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Report

High-Performance Liquid Chromatographic Assay of a Central Nervous System (CNS)-Directed Estradiol Chemical Delivery System and Its Application After Intravenous Administration to Rats

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A redox-based chemical delivery system for estradiol (E_2-CDS) has been shown capable of sustained and brain-selective delivery of estradiol (E_2). A reversed-phase high-performance liquid chromatographic (HPLC) method is presented for the analysis of E_2-CDS and its oxidized quaternary metabolite (E_2-Quat) in biological fluids or tissues. The assay utilizes a precolumn enrichment technique and detects plasma levels down to 10 ng/ml E_2-Quat and 20 ng/ml E_2-CDS. Sample preparation is rapid and simple. Samples are homogenized with acetonitrile, then centrifuged, and the supernatant is directly injected into the HPLC system. A water delivering pump injects the sample on a precolumn where the drug is concentrated. The mobile phase backflushes the retained compound onto the analytical column. At the same time, another sample can be injected onto a second precolumn. This alternating precolumn sample enrichment technique allows the injection of large volumes, up to 1800 μl. Plasma and tissue samples of rats collected after i.v. administration of a single 15-mg/kg E_2-CDS dose were analyzed for E_2-CDS and E_2-Quat by this procedure. The results show sustained brain levels of E_2-Quat and prolonged half-life in brain compared to six peripheral tissues measured. These data support the concept of brain-targeted delivery using redox carrier systems of this type.

KEY WORDS: estrogen; estradiol, dihydropyridines; quaternary pyridinium salts; chemical delivery system; brain-specific drug delivery; blood–brain barrier; high-performance liquid chromatography (HPLC) with precolumn enrichment; analysis of plasma and tissues.

INTRODUCTION

Dihydropyridine–pyridinium salt-type redox systems have been shown capable of delivering a number of pharmacologically active drugs to the central nervous system (CNS) in a specific and sustained manner (1–8). Recent application of this chemical drug delivery system to the estrogen estradiol (E_2) has resulted in selective and enhanced release of the steroid in brain tissue (9–11). Figure 1 schematically shows the mechanism for brain-directed drug delivery. The drug is chemically combined with the dihydropyridine derivative of N-methylated nicotinic acid. This chemical delivery system (E_2-CDS), when administered systemically *in vivo*, can easily pass through the blood–brain barrier due to its high lipophilicity. The carrier is then oxidized to the corresponding quaternary pyridinium salt (E_2-Quat), similar to the endogenous NADH–NAD^+ interconversion. The ionic hydrophilic character of E_2-Quat prevents its efflux through the blood–brain barrier, thus locking the carrier in the brain. Hydrolysis of E_2-Quat results in a sustained release of the active species estradiol in the brain. The rapid peripheral elimination of E_2-Quat permits only transient peripheral estrogen activity.

While the CDS was originally developed for drugs which do not cross the blood–brain barrier, application of the CDS to steroids such as estradiol which normally do penetrate the blood–brain barrier (12) offers the advantage of sustained brain-specific drug release. The possibility of avoiding the unwanted peripheral steroid effects, of increasing the dosing interval, and of decreasing the overall dose by the use of a CNS-targeted drug system would therefore allow a wider and safer use of estrogens in the treatment of gonadal steroid-dependent conditions including prostate hypertrophy (13) and menopausal hot flushing (14). After systemic administration of E_2-CDS to rats, sustained levels of the oxidized carrier E_2-Quat were found in brain tissue, while peripheral levels decreased rapidly (9). Furthermore, estradiol could be detected in the brain but not in the blood. Decreased luteinizing hormon (LH) serum levels were observed up to 24 days after a single dose of E_2-CDS (10). For

