INVITED REVIEW

# Effects of a recombinant humanized anti-cocaine monoclonal antibody on the metabolism and distribution of cocaine in vitro and in mice

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## Abstract

The anti-cocaine monoclonal antibody, h2E2, is a candidate for treating cocaine-use disorder. h2E2 binds to and sequesters cocaine in the plasma compartment, effectively decreasing cocaine concentrations in the brains of rats and mice. Despite the binding of cocaine to h2E2, plasma cocaine concentrations decline rapidly in rodents over time, but there was a drastic decrease in the urinary elimination of cocaine in the presence of h2E2. Since cocaine is not being renally excreted, the apparent disappearance of cocaine from the plasma must be explained by either metabolism or distribution. However, binding of cocaine to h2E2 may restrict the availability of cocaine for hydrolysis by endogenous esterases. Therefore, the antibody would be expected to extend the elimination half-life of cocaine. In contrast, previous studies reported h2E2 as having no effect on the rate of cocaine clearance. It is important to examine the ultimate clearance of the cocaine to ascertain its half-life and potential for re-intoxication. Therefore, we investigated the effects of h2E2 on cocaine hydrolysis in vitro and on cocaine metabolism and disposition in vivo over a 6-h time course. The spontaneous and enzyme-mediated in vitro hydrolysis of cocaine was drastically decreased in the presence of h2E2 in vitro. Additionally, in mice, h2E2 significantly increased the distribution and elimination half-lives of cocaine relative to vehicle controls over an extended time course. Therefore, we concluded that h2E2 slowing the distribution and elimination of cocaine is the most appropriate explanation for the initial disappearance of cocaine from the plasma in vivo.

## 1 | INTRODUCTION

Over the past decade, the United States experienced a substantial rise in cocaine-related overdoses,[1] and cocaine-use disorder is an immediate public health issue. Despite decades of research, there is no FDA-approved treatment for cocaine-use disorder. Our team generated and developed a humanized anti-cocaine monoclonal antibody (mAb), designated h2E2, that sequesters cocaine in the

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**Abbreviations:** BChE, butyrylcholinesterase; BE, benzoylecgonine; CES1, liver carboxylesterase 1; EME, ecgonine methyl ester; ECGO, ecgonine; h2E2, humanized anti-cocaine monoclonal antibody; HPLC-MS/MS, high performance liquid chromatography tandem mass spectrometry; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCP, phencyclidine; PK, pharmacokinetic.

plasma compartment and inhibits its entry into the brains of rats and mice.[2-4] The humanized h2E2 mAb has high affinity and selectivity for cocaine over its inactive metabolites.[4] h2E2 has been shown to decrease the probability of cocaine-induced relapse in rats,[5] making it a lead candidate for the immunotherapeutic treatment of cocaine abuse.

We have previously demonstrated that h2E2 has a marked effect on the pharmacokinetics (PKs) of cocaine. Peak plasma concentrations of cocaine were 11.3-fold higher in h2E2-treated mice compared to vehicle, resulting in a lower volume of distribution of cocaine in the presence of h2E2.[6] Despite the dramatic increase in plasma cocaine concentrations, PK studies in rats and mice treated with h2E2 and its recombinant predecessor, 2E2, reported an apparently unchanged elimination half-life of cocaine compared to vehicle controls.[2,3] The apparently unchanged rate of clearance of cocaine was previously explained by an unaltered metabolism and excretion of cocaine,[3] but this is not consistent with studies showing that h2E2 significantly decreased cocaine urinary excretion.[7] Furthermore, once bound to h2E2, cocaine is likely unavailable for binding to and subsequent hydrolysis by endogenous esterases. Therefore, in contrast to existing PK data, h2E2 would be expected to slow or prevent the clearance of cocaine and formation of its metabolites. As the antibody advances toward the clinical phase of its development, the effects of h2E2 on the distribution, metabolism, and excretion of cocaine should be elucidated.

Herein, we describe the effects of h2E2 on the in vitro hydrolysis of cocaine both nonenzymatically and enzymatically. The present study evaluated hydrolysis by the two main enzymes responsible for the degradation of cocaine into its inactive metabolites: benzoylecgonine (BE), formed primarily by liver carboxylesterase 1 (CES1),[8] and ecgonine methyl ester (EME), formed mainly by butyrylcholinesterase (BChE).[9,10] The interactions of cocaine and h2E2 were also examined in vivo. Previous PK studies only evaluated the effects of h2E2 for 60-min after cocaine injection.[2,6] Due to the long half-life of the h2E2 (days), the existing PK data may not have fully captured the length and extent of h2E2's effects on the disposition of cocaine. Therefore, we investigated the PKs of cocaine in the presence of h2E2 in mice over several hours.

