Original Article

Physiological and drug-induced changes in blood levels of adrenal steroids and their precursors in cynomolgus monkeys: An application of steroid profiling by LC–MS/MS for evaluation of the adrenal toxicity

Tomoaki Tochitani, Akihito Yamashita, Izumi Matsumoto, Mami Kouchi, Yuta Fujii,
Toru Yamada and Izuru Miyawaki

Preclinical Research Unit, Sumitomo Dainippon Pharma Co., Ltd.,
3-1-98, Kasugade-naka, Konohana-ku, Osaka 554-0022, Japan

(Received May 7, 2019; Accepted June 3, 2019)

ABSTRACT — The adrenal gland is the most common toxicological target of drugs within the endocrine system, and inhibition of adrenal steroidogenesis can be fatal in humans. However, methods to evaluate the adrenal toxicity are limited. The aim of the present study was to verify the usefulness of simultaneous measurement of blood levels of multiple adrenal steroids, including precursors, as a method to evaluate drug effects on adrenal steroidogenesis in cynomolgus monkeys. With this aim, physiological and drug-induced changes in blood levels of adrenal steroids, including cortisol, aldosterone, androgen, and their precursors were examined. First, for physiological changes, intraday and interday changes in blood steroid levels were examined in male and female cynomolgus monkeys. The animals showed circadian changes in steroid levels that are similar to those in humans, while interday changes were relatively small in males. Next, using males, changes in blood steroid levels induced by ketoconazole and metyrapone were examined, which suppress adrenal steroidogenesis via inhibition of CYP enzymes. Consistent with rats and humans, both ketoconazole and metyrapone increased the deoxycorticosterone and deoxycortisol levels, probably via CYP11B1 inhibition, and the increase was observed earlier and with greater dynamic range than the changes in cortisol level. Changes in other steroid levels reflecting the drug mechanisms were also observed. In conclusion, this study showed that in cynomolgus monkeys, simultaneous measurement of blood levels of adrenal steroids, including precursors, can be a valuable method to sensitively evaluate drug effects on adrenal steroidogenesis and to investigate the underlying mechanisms.

Key words: Adrenal steroidogenesis, Simultaneous measurement of multiple steroids, Cynomolgus monkey, *In vivo*, Liquid chromatography-tandem mass spectrometry

INTRODUCTION

The adrenal gland is the most common toxicological target of drugs within the endocrine system (Ribelin, 1984; Briggs *et al.*, 2015). Also, drug-induced inhibition of adrenal steroidogenesis can be fatal in humans (Harvey and Everett, 2003). However, methods to evaluate drug effects on adrenal steroidogenesis are limited, and novel methods are needed (Harvey and Everett, 2003; Hinson and Raven, 2006).

A traditionally used method to evaluate adrenal toxicity *in vivo* is to measure blood levels of adrenal steroids

such as corticosterone and cortisol (Inomata and Sasano, 2015). However, the basal level of corticosteroid alone is not a sensitive marker for impaired adrenal steroidogenesis (Yarrington and Reindel, 1996; Hinson and Raven, 2006). Recently, mass spectrometry has become increasingly used to measure steroids (Shackleton, 2010), and this has enabled simultaneous measurement of multiple steroids using a small amount of sample (Kushnir *et al.*, 2010; Maeda *et al.*, 2013).

In our previous studies in rats, we have shown that simultaneous measurement of blood levels of multiple adrenal steroids, including precursors, can be a sen-

sitive method to detect drug effects on adrenal steroidogenesis, and also can be useful to investigate the underlying mechanisms (Tochitani *et al.*, 2016, 2017). However, the rat (and mouse) adrenal gland is unique in that it does not express CYP17 in the zona fasciculata/reticularis, and therefore synthesizes corticosterone as the major glucocorticoid rather than cortisol, and does not synthesize androgen precursors dehydroepiandrosterone (DHEA) or androstenedione (Hanukoglu, 1992; Hinson and Raven, 2006; Inomata and Sasano, 2015). Thus, it was uncertain whether this method can be applied to experimental animals other than rats and mice.

The aim of the present study was to verify the usefulness of simultaneous measurement of blood levels of multiple adrenal steroids as a method to evaluate drug effects on adrenal steroidogenesis in the cynomolgus monkey, a non-rodent species that is frequently used in toxicity studies of drugs. With this aim, physiological changes in blood levels of adrenal steroids, including precursors, were first examined, since the information is scarce in cynomolgus monkeys. Then, drug-induced changes in blood levels of adrenal steroids and other toxicity parameters were examined. Drugs used were ketoconazole (KET) and metyrapone (MET), both of which inhibit adrenal steroidogenesis, and caused dynamic changes in blood levels of adrenal steroids in rats (Tochitani *et al.*, 2017).