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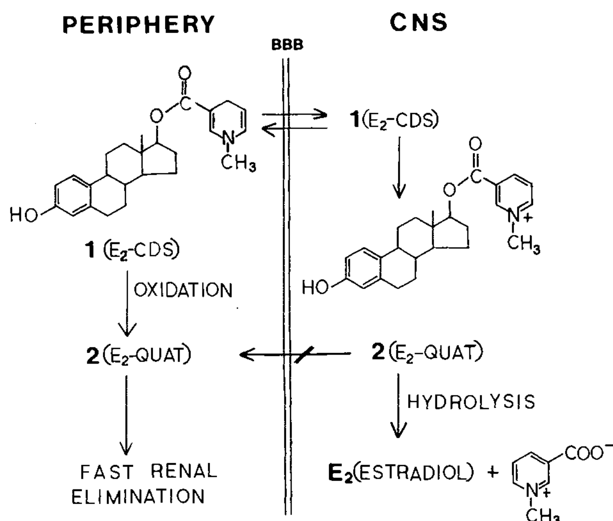

Fig. 1. Mechanism of brain-targeted drug delivery of estradiol.

future pharmacokinetic studies in laboratory animals a rapid and sensitive method of drug quantification in biological material is needed. This paper describes reversed-phase high-performance liquid chromatographic (HPLC) methods for the analysis of E_2-CDS and its oxidized metabolite E_2-Quat using a precolumn enrichment technique which is both rapid and specific in the determination of very low concentrations and can be used routinely.

MATERIALS

E_2-CDS (3-hydroxy-17β-{[(1-methyl-1,4-dihydropyridin-3-yl)carbonyl]oxy} estra-1,3,5(10)-triene) and E_2-Quat (1-methyl-3-{[(3-hydroxyestra - 1,3,5(10)-triene - 17β-yl) oxy]carbonyl} pyridinium iodide) were synthesized as previously reported (9,10). Both compounds were fully characterized by spectroscopic and elemental analysis prior to the development of the HPLC procedure. HPLC-grade acetonitrile and distilled, deionized water were used for the preparation of mobile phases. All other reagents used were of analytical grade.

INSTRUMENTATION

The HPLC system consisted of a LDC/Milton Roy Constametric III high-pressure pump, a LDC/Milton Roy variable-wavelength UV detector, a Perkin Elmer ISS-100 automatic injector equipped with a 2000-μl loop, and a Du Pont Zorbax ODS column, 15 cm × 4.6-mm i.d. (6-μm particle size). Vydac guard columns (5 cm × 3.2-mm i.d.), dry-packed with Du Pont Zorbax ODS material, were used. Chromatograms were recorded on a Hewlett–Packard Model 3390A computing integrator at a chart speed of 0.2 cm/min. In the precolumn enrichment system an enrichment injector (Rheodyne Model 7067-005) with two high-pressure switching valves, pneumatically turned by a tandem actuator (Rheodyne Model 7163), was inserted between the autoinjector and the analytical column. Switching of the valves was controlled via the autoinjector. This system also contained a Bodine Electric Co. RR/035 HPLC solvent pump for flushing the samples onto the enrichment columns.

METHODS

HPLC Conditions

An HPLC method was developed (15) that allows the injection of large volumes to optimize sensitivity based on alternating precolumn sample enrichment. Figure 2 schematically illustrates the apparatus we used. The sample containing the drug is injected with pump A, delivering pure water, onto one of two precolumns, which are alternatingly connected with the injection system by two pneumatically driven valves. Provided a certain lipophilicity, the drug is retained and concentrated on the precolumn, while accompanying water-soluble coproducts such as proteins are being washed out as long as water is pumped through the precolumn. This allows the direct injection of body fluids. After a certain enrichment time (6 and 8 min), simultaneous rotation of the two valves is induced, causing precolumn 1, where the injected drug has been absorbed, to be switched to the solvent stream of pump B. Also, at this point, the recording integrator is started. Pump B delivers the mobile phase, necessary for separation and chromatography, and backflushes the sample from precolumn 1 onto the analytical column. Parallel to this process, precolumn 2 is switched to the water stream of pump A so that a sample can be injected and enriched while the previous one is being eluted (alternating mode). Volumes up to 1800 μl can be injected due to the concentration effect of the enrichment phase.