## 2 | MATERIALS AND METHODS

### 2.1 | Enzymatic hydrolysis of cocaine in vitro

#### 2.1.1 | Materials and reference standards

Equine butyrylcholinesterase (BChE) and human liver carboxylesterase (CES1) were purchased from MilliporeSigma. All enzyme studies were performed using identical reagent lots. Enzymatic activity was determined by the vendor as 1 nmol/min hydrolysis of 4-nitrophenyl for CES1 and 1 μmol/min hydrolysis of butyrylcholine for BChE. Cocaine (1 mg/ml in acetonitrile) solution and the solutions used for internal standards (Coc-D3, BE-D8, and EME-D3 at concentrations

100 μg/ml) were obtained from Cerilliant. Methanol, 2-propanol, and 10 mM ammonium formate with 0.05% formic acid were all HPLC or HPLC-MS/MS grade (Thermo Fisher). A stock solution of 1 M phosphate-buffered saline (PBS; pH 7.4) (Sigma-Aldrich) was prepared for use throughout the duration of the study. Each chemical used was reagent grade or HPLC grade.

#### 2.1.2 | In vitro hydrolysis of cocaine by liver carboxylesterase I and BChE

At a volume of 2 ml in PBS (1 M, pH 7.4), cocaine degradation was evaluated with an initial concentration of 20 μM. The following reaction conditions were investigated in triplicate: (i) BChE (100 U/ml); (ii) BChE (100 U/ml) and h2E2 (10 μM); (iii) human liver CES1 (500 U/ml); (iv) CES1 (500 U/ml) and h2E2 (10 μM); and (v) PBS. These conditions were determined experimentally to have measurable enzymatic activity against cocaine. These conditions allowed for the testing of the effects of h2E2 on the metabolism of cocaine.

The resulting conditions were incubated at 37°C in an electronically controlled water bath (Branson 5510). The first aliquot was taken immediately after the addition of enzyme ($t = 0$). For the BChE reactions, aliquots were collected at 7.5-, 15-, 30-, 60-, 120-, 240-, 480-, 960-, 1680-, and 3120-min post-addition of enzyme. For the CES1 reactions, aliquots were collected at $t = 0$ and at 60-, 120-, 240-, 480-, 960-, 1680-, and 3120-min post-addition of enzyme. Enzyme activity was quenched by transferring these aliquots to Eppendorf microcentrifuge tubes containing extraction buffer (90% methanolic 50 mM $ZnSO_4$ containing internal standard at 100 ng/ml COC-D3 and 10 ng/ml BE-D8 and EME-D3). Aliquots from both the BChE and CES1 reactions were diluted 1:10 in extraction buffer and then were incubated for 15 min at 4°C. The extracts were centrifuged at 21 100× $g$ at 4°C for 30 min in a refrigerated centrifuge (Hermle Z126-MK). The supernatant was transferred to a clean glass autosampler vial and stored at 4°C until HPLC-MS/MS analysis. Cocaine and metabolite concentrations in each in vitro aliquot were quantified via HPLC-MS/MS within 1 day of collection.

### 2.2 | The PKs of cocaine in the presence of the mAb h2E2

#### 2.2.1 | Animals

Six-week-old male Swiss-Webster mice (19–22 g) were purchased from Taconic Farms. Mice were implanted with a jugular vein catheter under isoflurane anesthesia. Mice were housed individually on a 14/10 h light/dark cycle with ad libitum access to food and water. These studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals under a protocol approved by the Institutional Animal Care and Use Committee at the College of Medicine, University of Cincinnati (Cincinnati, OH).

### 2.2.2 | Materials and reagents

Recombinant h2E2 and its formulation buffer (10 mM Histidine, 10% Sucrose, 0.01% PS80, pH 6) were manufactured by Catalent Pharma Solutions. BD Microtainer® tubes coated with lithium heparin were obtained for trunk blood collection. Ceramic beads and garnet shards were prepackaged in 2.0 ml tubes from Benchmark Scientific and were transferred to screwcap 5 ml macrotubes (MTC BioTech) before brain extraction. 10 mM ammonium formate with 0.05% formic acid was obtained from Thermo Fisher Scientific.

