Chronopharmacological Study of Sodium Valproate in Mice: Dose-Concentration-Response Relationship†

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Abstract—Effects of the time-of-day of drug administration on the pharmacokinetics electroshock seizure (ES) threshold and acute toxicity were investigated in mice with sodium valproate (VPA). ICR male mice, housed under a light-dark (12:12) cycle, were orally administered 600 mg/kg VPA for anticonvulsant effect studies and administered 1500 mg/kg VPA for acute toxicity studies. A significant circadian rhythm was demonstrated for the ES threshold at 30 min after VPA administration, with the highest value in the light phase and the lowest in the dark phase, although no rhythm was shown in the nondrugged state. A significant circadian rhythm was also shown for plasma and brain VPA concentrations. This finding nicely corresponded to the circadian rhythm in the ES threshold. The positive relationship between the brain VPA concentration and the ES threshold was not different between the light phase and the dark phase. There was also a significant circadian rhythm in the acute toxicity induced by VPA, with the highest mortality in the light phase and the lowest in the dark phase. The results suggest the importance of time in the circadian stage at which VPA is administered in the experimental studies in mice and the significance of circadian rhythm in VPA kinetics in relation to the rhythm of ES threshold.

Responses to a variety of drugs follow circadian rhythms (1–3). These include the responses to phenobarbital, theophylline, gentamicin, apomorphine, ouabain and ethanol (4–9). The effectiveness and toxicity of drugs are determined by the sensitivity of the receptors of living organisms, but also by the pharmacokinetics of drugs. Therefore, underlying mechanisms of the circadian drug susceptibility rhythms should be explored on these aspects.

Sodium valproate (VPA) is the salt of a short-chain, branched, fatty acid and widely used as an anticonvulsant for the treatment of absence seizures and generalized tonic-clonic seizures. There have been some reports concerning the circadian stage-dependent changes in VPA kinetics in man as a function of the time of day of administration (10, 11). That is, the mean peak concentration of VPA is lower and the time of peak concentrations is slightly longer in the evening than in the morning. However, the significance of circadian stage-dependent changes in VPA kinetics is not clear to date, especially in relation to their pharmacologic actions.

In the nondrugged state, the light-dark difference in the electroshock seizure (ES) threshold, as a measurement of the changes in brain-activity in association with the circadian cycle, in rats has been reported (12). The susceptibility to audiogenic seizures in mice is severalfold greater in the dark than in the light (13). However, whether or not the circadian rhythm of the ES threshold, as a measurement of the pharmacologic effect, in mice with VPA can be observed is not known at present. In addition, the existence of

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circadian rhythm in the mortality, as a measurement of the acute toxicity, induced by VPA in mice has not been reported.

This study was designed to examine the existence of circadian rhythm in the ES threshold and the acute toxicity in mice with VPA and to elucidate the mechanism underlying this rhythm from the viewpoints of the dose-concentration and the concentration-response relationships of the drug by measuring the plasma and brain VPA concentrations and the ES threshold.

**Materials and Methods**

**Animals and treatments:** Male ICR mice (7 weeks old) were used. Mice were housed ten per cage in a light-controlled room (lights on from 0700 to 1900) at a room temperature of 24±1°C and a humidity of 60±10% with food and water ad libitum. A single oral dose of VPA (Valerin®, Dainippon, Japan) was used. In the study observing the circadian rhythm in the ES threshold and plasma VPA concentration, groups of 10 mice each were orally administered 600 mg/kg VPA at one of six times: 0900, 1300, 1700, 2100, 0100 and 0500. Mice were returned to their home cages after VPA administration. ES threshold was determined at 30 min after VPA administration. The plasma samples were obtained by orbital sinus collection immediately after ES threshold determination. To study the concentration-response relationship of VPA and to observe the time course of ES threshold and VPA concentrations in plasma and brain, groups of 4 mice each were orally administered 600 mg/kg VPA on two occasions, in the middle of the light phase (1300:midlight) or in the middle of the dark phase (0100:middark). The ES threshold, the plasma and brain VPA concentrations were determined at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120 and 180 min after VPA administration. In the acute toxicity study, a single oral dose of VPA was employed. Groups of 10 mice each were orally administered 1500 mg/kg VPA at one of six times as described above. After VPA administration, mice were returned to their home cages, and the VPA-induced mortality was observed for 24 hr after VPA administration. Dead mice were removed at each observation.

