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THE EFFECTS OF ANTICONVULSANT DRUGS ON VITAMIN D_3-ACTIVATING CYTOCHROME *P*-450-LINKED MONOOXYGENASE SYSTEMS

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Summary—The effects of two anticonvulsant drugs, phenytoin and sodium valproate, on the bioactivation of vitamin D_3 have been studied with respect to the microsomal and mitochondrial cytochrome *P*-450-linked monooxygenase systems that contribute to 25-hydroxylation of vitamin D_3 in rabbit liver, and the mitochondrial cytochrome *P*-450-linked monooxygenase system that catalyzes 1α-hydroxylation of 25-hydroxyvitamin D_3 in rabbit kidney. These anticonvulsant drugs were found to inhibit the 25-hydroxylase activity on vitamin D_3 in liver microsomes and mitochondria, respectively, but not to inhibit the 1α-hydroxylation of 25-hydroxyvitamin D_3, even over a wide concentration range. Moreover, the activities of the components of the cytochrome *P*-450-linked monooxygenase systems: NADPH–cytochrome *P*-450 reductase, NADPH–ferredoxin reductase and ferredoxin, were never inhibited by these drugs. It is possible that the inhibition of bioactivation of vitamin D_3 by these anticonvulsant drugs causes rickets and osteomalacia, and the site of inhibition is expected to be the cytochrome *P*-450 mediated reactions in liver mitochondria.

INTRODUCTION

Phenytoin (5,5-diphenyl-2,4-imidazolidinedione) and sodium valproate (sodium propyl valerate) have been widely used as anticonvulsant drugs. Since Schmid reported that long-term administration of these drugs to patients causes rickets [1], there have been many similar reports that side effects of these drugs: metabolic errors of calcium, phosphorus and vitamin D_3, cause osteomalacia and rickets [2–4]. The mechanism underlying these effects, however, remain to be elucidated.

There are microsomal and mitochondrial types of cytochrome *P*-450-linked monooxygenase systems that contribute to bioactivation of vitamin D_3; the former consists of cytochrome *P*-450_{D25} and NADPH–cytochrome *P*-450 reductase, and the latter of cytochrome *P*-450_{D25}, cytochrome *P*-$450_{D1}\alpha$, ferredoxin (iron–sulfur protein) and NADPH–ferredoxin reductase [5–7]. The 25-hydroxylation of vitamin D_3 is the first step in the bioconversion of vitamin D_3 to the renal steroid hormone, and these 25-hydroxylase systems are present in microsomes and mitochondria, mainly in the liver [8]. The 1α-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) is the second step in the conversion into the renal steroid hormone, and this 1α-hydroxylase system is present in mitochondria of the kidney cortex [6], pituitary gland [9] and placenta [10].

It is very important to investigate the effects of anticonvulsant drugs on the bioactivation of vitamin D_3 by these cytochrome *P*-450-linked monooxygenase systems. In the present study, the effects of phenytoin and sodium valproate on the activities of the vitamin D_3 25-hydroxylase and 25-OH-D_3 1α-hydroxylase systems of rabbit liver and kidney were examined. Moreover, to elucidate the site and mechanism of the inhibition, we studied the inhibitory effects of the drugs on the activities of components of the cytochrome *P*-450-linked monooxygenase systems. As a result, it was found that the inhibition mechanisms of both drugs are different in the microsomal and mitochondrial vitamin D_3 25-hydroxylase systems. These results are consistent with the finding that vitamin D_3 25-hydroxylase exhibits specificity for the structures of cytochrome *P*-450_{D25} of microsomes and mitochondria, as described previously [11]. We studied the effects of anticonvulsant drugs on the two vitamin D_3 25-hydroxylase systems

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because of reports that the subcellular distribution of the activity of 25-hydroxylase shows specificity as to species, sex or medication [11]. Furthermore, it has been reported that vitamin D_3 25-hydroxylase activity in human liver is only detected in mitochondria [12] and this study showed that two drugs inhibited mitochondrial vitamin D_3 25-hydroxylase activity.

These results are reasonable considering that the administration of anticonvulsant drugs decreases the 25-OH-D_3. Therefore, it is possible that rickets or osteomalacia results from the reduction of 25-hydroxylase activity on the administration of anticonvulsant drugs, being caused by the low and insufficient concentration of 25-OH-D_3 as a substrate for the 25-OH-D_3 1α-hydroxylase systems. The concentrations of phenytoin and sodium valproate are kept at about 0.1 and 0.8 mM in a patient's blood, respectively, and long-term administration is apt to cause an overdose. Therefore, it is possible that concentrations of anticonvulsant drugs that inhibit the activity of vitamin D_3 25-hydroxylase are reached.