### 2.2.3 | Cocaine PK studies

Before the start of the study, venous catheter patency was verified by injecting heparin saline solution via the catheter. Mice received an intravenous injection (*i.v.*) of h2E2 (120 mg/kg) or vehicle (10 mM Histidine, 10% Sucrose, 0.01% PS80, pH 6) at a rate of 0.3 ml/10 s, depending on the antibody concentration and the body weight of the animal, with the animal held under mild restraint. One hour after the h2E2 injection, mice received an injection of cocaine HCl (0.56 mg/kg *i.v.*) followed by a flush of heparin saline. Mice were anesthetized with pentobarbital sodium (50 mg/kg *i.p.*) 3 min prior to sacrifice. For the 0.75-min time point, mice received the cocaine infusion while under anesthesia. At 0.75, 5, 20, 60, 120, 240, 360, and 480 min, anesthetized mice were sacrificed by decapitation. Trunk blood (0.8–1.4 ml) was collected in an anti-coagulant-coated 1.5-ml polypropylene microcentrifuge tube. The blood samples were centrifuged at 5000× *g* for 3 min immediately following collection. Plasma (0.4–1 ml) was carefully separated from packed red blood cells and transferred to sterile 1.5-ml Eppendorf microcentrifuge tubes. The samples were rapidly frozen on dry ice and then stored at −80°C until extraction and analysis by HPLC-MS/MS. The whole brain was removed from each of the decapitated mice. Surface blood was blotted away, and the brain was placed in a polypropylene tube containing 1 ml 10 mM ammonium formate with 0.05% formic acid, a ceramic bead, and garnet shards. The subsequent solution was rapidly frozen on dry ice. Brains were stored at −80°C until extraction and HPLC-MS/MS analysis.

### 2.3 | HPLC-MS/MS analysis of cocaine and cocaine metabolites

### 2.3.1 | Sample preparation and extraction

Biological samples were thawed on the date of analysis. Plasma samples were diluted 1:10 in extraction buffer (90% methanolic 50 mM $ZnSO_4$ containing internal standard at 100 ng/ml COC-D3 and 10 ng/ml BE-D8 and EME-D3) to remove any interfering matrix elements. After dispensing the aliquots into extraction buffer, the resulting diluted sample was incubated for 15 min at 4°C. The samples were centrifuged at 21.1× *g*, 4°C, for 1 h. After centrifugation,

approximately 0.5 ml aliquots were carefully removed from the clear upper layer containing the analyte and were transferred to glass HPLC vials. Cocaine and metabolites were extracted from brains using a similar protocol. One whole mouse brain, 1 ml 10 mM ammonium formate in water, and a ceramic bead and garnet shards were transferred to 5 ml screwcap tubes. The tubes were loaded onto the bead homogenizer (BeadBlaster24 Model D2400) in a 12-position rotor. The locking ring was secured, and brains were homogenized at 6.0 m/s for 40 s for three cycles. The resulting brain homogenates were centrifuged at 10 rpm for 30 s to allow the garnet shards and the bead to settle at the bottom of the tubes. Then, each brain homogenate was removed and diluted 1:10 into extraction buffer. The resulting extract was incubated for 15 min at 4°C. Then, the extracted samples were centrifuged at 21.1× *g*, 4°C, for 2 h. The supernatant was transferred to a clear glass autosampler vial for analysis by HPLC-MS/MS.

### 2.3.2 | Preparation of calibrators and quality control samples

Calibration curves were generated with $1/x^2$ weighting of the ratio of the quantifier ion peak area: deuterated IS peak area. Calibration was performed using Cerilliant standards spiked into 10 mM ammonium formate with 0.05% formic acid at concentrations of 7000, 2333, 778, 260, 87, 29, 9.6, and 3.2 ng/ml for COC, BE, EME, and ECGO. Quality control material was independently prepared from calibrator solutions at concentrations of 5600, 145, 9, and 3 ng/ml. Calibrators and quality controls were diluted 1:10 in extraction buffer (90% methanolic 50 mM $ZnSO_4$ containing internal standard at 100 ng/ml COC-D3 and 10 ng/ml BE-D8 and EME-D3) and extracted according to the methods used on the biological samples. The acquisition parameters demonstrated adequate accuracy, precision, and process efficiency for plasma and brain homogenates for all analyte levels except ecgonine at 3 ng/ml. Additional method verification methods and results are presented in the Supporting Information section.

### 2.3.3 | Instrument and acquisition parameters

The HPLC-MS/MS system used in this study was an LC-20ADXR HPLC System (Shimadzu) coupled with an LCMS-8060 triple quadrupole mass spectrometer, CTO-20A column oven, Nexera SIL-30 AC autosampler, and CBM-20A controller (Shimadzu). Extracts (500 nl) were injected onto a Phenomenex Kinetex® Biphenyl HPLC column (2.6 μm, 2.1×150 mm; Phenomenex). Elution was performed with an aqueous solution containing 10 mM ammonium formate with 0.05% formic acid (Thermo) and an organic solution containing neat HPLC methanol (AlfaAesar). The HPLC flow rate was set at 0.5 ml/min. The HPLC column was maintained at 60°C for the duration of analysis. Over the course of 8 min, a gradient profile was used to wash, elute, and equilibrate the column: initial organic condition was set