**Determination of ES threshold:** The ES threshold was determined by a stimulator (E.C. stimulator model MK-800, Muromachi, Japan), which increased the direct current (0.1 mA) stepwise every prefixed period of time (0.2 sec). The stimulus was delivered through copper corneal electrodes placed on the eyes. The ES threshold was defined as the amount of current in milliamperes delivered through corneal electrodes, which resulted in a detectable tonic forepaws flexion.

**Determination of VPA concentrations:** One hundred µl 12 N hydrochloric acid and 100 µl chloroform containing diphenyl as an internal standard (30 µg/ml) were added to 100 µl plasma in a 400 µl tube. Three ml saline, 1 ml 12 N hydrochloric acid and 500 µl chloroform containing diphenyl as an internal standard (30 µg/ml or 150 µg/ml) were added to whole brain in a 10 ml tube. The brain samples were homogenized using a Kinematica Polytron. Then, both samples were shaken vigorously for 10 min and centrifuged at 3,000 rpm for 10 min (Kubota KN-70). The upper layer and the protein layer were removed and the organic phase was then taken as the sample for gas chromatography. The VPA concentrations in plasma and whole brain were determined by gas-liquid chromatography (Hitachi-163 type gas chromatography) using a modification of the procedure developed by Lüscher (14). Chromatographic separation was achieved on a glass column (2 m, 3 mm internal diameter) packed with 5% FFAP on 80/100 Gas-Chrom Q. The column temperature was maintained at 170°C, and the injector and detector temperatures were kept at 250°C. Nitrogen, hydrogen and air flow rates were 30, 30 and 300 ml/min, respectively. Peak height ratios (VPA to diphenyl) were calculated and converted to µg/ml of VPA using a calibration curve. The calibration curves were linear in the range of 10 to 150 µg/ml in the plasma and in the range of 10 to 150 µg/ml and 100 to 600 µg/ml in the brain sample.

**Statistical analysis:** Analysis of variance (ANOVA), Kruskal-Wallis one-way analysis of variance and the x²-test for k independent samples were applied for the statistical analysis of the experimental data.
Results

**Circadian rhythm of ES threshold:** There was a significant circadian rhythm in the ES threshold at 30 min after VPA administration [P<0.01 by the Kruskal-Wallis test (Fig. 1)]. The mean ES threshold values were higher when VPA was administered at 1300 (mean ±S.E.=9.7±0.7 mA), 1700 (8.9±0.7 mA) or 0500 (9.5±0.7 mA) than at 0900 (7.8±0.4 mA), 2100 (7.1±0.3 mA) or 0100 (7.1±0.2 mA). The highest mean ES threshold was found in mice administered the drug at 1300, the midlight phase. The lowest was found in mice administered the drug at 2100, the early dark phase, and 0100, the middark phase. However, no significant circadian rhythm was found for the ES threshold in the nondrugged state, although the ES threshold tended to be slightly higher in the midlight phase than in the middark one (0900: 5.0±0.2 mA, 1300: 5.2±0.2 mA, 1700: 4.9±0.2 mA, 2100: 4.6±0.2 mA, 0100: 4.7±0.2 mA, 0500: 5.2±0.2 mA) (Fig. 1).

**Circadian rhythm of plasma VPA concentrations:** A significant circadian rhythm was also found for plasma VPA concentrations at 30 min after VPA administration [P<0.01 by the Kruskal-Wallis test (Fig. 2)]. The mean plasma VPA concentrations were higher when the drug was administered at 1300 (1015.2±144.3 µg/ml), 1700 (928.2±171.5 µg/ml) or 0500 (1025.0±147.7 µg/ml) than at 0900 (517.9±88.4 µg/ml), 2100 (618.4±75.2 µg/ml) or 0100 (586.1±51.4 µg/ml). This finding nicely corresponded to the circadian rhythm in the ES threshold.