This system was applicable to the quantification of E_2, E_2-CDS, and E_2-Quat. The mobile phase for E_2-CDS was acetonitrile/water, 80:20, at a flow rate of 1.8 ml/min. Optimal peak shape and retention time for E_2 and E_2-Quat were obtained with pump B delivering a mixture of acetonitrile/

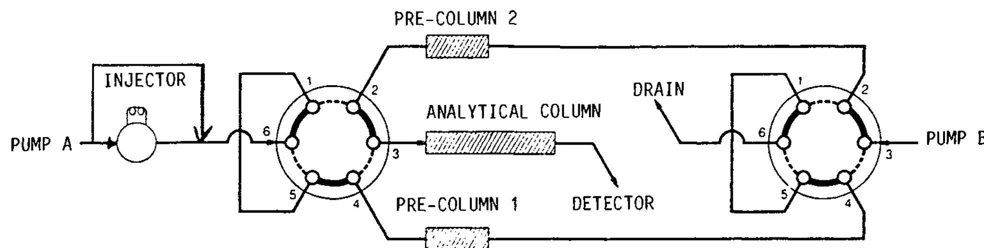

Fig. 2. Schematic representation of HPLC system with alternating precolumn enrichment. Pump A displaces sample from injector onto precolum for concentration. Pump B backflushes concentrated sample onto analytical column.

water, 42/58, which contained 0.025 *M* sodium salt of 1-octanesulfonic acid and 0.003 *M* tetrabutylammonium phosphate. The pH was adjusted to pH 5, and the flow rate was 1.5 ml/min.

Standard Solutions and Stability

Sample stock solutions of E_2-CDS, E_2-Quat, and E_2 were prepared in acetonitrile. All solutions were stored at 6°C. For E_2-CDS, the stock solution was prepared freshly every 2 weeks. All other solutions were stable over a period of at least 6 months. Spiked plasma samples containing all three compounds were frozen at −20°C and analyzed repeatedly at different time intervals. No loss of drug was found under these storage conditions during 2 months.

At room temperature, E_2-CDS was shown to be completely stable for 24 hr in solutions with a pH higher than 6.5. At pH 5, 88% loss was determined after 12 hr. It could be shown that Quat did not arise from acid degradation of the CDS.

Sample Preparation

Attempts to extract E_2-CDS from aqueous phases were not successful since the compound was shown to deteriorate unreproducibly during evaporation, even at room temperature and in the presence of oxygen-free nitrogen. Therefore, the compound had to be analyzed from biological fluids without an extraction procedure.

With the precolum enrichment technique described above, drugs can be detected from directly injected plasma without sample preparation. However, when large volumes are injected to obtain maximum sensitivity, it is desirable to remove proteins to a large extent prior to injection in order to prevent frequent precolumn packing.

Acidic precipitating agents, which remove proteins when only small volumes are added to biological fluids (18), could not be used because they induce degradative loss of E_2-CDS. Neutral or slightly basic aqueous reagents used efficiently for deproteinization such as $ZnSO_4$/NaOH, $CuSO_4$/Na_2WO_4, and saturated $(NH_4)_2SO_4$ (16) would be more suitable to be injected onto the enrichment columns than organic solvents. But all of these reagents were shown to adsorb E_2-CDS on the precipitate. Thus, the method of choice to avoid instability problems and at the same time keep all compounds in solution was deproteinization with acetonitrile.

The following sample preparation procedures were used for plasma and tissues. For plasma, 0.6 ml plasma was added to 1.2 ml acetonitrile. The mixture was vortexed for 5 sec and allowed to stand for 10 min at room temperature, vortexed again, and centrifuged for 10 min at 2000 rpm. Then 1000–1500 μl of the supernatant was injected into the precolumn enrichment system. For tissue (e.g., brain), 1 ml of water was added to one rat brain and the organ was thoroughly homogenized. After sonication for 2 min, 2 ml of acetonitrile was added. After centrifugation at 2000 rpm for 10 min, the supernatant (1000–1500 μl) was injected into the enrichment system.

