Placenta Transfer and Toxicokinetics of Valproic Acid in Pregnant Cynomolgus Monkeys

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Placenta transfer study in non-human primate (NHP) is one of the crucial components in the assessment of developmental toxicity because of the similarity between NHP and humans. To establish the method to determine placenta transfer in non-human primate, toxicokinetics of valproic acid (VPA), a drug used to treat epilepsy in pregnant women, were determined in pregnant cynomolgus monkeys. After mating, pregnancy-proven females were daily administered with VPA at dose levels of 0, 20, 60 and 180 mg/kg by oral route during the organogenesis period from gestation day (GD) 20 to 50. Concentrations of VPA and its metabolite, 4-ene-VPA, in maternal plasma on GDs 20 and 50, and concentrations of VPA and 4-ene-VPA in placenta, amniotic fluid and fetus on GD 50 were analyzed using LC/MS/MS. Following single oral administration of VPA to pregnant monkeys, concentrations of VPA and 4-ene-VPA were generally quantifiable in the plasma from all treatment groups up to 4-24 hours post-dose, demonstrating that VPA was absorbed and the monkeys were systemically exposed to VPA and 4-ene-VPA. After repeated administration of VPA to the monkeys, VPA was detected in amniotic fluid, placenta and fetus from all treatment groups, demonstrating that VPA was transferred via placenta and the fetus was exposed to VPA, and the exposures were increased with increasing dose. Concentrations of 4-ene-VPA in amniotic fluid and fetus were below the limit of quantification, but small amount of 4-ene-VPA was detected in placenta. In conclusion, pregnant monkeys were exposed to VPA and 4-ene-VPA after oral administration of VPA at dose levels of 20, 60 and 180 mg/kg during the organogenesis period. VPA was transferred via placenta and the fetus was exposed to VPA with dose-dependent exposure. The metabolite, 4-ene VPA, was not detected in both amniotic fluid and fetus, but small amount of 4-ene-VPA was detected in placenta. These results demonstrated that proper procedures to investigate placenta transfer in NHP, such as mating and diagnosis of pregnancy via examining gestational sac with ultrasonography, collection of amniotic fluid, placenta and fetus after Caesarean section followed by adequate bioanalysis and toxicokinetic analysis, were established in this study using cynomolugus monkeys.

Key words: Non-human primate, Cynomolgus monkey, Placenta transfer, Toxicokinetics, Valproic acid, 4-ene valproic acid, Organogenesis, Pregnant, LC/MS/MS

INTRODUCTION

Investigation of placenta transfer and exposure of fetus by potential toxicants in non-human primate (NHP) is one of the important components in the assessment of developmental toxicity and teratogenicity because of the similarity

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List of abbreviation: non-human primate (NHP), valproic acid (VPA), 4-ene valproic acid (4-ene VPA), gestation day (GD), Lower limit of quantification (LLOQ)

between NHP and humans and potential species differences in teratogenic manifestations.

Valproic acid (2-propyl-pentanoic acid, VPA) has been widely used as an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy, bipolar disorder and less commonly in major depression. The daily dose for seizure control is 300-2000 mg for aiming to achieve plasma concentration level of 50-100 µg/m*l* (Ornoy, 2006). These effects of VPA are shown through the inhibition of GABA transaminase and indirect action as GABA agonist. In addition, it was reported that VPA also blocks the volatage-gated sodium channels and T-type calcium channel. These mechanisms make VPA a broad spectrum anticonvulsant drug (Rosenberg, 2007). Recently, it was reported

that VPA has an anti-cancer effects on various tumors such as multiple myeloma, glioma and melanoma through inhibition of histone deacetylases and induction of DNA-methylation (Rosenberg, 2007). VPA treatment with an intensified antiviral therapy could reduce CD4+ T-cell infection of human immunodeficiency virus (HIV) through the inhibition of histone deacetylase HDAC1 which is needed for HIV to remain in infected cells. Therefore, the scope of VPA application on diseases is gradually getting wider. However, in spite of its usefulness on various diseases, there have been a lot of concerns because of the various side effects, especially such as teratogenesis and hepatotoxicity.

In human, prospective and retrospective epidemiological studies suggested that medication of VPA during early pregnancy may be associated with an increased incidence of spina bifida and autism (Wide *et al.*, 2004; Alsdorf and Wyszynski, 2005). In laboratory animals, *in utero* exposure during the organogenesis period resulted in skeletal and visceral abnormalities, growth retardation and embryonic death, and species differences in teratogenic manifestations of VPA has been reported. In mice, VPA treatment caused neural tube defect such as exencephaly (Nau *et al.*, 1991; Emmanouil-Nikoloussi *et al.*, 2004). On the other hand, in other laboratory animals such as rats and rhesus monkey, skeletal malformation was reported as the major teratogenic effect of VAP (Menegola *et al.*, 1996; Tong *et al.*, 2005; Nau, 1986). VPA itself was reported as responsible for the teratogenic effects in mice (Nau, 1986).

