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Report

A Redox-Based System that Enhances Delivery of Estradiol to the Brain: Pharmacokinetic Evaluation in the Dog

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The pharmacokinetics of a dihydropyridine–pyridinium salt-type chemical delivery system (CDS) for brain-targeted delivery of estradiol (E_2) were examined in dogs. Parameters evaluated *in vitro* included stability in buffers and biological fluids and plasma protein binding. *In vivo* studies examined drug and metabolite concentrations in plasma, urine, and cerebrospinal fluid as well as in selected brain regions. The administered lipophilic E_2-CDS disappeared very quickly from plasma and was not detected in urine. The oxidized drug form, E_2-Q^+, was excreted unchanged or as a conjugate in the urine for as long as 2 weeks. Plasma levels were below assay detection limits at later times. Pharmacokinetic analysis of urine E_2-Q^+ levels allowed estimation of a half-life of 2.2 days. Amounts of E_2-Q^+ excreted into the urine were proportional to the dose but averaged only 13.9% of the dose, indicating that other routes of excretion must be considered. CSF levels were below the limit of detection for both E_2-CDS and E_2-Q^+, however, brain tissue concentrations of E_2-Q^+ were similar in several brain regions of individual animals examined 1 or 3 days after drug dosing.

KEY WORDS: estradiol; chemical delivery system; brain enhanced drug delivery; blood–brain barrier; pharmacokinetics; redox drug delivery.

INTRODUCTION

The dihydropyridine–pyridinium salt-type redox system has been applied to a number of pharmacologically active drugs for targeted and sustained delivery to the central nervous system (1–8). Several studies showed that this chemical delivery system (CDS) can be successfully coupled with estradiol (E_2) (9–12).

The mechanism of the CDS for brain-targeted drug delivery has been described in detail (2,7,8). Briefly, the dihydropyridine derivative of *N*-methyl nicotinic acid is covalently attached to E_2 at the 17 position to result in E_2-CDS (Fig. 1). The highly lipophilic moiety, E_2-CDS, can easily penetrate the blood–brain barrier (BBB) after systemic administration. However, oxidation of the labile carrier yields the corresponding quarterly salt (E_2-Q^+), which is retarded from efflux through the BBB because of its ionic hydrophilic character. The drug carrier moiety thus is "trapped" behind the BBB as evidenced by sustained elevated brain drug concentrations compared to peripheral tissues (9,12). The second step requires cleavage or hydrolysis of the E_2-Q^+, to release estradiol and the trigonelline carrier. Small charged moieties including trigonelline are readily eliminated from the central nervous system (13,14). Steroids such as estradiol normally pass the BBB. Therefore circulating serum concentrations of the hormone are closely correlated to their central activity with available estrogen replacement therapies (15).

Application of the CDS to estradiol may offer the advantage of targeted treatment of centrally mediated impairments associated with estrogen withdrawal without excess peripheral estrogenization such as hot flushing. Additionally, this approach may be therapeutically useful in treatment of prostate cancer and endometriosis, for libido enhancement, and for contraception.

This paper describes initial pharmacokinetic studies of E_2-CDS in dogs. Plasma, urine, cerebrospinal fluid, and brain tissue were analyzed for the concentrations of E_2-CDS and E_2-Q^+ after intravenous administration of E_2-CDS.

MATERIALS AND METHODS

Chemicals. 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-hydroxyestra-1,3,5-(10triene-17β-yl)oxy]carbonyl)pyridinium iodide) were synthetized as previously reported (9,10). The highly lipophilic E_2-CDS was complexed with 2-hydroxypropyl-β-cyclodextrin to permit drug administration in an aqueous vehicle as previously described (16). HPLC-grade acetonitrile and distilled water

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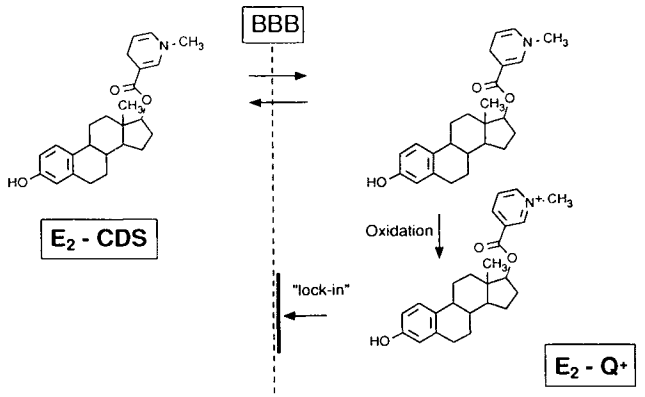

Fig. 1. Mechanism of brain-targeted drug delivery of estradiol. BBB, blood–brain barrier.

were used for the preparation of mobile phases. All other reagents were of analytical grade. β-Glucuronidase (Type H-1) containing sulfatase activity was purchased from Sigma (St. Louis, MO).