MATERIALS AND METHODS

Materials

Healthy male rabbits (Japan white, 6 months old, 2.0–2.5 kg), having no significant medication history, were obtained commercially. The animals were sacrificed by air embolism and their tissues were immediately used for this experiment.

Chemicals

Vitamin D_3 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 25-OH-D_3 and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2$-D_3] were kind gifts from Chugai Pharmacological Co. (Japan). [^{3}H]Vitamin D_3 and 25-hydroxy[23,24(N)-^{3}H]vitamin D_3 were obtained from Amersham-Japan Co. (Japan). Phenytoin and sodium valproate were obtained from Dainippon and Kyowa Pharmacological Co. (Japan), respectively. NADPH and glucose 6-phosphate were obtained from Oriental Yeast Co. (Japan). All other chemicals were of the highest purity available from commercial sources.

Preparation of microsomal and mitochondrial fractions

All manipulations were carried out at 4°C unless otherwise indicated. Rabbit liver was perfused with an ice-cold 1.15% KCl solution and then homogenized with 5 vol of a 0.25 M sucrose solution (adjusted to pH 7.4 with 1 M Tris), and mitochondria were prepared by the method of Johnson and Lardy [13]. Rabbit kidney was also homogenized with ice-cold 0.25 M sucrose (pH 7.4) and mitochondria were prepared by the method of Johnson and Lardy [13]. Rabbit liver microsomes were prepared by the method of Mitoma *et al.* [14].

Other enzymes

NADPH–cytochrome *P*-450 reductase was purified, to protect the enzyme from proteinase digestion, from untreated bovine liver microsomes in the presence of 0.4 mM phenylmethanesulfonylfluoride [15].

Other procedures were essentially the same as described in [16] and [17]. Ferredoxin and NADPH–ferredoxin reductase were purified from bovine adrenocortical mitochondria as described previously [18, 19].

Enzyme assays

The activity of vitamin D_3 25-hydroxylase of liver mitochondria and microsomes was measured in the absence and presence of anticonvulsant drugs by the method of Ichikawa *et al.* [11]. The assay mixture for the activity of the mitochondrial vitamin D_3 25-monooxygenase system contained, in a final vol of 1.0 ml, 20 mM potassium phosphate buffer (pH 7.4), vitamin D_3, at three concentrations (3, 2 and 1 nM; 35,000 dpm/n mol [^{3}H]vitamin D_3), 10 μM adrenodoxin, 0.5 μM NADPH–adrenodoxin reductase and 0.25 ml of the liver mitochondrial fraction (16–22 mg protein/ml) solubilized with 1% sodium cholate in the absence and presence of phenytoin or sodium valproate, at three different concentrations (0.5, 1 and 2 mM). In the same way, the assay mixture for the 25-OH-D_3 1α-monooxygenase system was prepared. The reaction mixture contained [^{3}H]25-OH-D_3 at three various concentrations (2, 1 and 0.5 μM; 87,000 dpm/10 nmol [^{3}H]25-OH-D_3). The assay mixture for the microsomal vitamin D_3 25-monooxygenase system contained, in a final vol of 1.0 ml, 20 mM potassium phosphate buffer (pH 7.4), vitamin D_3 (3, 2 and 1 nM), 0.5 ml of rabbit liver microsomes (14 mg protein/ml) and inhibitor (phenytoin or sodium valproate). After preincubation of a sample mixture for 1 min at 37°C, the reaction was started by adding 130 μl of

Table 1. Kinetic constants for vitamin D_3 activation catalyzed by cytochrome *P*-450-linked monooxygenase systems

	K_i (M) values for vitamin D_3 25 monooxygenase system	
Drug	Mitochondrial type	Microsomal type
Phenytoin	0.58×10^{-3}	1.89×10^{-3}
Sodium valproate	0.76×10^{-3}	0.77×10^{-3}

