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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ECLANAMINE AND ITS N-DESMETHYL AND N,N-DIDESMETHYL METABOLITES IN URINE

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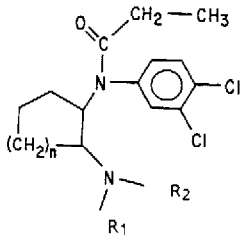
SUMMARY

A high-performance liquid chromatographic method has been defined for the determination of eclanamine (free base of eclanamine maleate) and two of its metabolites, N-desmethyleclanamine and N,N-didesmethyleclanamine in urine. The method employs 10-ml urine samples, has a linear range from 5 to 500 ng/ml for the three compounds, and has a detection limit of 0.5 ng/ml for each compound. Sample preparation uses a cyanopropylsilane extraction column with washes of water, acetonitrile–water (30:70, v/v), and acetonitrile, and elution with 2% trifluoroacetic acid in acetonitrile. The eluate is evaporated to dryness, the residue dissolved in 1.0 ml acetonitrile–water (10:90, v/v) and 100 μl are injected onto a Supelcosil LC-CN column. Eclanamine and its metabolites are eluted with an acetonitrile–water (35:65, v/v) eluent containing 0.01 *M* triethylamine and adjusted to pH 7.0 with phosphoric acid. The method has been validated by preparing and analyzing a series of fortified urines (range 2–500 ng/ml for each compound) on four separate days. Good linearity, precision, reproducibility, and specificity were obtained. Certification of the analytical method was accomplished by analyzing urine specimens collected from one volunteer administered a single oral dose of 45 mg eclanamine maleate. The data suggest that the metabolites of eclanamine have long elimination half-lives with levels still quantifiable in the 72–96 h collection interval.

INTRODUCTION

Eclanamine maleate has reported antidepressant-like activity in animal models [1,2]. This activity was studied by comparing the interactions of this drug with biogenic amine systems, and the data suggested that eclanamine (E, free base of eclanamine maleate, Fig. 1) was a more potent antidepressant than imipramine and had a more pronounced effect upon α_2-adrenergic receptor sensitivity.

To evaluate the pharmacokinetic and metabolic profile of this potential antidepressant drug, sensitive, precise, and specific analytical methodology is required since the expected therapeutic dose level is below 5 mg per day. Previous



E	$R_1, R_2 = CH_3$, n = 1
DME	R_1 = H, $R_2 = CH_3$, n = 1
DDME	R_1, R_2 = H, n = 1
IS	$R_1, R_2 = CH_3$, n = 2

Fig. 1. Structures of E, DME, DDME, and the I.S.

efforts to define analytical methods have been directed toward serum sample analysis [3–5]. These studies demonstrated that eclanamine maleate administered to animals was metabolized to at least two pharmacologically active metabolites, N-desmethyleclanamine (DME, Fig. 1) and N,N-didesmethyleclanamine (DDME, Fig. 1). Also, the apparent terminal disposition rate constants for the metabolites were shown to be small resulting in long half-lives, i.e. 24–48 h, in the animal models.

Little information on the elimination pathway for E and its known metabolites has been reported. Results from tracer studies in animals [6] suggested that urinary elimination is one pathway of elimination for drug-related material. The analysis of urine specimens for E, DME, and DDME levels would provide information on the elimination pathways for the drug candidate and its known metabolites, provide kinetic data on the rate and extent of urinary excretion, and possibly assist in the isolation and identification of other E metabolites. This report presents the development of a high-performance liquid chromatographic (HPLC) method for the quantitative determination of E, DME, and DDME in urine.

EXPERIMENTAL

Apparatus

The isocratic HPLC system employed to detect and quantify E, DME, and DDME in urine specimens consisted of a ConstaMetric III pump and a UV Monitor III detector with a 214-nm filter [Laboratory Data Control (LDC), Riviera Beach, FL, U.S.A.], a Model 7126 variable-loop (100 μl) injector (Rheodyne, Cotati, CA, U.S.A.) mounted on a μP autosampler (Upjohn, Kalamazoo, MI, U.S.A.), and a Model 585 dual-pen recorder (Linear Instruments, Irvine, CA, U.S.A.). The data were collected and processed using a Harris computer system.