Animal Study

Fifteen milligrams per kilogram of E_2-CDS dissolved in dimethyl sulfoxide (DMSO) was administered intravenously to conscious, restrained, male Sprague–Dawley rats (190–300 g). Animals were sacrificed in groups of four at 5, 15, and 30 min and at 1, 2, 4, 8, and 24 hr after drug injec-

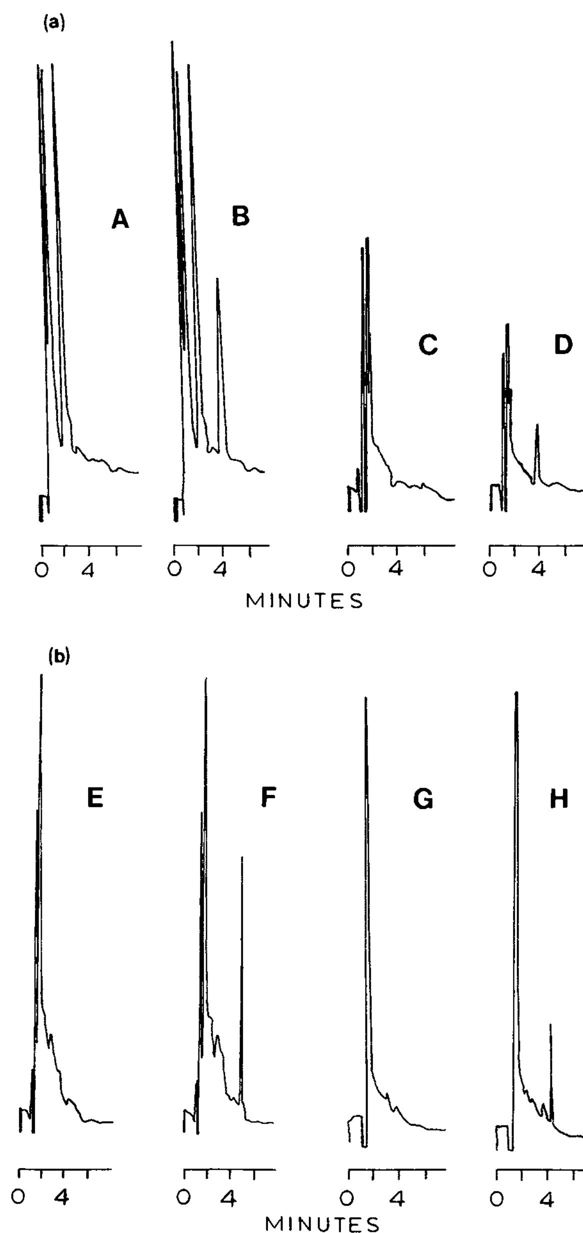

Fig. 3. Chromatograms of E_2-CDS and E_2-Quat in rat plasma and brain obtained after precolumn enrichment of the injected sample. Plasma was diluted 1:2 with acetonitrile and centrifuged prior to injection. Brains were homogenized in a mixture of 1 ml water and 2 ml acetonitrile. (a) E_2-CDS: (A) blank plasma; (B) plasma containing 200 ng E_2-CDS/ml; (C) blank brain; (D) brain containing 166 ng E_2-CDS/g brain tissue. (b) E_2-Quat: (E) blank plasma; (F) plasma containing 375 ng E_2-Quat/ml; (G) blank brain; (H) brain containing 128 ng E_2-Quat/g brain tissue. The injected volume was 1500 μl (A, B), 1200 μl (C, D, G, H), and 1000 μl (E, F).

tion. Trunk blood was collected into heparinized tubes, and plasma obtained and immediately frozen at −20°C until analysis. Organs were dissected and placed on dry ice within 2 min of death and stored at −20°C for later analysis.

