Approaches of validation of a 2-week combined repeated oral dose toxicity study with plasma micro sampling toxicokinetics (PMS-TK) in common marmosets

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ABSTRACT — We investigated the viability of a combined repeated dose toxicity study, including toxicokinetics (TK), in common marmosets according to the ICH-S4, ICH-S3A and ICH-S7A Guidelines using valsartan as test article whose non-clinical repeated dose toxicity studies had been conducted using this species for regulatory purpose. Valsartan was administered orally to 3 animals/sex at 200 mg/kg/day for 2 weeks. In addition to the routine parameters in repeated dose toxicity studies, safety pharmacology parameters (examinations of the central nervous, respiratory and cardiovascular systems) were also evaluated. The Plasma Micro Sampling Toxicokinetics (PMS-TK) method required ultrasensitive quantitation, was employed to evaluate the relationship between toxic changes and plasma concentrations as well as the effects of frequent blood sampling in individual animals. In valsartan, toxic findings (a deteriorated physical condition; moribundity of one male and one female on Day 14; sporadic vomitus; decreases in body weights and food consumption; decreases in erythrocytic parameters; and renal changes such as an increase in urea nitrogen, dilation of the tubules and hypertrophy of the tubular epithelium) were similar and plasma concentrations comparable to the results in the approval information. Furthermore, no side effects caused by frequent blood sampling were confirmed in the negative control group. Consequently, a combined repeated dose toxicity study including TK analysis using the PMS-TK method is viable in common marmosets and contributes to animal welfare.

Key words: Common marmoset, Combined repeated dose toxicity study, Valsartan, Plasma micro sampling toxicokinetics (PMS-TK) method, Ultrasensitive quantitation

INTRODUCTION

Prior to a First in Human (FIH) clinical trial, non-clinical studies that include 2-week repeated dose toxicity studies in one rodent and one non-rodent species, safety pharmacology core battery studies (CNS, respiratory, cardiovascular, telemetry, hERG), and genotoxicity studies (Ames, chromosomal aberration) are required (ICH, 2009). Meanwhile, the ICH-M3 (R2) and its corresponding Japanese guidance, “Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals”, stipulated that any in vivo safety pharmacology studies should be involved to the general toxicity studies (Ministry of Health, Labour and Welfare of Japan, 2010). This statement clarifies the 3R principles of animal experiments; to reduce the number of animals used, refine the pain, and replace with alternatives, so that it is important to materialize those principles.

Common marmosets (Callithrix jacchus; hereafter marmoset) belong to the same family as humans, anthropoids (haplorhines), and have a variety of similarities to our species in physiological and anatomical characteristics as well as in drug metabolism (Yamaguchi et al., 1986; Mansfield, 2003; Okano et al., 2012; ’t Hart et al., 2012; Uno et al., 2016). The fact that thalidomide-induced phocomelia, one of the most serious side-effects of medical drugs, had been reproduced in marmosets (Poswillo et
Marmosets are as small as adult rats (Orsi et al., 2011), and well known for their ease of handling among non-human primates (Layne and Power, 2003). However, their small size did cause limitations in the volume and frequency of blood sampling possible, unlike the macaque, and satellite groups are required of toxicokinetics in repeated dose toxicity studies (Smith et al., 2001; Orsi et al., 2011). This is a large concern in a combined toxicity study and safety pharmacology studies using the marmoset because reducing the number of animals might be difficult.

However, the latest technological advances in high-performance LC/MS/MS enabled us to analyze plasma drug concentration with only a minute volume of sample (ICH, 2017). We looked at these advances and considered if the PMS-TK method, a methodology combining with micro-blood-sampling and ultrasensitive quantitation, made it possible to conduct at once toxicity evaluation and TK in the same marmosets, which might result in satellite group elimination (Orsi et al., 2011).

To validate this possibility, a combined repeated dose toxicity study, including TK analysis, in marmosets was conducted in accordance with ICH guidelines (ICH-S4, ICH-S3A and ICH-S7A) using valsartan (Diovan® tablet, Novartis Pharmaceuticals; an angiotensin II receptor antagonist possessing an antihypertensive action), for which marmosets were used in a non-clinical study for approval information (FDA, 1996), to evaluate the reproducibility of data and the presence or absence of effects of the experimental procedures on animals.

MATERIALS AND METHODS

This study was conducted in compliance with the Japanese “Law for the Humane Treatment and Management of Animals” and the “Guidance for Animal Care and Use” of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee (IACUC) of Ina Research Inc., a facility which is fully accredited by AAALAC International.

