2-Hydroxyestradiol Is A Prodrug of 2-Methoxyestradiol

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ABSTRACT

Previous *in vivo* studies indicate that 2-hydroxyestradiol (2OHE) attenuates cardiovascular and renal diseases. In vitro studies suggest that the biological effects of 2OHE are mediated by 2-methoxyestradiol (2MEOE) following methylation of 2OHE by catechol-O-methyltransferase (COMT). This study tested the hypothesis that *in vivo* 2OHE is a prodrug of 2MEOE. We administered to male rats IV boluses of either 2OHE or 2MEOE and measured plasma levels of 2OHE and 2MEOE by gas chromatography-mass spectrometry at various time points after drug administration. Following administration of 2OHE, plasma levels of 2OHE declined extremely rapidly ($t_{1/2(1)}$ = 0.94 min and $t_{1/2(2)}$ = 10.2 min) becoming undetectable after 45 min. Concomitant with the disappearance of 2OHE, 2MEOE appeared and then declined ($t_{1/2(1)}$ = 7.9 min and $t_{1/2(2)}$ = 24.9 min). The peak concentration and total exposure (AUC) for 2OHE were much lower than for 2MEOE. 2OHE had a much higher plasma clearance (CL) and volume of distribution (V_d) compared with 2MEOE (2OHE: CL=1215 ml min^{-1} kg^{-1} and V_d= 17,875 ml/kg; 2MEOE: CL = 50 ml min^{-1} kg^{-1} and V_d = 1760 ml/kg). Following administration of 2MEOE, plasma levels of 2MEOE declined ($t_{1/2(1)}$ = 2.5 min and $t_{1/2(2)}$ = 20.2 min) with a plasma CL of 50 ml min^{-1} kg^{-1} and a V_d of 1500 ml/kg. We could not detect 2OHE in plasma from rats receiving 2MEOE. We conclude that the conversion of 2OHE to 2MEOE is so efficient that in terms of 2MEOE exposure, administration of 2OHE is bioequivalent to administration of 2MEOE itself.

2-Hydroxyestradiol (2OHE) is a metabolite of estradiol with low affinity for estrogen receptors (Ball and Knuppen, 1990). Our *in vivo* work demonstrates that 2OHE attenuates the development of obesity, the metabolic syndrome and vascular and renal dysfunction in obese ZSF1 rats (Tofovic et al., 2001). Moreover, our more recent studies indicate that 2OHE protects against puromycin aminonucleoside-induced nephropathy (Tofovic et al, 2002), monocrotaline-induced pulmonary hypertension (Tofovic et al., 2003b) and angiotensin II-induced renal and cardiovascular injury (Tofovic et al.,2003a).

Although the aforementioned *in vivo* studies were conducted with 2OHE, in point-of-fact 2OHE is readily oxidized and is therefore a poor candidate for drug development. 2MEOE, on the other hand, is less susceptible to oxidation, and *in vitro* evidence suggests that most of the cellular effects of 2OHE are mediated by 2-methoxyestradiol (2MEOE), a metabolite of 2OHE that is devoid of estrogenic activity. In this regard, inhibition of catechol-O-methyltransferase (COMT), the enzyme that methylates 2OHE and converts it to 2MEOE, blocks the ability of 2OHE to inhibit growth of vascular smooth muscle cells (Dubey et al., 2000), cardiac fibroblasts (Dubey et al., 2002b) and renal mesangial cells (Dubey et al., 2002a). Moreover, 2OHE inhibits vascular smooth muscle cell growth in cells obtained from wild-type mice but not in cells cultured from COMT knockout mice (Zacharia et al., 2004b). In contrast to 2OHE, treatment of vascular smooth muscle cells with 2MEOE inhibits serum-induced growth of cells from both wild-type and COMT- knockout mice (Zacharia et al., 2004b).