**Relationship between brain VPA concentration and ES threshold:** A significant positive linear relationship was found between brain VPA concentration and ES threshold [midlight: r=0.81, P<0.01; middark: r=0.67, P<0.01 (Fig. 3)]. However, the relationship was not different between mice administered the drug at the midlight phase and mice administered at the middark phase. That is, the slopes of the linear regression lines were essentially similar in the midlight phase and in the middark phase (0.0104 and 0.0109, respectively). The intercept of the linear regression line was slightly higher in the midlight phase than in the middark one (6.1 and 5.6, respectively). The lower intercept value in the latter was associated with a slightly rightward shift of the line and corresponded to the slight light-dark difference of the ES threshold in the nondrugged state.
Effect of time-of-day of drug administration on the time course of ES threshold and VPA concentrations in plasma and brain: The time course of mean VPA concentrations showed a pattern of the VPA appearing quickly after its administration and then gradually decreasing (Fig. 4). Mean VPA concentrations in plasma tended to be higher in the midlight phase than in the middark one, especially at 15 min (737.2±291.1 and 497.2±49.9 μg/ml), 30 min (1031.2±167.7 and 658.8±68.9 μg/ml) and 45 min (985.2±91.3 and 657.6±75.5 μg/ml) after VPA administration. Plasma VPA concentration reached a peak by 30 min after administration in the midlight phase. On the other hand, the time of peak plasma VPA concentration after administration in the middark phase was not clear. The time course of brain VPA concentrations was similar to that of plasma VPA concentrations (Fig. 5). Mean brain VPA concentrations tended to be higher in the midlight phase than in the middark one, especially at 15 min (187.0±104.1 and 105.0±4.3 μg/g), 30 min (294.3±66.5 and 132.6±16.1 μg/g) and 45 min (297.0±41.1 and 140.1±24.6 μg/g) after VPA administration. The time course of ES threshold nicely corresponded to those of VPA concentrations in plasma and brain (Fig. 6). Mean ES threshold tended to be higher in the midlight phase than in the middark one, especially at 15 min (8.6±1.2 and 6.7±0.2 mA), 30 min (10.1±1.1 and 7.8±0.5 mA) and 45 min (10.5±0.6 and 7.3±0.3 mA) after VPA administration.

Circadian rhythm of acute toxicity: There was a significant rhythm in mortality depending on the time of administration \( [x^2=11.55, df=5, P<0.05 (\text{Fig. 7})] \). Most of the dead mice died within 30 min after each adminis-

![Relationship between brain VPA concentration and ES threshold](image)

**Fig. 3.** Relationship between brain VPA concentration and ES threshold. The closed circle and solid line indicate the data from mice administered the drug at 1300 \( (Y=0.0104X+6.1, r=0.81, P<0.01, N=44) \). The open circle and dashed line indicate the data from mice administered the drug at 0100 \( (Y=0.0109X+5.6, r=0.67, P<0.01, N=44) \).
Fig. 4. The time course of plasma VPA concentrations after an oral administration of VPA (600 mg/kg) at 1300 or at 0100. Each point represents the mean±S.E. of 4 mice. ■—■, administration of VPA at 1300; ○—○, administration of VPA at 0100.

Fig. 5. The time course of brain VPA concentrations after an oral administration of VPA (600 mg/kg) at 1300 or at 0100. Each point represents the mean±S.E. of 4 mice. ■—■, administration of VPA at 1300; ○—○, administration of VPA at 0100.

Fig. 6. The time course of ES threshold after an oral administration of VPA (600 mg/kg) at 1300 or at 0100. Each point represents the mean±S.E. of 4 mice. ■—■, administration of VPA at 1300; ○—○, administration of VPA at 0100.

Fig. 7. Circadian rhythm of mortality after an oral administration of VPA (1500 mg/kg). Each point represents the data from 10 mice.
tion. All of the dead mice died within 18 hr after VPA administration. Peak mortality was found in mice administered the drug at 1700, toward the latter half of the light phase. The lowest mortality was found in mice administered the drug at 0100, toward the middle of the dark phase.

Discussion

In the present study, a significant circadian rhythm was demonstrated for ES threshold at 30 min after VPA administration, with the highest value in the light phase and the lowest in the dark phase. However, there was no significant circadian rhythm for ES threshold in the nondrugged state. Why does the definite rhythm exist only under the drugged state? Drug action is determined by a physicochemical interaction between the drug and functionally important molecule which is usually a receptor in the body. The magnitude of the response is closely related to the concentration of the drug at the site of action. Once the drug is administered, it is absorbed from the site of administration into the bloodstream and is distributed to the brain and other organs where the sites of action exist. Since the site of action of VPA is the brain, this study was performed to elucidate the mechanism underlying the circadian rhythm in the ES threshold in mice with VPA from the viewpoint of the dose-concentration-response relationship of the drug by measuring plasma and brain VPA concentrations and ES threshold in mice.