The urine samples were processed using a Spe™ cyano disposable column (J.T. Baker, Phillipsburg, NJ, U.S.A.) and a solid-phase extraction vacuum manifold

(Supelco, Bellefonte, PA, U.S.A.). Prepared samples were evaporated to dryness using a drying tray at 30°C and nitrogen gas with individual control for the gas stream.

HPLC conditions

The HPLC analytical column was a Supelcosil LC-CN, 5 μm, 250 mm × 4.6 mm I.D. (Supelco) combined with a Pelliguard LC-CN, 35 μm, 50 mm × 2.1 mm I.D. guard column (Whatman, Clifton, NJ, U.S.A.). The eluent was acetonitrile–water (35:65, v/v) containing 0.01 *M* triethylamine (TEA) and adjusted to pH 7.0 with phosphoric acid; the flow-rate was 1 ml/min; and the column temperature was ambient. The eluent was filtered through a Nylon-66, 0.2-μm filter (Rainin Instruments, Woburn, MA, U.S.A.) and helium-degassed prior to use. The prepared eluent was stable for a minimum of seven days.

Chemicals and reagents

E, DME, DDME, and the internal standard (I.S., a structural analogue of E, Fig. 1) were provided by The Upjohn Company. TEA (99%) and trifluoroacetic acid (TFA, 99%) were obtained from Aldrich (Milwaukee, WI, U.S.A.); phosphoric acid (85%) from Mallinckrodt (Paris, KY, U.S.A.); and acetonitrile (UV grade, distilled in glass) from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was filtered through a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Stock solutions

Approximately 10 mg of accurately weighed free base equivalents of E, DME, DDME, and I.S. were placed in individual 100-ml volumetric flasks, dissolved and diluted to volume with water. A working stock of E, DME, and DDME was prepared by quantitatively pipetting 10 ml of each free base stock into a 100-ml volumetric flask and diluting to volume with water. Concentrations of E, DME, and DDME in the working stock were 10 μg/ml. The working stock was used to prepare reference standards and to fortify urine samples. A working stock of I.S. was prepared in a similar procedure. The stock solutions of the compounds and the I.S. were stable for a minimum of three months when stored at 4°C.

Reference standard solutions were prepared by adding 2–500 μl of the working stock (20–5000 ng of E, DME, and DDME) and 100 μl of the working I.S. stock (1000 ng of I.S.) to a 1-ml autoinjector vial and diluting to 1 ml with acetonitrile–water (10:90, v/v).

Sample preparation

The procedure used to prepare urine samples for injection onto the HPLC system consisted of solid-phase extraction using cyano columns. Each column was activated with 5 ml acetonitrile and washed with 5 ml water. A 10-ml urine sample containing 1000 ng I.S. was loaded onto a column at a flow-rate of about 0.5 ml/min. After loading, the column was washed with 5 ml water, 5 ml acetonitrile–water (30:70, v/v), and 5 ml acetonitrile. The compounds and I.S. were eluted

from the column with 3 ml of 2% TFA in acetonitrile. The eluate was evaporated to dryness and the residue dissolved in 1 ml acetonitrile–water (10:90, v/v). After transferring to an autoinjector vial, 100 μl were assayed by HPLC.

Fortified urines were prepared by adding 2–500 μl of the working stock to 10-ml urine samples to give a concentration series containing between 2 and 500 ng/ml E, DME, and DDME.

Urine samples

The sample preparation procedure and HPLC analytical technique were validated using control human urine. The method was certified using human urine specimens obtained from a volunteer receiving a 45-mg single dose of eclanamine maleate (32 mg E). Urine specimens were collected prior to dosing and at 0–1, 1–2, 2–4, 4–8, 8–12, 12–24, 24–36, 36–48, 48–72, and 72–96 h intervals after dosing. The volume from each interval was measured, an aliquot taken, frozen, and maintained at −20°C until analysis. Reanalyses of the selected specimens after storage at −20°C for three months showed similar levels for E, DME, and DDME. Thus, no degradation of the parent drug or metabolites occurred during storage for the three-month period.

Calculations

The concentrations of E, DME, and DDME in fortified urines and in urine specimens obtained from a human volunteer were calculated using the relative weight response (RWR) to an internal standard method.