Animals and housing conditions

Marmosets (3 animals/sex/group; males 1-9 years, females 5-8 years) were purchased from CLEA Japan Inc. (Tokyo, Japan). The number of animals per group was selected based upon general information available in the applications for Diovan®, accepted by the US FDA (FDA, 1996) and the Japanese PMDA (PMDA, 2000), in accordance with ICH guideline (ICH-S4A) recommendations for 3-month repeated dose toxicity studies. In consideration of animal welfare, only 1 group was set in this study, since the toxic profile of the test article has already been established. The animals were housed individually in stainless steel wire mesh cages (39W × 56.5D × 70H cm) fitted with wooden perches for enrichment. The temperature in the animal room was maintained between 25.0-29.0°C and humidity between 30.0-70.0%. Lighting was set on a 12-hr light-dark cycle (fluorescent lighting from 7:00 to 19:00). Each animal was provided with 20 g of feed (CMS-1M, CLEA Japan Inc.) daily and drinking water ad libitum via an automatic watering system, with the exception that polypropylene water bottles were used during measurements of water consumption.

Dosing articles

Valsartan, JP (Diovan® OD tablet 160 mg; Novartis Pharma K.K, Tokyo, Japan) was suspended in a 0.5 w/v% carmellose sodium aqueous solution containing 0.1% Tween 80 at concentrations to achieve a dose volume of 1 mL/100 g of body weight. The vehicle was used as the negative control.

Justification for selection of the dose level

In a 3-month subacute toxicity study of Diovan® OD tablets in marmosets via oral gavage, vomiting associated with the bitter taste of the test article, decreases in skin elasticity and erythrocytic parameters, a tendency to decrease in body weights and food consumption and increases in urea nitrogen and the creatinine content, as well as degeneration of the tubular epithelium, basophilic changes and interstitial nephritis in the kidneys were noted at 200 mg/kg and above. Furthermore, 1 of the 6 females receiving 200 mg/kg exhibited a deteriorated physical condition and was prematurely euthanized on Day 20 (FDA, 1996). Based on this information, 1 female animal/dose level was administered with valsartan at 200, 300 or 400 mg/kg/day in a preliminary 2-week dose range-finding study (PMS-TK not conducted). As a result, clear decreases in body weights and food consumption were noted at 300 and 400 mg/kg/day. Although no death occurred, emaciation was so severe at 400 mg/kg/day that the animal was euthanized at completion of study procedures. Therefore, 200 mg/kg/day, a dose level at which similar changes could be expected but death would not occur after 14-day administration, was selected as the dose level for the current study.
Dosing method and duration

The dosing formulation was prepared at concentrations to achieve a dose volume of 1 mL/100 g of body weight. Dosing was conducted once daily for 14 days via oral gavage using nutritional catheters. Animals in the negative control group received a 0.5 w/v% carmellose sodium aqueous solution containing 0.1% Tween 80 in a similar manner. A dosing period of 14 days was selected based on the ICH guideline [ICH-M3 (R2)], which recommends a nonclinical repeated dose toxicity study of at least 2 weeks be completed prior to initiation of FIH clinical trials.

Observations and examinations (Table 1)

Clinical observations, body weights and food and water consumption

Clinical observations were conducted daily, measurements of body weights and food consumption were conducted twice/week and measurements of water consumption were conducted during Week 2.

Ophthalmology

After instillation of a mydriatic (Mydrin-P; Santen Pharmaceutical Co., Ltd., Osaka, Japan) during pretest and Week 2, the animals were anesthetized with 0.15 mL/kg of ketamine hydrochloride (KETAMINE INJ. 5%; Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) and 0.03 mL/kg of xylazine (Celactal® 2% injection; Bayer Yakuhin, Ltd., Osaka, Japan) by intramuscular injection. Then, the anterior portions of the eyes, optic media and ocular fundi were examined using a slit-lamp (Kowa Company, Ltd., Nagoya, Japan or Takagi Seiko Co., Ltd., Toyama, Japan) and a binocular indirect ophthalmoscope (Neitz Instruments Co., Ltd., Tokyo, Japan or Heine Optotechnik GmbH, Herrsching, Germany).

Hematology and clinical chemistry

Animals were anesthetized by inhalation of isoflurane, JP (Mylan Seiyaku Ltd., Tokyo, Japan; hereafter referred to as “isoflurane”) and blood samples of approximately 1.2 mL were collected from the femoral vein during pretest and Week 2. The ADVIA 120 Hematology System (Siemens Healthcare Diagnostics K.K., Tokyo, Japan) and Automated Blood Coagulation Analyzer CA-510 (Sysmex Corporation, Hyogo, Japan) were used for analyses of RBC, HGB, HCT, MCV, MCH, MCHC, Retic (ratio and count), PLT, WBC, differential WBCs (ratio and count), PT and APTT. Differential WBCs were determined under a microscope after blood smear slides were prepared and stained with May-Grunwald-Giemsa. Blood samples were then centrifuged (approx. 1,600 × g, 10 min, 4°C) to obtain plasma. Plasma samples were analyzed for AST, ALT, ALP, LDH, CK, GLU, T-Bil, BUN, CRE, T-Chol, TG, PL, P, K, Ca, Na, K, Cl, TP, ALB and A/G using the Clinical Analyzer Model 7180 (Hitachi High-Technologies Corporation, Tokyo, Japan; hereafter referred to as “Hitachi 7180”).