Many animal studies were conducted to elucidate action mechanisms on teratogensis in human and to mimic the effects of VPA on human embryo (Emmanouil-Nikoloussi *et al.*, 2004; Menegola *et al.*, 1996; Vorhees, 1987, 1991; Mast *et al.*, 1986). A lot of studies on embryo-fetal toxicity and toxicokinetics of VPA have been conducted using various species, such as mice, rats, sheeps and rhesus monkeys (Nau, 1986; Nau *et al.*, 1991; Binkerd *et al.*, 1988; Semmes and Shen, 1990; Ohdo *et al.*, 1996; Dickinson *et al.*, 1979, 1980), and it has been reported that there are species differences in pharmacokinetics and teratogenesis of VPA (Nau, 1986; Hendrickx *et al.*, 1988). It was pointed out that the half-life VPA in experimental animals (0.3~4 h) is much shorter than that in humans (9~18 h) (Hendrickx *et al.*, 1988). However, there was no information on the placenta transfer and toxicokinetics of VPA in cynomolgus money, which is one of the mostly used animal strains for reproductive toxicity studies using NHP.

Therefore, this study was conducted to establish the method and to determine placenta transfer and toxicokinetics of VPA and its major toxic metabolite, 4-ene valproic acid (2-n-propyl-4-pentanoic acid, 4-ene-VPA) in cynomolgus monkeys (Hauck and Nau, 1989). This study is the first report for the toxicokinetics of VPA using pregnant cynomolgus monkeys.

MATERIALS AND METHODS

Animals. The cynomolgus monkey (*Macaca fascicularis*) was used in this study. Six healthy males, aged 6~7 years, weighing 4~6 kg and 18 healthy females showing regular menstrual cycle, aged 5~7 years, weighing 2.5~4.5 kg were selected among female monkeys maintained in this facility. The animals were maintained in an air-conditioned room at 20~26°C, with a relative humidity of 45~65%, under a controlled 12/12 light/dark cycle, with a ventilation rate of 10~20 air changes/hour, and were housed individually, except for mating. The monkeys were fed 120 g/day of diet (Oriental Yeast Co., Ltd., Japan). Tab water was given to rat ad libitum, following the UV-irradiation and filtration. This study has been approved by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology (KIT) and performed in accordance with the ethics criteria contained in the bylaws of the committee of KIT.

Mating and diagnosis of pr egnancy. Pair of adult male and female monkey was cohabitated for mating. The visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the median day of the mating period was regarded as day 0 of gestation. Pregnancy was confirmed on day 18 or 19 of gestation by ultrasonography (SA-9900, Medison Co. Ltd., Korea). Pregnant females, weighing 2.51~4.50 kg on day 0 of gestation, were allocated randomly to four groups, and housed individually (Fig. 1).

Administration of VPA. The pregnant monkeys were dosed once daily with valproic acid sodium salt (lot no. 036K0731, Sigma-Aldrich Co. Ltd., USA) at 0, 20, 60 and 180 mg/kg by nasogastric intubation on days 20~50 of gestation, i.e., during the entire period of organogenesis. Dosing was terminated in the dams when embryonic/fetal loss occurred. The dose volume was adjusted to 0.5 m*l*/kg of the most recent body weight. Saline was administered to the monkeys in the vehicle control group.

In-life observation of pregnant monkeys. The pregnant monkeys were observed for clinical signs twice a day
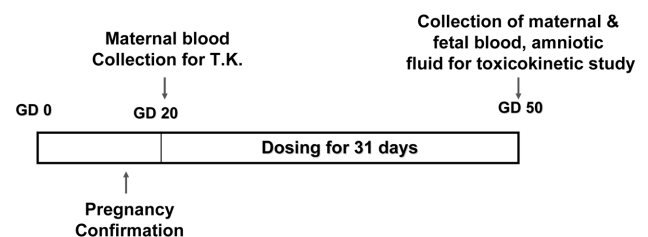

Fig. 1. Schematic representation of the placental transfer study in cynomolgus monkeys.

during the administration period and once a day during the non-administration period. The body weight was recorded on days 0, 20, 27, 34, 41 and 50 of gestation. The food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 47 and 50 of gestation. Embryonic heartbeat and growth were monitored using ultrasonography under restraint in a monkey chair on days 25, 30, 35, 40 and 50 of gestation. When embryonic cardiac arrest was confirmed by ultrasonography, dosing for dams was terminated and these dams were excluded from the study.

Sampling of maternal blood. Blood samples (approximately 1 m*l*) were obtained from cephalic vein or femoral vain at 0 (before treatment), 0.5, 1, 2, 4, 8 and 24 h after administration of VPA on day 20 of gestation (dosing initiation day), and at 0 and 1 h after administration of VPA on day 50 of gestation (dosing termination day), respectively. Blood samples were collected into pre-labeled tubes containing heparin (6 IU/tube). Plasma samples were obtained after centrifuging the blood samples at 12,000 rpm for 3 min, transferred to pre-labeled cryo-tubes, and kept at approximately −80°C until analyzed.