RESULTS AND DISCUSSION

HPLC parameter definition

The HPLC conditions selected for the analysis of E, DME, and DDME in urine were defined using a gradient system. Initial evaluations using an octadecylsilyl column without ion-pairing agents gave poor peak shapes and long retention times for the three compounds. Addition of a cationic (TEA) or an anionic (hexanesulfonic acid) ion-pairing agent gave much improved peak shapes but did not substantially reduce the retention volume. By increasing the polarity of the HPLC column by using a cyano column in the reversed-phase mode, the retention time was reduced without affecting the peak shape. The LC-CN column gave sharper peaks than a Supelcosil LC-18 or an IBM ODS column and was selected for further studies. TEA was acceptable as a tertiary amine for the eluent and at a neutral pH, complete separation of E, DME, DDME, and the I.S. was achieved. After the general characteristics of the analytical system had been selected, optimization studies of the organic modifier, TEA, and pH for the eluent were conducted. The TEA content did not greatly affect the elution volume, peak shape, or separation when above 0.002 *M* (0.25 ml TEA per liter eluent). The pH also did not affect the elution characteristics of the four compounds when maintained above pH 5.5 and below pH 8.0. At pH values below 5.5, the DME and DDME were retained by the column and had poor peak shape. Changes in the organic modifier level resulted in increased or decreased retention volumes but did not

change the elution order or the peak shape. To allow complete elution of potential interfering components from urine, a total chromatographic time of 20 min was selected. The eluent described in Experimental gave these characteristics.

The UV spectra of E, DME, and DDME do not contain an absorbance maximum above 230 nm with sufficient intensity for quantitation at the low nanogram on-column level. A wavelength of 214 nm was selected for quantitation in order to use a fixed-wavelength detector. This wavelength was on the slope of the UV spectra for each compound and thus slight changes in the wavelength might result in increased or decreased response for the four compounds.

Stability

The stability of E, DME, and DDME was evaluated in water and HPLC eluent at room temperature, in water and urine at 4 °C, and in urine at −20 °C. No loss of the three compounds was observed when stored in water or eluent at room temperature and in the light for seven days. Water or urine stored at 4 °C showed no apparent loss for the three compounds after 60 days of storage, and urine specimens stored at −20 °C were stable for at least 120 days.

Urine sample preparation

Procedures utilized to prepare serum samples for E, DME, and DDME analysis included liquid–liquid extraction [3], solid-phase extraction [4], or a combination of the two [5]. Since a relatively large urine specimen was considered necessary to obtain the desired sensitivity, i.e. 2–10 ng/ml, liquid–liquid extraction was not considered the method of choice. Solid-phase extraction with C_{18}, C_8, CN, and phenol cartridges was evaluated. With each column type, the three compounds were completely retained and could not be eluted even with 100% organic eluents. Because the analytical column was CN, additional studies were conducted using the CN cartridge. HPLC eluent did not elute the compounds from the CN cartridge; however, when the pH of the eluent was adjusted to 2.0, the compounds were partially eluted. Further evaluations showed that complete elution was obtained using 2% TFA in acetonitrile. When the level of TFA was reduced to 0.5%, elution was not complete. Thus, E, DME, and DDME were retained on the CN cartridge most likely through interaction with free silanol groups and could be washed with acetonitrile prior to elution with 2% TFA in acetonitrile. The procedure resulted in injecting the equivalent of 1.0 ml of urine on-column when the initial sample size was 10 ml urine.

Validation

The analytical method for the HPLC–UV determination of E, DME, and DDME was validated by preparing and analyzing a series of fortified control urines on four separate days. The fortification levels were 2, 5, 10, 15, 25, 50, 75, 100, 300, and 500 ng/ml and the urine sample size was 10 ml resulting in sample range of 20–5000 ng of each compound per sample. The results of the validation studies are summarized in Table I. Least-squares linear regression evaluation of the validation results gave the following equations:

TABLE I

ACCURACY AND PRECISION OF URINE ANALYSIS FOR E, DME, AND, DDME

N.D. = not detectable, less than 0.5 ng/ml; R.S.D. = relative standard deviation; in all cases, $n = 4$.