Table 1. Study schedule.

| Category            | Examination                      | Period                                                                 |
|---------------------|----------------------------------|------------------------------------------------------------------------|
|                     |                                  | Prior to dosing  | Dosing |
|                     |                                  | Week  -1         | 1 - 15 |
|                     |                                  | Day   -7 -6 -5 -4 -3 -2 -1     | 1  2  3  4  5  6  7  8  9  10  11 12 13 14 15 |
| Toxicty             | Clinical observations            | ✓✓✓✓✓✓✓✓✓✓         |        |
|                     | Food consumption                 | ✓✓✓✓✓✓✓✓        |        |
|                     | Body weight                      | ✓✓✓✓✓✓         |        |
|                     | Water consumption                | ✓✓✓✓✓         |        |
|                     | Urinalysis                       | ✓✓✓✓         |        |
| Ophthalmology       | Ophthalmology                    | ✓✓✓         |        |
| Hematology          | Clinical chemistry               | ✓✓         |        |
|                     | Gross pathology                 | ✓✓         |        |
| Toxicokinetics (TK) |                                  | ✓✓         |        |
| Safety pharmacology | Functional observational battery | ✓✓         |        |
|                     | Blood pressure and heart rate   | ✓✓         |        |
|                     | Blood gases                      | ✓✓         |        |

✓ : Day of test
Blood sample volume:
| a) 1.2 mL |
| b) 50 μL × 7 points |
| c) 0.5 mL |

Combined toxicity study in common marmosets
Urinalysis

Animals were transferred to metabolic cages for urine collection during pretest and Week 2. The urine accumulated over 4-8 hr after the start of urine collection was used as fresh urine for analyses of the pH, Pro., Glu., Ket., Bil., Occ., Uro. and sediments. The urine collected over 24 hr after the start of urine collection was used as cumulative urine for analyses of Vol., Col., S.G., NA, K and CL. Analyses were conducted using the Urine S.G. Refractometer URC-JE (Atago Co., Ltd., Tokyo, Japan), Hitachi 7180 and Urine Chemistry Analyzer Clinitek 500 (Siemens Healthcare Diagnostics K.K.).

Functional Observational Battery

Home cage observations (posture/position, vocalization, piloerection, tremors, convulsions, respiration, stereotypy, abnormal behavior, palpebral closure, feces, urine, ataxia, bradykinesia, continual movement and hypoactivity), hand-held observations (ease of handling and removal of the cage, aggression to the observer, hyporeactivity to the observer, muscle twitching, muscle tone, skin, pupil size, lacrimation, eyelid reflex and salivation), body temperature measurements (rectal temperature) and observations of sensorimotor function and reflexes (catalepsy, auditory response, grip strength of all 4 limbs and pain response) were conducted once during pretest (at the time corresponding to 1 hr post-dosing during the dosing period) and at 7 time points (pre-dosing and at 0.5, 1, 2, 4, 8 and 24 hr post-dosing) on Days 1 and 14.

Blood pressure and heart rate

During pretest and at 1-2 hr post-dosing on Days 1 and 14, unanesthetized animals were kept in non-load ed restrainers and the cuff of an automatic sphygmomanometer (BP-98AL, Softron Co., Ltd., Tokyo, Japan) was placed around the tail to measure blood pressure (systolic, diastolic and mean) and heart rate non-invasively.

Blood gases

Blood samples (0.5 mL) were collected from the femoral artery of the animals using arterial blood sampling kits (Rapidlyte, Siemens Healthcare Diagnostics K.K.) and analyzed using the Ciba Corning 248 (Siemens Healthcare Diagnostics K.K.) for analyses of partial pressure of oxygen, partial pressure of carbon dioxide and oxygen saturation during pretest and Week 2 (1 hr post-dosing).

Gross pathology, organ weights and histopathology

One day after the last dosing, the animals were anesthetized by inhalation of isoflurane and euthanized by exsanguination from the axillary and femoral arteries and veins for complete macroscopic postmortem examinations. The major organs were removed and fixed in 10 vol% neutral buffered formalin after recording the organ weights. However, the testes were fixed in formalin-sucrose-acetic acid (FSA) solution, the eyes with optic nerves were fixed in 1% formaldehyde-2.5% glutaraldehyde in phosphate buffer and both organs were post-fixed in 10 vol% neutral buffered formalin. Following routine paraffin embedding, the organs were sectioned, stained with hematoxylin and eosin (HE) and examined histopathologically.

PMS-TK method

At pre-dosing and 0.5, 1, 2, 4, 8 and 24 hr post-dosing (7 time points) on Days 1 and 14, blood samples of 50 μL/time point were collected from the tail or femoral vein of unanesthetized animals using the PMS method using polypropylene syringes treated with heparin sodium and fitted with 25 or 26 G needles. Blood samples were promptly chilled in ice water and centrifuged (approx. 1,600 × g, 10 min, 4°C) within 30 min of sampling to obtain plasma. Plasma samples were stored frozen at −80°C in Ina Research Inc and transferred to Sumika Chemical Analysis Service, Ltd and stored at −20°C until analysis.