to 1% to allow the column to wash, from 0.26 to 5.00 min a linear gradient from 25% to 55% organic was applied to elute the analytes, the column was then washed from 1 min at 99% organic and an additional minute at 1% organic to achieve equilibrium (total analysis time: 8 min). The 8060 triple quadrupole mass spectrometer was equipped with a heated electrospray ionization source and operated in positive ionization mode with nitrogen sheath gas. Collision gas was 0.99999 nitrogen. The electrospray source operating conditions were nebulizing gas flow 3 L/min, heating gas flow 10 L/min, interface temperature 300°C, desolvation temperature 526°C, desolvation line (DL) temperature 250°C, heat block temperature 400°C, and drying gas flow rate 10 L/min. Relevant analyte-specific acquisition parameters are listed in Table S1. Data acquisition and instrument control were performed using Labsolutions Realtime Analysis 5.97 (Shimadzu). Data handling were performed using Labsolutions Insight 3.5 (Shimadzu).

## 2.4 | Statistical analysis

Significances for the hydrolysis of cocaine and formation of metabolites were determined using paired $t$-tests comparing the concentrations of analyte at respective time points from the antibody-positive reactions to the respective antibody negative control reaction over time. If the data passed normality (as assessed by a Shapiro–Wilks normality test), a Welch's $t$-test was used. Otherwise, data were compared using a Mann–Whitney $U$ test. To correct for multiple comparisons, a false-discovery rate procedure was applied to all behavioral data. Regression analyses were performed using SigmaPlot (Systat Software, Inc.). Due to catheter failure, one of the mice in the h2E2-treated cohort was eliminated from the experiment, thereby reducing the 0.75 time point group to $n = 2$. All other mice were included ($n = 3$) for each timepoint. In vivo PK data were fit using an intravenous bolus injection into a two-compartment model in Phoenix WinNonlin.

## 3 | RESULTS

### 3.1 | The effects of h2E2 on the in vitro enzymatic hydrolysis of cocaine by liver carboxylesterase I and BChE

h2E2 dramatically decelerated enzyme-mediated and spontaneous cocaine hydrolysis by both BChE and CES1 (Figures 1A,B and 2A,B). In the presence of the h2E2, cocaine concentrations were maintained near 5000 ng/ml throughout the entire sampling periods. Under the conditions of the BChE reactions, 50% of the initial cocaine was hydrolyzed for 38.3 min. There was no detectable cocaine in the BChE reaction after the 8-h timepoint. The addition of h2E2 drastically slowed hydrolysis with only 5% of cocaine hydrolyzed by 38.3 min. Peak EME concentration differed significantly in h2E2-treated reactions (237 ± 1 ng/ml; mean ± SD) compared to controls
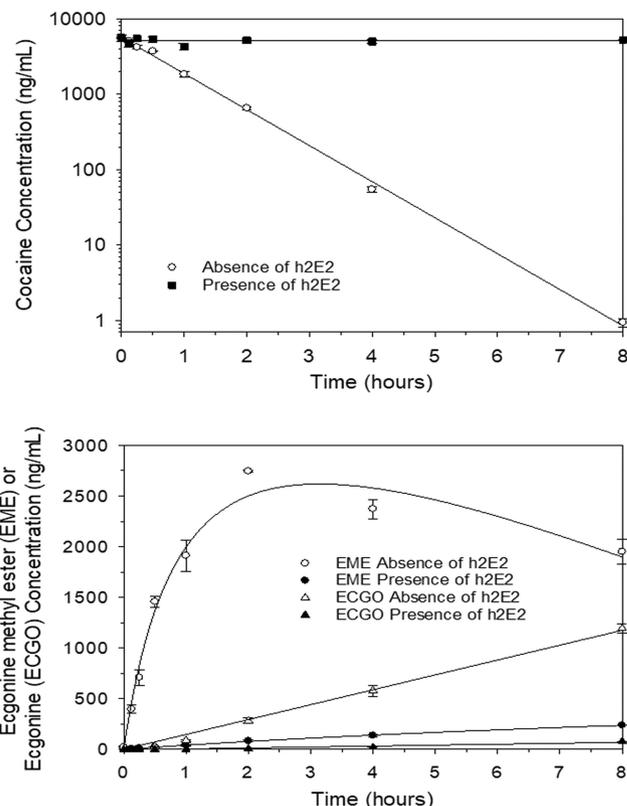


**FIGURE 1** Degradation of cocaine by butyrylcholinesterase (equine) into EME and ecgonine in the presence and absence of h2E2. Cocaine was added in both reaction conditions. Concentrations in ng/ml of cocaine (upper panel, (A)) or EME and ecgonine (lower panel, (B)) are displayed for the 8-h time course following the addition of enzyme. No detectable cocaine was present past 8 h, so the graph was truncated at 8 h. Symbols represent the mean ± SD from three reactions. The line through the data points represents the best fit according to exponential or hyperbolic functions. Cocaine and cocaine metabolite concentrations were measured using liquid chromatography tandem mass spectrometry. The hydrolysis of cocaine is slowed significantly ($p < .05$ Welch's $t$-test) in the presence of the anti-cocaine mAb h2E2 compared to controls. EME, ecgonine methyl ester; h2E2, humanized anticocaine monoclonal antibody.