Amount added (ng/ml)	Average amount found (ng/ml)					
	E		DME		DDME	
	Level	R S.D. (%)	Level	R.S.D. (%)	Level	R.S.D. (%)
0	N.D.	–	N.D.	–	N.D.	–
2	2.2	6.8	3.0	17.2	2.6	23.2
5	5 1	5.8	5.6	7.8	5.4	11.4
10	9.7	3.9	10.6	6.3	10.8	7.7
15	14.6	2.8	15.4	3.1	16.0	7.8
25	24.6	4.8	25.4	3.7	26.4	7.1
50	50.0	2.1	52.0	5.9	52.9	7.5
75	76.3	5.2	77.3	3.7	79.2	7.5
100	102	3.4	102	3.0	106	7.1
300	305	3.8	310	4.0	320	7.9
500	511	1 9	521	2.1	535	6.2

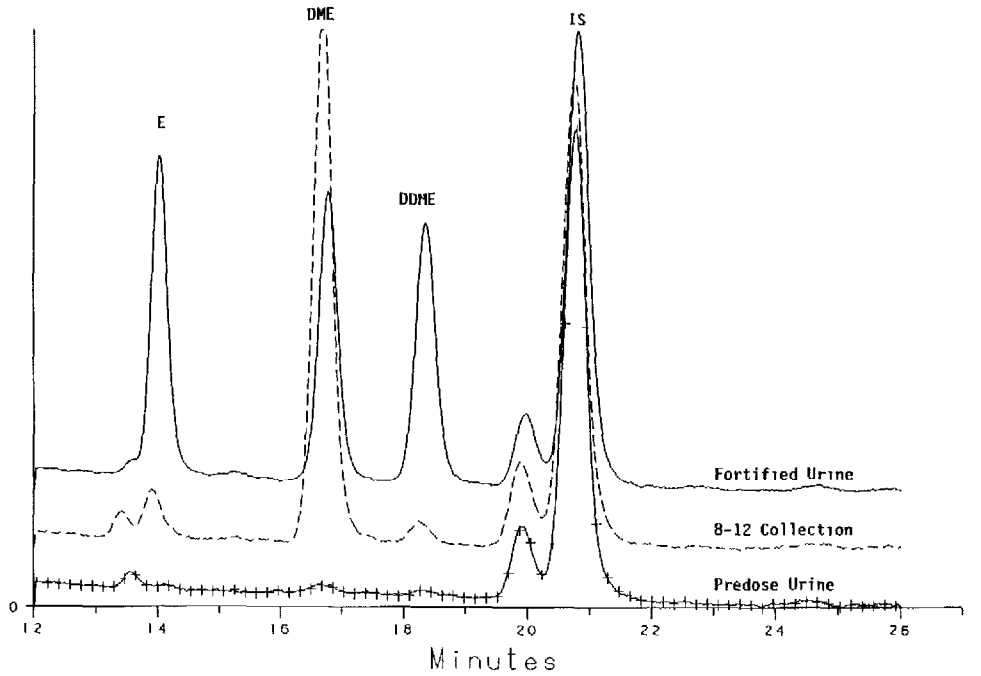


Fig. 2. Representative HPLC-UV (214 nm) profiles of a urine fortified with 50 ng/ml E, DME, and DDME (——), the predose urine (+ + + +), and the 8–12 h collection urine (- - - -) from a volunteer administered 45 mg eclanamine maleate.

TABLE II

N, DME AND DDME LEVELS IN HUMAN URINE SPECIMENS

Dose 45 mg eclanamine maleate. N.D. = not detectable, less than 5 ng per sample; tr = trace, between 5 and 50 ng per sample

Collection interval (h)	Volume (ml)	E		DME		DDME		Cumulative	
		Collection (μg)	Cumulative (μg)	Collection (μg)	Cumulative (μg)	Collection (μg)	Cumulative (μg)	Total* (μg)	Percentage of dose
−12- 0	660	tr	–	N.D.	–	N.D.	–	–	
0- 1	285	N.D.	0	N D.	0	N.D.	0	0	–
1- 2	280	3.8	3 8	32.5	32 5	5 2	5.2	43.5	0 1
2- 4	525	5 4	9 2	192.7	225.2	25 7	30.9	278	0 8
4- 8	620	6 0	15.2	362 2	587 4	67.4	98.3	736	2 2
8-12	520	9 5	24.7	529 0	1116	122.2	220 5	1432	4 4
12-24	694	10.2	34.9	1161	2277	391.2	611 7	3082	9.4
24-36	2070	N.D.	34.9	748 9	3026	376.5	988 2	4276	13.0
36-48	590	8.0	42.9	597.4	3623	435.3	1424	5384	16.4
48-72	270	N.D.	42.9	276.8	3900	399.2	1763	6044	18.4
72-96	650	N.D.	42.9	345 6	4246	580.2	2343	7040	21.5