Valsartan concentrations in plasma were analyzed by LC/MS/MS, consisting of Nexera X2 (Shimazu Corporation, Kyoto, Japan), a column (Shim-Pack XR-ODS, 75 mm L × 3.0 mm I.D., 2.2 μm, Shimazu Corporation) and Triple Quad 6500 (AB Sciex Pte. Ltd., Framingham, MA, USA). Gradient elution was performed with mobile phase A (water:formic acid (1000:5, v/v)) and mobile phase B (acetonitrile:formic acid (1000:5, v/v)). The flow rate was 0.7 mL/min. Quantification of ion was performed in the MRM (Multiple Reaction Monitoring) mode using the internal standard method based on the peak area ratio. The MRM transitions of m/z 436→m/z 207 were chosen for valsartan. TK parameters (Cmax, Tmax and AUC0-24hr) were calculated using Phoenix WinNonlin software (Certara, LP, Princeton, NJ, USA). Samples of 2.5 μL were used for analysis at each time point.

One female in the test article-treated group was euthanized prior to blood sampling for the PMS-TK method on Day 14; therefore, analysis of plasma valsartan concentrations was not conducted for this animal. In addition, the AUC value for 1 male in the test article-treated group that was euthanized at 8 hr post-dosing on Day 14 was calculated using the plasma concentrations obtained between 0 and 8 hr post-dosing.
Statistical analysis
Mean group values and standard deviations were calculated for the quantitative data. A two-tailed Student's t-test was conducted between the control and test article-treated groups to evaluate the effects of the test article. Furthermore, to evaluate the effects of blood sampling, control group data for hematology, clinical chemistry, urinalysis and blood gas measurements were compared between pretest and Week 2 and those for blood pressure and heart rate were compared between Days 1 and 14 using a one-tailed Student’s t-test. Differences from the control group were evaluated at the 5% level of significance.

RESULTS

Clinical signs, body weights and food and water consumption
One female in the test article-treated group exhibited a deteriorated physical condition, evidenced by decreases in movement and bradypnea, from pre-dosing on Day 11 and was euthanized on Day 14. In addition, 1 male, in which no clinical signs had been observed, was euthanized on Day 14 after suddenly showing decreases in movement and body temperature as well as bradypnea. Likewise, the physical condition of the surviving female in the treated group, in which no clinical signs had been observed until the last day of dosing, suddenly deteriorated just prior to necropsy. Vomiting of undigested feed was sporadically noted for 1 male and 1 female in the treated group.

Decreases in food consumption and body weights were noted in the abovementioned animals, to a marked degree in the moribund animals in particular. The moribund female also exhibited a marked decrease in water consumption (Fig. 1).

Ophthalmology
No treatment-related changes were observed.

Hematology and clinical chemistry
In the hematology, decreases in the erythrocytic parameters were noted in the test article-treated group, accompanied by a related increase in the reticulocyte count. In the clinical chemistry, increases in urea nitrogen were noted in males and females in the test article-treated group.

Fig. 1. Body weight, food and water consumption. Valsartan at 200 mg/kg/day or vehicle was given by oral gavage once/day. Graphs A-C represent mean body weight, food and water consumption (n = 3), respectively. Body weight: vehicle vs. valsartan; vehicle on Day 1 vs. Day 14, no significant differences. Food consumption: vehicle vs. valsartan; vehicle on Day -1 vs. Day 13, no significant differences. Water consumption: vehicle vs. valsartan, vehicle on pre vs. Week 2, no significant differences.
No changes considered to be a direct effect of treatment with the test article were noted in any other parameter. In the negative control group, no changes suspected to be the effects of blood sampling were noted in any parameter between the pretest and Week 2.

**Urinalysis**

No effects of treatment with the test article or blood sampling were observed.

**Functional observational battery**

Other than the changes attributable to the deteriorated physical condition noted in 1 male and 1 female on Day 14, no pharmacological effects were noted in the test article-treated group. In the negative control group, no effects associated with blood sampling were noted.

**Blood pressure and heart rate**

Decreases or a tendency to decrease in the systolic, diastolic and mean blood pressure values were noted in males and females in the test article-treated group on Days 1 and 14 as compared to pretest values. In females, a compensatory increase in the heart rate was also noted (Table 3). Compared to the pretest values, no statistically significant changes were noted in any parameter in the negative control group on Day 1 or 14.

**Blood gases**

No statistically significant changes were noted in either sex in the test article-treated group as compared to the negative control group. Furthermore, compared to the pretest values, no changes associated with blood sampling were noted in any parameter in the negative control group on Day 1 or 14.