Cesarean section and sampling of amniotic fluid, placenta and fetus. Cesarean section was performed after collecting maternal blood samples at approximately 1 h after dosing on day 50 of gestation, under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1~ 0.2 m*l*/kg) and inhalation of isoflurane gas (0.5~2.0%). Contraction was induced with intravenous injection of atropine (0.01 mg/kg, Tanabe Seiyaku Co. Ltd., Osaka, Japan). After the Caesarean section, amniotic fluid (approximately 0.5 m*l*) was carefully collected from the sac to prevent contamination by the maternal blood. Then fetus and placenta were collected from the dam with caution as quickly as practically possible. The placenta and fetus was washed with normal saline, blot dried and the weight was measured. The samples were stored at approximately −80°C in pre-labeled tubes until analyzed. Dams were allowed to be recovered after the Cesarean section and collection of samples.
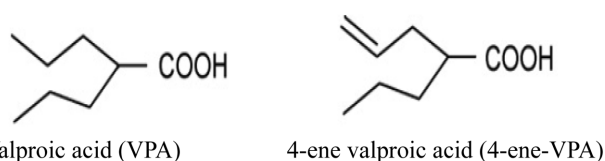
Fig. 2. Chemical structure of valproic acid (VPA) and 4-ene valproic acid (4-ene-VPA).

Bioanalysis of VPA and 4-ene-VPA in plasma, aminiotic fluid, placenta and fetus. Quantification of VPA and 4-ene-VPA (Fig. 2) was achieved by liquid chromatography/mass spectroscopy/mass spectroscopy (LC/MS/MS) using Agilent 1100 HPLC system (Agilent, USA) and API 3200 Qtrap (Applied Biosystems, USA). Fetus and placenta samples were homogenized with the addition of deionized distilled water (4X v/w). After acidifying the plasma, amniotic fluid, fetus homogenate samples with the addition of 10% HCl, samples were extracted by solid-phase extraction using OASIS HLB cartridge(Waters, USA). Hydrothiazide was used as an internal standard. VPA and 4-ene-VPA was analyzed using 5 mM Ammonium Formate/AcCN (50/ 50) as a mobile phase (Table 1). Lower limit of quantification (LLOQ) of VPA in plasma, amniotic fluid, placenta and fetus was 1 μg/m*l* or 1 μg/g and LLOQ of 4-ene-VPA in plasma, amniotic fluid, placenta and fetus was 0.5 μg/m*l* or 0.5 μg/g, respectively.

Pharmcokinetic analysis of plasma concentration data. Non-compartmental pharmacokinetic analysis was performed on individual plasma concentration data using a validated software WinNonlin®, (version 5.2.1, Pharsight Corp., USA). Where practical, following pharmacokinetic parameters were accessed: The apparent terminal elimination rate constant (λ_z) was determined using the non-compartmental model in WinNonlin®, and the apparent terminal half life ($t_{1/2,z}$) associated with the apparent terminal elimination phase was calculated from the equation, $t_{1/2,z} = 0.693/\lambda_z$. The maximum observed peak plasma concentration (C_{max}) and the time at which it is observed (T_{max}) were obtained directly from the concentration-time data. The area under

Table 1. Analytical condition of LC/MS/MS for simultaneous determination of valproic acid and 4-ene valproic acid

HPLC	MS/MS

- Mobile phase: 5mM Ammonium Formate/AcCN (1 : 1)
 - Column: C$_{18}$, 2.1 × 100 mm, 3.5 μm
 - Flow rate: 0.2 m*l*/min
 - Injection volume: 2 μ*l*
 - Column oven: 40°C
 - Internal standard: Hydrothiazide
	- Ionization mode: ESI negative mode
	 - Curtain gas: 15 psi
	 - Source temperature: 450°C
	 - Nebulizer gas: 15 psi
	 - Heating gas: 15 psi
	 - Declustering potential: −30 V
	 - SIM (selected ion monitoring) method
	   Valproic acid (143.3, EP −7 V)
	   4-ene valproic acid (141.3, EP −7 V)
	   Hydrothiazide (296.1, EP −10 V)

the plasma concentration-time curve (AUC) from the start of dosing to the last quantifiable sampling time point (AUC$_{last}$) was calculated using linear-logarithmic trapezoidal rule, and the AUC from time zero to infinity (AUC$_{inf}$) was calculated with linear-logarithmic trapezoidal rule and extrapolation to infinity by dividing the final concentration by λ_z. The mean residence time (MRT) from the start of dosing to the last quantifiable sampling time point (MRT$_{last}$) and the MRT from time zero to infinity (MRT$_{inf}$) were calculated from MRT$_{last}$ = AUMC$_{last}$/AUC$_{last}$ and MRT$_{inf}$ = AUMC$_{inf}$/AUC$_{inf}$, respectively; where AUMC$_{last}$ and AUMC$_{inf}$ are the area under the first moment curve from time 0 to the time of last sampling and AUMC from time 0 to infinity, respectively. Total body clearance (CL) was calculated from CL = dose/AUC$_{inf}$ and the volume of distribution based on the terminal phase (V$_z$) was calculated from V$_z$ = CL/λ_z. Since the administration route of this study is extravascular, the fraction of dose absorbed cannot be estimated. Therefore, V$_z$/F and CL/F were reported. Concentrations below LLOQ were treated as zero for calculating parameters. For the estimate of λ_z to be accepted as reliable, the following criteria were imposed:

1. The terminal data points were apparently randomly distributed about a single straight line (on visual inspection).

2. A minimum of 3 data points were available for the regression.

3. The regression coefficient was 0.85.

4. The interval including the data points chosen for the regression was at least two-fold greater than the half-life itself.

