A Redox-Based Chemical Delivery System that Enhances Estradiol Distribution to the Brain: Disposition Studies in the Rat

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The disposition of a chemical delivery system for estradiol (E_2-CDS) which is based on a redox dihydropyridine–pyridinium salt conversion was investigated in rats. Tissue and plasma concentrations of E_2-CDS and the oxidized metabolite (E_2-Q^+) were evaluated at times ranging from 1 to 14 days after intravenous administration of E_2-CDS formulated as a modified cyclodextrin inclusion complex. While E_2-CDS levels were below HPLC assay detection limits for all samples by 1 day postdosing, E_2-Q^+ was readily quantified. The calculated half-life of E_2-Q^+ was longest in brain tissue, significantly shorter in heart, lung, and kidney tissues, and shortest in plasma. There was a linear relationship between administered E_2-CDS dose and oxidized metabolite measured in brain as well as in other tissues collected 24 hr after drug administration. Coadministration of high doses of a similarly oxidizable dihydropyridine, 1-methyl-1,4-dihydronicotinamide (NMN), in a dimethylsulfoxide (DMSO) vehicle decreased E_2-Q^+ measured in brain and other tissues without significantly affecting the relative patterns of distribution in these tissues. Brain tissue E_2Q^+ levels were not detected after dosing with the oxidized metabolite.

KEY WORDS: estradiol; chemical delivery system; central nervous system (CNS)-targeted drug delivery.

INTRODUCTION

An approach to organ-specific drug delivery which uses a redox-based chemical delivery system (CDS) is capable of providing brain-enhanced and sustained drug levels for a variety of therapeutic agents (1–8). Several studies have indicated that this system can be successfully applied to estradiol to stimulate preferentially centrally mediated estrogen responses and minimize peripheral estrogenization (9–12). The approach covalently links estradiol with the dihydropyridine derivative of 1-methyl-nicotinic acid through the C-17 position of the steroid, thus blocking the estrogen receptor recognition site (13). The system requires a two-step process where the lipophilic carrier–drug moiety, E_2-CDS, penetrates the blood–brain barrier (BBB) as well as other perfused body compartments upon systemic administration. Then, the drug–carrier complex is oxidized to the corresponding charged quaternary salt (E_2-Q^+). The charged moiety is retarded from efflux through the BBB but is excreted from the systemic circulation, thus E_2-Q^+ is "trapped" within the central nervous system. The second step is a slower hydrolysis of E_2-Q^+ which releases estradiol for sustained receptor stimulation and trigonelline (7,8). Although estradiol rapidly penetrates the BBB, relatively high doses are required for centrally mediated estrogenic effects such as alleviation of menopausal or castration-associated hot flushes (14,15). Further, high-dose estrogen treatment is associated with peripheral toxicities (16). Animal studies have shown that central receptors are less sensitive to estradiol compared with peripheral receptors (17). An agent capable of preferential central estrogenization may prove therapeutically advantageous for treating several centrally mediated anomalies including hot flushing and depression. Endometriosis, prostate hypertrophy, and fertility may also be controlled with E_2-CDS.

This report deals with disposition of E_2-CDS under various conditions in rats. First, tissue levels of E_2-CDS and E_2-Q^+ were evaluated from 1 to 14 days after intravenous (i.v.) dosing with E_2-CDS. Second, tissue E_2-Q^+ was measured 1 day after i.v. administration of various doses of E_2-CDS to evaluate the dose–tissue concentration relationship. Next, the effect of simultaneous administration with various doses of 1-methyl-1,4-dihydronicotinamide (NMN) with E_2-CDS on E_2-Q^+ tissue levels was characterized. This study was intended to examine the nature and robustness of E_2-CDS oxidation.

