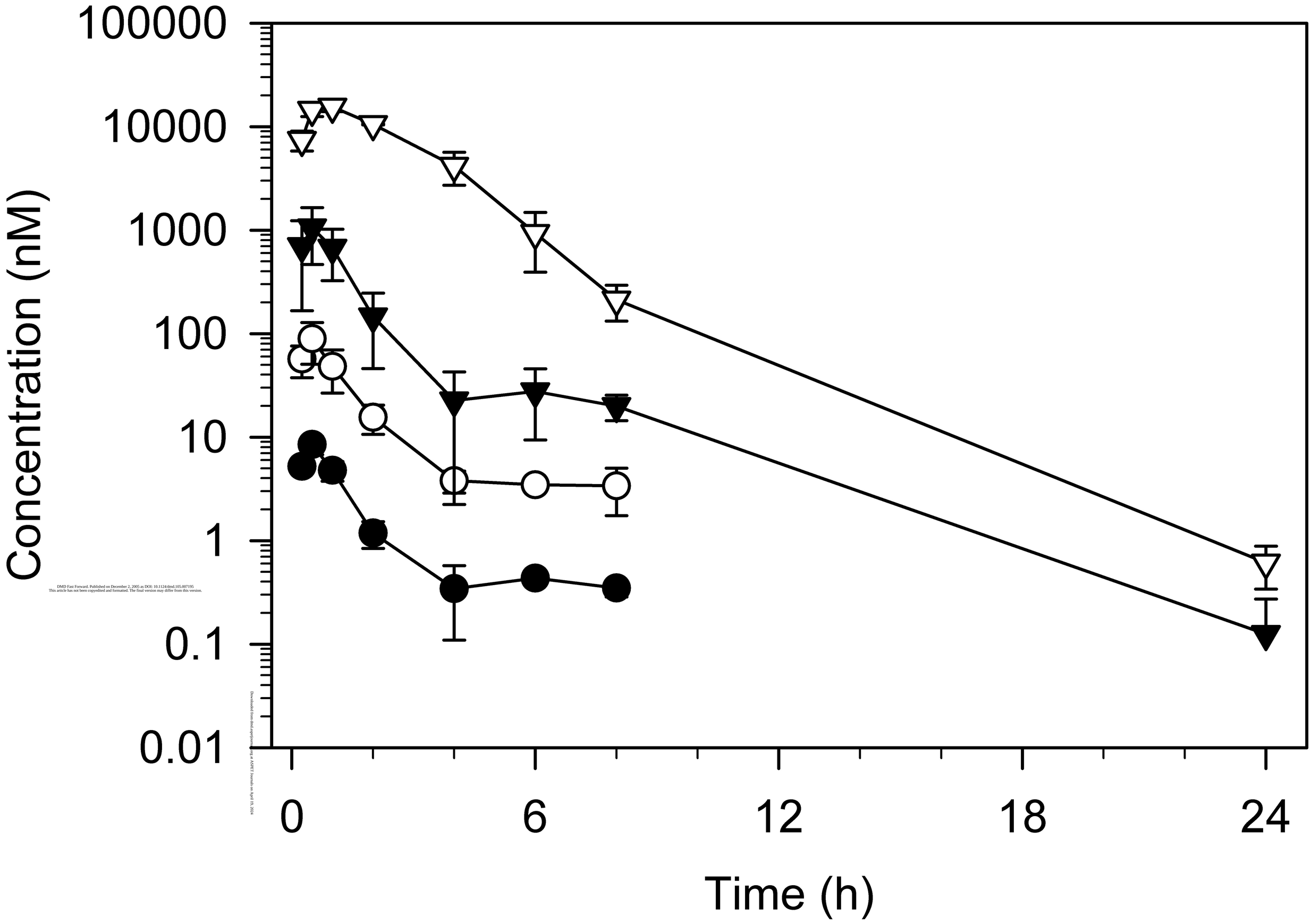

Fig. 4