(2745 ± 35 ng/ml; mean ± SD) (Figure 1A,B). By 120 min, EME concentrations were 97% higher in the absence of h2E2. As EME concentration decreased, ecgonine concentration increased. Relative to EME and ecgonine, minimal BE was formed.

In the CES1 reactions, in the absence of h2E2, the cocaine concentration decreased and a gradual increase in BE concentration through spontaneous and enzyme-mediated hydrolysis was observed. In the absence of h2E2, the reaction half-life was 832 min. In contrast, less than 1% cocaine was hydrolyzed at 832 min in the presence of h2E2. In the CES1 reactions, BE formation was significantly decreased in the presence of h2E2 (peak of 3600 ± 35 ng/ml; mean ± SD) by 92% compared to controls (peak of 279 ± 11 ng/ml; mean ± SD) as detected at the final timepoint (Figure 2A,B). EME concentration was rarely above the limit of detection in the presence of h2E2. Without the addition of h2E2, EME was detected with
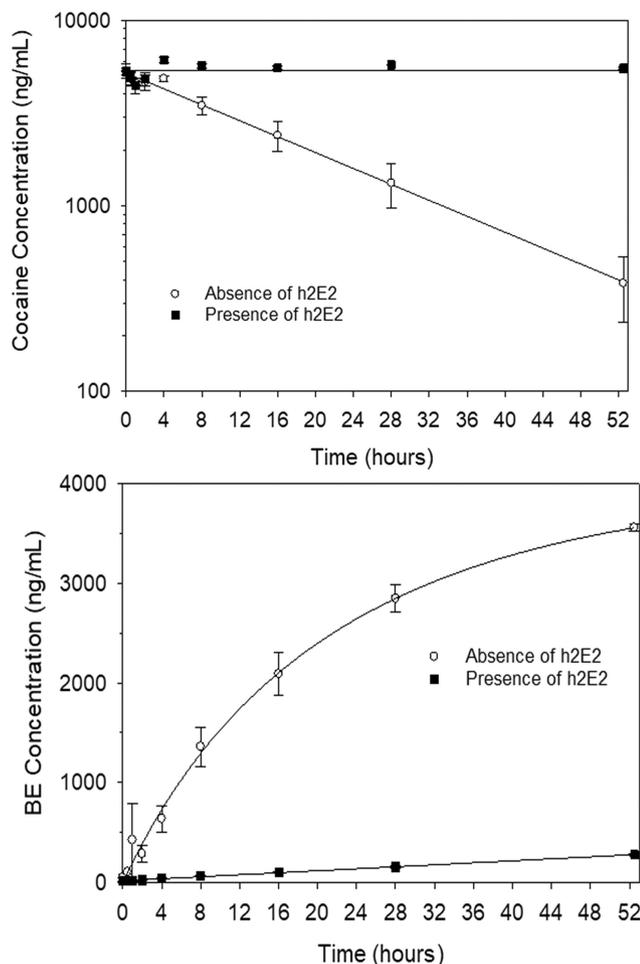
FIGURE 2   Degradation of cocaine by recombinant human carboxylesterase 1 into benzoylecgonine in the presence and absence of h2E2. Cocaine was added in both reaction conditions. Concentrations in ng/ml of cocaine (upper panel, (A)) or benzoylecgonine (lower panel, (B)) are displayed for the 52-h time course following the addition of enzyme. Symbols represent the mean ± SD from three reactions. The line through the data points represents the best fit according to an exponential function. Cocaine and cocaine metabolite concentrations were measured using high performance liquid chromatography tandem mass spectrometry. The hydrolysis of cocaine is slowed significantly ($p < .05$ Welch's $t$-test) in the presence of the anti-cocaine mAb h2E2 compared to controls. h2E2, humanized anticocaine monoclonal antibody.

a peak concentration of $627 \pm 57$ ng/ml; mean ± SD, which was significantly less than the peak BE concentration.