*Total expressed as E equivalents

E: $y = (1.022 \pm 0.028)x - (0.50 \pm 5.1)$, $r^2 = 0.999$; DME: $y = (1.040 \pm 0.030)x - (0.20 \pm 5.5)$, $r^2 = 0.999$; DDME: $y = (1.068 \pm 0.075)x - (0.29 \pm 13.7)$, $r^2 = 0.995$, where y is the concentration found (ng/ml) and x is the concentration added (ng/ml).

Thus, excellent linearity and relative recovery were observed for the three compounds from 5 to 500 ng/ml and the intercepts of the linear regression equations were not statistically different from zero. The precision of the 2 ng/ml fortified samples was not considered sufficient to quantitate at that level. Based on these data, the quantification range for the determination of E, DME, and DDME in urine was 5–500 ng/ml. Levels at 0.5 ng/ml were detectable; thus, between 0.5 and 5 ng/ml was considered as trace levels. Urine from four individuals was evaluated for extraneous peaks eluting with the same retention characteristics as E, DME, and DDME, or the I.S. For each individual, no peaks were detected at levels greater than 2 ng equivalents per ml for the four compounds indicating good specificity for the method. Representative HPLC–UV (214 nm) profiles of a fortified urine and the predose urine and 8–12 h urine collection from a human volunteer receiving 45 mg eclanamine maleate are shown in Fig. 2.

Certification

The defined analytical method was used to analyze the urinary levels of E, DME, and DDME in a human volunteer receiving a single oral dose of 45 mg eclanamine maleate (32 mg E). Urine specimens were collected prior to dosing and at 0–1, 1–2, 2–4, 4–8, 8–12, 12–24, 24–36, 36–48, 48–72, and 72–96 h after dosing. The levels of E, DME, and DDME in the urine specimens are given in Table II. The cumulative urinary excretion levels of E, DME, and DDME and total are shown in Fig. 3. The total was calculated by combining the levels of E and of DME and DDME corrected to E equivalents. Thus, the total was in E