5. The % extrapolation value [(AUC$_{inf}$ − AUC$_{last}$)/AUC$_{inf}$ × 100] was < 20%.

Evaluation of placenta transfer. Placenta to maternal plasma ratio, amniotic fluid to maternal plasma ratio, and fetus to maternal plasma ratio were calculated using following equations.

Placenta to maternal plasma ratio
 = concentration in placenta (ng/g)
 /concentration in corresponding
 maternal plasma (ng/mL)
Amniotic fluid to maternal plasma ratio
 = concentration in amniotic fluid (ng/mL)
 /concentration in corresponding
 maternal plasma (ng/mL)
Fetus to maternal plasma ratio
 = concentration in fetus (ng/g)
 / concentration in corresponding
 maternal plasma (ng/mL)

RESULTS

Clinical signs, body weight and food consumption of pregnant monkeys. No treatment related abnormal clinical signs were observed in dams treated with VPA at doses of 20, 60 and 180 mg/kg/day. Although temporal vaginal hemorrhage was found in one dam from the vehicle control

Table 2. Pharmacokinetic parameters of valproic acid (VPA) after single oral administration of VPA in pregnant cynomolgus monkeys on the day 20 of gestation (n = 2)

| Dose (mg/kg/day)		λ_z (1/h)	$t_{1/2,z}$ (h)	T_{max} (h)	C_{max} (ng/ml)	AUC$_{last}$ (ng·h/ml)	AUC$_{inf}$ (ng·h/ml)	V$_z$/F (ml/kg)	CL/F (ml/h/kg)	MRT$_{last}$ (h)	MRT$_{inf}$ (h)
20	Mean	0.104	6.8	0.8	166.50	426	448	447	45.6	4.4	5.9
	SD	0.010	0.6	0.4	54.45	94	93	134	9.4	0.3	0.8
60	Mean	0.370	2.0	0.5	201.75	387	409	756	230.4	2.0	2.6
	SD	0.125	0.7	0.0	165.82	344	348	786	196.2	0.6	1.1
180	Mean	0.199	3.5	2.0	559.00	2290	2309	392	77.9	4.1	4.3
	SD	0.015	0.3	0.0	97.58	7	8	30	0.3	0.6	0.6

Table 3. Pharmacokinetic parameters of 4-ene valproic acid (4-ene-VPA) after single oral administration of VPA in pregnant cynomolgus monkeys on the day 20 of gestation (n = 2)

| Dose (mg/kg/day)		λ_z (1/h)	$t_{1/2,z}$ (h)	T_{max} (h)	C_{max} (ng/ml)	AUC$_{last}$ (ng·h/ml)	AUC$_{inf}$ (ng·h/ml)	V$_z$/F (ml/kg)	CL/F (ml/h/kg)	MRT$_{last}$ (h)	MRT$_{inf}$ (h)
20	Mean	0.078	11.0	2.0	1.05	5.87	17.11	18116	1381.8	4.2	17.0
	SD	0.048	6.7	0.0	0.01	0.42	9.50	1222	767.0	0.1	9.7
60	Mean	NA	NA	1.5	0.86	1.51	NA	NA	NA	2.1	NA
	SD	NA	NA	0.7	0.04	0.74	NA	NA	NA	1.0	NA
180	Mean	0.037*	18.7*	3.0	1.54	15.24	36.89*	43804*	1626.4*	7.9	27.3*
	SD	NA	NA	1.4	0.36	9.21	NA	NA	NA	4.2	NA