We hypothesize that *in vivo* 2OHE is essentially a prodrug of 2MEOE and is converted so rapidly to 2MEOE as to be essentially bioequivalent with administration of 2MEOE. This hypothesis is supported indirectly by our recent findings that 2OHE is

converted to 2MEOE very efficiently by the perfused rat kidney and heart (Zacharia et al., 2003). The purpose of the present study was to directly test this hypothesis by carefully examining and comparing the pharmacokinetic behavior of both 2OHE and 2MEOE. The importance of testing this hypothesis is that if 2OHE is bioequivalent to the more stable metabolite 2MEOE, then 2MEOE would be a better drug candidate.

METHODS

Animals: Adult male Sprague Dawley rats (260-310 grams) were acclimated for at least 5 days before use. Male rats were used to avoid endogenous 2OHE and 2MEOE that would be present in female rats. The rats were fed a laboratory animal feed and were provided tap water ad libitum. Lighting was on a standard 12-hours on, 12-hours off cycle. The humidity in the housing area during the study was 50% and the temperature was 68^0 F.

Drugs: 2OHE and 2MEOE were obtained from Steraloids (Newport, RI) and dissolved in PEG200 at a concentration of 1 mg/ml.

Protocol: Rats (n = 84) were anesthetized with isoflurane and received a single IV injection of 0.5 mg/kg of either 2OHE (n = 42) or 2MEOE (n = 42). Blood samples (~6 mL) were obtained at 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 360 minutes after dosing. Animals were anesthetized with isoflurane for blood sampling and samples were obtained by cardiac stick prior to euthanasia. Ascorbic acid (final concentration = 1 mM) was added to each sample to prevent oxidation of 2OHE and 2MEOE. Only a single blood sample was taken per rat, and three rats were used for each time point. After the blood samples were obtained, animals were euthanized with CO_2.

Assay for 2OHE and 2MEOE: We recently developed and validated a GC/MS assay for 2OHE and 2MEOE in rat plasma (Zacharia et al., 2004a), and this assay was used in the current study. Briefly, plasma samples were deprotonated using acetone, dried, reconstituted in acetonitrile, derivatized with pentafluorpropionic acid anhydride and analyzed by negative chemical ionization on a ThermoQuest Finnigan Trace GC/MS. The metabolites 2OHE and 2MEOE were assayed simultaneously, and levels determined

from a standard curve prepared in rat plasma. The detection limit for both metabolites was 0.25 pg/µl. Inter-assay coefficients of variation were for 2OHE 7.1%, 6.6% and 3.4% and for 2MEOE 16.7%, 16.6%, and 12% for the low (0.375pg/µl), medium (1.875pg/µl) and high (4.375pg/µl) quality control samples, respectively. Intra-assay coefficients of variation were for 2OHE 15.6%, 26.2%, 25.6% and for 2MEOE 16.5%, 11.78%, 11.23% for the low medium and high quality control samples, respectively.

Statistical Methods: Standard pharmacokinetic parameters for 2OHE and 2MEOE were determined from mean plasma concentration versus time data. Plots of mean plasma concentration versus time data include ± standard deviation for each mean plasma concentration data point. Because of the rapid and extensive metabolic conversion of 2OHE to 2MEOE observed in this study, pharmacokinetic analysis was also applied to the 2MEOE plasma data following administration of 2OHE. Areas under the concentration versus time curve (AUC) were calculated from time zero to the last measurable time point (360 min). Plasma concentration data were fit to poly-exponential equations, using a non-linear, least-squares method (RSTRIP, MicroMath) to determine the apparent half-lives ($t_{1/2}$) of each phase, and time zero plasma concentrations (C_0).