**Gross pathology, organ weights and histopathology**

In the moribund animals, dilation of the tubules and hypertrophy of the tubular epithelium were observed in the kidneys. Chronic inflammation of the small intestine and acute or chronic inflammation of the large intestine were also observed in these animals (Fig. 2, Table 4). Other than the above lesions, adrenal cortical hyperplasia was noted.

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**Table 2.** Hematology and clinical chemistry.

| Sex    | Group               | Sex Group | RBC (10^6/μL) Pre | RBC (10^6/μL) Week 2 | HGB (g/dL) Pre | HGB (g/dL) Week 2 | HCT (%) Pre | HCT (%) Week 2 |
|--------|---------------------|-----------|-------------------|----------------------|----------------|------------------|-------------|----------------|
| Male   | Vehicle             | Male      | 5.17 ± 1.85       | 6.03 ± 1.69          | 11.1 ± 4.0     | 12.7 ± 3.4       | 40.2 ± 12.2 | 46.2 ± 10.9    |
|        | Valsartan 200 mg/kg/day | Male      | 5.08 ± 1.45       | 4.63 ± 1.12          | 11.7 ± 3.0     | 10.2 ± 2.3       | 40.2 ± 10.2 | 37 ± 6.7       |
| Female | Vehicle             | Female    | 4.19 ± 1.23       | 4.36 ± 1.19          | 9.4 ± 2.2      | 9.5 ± 2.2        | 34.1 ± 7.7  | 35 ± 8.5       |
|        | Valsartan 200 mg/kg/day | Female  | 4.79 ± 0.88       | 3.88 ± 0.92          | 10.3 ± 1.3     | 8.2 ± 1.9        | 36.8 ± 5.3  | 29.9 ± 7.3     |

*: p < 0.05, pre vs. Week 2 in vehicle

**Table 3.** Blood pressure and heart rate.

| Group          | Sex   | SBP (mmHg) Day | MBP (mmHg) Day | DBP (mmHg) Day | HR (beats/min) Day |
|----------------|-------|---------------|---------------|---------------|-------------------|
|                | Pretest 1 | 14  | Pretest 1 | 14 | Pretest 1 | 14 | Pretest 1 | 14 | Pretest 1 | 14 |
| Vehicle        | Male   | 117±9   | 81±6 | 61±7   | 253±31 | 323±12 | 283±35 |
|                | Female | 104±6   | 73±15 | 58±21  | 313±20 | 331±25 | 312±32 |
| Valsartan 200 mg/kg/day | Male   | 108±17  | 84±11 | 73±9   | 269±16 | 272±51 | 273±84 |
|                | Female | 106±13  | 73±12 | 56±15  | 269±34 | 268±72 | 325±1  |

Pretest and Day 1: N = 3   Day 14: Vehicle groups; N = 3, Valsartan groups; N = 2
*: p < 0.05 vs. vehicle
No significant differences: vehicle on pretest vs. Day 1 and 14

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(Table 2). No changes considered to be a direct effect of treatment with the test article were noted in any other parameter.

In the negative control group, no changes suspected to be the effects of blood sampling were noted in any parameter between the pretest and Week 2.
sia, thought to be attributable to stress, and thymic atrophy, found at a similar severity in the control group, were also observed.

In the remaining animals in the test article-treated group and the animals in the negative control group, inflammatory changes were noted from the duodenum to the rectum; however, the degree tended to be more severe in the test article-treated group.

Changes associated with blood sampling, such as extramedullary hematopoiesis, were not noted in the negative control group.

**PMS-TK**

Clear individual differences were noted in plasma concentrations on Days 1 and 14.

On Day 1, plasma concentrations reached the $C_{\text{max}}$ (males: 61,300 ng/mL, females: 61,900 ng/mL) between 0.5 and 1.5 hr post-dosing and decreased gradually thereafter. The $\text{AUC}_{0-24\text{hr}}$ values were 217,000 and 200,000 ng·hr/mL in males and females, respectively. In the application for Diovan® (PMDA, 2000), the $\text{AUC}_{0-24\text{hr}}$ values were noted as 115,000 ng·hr/mL (264 μmol·hr/L) in males and 90,000 ng·hr/mL (207 μmol·hr/L) in females at 200 mg/kg on Day 1, meaning the $\text{AUC}_{0-24\text{hr}}$ values in the current study were approximately 2-fold the application values.

Plasma concentrations on Day 14 were comparable to those on Day 1, indicating no effects of repeated dosing.

Fig. 2. Histopathology (HE stain). Histopathological features in the kidneys treated with Valsartan at 200 mg/kg/day (Animal No. 2M02). The photographs show (A) dilation, tubule, (B) hypertrophy, tubular epithelium.