RESULTS AND DISCUSSION

Chromatography

The determination of the optimum conditions for mobile-phase composition demonstrated that retention times of estradiol and E_2-CDS were affected only by the acetonitrile/water ratio in the mobile phase. Retention time and peak shape of E_2-Quat were significantly influenced by the concentration and composition of salts. An anion has to be added to form an ion pair with E_2-Quat and to shift its peak from the solvent front. However, with anions alone, relatively broad peaks were eluted. The addition of tetrabutylammonium ions, which are known to saturate unbound silanol groups of the stationary phase, markedly improved the peak shape and also decreased the retention time. Thus, the E_2-Quat peak could be shifted over a wide range of retention times by variation of the octanesulfonic acid/tetrabutylammonium ratio. With the mobile phase finally chosen for analysis, it was possible to obtain extremely sharp E_2-Quat peaks with very high sensitivity.

When standard solutions of E_2, E_2-CDS, and E_2-Quat in acetonitrile were first injected into the precolumn enrichment system, the measured peak areas decreased markedly when either one of the following three parameters was increased: (a) the time of sample concentration on the precolumn, (b) the flow rate of pump A delivering water, and (c) the injection volume of the sample. Keeping all other parameters constant, the injection of 30, 60, and 150 μl of a 50-μg/ml E_2-CDS solution resulted in peak area values of 1561, 2163, and 2463, respectively. The reason for this was an effect of the injected solution containing acetonitrile, which led to the formation of a pseudo-mobile phase on the precolumn. Consequently, the more acetonitrile was flushed through the enrichment column, the higher the degree of partial elution and thereby the loss of compounds. One necessary improvement made was the packing of the enrichment columns with the same absorption material used for the analytical columns instead of the larger-particle-size pellicular bulk packing material conventionally applied for guard columns. However, even with the higher retention capacity of this material, it was not possible to obtain a linear correlation between injection volume and area for volumes over 500 μl. The only successful approach seemed to be the reduction of acetonitrile in the injected solution and its replacement by water. It could be shown that volumes up to 1300 μl were injectable without area loss, if the amount of water in the injection vial was at least 70%. However, it was not possible to inject CH_3CN/H_2O mixtures because the low solubility of E_2-CDS would lead to precipitation and unreproducible results. Furthermore, the preparation procedure for plasma and biological fluids without extraction required more than 50% acetonitrile in the supernatant to be injected for efficient removal of proteins. A washout of compounds from such a supernatant during the enrichment phase could finally be avoided by the use of a tee piece that added a water flow to the sample immediately after its displacement from the injector but prior to the precolumn (Fig. 1). The flow rates of water pumped to the injector and added after injection were adjusted so that up to 1800 μl of all the investigated compounds could be analyzed without any loss.

Chromatograms of E_2-CDS and E_2-Quat obtained by this technique after rat plasma and brain preparation are shown in Fig. 3. E_2-CDS eluted at 4.1 min. and the retention time of Quat was 4.75 min. Interestingly, unlike for E_2-CDS, E_2-Quat retention was extremely sensitive to variabilities in the chromatography such as change of the mobile-phase composition, percentage of water in the injected sample, injection volume, or packing of precolumns. Increasing the percentage of water in the injection vial resulted, e.g., in earlier and sharper peaks, which is due to the drug being absorbed earlier on the enrichment column and thereby eluted faster in the backflush mode. These variations did not, however, affect the assay accuracy, since fresh calibration curves were injected on each day of sample analysis.

Estradiol could also be quantified by this method, but the sensitivity was not sufficient in any of the rat samples analyzed after a high-dose E_2-CDS administration. An extremely fast metabolic clearance has been reported for estradiol in rats (17) and makes HPLC detection in lower-dose

Table I. Accuracy and Precision for Spiked Plasma Samples

Compound	Medium	Sample concentration (μg/ml or μg/g)	Assayed concentration [mean (SD) $N = 4$] (μg/ml or μg/g)		Coefficient of variation (%)
E_2-CDS	Plasma	0.020	0.0196	(0.0011)	5.8
		0.2	0.19	(0.0099)	5.2
		1.0	0.95	(0.0655)	6.9
		3.0	3.09	(0.123)	4.0
	Brain	0.5	0.52	(0.04)	7.8
E_2-Quat	Plasma	0.010	0.010	(0.007)	7.5
		0.05	0.046	(0.0017)	3.8
		0.5	0.49	(0.010)	2.0
		5.0	5.28	(0.22)	4.2
	Brain	1.0	1.07	(0.072)	6.7

pharmacokinetic studies difficult. Therefore, investigation of estradiol was not pursued further in reproducibility and linearity studies.