Note: NA; Not available, *; result was obtained from one animal sine the data from one animal did not meet the acceptance criteria described in materials and methods.
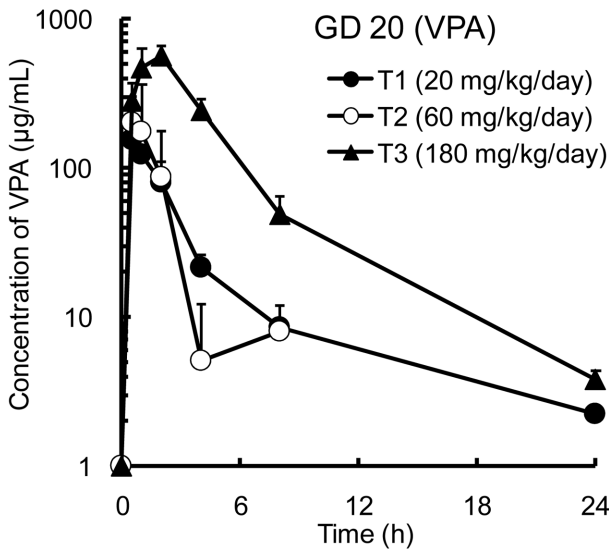
Fig. 3. Plasma concentration of valproic acid (VPA) after oral administration of valproic acid at doses of 20, 60 and 180 mg/kg in pregnant cynomolgus monkeys on day 20 of gestation (GD 20).
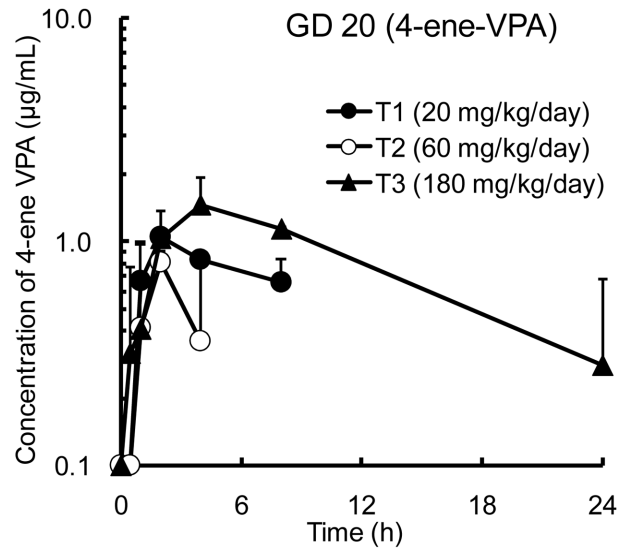
Fig. 4. Plasma concentration of 4-ene valproic acid (4-ene-VPA) after oral administration of valproic acid at doses of 20, 60 and 180 mg/kg in pregnant cynomolgus monkeys on day 20 of gestation (GD 20).
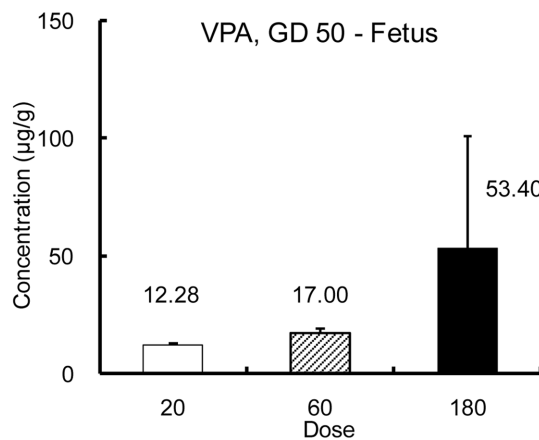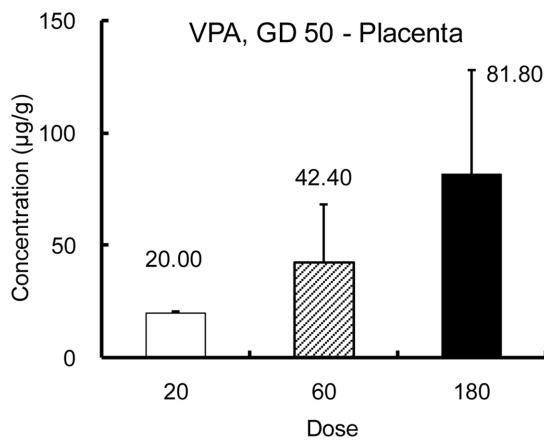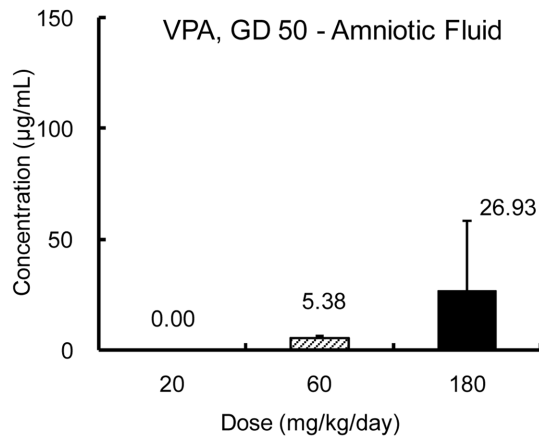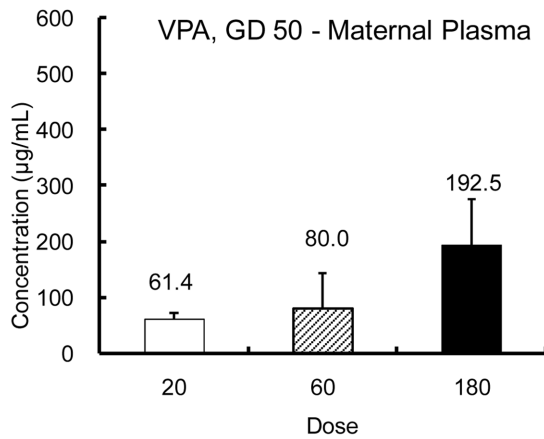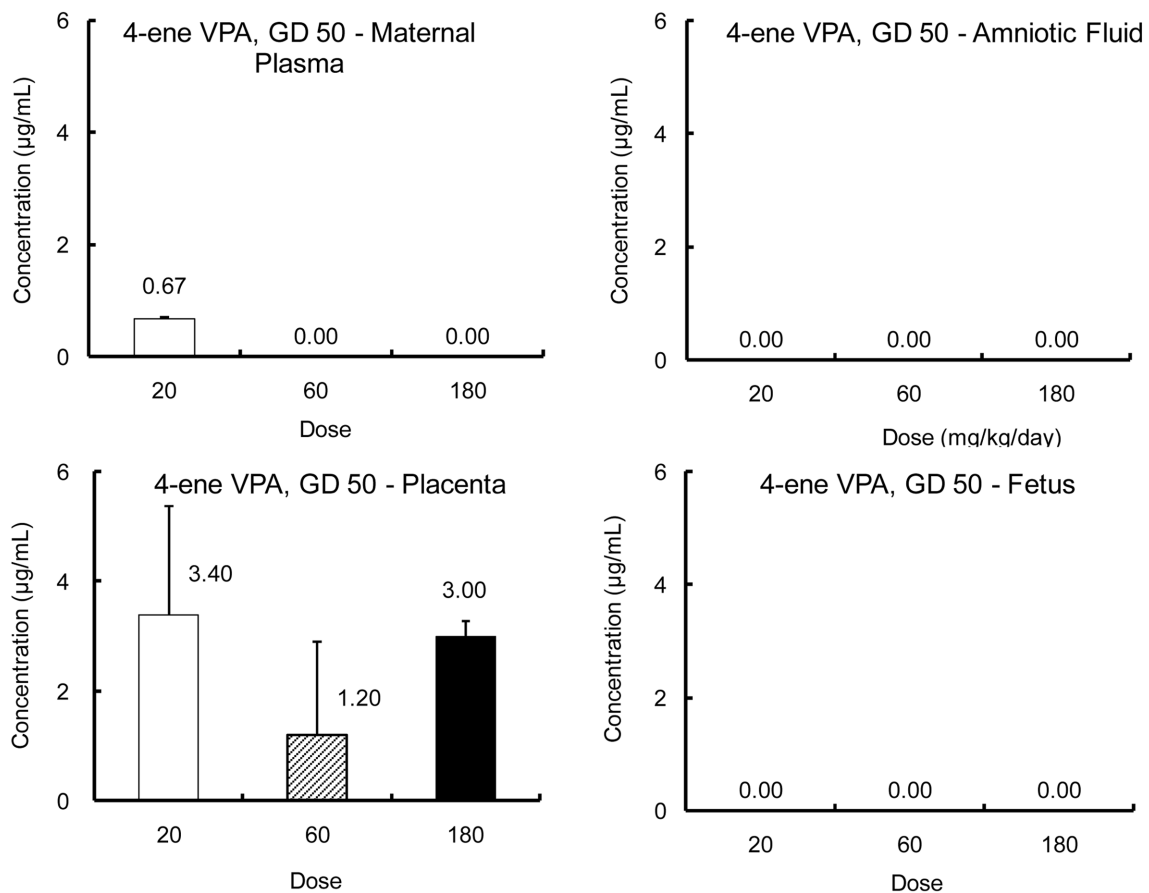

