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Plasma and Brain Concentrations of Doxycycline after Single and Repeated Doses in Wild-Type and APP23 Mice[S]

Jacopo Lucchetti, Claudia Fracasso, Claudia Balducci, Alice Passoni, Gianluigi Forloni, Mario Salmona, and Marco Gobbi

Departments of Molecular Biochemistry and Pharmacology (J.L., C.F., M.S., M.G.), Neuroscience (C.B., G.F.), and Environmental Health Science (A.P.), Instituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy

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ABSTRACT

Repurposing doxycycline for the treatment of amyloidosis has recently been put forward because of the antiaggregating and anti-inflammatory properties of the drug. Most of the investigations of the therapeutic potential of doxycycline for neurodegenerative amyloidosis, e.g., prion and Alzheimer disease (AD), have been carried out in mouse models, but surprisingly no data are available regarding the concentrations reached in the brain after systemic administration. We filled this gap by analyzing the pharmacokinetic profile of doxycycline in plasma and brain after single and repeated intraperitoneal injections of 10 and 100 mg/kg, in wild-type mice and the APP23 mouse model of AD. The main outcomes of our study are: 1) Peak plasma concentrations ranged from 2 to10 μg/ml, superimposable to those in humans; 2) brain-to-plasma ratio was ~0.2, comparable to the cerebrospinal fluid/serum ratios in humans; 3) brain C_{max} 4–6 hours after a single dose was ~0.5 (10 mg/kg) and ~5 μM (100 mg/kg). Notably, these concentrations are lower than those required for the drug's antiaggregating properties as observed in cell-free studies, suggesting that other features underlie the positive cognitive effects in AD mice; 4) elimination half-life was shorter than in humans (3–6 vs. 15–30 hours), therefore no significant accumulation was observed in mouse brain following repeated treatments; and 5) there were no differences between doxycycline concentrations in brain areas of age-matched wild-type and APP23 mice. These data are useful for planning preclinical studies with translational validity, and to identify more reliably the mechanism(s) of action underlying the central in vivo effects of doxycycline.

Introduction

Tetracyclines are a well known class of broad-spectrum antibiotics discovered in the late 1940s (Chopra and Roberts, 2001). More recent, extensive evidence suggests other interesting properties of these drugs, including antiapoptotic, anti-inflammatory, and antiaggregating activities (Griffin et al., 2010; Stoilova et al., 2013), which has prompted their repurposing to treat amyloidosis, a group of diseases caused by misfolding and abnormal aggregation of specific proteins that eventually accumulate as insoluble amyloid deposits (Chiti and Dobson, 2006). Doxycycline is a tetracycline derivative widely studied in these conditions.

Doxycycline disrupts transthyretin amyloid in vitro (Cardoso et al., 2003) and in vivo in animal models (Cardoso and Saraiva, 2006); in a phase 2 clinical trial, doxycycline plus tauroursodeoxycholic acid stabilized the disease in the majority of patients with hereditary transthyretin amyloidosis (Obici et al., 2012). In vitro and in vivo preclinical data also showed positive effects of doxycycline on amyloid light chain (AL) amyloidosis (Ward et al., 2011); this was followed by a report that the supplementation with doxycycline of standard chemotherapy in AL patients reduced early cardiac mortality (Wechalekar and Whelan, 2017). Doxycycline also inhibits β2-microglobulin (β2M) fibrillogenesis (Giorgetti et al., 2011), prompting the proposal of "off-label" treatment of a severe form of dialysis-related β2M amyloidosis in three patients who displayed a significant reduction in articular pain (Montagna et al., 2013). The use of doxycycline as an orphan drug for the treatment of hereditary amyloid polyneuropathy caused by β2M was recently approved by the European Committee for Orphan Medicinal Products (http://www.emea.europa.eu/docs/en_GB/document_library/Orphan_designation/2012/05/WC500127736.pdf). Doxycycline might also have therapeutic potential for neurodegenerative amyloidosis, such as in prion diseases and Alzheimer disease (AD), according to in vitro and animal data (Forloni et al., 2001, 2002; De Luigi et al., 2008; Diomede et al., 2010). A recent phase 2 study in Creutzfeldt-Jakob disease (CJD) patients at an early stage demonstrated the superiority of doxycycline over control (Varges et al., 2017), contrasting with the negative results of an earlier study (Haik et al., 2014) carried out however in CJD patients at a very late stage. Contrasting results were also obtained in clinical trials with doxycycline in AD patients (Loeb et al., 2004; Molloy et al., 2013). A preventive clinical trial in patients with fatal familial insomnia, a genetic prion disease, is currently ongoing (Forloni et al., 2015).

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ABBREVIATIONS: AUC, area under the curve; BBB, blood-brain-barrier; CJD, Creutzfeldt-Jakob disease; C_{max}, peak concentration; HCOOH, formic acid; HPLC, high-performance liquid chromatography; IS, internal standard; MS/MS, tandem mass spectrometry; PK, pharmacokinetic(s); $t_{1/2}$, elimination half-life; WT, wild type.

Different variables affect the in vivo activity of doxycycline for treating amyloidosis, including the stage of the disease when treatment starts, and whether the treatment regimen can achieve adequate drug concentrations at the active site (e.g., in the brain for neurodegenerative amyloidosis).

Many of the main pharmacokinetic (PK) features of doxycycline in humans have already been investigated in detail (Cunha et al., 1982; Saivin and Houin, 1988): At the usual oral doses of 100–200 mg/day, it is absorbed rapidly and almost completely, with peak serum concentrations from 1.7 to 5.9 μg/ml 2–3 hours after dosing, and elimination half-life of 15–30 hours (Saivin and Houin, 1988; Binh et al., 2009; Montagna et al., 2013). After long-term administration of 100 mg/day, concentrations at steady-state reach 0.7–1.5 μg/ml (Binh et al., 2009; Montagna et al., 2013). Plasma protein binding of doxycycline is 80%–90% (Cunha et al., 1982; Saivin and Houin, 1988) and there is no significant metabolism (Yim et al., 1985; Saivin and Houin, 1988). Because of its lipophilicity—much higher than that of tetracycline—doxycycline easily penetrates and distributes within body tissues. However, very few data are available on its ability to cross the blood-brain barrier (BBB): In patients with neurosyphilis (Yim et al., 1985) or suspected tick-borne neuroborreliosis (Dotevall and Hagberg, 1989), cerebrospinal fluid concentrations were 15%–25% of serum levels. Brain concentrations of doxycycline were also measured in autopsy samples from late-stage CJD patients given 100 mg daily (Haik et al., 2014): The drug crossed the BBB and persisted in the brain for days after the end of treatment, possibly because of its ability to bind the prion protein aggregates present in the brain of CJD patients.

A more detailed analysis of BBB passage can be obtained in animal models, particularly rodents, which also offer the possibility of correlating a pharmacological effect (e.g., on amyloid load or neuroprotection) with the actual brain concentrations. When planning treatment schedules in animals, one must also take into account that the drug's PK profile varies as a function of body weight (Boxenbaum, 1982).

Very few reports describe the PK of doxycycline in rodents. The half-time of elimination from serum is 3–4 hours (Schach won Wittenau et al., 1972; Bocker et al., 1981), i.e., significantly faster than in humans; the brain-to-plasma concentration ratio was 0.31 at a single time point (4 hours) after intravenous injection of 25 mg/kg in rats (Colovic and Caccia, 2003). To our knowledge, no data are available on BBB passage in mice.

Given the paucity of published data and the importance of this information for planning preclinical studies with translational validity, we carried out ad hoc PK studies in mice, measuring plasma and brain levels of doxycycline after single and repeated intraperitoneal injections. We also did a single-dose study to measure plasma and brain levels of doxycycline in APP23 mice, a transgenic model of AD. The analytical method, employing high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS), was developed and validated according to accepted guidelines.

Materials and Methods

Mice

Three groups of mice were used: 1) 7-week-old, male C57Bl/6 CRI mice (Charles River Laboratories Italia, Calco, Italy), 2) 20-month-old, female and male APP23 transgenic (Tg) mice, and 3) sex- and age-matched wild-type (WT) female and male littermates. Mice were housed three to four per cage at constant room temperature (21 ± 1°C) and relative humidity (60%) with a 12-hour light cycle (lights on 7:00 AM to 7:00 PM) with food and water ad libitum (Global Diet 2018S; Envigo, Somerset, NJ). Procedures involving animals were conducted at the Instituto di Ricerche Farmacologiche "Mario Negri" (IRCCS), which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments which includes ad hoc members for ethical issues (authorization code 1/05-D) (Quality Management System Certificate—UNI EN ISO 9001:2008—Reg. N° 6121). The NIH *Guide for the Care and Use of Laboratory Animals* (2011 edition), and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and expired on September 30, 2019 (Animal Welfare Assurance no. A5023-01).

Drug Treatments

Doxycycline hyclate HCl (MilliporeSigma, St. Louis, MO) was dissolved in sterile 0.9% saline at 10 and 100 mg/ml (free base) and injected intraperitoneally in volumes of 10 ml/kg to have final doses of 10 and 100 mg/kg.

Single Treatment in 7-Week-Old Male C57Bl/6 Mice. Mice weighing 24.4 ± 1.5 g were treated with 10 and 100 mg/kg (36 mice for each dose) and killed by decapitation at 0.5, 1, 2 4, 6, 8, 10, 20, and 24 hours after the injection (4/time point/dose).

Repeated Treatment in 7-Week-Old Male C57Bl/6 Mice. Mice weighing 25.3 ± 1.3 g were given 10 and 100 mg/kg doxycycline (24 mice/dose) once a day (9:00 AM) for four injections (12) or twice a day (17:00 and 9:00) for eight injections (12). Mice were killed just before, or 2 and 6 hours after, the last injection (4 mice/time point).

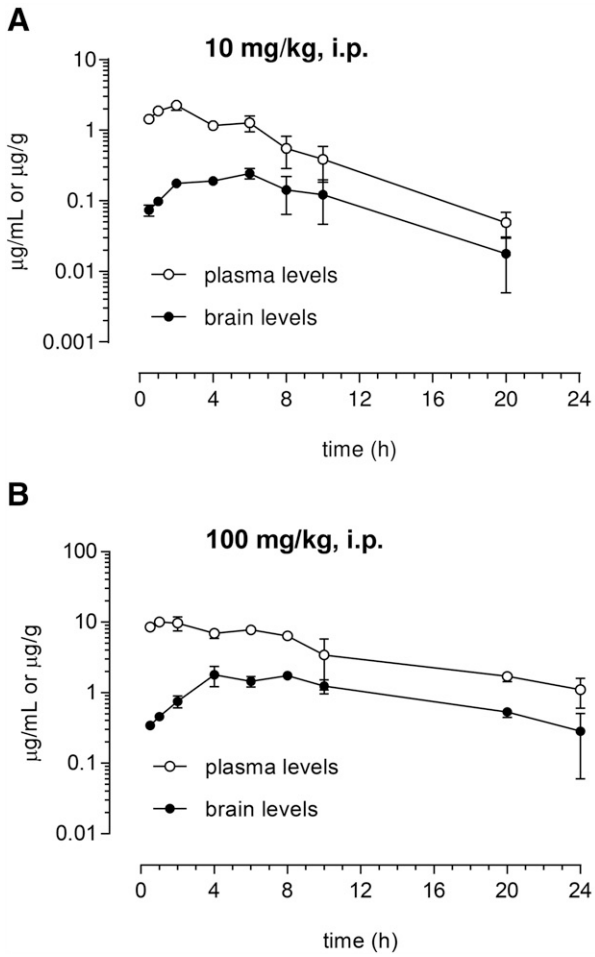
Single Treatment in 20-Month-Old Male and Female WT Mice. Mice weighing 39.8 ± 10.8 g were treated with 100 mg/kg and killed by decapitation at 2, 6, 8, 10, 16, and 24 hours after the injection (n = 3–6/time point).