Analytical Method. The HPLC system for the quantification of E_2-CDS and E_2-Q^+ utilized an on-line precolumn enrichment technique as described earlier (17) with slight modifications in order to improve peak shape. For enrichment a mobile phase consisting of acetonitrile/water, 20:80, was used at a flow rate of 1.5 ml/min. The mobile phase for backflushing and analytical separation was a mixture of acetonitrile/water, 42:58, which contained 0.025 *M* sodium 1-octanesulfonate and 0.0035 *M* tetrabutylammonium phosphate. The flow rate was 1.4 ml/min.

The procedure for the extraction of plasma and tissues was slightly modified to improve chromatography (17). After homogenization and centrifugation the acetonitrile supernatant from the plasma or tissue was held at −20°C to separate the organic and aqueous phases. The limit of sensitivity using 1 ml of plasma for analysis of E_2-Q^+ and E_2-CDS is about 10 ng/ml.

The reported analytical method was expanded to include the determination of E_2-Q^+-conjugates in urine. To 0.5 ml of urine, 100 μl of a 0.1 M acetate buffer (pH 5.0) and 2500 U of β-glucuronidase containing 275 U of sulfatase were added. The mixture was vortexed and incubated at 37°C for 45 min. After the addition of 1.8 ml of acetonitrile the samples were frozen overnight. One and six-tenths milliliters of the clear supernatant was injected into the HPLC system.

Plasma Protein Binding. Plasma (1 ml) was spiked with different amounts of E_2-Q^+ to give concentrations between 100 and 2000 ng/ml. The plasma was transferred into Amicon centrifugal concentrator (Danvers, MA) equipped with a low-adsorption, hydrophilic YM membrane, with a 10,000 molecular weight cutoff, and centrifuged for 5 min at approximately 3000*g* at 27°C. The filtrate (0.2 ml) was extracted with acetonitrile and injected into the HPLC. Different solutions of E_2-Q^+ in isotonic phosphate buffer (pH 7.4) were also filtered to examine the binding of E_2-Q^+ to the filter membrane. The fraction bound to proteins (f_B) was calculated as $f_B = (C_{tot} - C_f)/C_{tot}$, where C_{tot} is the total drug concentration and C_f is the unbound drug concentration.

Stability of E_2-Q^+. The purpose of the stability studies was to assure optimum adequate urine collection intervals and sample storing conditions for estimation of E_2-Q^+ excretion. Stability was studied as a function of pH and temperature. For the evaluation of the stability of E_2-Q^+ the drug was incubated in 0.05 *M* buffers at room temperature (25°C) and at 37°C. Samples were taken at defined time intervals over the 16-hr incubation period and injected directly into the HPLC system.

Animal Studies. The pharmacokinetic and distribution studies in male mongrel dogs (19.5–25 kg) were conducted according to protocols approved by the University of Florida Animal Care and Use Committee. Animals were housed in the Department of Animal Resources (University of Florida) and acclimated for at least 4 weeks prior to study. Dogs had free access to diets of Purina Kennel Chow and tap water.

For pharmacokinetic studies dogs were fitted with venous cannulas and urinary catheters on the morning of the experimental day. Lactated Ringer's solution or sterile normal saline was infused at a rate adequate to maintain urine flow. Dogs were comfortably restrained during the experimental procedures to prevent removal of catheters.

Distribution Studies and Basic Pharmacokinetics of E_2-CDS. E_2-CDS (1 mg/kg) was administered intravenously in 20% (w:v) hydroxypropyl-β-cyclodextrin vehicle (16). Blood was withdrawn over 6 hr and analyzed for E_2-CDS and E_2-Q^+. Two dogs were anesthetized with thiamylal and halothane to permit sampling of cerebrospinal fluid from the cisterna magna. Samples of CSF (0.4 ml) were collected at 30-min intervals over 4 hr. Four dogs were housed in metabolic cages to permit urine collection for 2 weeks after a dose of 1 mg/kg E_2-CDS. Tissue concentrations of E_2-Q^+ were determined from 10 different brain regions from individual dogs 1 or 3 days after treatment with 1 or 5 mg/kg E_2-CDS.