Fig. 5. Exposure of valproic acid (VPA) in maternal plasma, amniotic fluid, placenta and fetus on day 50 of gestation (GD 50) after oral administration of valproic acid at doses of 20, 60 and 180 mg/kg in pregnant cynomolgus monkeys in organogenesis period (from day 20 of gestation to day 50 of gestation).

group on day 34 of gestation, the animal was recovered. Slight decrease of body weight gain was observed in the animals in the treatment groups, without dose-relation, compared to the animals in the control group. On the other hand, a decrease in body weight gain was observed in the treatment groups. However, it was not considered to be treatment-related, since there was no dose-relation. There was no significant difference in food consumption between the vehicle control and the treatment groups during the study period.

Exposure of VPA in the plasma of pregnant cynomolgus monkeys. Following single administration of VPA at doses of 20, 60 and 180 mg/kg/day to pregnant monkeys on day 20 of gestation, concentrations of VPA were generally quantifiable in the maternal plasma from all treatment groups up to 8~24 hours post-dose, demonstrating that the pregnant monkeys were systemically exposed to VPA. Peak plasma concentration was reached at 0.5~1 h after dosing in animals dosed at 20 and 60 mg/kg/day, and at 2 h after dosing in animals dosed at 180 mg/kg/day, respectively. The

plasma concentration-time profile was apparently bi-exponential decline after the absorption phase. There was no remarkable difference in the maternal systemic exposure (determined by AUC_{last} and C_{max}) between the monkeys dosed at 20 and 60 mg/kg/day, but systemic exposure to VPA was increased by approximately 3~4 fold between animals dosed at 60 and 180 mg/kg/day. Apparent elimination half life ($T_{1/2,z}$) was 6.8, 2.0 and 3.5 h, and mean resident time (MRT_{last}) was 4.4, 2.0 and 4.1 h at 20, 60 and 180 mg/kg/day, respectively.