Repeated Treatments in 20-Month-Old Male and Female APP23 and WT Mice. WT and APP23 mice weighing 33.0 ± 9.8 g were given 100 mg/kg (three for group) once a day for four consecutive days, and killed by decapitation 24 hours after the last injection.

Blood samples were transferred to heparinized tubes and kept in ice until centrifugation at 2000g for 15 minutes (4°C), to obtain plasma that was stored at −80°C until HPLC-MS/MS analysis. Immediately after death brain tissues were removed, dissected into two halves, and kept on dry ice before storage at −80°C. The day of analysis, the brain tissues and dissected cerebral areas were homogenized in phosphate buffer 0.01 M pH 7.4 (1 g in 6 ml) with an Ultra Turrax T10 basic (IKA-Werke GmbH & Co., Staufen, DE) and processed as described below.

Doxycycline Concentrations in Plasma and Brain

The analytical method and procedures for its validation are described fully in the Supplemental Material. Briefly, after addition of internal standard (IS, demeclocycline), 50 μl of plasma or 200 μl brain homogenate were purified by protein precipitation with five volumes of cold acetonitrile (with 0.1% of formic acid), and centrifuged. The supernatants were evaporated under a nitrogen flow, and the residues resuspended in 0.1% HCOOH in water/acetonitrile (98/2, v/v) and injected into the HPLC-MS/MS. Separation was done on a Kinetex EVO C18 column with HCOOH 0.1% in water (mobile phase A, MP-A) and acetonitrile (mobile phase B, MP-B); elution started with 98% of MP-A held for 2 minutes followed by a 10-minute nonlinear gradient (curve 8) to 98% of MP-B. Doxycycline and the IS were acquired in positive multiple reaction mode monitoring the quantitative ion transitions mass-to-charge ratio (m/z) 445.1 → m/z 428.1 (collision



PK parameters	Plasma	Brain
C_{max} (µg/mL or µg/g)	2.26 ± 0.34	0.22 ± 0.02
t_{max} (h)	2	6
$t_{1/2}$ (h)	3.2	3.9
$AUC_{0\text{-}20h}$ (µg*h/mL or µg*h/g)	14.02	2.35
$AUC_{0\text{-}inf}$ (µg*h/mL or µg*h/g)	14.22	2.45
Vz/F (mL/kg)	3283	
Cl/F (mL/h*kg)	703	

PK parameters	Plasma	Brain
C_{max} (µg/mL or µg/g)	10.04 ± 0.91	1.79 ± 0.57
t_{max} (h)	1	4
$t_{1/2}$ (h)	6.5	6.5
$AUC_{0\text{-}24h}$ (µg*h/mL or µg*h/g)	110.22	23.41
$AUC_{0\text{-}inf}$ (µg*h/mL or µg*h/g)	120.55	26.08
Vz/F (mL/kg)	7785	
Cl/F (mL/h*kg)	830	

Fig. 1. Time course of doxycycline concentrations in plasma (white circles) and brain (black circles) of 7-week-old male WT mice injected with (A) 10 or (B) 100 mg/kg, i.p. Each point is the mean ± S.D. of four mice. Tables report the corresponding PK parameters calculated with PKSolver.

energy 20 eV) and *m/z* 465.1 → *m/z* 448.1 (collision energy 15 eV), respectively.

Plasma and brain samples of treated mice were analyzed in parallel with quality control samples (two replicates at three concentrations) and with freshly prepared calibration curves linear in the range 0.1–10 μg/ml for plasma and 60–6000 ng/g for brain.

The GraphPad Prism program (GraphPad Software, Inc. La Jolla, CA), was used for plotting the calibration curves and for quantifying the unknown concentrations of doxycycline in plasma and brain. The same software was used for statistical analysis and graphics. Plasma and brain PK profiles were analyzed using a noncompartmental model for extravascular administration to obtain the main PK parameters. The peak concentration (C_{max}) and the time taken to reach it (T_{max}) were taken directly from the data; elimination half-life ($t_{1/2}$), area under the curve from 0 to the last time point (AUC_{0-t}) and from 0 to infinity (AUC_{0-inf}), apparent volume of distribution (V/F), and apparent clearance (Cl/F) were obtained with PKSolver, a freely available menu-driven add-in program for Microsoft Excel (Zhang et al., 2010).

Identification and Analysis of Metabolites in Plasma and Brain

In vivo formation of doxycycline metabolites was investigated using a high-resolution mass spectrometer (Q Exactive Orbitrap; Thermo Fisher Scientific, Waltham, MA) coupled with a liquid chromatographic system (1200 series; Agilent Technologies, Santa Clara, CA). Extraction procedure from biologic matrices and HPLC-MS/MS analysis is described in the Supplemental Material.

Results

HPLC-MS/MS Method Validation

We developed an HPLC-MS/MS method and validated it following EMA guidelines (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf).

Analysis of plasma and brain homogenates spiked with different amounts of doxycycline (respectively, 0.1–10 μg/ml and 60–6000 ng/g) allowed evaluation of the linearity of response and limits of quantifications (0.1 μg/ml and 60 ng/g), the selectivity (i.e., the ability of the method to distinguish doxycycline and the IS from other components of the biologic matrices), the recovery and matrix effects, accuracy and precision, carryover, and analyte stability in the different experimental conditions. All these validation data are detailed in the Supplemental Material.

Pharmacokinetic Studies

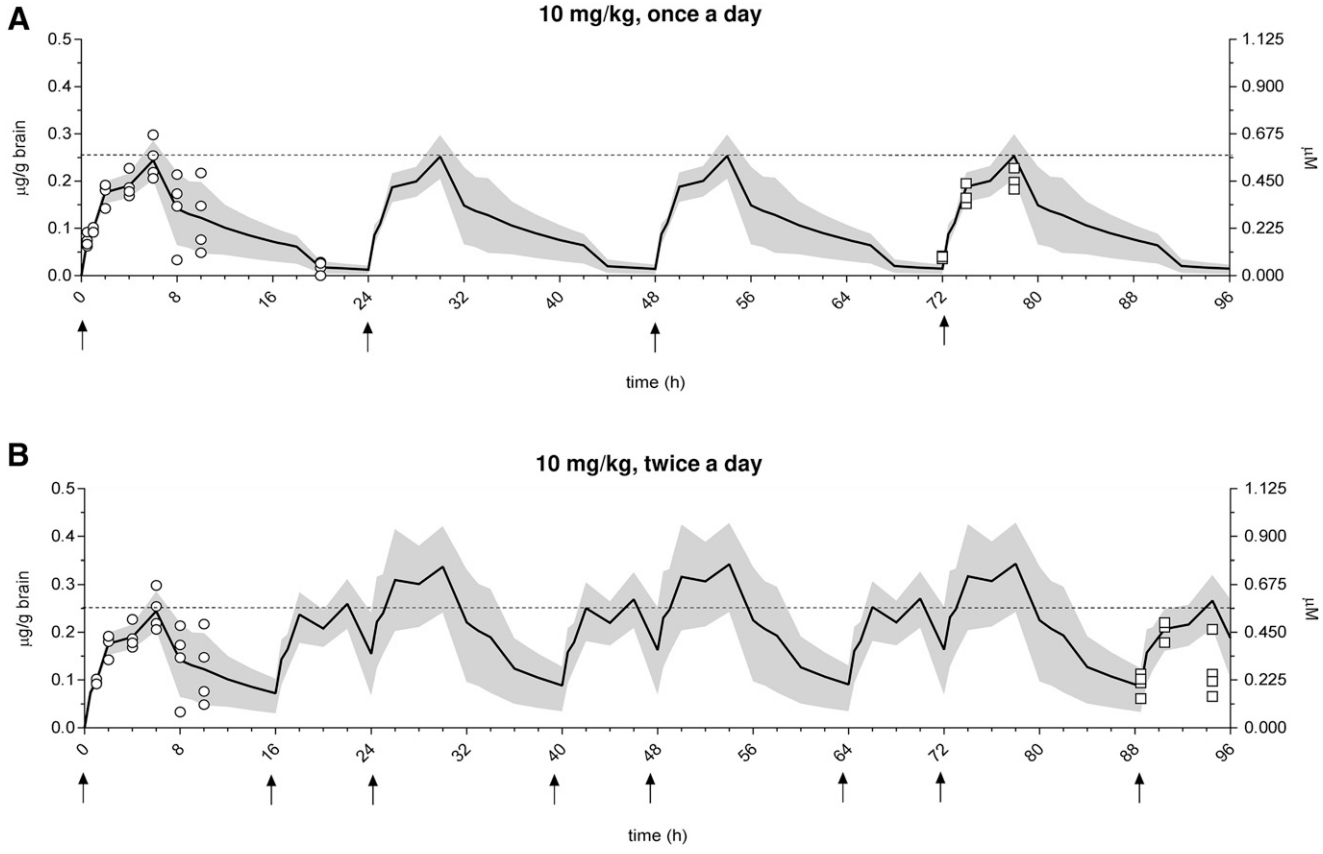
Single Treatment in 7-Week-Old C57Bl/6 Mice. Figure 1 shows the pharmacokinetic profile of doxycycline in plasma and brain of male C57Bl/6 mice, at different times after a single i.p. dose of 10 or 100 mg/kg. After the lowest dose (Fig. 1A), plasma concentration (C_{max}) of 2.26 ± 0.34 μg/ml (mean ± S.D., four mice) peaked at 2 hours. The terminal phase had a half-life of 3.2 hours; concentrations were not detectable at

24 hours. $AUC_{0–20h}$ and $AUC_{0–inf}$ were similar. The analysis of brain tissues (Fig. 1A) indicated a rapid distribution of doxycycline, with quantifiable levels 30 minutes after the dose; the C_{max} (0.22 ± 0.04 μg/g) was reached at 6 hours after the treatment. Brain doxycycline declined with a $t_{1/2}$ of 3.9 hours. $AUC_{0–24\ hours}$ and $AUC_{0–inf}$ were also closed. The ratio of $AUC_{0–inf\ brain}$ to $AUC_{0–inf\ plasma}$ was 0.17.

After the highest dose plasma C_{max} was reached at 1 hour (Fig. 1B), the terminal half-life calculated on the last three points was 6.5 hours. In the brain, C_{max} was reached after 4 hours and concentrations declined then with a $t_{1/2}$ of 6.5 hours. At this dose, the ratio of $AUC_{0–inf\ brain}$ to $AUC_{0–inf\ plasma}$ was 0.22. The ratio of $AUC_{0–inf}$ (100 mg/kg) to $AUC_{0–inf}$ (10 mg/kg) was 8.5 in plasma and 10.6 in brain tissue, indicating dose-proportionality.