RESULTS

Pharmacokinetic Analysis of 2MEOE Following Bolus Dosing of 2MEOE: The pharmacokinetic parameters for 2MEOE calculated from a study using intravenous boluses of 2MEOE in male rats are shown in Table 1. After IV injection, 2MEOE exhibited a multi-phasic serum versus time profile (Figure 1). Peak plasma concentrations of > 500 ng/ml were observed immediately after the injection (C_{max} = 584 ng/ml). Plasma levels initially fell rapidly ($t_{1/2(1)}$ = 2.5 min), then more slowly ($t_{1/2(2)}$ = 20.2 min) over the first 2 hours. The initial concentration of 2MEOE achieved (C_0), was 751 ng/ml with an initial volume of distribution, Vc (volume of the central compartment), of 666 ml/kg. 2MEOE was rapidly eliminated over the next 2 hours with concentrations of plasma falling nearly 500-fold in 360 minutes. Although the shape of the plasma concentration versus time curve prevented accurate determination of the terminal elimination rate, the AUC calculated from time 0 to 360 minutes is likely to be a close approximation to the total AUC due to the low levels observed at the last time point. Based on the AUC from 0-360 minutes, the clearance of 2MEOE was 50 ml min^{-1} kg^{-1}. Assuming that the elimination half-life is that of the second phase observed in this study ($t_{1/2(2)}$ = 20.2 min), then a volume of distribution (V_d; volume of distribution during elimination) of approximately 1500 ml/kg would be predicted during the elimination phase.

Pharmacokinetic Analysis of 2OHE Following Bolus Dosing of 2OHE: The pharmacokinetic parameters of 2OHE after intravenous administration are shown in Table 1. After IV injection, 2OHE exhibited a biphasic serum versus time profile (Figure

2). Plasma 2OHE concentrations peaked at approximately 100 ng/ml immediately after injection (C_{max} = 107 ng/ml). Plasma levels fell extremely rapidly ($t_{1/2(1)}$ = 0.94 min) for the first few minutes, then more slowly, but still very rapidly ($t_{1/2(2)}$ = 10.2 min) over the next half-hour. Plasma levels were not detectable at time points more than 45 minutes after dosing. The initial concentration of 2OHE, $C_{0,}$ was 216 ng/ml with an initial volume of distribution, V_C, of 2315 ml/kg. The AUC from time 0 to 45 minutes is likely to be a close approximation to the total AUC due to the low levels observed at the last time point. Based on this AUC, the clearance of 2OHE was 1215 ml min^{-1} kg^{-1}. Based on the observed terminal half-life in this study ($t_{1/2(2)}$ = 10.2 min), 2OHE has a very large volume of distribution (V_d) of approximately 18,000 ml/kg.

Pharmacokinetic Analysis of 2MEOE Following Bolus Doses of 2OHE: The pharmacokinetic parameters of the metabolite 2MEOE after 2OHE administration are shown in Table 1. After IV injection of 2OHE, plasma concentrations of the metabolite rose rapidly (C_{max}, 494 ng/ml at 2 min), then fell in a bi-exponential manner over the next 3 hours. (Figure 3). Both the initial and terminal half-lives of 2MEOE ($t_{1/2(1)}$ = 7.9 min and $t_{1/2(2)}$ = 24.9 min, respectively) were longer than those observed for the parent 2OHE. The plasma concentration versus time profile of 2MEOE after 2OHE injection was nearly identical to that observed after injection of the 2MEOE itself at the same dose (Figure 4). The AUCs of 2MEOE observed after 2OHE administration were nearly identical after administration of 2OHE or 2MEOE, with similar peak plasma levels and half-lives. The initial concentration of 2MEOE achieved in this study, C_0, was 495 ng/ml with an initial volume of distribution, Vc, of 1010 mg/kg. From the AUCs in plasma observed after 2OHE administration, the clearance of 2MEOE was approximated to be 50 ml min^{-1} kg^{-1}.

Based on the observed terminal half-life in this study ($t_{1/2(2)}$ = 24.9 min), 2MEOE has a volume of distribution (V_d) of approximately 1760 ml/kg. Figure 5 shows the plasma concentration versus time relationships on the same graph for 2OHE and 2MEOE after administration of 2OHE.

