Time-Dependent Kinetics II: Diurnal Oscillations in Steady-State Plasma Ethosuximide Levels in Rhesus Monkeys

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Abstract □ Morning steady-state (9 am) plasma levels were significantly higher than the corresponding evening (5 pm) plasma levels during a 3-week zero-order infusion of ethosuximide to six monkeys. These differences could not be explained by experimental variables such as GLC assay and infusion pump. Circadian periodicity in steady-state plasma levels was investigated in three monkeys over 4 months under controlled experimental conditions: blood sampling at 2-hr intervals for 26 hr, 1 day/week; fixed lighting, feeding, and noise schedules; and electroencephalogram monitoring. The plasma concentration–time curves showed two minima in the 12 noon–2 pm and 8 pm–12 midnight periods, and the later involved the largest percent change in plasma levels (4–8%). The plasma concentration–time data were subjected to cross-correlation analysis, which indicated a circadian rhythm in steady-state plasma levels with a period of 24–26 hr.

Keyphrases □ Time-dependent kinetics—ethosuximide, circadian rhythm in steady-state plasma levels, monkeys □ Ethosuximide—circadian rhythm in steady-state plasma levels, monkeys □ Pharmacokinetics—ethosuximide, circadian rhythm in steady-state plasma levels, monkeys □ Circadian rhythm—in steady-state plasma levels of ethosuximide, monkeys □ Anticonvulsants—ethosuximide, circadian rhythm in steady-state plasma levels, monkeys

Time dependency in pharmacokinetics involves several types of phenomena. Previous studies dealt with the self-induced decreases in steady-state concentrations of carbamazepine during chronic administration of the drug to humans (1) and monkeys (2). More recently, the terms "cronodisponibilita" (3) (equivalent to chronoavailability) and "chronopharmacokinetics" (4) have been coined to stress the importance of temporal changes in bioavailability and pharmacokinetics, respectively.

Diurnal variations in excretion rates of several acidic and basic drugs (*e.g.*, salicylate, sulfasymazine, and amphetamine) were reported (5–7). In several instances, these variations could be explained on the basis of circadian rhythms in kidney functions (8–11). Diurnal fluctuations in plasma levels of dipyridamole could be related to circadian changes in GI absorption (12).

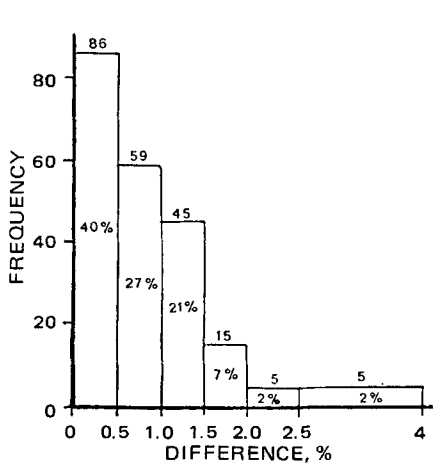

Figure 1—*Plot of frequency* versus *difference (percent) between duplicate assay determinations.*

In a previous study (13) designed to test for time dependency in elimination kinetics of ethosuximide in monkeys, the drug was chronically infused for 22 days to six animals. Morning (9 am) plasma concentrations were consistently higher than evening (5 pm) concentrations, and the presence of circadian rhythm in steady-state plasma levels was suspected. The present study was undertaken to elucidate this phenomenon under more rigorously controlled experimental and, particularly, environmental conditions. These conditions included frequent blood sampling (2-hr intervals); duplicate GLC assays; infusion pump monitoring; fixed lighting, feeding, and noise schedules; and electroencephalogram (EEG) monitoring (sleep staging).

EXPERIMENTAL

Experimental Subjects—Three chronically catheterized male rhesus monkeys (Animals 903, 923, and 983) were used. These monkeys were previously used to determine the pharmacokinetics of ethosuximide under single-dose and chronic dosing conditions (13, 14).

EEG Monitoring—Screw electrodes[1], 3 mm, were bilaterally implanted in the mastoid, occipital, central, and inferior lateral orbital regions of the skull. The left mastoid electrode served as a reference electrode. Electroculograms were recorded for each eye with respect to the reference electrode. Electromyograms were recorded from the temporal muscles. Electroencephalograms (EEG) were monitored from the right central electrode to the left mastoid electrode.