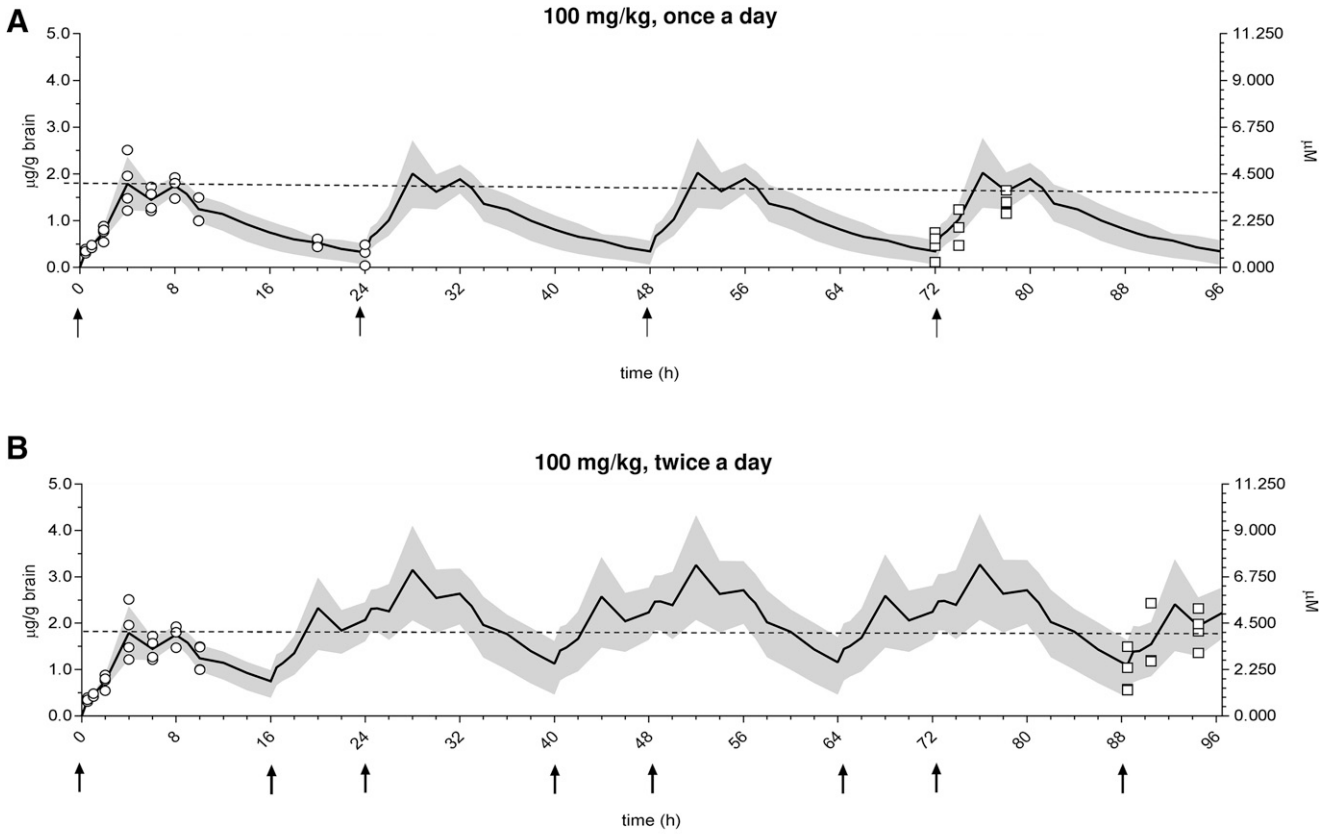
Repeated Treatment in 7-Week-Old C57Bl/6 Mice. Data after a single intraperitoneal injection were used to simulate the expected PK profiles after repeated doses of 10 and 100 mg/kg doxycycline, once or twice a day. These simulations are shown in Fig. 2 (10 mg/kg) and Fig. 3 (100 mg/kg) for brain and in Supplemental Figures 4 and 5 for plasma, with their variability (as S.D.). We considered these simulations useful 1) to visually show that the fast elimination of doxycycline does not allow significant accumulation of the drug during repeated treatment, with the partial exception of the higher dose twice a day, and 2) to verify experimentally that the concentrations after repeated administrations are as expected, thus excluding possible changes in the PK profile during chronic treatment (e.g., in absorption or elimination mechanisms).

Figures 2 and 3 show that brain doxycycline concentrations measured just before, or 2 and 6 hours after, the last of four once-a-day injections or eight twice-a-day injections, are mostly within the expected values, and not significantly different from the concentrations after single injections, although we noticed a slight accumulation after eight twice-a-day injections with both doses. Plasma doxycycline



Time after last dose (h)	Single dose	Multiple doses, once a day (4 injections)		Multiple doses , twice a day (8 injections)	
	µg/g (mean ± SD)	*µg/g (mean ± SD)*	*P value*	*µg/g (mean ± SD)*	*P value*
2	0.176 ± 0.023	0.141 ± 0.062	0.6857	0.203 ± 0.022	0.4000
6	0.244 ± 0.041	0.203 ± 0.023	0.2286	0.120 ± 0.061	0.0571

Fig. 2. Experimental data and simulation of the time course of doxycycline levels in the brain after repeated doses of doxycycline 10 mg/kg, once (A) or twice (B) a day in 7-week-old male WT mice. Empty symbols are the experimental values measured after single injections (circles, from Fig. 1), or after repeated injections (squares). Solid lines represent the PK profile simulated with the single-dose data; the gray area is the S.D., the dotted line the mean C_{max} after the single injection and arrows indicate the injection time. The table shows the doxycycline brain concentrations and the *P* value for the difference from the single dose (Mann Whitney test).



Time after last dose (h)	Single dose	Multiple doses, once a day (4 injections)		Multiple doses , twice a day (8 injections)	
	µg/g (mean ± SD)	*µg/g (mean ± SD)*	*P value*	*µg/g (mean ± SD)*	*P value*
2	0.750 ± 0.145	0.857 ± 0.383	0.8571	1.604 ± 0.719	0.0571
6	1.447 ± 0.244	1.396 ± 0.204	0.8857	1.874 ± 0.400	0.1143

Fig. 3. Experimental data and simulation of the time course of doxycycline levels in the brain after repeated doses of doxycycline 100 mg/kg, once (A) or twice (B) a day in 7-week-old male WT mice. Empty symbols are the experimental values measured after single injections (circles, from Fig. 1), or after repeated injections (squares). Solid lines represent the PK profile simulated with the single-dose data; the gray area is the S.D., the dotted line the mean C_{max} after the single injection and arrows indicate the injection time. The table shows the doxycycline brain concentrations and the *P* value for the difference from the single dose (Mann Whitney test).

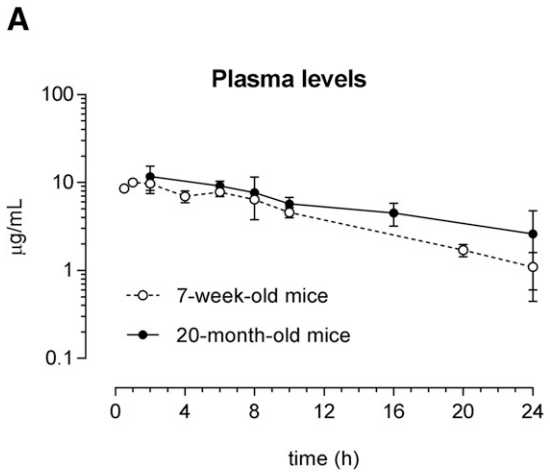
concentrations after repeated treatment were mostly in line with expected values (Supplemental Figures 4 and 5).

Single Treatment in 20-Month-Old C57Bl/6 Mice

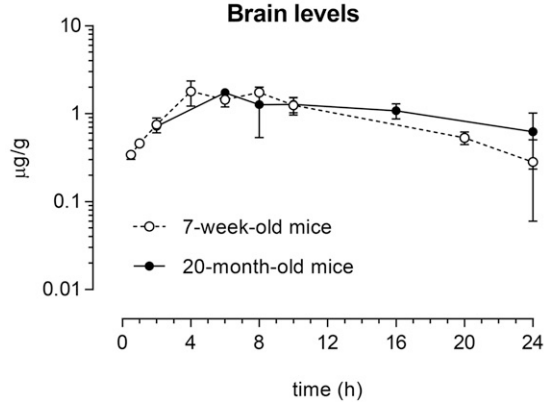
Figure 4 shows the PK profile of doxycycline in plasma and brain of 20-month-old C57Bl/6 mice at different times after a single i.p. dose of 100 mg/kg. The aim was to gain PK information for the best design of studies in age-matched APP23 mice; thus, this analysis included both male (n = 12) and female (n = 10) mice, comparably distributed at the different time points. No clear-cut differences were noted (data not shown), so the values for the two genders were combined.

The time courses of plasma and brain levels in these 20-month-old mice were superimposable on those of younger mice, except at the longest time points (Fig. 4). Analysis of the last four points gave $t_{1/2}$ of 11.1 (plasma) and 15.5 hours (brain), about double those in younger mice. The absolute Cl/F were comparable, whereas the absolute apparent volume of distribution during terminal phase after nonintravenous injection (Vz/F) was higher in older mice (332 ml vs. 180 ml in younger mice). The brain-to-plasma ratio ($AUC_{0-inf\ brain}/AUC_{0-inf\ plasma}$) was about 20% for both ages.

Repeated Treatment in 20-Month-Old APP23 Mice. On the basis of the data in 20-month-old WT mice, doxycycline levels were measured 24 hours after the fourth i.p. injection (1/day) of 100 mg/kg in three APP23 mice (two females and one male) and three sex- and age-matched WT mice, in several brains areas: cortex, hippocampus, cerebellum, striatum, and the "rest of the brain." Doxycycline levels were measured at this time point on the basis of the PK profile observed in age-matched mice treated once (Fig. 4); in fact, distribution equilibrium is more probably achieved at the end of the dosing interval and, therefore, a trough value allows a better estimation of the degree of accumulation than C_{max} (Rowland and Tozer, 2011). Moreover, mice were treated with the highest doxycycline dose (100 mg/kg)



Plasma PK parameters	7-week-old mice	20-month-old mice
Weight (g)	24.4 ± 1.5	39.8 ± 10.8
mean dose (mg/mice)	2.44	3.98
k_{el} (1/h)	0.107	0.063
$t_{1/2}$ (h)	6.5	11.1
$AUC_{0\text{-}24h}$ (µg*h/mL)	110.22	142.8
$AUC_{0\text{-}inf}$ (µg*h/mL)	120.55	184.4
Vz/F (mL)	190.0	344.5
weight-adjusted Vz/F (mL/kg)	7785	8655
Cl/F (mL/h)	20.3	21.6
weight-adjusted Cl/F (mL/h*kg)	830	542



Brain PK parameters	7-week-old mice	20-month-old mice
Weight (g)	24.4 ± 1.5	39.8 ± 10.8
mean dose (mg/mice)	2.44	3.98
k_{el} (1/h)	0.106	0.045
$t_{1/2}$ (h)	6.5	15.4
$AUC_{0\text{-}24h}$ (µg*h/g)	23.41	25.12
$AUC_{0\text{-}inf}$ (µg*h/g)	26.08	38.96
Brain $AUC_{0\text{-}inf}$ / Plasma $AUC_{0\text{-}inf}$	0.22	0.21

Fig. 4. Time course of doxycycline concentrations in (A) plasma and (B) brain measured in 20-month-old WT mice (black circles and solid lines) injected once with 100 mg/kg, i.p. Each point is the mean ± S.D. of three to six mice (both males and females). Tables report the corresponding PK parameters calculated with PKSolver. For comparison, the time course of doxycycline concentrations (white circles and dotted line), are reported with the corresponding PK parameters in 7-week-old male WT mice (from Fig. 1).

to have clearly measurable levels after 24 hours. Since preliminary studies in old WT mice showed some toxicity at this dose twice a day, we decided to treat APP23 mice once a day.

Brain levels of doxycycline were similar in WT and APP23 mice in all the regions considered (Table 1).

Identification and Analysis of In Vivo Doxycycline Metabolite. Since previous data suggested minimal metabolism of doxycycline (Bocker, 1983), initial analyses were carried out in a pool of plasma and brain samples obtained from the young WT mice chronically treated with the highest dose of doxycycline. *N*-demethylated doxycycline was the only metabolite identified in plasma (Supplemental Figure 6), whereas no metabolites were found in the brain samples. Subsequent semiquantitative analysis in all the plasma samples of the mice treated with 100 mg/kg doxycycline (all time points after single or repeated doses, in both young and old WT mice) showed that *N*-demethylated metabolite never exceed 5% of the parent drug (Supplemental Figure 7) (more details can be found in the Supplemental Material).