Sensitivity and Linearity

The limits of accurate determination for plasma samples were 10 ng/ml for E_2-Quat, 20 ng/ml for E_2-CDS, and 50 ng/ml for estradiol. A comparison of the recovery of these drugs after the injection of spiked water and spiked plasma with different drug concentrations did not indicate any loss in plasma due to the deproteinization. Calibration curves for this method showed a linear relationship for CDS and Quat up to 30 μg/ml plasma.

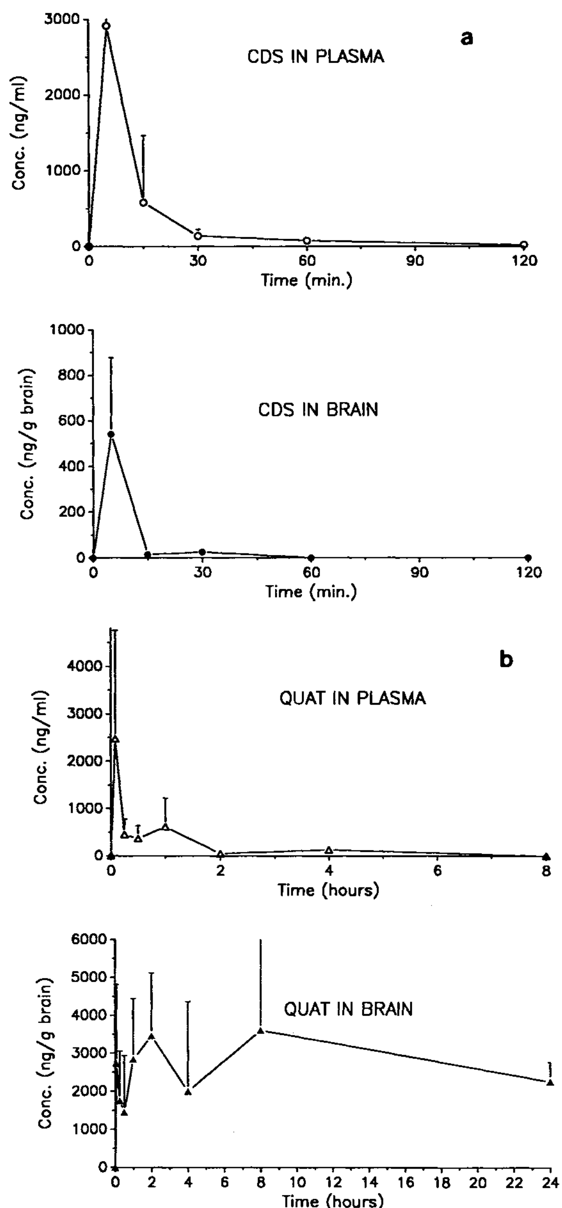

Fig. 4. Plasma and brain levels of (a) E_2-CDS and (b) E_2-Quat following intravenous administration of 15 mg/kg E_2-CDS in DMSO to rats. Each point represents the mean concentration of four different animals (+SD).

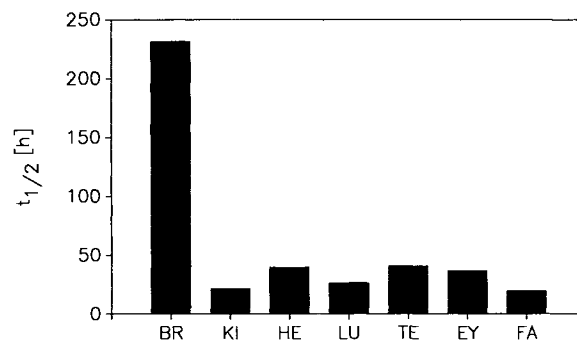

Fig. 5. Half-life of E_2-Quat in the various tissues analyzed: brain (BR), kidney (KI), heart (HE), lungs (LU), testes (TE), eyes (EY), and fat (FA).