All leads were crimped to a miniature 16-pin socket, and the assembly was fixed to the skull with 3-mm screws[1] and cranioplastic fluid[2]. A chronic, shielded, flexible cable connected the leads of the socket assembly to the four-channel EEG machine[3].

The sleep stages were determined from the EEG data using a previously described procedure (15).

Environmental Conditions—The animals were housed in a room with glass windows and diurnal lighting (light during "day" period, 6 am–6 pm; dark during "night" period, 6 pm–6 am). They were fed twice a day, at 6 am (monkey chow) and 5:30 pm (monkey chow and fresh fruit). The glass windows were covered to prevent penetration of external light. The jugular and femoral catheters, as well as the EEG cable of each animal, were carried to an adjacent room through holes drilled in the glass window.

Infusion of drug solutions, blood sampling, and EEG monitoring were performed in the adjacent room. Continuous 24-hr music (moderately high volume, 6 am–6 pm; low volume, 6 pm–6 am) aided in the masking of external noise during the study. Activities in the monkey room and the adjacent room were kept to a minimum.

Experimental Protocol—The animals received a continuous zero-order infusion[4] of ethosuximide in normal saline over 4 months (infusion rate, 3.72–4.74 mg/hr; steady-state plasma concentration, 30–55 μg/ml). Plasma samples of 0.4 ml were collected 1 day/week at 2-hr intervals for 26 hr for 5 weeks. Fractions of 0.1 ml of the plasma samples were assayed for ethosuximide in duplicate using the GLC assay of Dill *et al.* (16). Only

[1] Vitallium, Howmedica Inc., Rutherford, N.J.
[2] Codman and Shartleff, Inc., Randolph, Mass.
[3] Grass model III-D, Grass Instruments Co., Quincy, Mass.
[4] Holter infusion pump, Extracorporeal Medical Specialties, King of Prussia, Pa.

Table I—Statistical Comparison[a] of Morning (9 am) and Evening (5 pm) Plasma Concentrations[b] (Micrograms per Milliliter) of Ethosuximide in Monkeys

	Stage 1			Stage 2			Stage 3		
Animal	Morning	Evening	p	Morning	Evening	p	Morning	Evening	p
733	23.24	22.20	0.05	—	—	—	—	—	—
743	26.30	24.85	0.01	53.84	51.40	0.01	86.00	79.29	0.05
753	27.21	26.00	0.05	50.12	49.93	NS	78.67	78.54	NS
903	25.10	23.17	0.01	50.08	46.01	0.01	76.97	72.05	0.01
923	29.90	28.75	NS[c]	56.10	54.12	0.01	89.40	86.45	0.01
983	28.99	27.51	NS	—	—	—	—	—	—
Mean	26.79	25.41	0.01	52.54	50.37	0.01	82.76	79.09	0.01

[a] Paired t-test. [b] Each concentration value represents a mean of six to eight determinations, excluding the concentrations obtained during the first 24 hr of each stage. [c] Not significant at $p = 0.05$.

3 weeks of data for Animal 983 could be obtained because of technical difficulties with the catheters.

Continuous EEG monitoring was performed from 10 am to 10 am or from 10 pm to 6 am with and without blood sampling.

RESULTS

Preliminary Observations—Previously (13), it was pointed out that morning steady-state plasma concentrations of ethosuximide were consistently higher than the corresponding evening concentrations during a three-stage zero-order infusion of the drug to six monkeys. A statistical evaluation of the differences between morning and evening concentrations was performed (Table I). The differences between morning and evening plasma concentrations ranged from 3.8 to 7.7% in Stage 1, from 0.4 to 8.1% in Stage 2, and from 0.2 to 7.8% in Stage 3. These differences were significant in the majority of animals in each stage. However, these differences were of a small magnitude and could be explained by fluctuations in several experimental variables (*e.g.*, drug assay and infusion pump). Thus, it became necessary to evaluate the potential contribution of these experimental factors to the observed differences in plasma concentrations.