MATERIALS AND METHODS

Compounds

KET and MET were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich Co. LLC (St. Louis, MO, USA), respectively.

Animals and husbandry

Animal usage was approved by the Institutional Animal Care and Use Committee of Drug Research Division of Sumitomo Dainippon Pharma Co., Ltd.

In Experiments 1 and 2, cynomolgus monkeys aged 4 to 5 years purchased from Simian Conservation Breeding and Research Center, Inc. (Manila, Philippines) were used, with 5 males and 5 females in Experiment 1, and 4 males in Experiment 2. The animals were housed individually in stainless steel cages in a conventional system room with controlled temperature of $24 \pm 2^\circ\text{C}$ and relative humidity of $60 \pm 20\%$ and a 12-hr light (clock time: 8-20)/dark cycle.

In Experiment 3, 3 male cynomolgus monkeys aged 8 to 9 years purchased from Hainan Jingang Laboratory Animal Co. Ltd. (Hainan, China) were used. The animals were housed individually in stainless steel cages in

a conventional system room with controlled temperature of $18\text{--}20^\circ\text{C}$ and relative humidity of 30-80% and a 12-hr light (clock time: 7-19)/dark cycle.

The animals were fed about 100 g of commercial pellet diet (PS-A, Oriental Yeast Co., Ltd., Tokyo, Japan) and a piece of apple or other treat once daily before noon, and tap water was provided *ad libitum*. To minimize handling-related stress, the animals were acclimated to dosing, and dosing and blood sampling were performed by experienced technicians.

Animal experiments

Experiment 1: physiological changes in blood steroid levels

About 1 mL of blood was sampled from each animal at clock times 9, 11, 13, and 17 on the first day (Day 1) of blood sampling, from cephalic vein using a syringe with a needle. Blood was sampled at 9 on Days 3 and 7, in the same manner as on Day 1. Plasma was prepared by centrifuging the blood samples using heparin as anticoagulant, and stored at -80°C until use.

Experiment 2: KET-induced changes in blood steroid levels and other toxicity parameters

The animals were orally dosed with 100 mg/kg of KET once daily at 10 a.m. for 7 days. KET was suspended in 0.5% methylcellulose solution at a concentration of 20 mg/mL, and the dosing volume was 5 mL/kg. The dose level was selected based on the dose level used in rats (Tochitani *et al.*, 2017).

During the dosing period, daily observation for clinical signs was performed before and just after dosing, and 1 to 2 hr after dosing. Body weights were measured before dosing on the first day of dosing (Day 1), Day 4, and the day of necropsy (the day after the final dosing).

For steroid measurement, 1.3 mL of blood was sampled from each animal at clock times 10, 11, 13, and 17 on the day before the initiation of dosing (Day -1), and 1, 3, 7, and 24 hr after dosing on Days 1 and 7 (each at clock times 11, 13, 17, and at 10 of next day), from cephalic vein using a syringe with a needle. Plasma was prepared by centrifuging the blood sample using heparin as anticoagulant. Also for blood chemistry, 0.6 mL of blood was sampled from each animal at 10 a.m. on Day -1 and on the day of necropsy, and serum was prepared. The plasma and serum samples were stored at -80°C until use.

After the blood sampling on the day after the final dosing, the animals were euthanized by exsanguination and subjected to necropsy. The bilateral adrenal glands were rapidly removed, grossly examined, and fixed in 10% neutral-buffered formalin (NBF).

Experiment 3: MET-induced changes in blood steroid levels

The animals were orally dosed with 90 mg/kg of MET at 9 a.m. on the first day of dosing (Day 1), and 180 mg/kg of MET at 9 a.m. on Day 2. MET was suspended in 0.5% methylcellulose solution at a concentration of 18 and 36 mg/mL, and the dosing volume was 5 mL/kg. The dose level was selected based on the clinical dose of MET.

Clinical observation was performed before and after dosing, and before blood sampling at each time point. Body weights were measured before dosing on Day 1.

For steroid measurement, 1.1 mL of blood was sampled from each animal at clock times 9, 10, 12, and 16 on the day before the initiation of dosing (Day -1), and 1, 3, 7 and 24 hr after dosing on Days 1 and 2 (each at clock times 10, 12, 16, and at 9 of next day). Plasma was prepared by centrifuging the blood sample using heparin as anticoagulant. The plasma samples were stored at -80°C until use.