**Table 4. Histopathology.**

| Group       | sex | Animal No. | Kidney | Ileum | Cecum | Colon |
|-------------|-----|------------|--------|-------|-------|-------|
|             |     |            | dilation, tubule | hypertrophy, tubular epithelium | inflammation, chronic | inflammation, acute/chronic | inflammation, acute/chronic |
| Vehicle     | Male | 1M01       | -       | -     | +     | ±     | -     |
|             |     | 1M02       | -       | -     | -     | ±     | ±     |
|             |     | 1M03       | -       | -     | ±     | ±     | ±     |
|             | Female | 1F01       | -       | -     | ±     | +     | +     |
|             |     | 1F02       | -       | -     | +     | +     | ±     |
|             |     | 1F03       | -       | -     | +     | ±     | ±     |
| Valsartan 200 mg/kg/day | Male | 2M01       | -       | -     | ±     | +     | +     |
|             |     | 2M02 a)    | +       | ±     | ++    | ++    | ++    |
|             |     | 2M03       | -       | -     | ++    | ±     | -     |
|             | Female | 2F01       | -       | -     | ±     | -     | -     |
|             |     | 2F02 a)    | +       | -     | -     | ++    | ++    |
|             |     | 2F03       | -       | -     | +     | ±     | ±     |

- None/Negative ±: Slight +: Moderate ++: Severe +++: Very severe

a) Necropsied on Day 14 due to a moribund condition
Furthermore, there were no sex differences noted on Day 1 or 14, and plasma concentrations in the negative control group were below the lower limit of quantification (10 ng/mL) at all time points on both days.

In the male that became moribund during blood sampling at 24-hr post-dosing on Day 14 in the test article-treated group, the Cmax and AUC0-24hr values were almost 10-fold those of the other animals (Fig. 3, Table 5).

Results of incurred sample reanalysis (ISR) conformed to the criteria for acceptability stipulated in the relevant guidelines, confirming the validity of the analytical method used in this study.

**DISCUSSION**

Changes observed in the test article-treated group, such as decreases in movement and body temperature and bradypnea, were surmised to be due to strong toxic effects of the test article; however, vomiting was judged to be a result of its bitter taste, as noted in the approval information. Decreases in the blood pressure and compensatory increases in the heart rate were judged to be due to the pharmacological action of the test article. The decreases in the erythrocytic parameters and increases in the reticuloocyte count in the hematology, increases in urea nitrogen in the clinical chemistry, and histopathological changes in the kidneys (dilation of the tubules and hypertrophy of the tubular epithelium) observed in this study were similar to those described in the approval information. In other words, the toxic changes were all comparable with the approval information. These toxic changes, as well as the pharmacological effects of the test article, were replicated in the present study. On the other hand, plasma concentrations obtained using the PMS-TK method were almost 2-fold those of the approval information. This disparity is thought to be due to improvement in the oral absorbency of the commercially available formulation of the test article, accompanied by mainstream closed-breeding practices (Smith et al., 2001; Orsi et al., 2011), meaning differences between colonies cannot be denied.

Inflammation observed in the digestive tract of the moribund animals appeared to be chronic; however, since an acute lesion was observed in the large intestine, the inflammatory changes may have been recurring. However,

![Fig. 3. TK of valsartan 200 mg/kg/day on Days 1 and 14. Animal No. 2M02: Necropsied on Day 14 due to a moribund condition.](image)

![Table 5. PMS-TK.](image)

| Group          | Sex | Day | TK parameter | Cmax (ng/mL) | Tmax (hr) | AUC0-24hr (ng·hr/mL) |
|---------------|-----|-----|--------------|--------------|-----------|----------------------|
| Valsartan 200 mg/kg/day | Male | 1   | Cmax         | 61,300 ± 12,700 | 1.5 ± 0.9 | 217,000 ± 73,000     |
|                |     | 14  |              | 54,500 ± 7,637 | 1.3 ± 1.1 | 196,000 ± 6,225       |
|                | Female | 1   |              | 61,900 ± 31,500 | 1.0 ± 0.9 | 200,000 ± 128,000     |
|                |     | 14  |              | 22,500 ± 10,324 | 0.5 ± 0.0 | 74,400 ± 15,556       |

*Data of 1 animal was excluded because values were 5 to 10-fold those of other animals. See Fig. 2 for details.

* Data of 2 animals were represented because 1 animal was euthanized on Day 14.
since inflammation was also noted to a severe degree in a surviving animal and to a moderate degree in control animals, and inflammation of the large intestine is a known non-specific lesion in this species (David et al., 2009), it is unlikely to have been a direct cause of death. Furthermore, the tissue changes in the kidneys and increases in urea nitrogen concentrations in the blood were not always severe. The $C_{\text{max}}$ and AUC values in 1 moribund animal obtained using the PMS-TK method were approximately 10-fold those of the other animals in the same group. In the moribund animals, marked decreases in feed and water consumption were noted, as well as adrenal cortical hyperplasia, a lesion thought to be indicative of stress, in some of these animals. Based on these findings, it was considered likely that the animals became moribund due to a deteriorated physical condition caused by decreased feed and water consumption. Furthermore, since thymic atrophy was observed at a similar severity in the control group, this lesion was considered not to be directly attributable to death. Since plasma drug concentrations could not be determined in most of these animals because they had become moribund prior to blood sampling, the reason for such high values in 1 of the animals could not be established definitively. It is possible that a moribund state may have been induced at plasma concentrations 2-fold higher than the application data (PMDA, 2000), however, the probable cause of this 10-fold increase was thought to be metabolic failure during the throws of death. Similar accounts to the death and moribundity that occurred in the current study were noted in the Diovan® application forms (PMDA, 2000). In other words, these instances were due to a deteriorated physical condition that resulted from no food consumption. The thymic atrophy, adrenal cortical hyperplasia, erosion in the stomach and ulceration where caused by stress from being in a moribund state, which was in turn caused by a deteriorated physical condition.