Discussion

Preclinical studies are often limited by inadequate evaluation of whether the drug dose actually achieves plasma and/or tissue (e.g., brain) concentrations with translational validity (meaning, that result in a similar exposure), and are

TABLE 1

Doxycycline levels in cortex (CTX), hippocampus (HIPP), cerebellum (CB), striatum (STR), and in the "rest of the brain" (ROB) of WT and APP23 mice 24 hours after the last of four daily intraperitoneal injections (mean ± S.D. of three mice; two females and one male per group)

Two-way analysis of variance indicated no significant overall difference between WT and APP23 mice and multiple comparison test indicated no significant differences for the single brain areas.

Brain Area	WT Mice	APP23 Mice
	µg/g	*µg/g*
CTX	0.359 ± 0.227	0.365 ± 0.287
HIPP	0.707 ± 0.391	0.870 ± 0.719
CB	0.499 ± 0.263	0.685 ± 0.564
STR	0.482 ± 0.322	0.470 ± 0.360
ROB	0.519 ± 0.451	0.447 ± 0.320

compatible with the desired pharmacological effect. We reassessed the PK parameters of doxycycline in mice with the main aim of providing the information needed to fine-tune preclinical studies in mouse models of neurodegenerative amyloidosis.

The peak plasma concentrations of doxycycline in the 7-week-old male C57BL76 mice, observed 1–2 hours after single intraperitoneal injections of 10 or 100 mg/kg, were 2–10 μg/ml, superimposable with those in humans taking the usual oral doses of 100–200 mg [1.7–5.9 μg/ml; (Saivin and Houin, 1988; Binh et al., 2009; Montagna et al., 2013)]. This is consistent with empirical allometric extrapolations (McCann and Ricaurte, 2001), following the principle of interspecies drug dose scaling, which suggests that a dose of 100 mg in humans should correspond approximately to a dose of 15 mg/kg in mice. A nonsignificant increase in drug concentrations appeared 6 hours after the treatment with both 10 and 100 mg/kg, in comparison with the concentrations measured after 4 hours. The presence of a second peak may be consistent with the enterohepatic recycling previously described for doxycycline in mice (Bocker at al., 1981) and tetracycline in rat (Adir, 1975); a second peak had also been reported in the plasma of patients treated with doxycycline (Fabre et al., 1966; Pedersen and Miller, 1980; Malmborg, 1984). We also found that the AUC for doxycycline in mice was proportional to the dose administered, at least within the 10–100 mg/kg range.

A slower $t_{1/2}$ was observed after treatment with 100 mg/kg than after 10 mg/kg (6.5 vs. 3.2 hours, respectively). Since this is accompanied by a less-than-proportional C_{max} (10 vs. 2 μg/ml with 100 and 10 mg/kg), we suggest that at the highest dose absorption became a limiting factor, a condition in which the terminal half-life also reflects rate and extent of absorption and not just the elimination process (Toutain and Bousquet-Mélou, 2004). However, plasma clearance was similar for the two doses.

There was a remarkable difference, however, in the half-time of elimination from plasma, which is 15–30 hours in humans (Saivin and Houin, 1988; Binh et al., 2009; Montagna et al., 2013) but significantly shorter in young mice [3–6 hours, in line with previous data (Schach won Wittenau et al., 1972; Bocker et al., 1981)]. Our old mice had an intermediate value of 11 hours (this is discussed below). These results have implications for treatment schedules, since a single daily dose may be justified in humans but not for mice. Doxycycline metabolism appeared negligible, confirming previous data (Bocker, 1983). In fact, plasma levels of *N*-demethyl-doxycycline, the only detectable metabolite, never exceed 5% of the levels of the parent drug; no metabolites were detected in brain samples of doxycycline-treated mice.

Mean maximum brain concentrations of 0.24 and 1.79 μg doxycycline/g of brain were reached within 4–6 hours after 10 and 100 mg/kg doxycycline. Interestingly, the values in mice are comparable to those in autopsy samples from CJD patients chronically treated with 100 mg/day: In individuals who received the last dose within 24 hours of death, levels were 0.6–3.0 μg/g (Haik et al., 2014).

The present study describes for the first time the brain-to-plasma ratio of doxycycline in mice, which was about 0.2 (calculated on the AUC values), after either 10 or 100 mg/kg, in both young and old mice. Assuming that cerebral blood accounts for 2% of total blood (Edvinsson et al., 1973; Modak et al., 1978; Chugh et al., 2009), it follows that brain levels cannot be accounted for by the residual cerebral blood. This value is very similar to the cerebrospinal fluid/serum ratios in patients with neurosyphilis (Yim et al., 1985) or suspected tick-borne neuroborreliosis (Dotevall and Hagberg, 1989). Assuming a mean plasma protein binding of 80%–95% (Saivin and Houin, 1988; Riond and Riviere, 1989, 1990; Riond et al., 1990; Davis et al., 2006), the concentration gradient of doxycycline—i.e., the ratio of brain to free plasma concentration—is 1 to 2 with both doses, in agreement with that measured in rats (Colovic and Caccia, 2003), dogs (Barza et al., 1975), and humans (Yim et al., 1985; Dotevall and Hagberg, 1989).

The mean elimination half-times from the brain of young mice (3.9 and 6.5 hours, after 10 and 100 mg/kg) were identical to those in plasma. This fast elimination suggests that no significant drug accumulation can be expected after repeated doses, even twice a day, as can be visually appreciated in the simulations shown in Figs. 2 and 3. Accordingly, the brain concentrations after 4-day treatment with 10 or 100 mg/kg were not significantly different from those after a single dose. These data show that maximal brain concentrations (C_{max}) approaching 7 μM on average can only be reached after 100 mg/kg doxycycline twice a day, whereas one dose a day gives up to 5 μM. After repeated doses of 10 mg/kg, either once or twice a day, C_{max} remains at ~0.5 μM.

Elimination was slower from both plasma and brain of 20-month-old mice (11.1 and 15.4 hours, respectively); these data led us to estimate that brain concentrations up to 10 μM might be reached after repeated doses (data not shown). Analysis of PK data also indicated greater exposure to the drug in older mice owing to the higher apparent volume of distribution as a consequence of the different weight (i.e., more adipose tissue), and this is a common observation for lipophilic drugs like doxycycline (Hanley et al., 2010).

Finally, doxycycline levels were similar in brain areas of 20-month-old WT and APP23 mice 24 hours after the fourth intraperitoneal injection, indicating no significant changes in BBB passage of the drug in AD mice.

The new details on doxycycline pharmacokinetics in the present study must be taken into account for fine tuning in vivo animal studies. In addition, the drug concentrations reached in the brain under specific treatment conditions may help identify the mechanism(s) of action of the central in vivo effects of doxycycline in mice. For example, it was recently shown (Balducci et al., 2018) that memory deficits in APP/PS1Tg mice are significantly rescued by single or repeated intraperitoneal doses of 10 mg/kg doxycycline, which results in brain concentrations always lower than 1 μM, i.e., lower than the concentrations required for antiaggregating effects in vitro ($\geq$10 μM) (Forloni et al., 2001, 2002; Cardoso et al., 2003; Giorgetti et al., 2011; Ward et al., 2011; De Luigi et al., 2015; Gonzalez-Lizarraga et al., 2017). This suggests that other properties of doxycycline might be at play, in accordance with the drug's pleiotropic activities (Stoilova et al., 2013). In fact, the positive cognitive effects of doxycycline in AD mice were not associated with any reduction of the Aβ plaque load, but there was a significant normalization effect on glial cells, whose activation in the AD mouse brain contributes to the memory impairment (Balducci et al., 2017), thus highlighting an important contribution of the drug's anti-inflammatory effects (Balducci et al., 2018). It has also been shown that the inhibitory effect of doxycycline on poly (ADP-ribose)

polymerase-1 (PARP-1), involved in microglial activation, inflammation, and cell death (Kauppinen and Swanson, 2005), also induced by Aβ (Kauppinen et al., 2011), occurs at submicromolar concentrations (Alano et al., 2006), consistently with the concentrations actually measured in the brain of our mice.

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Authorship Contributions

Participated in research design: Lucchetti, Fracasso, Balducci, Forloni, Salmona, Gobbi.

Conducted experiments: Lucchetti, Fracasso, Passoni, Balducci.

Performed data analysis: Lucchetti, Fracasso, Gobbi.

Wrote or contributed to the writing of the manuscript: Lucchetti, Gobbi.

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Address correspondence to: Dr. Jacopo Lucchetti, Laboratory of Pharmacodynamics and Pharmacokinetics, Department of Molecular Biochemistry and Pharmacology, Instituto di Ricerche Farmacologiche Mario Negri IRCCS, via La Masa 19, 20156 Milan, Italy. E-mail: jacopo.lucchetti@marionegri.it; or Dr. Marco Gobbi, Laboratory of Pharmacodynamics and Pharmacokinetics, Department of Molecular Biochemistry and Pharmacology, Instituto di Ricerche Farmacologiche Mario Negri IRCCS, via La Masa 19, 20156 Milan, Italy. E-mail: marco.gobbi@marionegri.it

Supplemental Data Files

Plasma and brain concentrations of doxycycline after single and repeated doses in wild-type and APP23 mice

Jacopo Lucchetti, Claudia Fracasso, Claudia Balducci, Alice Passoni, Gianluigi Forloni, Mario Salmona and Marco Gobbi

Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy (J.L., C.F., M.S., M.G.); Department of Neuroscience, IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy (C.B., G.F.); Department of Environmental Health Science (A.P.)

Address correspondence to:

- Jacopo Lucchetti, Laboratory of Pharmacodynamics and Pharmacokinetics, Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via La Masa 19, 20156 Milan, Italy. Telephone: +39 02 3901 4461. E-mail: jacopo.lucchetti@marionegri.it

- Marco Gobbi, Laboratory of Pharmacodynamics and Pharmacokinetics, Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via La Masa 19, 20156 Milan, Italy. Telephone: +39 02 3901 4570. E-mail: marco.gobbi@marionegri.it

Contents

1) Analytical method for doxycycline concentrations in plasma and brain

Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH) and formic acid (HCOOH) were from Sigma-Aldrich Co. (Milan, Italy); all solvents were of liquid chromatography-mass spectrometry (LC-MS) grade. LC-MS grade water was obtained in-house with a Milli-Q system (Millipore, Bedford, MA, USA). Doxycycline hyclate powder used for the *in vivo* treatments was purchased from Sigma Aldrich (St Louis, MO, USA) and stored at -20°C. The internal standard (IS) demeclocycline, was obtained from Sigma Aldrich (St Louis, MO, USA).

Stock and working solutions

A stock solution of doxycycline reference standard was prepared in MeOH at a concentration of 1 mg/mL. The stock solution was diluted in MeOH to obtain seven working solutions at 0.5, 1.25, 2.5, 5, 12.5, 25.0 and 50.0 µg/mL (for the calibration curve). Four quality control (QC) working solutions at 0.5, 1.25, 12.5 and 37.5 µg/mL were used to prepare QC samples for method validation and sample analysis. An internal standard stock solution was also prepared in MeOH at the concentration of 1 mg/mL, and then diluted to 5 µg/mL in the same solvent. Stock and working solutions were stored at -20°C.