The precision of the GLC assay was calculated from duplicate determinations of approximately 200 plasma samples (concentration range of 30–55 μg/ml), and a frequency plot was constructed from these data (Fig. 1). The percent difference was less than 1% in 67% of the cases, 1–2% in 28% of the cases, and 2–4% in the remaining instances. These results indicated that the GLC procedure can establish differences in plasma concentrations of 4–7%. Furthermore, morning and evening plasma samples were always assayed simultaneously (within the same run). Since assay error would tend to be random, it could not explain the observed systematic differences between morning and evening plasma concentrations.

Fluctuations in the voltage of the electrical line could influence the operational pump voltage, rotational speed, and, consequently, delivery rate. The delivery rates of two infusion pumps were determined by recording the volume delivered between 9 am and 5 pm and between 5 pm and 9 am for 5–10 consecutive days. These measurements utilized six different pump chambers. In three instances, delivery rates increased during the 5 pm–9 am period (0.3–0.7%); in the other three, they decreased (0.5–2.4%). There were no significant differences between the delivery rates of the 9 am–5 pm and 5 pm–9 am periods in five of six cases.

These experiments suggested that experimental variables such as infusion pump and drug assay did not contribute to the observed fluctuations in steady-state plasma levels. However, the sampling schedule employed in the previous study (13) was limited. Therefore, additional studies were undertaken where steady-state plasma levels were obtained at 2-hr intervals over 26 hr under rigorously controlled experimental conditions.

EEG–Time and Plasma Concentration–Time Data—EEG data were collected to determine the sleeping behavior of monkeys during day and night periods. The data indicated that the animals were awake during most of the day periods and slept an appreciable portion of the night periods (Fig. 2 and Table II).

Typical diurnal variations in steady-state plasma levels for Animals 903, 923, and 983 during a single sampling period are represented in Figs. 3A–5A. The observed oscillations were reproducible within a given animal over 3–5 weeks. Consequently, plasma concentrations were averaged, and plots of mean percent change in plasma level (using the 8 am concentration as a reference value) as a function of time were constructed (Figs. 3B–5B). Certain features were present in the profiles of all animals: a decline from 8 am to 2–4 pm, a small rise or plateau between 4 and 6 pm, and a steady decline until 8 pm–12 midnight followed by a steady rise until 8–10 am (next day). Thus, in each plot, there appeared to be two minima, in the 12 noon–2 pm and 8 pm–12 midnight time periods. However, the minimum occurring during the night period involved the largest percent change in plasma levels (4–8%).

The evidence suggesting a circadian rhythm in steady-state plasma levels can be summarized as follows:

1. The earlier multistage infusion studies (13) indicated that the pattern of oscillations in plasma levels was similar on several consecutive days (up to 3 weeks).
2. The percent change in plasma concentration–time profiles obtained in the present study defined the actual time course of steady-state plasma levels within a 26-hr period.
3. The concentrations observed in the 8–10 am period on 2 consecutive days were quite close.
4. The time course of steady-state plasma levels within a 26-hr period was reproducible on successive weeks (on the same day of the week).

Several techniques (moving variate analysis, Fourier analysis, auto- and cross-correlation, and cosinor analysis) are available to estimate the

Table II—Sleeping Behavior of Rhesus Monkeys during Night Period[a], 10 pm–6 am

	Percent of Night Period Spent in Stage					
Animal	0	1	2	3	4	REM
903[b]	31	27	15	10	9	8
923[b]	28	27	20	10	7	9
983[c]	38	22	2	14	17	7

[a] Calculated from continuous EEG data obtained from 10 pm to 6 am. [b] The values shown for Animals 903 and 923 represent the mean values of five night periods. [c] The values obtained for Animal 983 are from a single night period.