DISCUSSION

The hypothesis tested by this study is that 2OHE is a prodrug of 2MEOE. Our data demonstrate that administration of 2OHE is equivalent to the administration of 2MEOE with regard to the total exposure of the body to 2MEOE. Moreover, based on the clearances observed in this study, a constant rate infusion of 2OHE would result in steady-state concentrations of 2MEOE that are approximately 25-fold higher than steady-state concentrations of 2OHE. *In vitro*, 2MEOE is more potent than 2OHE with regard to inhibiting growth of vascular smooth muscle cells (Dubey et al., 2000), cardiac fibroblasts (Dubey et al., 2002b) and renal mesangial cells (Dubey et al., 2002a). Moreover, 2OHE has little estrogenic activity, and its *in vitro* effects are not blocked by estrogen receptor antagonist ICI 182,780 (Dubey et al., 2000). Because 2OHE is nearly quantitatively converted to 2MEOE, because constant rate infusions of 2OHE would produce steady-state levels of 2MEOE that are 25-fold higher than steady state levels of 2OHE, and because the cellular effects of 2MEOE are more potent than 2OHE, taken together our findings indicate that 2OHE is a prodrug of 2MEOE. It is conceivable, however, that at extremely high infusion rates of 2OHE, steady state concentrations of 2OHE may reach sufficiently high levels to have direct biological activity. Nonetheless, given the pharmacokinetic and pharmacodynamic profiles of 2OHE versus 2MEOE, it seems appropriate to call 2OHE a prodrug of 2MEOE.

The conclusion that administration of 2OHE is equivalent to the administration of 2MEOE with regard to the total exposure of the body to 2MEOE is based on the following considerations. The plasma concentration versus time profile of 2MEOE after

IV injection of 2OHE is nearly identical to the profile after IV injection of the same dose of 2MEOE. Moreover, the AUC for 2MEOE following IV administration of 2OHE is very similar to the AUC following IV administration of the same dose of 2MEOE. Also, the peak plasma levels and half-lives of 2MEOE following IV administration of 2MEOE are similar to those observed after IV administration of the same dose of 2OHE. Thus, in terms of exposure to 2MEOE, 2OHE and 2MEOE injections appear to be equivalent.

The high clearance of 2OHE (1215 ml min^{-1} kg^{-1}) exceeds the rat's liver blood flow (approximately 55 ml min^{-1} kg^{-1}), suggesting that 2OHE is subject to extensive extrahepatic metabolism to 2MEOE. Indeed the clearance of 2OHE even exceeds the rat's cardiac output (approximately 300 ml min^{-1} kg^{-1}). This exceedingly high clearance of 2OHE is most consistent with the concept that 2OHE partitions rapidly and extensively into erythrocytes where it is quickly converted to 2MEOE by COMT. The high COMT content of erythrocytes (Mannisto and Kaakkola, 1999) is supportive of this hypothesis. Based on the observed terminal half-life in this study (10.2 min), 2OHE has an extremely large volume of distribution (approximately 18,000 ml/kg). This finding indicates that 2OHE is extensively distributed to sites outside the plasma compartment (including blood cells). This high volume of distribution provides ample encounters with COMT to methylate 2OHE to 2MEOE.

The sample size per time point (3 animals per time point) was adequate for the overall characterization of 2MEOE serum pharmacokinetics. The half-lives we report, from curve fits to mean serum data, are estimates whose values are clearly subject to variability observed within the sample population. The pharmacokinetic profile of 2MEOE was "multiphasic", and this is consistent with the most important

characterization of the kinetic profile, i.e., 2MEOE disappears rapidly from serum after administration in a manner suggesting that both distributional and metabolic components are involved.