First, the dose-concentration relationship of VPA was investigated. A significant circadian rhythm was shown for plasma VPA concentrations at 30 min after VPA administration, with a higher level in the light phase as compared with that in the dark phase. This finding nicely corresponded to the circadian rhythm in the ES threshold. The circadian rhythm of plasma VPA concentrations showed pattern similar to that of the ES threshold. Mean brain VPA concentrations were also significantly higher in the midlight than in the middark. The time course of brain VPA concentrations nicely corresponded to those of plasma VPA concentrations and of the ES threshold. The plasma VPA concentration seems to reflect the VPA concentration in the brain. This finding is similar to the report that a positive linear relationship was demonstrated between the two concentrations (r=0.889, P<0.001) (15). Thus, there is a circadian rhythm of VPA concentrations in the brain as well as in the plasma.

Secondly, the concentration-response relationship of VPA was observed to clarify the light-dark difference in the response of living organisms to VPA. A positive relationship was demonstrated between the brain VPA concentration and the ES threshold. However, the relationship was not different between the midlight phase and the middark one, although the intercept value of the linear regression line was slightly higher in the former. The lower intercept value in the latter seems to be due to the slight changes in brain activity in association with the circadian cycle, since in the nondrugged state, the ES threshold was slightly but not significantly lower in the middark phase. This probably caused the slightly rightward shift of the linear regression line obtained in the middark phase. The lack of any difference in the slope of the linear regression line between the midlight phase and the middark one suggests that the sensitivity to VPA in mice is not influenced by the time of VPA administration when the ES threshold is used as a measurement of the pharmacologic action.

Although in the nondrugged state, the light-dark difference in the ES threshold of rats (12) and in the audiogenic seizures of mice (13) have been reported, the discrepancy between these and our results may be due to the different assays used for quantitating the seizure threshold and/or the strain difference in the seizure threshold of the rodents.

VPA has more than eight active metabolites, of which two unsaturated compounds, 2-propyl-2-pentenoic acid (2-en-VPA) and 2-propyl-4-pentenoic acid, are the most active (16). The study in mice indicates that 2-en-VPA is about 1.3 times more potent than VPA when anticonvulsant potency is derived from the increase in the ES threshold taking into consideration the difference in brain VPA concentrations (17). However, the only metabolite which is detectable in the mouse brain after injection of 200 mg/kg, i.p.
is 2-en-VPA and the concentrations found up to 2 hr after injection are 1–50 μg/g (VPA) and 0.1–0.2 μg/g (2-en-VPA). Thus, the 2-en-VPA/VPA ratio in the brain is below 0.1 at the early stage, 0–2 hr, after injection (18). The metabolite 2-en-VPA may not add more than 13% to the anticonvulsant effect of VPA in mice. Therefore, VPA seems responsible for more than 87% of the antiepileptic effect after single VPA administration.

Considering the small circadian variations of the ES threshold in the nondrugged state and those of the concentration-response relationship of VPA, the results obtained in the present study show that the circadian rhythm of ES threshold in mice with VPA can be explained mainly by the circadian rhythm in VPA kinetics. Drug concentrations in the plasma and also in the brain are influenced by the rates of four pharmacokinetic factors: absorption, distribution, metabolism and excretion of the drug. Therefore, underlying mechanisms of circadian rhythm in plasma and brain VPA concentrations in mice were then investigated to clarify which factor was mainly responsible for the circadian rhythm in VPA kinetics.

Up to 1 hr after administration, mean plasma VPA concentrations were higher in the midlight phase than in the middark one. Plasma VPA concentration reached the peak by 30 min after administration in the midlight phase. On the other hand, the time to attain a peak plasma VPA concentration was not clear after administration at the middark phase. Plasma VPA concentrations at 30 min after administration seem to reflect the absorption process of the drug. In the intraperitoneal administration study designed to eliminate factors influencing the absorption process from the gastrointestinal tract, the plasma VPA concentrations did not differ at 30 min (640.9±31.7 and 625.4±43.8 μg/ml) after administration of 300 mg/kg VPA between the midlight phase and the middark one (S. Ohdo et al., unpublished results). Therefore, the difference in mean plasma VPA concentrations from 10 to 60 min after administration between the midlight phase and the middark phase seems to be due to the difference in the absorption rate from the gastrointestinal tract.