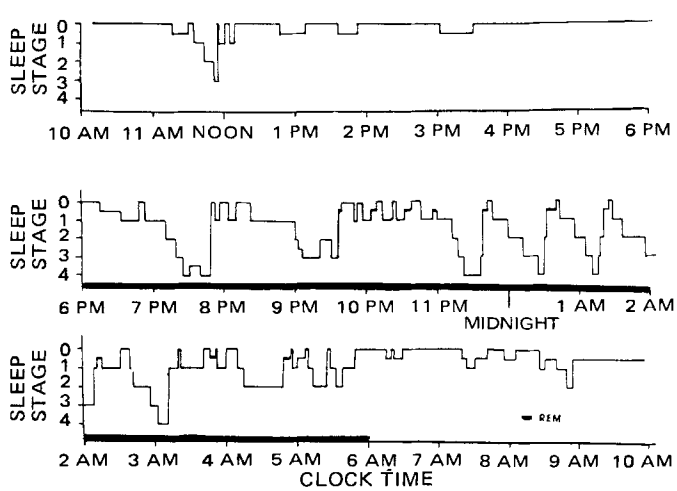

Figure 2—*The 24-hr sleep pattern of Animal 903.*

Table III—Cross-Correlation Analysis of Experimental Data

Animal	Week	Estimated Value of Period, hr	Estimated Value of Amplitude, %
903	1[a]	34.5	0.92
	2	26.0	2.62
	3	29.5	1.77
	4	25.5	1.13
	5	24.5	1.55
	Mean	28.0	1.60
923	1[a]	27.0	3.86
	2	24.0	4.50
	3	25.5	2.28
	4	26.0	2.75
	5	21.5	2.80
	Mean	24.8	3.24
983	1[a]	21.0	2.09
	2	39.5	2.65
	3	25.0	3.99
	Mean	28.5	2.91
903	Mean profile	25.5	1.24
923	Mean profile	25.5	3.05
983	Mean profile	24.5	2.49

[a] Plasma samples were collected over 22 hr. Mean values obtained at 24 and 26 hr were substituted for the missing values.

parameters of a periodic function, namely, period and amplitude (17–24). However, a literature review failed to demonstrate applications of these analyses to blood level data of exogenous compounds such as drugs. In the present study, an attempt was made to analyze the observed data by cross-correlation analysis (22).

In view of the relatively small oscillations in plasma levels found in this study, the cross-correlation method was first evaluated with generated data using the equation $y = a + b\cos(cX)$. The following input values were used: period = 24 hr, amplitude = 5%, sampling interval = 2 hr, and observation timespan = 26 hr. The method yielded values of the period within 2% and values of the amplitude within 14% of the input values even with 1–4% random error in the data. These results indicated that the cross-correlation method could be applied to the experimental observations.

Sets of plasma concentration–time data of individual animals, as well as the mean set of data of each animal, were subjected to the cross-correlation analysis (Table III). The mean periods obtained from individual sets of data were 28.0, 24.8, and 28.5 hr for Animals 903, 923, and 983, respectively. The corresponding periods obtained from mean plasma concentration–time data were 25.5, 25.5, and 24.5 hr. These values are close to the circadian period (24 hr) and provide further support for the hypothesis of a circadian rhythm in steady-state plasma levels.

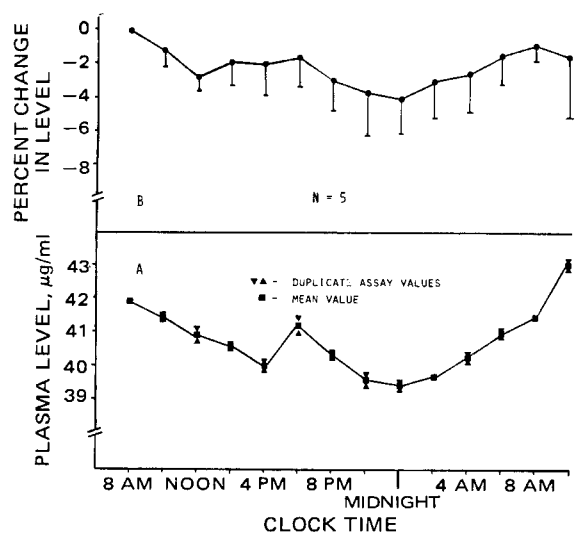

Figure 3—*Plots of typical diurnal changes in steady-state plasma levels observed during a given sampling period (A) and mean (±SD) percent changes in plasma levels* versus *time (B) for Animal 903.*