The reproducibility of the deproteinization method with subsequent precolumn enrichment chromatography was evaluated for E_2-CDS and E_2-Quat by replicate analysis of spiked plasma and brain samples at different drug levels. The results in Table I demonstrate that the applied method is accurate and precise for quantification of both drugs in plasma and brain tissue.

Application

The precolumn enrichment technique was applied to determine E_2-CDS and E_2-Quat in rat plasma and brain after the intravenous administration of 15 mg/kg E_2-CDS in DMSO. Figure 4 shows the mean concentration–time curves obtained. At each time point, four animals were sacrificed. E_2-CDS was detected only up to 2 hr. Peak levels were about six times higher in plasma than in brain and dropped very fast in both of these media. The oxidized metabolite E_2-Quat showed high plasma concentrations after 5 min (up to 5 μg/ml) which decreased rapidly to low nanogram levels. Quat was detected in plasma up to 4 hr. In contrast, Quat levels in brain were fairly consistent over the whole time period studied. Even after 24 hr, a mean concentration of 2.26 μg/ml was maintained. The half-life in brain was estimated to be significantly longer than in the other tissues studied (Fig. 5).

This study clearly indicates that E_2-Quat is retained in the brain for a much longer period than in the periphery after a single administration of the CDS. Our data confirm the postulated concept of CNS-targeted estrogen delivery and anticipate promising results in further and more detailed pharmacokinetic studies.

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REFERENCES

1. N. Bodor, H. H. Farag, and M. E. Brewster. *Science* 214:1370–1373 (1981).
2. N. Bodor and M. E. Brewster. *Pharmacol. Ther.* 19:337–386 (1983).

3. N. Bodor and H. H. Farag. *J. Med. Chem.* **26**:313–318 (1983).
4. N. Bodor and J. W. Simpkins. *Science* 221:65–67 (1983).
5. N. Bodor and H. H. Farag. *J. Pharm. Sci.* 73:385–389 (1984).
6. J. W. Simpkins, N. Bodor, and A. Enz. *J. Pharm. Sci.* 74:1033–1036 (1985).
7. N. Bodor. In H. Bundgaard (ed.), *Design of Prodrugs,* Elsevier, Amsterdam, 1985, pp. 333–354.
8. N. Bodor. In K. Widder (ed.), *Methods in Enzymology, Drug and Enzyme Targeting,* Academic Press, New York, 1985, pp. 381–396.
9. N. Bodor, J. McCornack, and M. E. Brewster. *Int. J. Pharm.* **35**:47–59 (1987).
10. J. W. Simpkins, J. McCornack, K. S. Estes, M. E. Brewster, E. Shek, and N. Bodor. *J. Med. Chem.* **29**:1809–1812 (1986).
11. K. Estes, M. E. Brewster, J. Simpkins, and N. Bodor. *Life Sci.* **40**:1327–1334 (1987).
12. R. L. Goodman, and E. Knobil. *Neuroendocrinology* **32**:57–63 (1981).
13. F. Labrie, A. Dupont, A. Belanger, F. A. Lefebyre, L. Cusan, G. Monfette, J. G. Leberge, J. P. Emond, J. P. Raymond, J. M. Husson, and A. T. A. Fazekas. *Steroid Biochem.* **19**:999–1007 (1983).
14. S. S. C. Yen. *J. Reprod. Med.* **18**:287–289 (1977).
15. W. Roth, K. Beschke, R. Jauch, A. Zimmer, and F. W. Koss. *J. Chromatogr.* **222**:13–22 (1981).
16. J. Blanchard. *J. Chromatogr.* **226**:455–460 (1981).
17. J. M. Larner and R. B. Hochberg. *Endocrinology* **117**:1209–1214 (1985).