The pharmacokinetics of 2OHE and 2MEOE after 2OHE administration has been previously examined in humans using radioimmunoassay as the detection method (Kono et al., 1982). The current study employed a highly sensitive and specific gas chromatographic-mass spectrometric assay and utilized an experimental design that allowed a comprehensive comparison of the pharmacokinetic profiles of 2OHE versus 2MEOE. Importantly, there were no major differences in the pharmacokinetic parameters in our studies in rats and versus the previous study in humans, indicating that the conclusions of the present study most likely hold for human beings as well.

The results of the present study have important implications. In this regard, endogenous estradiol can be hydroxylated by cytochrome P450s in the liver (Martucci and Fishman, 1993; Ball and Knuppen, 1978) and in non-hepatic tissues (Martucci and Fishman, 1993; Ball and Knuppen, 1978) to form 2OHE. The fact that administration of 2OHE behaves very similar to administration of 2MEOE suggests that endogenously synthesized 2OHE is converted rapidly, locally and mostly to 2MEOE. Rapid methylation of 2OHE to 2MEOE is consistent with the possibility that 2MEOE is an active endogenous compound.

Inasmuch as 2MEOE inhibits growth of vascular smooth muscle cells (Dubey et al., 2000), cardiac fibroblasts (Dubey et al., 2002b), renal mesangial cells (Dubey et al., 2002a) and cancer cells and is an anti-mitogenic agent, the present data suggest that metabolism of endogenous 2OHE to 2MEOE may importantly contribute to protection

against cardiovascular/renal diseases and cancer. Any reduction in the efficacy of COMT-mediated methylation of 2OHE could lead to increased susceptibility to disease. In this regard, because catecholamines inhibit methylation of 2OHE (Zacharia et al., 2001), stress-induced activation of the sympathoadrenal axis may limit the protection afforded by this biochemical pathway. Also, the COMT gene has low, intermediate and high efficiencies due to genetic polymorphisms (Boudikova et al., 1990), and therefore individuals may be protected differently by endogenous 2OHE depending on their genotype.

From the therapeutic perspective, because the administration of 2OHE is equivalent to administering 2MEOE, our data imply that the pharmacology of 2MEOE would be similar to that of 2OHE. This may be advantageous because 2OHE is less chemically stable (more readily oxidized) compared with 2MEOE and therefore 2MEOE may be more easily formulated as a drug. Indeed, we have successfully formulated 2MEOE in biodegradable microparticles for sustained release resulting in pharmacologically active plasma levels of 2MEOE in rats for up to one month following a single subcutaneous injection (work in progress). The potential use of 2MEOE is currently being evaluated in Phase I and Phase II clinical trials for the treatment of multiple types of cancer including breast cancer, advanced refractory metastatic breast cancer and hormone-refractory prostate cancer (Lakhani et al., 2003). In addition to being a potent anti-carcinogenic agent, animal studies provide evidence that 2MEOE may effectively protect against proliferative disorders associated with cardiovascular disease, renal disease and obesity (Tofovic et al., 2001 and 2002).

In conclusion, the results of the present study strongly support the hypothesis that 2OHE is converted mostly to 2MEOE and that 2OHE is, for all practical purposes, a prodrug of 2MEOE.

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FIGURE LEGENDS

Figure 1. Line graph illustrating the relationship between mean (±SD) plasma levels of 2-methoxyestrtadiol (2MEOE) concentrations versus time after IV administration of 0.5 mg/kg of 2-methoxyestradiol. The insert represents the fitted curve using a bi-exponential equation.

Figure 2. Line graph illustrating the relationship between mean (±SD) plasma levels of 2-hydroxyestradiol (2OHE) versus time after IV administration of 0.5 mg/kg of 2-hydroxyestradiol. The insert represents the fitted curve using a bi-exponential equation.

Figure 3. Line graph illustrating the relationship between mean (±SD) plasma levels of 2-methoxyestrtadiol (2MEOE) versus time after IV administration of 0.5 mg/kg of 2-hydroxyestradiol. The insert represents the fitted curve using a bi-exponential equation.