MATERIALS AND METHODS

Chemicals. Both E_2-CDS (3-hydroxy-17β{[(1-methyl-1,4-dihydropyridine-3-yl)carbonyl]oxy}estra-1,3,5(10)-triene) and E_2-Q^+ (1-methyl-3-{[(3-hydroxy estra-1,3,5(10)-triene-17β-yl)oxy]carbonyl}pyridinium) were synthesized and characterized as previously reported (9,10). For E_2-CDS, the final synthetic step was reduction of E_2-Q^+ in aqueous basic sodium dithionate. For most animal studies both E_2-CDS and E_2-Q^+ were complexed with 2-hydroxypropyl-β-cyclodextrin (HPCD) to enhance aqueous solubility of the compounds and permit administration in a nonirritating vehicle (17). The competing dihydro compound, NMN, was synthesized by refluxing with nicotinamide and methyl iodide in methanol followed by reduction in aqueous basic sodium dithionate to yield the dihydropyridine product (18). For animal studies the NMN, various amounts of the oil were dissolved in dimethylsulfoxide (DMSO) containing 10 mg/ml of E_2-CDS. The DMSO vehicle permitted dosing with higher levels of NMN in small volumes than use of HPCD. All reagents and chemicals used were of analytical grade except acetonitrile and distilled water used to prepare mobile phases which were of HPLC grade.

Animals and Treatments. Male Sprague Dawley rats (180–200 g) were purchased from Charles River Laboratories (Wilmington, MA) and housed under standard conditions in the temperature (22 ± 2°C)- and light (14 hr light, 10 hr dark)-controlled vivarium. Animals were provided free access to tap water and Purina Rat Chow.

To examine the distribution of drug following E_2-CDS, 30 rats (220–250 g) were treated with 5 mg/kg E_2-CDS in an

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aqueous vehicle containing 20% (w/v) HPCD via tail vein injection (1 ml/kg). Groups of five rats were killed on days 1, 2, 4, 6, 10, and 14 postdosing at the same hour each morning. Brain, liver, lung, heart, testes, kidney, and peritoneal fat were dissected, weighed, and placed on dry ice within 90 sec of decapitation. Plasma was separated from collected trunk blood by centrifugation (2000*g*). Tissues were stored at −20°C until processed for HPLC assay.

To examine the effect of dose on drug distribution, animals (250–280 g) were treated with 0.5, 1.0, 2.5, or 5.0 mg/kg E_2-CDS in 20% HPCD via tail vein injection ($n = 6$) and killed 24 hr postdosing. The following tissues were rapidly dissected, weighed, and frozen on dry ice: brain, liver, heart, kidneys, testes, peritoneal fat, spleen, and duodenum. Plasma was separated from trunk blood.

To compare the disposition of oxidized metabolite following i.v. dosing with the lipophilic E_2-CDS and quaternary salt E_2-Q^+, rats received 1 mg/kg of E_2-CDS or E_2-Q^+ via tail vein injection in a vehicle of 20% HPCD (w/v). Rats were killed 24 or 72 hr later ($n = 6$ animals/group), with plasma, brain, liver kidney, heart, lung, testes, fat, spleen, and duodenum collected and frozen for subsequent analysis of E_2-Q^+.

In an initial study to examine the nature and selectivity of the oxidation mechanisms *in vivo*, a large dose of NMN (500 mg/kg) was coadministered with E_2-CDS (5 mg/kg) i.v. in a DMSO vehicle (0.5 mg/kg). Tissues were collected 6 hr posttreatment for E_2-Q^+ analysis. Because significant decreases in brain E_2-Q^+ levels were seen in NMN-treated rats, the next study similarly coadministered NMN in doses of 12.5, 25, 50, 125, and 250 mg/kg ($n = 6$ animals/group) with E_2-CDS (5 mg/kg). Tissues were collected 5 hr postdosing for E_2-Q^+ analysis. Previous studies had shown that E_2-CDS was below detection (20 ng/ml) in plasma and brain by 4 hr after E_2-CDS (9,19).

Assay System

The HPLC system for the quantification of E_2-CDS and E_2-Q^+ utilized an on-line precolumn enrichment technique which was described earlier (19). Briefly, a mixture of acetonitrile/water 20:80 was used at a flow rate of 1.5 ml/min to flush the sample onto the precolumn. For separation of E_2-Q^+ on a reversed-phase Spherisorb S5 ODSII column (15 cm × 4.6-mm i.d., Regis Chemical, Morton Grove, IL), a mobile phase was used consisting of acetonitrile/water, 42:58, containing 0.025 *M* sodium 1-octanesulfonate and 0.0035 *M* tetrabutylammonium phosphate. The flow rate was 1.4 ml/min. For the separation of E_2-CDS a mixture of acetonitrile/water, 80:20, at a flow rate of 1.8 ml/min was used.