		Vitamin D_3 25 monooxygenase system	
Value	Substrate	Mitochondrial type	Microsomal type
K_m (M)	Vitamin D_3	1.66×10^{-9}	7.25×10^{-9}
V_{max} (pmol/min/mg protein)	Vitamin D_3	2.09	0.43

a 5 mM NADPH and NADPH-generating system. The NADPH-generating system consisted of 50 μl of 100 mM glucose-6-phosphate, 50 μl of 20 mM NADPH, 20 μl of 100 mM $MgCl_2$ and 10 μl of glucose 6-phosphate dehydrogenase (0.5 U). The reaction was performed for 30 min at 37°C and then terminated by the addition of 1.5 ml of isopropanol–hexane (2:1, v/v). After extraction of the products by the addition of 2 ml of hexane, the lyophilized extract was dissolved in 40 μl of ethanol and then spotted onto thin layer chromatography plates. Development was performed with chloroform–acetone (85:15, v/v and 70:30, v/v) for 25-OH-D_3 and 1α,25-$(OH)_2$-D_3, respectively. Each spot was scraped off and counted with a liquid scintillation spectrometer. The enzymatic reactions of the vitamin D_3 25 and 1α-monooxygenase systems were studied kinetically, and the results are shown in Table 1.

Effects of anticonvulsant drugs on the enzymatic activities of components of the cytochrome P-450-linked monooxygenase systems in mitochondria and microsomes

The effects of inhibitors at three concentrations (0.5, 1 and 2 mM) on the activities of NADPH–cytochrome c reductase of NADPH–cytochrome *P*-450 reductase or NADPH–ferredoxin reductase complex with ferredoxin and NADPH–ferricyanide reductase of NADPH–cytochrome *P*-450 reductase or NADPH–ferredoxin reductase were measured by the method of Hiwatashi *et al.* [18]. To investigate the mechanism underlying the inhibitory effects of anticonvulsant drugs on the cytochrome *P*-450-linked monooxygenase system, and to determine kinetic and inhibition constants, Lineweaver–Burk plotting of the hydroxylase activities in the absence and presence of an inhibitor was performed.

RESULTS

Effects of anticonvulsant drugs on the activities of the vitamin D_3 25-monooxygenase system in microsomes

The activity of vitamin D_3 25-monooxygenase was studied kinetically in the absence and presence of anticonvulsant drugs: phenytoin and sodium valproate. Kinetic tests were performed to determine whether the inhibition of the 25-monooxygenase activity was competitive, noncompetitive or uncompetitive.

Figure 1 shows double-reciprocal plots of microsomal vitamin D_3 25-monooxygenase activity in the absence and presence of various concentrations of phenytoin. The lines have a common intercept on the 1/v axis, but different slopes. The inhibition type is competitive and the K_i value for phenytoin is 1.89 mM. Similarly, the effect of sodium valproate was studied kinetically with respect to the activity of vitamin D_3 25-monooxygenase. Double-reciprocal plots of microsomal vitamin D_3 25-monooxygenase activity had a common intercept on the 1/(S) axis. The inhibition type was typically noncompetitive, as shown in Fig. 2. The K_i was calculated from the figure to be 0.77 mM.

Effects of anticonvulsant drugs on vitamin D_3 25-monooxygenase in mitochondria

The activity of vitamin D_3 25-monooxygenase in mitochondria, as that in microsomes was inhibited by phenytoin or sodium valproate. The results are shown in Figs 3 and 4, as double-reciprocal plots. The inhibition mechanism of both inhibitors was noncompetitive, unlike in the case of microsomes. The K_i values were 0.58 mM for phenytoin and 0.76 mM for sodium valproate.

Effects of anticonvulsant drugs on 25-OH-D_3 1α-monooxygenase in kidney mitochondria

The activity of 25-OH-D_3 or vitamin D_3 1α-monooxygenase in rabbit kidney mitochondria