Blood chemistry

In Experiment 2, using the serum sample, glucose, total cholesterol, triglyceride, phospholipid, sodium (Na) and potassium (K) were measured using an automated analyzer JCA-BM1650 (JEOL Ltd., Tokyo, Japan).

Histopathology

In Experiment 2, the adrenal glands fixed in 10% NBF were embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and examined with light microscopy by a pathologist certified by the Japanese Society of Toxicologic Pathology.

Measurement of plasma concentrations of steroid hormones and its precursors

In Experiments 1 and 2, plasma concentrations of pregnenolone, progesterone, deoxycorticosterone, corticosterone, aldosterone, 17-hydroxypregnenolone (17-OH pregnenolone), 17-hydroxyprogesterone (17-OH progesterone), deoxycortisol, cortisol, DHEA, androstenedione, and testosterone were simultaneously measured, using a liquid chromatograph (Nexera, Shimadzu, Kyoto, Japan) coupled with a tandem mass spectrometer (LC-MS/MS; Triple Quad 6500, AB Sciex, Framingham, MA, USA).

In Experiment 3, plasma concentrations of above steroids except for pregnenolone, 17-OH pregnenolone, and DHEA were simultaneously measured, using a liquid chromatograph (Nexera, Shimadzu) coupled with a tandem mass spectrometer (QTRAP 5500, AB Sciex).

The lower limit of quantification (LLOQ) in steroid measurement in each experiment is shown in Supplemen-

tal Table 1.

Statistical analysis

In Experiments 2 and 3, statistical analysis was performed using SAS v9.4 software (SAS Institute Inc., NC, USA). Pre- and post-dosing values in the blood chemistry and blood steroid levels were compared using paired *t*-test (two-tailed; significance level of 5%). For blood steroid levels, multiple comparison was performed using Bonferroni correction.

Before the statistical analysis, values of blood steroid levels were log-transformed. Also, values of blood steroid levels below the LLOQ were set at LLOQ/2. In Experiment 2, blood steroid levels of Animal No. 1 on Day 1 were excluded from the statistical analysis, because the animal vomited test compound-like substance soon after the dosing on Day 1.

RESULTS**Experiment 1: physiological changes in blood steroid levels***Intraday changes*

Figure 1 shows intraday changes in blood steroid levels in males and females. Sex differences were not obvious except in testosterone. Generally, levels of corticosterone, cortisol, and their precursors peaked at clock time 11, decreased by 13, and stayed low until, or increased again by 17. In contrast to the cortisol level, the aldosterone level did not have a peak at 11, and was generally highest at 9, decreased by 11 or 13, and stayed low until or increased again by 17.

Though the levels of DHEA and androstenedione showed similar tendency as that of cortisol, there was no such tendency in the testosterone level.

Interday changes

Figure 2 shows interday changes in blood steroid levels in males and females. In males, the interday chang-

Table 1. Blood chemistry before and after 7-day repeated dosing of KET.

Test item	Day -1	Day 8
Glucose (mg/dL)	50 ± 8	60 ± 9
Total cholesterol (mg/dL)	103 ± 20	96 ± 24
Triglyceride (mg/dL)	30 ± 10	33 ± 13
Phospholipid (mg/dL)	144 ± 29	145 ± 37
Sodium (Na) (mEq/L)	149 ± 2	149 ± 1
Potassium (K) (mEq/L)	4.4 ± 0.6	4.2 ± 0.2

The values are shown as the group mean ± standard deviation (N = 4).

Fig. 1. Blood steroid levels in males and females at different time points within a day. Each dot shows an individual animal value at a time point (N = 5/sex). The Y-axis is logarithmic. 17-OH PREG = 17-OH pregnenolone; 17-OH PROG = 17-OH progesterone.

Fig. 2. Blood steroid levels in males and females at the same time point (9 a.m.) on different days within a week. Each dot shows an individual animal value on a given day (N = 5/sex). The Y-axis is logarithmic.

Steroid profiling for adrenal toxicity evaluation in monkeys

es were not as dynamic as the intraday changes. On the other hand, in females the changes were relatively large. The changes were inconsistent among animals, but within each animal, they were seemingly correlated with each other except for androstenedione, testosterone, and aldosterone.