## 3.2  |  In vivo

Both h2E2 and control-treated datasets were readily fit with a two-compartment model. The model generated a distribution half-life of 1.5 min, an elimination half-life of 17 min, and a $V_{dss}$ of 9.1 L/kg of cocaine in the control cohort. In both the control and h2E2 treatment groups, the highest plasma cocaine concentrations were
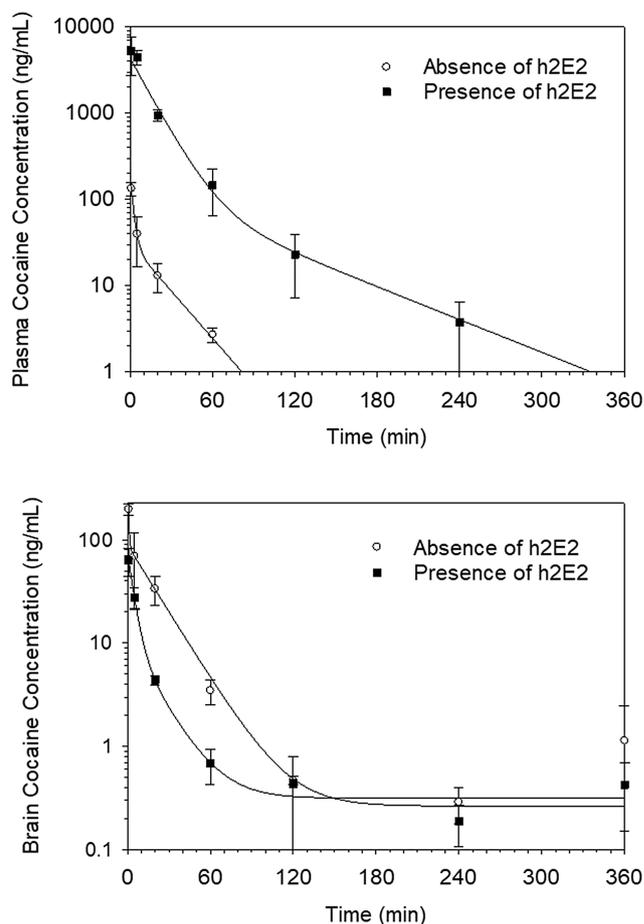
FIGURE 3   Effect of h2E2 on the pharmacokinetics of cocaine in plasma (upper panel, (A)) and brain (lower panel, (B)). Mice received an infusion of 120 mg/kg h2E2. One-hour post-infusion, mice received an equimolar (0.5 mg/kg) intravenous dose of cocaine (0.56 mg/kg cocaine HCl). Cocaine concentrations were determined using high performance liquid chromatography tandem mass spectrometry. Symbols represent the mean ± SD from three mice from all timepoints except for 45 s, where only two mice were included due to a catheter failure. In the absence of the h2E2 (open circles), the cocaine concentration-time profile in the plasma was described by a two-compartment model with a $t_{1/2\alpha}$ of 1.5 min, a $t_{1/2\beta}$ of 17 min, and a $V_{dss}$ of 9.1 L/kg. In the presence of h2E2 (black squares), a two-compartment model indicated a $t_{1/2\alpha}$ of 10 min, a $t_{1/2\beta}$ of 47 min, and a $V_{dss}$ of 0.2 L/kg. h2E2, humanized anticocaine monoclonal antibody.

observed at the first timepoint (45 s). In the absence of h2E2, the mean ± SD plasma cocaine concentration at 45 s post-infusion of cocaine was $134 \pm 20$ ng/ml ($n = 3$). However, pretreatment with h2E2 resulted in a substantial 39-fold increase in the plasma concentrations of cocaine, with a peak concentration of $5255 \pm 359$ ng/ml at 45 s ($n = 2$) (Figure 3A). The model also resolved two distinct phases of cocaine PKs in the presence of h2E2, and h2E2 significantly increased the distribution and elimination half-lives of cocaine relative to non-treated controls (Figure 3A). The model resolved a distribution half-life of 10 min, an elimination half-life of 47 min, and a $V_{dss}$ of 0.2 L/kg of cocaine in the presence of the antibody. Cocaine distribution to the brains of control mice was rapid and the highest
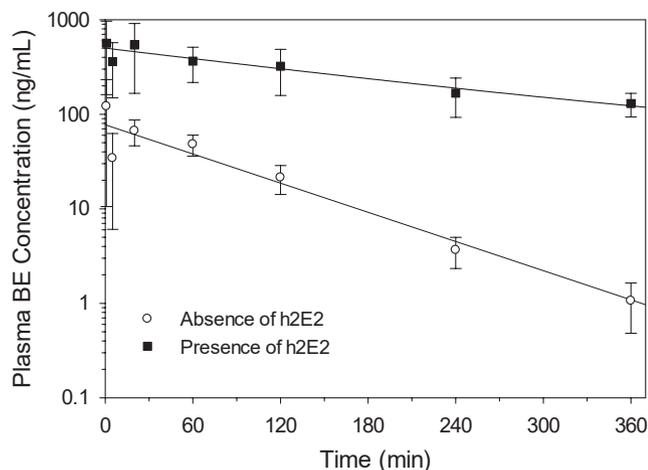
**FIGURE 4** BE concentrations in plasma in the presence and absence of h2E2. Mice received an infusion of 120 mg/kg h2E2 or vehicle. One-hour post-infusion, mice received an equimolar (0.5 mg/kg) intravenous dose of cocaine (0.56 mg/kg cocaine HCl). BE concentrations were determined using high performance liquid chromatography tandem mass spectrometry. Symbols represent the mean ± SD from three mice from all timepoints except for 45 s, where only two mice were included due to a catheter failure. BE, benzoylecgonine; h2E2, humanized anticocaine monoclonal antibody.