Figure 4. Line graph illustrating the relationship between mean (±SD) plasma levels of 2-methoxyestrtadiol (2MEOE) versus time after IV administration of either 2-methoxyestradiol or 2-hydroxyestradiol (2OHE), each at 0.5 mg/kg.

Figure 5. Line graphs illustrating the relationship between mean (±SD) plasma levels of either 2-hydroxyestradiol (2OHE) or 2-methoxyestradiol (2MEOE) after IV administration of 2-hydroxyestradiol (0.5mg/kg).

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Table 1. Pharmacokinetic Parameters of Intravenous 2-hydroxyestradiol (2OHE) and 2-methoxyestradiol (2MEOE) in male rats.

Parameter	*Unit*	*2OHE*	*2MEOE*	*2MEOE*
Drug Infused	NA	2OHE	2OHE	2MEOE
Dose	mg/kg	0.5	0.5	0.5
AUC	ng/min/ml	411	10,182	10,066
CL	ml/min kg	1,215	49.1	49.7
C_{max}	ng/ml	107	494	584
C_0	ng/ml	216	495	751
V_c	ml/kg	2,315	1,010	666
$t_{1/2\ (1)}$	min	0.94	7.9	2.5
$t_{1/2\ (2)}$	min	10.2	24.9	20.2
V_d	ml/kg	17,875	1,760	1,500

AUC, area under the curve

CL, intravenous clearance based on AUC from 0-last measurable time point

C_0, plasma concentration extrapolated to time zero

C_{max}, maximum observed concentration in plasma

V_c, volume of the central compartment

$t_{1/2}$, half-lives based on exponential curve fits to concentration versus time data