Biological sample preparation

Seven-point calibration curves were generated by spiking 50 µL of control plasma or 200 µL brain homogenate (1g in 6 mL of PBS 1x pH 7.4) with 10 µL or 4 µL, respectively, of doxycycline working solutions, to final concentrations in the range of 0.1-10 µg/mL for plasma and 0.06–6 µg/g for brain. QC samples were prepared the same way to final concentrations of 0.10 µg/mL or 0.06 µg/g (lower quality control, LLQC), 0.25 µg/mL or 0.15 µg/g (low quality control, LQC), 2.5 µg/mL or 1.5 µg/g (mid-quality control, MQC) and 7.5 µg/mL or 4.5 µg/g (high quality control, HQC).
After the addition of 10 µL (plasma) or 4 µL (brain homogenate) of IS working solution (final concentration in the biological matrices 1 µg/mL or 0.6 µg/g) the samples were mixed with 250 µL (plasma) or 1000 µL (brain homogenate) of cold ACN (with 1% HCOOH) and vortexed; then the mixtures were centrifuged at 4°C for 10 min at 13,000g. The supernatants were recovered and dried under a nitrogen flow; the residues were re-suspended in 400 µL (plasma) or 250 µL (brain) of HCOOH 0.1% in water / ACN (98/2, v/v) and 30 µL were injected into the HPLC-MS/MS system.

Quantitative HPLC-ESI-TripleQ method

Mass spectrometric analyses were done using multiple reaction monitoring (MRM) mode, measuring the fragmentation products of the protonated pseudo-molecular ions of doxycycline and the IS. The fragmentation products for doxycycline and IS and the optimization of collision-induced dissociation

energies and other instrumental parameters were selected in continuous-flow mode, using standard solutions at 1 ng/µL in 0.1 % HCOOH in water / ACN (50:50, v/v) in a Quattro Micro API triple quadrupole instrument (Waters Corp., Manchester, UK). The mass spectrometer was equipped with an electrospray ionization source (ESI) operating in positive ion mode. The mass transitions for MRM acquisition of doxycycline were *m/z* 445.1 → *m/z* 428.1 (quantitative ion transition) and *m/z* 445.1 → *m/z* 154.1 (qualitative ion transition) with collision energies of 20 eV and 30 eV, respectively. For the IS, the quantification ion transition was *m/z* 465.1 → *m/z* 448.1 (collision energy 15 eV) and the qualitative ion transition *m/z* 465.1 → *m/z* 154.1 (collision energy 25 eV). The optimized mass spectrometric parameters for capillary, cone, extractor and RF lens voltages were respectively 3.0 kV, 25 V, 3.0 V and 0.1 V. The source temperature and desolvation temperature were 120 and 420°C. The desolvation and cone gas flows were 600 and 60 L/h. Argon was used as collision gas.

Chromatographic separation was done on an Alliance 2695 (Waters Corp.) using a Kinetex EVO C18 column (150 × 2.1 mm, 5 µm particle size; Phenomenex Inc. USA) at 30°C, with SecurityGuard™ ULTRA cartridges EVOC18 (Phenomenex Inc.). The elution solvents used were 0.1% HCOOH in water (mobile phase A, MP-A) and 0.1% HCOOH in acetonitrile (mobile phase B, MP-B). The injection volume was 30 µL and the flow rate 400 µL/min. The auto-sampler temperature was maintained at 6°C. Elution started with 98% of MP-A and 2% MP-B for 2 min, followed by a 10-min non-linear gradient (curve 8) to 98% of MP-B which was maintained for 2 min; thereafter, a 1-minutes linear gradient bring to 98% of MP-A that was maintained for 5 min to equilibrate the column. The total run time was 20 min. Retention times for doxycycline and IS were 10.8 min and 10.1 min, respectively. The HPLC-MS/MS system was controlled by the MassLynx® version 4.1 (Waters Corp.) and data were collected with the same software.

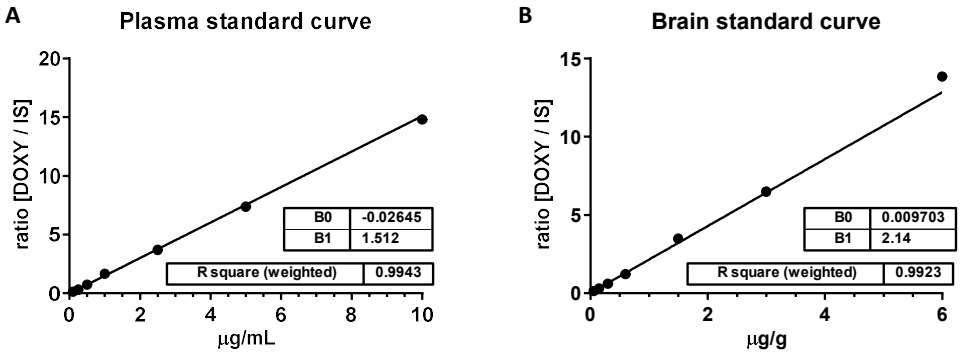
Method validation

The HPLC-MS/MS method was validated in accordance with EMA guidelines for bioanalytical method validation. For all tests, accuracy and precision were evaluated. Accuracy was determined by expressing the calculated concentration as a percentage of the nominal concentration and had to be within 15% of the nominal value for each concentration (±20% for the LLOQ as an exception). Precision, expressed by the CV (%), had not to exceed 15% for all concentrations (20% for the LLOQ as an exception). A calibration curve was analyzed in each validation run.

Linearity, LLOQ and selectivity

All calibration curves analyzed throughout the validation process included one blank plasma or brain homogenate sample , one “zero” sample (blank plasma or brain homogenate with only IS) and seven-point calibration standards at 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 µg/mL (plasma) or 0.06, 0.15, 0.3, 0.6, 1.5, 3.0, and 6.0 µg/g (brain). Responses, expressed as the peak area ratio of the analyte to IS, were plotted against the

corresponding drug concentration and the data were fitted with a weighted ($1/x^2$) linear regression curve (Supplemental Figure 1).



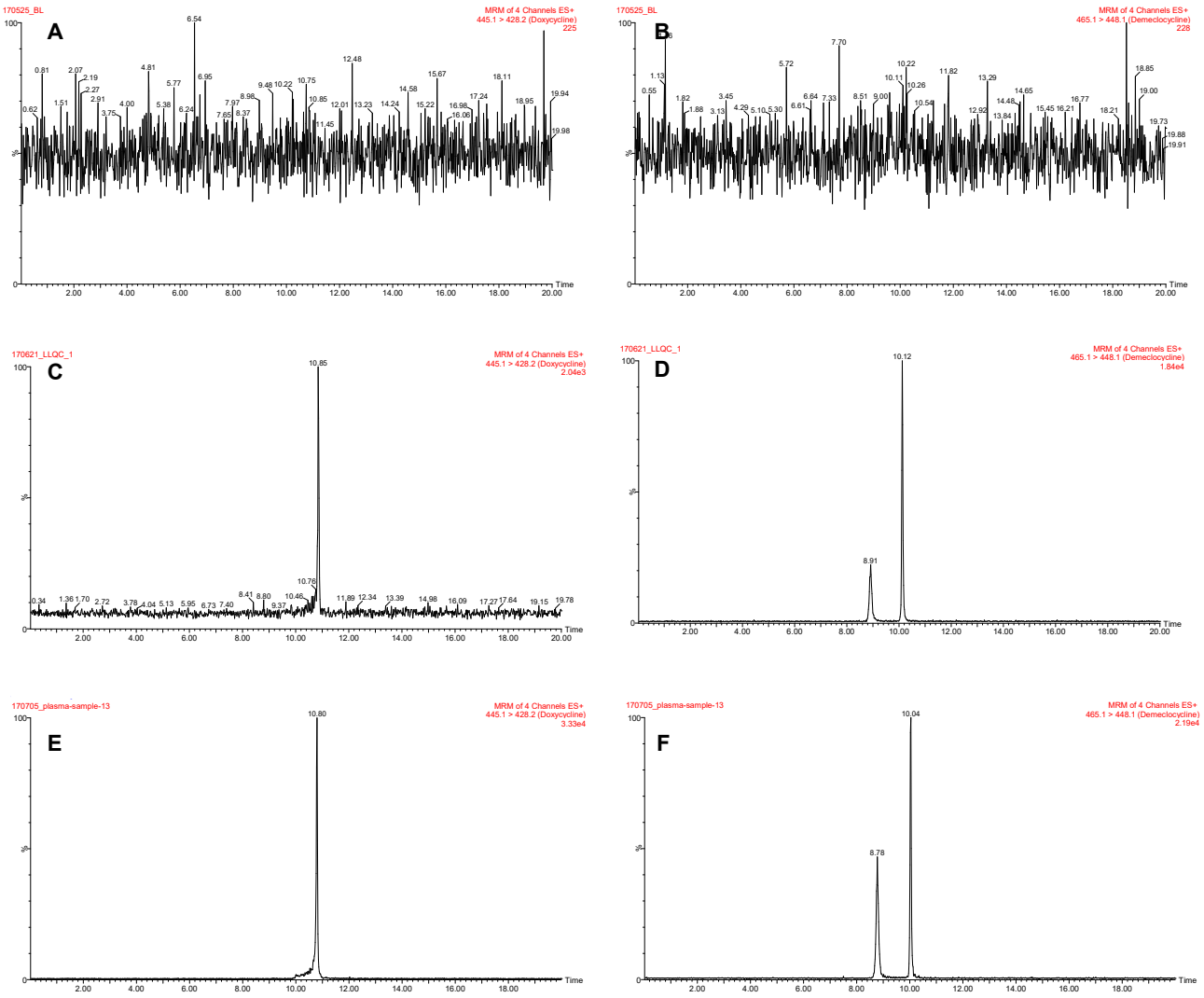
Supplemental Figure 1. Indicative calibration curves of doxycycline in (A) plasma (0.1-10 µg/mL) and (B) brain (0.06-6 µg/g).

All calibration curves analyzed during method validation (5) had highly reproducible slopes, with average determination coefficients (r^2) of 0.9920 and 0.9934 in plasma and brain homogenate, respectively; the accuracy of the back-calculated concentrations was within the acceptance limits (Supplemental Table 1).

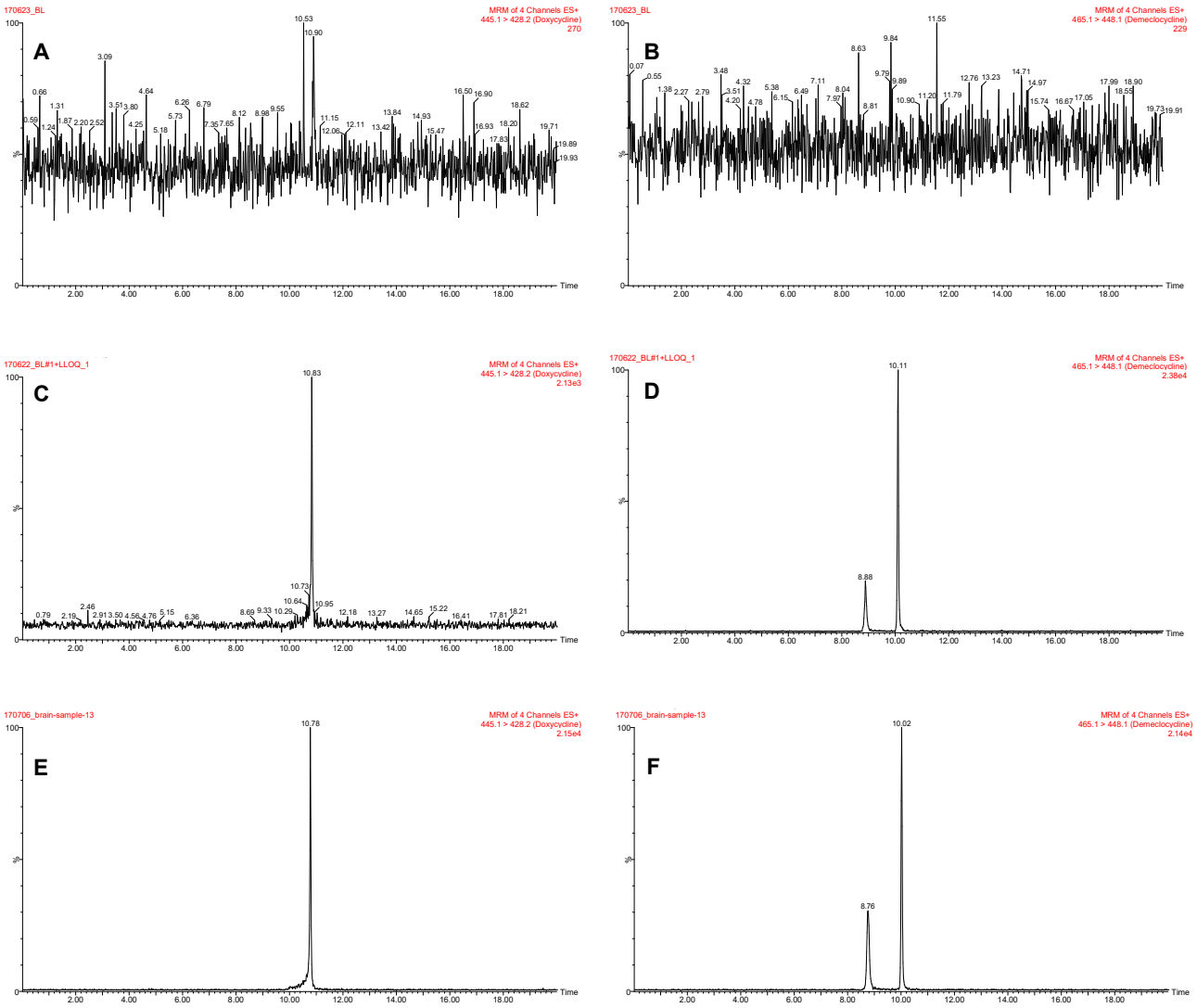
Supplemental Table 1. Linearity and LLOQ (0.1 µg/mL or 0.06 µg/g) of doxycycline in plasma and brain