Exposure of 4-ene-VPA in the plasma of pregnant cynomolgus monkeys. Following single administration of VPA at doses of 20, 60 and 180 mg/kg/day to pregnant monkeys on day 20 of gestation, concentrations of 4-ene-VPA were generally quantifiable in the maternal plasma from all treatment groups up to 4~24 hours post-dose, and the plasma concentrations of 4-ene VPA were very low compared to those of VPA, the parent drug. Peak plasma concentration was 0.3~0.7%, and AUC_{last} was 0.3~1.5% of corresponding values of VPA. Peak plasma concentration

Fig. 6. Exposure of 4-ene valproic acid (4-ene-VPA) in maternal plasma, amniotic fluid, placenta and fetus on day 50 of gestation (GD 50) after oral administration of valproic acid at doses of 20, 60 and 180 mg/kg in pregnant cynomolgus monkeys in organogenesis period (from day 20 to day 50 of gestation).

was reached at 1~2 h after dosing in animals dosed at 20 and 60 mg/kg/day, and at 2~4 h after dosing in animals dosed at 180 mg/kg/day, respectively, which was longer than the corresponding values of VPA. Mean resident time (MRT$_{last}$) was 4.2, 2.1 and 7.9 h at 20, 60 and 180 mg/kg/day, respectively, which was similar as the corresponding values of VPA.

Placenta transfer of VPA after repeated oral administration during organogenesis period. After repeated administration of VPA at doses of 20, 60 and 180 mg/kg/day from day 20 to day 50 of gestation, VPA was detected in amniotic fluid, placenta and fetus taken after Cesarean section at approximately 1 h after dosing on day 50 of gestation. The concentrations of VPA were quantifiable in amniotic fluid, placenta and fetus from all treatment groups, and the exposures in amniotic fluid, placenta and fetus were increased with increasing dose. Amniotic fluid to maternal plasma ratio was 0.0, 6.7 and 14.0%, placenta to maternal plasma ratio was 32.6, 53.0 and 42.5%, and fetus to maternal plasma ratio was 20.0, 21.3 and 27.7% at 20, 60 and 180 mg/kg/day, respectively.

Exposure of 4-ene-VPA in placenta, amniotic fluid and fetus after repeated oral administration during organogenesis period. After repeated administration of VPA at doses of 20, 60 and 180 mg/kg/day from day 20 to day 50 of gestation, the concentrations of 4-ene-VPA in both amniotic fluid and fetus on day 50 of gestation were under LLOQ (0.5 μg/m*l* amniotic fluid or 0.5 μg/g fetus), but small amount of 4-ene-VPA was detected in placenta at all the doses studied (20, 60 and 180 mg/kg/day), although the maternal plasma concentration was slightly over or under LLOQ (0.5 μg/m*l* plasma.)

DISCUSSION

There are close relation between the species differences in pharmacokinetics and drug teratogenesis (Nau, 1986), and a lot of studies have been performed in this area. Because of the similarity between humans and NHP, there have been growing needs for the toxicity and pharmacokinetic study in pregnant NHP. Cynomolgus monkeys are one of the most commonly used NHP in toxicity and pharmacokinetics assessment. The primary object of the present study was to establish the methods for placenta transfer study in cynomolgus monkeys. VPA was selected as a model drug because firstly, VPA is a well known teratogen in humans and experimental animals but still is used in humans including pregnant population and application of VPA is getting wider (Alsdorf and Wyszynski, 2005, Ornoy, 2009), and secondly embryo-fetal toxicity and toxicokinetics of VPA have been conducted using various species, such as mice, rats, sheep and rhesus monkeys but not in cynomolgus

monkeys.

In this study, procedures to investigate placenta transfer in cynomolgus monkeys, e.g., mating and non-invasive diagnosis of pregnancy via examining gestational sac with ultrasonography, collection of amniotic fluid, placenta and fetus after Caesarean section were established. Animals were allowed to be recovered after Caesarean section.

In maternal toxicities, no treatment-related changes in general findings, such as clinical signs, body weight and food consumption, were noted after repeated administration of VPA at doses of 20, 60 and 180 mg/kg/day from day 20 to day 50 of gestation, which is generally comparable to those previously reported in rhesus monkeys (Hendrickx *et al*., 1988); minimal and dose independent toxicities were observed in pregnant rhesus monkeys treated with VPA at dose levels of 100~600 mg/kg/day during the organogenesis period from day 21 to day 50 of gestation (Hendrickx *et al*., 1988). Although this study was not intended to elucidate teratogenic effect of VPA in cynomolgus monkeys, no external abnormality was noted in fetus, which were sampled for toxicokinetic analysis, in contrast to the previously reported in rhesus monkeys, where dose-dependent developmental toxicity was observed at dose levels of 20~600 mg/kg/day (Hendrickx *et al*., 1988).

In pharmacokinetic analysis of maternal plasma after single administration on day 20 of gestation, pregnant monkeys were exposed to VPA and 4-ene-VPA after oral administration of VPA at 20~180 mg/kg/day. The maternal plasma concentration-time profile showed bi-exponential decline, which was similar as previously reported concentration-time profile in rhesus monkeys of late pregnancy (day 141 of gestation) after intravenous administration of VPA at 50 mg/kg (Dickinson *et al*., 1979; 1980), and in rhesus monkeys of organogenesis period after oral administration of VPA at 100 or 200 mg/kg (Hendrickx *et al*., 1988). At 180 mg/kg/day, the apparent elimination half life was slightly longer, but C$_{max}$ and AUC wereslightly higher than those reported in rhesus monkeys after oral administration of VPA at 200 mg/kg (Hendrickx *et al*., 1988). Peak VPA concentration was considered to be an important aspect of VPA teratogenicity (Nau, 1986), but higher C$_{max}$ in the present study was not accompanied by higher teratogenicity, suggesting involvement of other complex factors in teratogenic effect of VPA. Species difference in maternal plasma protein binding was pointed as one of the important factors affecting the teratogenicity and toxicity of VPA and other drugs (Nau, 1986; Simmes and Shen, 1990). In the present study, free VPA concentration was measured using solid phase extraction of plasma samples followed by LC/MS/MS analysis, but plasma protein binding study was not conducted since it was out of scope. Further study may be needed in the future.