cocaine concentration (198 ± 24 ng/ml) was observed at the first timepoint whereas h2E2-treated mice had a threefold lower brain cocaine concentration (64 ± 20 ng/ml) (Figure 3B). After the initial timepoint, both treatment groups saw monotonically decreasing concentrations of cocaine in the brain. Cocaine was not detected above the limit of detection in the brains of the h2E2-treated animals after 20 min (4.4 ng/ml). No metabolites were detected in the brain in either treatment group. As shown in Figure 4, BE was detected in the plasma of h2E2-treated and vehicle-treated mice during the time course. However, in the presence of the antibody, the plasma BE concentrations were 4.6-fold higher than in the control cohort at 45 s. After the initial timepoint, BE was rapidly cleared from the plasma of control mice. In the presence of h2E2, BE was detected above the limit of quantification at all timepoints. Further, the plasma BE concentrations in h2E2-treated mice were at least five-fold higher than the control plasma BE concentration at any time point. EME and ecgonine concentrations were rarely above our limit of quantification throughout the entire time course for both h2E2- and vehicle-treated mice (data not shown).

## 4 | DISCUSSION

We first determined the effects of an equimolar binding site concentration of h2E2 on cocaine hydrolysis in vitro. The presence of h2E2 drastically slows spontaneous hydrolysis as well as enzymatic hydrolysis by liver CES1 and BChE. We hypothesize that the slowed hydrolysis is a consequence of h2E2 decreasing the free cocaine available to bind to the enzyme's active site. The in vitro

data suggested that h2E2 inhibits enzymatic hydrolysis through chemical antagonism. This is consistent with experiments showing that nicotine binding to P-450 enzymes is competitively removed by antibody binding to nicotine.[11] In biologic systems, cocaine may be hydrolyzed by both nonenzymatic and enzymatic mechanisms.[12] The in vitro data show that h2E2 inhibited the formation of EME and BE by enzymatic hydrolysis as well as BE formed as a nonenzymatic hydrolysis product. These experiments were done at physiological temperature and pH, and enzyme concentrations were titrated to have measurable activity with a metabolite profile that would be expected in vivo. While these conditions were different, the difference between the degradation of cocaine in the presence and absence of h2E2 was pronounced. This finding suggests that the in vivo mechanisms of cocaine hydrolysis, and therefore cocaine metabolite formation, would be slowed.

The PKs of h2E2 in mouse plasma have been characterized in detail. PK analysis of the mAb reveals three distinct phases: an initial rapid distribution phase (4.4 min) followed by a longer distribution phase (4.2 h) then a terminal elimination phase (7.8 days).[13] The elimination half-life of cocaine is nearly 300-fold faster than the elimination of the mAb. This suggests that the effects of h2E2 on cocaine PKs could persist for several days. However, previous studies concluded that the terminal elimination half-life of cocaine is unchanged in the presence of 2E2 over 60 min.[3] According to the present extended time course study, the terminal elimination half-life of cocaine is 47 min in the presence of h2E2. The prolonged distribution half-life of cocaine in the presence of h2E2 (10 min) closely resembles the elimination half-life of cocaine in controls (17 min) (Figure 3A). The close resemblance likely caused the two half-lives to be conflated in previous studies which only evaluated these effects up to 60 min. Therefore, we conclude that the initial disappearance of cocaine from the plasma of h2E2-treated mice is best explained by a prolonged distribution phase of the h2E2-bound cocaine. This finding was consistent with a mathematical model of h2E2's effects on cocaine PKs in mice that explained the disappearance of cocaine from the plasma as an extended distribution phase.[6] However, the 47-min elimination half-life in the presence of h2E2 is still not fully explained. The in vivo plasma half-life of cocaine (free and h2E2-bound) was fast considering the results from the in vitro experiments showing minimal enzymatic degradation of cocaine in the presence of h2E2. Given the relatively large volume of distribution of cocaine compared to h2E2, a possible explanation for this observation is the presence of compartments within the body that are inaccessible to the antibody where substantial clearance of cocaine may occur when bound cocaine dissociates from the h2E2 cocaine binding site and some fraction enters these h2E2-inaccessible compartments.