The biggest advantage of the PMS-TK method is the ability to collect samples from individual animals at multiple time points whilst also reducing the number of animals required. In the application for Diovan® (PMDA, 2000), 3 animals/sex/group were used for toxicological evaluation, but an additional 6 animals/sex/group were assigned for the TK satellite. It is not clear from the general information available in the application forms exactly how much blood or plasma were required for analysis of the plasma drug concentrations. However, for analysis of Valsartan in clinical use (Shah et al., 2017), plasma samples of 100 μL/time point were used to determine plasma drug concentrations. Since sampling is generally conducted assuming 1 sample will be analyzed several times, a volume of approximately 1 mL/time point would be necessary. This means that for the current study design, a sample volume totaling 7 mL would be required over the 7 scheduled analytical time points. This volume is equal to 28% to 35% of the total blood volume (20 to 25mL) of the marmoset, and the sampling of such a large volume is impossible on animal welfare grounds (Diehl et al. 2001). If such sampling was conducted in animals used for toxicological assessment, aside from any effects of the test article, effects of sampling would also be a concern. It is for this reason that a TK satellite group was probably added to the Diovan® study design. If the PMS-TK method developed in this study had been applied, a plasma volume of 2.5 μL/time point, a sampling volume of 50 μL/time point, totaling 0.35 mL for all 7 time points would have been enough. Since this is equivalent to only a small percentage of the total blood volume of the marmoset, between 1.4% and 1.75%, it is thought that blood volumes would recover in less than 1 week (Diehl et al., 2001). In actual practice, there were no clinical signs attributable to blood sampling observed in the current study. Using the PMS-TK method, there was no need for a TK satellite group, evaluation was possible in the same individual animals, and we succeeded in reducing the number of animals required.

In the present study, 2.9 mL of blood [equivalent to approximately 14% of the total blood volume of the marmoset (Diehl et al., 2001)] were collected from each animal during the 2-week dosing period in a sequence of repeated sampling. However, based on the results of the negative control group, no side-effects of repeated blood sampling were evident. In order to further reduce the volume of blood required, it has also been suggested that respiratory function could be analyzed by the whole body plethysmograph method (Bergers et al., 2004), rather than by evaluation of blood gases. If applied to the current study design, 0.5 mL x 2 time points (1 mL in total) could be reduced from the total volume of blood required, further reducing the possible effects of sampling.

Nonclinical studies in marmosets have been thoroughly reviewed by Smith et al. (2001) and Orsi et al. (2011). Smith et al. (2001) indicate the necessity for a satellite group for TK analysis and recommend sampling volumes and animal numbers/group for repeated dose toxicity studies. Orsi et al. (2011) suggest the PMS method and combination toxicity studies. In the current study, a combination toxicity study was successfully completed using the PMS method, which allowed the elimination of a TK satellite group, providing further encouragement for the use of marmosets in toxicity studies in the future. It is necessary to note, however, that when using low-molecu-
lar substances, as in this case, development and application of the high-performance LC/MS/MS analysis method was possible. However, for biopharmaceutical research, for which marmosets are thought to be best suited, satellite groups are still required for analysis using the ELISA and LC/MS/MS methods currently capable of detection.

This investigation was undertaken to demonstrate the value of a small species of monkey, the marmoset, in non-clinical safety research for FIH trials during the early stages of development, when limited amounts of test article are available. Accordingly, a dosing period of 2 weeks, the minimum requirement set out in the ICH-M3 guideline for non-clinical studies required prior to FIH trials, was selected. Since the toxic profile of the test article had already been established in the application data for Diovan, only 1 group was set in the current study, in the interests of animal welfare. The dose level was set at 200 mg/kg, based on the results of the preliminary toxicity test. The number of animals/group was set at 3, according to the ICH-S4 guidelines and the number used in studies for regulatory approval of Diovan. However, it cannot be denied that this number is insufficient for the evaluation of the dose-relationship of plasma concentrations and toxic changes, as well as individual variance.

By correlating toxic lesion and TK data obtained in a preliminary dose range-finding study employing PMS-TK analysis, a sufficient number of groups with a sufficient number of animals/group for analysis of statistical significance can be established. This will allow evaluation of the dose-relationship of plasma concentrations and toxic changes, as well as individual variance.