The tissue samples were homogenized and extracted by adding 1 ml of saturated sodium chloride solution and 4 ml of acetonitrile to 1 g of tissue or 1 ml of plasma. The acetonitrile phase was separated at −20°C and injected without further purification. E_2-Q^+ and E_2-CDS were detected at 224 and 360 nm, respectively. The limits of detection for E_2-Q^+ and E_2-CDS under these conditions, expressed as nanograms per gram of tissue or milliliter of plasma, were 10 and 20, respectively. The coefficient of variation was less than 5% for standards as well as blank E_2-Q^+ and E_2-CDS-spiked tissue samples.

Data Analysis

The half-life of E_2-Q^+ was calculated for each tissue using nonlinear regression analysis. Mean differences between treatment groups were analyzed using ANOVA followed by Student Newman Keuls tests and were considered significant at $P < 0.05$.

RESULTS

Analysis of tissue E_2-CDS concentrations following a 5 mg/kg i.v. dose of E_2-CDS showed that the drug levels were less than the detectable limits for the HPLC method used (20 ng/g) in all tissues by day 1. In contrast, and as predicted from previous studies with this redox-based chemical delivery system (20), the oxidized metabolite, E_2-Q^+, was readily quantified in all tissues. Results are shown in Fig. 1. Brain concentrations of E_2-Q^+ were measurable throughout the study and decreased from 1.54 μg/g on day 1 to 0.15 μg/g on day 14, with a half-life of 4.1 days. The estimated brain half-life was close to twice that estimated for other tissues which initially had higher metabolite levels. Specifically heart, lung, and kidney tissue E_2-Q^+ half-life estimates were 2.4, 2.5, and 2.5 days, respectively. At the end of the study the E_2-Q^+ concentration in brain tissue exceeded levels measured in all other tissues at least twofold (Fig. 2). These results are consistent with retarded efflux of E_2-Q^+ through the BBB.

Results of the dose disposition study indicated that the tissue concentration of oxidized metabolite is linearly related to the i.v. dose of E_2-CDS. Results of this study are shown in Fig. 3 for brain tissue, where $r = 0.9932$. Other tissues had similar linear dose–metabolite concentrations, with heart, lung, kidney, spleen, testes, and duodenum having $r^2 > 0.95$.

Tissue E_2-Q^+ levels measured 1 and 3 days after dosing with 1 mg/kg E_2-CDS or an equimolar dose of the oxidized metabolite are shown in Table I. As in previous studies, E_2-CDS was not detected in any tissue 24 hr postdosing. The most striking differences between treatment groups are the low metabolite levels measured in heart, kidney, and lungs as well as the undetectable level in brain tissue after E_2-Q^+ compared with E_2-CDS administration. This suggests that while E_2-Q^+ can partition to systemic tissues, the majority of metabolite measured in heart, kidney, and lungs after E_2-

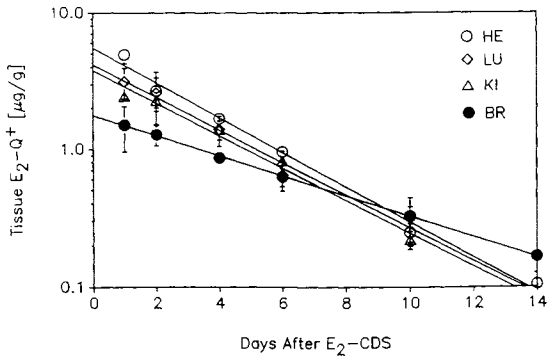

Fig. 1. Rate of E_2-Q^+ decrease in lung (LU), heart (HE), brain (BR), and kidney (KI) tissues from 1 through 14 days after E_2-CDS (5 mg/kg, i.v.). Each point represents the mean of five animals, with vertical bars indicating standard deviation.

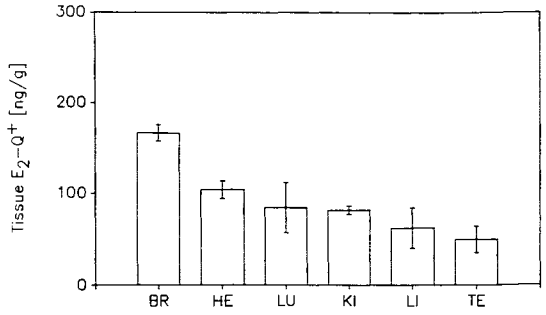

Fig. 2. Level of E_2-Q^+ assayed in tissues 14 days after a single dose of E_2-CDS (5 mg/kg, i.v.). Shown are means of five animals, with vertical bars representing SE for brain (BR), heart (HE), kidney (KI), liver (LI), lung (LU), and testes (TE).