Experiment 2: KET-induced changes in blood steroid levels and other toxicity parameters

Clinical signs, body weights, and blood chemistry

During the dosing period, sporadic vomiting was observed in all animals except in one animal (Animal No. 2). Notably, in one animal (Animal No. 1), vomiting of test compound-like substance was observed soon after the dosing on Day 1, on which steroid-level measurement was performed. There were no effects on body weights in any animal.

In the blood chemistry, no obvious changes were observed (Table 1).

Necropsy and histopathology

At necropsy, no obvious changes were observed. Histopathologically, minimal increase of vacuolation of the zona reticularis in the adrenal gland was observed (Fig. 3). The vacuoles were thought to be lipid droplets based on their morphology. No obvious changes were observed in the zona fasciculata or zona glomerulosa.

Blood steroid levels

Figure 4 shows changes in blood steroid levels after KET administration. The corticosterone level was significantly higher 3 hr after dosing on Day 7, while the cortisol level was slightly but significantly lower at 1 and 3 hr after dosing on Day 7. The aldosterone level was significantly

cantly lower 24 hr after dosing on Day 7.

The deoxycorticosterone level was significantly higher 3 hr after dosing on Day 7, and tended to be higher at the same time point on Day 1. The dynamic range was from 5- to 10-fold and was greater than that of cortisol. The deoxycortisol level was significantly higher 3 hr after dosing on Day 1, but the change was not obvious on Day 7. Though the deoxycortisol level was significantly lower 24 hr after dosing on Day 1, the change was minimal and thought to be incidental.

The progesterone and 17-OH progesterone levels were significantly lower 1 hr after dosing on Day 1; however, since similar tendency was no longer observed at the later time points on Day 1 nor at the same time point on Day 7, and similar tendency was observed even in the animal (Animal No. 1) that vomited test compound-like substance just after dosing, the changes were considered to be incidental.

The pregnenolone level was significantly higher at 3, 7, and 24 hr after dosing on Day 7.

The DHEA level was significantly lower 1 hr after dosing on Day 7; however, the change was minimal and thought to be incidental.

Experiment 3: MET-induced changes in blood steroid levels

Clinical signs

At the 180 mg/kg dose of MET, vomiting was observed in one animal (Animal No. 2) about 30 min after the dosing.

Blood steroid levels

Figure 5 shows changes in blood steroid levels after MET administration. The corticosterone, cortisol, and

Fig. 3. Histopathological images of the adrenal glands of a KET-treated male (a) and an age-matched normal male (b). The medulla and zona reticularis are shown in the lower left and upper right, respectively. Cells in the zona reticularis are minimally vacuolated in the KET-treated animal, compared to those in the normal animal.

Fig. 4. Blood steroid levels in males after single or 7-day repeated dosing of KET. Each dot shows an individual animal value at a time point on a given day (N = 4). The Y-axis is logarithmic. Asterisks indicate statistically significant difference from the values on Day -1 of dosing (* $p < 0.05$). Note that animal No. 1 vomited test compound-like substance just after the dosing on Day 1, and the values for this animal on Day 1 were excluded from the statistical analysis.

Fig. 5. Blood steroid levels in males after single dosing of MET at 90 and 180 mg/kg. Each dot shows an individual animal value at a time point on a given day (N = 3). The Y-axis is logarithmic. Asterisks indicate statistically significant difference from the pre-dosing values (* $p < 0.05$).

Steroid profiling for adrenal toxicity evaluation in monkeys

aldosterone levels were significantly lower from 1 or 3 hr after dosing at 90 and 180 mg/kg. The corticosterone and cortisol levels recovered to pre-dosing levels by 24 hr after treatment, while the aldosterone level stayed decreased.

The deoxycorticosterone and deoxycortisol levels were significantly higher from 1 hr after dosing at 90 and 180 mg/kg. The increases were greatest 3 or 7 hr after dosing, with dynamic ranges of more than 1000- and 100-fold in the deoxycorticosterone and deoxycortisol levels, respectively. The increase tended to recover by 24 hr after dosing, but the levels were still high compared to the pre-dosing levels.

The progesterone and 17-OH progesterone levels were significantly higher from 3 hr after dosing at 90 and 180 mg/kg. Also, the androstenedione levels were significantly higher 3 hr after dosing at 90 mg/kg.

DISCUSSION

In this study, we examined the physiological and drug-induced changes in blood levels of adrenal steroids and their precursors in cynomolgus monkeys.