V_d, estimated volume of distribution during elimination

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Figure 1

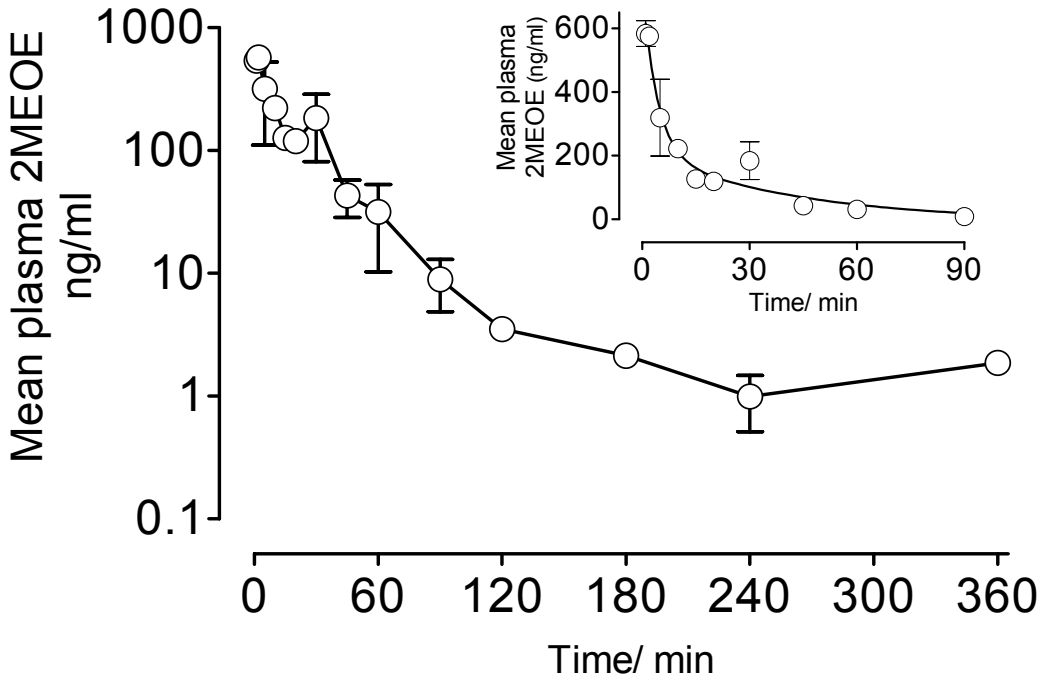

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Figure 2

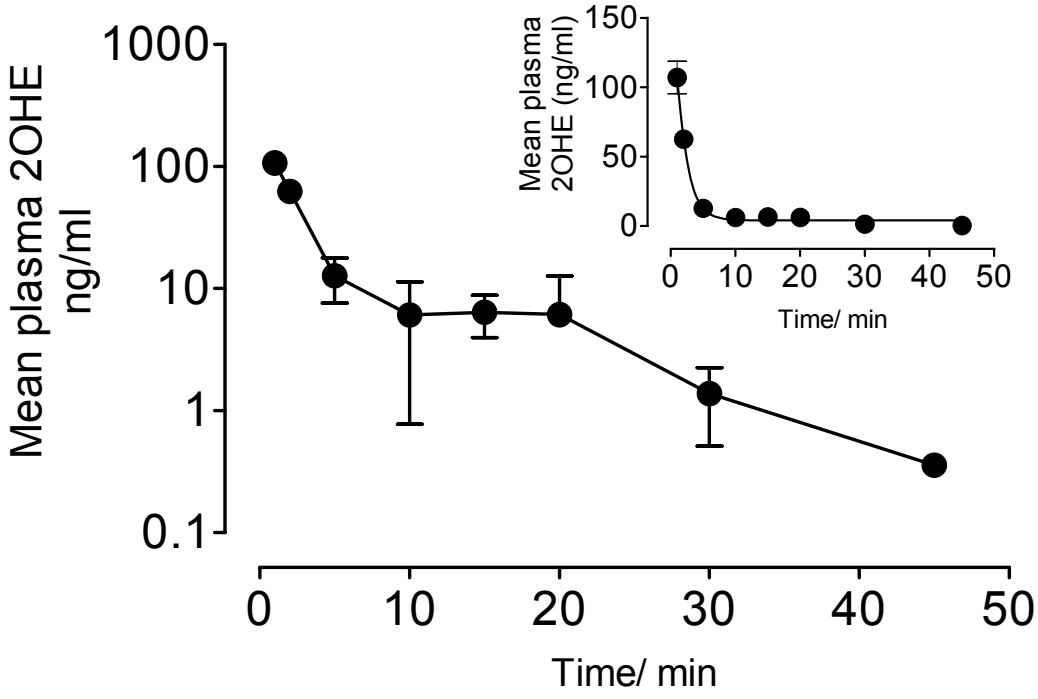