Drug absorption is often influenced by the amount of food in the stomach, since food induces changes in gastric secretion of hydrochloric acid, the rate of gastric emptying and intestinal transit time (19). The food and water intake of nocturnal animals such as rodents is confined mostly to the dark phase, especially the first half of the dark phase (20–22). Furthermore, mean plasma VPA concentration at 60 min after intragastric administration in rats with the stomach isolated from the intestine by ligating the pylorus is about 50% of the corresponding value of intraintestinal administration and thus VPA is absorbed from rat stomach at a slower rate than from the whole intestine (23). Therefore, the difference in food intake between the light phase and dark phase may influence the rate of gastric emptying and intestinal transit time and also the absorption process of VPA kinetics. The different amount of food intake between the first half and the latter half of the dark phase might become the critical factor influencing the drug absorption and might produce the two peaks observed in the circadian rhythm in VPA kinetics and in the ES threshold in the present study.

As the results of the present study in mice, the circadian changes in VPA kinetics have also been demonstrated in our human studies under meal conditions controlled to fit the subjects’ usual food intake. When a 800 mg dose of VPA was orally administered on two occasions in the morning (0830) or in the evening (2030), a significant difference was demonstrated in the pharmacokinetic parameters of VPA related to drug absorption such as absorption rate constant (Ka) and peak plasma concentration (Cmax), showing higher Cmax (P<0.05) and larger Ka of VPA (P<0.05) in the morning than in the evening (24). A similar finding has been reported for theophylline and diazepam kinetics at the absorption phase after a single oral administration (25, 26). However, the significant difference in VPA kinetics between morning and evening disappears when both the amount of breakfast and the amount of dinner are the same as that of the standard breakfast for the subject (S. Ohdo et al., unpublished data). These results suggest that the different amount of food between breakfast and dinner.
plays a major role in circadian stage-dependent changes in VPA kinetics after oral administration in man. The same may also exist in mice.

In the present study, a significant circadian rhythm was also demonstrated for acute toxicity measured by VPA induced-mortality, with the highest value in mice administered the drug at 1700 and the lowest at 0100. Most of the dead mice died within 30 min after each administration. Although 1500 mg/kg of VPA, the dose used for the present acute toxicity study, is too high to perform the pharmacokinetic study in mice, the plasma and the brain VPA concentrations up to 30 min after oral administration reflect the absorption process of the drug in view of VPA kinetics at the dose of 600 mg/kg. Therefore, the peak and trough in the mortality induced by VPA may be related to the circadian rhythm in the pharmacokinetics, especially in the absorption phase, of VPA. Since the pattern of the circadian rhythm of acute toxicity is not identical to those of the ES threshold and the plasma VPA concentrations after the dose of 600 mg/kg, the circadian rhythm of VPA toxicity may not be completely explained by the rhythm in the pharmacokinetics. The circadian rhythm in the ES threshold is closely related to the circadian rhythm in the dose-concentration relationship. On the other hand, the rhythm in the toxicity might be due to the circadian rhythm in the concentration-response relationship in addition to the circadian rhythm in the dose-concentration relationship. Further studies are necessary to clarify the role of pharmacokinetics in the chronic toxicity of VPA.

The circadian rhythms in drug effectiveness and toxicity differ depending on the dose. For example, the sedative effects induced by haloperidol and chlorpromazine show a different circadian rhythm according to the dose (27, 28). The result obtained in the present study also shows a similar but not identical circadian rhythm between the drug effectiveness and the toxicity depending on the dose. The mechanisms underlying the action of VPA may differ between the effectiveness and the toxicity. Although VPA is thought to act on the inhibitory central nervous system including the GABA system, the effective dose of VPA does not cause respiratory failure. However, the toxic dose of VPA acts on the system controlling respiration and produces respiratory failure. The respiratory failure induced by the toxic dose of VPA may be attributed to the central inhibitory effect, especially in the medulla oblongata controlling respiration, because VPA has no effects on visceral functions such as neuromuscular blockade. The different mechanisms underlying the action of VPA depending on the dose may cause the difference between the rhythms induced by the effective dose and by the toxic dose.

Circadian rhythms have shown in the pharmacologic actions of a wide variety of drugs (1, 29). In some of them, the time-dependent absorption rate of drugs contributes to circadian variations in drug actions. These include the enhanced effects of the sedation elicited by diazepam or amitriptyline and of the anticholinergic actions induced by amitriptyline in the morning in man (30, 31). VPA seems to be a drug of this type. The results obtained in the present study suggest the importance of time in the circadian stage at which VPA is administered in the experimental studies with VPA in mice and the significance of the circadian rhythm in VPA kinetics in relation to the rhythm in the ES threshold.

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