We expect that most of the cocaine in this study was bound to h2E2. Previous studies using mathematical modeling predicted that, following an equimolar dose, the vast majority of cocaine would be bound to h2E2.[6] However, we were unable to experimentally distinguish between bound and unbound cocaine in this study. The binding of h2E2 to cocaine exhibits unusually rapid kon

and koff rates,[6] making quantification of bound versus unbound fractions challenging. If we assume that most of the cocaine is bound to h2E2, antibody binding may also mitigate the effects of cocaine in the periphery, including its potentially toxic effects on the cardiac and skeletal muscles. Additional studies are necessary to determine whether h2E2 ameliorates the peripheral effects of cocaine.

The present study demonstrates that h2E2 dramatically slows the disappearance of cocaine from plasma. Due to the moderate binding affinity of cocaine for BE, the antibody may potentially extend the presence of BE in plasma as well. In fact, increased plasma concentrations of BE were observed, congruent with previous PK studies that evaluated plasma cocaine and metabolite concentrations in the presence and absence of h2E2.[6] It is likely that BE-h2E2 binding accounts for the increased BE concentrations, where any BE formed by metabolism or spontaneous hydrolysis is sequestered in the plasma. This explanation is also consistent with the decrease in the urinary excretion of BE in the presence of h2E2.[7] As the development of immunotherapies for the treatment of substance use disorders progresses, it must be considered that both monoclonal antibodies and polyclonal antibodies produced by active immunization may significantly alter the PKs of their target drugs.

Benzoylecgonine and cocaine urine tests are the primary mechanism of testing for cocaine intake. The clinical effectiveness of h2E2 would likely have been determined by patient urine testing negative for BE and cocaine. In fact, this metric was used in determining the clinical effectiveness of cocaine vaccines.[14] Active immunization was used to induce a polyclonal response, which produced antibodies with varying affinities for cocaine and inconsistent antibody titers between patients.[15] It would therefore be difficult to predict the effect the antibodies generated through immunization would have on cocaine metabolism and excretion. Consequentially, care must be taken when interpreting the results of efficacy studies for these immunotherapies as assessed by cocaine and BE in urine samples.

We also examined cocaine and cocaine metabolite concentrations in mouse brains. Only cocaine was recovered from the brain; no metabolites were present at concentrations greater than our limit of quantification. The h2E2-treated mice demonstrated a monotonically decreasing concentration of cocaine during the entire 8-h time course. Importantly, there was no evidence for increasing brain concentrations over time, which indicates sustained protection of the brain from cocaine despite prolonged peripheral exposure. These data suggest there is no evidence for the re-intoxication, or re-penetration of drug into the brain for hours after antibody administration consistent with observations for phencyclidine (PCP) in the presence of monoclonal anti-PCP antibodies.[16]

In summary, the high affinity humanized anti-cocaine mAb, h2E2, significantly slows the clearance of cocaine while preventing cocaine entry into the brain. At an equimolar binding site concentration in vitro, h2E2 drastically decreased the rate of hydrolysis of cocaine.

The decreased rate of hydrolysis suggested that the apparent disappearance of cocaine from the plasma in h2E2-treated mice could not be explained by metabolism but rather prolonged distribution. Indeed, the findings of the in vivo study confirm that in the presence of h2E2, cocaine's distribution is significantly slowed. Despite this, brain cocaine concentrations remain very low. The findings of this study have useful implications for the pre-clinical development of and clinical trial design for future antibody therapies.

## AUTHOR CONTRIBUTIONS

Participated in research design: Turner, Wetzel, and Norman. Conducted experiments and data collection: Turner, Zinani. Validated analytical methods: Turner, Crutchfield. Performed data analysis: Turner, Wetzel. Supervised the research: Wetzel, Norman. Wrote the manuscript: Turner. All authors revised the draft manuscript.

## ETHICS STATEMENT

These studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals under a protocol approved by the Institutional Animal Care and Use Committee at the College of Medicine, University of Cincinnati (Cincinnati, OH).

## DATA AVAILABILITY

The data reported in this study are available from the corresponding author, Andrew B. Norman, upon reasonable request.

## DISCLOSURE

ABN is a co-inventor on a series of patents on the matter and use of the h2E2 monoclonal antibody.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.