	day	Nominal concentration (µg/mL)							Calibration curve		
		0.100	0.250	0.500	1.000	2.500	5.000	10.000	slope	intercept	r^2
		Calculated concentration (µg/mL									
PLASMA	day 1	0.099	0.252	*	1.153	2.401	4.637	9.668	1.5341	0.086	0.992
	day 2	0.100	0.230	0.542	1.149	2.544	4.801	8.675	1.2649	0.085	0.988
	day 3	0.101	0.226	0.575	*	2.473	4.837	9.790	1.3654	0.083	0.991
	day 4	0.098	0.251	0.545	1.061	2.424	4.920	9.151	1.4892	0.091	0.995
	day 5	0.103	0.224	0.511	1.110	2.464	4.900	9.809	1.5118	-0.026	0.994
	Mean (µg/mL)	0.100	0.237	0.543	1.118	2.461	4.819	9.419	1.4331		0.9920
	±SD (µg/mL)	0.002	0.014	0.026	0.043	0.055	0.112	0.494	0.1145		0.0030
	Accuracy (%)	100.0%	94.6%	108.6%	111.8%	98.4%	96.4%	94.2%			
	Precision (CV%)	2.0%	5.8%	4.8%	3.8%	2.2%	2.3%	5.2%	8.0%		
	day	Nominal concentration (µg/g)							Calibration curve		
		0.060	0.150	0.300	0.600	1.500	3.000	6.000	slope	intercept	r^2
		Calculated concentration (µg/g)									
BRAIN	day 1	0.062	0.147	0.262	0.637	1.488	2.879	6.641	1.8812	0.026	0.992
	day 2	0.062	0.147	0.266	0.578	1.630	3.057	6.193	1.6285	-0.002	0.995
	day 3	0.060	0.154	0.277	0.579	1.556	3.036	6.193	1.6489	-0.002	0.998
	day 4	0.063	0.136	0.278	0.564	1.629	3.029	6.470	2.1399	0.010	0.992
	day 5	0.063	0.135	0.282	0.566	1.599	2.902	6.788	1.5675	0.220	0.990
	Mean (µg/g)	0.062	0.144	0.273	0.585	1.580	2.980	6.457	1.7732		0.9934
	±SD (µg/g)	0.001	0.008	0.008	0.030	0.060	0.083	0.266	0.2371		0.0028
	Accuracy (%)	103.5%	95.8%	91.0%	97.4%	105.4%	99.3%	107.6%			
	Precision (CV%)	1.9%	5.6%	3.0%	5.1%	3.8%	2.8%	4.1%	13.4%		

The lower limit of quantification (LLOQ) was set at 0.1 µg/mL and 0.06 µg/g after confirmation that its signal was at least five times the signal of the blank sample; selectivity of the method was confirmed in six different matrices with and without fortification with doxycycline at the LLOQ and IS at the concentration used (Supplemental Figure 2).



Supplemental Figure 2. Extracted HPLC-MS/MS chromatograms in plasma of i) indicative a blank matrix (panels A and B), ii) doxycycline spiked in a blank matrix at the LLOQ (0.1 µg/mL) (panels C and D) and iii) mice injected i.p. with 100 mg/kg of doxycycline and killed 2h later (panels E and F). IS was spiked at 1 µg/mL.



Supplemental Figure 3. Extracted HPLC-MS/MS chromatograms in brain homogenate of i) indicative blank matrix (panels A and B), ii) doxycycline spiked in a blank matrix at the LLOQ (0.06 µg/g) (panels C and D) and iii) mice injected i.p. with 100 mg/kg of doxycycline and killed 2h later (panels E and F). IS was spiked at 0.6 µg/g.

Carry-over

Carry-over was investigated by injecting doxycycline at the highest concentration (upper limit of quantification, ULOQ, 10 µg/mL or 6.0 µg/g) following by repeated injections of blank plasma or brain homogenate. Using a needle-wash solution composed of MeOH:ACN:2-Prop:H_2O (1:1:1:1 v/v) with HCOOH 1%, carry-over was negligible (<20% of the LLOQ signal).

Accuracy and precision

Intra-day accuracy and precision were calculated by analyzing in a single run six replicates of QC at each concentration (0.1, 0.25, 2.5 and 7.5 µg/mL or 0.06, 0.15, 1.5 and 4.5 µg/g) (Supplemental Table 2); inter-day accuracy and precision were obtained by analysis of six replicates of each QC (10.1, 0.25, 2.5 and 7.5 µg/mL or 0.06, 0.15, 1.5 and 4.5 µg/g) over three separate days (Supplemental Table 3). Accuracy of the method was within the range 89.1-100.8% (plasma) and 96.4-102.2% (brain) in the same analytical run (intra-day), and 95.1-97.9% (plasma) and 100.0-107.2% (brain) over different days (inter-day). Precision was in the range 1.5-6.6% (plasma) and 4.6-6.8% (brain) in the same analytical run (intra-day) and 7.1-8.9% (plasma) and 4.3-7.8% (brain) over different days (inter-day).

Supplemental Table 2. Intra-day accuracy and precision of doxycycline

	QCs nominal concentration (µg/mL)	QCs calculated concentration (µg/mL)						mean (µg/mL)	±SD (µg/mL)	Accuracy (%)	Precision (CV%)
PLASMA	0.100	0.108	0.099	0.099	0.107	0.101	0.090	0.101	0.007	100.8%	6.6%
	0.250	0.252	0.229	0.239	0.256	0.265	0.230	0.245	0.015	98.1%	6.0%
	2.500	2.248	2.196	2.252	*	2.257	2.190	2.229	0.033	89.1%	1.5%
	7.500	6.721	7.125	7.304	7.411	7.002	7.463	7.171	0.280	95.6%	3.9%
	QCs nominal concentration (µg/g)	**QCs calculated concentration (µg/g)**						**mean (µg/g)**	**±SD (µg/g)**	**Accuracy (%)**	**Precision (CV%)**
BRAIN	0.060	0.064	0.065	0.061	0.058	0.061	0.058	0.061	0.003	102.1%	4.9%
	0.150	0.154	0.152	0.141	0.136	0.138	0.147	0.145	0.007	96.4%	5.1%
	1.500	1.488	1.628	1.497	*	1.456	1.572	1.528	0.070	101.9%	4.6%
	4.500	4.371	4.621	4.939	4.745	4.810	4.099	4.598	0.311	102.2%	6.8%

* excluded

Supplemental Table 3. Inter-day accuracy and precision of doxycycline

	QC nominal concentration (µg/mL)	QC calculated concentration (µg/mL)						Mean (µg/mL)	±SD (µg/mL)	Accuracy (%)	Precision (CV%)
PLASMA	0.100	0.108	0.099	0.088	0.098	0.093	0.085	0.095	0.008	95.1%	8.9%
	0.250	0.252	0.229	*	0.263	0.223	0.254	0.244	0.017	97.7%	7.1%
	2.500	2.758	2.599	2.362	2.529	2.248	2.196	2.449	0.217	97.9%	8.9%
	7.500	6.721	7.125	7.944	8.169	6.585	7.192	7.289	0.641	97.2%	8.8%
	QC nominal concentration (µg/g)	**QC calculated concentration (µg/g)**						**Mean (µg/g)**	**±SD (µg/g)**	**Accuracy (%)**	**Precision (CV%)**
BRAIN	0.060	0.064	0.065	0.058	0.063	0.072	*	0.064	0.005	107.2%	7.8%
	0.150	0.154	0.152	0.161	0.162	0.138	0.159	0.154	0.009	102.8%	5.8%
	1.500	1.488	1.628	1.665	*	1.590	1.556	1.585	0.068	105.7%	4.3%
	4.500	4.371	4.621	4.148	4.386	4.796	4.681	4.501	0.240	100.0%	5.3%

* excluded

Matrix factor and recovery

Matrix factor (MF, as a numerical measurement of the matrix effect) was evaluated in six different matrices analyzing the QC samples at three concentrations (0.25, 2.5 and 7.5 µg/mL or 0.15, 1.5 and 4.5 µg/g). MF was calculated as the ratio of (A) the peak area of the doxycycline added to the biological matrix after extraction, to (B) the peak area of the analyte without the matrix (pure solution of the analyte) (MF = A/B). The IS-normalized MF was obtained by dividing the MF of the analyte by the MF of the IS, then calculating the CV% at each concentration. As shown in Table S4, The IS-normalized MF is reliable across the concentrations in the six matrices tested, with CV% in the range 4.4-6.5% (plasma) and 2.1-14.7% (brain).

Supplemental Table 4. Doxycycline recovery and matrix effect in plasma and brain calculated on six different matrices

PLASMA												
QC levels (µg/mL)	Doxycycline recovery (%)			IS recovery (%)			Doxycycline MF			IS-normalized MF		
	mean	±SD	CV	mean	±SD	CV	mean	±SD	CV	mean	±SD	CV
0.25 (6)	69.8%	2.1%	3.0%				0.971	0.044	4.5%	1.469	0.096	6.5%
2.50 (6)	74.8%	3.5%	4.6%	68.4%	4.1%	6.0%	0.971	0.027	2.7%	1.508	0.079	5.2%
7.50 (6)	74.2%	3.3%	4.4%				0.930	0.030	3.2%	1.508	0.067	4.4%
BRAIN												
QC levels (µg/g)	Doxycycline recovery (%)			IS recovery (%)			Doxycycline MF			IS-normalized MF		
	mean	±SD	CV	mean	±SD	CV	mean	±SD	CV	mean	±SD	CV
0.15 (6)	92.2%	11.8%	12.8%				0.921	0.113	12.3%	1.083	0.159	14.7%
1.50 (6)	97.9%	11.5%	11.8%	72.1%	7.0%	9.7%	0.832	0.067	8.1%	1.013	0.022	2.1%
4.50 (6)	85.7%	5.1%	6.0%				0.832	0.062	7.4%	1.015	0.062	6.1%

The extraction efficiency of the method (recovery) was determined by comparing (C) the peak area of doxycycline spiked to matrix samples before extraction and (A) the peak area of the analyte spiked to the matrix after extraction. Recovery of doxycycline (Rec% = C/A*100) was calculated in plasma and brain homogenate at three concentrations (0.25, 2.5 and 7.5 µg/mL or 0.15, 1.5 and 4.5 µg/g) for each of the six different matrices tested, and gave reproducible values within the range 85.7-97.9% (plasma) and 91.2-92.7% (brain) (Table S4). The same was done for the IS at the concentration of used: recovery was, in average, 68.4% (plasma) and 72.1% (brain homogenate).