By conducting even longer studies, it may be possible to investigate the incidence of chronic toxicity. However, it became apparent in this study that the time taken from initial deterioration of the animals’ health to moribundity is very short in the marmoset. Therefore, in order for an investigation of chronic toxicity to be possible, clinical observations must facilitate the early detection of deterioration (decreases in body weights and food consumption etc.). In addition, appropriate veterinary care, which allows the animals to recovery from emaciation, is vital.

Based on the results of this investigation, it was confirmed that, even with the addition of safety pharmacology parameters to a general repeated dose toxicity study, evaluation of not only toxicity but also TK in the same animals is feasible using the PMS-TK method, a combination of micro-blood-sampling technology and ultrasensitive quantitation using high-performance LC/MS/MS.

Where experimental animal welfare is concerned, this method contributes to the 3R principles of reduction (in the number of animals, as no satellite group is required) and refinement (reducing pain through sampling of minute volumes of blood). Furthermore, during early exploratory clinical trials (ICH-M3), in which large amounts of test articles are usually difficult to obtain, the current study re-establishes the marmoset (required test article volume: 1/10-1/15 of macaques, 1/30 of dogs (Mansfield, 2003; Orsi et al., 2011; ’t Hart et al., 2012) as a highly valuable test system.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Bergers, W.W., van de Meent-van der Horst, D. and Joosens, M.J. (2004): Respiration-based measurement of lung deposition of a fluorescent dextrane aerosol in a marmoset monkey. Inhal. Toxicol., 16, 141-146.

Diehl, K.H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.M. and van de Vorstenbosch, C.; European Federation of Pharmaceutical Industries Association and European Centre for the Validation of Alternative Methods. (2001): A good practice guide to the administration of substances and removal of blood, including routes and volumes. J. Appl. Toxicol., 21, 15-23.

David, J.M., Dick, E.J. Jr. and Hubbard, G.B. (2009): Spontaneous pathology of the common marmoset (Callithrix jacchus) and tamarins (Saguinus oedipus, Saguinus mystax). J. Med. Primatol., 38, 347-359.

FDA. (1996): NDA#020665, Review and evaluation of pharmacology and toxicology data. (https://www.accessdata.fda.gov/drugsatfda_docs/nda/96/020665_s000.pdf)

ICH. (2009): Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2).

ICH. (2017): Questions and answers to ICH S3A: Note for guidance on toxicokinetics: The assessment of systemic exposure in toxicity studies focus on microsampling S3A Q&As.

Layne, D.G. and Power, R.A. (2003): Husbandry, handling, and nutrition for marmosets. Comp. Med., 53, 351-359.

Mansfield, K. (2003): Marmoset models commonly used in biomedical research. Comp. Med., 53, 383-392.

Ministry of Health, Labour and Welfare of Japan. (2010): Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3(R2).

Okano, H., Hikishima, K., Iriki, A. and Sasaki, E. (2012): The common marmoset as a novel animal model system for biomedical and neuroscience research applications. Semin. Fetal Neonatal Med., 17, 336-340.

Orsi, A., Rees, D., Andreini, I., Venturella, S., Cinelli, S. and Oberto, G. (2011): Overview of the marmoset as a model in nonclinical development of pharmaceutical products. Regul. Toxicol. Pharmacol., 59, 19-27.
PMDA. (2000): Diovan® tablet regulatory application information, general information available for Diovan® tablet 20 mg, 40 mg, 80 mg (valsartan). 217-219. (http://www.pmda.go.jp/drugs/2000/g000906/index.html)

Poswillo, D.E., Hamilton, W.J. and Sopher, D. (1972): The marmoset as an animal model for teratological research. Nature, 239, 460-462.

Shah, J.V., Parekh, J.M., Shah, P.A., Shah, P.V., Sanyal, M. and Shrivastav, P.S. (2017): Application of an LC-MS/MS method for the analysis of amlodipine, valsartan and hydrochlorothiazide in polypill for a bioequivalence study. J. Pharm. Anal., 7, 309-316.

Smith, D., Trenery, P., Farningham, D. and Klapwijk, J. (2001): The selection of marmoset monkeys (Callithrix jacchus) in pharmaceutical toxicology. Lab. Anim., 35, 117-130.

't Hart, B.A., Abbott, D.H., Nakamura, K. and Fuchs, E. (2012): The marmoset monkey: a multi-purpose preclinical and translational model of human biology and disease. Drug Discov. Today, 17, 1160-1165.

Uno, Y., Uehara, S. and Yamazaki, H. (2016): Utility of non-human primates in drug development: comparison of non-human primate and human drug-metabolizing cytochrome P450 enzymes. Biochem. Pharmacol., 121, 1-7.

Yamaguchi, A., Kohno, Y., Yamazaki, T., Takahashi, N., Shinki, T., Horiuchi, N., Suda, T., Koizumi, H., Tanioka, Y. and Yoshiki, S. (1986): Bone in the marmoset: a resemblance to vitamin D-dependent rickets, type II. Calcif. Tissue Int., 39, 22-27.