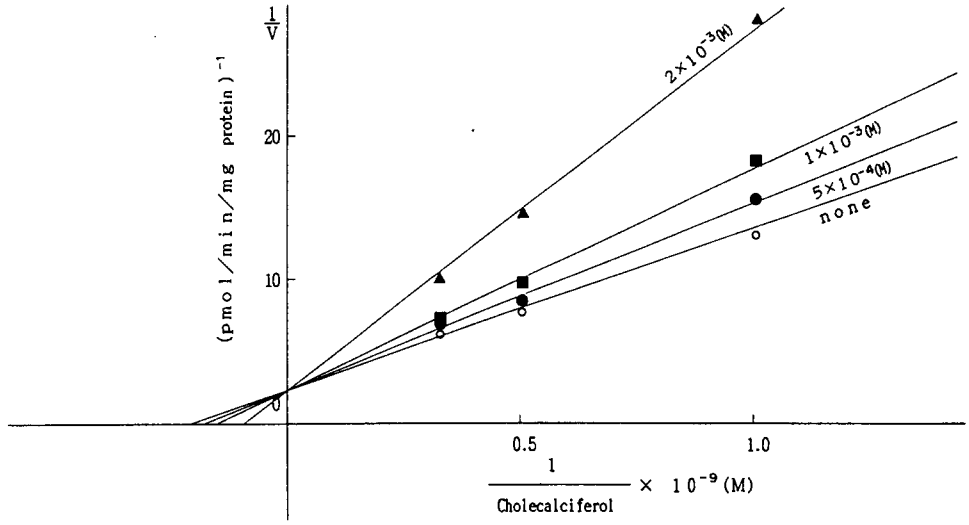

Fig. 1. Effects of phenytoin on the activity of vitamin D_3 25-monooxygenase in liver microsomes. The activity of vitamin D_3 25-monooxygenase was assayed with various concentrations of phenytoin, as described under 'Materials and Methods'. Double reciprocal plots of the initial velocity of the vitamin D_3 25-monooxygenase reaction with vitamin D_3 at various concentrations and a series of fixed concentrations of phenytoin are presented. ○, phenytoin-free; ▲, ■, ●, with phenytoin.

was not inhibited by 0.1 M phenytoin or 0.1 M sodium valproate. This indicates that the anticonvulsant drugs are not inhibitors of 25-OH-D_3 1α-monooxygenase activity (Table 2). No inhibitory effect was observed for either anticonvulsant drug.

Effects of anticonvulsant drugs on the components of the mitochondrial and microsomal cytochrome P-450-linked monooxygenase systems

The cytochrome *P*-450-linked monooxygenase system of mitochondria is composed of three components, NADPH–ferredoxin reductase, ferredoxin and cytochrome *P*-450. That of the microsomal type is composed of two components, NADPH–cytochrome *P*-450 reductase and cytochrome *P*-450. The activities of NADPH–ferricyanide reductase of NADPH–ferredoxin reductase and NADPH–cytochrome *P*-450 reductase, and the activities of NADPH–cytochrome c reductase of NADPH–ferredoxin reductase, complex with ferredoxin and NADPH–cytochrome *P*-450 reductase, were examined in the absence and presence of the anticonvulsant drugs: phenytoin and sodium valproate. The activities showed no appreciable

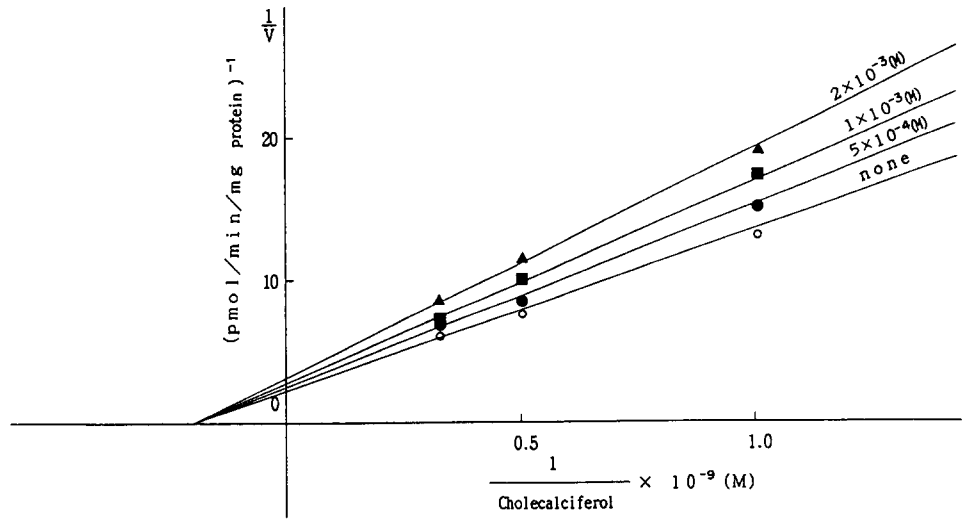

Fig. 2. Effects of sodium valproate on the activity of vitamin D_3 25-monooxygenase in liver microsomes. The activity was assayed and expressed as indicated in the legend to Fig. 1, except that sodium valproate was used as an inhibitor instead of phenytoin. ○, sodium valproate-free; ▲, ■, ●, with sodium valproate.