Analyte stability

The stability of doxycycline in plasma or brain homogenate was evaluated at two concentrations (0.25 and 7.5 µg/mL or 0.15 and 4.5 µg/g) under four conditions: i) bench-top stability (2h at room temperature), ii) long-term storage stability (two weeks at -20°C), iii) stability after two freeze-thaw cycles, and iv) auto-sampler stability (48h at 6°C) (Supplemental Table 5). All stability data, in plasma and brain, were consistent with the requirements indicated in the EMA guidelines, since the accuracy and precision of tested QCs were always <15%.

Supplemental Table 5. Stability of doxycycline in plasma and brain under different conditions.

PLASMA								
Stability conditions	**LQC - 0.250 µg/mL**				**HQC - 7.500 µg/mL**			
	Mean (µg/mL)	**±SD (µg/mL)**	**Deviation (%)**	**Precision (CV%)**	**Mean (µg/mL)**	**±SD (µg/mL)**	**Deviation (%)**	**Precision (CV%)**
Freshly prepared (t=0) (6) [a]	0.245	0.015	-1.9%	6.0%	7.171	0.258	-4.4%	3.6%
Bench-top (4h RT) (6) [b]	0.236	0.019	-3.6%	8.2%	6.945	0.087	-3.2%	1.3%
Freeze and thaw 2nd cycle (6) [b]	0.231	0.009	-5.7%	4.1%	6.770	0.115	-5.6%	1.7%
Autosampler (6°C) 24h (6) [c]	0.229	0.014	-1.1%	6.2%	6.661	0.247	-1.6%	3.7%
Autosampler (6°C) 48h (6) [c]	0.244	0.022	5.7%	9.1%	6.994	0.236	3.3%	3.4%
BRAIN								
Stability conditions	**LQC - 0.150 µg/g**				**HQC - 4.500 µg/g**			
	Mean (µg/g)	**±SD (µg/g)**	**Deviation (%)**	**Precision (CV%)**	**Mean (µg/g)**	**±SD (µg/g)**	**Deviation (%)**	**Precision (CV%)**
Freshly prepared (t=0) (6) [a]	146.25	6.98	-2.5%	4.8%	4598.09	242.09	2.2%	5.3%
Bench-top (4h RT) (6) [b]	130.40	12.72	-10.8%	9.8%	4550.82	178.63	-1.0%	3.9%
Freeze and thaw 2nd cycle (6) [b]	136.94	7.14	-6.4%	5.2%	4662.01	199.28	1.4%	4.3%
Autosampler (6°C) 24h (6) [c]	142.95	19.76	4.4%	13.8%	4820.54	273.62	3.4%	5.7%
Autosampler (6°C) 48h (6) [c]	136.94	16.50	0.0%	12.1%	5066.60	102.01	8.7%	2.0%

[a] Deviation (%) was calculated by comparison with the nominal concentration

[b] Deviation (%) was calculated by comparison with the concentration calculated in “freshly prepared” samples

[c] Deviation (%) was calculated by comparison with the concentration calculated in “freeze-thaw 2nd cycle” samples

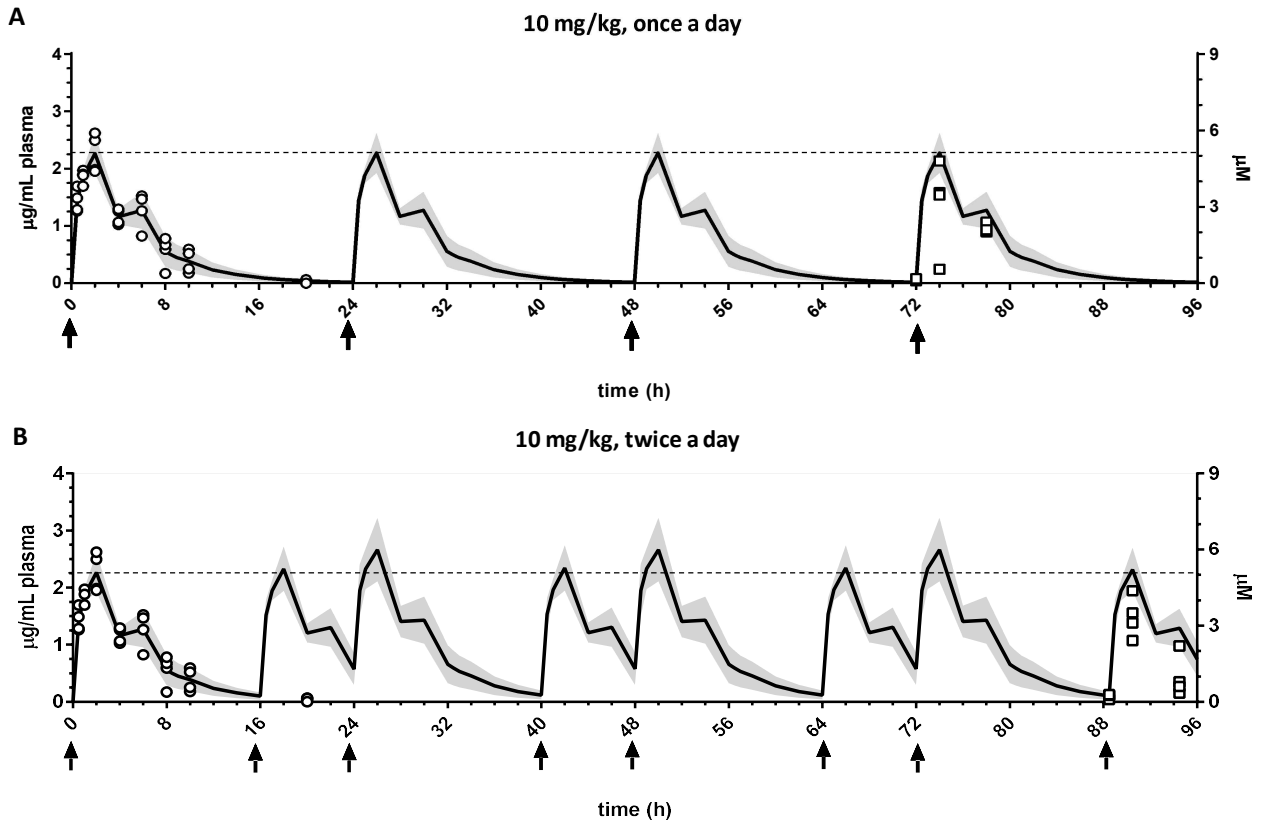
We also confirmed the stability of stock solutions of doxycycline and IS in the storage condition (-20°C for one week) (Supplemental Table 6).

We also tested the stability of the working solutions of both substances at room temperature for 2 hours (bench-top) and in the storage condition (-20°C for one week), investigating six replicates of the lowest and highest calibration standards (for doxycycline) and the IS (Supplemental Table 6). The data indicated degradation of doxycycline in the working solution of the lowest calibration standard (0.5 µg/mL) at the storage condition. This means working solutions of doxycycline should be prepared freshly for each day of analysis.

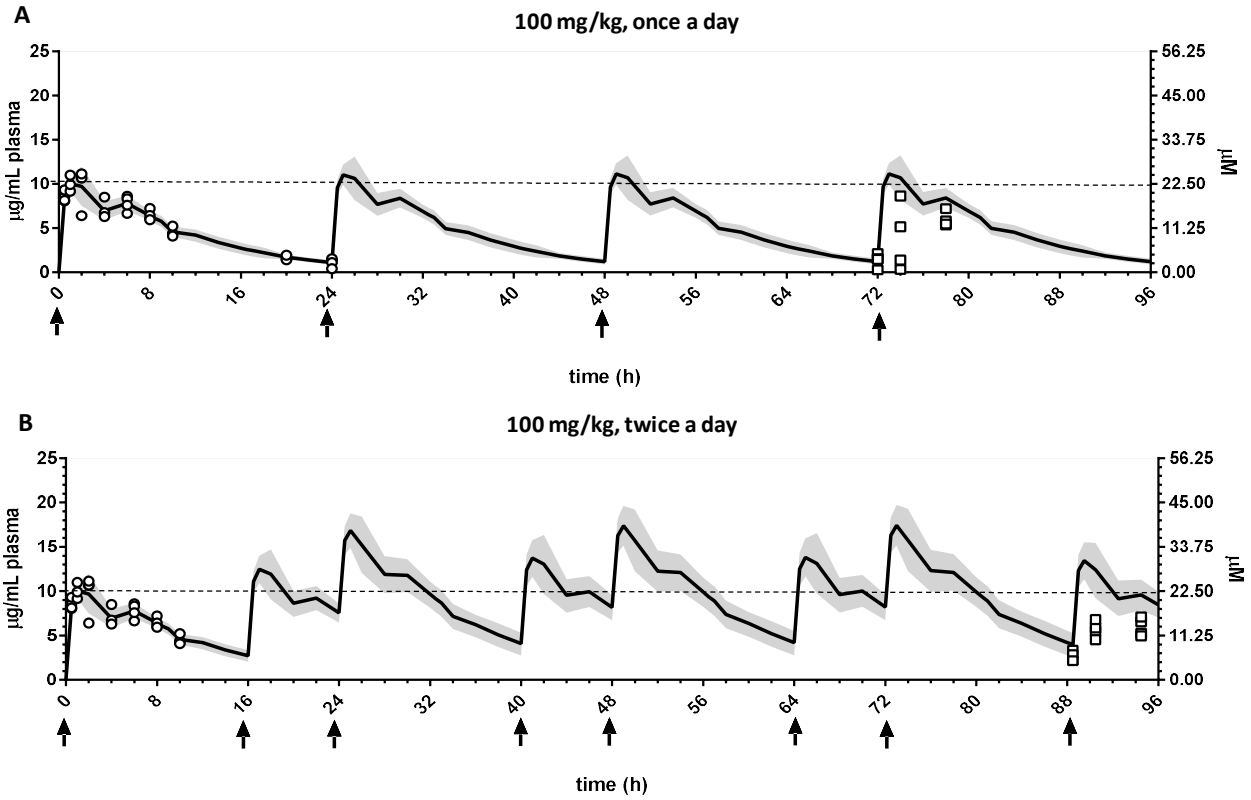
Supplemental Table 6. Stability of doxycycline (DOXY) and IS working solution

Stability of stock and working solution							
Tested solutions	**t = 0**			**t = 1 week at -20°C**			
	Mean peak area	**±SD**	**Precision (CV)**	**Mean peak area**	**±SD**	**Deviation from t=0**	**Precision (CV)**
DOXY stock solution 1 mg/mL diluted 1:100 (6)	6044.20	369.33	6.1%	5895.34	181.63	-2.5%	3.1%
DOXY working solution 0.5 µg/mL (6)	215.47	10.01	4.6%	141.88	12.71	**-34.2%**	3.6%
DOXY working solution 50 µg/mL (6)	34190.28	1280.15	3.7%	33903.24	1164.04	-0.8%	3.4%
IS stock solution 1mg/mL diluted 1:100 (6)	2777.53	53.25	1.9%	2826.33	92.81	1.8%	3.3%
IS working solution 10 µg/mL (6)	3234.37	96.15	3.0%	3470.89	37.41	7.3%	1.1%

3) Plasma levels after repeated treatment in 7-week-old male C57Bl/6 mice.