CDS dosing results from oxidation of the drug in these tissues. However, only brain tissue levels remain relatively stable between 1 and 3 days postdosing, with a much more rapid E_2-Q^+ decrease in heart, kidney, and lung tissues. These results support a bidirectional blood–brain barrier for the quaternary pyridinium metabolite. The metabolite is readily eliminated from the circulation as evidenced in this and previous studies (10,17,19) which found undetectable plasma levels.

As shown in Table II, coadministration of a high dose of NMN (500 mg/kg) with E_2-CDS (5 mg/kg) significantly decreased E_2-Q^+ in brain and kidney tissue collected 6 hr posttreatment. Results of the next study, which examined E_2-Q^+ tissue levels 5 hr following various doses of NMN, are shown in Table III. Only brain and heart E_2-Q^+ levels were significantly reduced by coadministration of 125 and 250 mg/kg doses of NMN. As reflected in the large standard deviations, tissue levels measured after i.v. dosing with E_2-CDS in a DMSO vehicle were considerably more variable than tissue levels monitored after i.v. dosing with an HPCD vehicle.

DISCUSSION

The results of these studies confirm and extend previous reports examining the redox-based chemical delivery system for brain enhanced drug delivery (1–12). The first of the present studies characterizes the disposition of the oxidized metabolite from 1 to 14 days postdosing. Although tissue concentrations of the oxidized metabolite were initially concentrated in heart, lung, and kidney tissues compared to brain, only the brain maintained high metabolite levels for the duration of the study. Thus absolute E_2-Q^+ levels are highest in brain by the end of the study. The data therefore support the bidirectional nature of the blood–brain barrier with respect to the oxidized pyridinium-linked drug metabolite, E_2-Q^+. Previous studies similarly estimated longer half-life values for E_2-Q^+ in brain compared with other tissues (9,19). However, these studies used a DMSO vehicle and the tissue sampling times did not extend beyond one half-life. These factors may have contributed to some differences in the estimated value of tissue E_2-Q^+ half-life between studies. The consistent finding among these studies is that the decline in brain E_2-Q^+ is considerably slower compared to other tissues with relatively large initial concentrations such as the lung, kidney, and heart.

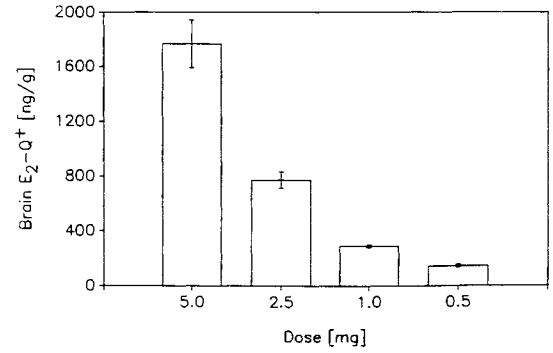

Fig. 3. Effect of E_2-CDS dose on brain concentrations of E_2-Q^+ measured 24 hr after an i.v. dose. Shown are means of six animals, with vertical bars representing standard deviation.

Enhanced tissue concentrations of E_2-Q^+ alone do not indicate significant estrogenic stimulation of the tissue. Indeed, esterification of estradiol with the pyridinium moiety through the C-17 position blocks the site of estradiol receptor binding (13). Estrogenic activity should require hydrolysis to release estradiol and trigonelline. Results from two laboratories indicate that this hydrolysis takes place and is sustained in rat brain tissue after E_2-CDS dosing. These previous reports showed that estradiol, measured with radioimmunoassay techniques, was significantly higher in brain compared to peripheral immunoassayable estradiol after E_2-CDS (12,20). The redox approach to targeted drug delivery predicts that the rate of E_2-Q^+ decrease in brain tissue corresponds to the rate of hydrolysis. While the present data are consistent with the concept, further studies are required to evaluate more directly the rate of free drug release relative to the hydrolysis rate of the quaternized drug among tissues.