The steroidogenic pathway in the adrenal gland is

illustrated in Fig. 6. Unlike rat adrenal glands, human and monkey adrenal glands express CYP17 in the zona fasciculata/reticularis, and synthesize cortisol as the major glucocorticoid, as well as androgen precursors DHEA and androstenedione (Hanukoglu, 1992; Hinson and Raven, 2006; Inomata and Sasano, 2015). Glucocorticoid synthesis is upregulated by adrenocorticotrophic hormone (ACTH), which promotes expression of the CYP enzymes (especially CYP11A), and increases availability of cholesterol to CYP11A (Rosol *et al.*, 2001; Miller and Bose, 2011; Inomata and Sasano, 2015). On the other hand, aldosterone synthesis is mainly upregulated by the renin-angiotensin system (Cooke *et al.*, 1979; Rosol *et al.*, 2001).

First, the intraday changes in blood steroid levels were examined in males and females. Generally, levels of corticosterone, cortisol, and their precursors peaked late in the morning, decreased in the afternoon, and stayed low until, or increased again by the evening. It is known that in diurnal species such as humans and monkeys, blood glucocorticoid levels peak at beginning of the activity period, or morning (Umerkoman-Wiita *et al.*, 1981; Chung *et al.*, 2011). In contrast to the cortisol level, the aldosterone level did not have a peak late in the morning, but was

Fig. 6. Steroidogenic pathway in monkey adrenal glands. In the testis, androstenedione is further metabolized into testosterone by 17 β -HSD. The rat adrenal gland does not express CYP17, and thus does not synthesize cortisol or androgen precursors. Enzymes are in *italics*, hormones are in **bold**, and arrows indicate the direction of metabolism. HSD = hydroxysteroid dehydrogenase.

generally highest in earliest morning, decreased by noon, and stayed low until or increased again by the evening. In humans, aldosterone levels show diurnal variation similar to cortisol levels, but there is dissociation between them, due to the difference in the regulation mechanism (Cooke *et al.*, 1979). Thus, cynomolgus monkeys showed circadian changes in blood steroid levels similar to those in humans.

Though the levels of DHEA and androstenedione showed similar tendency as those of the adrenal steroids, there was no such tendency in the testosterone level. Considering that there was no obvious sex difference in the DHEA or androstenedione levels, and the lower levels of testosterone than those of cortisol, it was suspected that blood levels of DHEA and androstenedione mainly reflect synthesis in the adrenal gland, rather than in the gonad.

Next, interday changes in blood steroid levels in males and females were examined. In males, the interday changes were not as dynamic as the intraday changes. On the other hand, in females the changes were relatively large. These interday changes in females may be due to the estrous cycle, but a correlation with estrone or estradiol levels was not obvious (data not shown). These interday changes should be considered when drug-induced changes in blood steroid levels are evaluated in females.

Moreover, using males, we examined changes in blood steroid levels and other toxicity parameters induced by KET. Due to the imidazole ring in its structure, KET inhibits multiple CYP enzymes, including CYP11A, CYP17, CYP21, and CYP11B1/2 (Engelhardt *et al.*, 1991; Johansson *et al.*, 2002; Hille *et al.*, 2011).

Histopathologically, repeated dosing of KET increased vacuolation (lipidosis) of the zona reticularis. Lipidosis of the zona fasciculata/reticularis can also be induced in rats by KET, probably due to CYP11A inhibition (Tochitani *et al.*, 2017). Unlike in rats, lipidosis of the zona fasciculata was not obvious in cynomolgus monkeys, since the zona fasciculata in monkeys is filled with lipid droplets even in a normal state.

There were increases or tendency toward increase in the deoxycorticosterone and deoxycortisol levels from Day 1 of dosing. On the other hand, the decrease in cortisol level was not obvious until Day 7, and the dynamic range was smaller than that of deoxycorticosterone. In previous studies in rats, KET increased the deoxycorticosterone level, probably via CYP11B1 inhibition (the deoxycortisol level was not evaluated, because it is not synthesized in rats), while effects on the corticosterone level were not significant (Tochitani *et al.*, 2017). Also, it has been reported that KET increased blood levels of deoxycorticosterone and deoxycortisol in humans, while

its effect on basal cortisol level was inconsistent among patients (Engelhardt *et al.*, 1985). Thus, KET-induced changes in adrenal histopathology and blood steroid levels in rats were reproduced in cynomolgus monkeys, and the pattern of steroid changes was similar to that in humans. These results suggest that blood levels of deoxycorticosterone and deoxycortisol are sensitive markers of KET effect on the adrenal steroidogenesis across these species.