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Figure 3

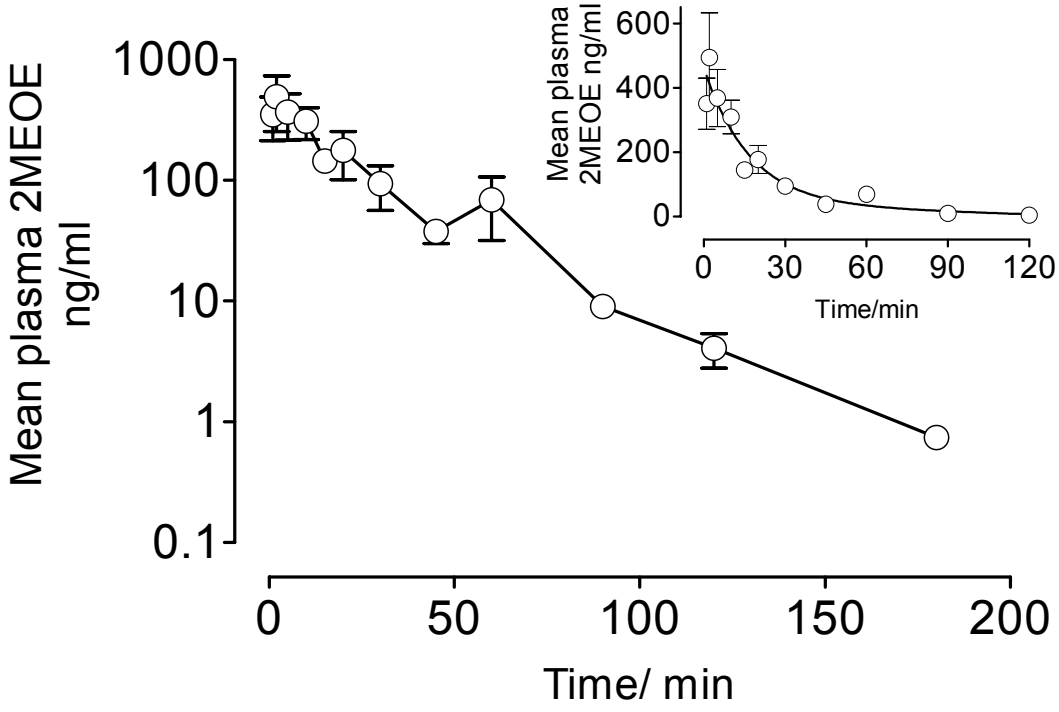

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Figure 4

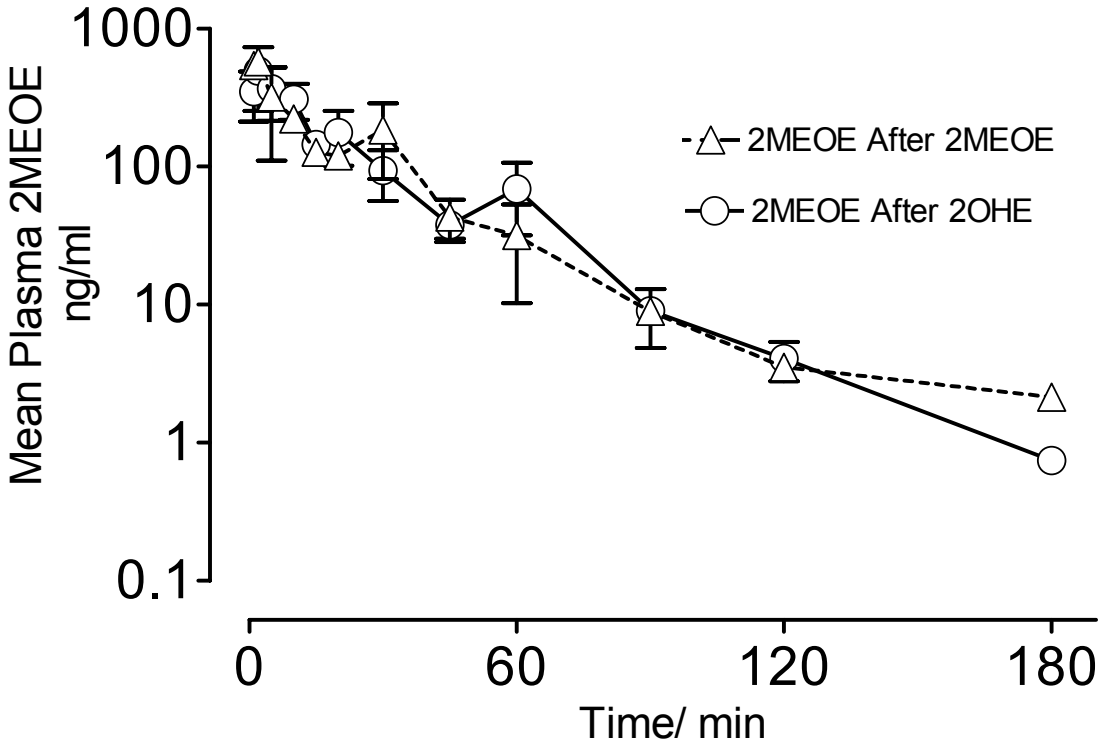

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Figure 5