Several lines of evidence indicate that hydrolysis is not the primary mechanism for elimination of E_2-Q^+ from peripheral tissues. Kinetic studies in dogs showed that E_2-Q^+ is excreted in urine for 2 weeks following administration of E_2-CDS, although plasma levels were below assay detection limits (21). The present studies found that while considerable E_2-Q^+ was measured in duodenal tissue after dosing with the E_2-CDS, the highest metabolite concentrations were found in the duodenum 24 hr after i.v. administration of the oxidized pyridinium salt metabolite. Our studies in bile duct-cannulated dogs showed that biliary excretion was greater after E_2-Q^+ dosing compared to E_2-CDS (Derendorf *et al.* unpublished observations). Finally, the sustained centrally mediated pharmacological activity after a single dose of E_2-CDS shown in the previous studies supports central hydrolysis of E_2-Q^+. Thus luteinizing hormone (LH) was suppressed in castrated male and female rats for more than 3 weeks (10,11), copulatory behavior was stimulated in male castrate rats for 4 weeks (22), and body weight gain was suppressed for 5 weeks (23) after a single 1–3 mg/kg dose of E_2-CDS. Peripheral or circulating estradiol concentrations could not account for the monitored pharmacological activity. This is consistent with central estrogenization and does not indicate significant peripheral hydrolysis (24).

The significance of the oxidation step for the chemical delivery system is substantiated in the study, which compared drug metabolite levels after i.v. administration of E_2-CDS or the same dose of the corresponding quaternary salt.

Table I. Tissue E_2-Q^+ Levels 1 and 3 Days After E_2-CDS or E_2-Q^+ (1 mg/kg, i.v.) in Male Rats

	E_2-Q^+ (μg/g) Treatment			
	E_2-CDS		E_2-Q^+	
Tissue	24 hr	72 hr	24 hr	72 hr
Brain	0.24 ± 0.03[a]	0.22 ± 0.03	ND[b]	ND
Heart	5.08 ± 0.59	1.67 ± 0.25	0.17 ± 0.08	ND
Kidney	1.59 ± 0.29	0.69 ± 0.10	0.07 ± 0.03	ND
Lung	0.93 ± 0.39	0.34 ± 0.23	0.11 ± 0.04	0.10 ± 0.04
Duodenum	0.72 ± 0.31	0.33 ± 0.04	0.36 ± 0.13	0.09 ± 0.02
Spleen	0.29 ± 0.06	0.15 ± 0.06	0.14 ± 0.06	0.09 ± 0.02
Liver	0.09 ± 0.05	0.06 ± 0.01	0.04 ± 0.02	0.04 ± 0.01
Testes	0.07 ± 0.03	0.09 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Fat	0.08 ± 0.01	0.11 ± 0.04	0.11 ± 0.03	0.08 ± 0.03
Plasma	ND	ND	ND	ND

[a] Mean ± SD, n = 4–6 animals/group.
[b] Less than detectable, 0.01 μg/g.

The brain is the only tissue which effectively excludes E_2-Q^+. However, the vast majority of E_2-Q^+ measured in heart, kidney, and lung tissue following E_2-CDS administration was not found after E_2-Q^+ dosing, which strongly indicates that the metabolite results from *in situ* oxidation in peripheral tissues as well as brain tissue. The high metabolite levels present in heart, kidney, and lung tissues cannot readily be explained on the basis of blood perfusion to these organs during the initial postinjection period prior to E_2-CDS oxidation. If the amount of oxidized metabolite in tissues was directly related to blood perfusion, the concentration of E_2-Q^+ in heart should be about one-third of that found in brain based on the proportion of cardiac output in the animal at rest (26).

An alternative explanation may be found in the relative oxidation capacity among these tissues. To examine this possibility *in vivo*, animals were simultaneously treated with E_2-CDS and a simple nontoxic dihydropyridine which *in vitro* studies indicated is similarly oxidized to a pyridinium salt, NMN (2). The drugs were administered as an i.v. bolus in a DMSO vehicle containing constant E_2-CDS (5 mg/kg/0.5 ml) and varying NMN (12.5 to 500 mg/kg/0.5 ml) doses. Results showed that the highest dose of NMN significantly decreased brain and kidney concentrations of E_2-Q^+ measured 6 hr postdosing by 72 and 61%, respectively. In other tissues, results suggested mean decreases ranging from 18 to 75%, which were not statistically significant. Interestingly, liver E_2-Q^+ levels were not affected by any dose of NMN in the subsequent dose comparison study. Only brain and heart tissue E_2-Q^+ levels were significantly decreased in this study, with both 125 and 250 mg/kg NMN doses reducing measured metabolite levels in these tissues. However, the decreased trend appeared at the lowest dose in brain tissue, which suggests lower oxidative capacity in brain tissue rather than the step response seen in heart tissue. No significant effects of NMN coadministration on E_2-Q^+ levels were seen in other tissues. The significance of these findings for the mechanism of oxidation and targeted redox-based drug delivery is unclear at present. Further studies are required to examine whether the rate of peripheral tissue oxidation of E_2-CDS can be controlled or may be altered in various physiological states.