Evaluation of Linear Pharmacokinetics. Four dogs each received three doses (0.1, 0.5, and 1.0 mg/kg) of E_2-CDS intravenously in hydroxypropyl-β-cyclodextrin as described above. Blood samples were collected into heparinized tubes through a jugular catheter at 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, and 480 min after drug administration. In addition, blood was collected after 1, 3, 4, and 7 days. The plasma was separated, frozen, and stored until analysis. Urine samples were collected from the urinary catheter at 0, 30, 60, 90, 120, 180, 240, 300, 360, and 480 min. A 24-hr urine sample was collected using a metabolic cage. Urine samples (10 ml) were frozen after recording total urine volumes and pH. Plasma and urine concentrations of E_2-Q^+ were measured. In addition, the amount of E_2-Q^+ eliminated as conjugates in urine was estimated.

RESULTS

Plasma Protein Binding of E_2-Q^+. The plasma protein binding of E_2-Q^+ was 87.0 ± 1.7%. The amount of the drug absorbed to the filter membrane was negligible. No concentration dependence of plasma protein binding was observed in the concentration range studied (100–2000 ng/ml).

Stability of E_2-Q^+. Results of the stability studies of E_2-Q^+ showed that only a negligible amount of degradation

was observed at pH 6 and 7 at 37°C. The degradation rate increased rapidly with increasing pH of the buffer solutions. At 25°C, the degradation half life of E_2-Q^+ at pH 7.5, 8, and 9 was 749, 316, and 82 min, respectively. At lower pH values (pH 1–3) no degradation of E_2-Q^+ could be observed over a time period of 16 hr. Consequently, urine samples containing E_2-Q^+ should be collected over relatively short time intervals of 1 hr or less and frozen immediately. Alternatively, the urine samples may be acidified to a pH range at which the compound is sufficiently stable during the collection process.

Plasma and Urine Levels of E_2-CDS and E_2-Q^+. E_2-CDS (dose 1 mg/kg) disappeared rapidly from plasma and was below detection limit after 2 hr. The total-body clearance (Cl_{tot}) averaged 11.9 ± 4.7 liters/min, indicating rapid oxidation of E_2-CDS. The mean residence time (MRT), calculated as the ratio of AUC and AUMC, was 23 ± 11 min. The volume of distribution ($V_{d_{ss}}$) was calculated to be 286 ± 204 l. This is equivalent to 12.1 ± 8.6 liters/kg, indicating excessive tissue distribution of E_2-CDS. No E_2-CDS could be detected in urine.

E_2-Q^+ plasma concentrations were higher than the E_2-CDS concentrations (Fig. 2) and could be monitored for all doses studied. However, the E_2-Q^+ concentrations after 0.1 mg/kg were only detectable for 2 hr. After the 0.5- and 1.0-mg/kg doses, plasma levels could be followed for 8 hr. Between 2 and 8 hr, no significant change in E_2-Q^+ concentrations could be observed, allowing no reliable estimation of plasma half-life. Areas under the curve up to 8 hr were 291 ± 23 ng/ml · hr for 0.5 mg/kg and 451 ± 20 ng/ml · hr for 1.0 mg/kg. Furthermore, E_2-Q^+ was excreted into the urine in dose proportional amounts. After 24 hr 97 ± 20, 475 ± 130, and 1025 ± 143 μg were excreted for the 0.1-, 0.5-, and 1-mg/kg doses, respectively. This is equivalent to an average of 4.5 ± 1.0% of the administered dose. Because a urinary excretion of E_2-Q^+ was maintained throughout the initial study period, the parameter was studied in four dogs over a period of 2 weeks after a dose of 1 mg/kg E_2-CDS. E_2-Q^+ was detected in urine over the entire period studied. After 14 days the cumulative amount excreted in urine was 2003 ± 385 μg or 8.9 ± 1.3% of the administered dose.