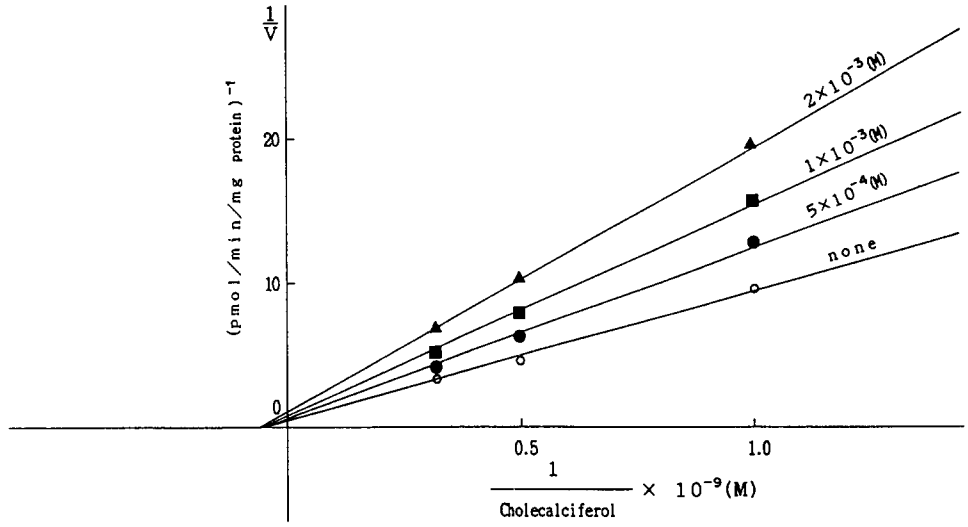

Fig. 3. Effects of phenytoin on the activity of vitamin D_3 25-monooxygenase in liver mitochondria. The activity of vitamin D_3 25-monooxygenase was assayed with various concentrations of phenytoin, as described under 'Materials and Methods'. Double reciprocal plots of the initial velocity of the vitamin D_3 25-monooxygenase reaction with vitamin D_3 in the absence and presence of 50 mM phenytoin. ○, phenytoin-free; ▲, ■, ●, with phenytoin.

changes, even with concentrations of 100 mM of these drugs (data not shown).

DISCUSSION

It is well accepted that 1α,25-$(OH)_2$-D_3 is the renal steroid hormone involved in bone calcium mobilization and intestinal calcium transport [20, 21]. It has been reported that long-term administration of anticonvulsant drugs caused a decrease in the plasma 25-OH-D_3 concentration [3, 22, 23], but it did not affect the plasma 1α,25-$(OH)_2$-D_3 concentration in the same patients [24]. In this study, we investigated the activities of cytochrome *P*-450-linked monooxygenase systems related to biosynthesis of this active form vitamin D_3 to explain the relationship between prescription of anticonvulsant drugs and bone metabolic errors. We found that both phenytoin and sodium valproate inhibit the activity of vitamin D_3 25-monooxygenase in liver mitochondria and microsomes, but not that of 25-OH-D_3 1α-monooxygenase in kidney mitochondria, as shown in Table 2. The