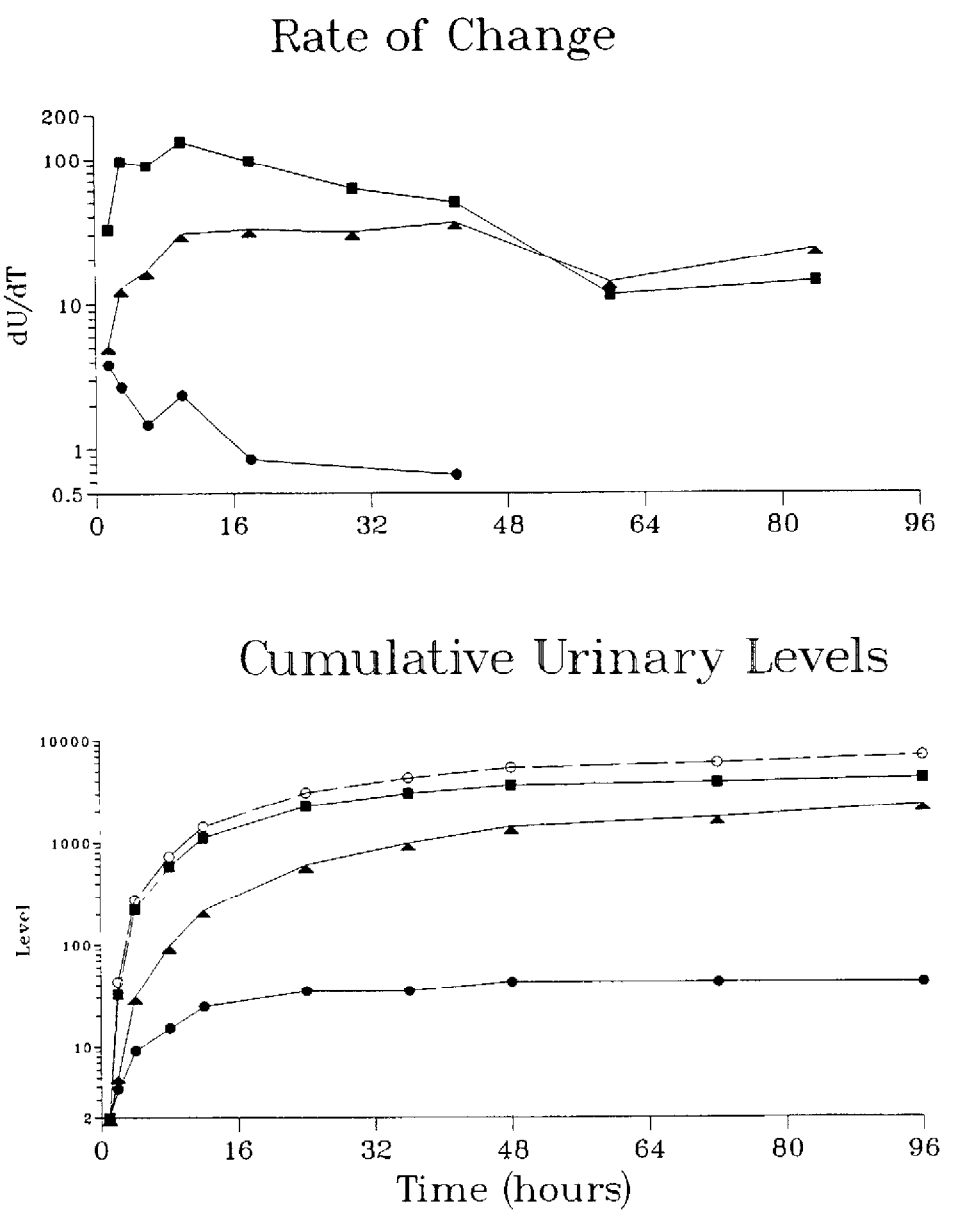


Fig. 3. Cumulative urinary excretion levels and the rate of change for E, DME, and DDME in a volunteer receiving 45 mg eclanamine maleate. Level in micrograms. ●, E; ■, DME; ▲, DDME; ○, total (E equivalents)

equivalents and the percentage of dose was determined using the amount of E administered. After 96 h, 21.5% of the administered dose was eliminated in the urine as the total of the three compounds. Also in Fig. 3 are the rates of change (dU/dT, the difference of two urinary levels $U_2 - U_1$, over a time interval, $T_2 - T_1$) for the urinary excretion of E, DME, and DDME. Least-squares linear regression of the log of dU/dT versus the mid point of the collection interval for the points after absorption for E and after formation for DME and DDME provided esti-

TABLE III

PHARMACOKINETIC PARAMETER ESTIMATES

Parameter	E	DME	DDME
U_T (0–96 h) (μg)	42.9	4246	2343
Percentage of dose*	0.1	13.5	7.8
k_e (h^{-1})	0.038	0.034	0.008
$t_{1/2}k_e$ (h)	18	20	85

*Each compound converted to eclanamine maleate equivalents to calculate percentage of dose excreted.

mates of the elimination rate constants (k_e) for the metabolites [7]. The curves in Fig. 3 suggest that 96 h after dosing the elimination of DME and DDME was not complete. The amount of each compound excreted, the percentage of dose, k_e, and the half-life ($t_{\frac{1}{2}}k_e$) are listed in Table III for this subject.

CONCLUSION

The results obtained from the human volunteer indicate that the developed analytical method has the necessary sensitivity to detect levels of E, DME, and DDME excreted in the urine. The results from the volunteer suggest that the method may be used to determine pharmacokinetic parameters such as estimates of the apparent terminal disposition rate constant and its half-life and the percentage of the administered dose eliminated in the urine. However, the total collection period after the single oral dose was not sufficient for the complete elimination of the two E metabolites, DME and DDME, from the body.

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