There was no remarkable difference in the apparent terminal elimination rate constant, apparent terminal half life, total body clearance and the volume of distribution between

animals dosed at 60 and 180 mg/kg/day, and systemic exposure (determined by AUC_{last} and C_{max}) was increased approximately dose proportionally between 60 and 180 mg/kg/day. These results suggests linear pharmacokinetics of VPA at dose range between 60 and 180 mg/kg/day. Linear elimination kinetics was also reported in rhesus monkeys of organogenesis period after oral administration of VPA at 100~200 mg/kg (Hendrickx *et al.*, 1988). There was no remarkable difference in the maternal systemic exposure (determined by AUC_{last} and C_{max}) between the monkeys dosed at 20 and 60 mg/kg/day. These results might be partially due to the relatively low plasma concentrations and high individual variance at these doses.

Among the metabolites of VPA, exposure of 4-ene-VPA was determined based on the toxicity, commercial availability and easiness of simultaneous analysis with VPA. 4-ene-VPA is known as a hepatotoxic metabolite of VPA which generates a chemically reactive intermediate(s) that alkylate(s) cellular macromolecules (Rettenmeier *et al.*, 1986a, b).

4-ene-VPA was detected in the plasma with lower concentrations than those of VPA with less than 1% of VPA in C_{max} at all dose levels studied (20~180 mg/kg/day), which was higher than the result in rhesus monkeys, where C_{max} of 4-ene-VPA did not exceed 1 μg/m*l* at 200 mg/kg/day (Hendrickx *et al.*, 1988).

In placenta transfer analysis on day 50 of gestation, VPA was transferred via placenta, and the fetus was exposed to VPA after oral administration of VPA at 20~180 mg/kg/day during the organogenesis period. The exposures in amniotic fluid, placenta and fetus were increased with increasing dose between 20 and 180 mg/kg/day. The fetus to maternal plasma ratio was approximately 20~30%, which was lower than the previously reported ratios in rhesus monkeys and humans, although direct comparison is difficult since different methods such as, dose, administration route, duration of administration, timing of sampling and bioanalytical methods, were used in each study. The fetus to maternal plasma ratio in rhesus monkeys dosed at 200 mg/kg/day during organogenesis period (day 21 to 37 of gestation) and determined on day 37 of gestation was approximately 50% (Hendrickx *et al.*, 1988), and the fetal blood to maternal blood ratio in rhesus monkeys dosed at 50 mg/kg in terminal pregnancy (day 141 and 142 of gestation) and determined on day 142 of gestation was approximately 125~145% (Dickinson *et al.*, 1980), and the new born to mother blood ratio in an epileptic mother was approximately 140% (Dickinson *et al.*, 1979). Although the concentrations of 4-ene-VPA in fetus and amniotic fluid were lower than LLOQ (0.5 μg/g or m*l*), 4-ene-VPA was detected in the placenta samples, suggesting the possibility of placenta transfer of 4-ene-VPA in cynomolgus monkeys at dose levels of 20~180 mg/kg/day.

In summary, when VPA was repeatedly administered to the pregnant monkeys during entire organogenesis period

(day 20 of gestation to day 50 of gestation) at doses of 20, 60 and 180 mg/kg/day via oral route, maternal plasma was exposed to VPA and 4-ene-VPA, one of the toxic metabolites of VPA. VPA was transferred via placenta, and the fetus was exposed to VPA with fetus to maternal plasma ratio of 20~30%, which was lower than reported values in rhesus monkeys and human. The exposure of VPA in fetus was increased with increasing dose. Although concentrations of 4-ene VPA in fetus were under the lower limit of quantification (0.5 μg/g), the presence of 4-ene-VPA in the placenta samples suggested the possibility of placenta transfer of 4-ene-VPA in cynomolgus monkeys at dose levels of 20~180 mg/kg/day. The maternal plasma concentration-time profile was similar as in rhesus monkeys as previously reported, with higher peak VPA concentration.

The present study is the first one to report the toxicokinetics and placenta transfer of VPA in cynomolgus monkeys. Considering the present situation that only limited numbers of placenta transfer studies in NHP are available, the method and results reported in the present study provides basic information on the toxicokinetics and placenta transfer of VPA in cynomolgus monkeys.

In conclusion, we established the method to study placenta transfer in cynomolgus monkeys using VPA as a model drug, and demonstrated that VPA was transferred via placenta, and the fetus was exposed to VPA after repeated oral administration of VPA at doses of 20, 60 and 180 mg/kg/day in pregnant monkeys during the entire organogenesis period (day 20 of gestation to day 50 of gestation).

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