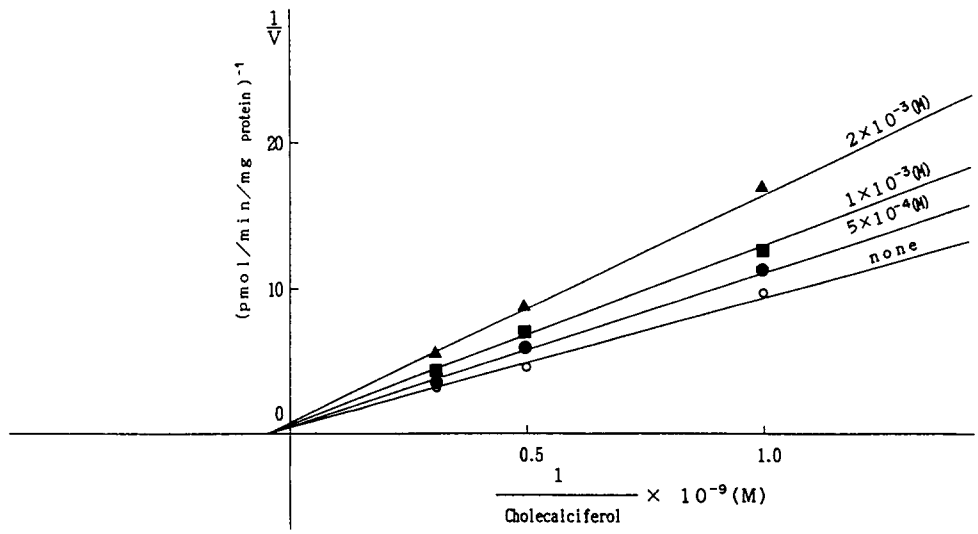

Fig. 4. Effects of sodium valproate on the activity of vitamin D_3 25-monooxygenase in liver mitochondria. The assaying of the enzymatic activity and double reciprocal plotting of the initial velocity of the vitamin D_3 25-monooxygenase reaction were performed as indicated in the legend to Fig. 3, except that 50 mM sodium valproate was used as an inhibitor. ○, sodium valproate-free; ▲, ■, ●, with sodium valproate.

Table 2. Effects of inhibitors on the vitamin D_3 25-hydroxylase-activities of cytochrome *P*-450-linked monooxygenase systems

	% Activities of vitamin D_3 25-hydroxylation [pmol/min/mg protein] (%)		% Activities of 25-OH-D_3 1α-hydroxylation [pmol/min/mg protein] (%)
	Mitochondrial type	Microsomal type	
Control	0.107 (100)	0.075 (100)	0.017 (100)
Phenytoin (mM)			
0.5	0.078 (73.2)	0.065 (85.7)	0.017 (101)
1	0.064 (60.0)	0.055 (72.4)	0.018 (103)
2	0.052 (48.4)	0.036 (47.1)	0.017 (100)
Sodium Valproate (mM)			
0.5	0.090 (83.8)	0.066 (87.3)	0.018 (104)
1	0.081 (75.6)	0.059 (75.5)	0.017 (100)
2	0.061 (57.3)	0.052 (69.0)	0.018 (102)

Value	Substrate	25-OH-D_3 1α-monooxygenase system
K_m (M)	25-OH-D_3	2.70×10^{-6}
V_{max} (pmol/min/mg protein)	25-OH-D_3	0.12

Vitamin D_3 25-hydroxylase and 25-OH-D_3 1α-hydroxylase activities were expressed as produced, the 25-OH-D_3 and 1α,25-$(OH)_2$-D_3, respectively.

inhibition mechanisms of both drugs were different in the microsomal and mitochondrial vitamin D_3 25-monooxygenase systems. These results are consistent with the finding that vitamin D_3 25-monooxygenase exhibits specificity for the structures of cytochrome $P\text{-}450_{D25}$ of microsomal and mitochondrial types, as reported previously [11]. We studied the effects of anticonvulsant drugs on both types of vitamin D_3 25-monooxygenase system, because the subcellular distribution of the activity of vitamin D_3 25-monooxygenase shows specificity as to species, sex or medication [11]. Furthermore, it has been reported that vitamin D_3 25-monooxygenase activity in human liver was only detected in mitochondria [12], and this study showed that two drugs inhibited mitochondrial vitamin D_3 25-monooxygenase activity. The results possibly demonstrate that administration of anticonvulsant drugs decreases the amount of 25-OH-D_3 in plasma, which then decreases the amount of 1α,25-$(OH)_2$-D_3 in kidney. Therefore, it is possible that rickets or osteomalacia does not appear until the low and insufficient concentration of 25-OH-D_3 in plasma, resulting on administration of anticonvulsant drugs, as a substrate for the 25-OH-D_3 1α-monooxygenase system in kidney mitochondria, is reached.

The concentrations of phenytoin and sodium valproate are kept at about 0.1 and 0.8 mM in a patient's blood, respectively, and long-term administration is apt to cause an overdose. Therefore, it is possible that concentrations of anticonvulsant drugs that inhibit the activity of vitamin D_3 25-monooxygenase are reached.

In addition, it is well known that bone metabolism is affected indirectly by various factors such as calcitonin, parathyroid hormone and estrogen. For example estrogen biosynthesized with cytochrome $P\text{-}450_{arom}$ (*P*-450 XIXAI, CYP19)-linked monooxygenase system from androgen, and promotes transcriptionally to biosynthesize insulin-like growth factor-I in osteoblasts [25, 26]. The cytochrome $P\text{-}450_{arom}$ is localized in adrenal gland, ovary, testis, placenta and fat tissue. Accordingly, it is also possible that the various factors are affected by the anticonvulsant drugs.

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