E_2-Q^+ Conjugates in Urine. Estradiol is metabolized primarily to estrone, which is excreted after conjugation to glucuronide or sulfate (18,19). Since in E_2-CDS, estradiol is coupled with *N*-methyl nicotinic acid at C-17, the conversion to estrone can take place only after cleavage of the molecule. Therefore, a likely metabolic pathway of E_2-Q^+ will be glucuronidation and sulfatation at C-3. The urine samples were treated with a mixture of β-glucuronidase and sulfatase. No degradation of the E_2-Q^+ could be observed under the conditions of the enzymatic assay, indicating sufficient specifity of the enzymes and sufficient stability of the compound itself. In the dose linearity study the cumulative amounts of E_2-Q^+ up to 8 hr found after hydrolysis were 308 ± 54, 1107 ± 361, and 1698 ± 246 μg for the 0.1-, 0.5-, and 1.0-mg/kg doses, respectively. This is equivalent to an average of 10.6 ± 3.7% of the given dose. Conjugate excretion continued over 2 weeks, when the average amount of E_2-Q^+ after hydrolysis was 3131 ± 896 μg or 13.9 ± 3.8% of the given dose.

Pharmacokinetic Evaluation of Urinary Data. The urinary data allowed calculation of the renal clearance from $Cl_{ren} = U_t/AUC_t$, where U_t is the total amount excreted up to time t and AUC_t is the area under the curve up to time t. Cl_{ren} of E_2-Q^+ was found to be 37.1 ± 1.8 ml/min, which is similar to or lower than the glomerular filtration rate in dogs (20). However, since only the free fraction in plasma can be filtered in the glomeruli, this value for Cl_{ren} indicates tubular secretion for E_2-Q^+. Pharmacokinetic analysis of the urinary data using a sigma-minus plot allowed estimation of the overall elimination rate constant k_e to be 0.315 day^{-1}. This is equivalent to a half-life of 2.2 days. Assuming a constant renal clearance in the lower plasma concentration range below 10 ng/ml permits calculation of plasma concentrations below the limit of detection for the HPLC method using the relationship $dU/dt = Cl_{ren} \cdot C_p$. This predicts that plasma levels of E_2-Q^+ will decrease slowly to about 100 pg/ml after 2 weeks.

CSF Levels of E_2-Q^+. Levels of E_2-CDS and E_2-Q^+ were below the limit of detection (10 ng/200 μl) in CSF samples obtained 30 to 240 min after dosing with 1 mg/kg E_2-CDS. Larger samples of CSF obtained in terminal studies from dogs dosed 1 day previously with 1 and 5 mg/kg contained 22 and 28 ng/ml of E_2-Q^+, respectively. Three days postdosing levels of E_2-Q^+ in CSF were close to the assay detection limit (8 and 15 ng/ml).

Distribution of E_2-Q^+ in Brain Tissue. Results of regional concentrations of E_2-Q^+ in individual animals in tissues collected 1 or 3 days after treatment with 1 or 5 mg/kg E_2-CDS are shown in Fig. 3. No site-specific enrichment among brain structures is apparent. Comparison of the 1 and 5-mg/kg doses suggests some dose nonlinearity, as brain tissue levels were increased only about twofold after 1 or 3 days posttreatment. Interestingly, levels were comparable in most brain regions after 1 or 3 days.

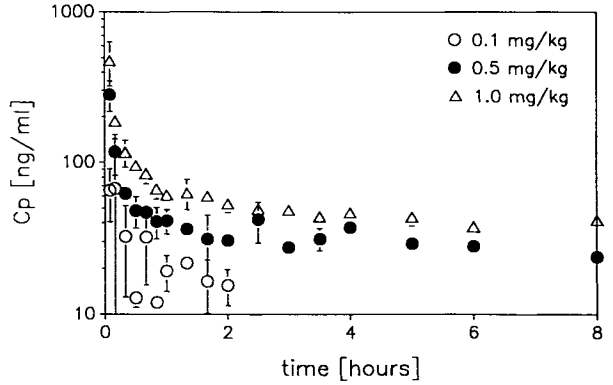

Fig. 2. E_2-Q^+ plasma concentrations after intravenous administration of 0.1, 0.5, and 1 mg/kg E_2-CDS (means ± SD; n = 4).

DISCUSSION

Results of these studies demonstrate that the dihydropyridine–pyridinium salt-type chemical delivery system for estradiol provides for extremely long residence times of the oxidized metabolite E_2-Q^+. The studies extend to the dog our previous conclusions based on rodent models. The results show that in plasma both E_2-CDS and E_2-Q^+ rapidly fall below detectable levels following intravenous administration of the reduced chemical delivery system (9,17,21).