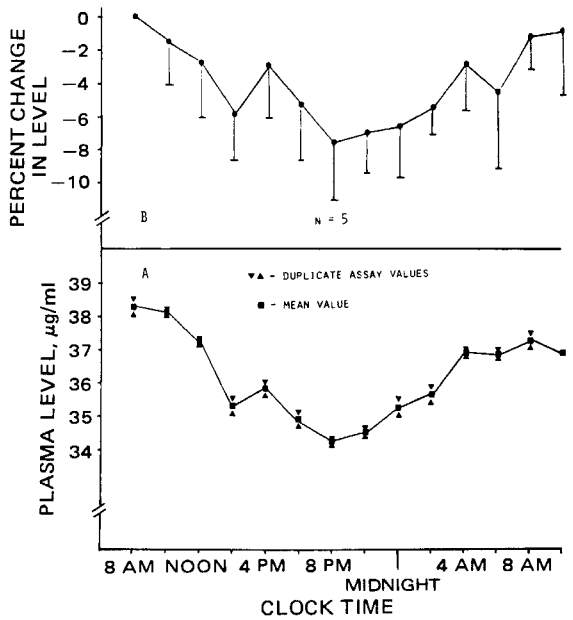

Figure 4—*Plots of typical diurnal changes in steady-state plasma levels observed during a given sampling period (A) and mean (±SD) percent changes in plasma levels* versus *time (B) for Animal 923.*

DISCUSSION

The present study revealed diurnal fluctuations (circadian periodicity) in steady-state plasma levels of ethosuximide in monkeys. An analysis of the possible causes for fluctuations in plasma levels during zero-order infusion suggested a circadian rhythm in total body clearance. Diurnal variations in the volume of distribution, renal excretion, and/or metabolism could explain the observed periodicity in plasma levels.

Circadian variations in the excretion rates of several drugs (*e.g.*, salicylate, sulfasymazine, and amphetamine) have been reported in humans and were believed to be associated with the diurnal urinary pH rhythm (5–7). It is not known whether such a circadian rhythm in urine pH exists in monkeys. However, even if such a cycle exists, it should include urine pH values close to 9 to influence the urinary excretion of ethosuximide (pKa = 9.4). In the present study, it was not possible to determine the existence of a circadian rhythm in the excretion rate of ethosuximide,

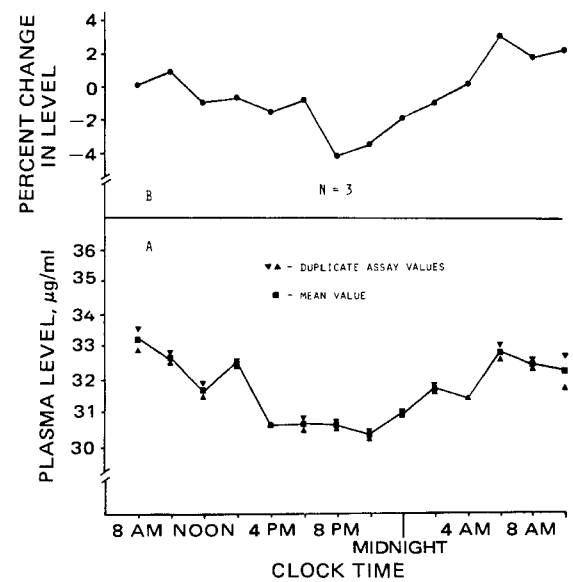

Figure 5—*Plots of typical diurnal changes in steady-state plasma levels observed during a given sampling period (A) and mean (±SD) percent changes in plasma levels* versus *time (B) for Animal 983.*

primarily because of the additional technical problems (trauma and infection) posed by chronic urethral catheters.

Circadian variations in the rates of *in vitro* metabolism of drugs (hexobarbital, aminopyrine, *p*-nitroanisole, and 4-dimethylaminoazobenzene) have been documented (25–32). However, whenever a circadian rhythm in metabolism has been reported, it has usually been associated with an oxidative metabolic process (33). Ethosuximide is metabolized to a significant extent (60–65%) in monkeys (13, 14, 34), and all known metabolic pathways (in rats, monkeys, and humans) involve oxidative processes (35–38). Thus, the possibility of circadian variations in the activities of hepatic drug-metabolizing enzymes in monkeys is not unrealistic.

This study shows that the phenomenon of circadian rhythm in steady-state levels is real. Although the extent of oscillations reported here is limited, ongoing studies show that other anticonvulsants (valproic acid and clonazepam) exhibit diurnal oscillations in plasma levels likely to have therapeutic consequences.

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