Table II. Effect of *N*-Methyldihydronicotinamide (NMN) on Tissue E_2-Q^+ Levels 6 hr After Coadministration with E_2-CDS in DMSO

	E_2-Q^+ (μg/g)	
Tissue	E_2-CDS (5 mg/kg, i.v.)	E_2-CDS + NMN (500 mg/kg)
Brain	4.51 ± 2.46[a]	1.28 ± 0.50*
Heart	19.22 ± 10.45	15.76 ± 7.20
Kidney	8.93 ± 2.49	3.49 ± 0.59*
Lung	10.13 ± 4.14	7.63 ± 2.43
Liver	0.38 ± 0.06	0.43 ± 0.09
Testes	0.68 ± 0.50	0.19 ± 0.09
Fat	0.37 ± 0.15	0.26 ± 0.06

[a] Mean ± SD, n = 6–8 animals/group.
* Significantly different from E_2-CDS alone, $P < 0.05$.

Development of E_2-CDS requires a reasonably stable formulation for drug dosing via an acceptable administration route. While initial studies utilized a DMSO vehicle to demonstrate the feasibility of the redox-based approach, this vehicle is not optimal for formulation development. Complexation with amorphous cyclodextrins provided enhanced stability and permitted aqueous solubilization of E_2-CDS in a vehicle with low toxicity and negligible venous irritation (17,26). Our previous studies showed that an additional advantage with i.v. administration of E_2-CDS as an inclusion complex was increased precision or decreased variability among samples compared to results using a DMSO vehicle (19). This effect was also seen in the present studies, where variability was considerably greater in the NMN study, which required a DMSO vehicle to solubilize large amounts of compound in a small volume.

Results of these studies in rats confirm and extend previous studies with E_2-CDS. Linear pharmacokinetic profiles were shown for all examined tissues over the dose range studied. The brain consistently was shown to have the long-

Table III. Effect of NMN Dose on Tissue E_2-Q^+ Levels 5 hr After Coadministration with E_2-CDS (5 mg/kg) in DMSO

	E_2-Q^+ (μg/g)					
	Dose of NMN (mg/kg)					
Tissue	0	12.5	25	50	125	250
Brain	4.17 ± 1.7[a]	3.3 ± 0.7	3.5 ± 0.5	3.6 ± 0.6	1.6 ± 0.7*	2.3 ± 0.3*
Heart	25.3 ± 3.59	22.7 ± 1.8	25.2 ± 2.2	24.1 ± 1.6	17.2 ± 5.7*	16.0 ± 3.2*
Kidney	6.8 ± 1.8	6.9 ± 0.4	6.3 ± 1.9	5.6 ± 2.4	5.5 ± 1.8	7.8 ± 1.8
Lung	21.0 ± 8.0	20.1 ± 3.4	12.0 ± 0.9	21.0 ± 10.3	12.2 ± 4.3	11.6 ± 1.9
Liver	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.3	0.7 ± 0.3	0.6 ± 0.2
Testes	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
Fat	0.8 ± 0.4	0.3 ± 0.1	1.0 ± 0.6	0.4 ± 0.1	0.8 ± 0.3	0.8 ± 0.2
Plasma	ND[b]	ND	ND	ND	ND	ND

[a] Mean ± SD, $n = 5$ animals/group.
[b] Less than 0.01 μg/ml.
* Significantly different from E_2-CDS alone (0 mg/kg NMN group), $P < 0.05$.

est half-life for E_2-Q^+ but E_2-Q^+ does not distribute to brain tissues after systemic administration. These preliminary studies indicate that oxidation of E_2-CDS is robust in all tissues. However, the oxidation of E_2-CDS in brain tissue may be perturbed in some circumstances. Further studies are required to evaluate the significance of these findings.

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