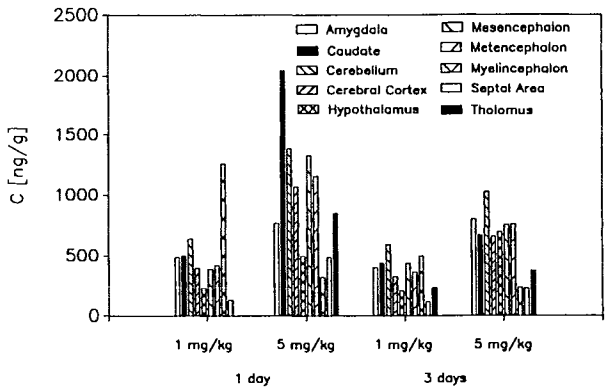

Fig. 3. Brain distribution of E_2-Q^+ in different regions of the dog brain.

Additionally, preliminary evidence is presented showing the sustained nature of brain E_2-Q^+-levels in dogs after E_2-CDS administration. Unchanged E_2-Q^+ and its glucuronide and sulfate conjugates were excreted in urine for 2 weeks after i.v. dosing in dogs. Since E_2-Q^+ needs to be present in plasma in order to be excreted by the kidneys, it is likely that the source of sustained urinary E_2-Q^+-levels is redistribution from the tissues.

Several lines of evidence indicate that the excreted drug measured represents preferential peripheral elimination of the oxidized drug form. Previous studies in rats showed that the rate of disappearance of E_2-Q^+ from peripheral tissues is 2 to 10 times faster compared to decreases from brain tissues after i.v. dosing the E_2-CDS (9,16,17,21). Second, our studies showed that systemic administration of the oxidized drug form did not result in detectable brain tissue levels of E_2-Q^+ in rats as expected for a quarternary charged moiety (22). Third, although small quarternary amines are readily eliminated from the central nervous system (13,14), no transport mechanisms have been identified for large quarternary amines corresponding to moieties the size of E_2-Q^+ (23). The relative rates of disappearance of E_2-Q^+ from peripheral and brain tissue compartments show that the "lock-in" mechanism results in enhanced brain levels of E_2-Q^+. Pharmacological activity requires cleavage of this locked-in drug form.

Recent studies using radioimmunoassay techniques show that free estradiol is enhanced in brain tissue after E_2-CDS, which indicates that the primary route of excretion from brain tissue is via hydrolysis of E_2-Q^+ (12,21). Investigations using the more sensitive, but less specific radioimmunoassay methods show that brain estradiol levels in rats remain increased compared to plasma levels for at least 14 days after E_2-CDS administration (12,21). The measured brain tissue estradiol levels were adequate to exert pharmacological activity in these studies. Further evidence for central estrogenic activity after E_2-CDS was shown in studies which evaluated various pharmacological end points including behavior, feeding, libido, locomotion and luteinizing hormone suppression (10,11,24). Attachment of the nicotinate carrier at the C_{17} position on the steroid structure renders estrogen receptor binding with E_2-Q^+ extremely unlikely (25), thus further supporting hydrolysis as the primary avenue for E_2-Q^+ efflux from the CNS.

Another factor which supports the integrity of the blood–brain barrier with respect to E_2-Q^+ is the relatively low level of E_2-Q^+ detected in CSF. Detectable levels could be found only in large CSF samples collected from the four necropsied animals. Interestingly, these levels were higher than plasma E_2-Q^+. This study was preliminary, and single-animal dosing could not estimate the statistical significance of these differences in CSF and plasma. Further studies are required to estimate pharmacokinetic parameters for CSF.

Results of the ranging dose studies showed a linear pharmacokinetic profile for single doses between 0.1 and 1.0 mg/kg. However, brain tissue concentrations did not indicate a linear relationship at higher doses (1 and 5 mg/kg) in dogs. A fivefold increase in dose resulted in only a twofold average increase in brain levels. Similar results were obtained in a multiple dosing study which included small groups of monkeys. While a clear difference in brain drug levels was found between dose groups, the dose relationship was not linear in monkeys (26).

Results of these studies indicate that urinary excretion is not the primary elimination route for E_2-CDS. Further studies are currently in progress to investigate the biliary excretion of the drug and its metabolites in dogs.

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