Supplemental Figure 4. Experimental data and simulation of the time-course of doxycycline levels in plasma after repeated doses of 10 mg/kg, once **(A)** or twice **(B)** a day in 7-week-old male WT mice. Empty symbols are the experimental values measured after single injections (circles, from Fig. 1), or repeated injections (squares). Solid lines represent the PK profile simulated with the single-dose data; grey area is the SD; dotted line the mean C_{max} reached after the single injection and arrows indicate injection time.



Supplemental Figure 5. Experimental data and simulation of the time-course of doxycycline levels in plasma after repeated doses of 100 mg/kg, once **(A)** or twice **(B)** a day in 7-week-old male WT mice. Empty symbols are the experimental values measured after single injections (circles, from Fig. 1), or repeated injections (squares). Solid lines represent the PK profile simulated with the single-dose data; grey area is the SD; dotted line the mean C_{max} reached after the single injection and arrows indicate injection time.

3) Identification and semi-quantitative analysis of doxycycline metabolites

Biological sample preparation

After the addition of 10 µL (plasma) or 4 µL (brain homogenate) of IS working solution to 50µL plasma or 200 µL brain homogenate (final concentration in the biological matrices 1 µg/mL or 0.6 µg/g), the samples were mixed with 250 µL (plasma) or 1000 µL (brain homogenate) of cold ACN (with 1% HCOOH) and vortexed; then the mixtures were centrifuged at 4°C for 10 min at 13,000g. The supernatants were recovered and dried under a nitrogen flow; the residues were re-suspended in 100 µL of HCOOH 0.1% in water / ACN (98/2, v/v) and 4 µL were analysed using high-resolution mass spectrometry (HRMS).

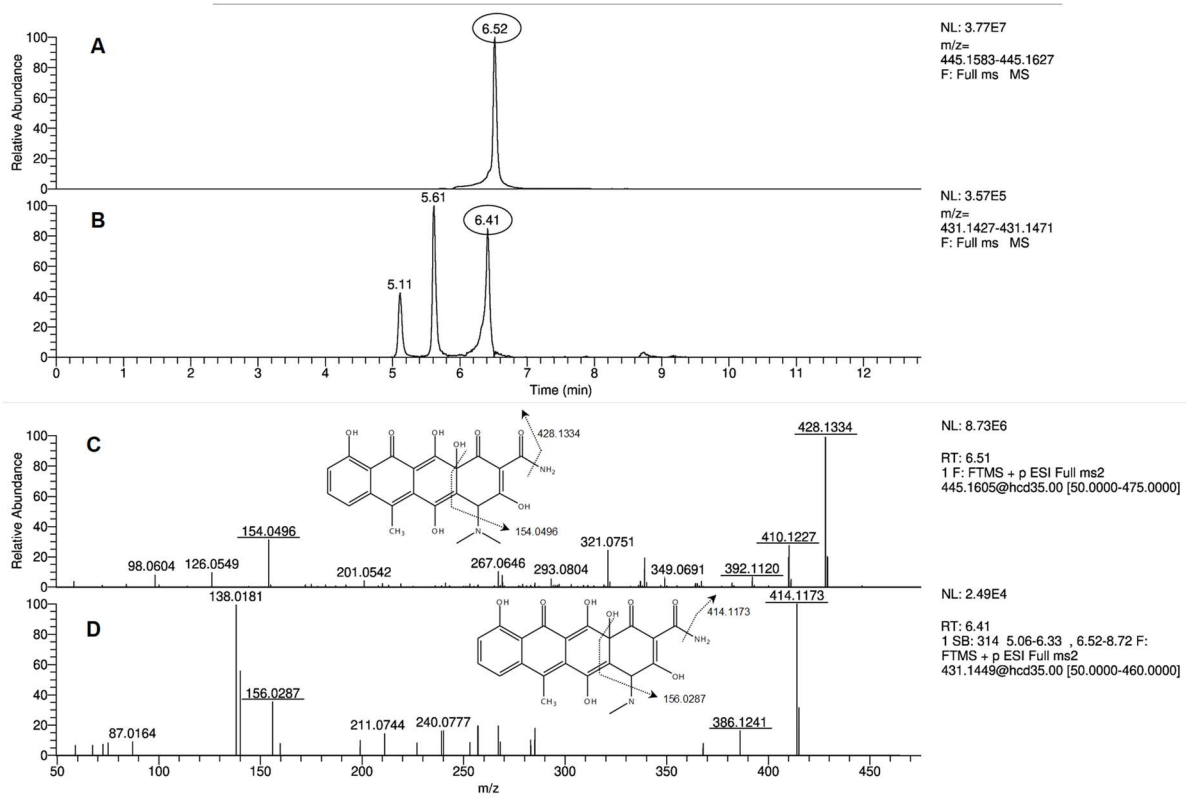
HPLC-HRMS method

The mass spectrometric analysis was performed using a hybrid quadrupole Orbitrap Q-Exactive instrument (Thermo Scientific, USA) equipped with a HESI-II ion source and High energy Collision-induced Dissociation (HCD) cell. A full-scan analysis at 70,000 resolving power, in the 200-800 m/z range, was done on extracted plasma and brain samples of doxycycline-treated and untreated mice. MS/MS fragmentation spectra (CID 35eV, resolving power 35,000) were acquired in the same analytical run using a data-dependent method, which selected the most abundant ions of each MS scan in real time in order to confirm the structure of identified metabolites.

Chromatographic separation was done on an Agilent 1200 series LC system (Agilent Technologies, USA) using a Kinetex EVO C18 column (150 × 2.1 mm, 5 µm particle size; Phenomenex Inc. USA) at 30°C, with SecurityGuard™ ULTRA cartridges EVOC18 (Phenomenex Inc.). The elution solvents used 0.1% HCOOH in water (mobile phase A, MP-A) and 0.1% HCOOH in acetonitrile (mobile phase B, MP-B). The injection volume was 4 µL and the flow rate 300 µL/min. The auto-sampler temperature was maintained at 6°C. Elution started with 98% of MP-A and 2% MP-B for 2 min, followed by a 10-min linear gradient to 98% of MP-B which was maintained for 2 min; thereafter, a 1-minutes linear gradient bring to 98% of MP-A that was maintained for 5 min to equilibrate the column. The total run time was 20 min. The HPLC-HRMS system was controlled by the Xcalibur 4.0.27.19 (Thermo Scientific) and data were collected with the same software.

Identification of doxycycline metabolites in plasma and brain

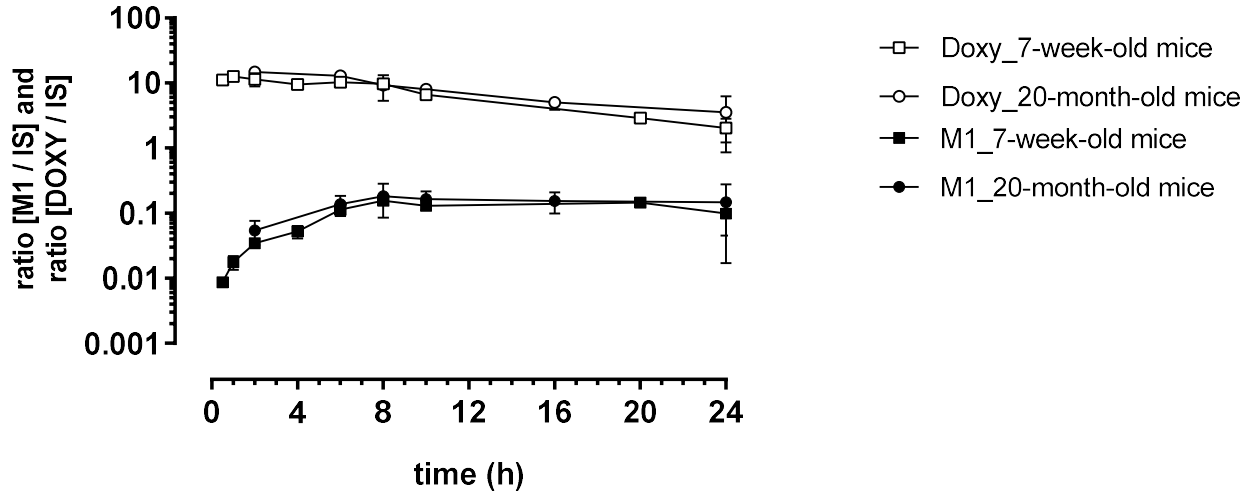
We looked for metabolites of doxycycline in a pool of plasma and brain homogenate samples just before, and 2h and 6h after, the last dose in 7-week-old male mice treated twice a day for 4 days with 100 mg/kg doxycycline. To filter out interfering signals, plasma and brain samples of untreated mice were acquired in the same way and common background peaks were not considered. We find only the *N*-demethylated metabolite (M1) of doxycycline in plasma, but not in the brain homogenate. As shown by the extracted ion chromatograms (EIC) (Supplemental Figure 6B), the chromatographic peak with retention time of 6.41 min corresponded to the m/z calculated for metabolite originating from N-demethylation (431.1449 ± 5 ppm) (doxycycline retention time of 6.51 min, Supplemental Figure 6A). Based on the MS/MS spectra of each precursor ion we confirmed the structure of doxycycline (Supplemental Figure 6C) and of its metabolite (Supplemental Figure 6D).



Supplemental Figure 6. Indicative extracted ion chromatograms (EIC) using the *m/z* values of (**panel A**) doxycycline (retention time 6.52 min) and **(panel B)** *N*-demethylated (M1, retention time 6.41 min) obtained in a pool of plasma samples from mice treated twice a day for 4 days with the dose of 100 mg/kg, and killed 6h after the last dose. **Panels C** and **D** show respectively the MS/MS spectra (CID 35eV, resolving power 35,000) of doxycycline and the metabolite M1 acquired for the corresponding EIC**.** In particular, **panel C** shows the product ion spectrum of doxycycline highlighting the diagnostic ions at *m/z* 428.1334 and 154.0496 (ions at *m/z* 410.1227 and 392.1120 are due to progressive loss of -OH groups from the product ion *m/z* 428.1334 during fragmentation process). **Panel D** shows the product ion spectra of metabolite originating from *N*-demethylation (M1), highlighting the diagnostic ions *m/z* 414.1173 and 156.0287 (ion at *m/z* 386.1241 derived by loss of –OH group from the product ion *m/z* 414.1173 during fragmentation process).

Semi-quantitative analysis of N-demethylated metabolite in plasma

Absolute plasma concentrations of *N*-demethylated metabolite (M1) could not be determined due to the lack of the reference standard; for this reason, comparisons were made on the M1 peak areas, normalized for the IS peak area (acquired in full-scan). Supplemental Figure 7 shows the time-course profiles of M1, and of doxycycline for comparison, in plasma of the 7-week-old and 20-month-old mice treated with 100 mg/kg doxycycline i.p. In both groups, *M1* was already detected at the first time-point (Supplemental Figure 7), reaching the C_{max} at 8 hours, with a slight decrease thereafter. M1 levels were however very low, ranging 0.1-5% of the parent drug. Chronic treatment in 7-week-old mice (100 mg/kg once or twice a day) did not result in plasma accumulation of the *N*-demethylated metabolite (data not shown).



Supplemental Figure 7. Time-course of the plasmatic levels of *N*-demethylated metabolite (M1), after i.p. injection with 100 mg/kg doxycycline in 7-week-old mice (filled squares) and in 20-month-old-mice (filled circles). The graph shows the peak area ratio of the analyte to IS. For comparison, time-course of doxycycline ratios are reported in parallel. Each point is the mean ± SD of 3-4 mice.