The aldosterone level was decreased 24 hr after dosing on Day 7. This may be the result of direct inhibition of synthesis via CYP11B1/2 inhibition by KET, as well as suppression of aldosterone synthesis by the increased level of deoxycorticosterone, which has mineralocorticoid activity and suppresses renin production (Nimkarn and New, 2008).

The pregnenolone level was also increased on Day 7. This may be due to compensatory changes to the decreased cortisol level, considering that the effect of KET on CYP is reversible (Yan *et al.*, 2002), the effect on CYP11A is weaker than that on CYP11B1 (Johansson *et al.*, 2002), and pregnenolone synthesis is a rate-limiting step in the adrenal steroidogenesis that is rapidly upregulated by ACTH (Rosol *et al.*, 2001; Miller and Bose, 2011). The corticosterone level was increased 3 hr after dosing on Day 7. KET-induced increase in a corticosterone level has also been reported in humans (Engelhardt *et al.*, 1985), but the mechanism was uncertain.

Furthermore, we examined changes in blood steroid levels induced by MET. Unlike KET, MET is not a imidazole derivative, and inhibits CYP11B1 more specifically (Johansson *et al.*, 2002; Hille *et al.*, 2011). Consistent with KET, MET increased the deoxycorticosterone and deoxycortisol levels. Though the corticosterone and cortisol levels were also decreased, the dynamic ranges were much smaller than those of the deoxycorticosterone and deoxycortisol levels. MET-induced increases in deoxycorticosterone and/or deoxycortisol level have been reported in rats (Tochitani *et al.*, 2017) and in humans (Schöneshöfer *et al.*, 1980; Koal *et al.*, 2012). Thus, it was further suggested that the deoxycorticosterone and deoxycortisol levels can be sensitive markers of CYP11B1 inhibition in cynomolgus monkeys, as well as in rats and humans.

The aldosterone level decreased from Day 1 of dosing, probably due to the same mechanism as KET. Also, the progesterone, 17-OH progesterone, and androstenedione levels significantly increased after MET dosing. MET-induced increases in progesterone and 17-OH progesterone levels have been reported in humans (Schöneshöfer *et al.*,

Steroid profiling for adrenal toxicity evaluation in monkeys

Table 2. Summary of drug-induced changes in blood steroid levels observed in the present study in cynomolgus monkeys, and those reported in rats and in humans.

Drug	Cynomolgus monkey	Rat (Tochitani <i>et al.</i> , 2017)	Humans (Engelhardt <i>et al.</i> , 1985; Schöneshöfer <i>et al.</i> , 1980; Koal <i>et al.</i> , 2012)
KET	↑Deoxycorticosterone	↑Deoxycorticosterone	↑Deoxycorticosterone
	↑Deoxycortisol		↑Deoxycortisol
	↑Pregnenolone		
	↑Corticosterone		↑Corticosterone
	↓Cortisol		↓Cortisol
MET	↓Aldosterone		
	↑Deoxycorticosterone	↑Deoxycorticosterone	↑Deoxycorticosterone
	↑Deoxycortisol		↑Deoxycortisol
	↑Progesterone	↑Progesterone	↑Progesterone
	↑17-OH progesterone		↑17-OH progesterone
	↑Androstenedione		
	↓Corticosterone		↓Corticosterone
	↓Cortisol		↓Cortisol
	↓Aldosterone		↓Aldosterone

↑ = increase; ↓ = decrease; Note that steroids measured are not consistent among species.

1980), and this may be due to compensatory changes to the decreased cortisol level. It is known that in humans with CYP11B1 deficiency, reduced cortisol feedback increases ACTH secretion from the pituitary gland, leading to increased synthesis of steroid precursors, including 17-OH progesterone. Some of the increased precursors are shunted into androgen synthesis, causing virilization (White *et al.*, 1994; Nimkarn and New, 2008).

KET- and MET-induced changes in blood steroid levels that were observed in cynomolgus monkeys in the present study and those reported in rats and in humans are summarized in Table 2. Though the pattern of changes in blood steroid levels were different between cynomolgus monkeys and rats due to the difference in the steroidogenic pathway, increased level of deoxycorticosterone that can be expected as a result of CYP11B1 inhibition was observed in common. Also, the changes in cynomolgus monkeys were generally consistent with those in humans. These results suggest that this method can be used across these species to sensitively detect drug effects on adrenal steroidogenesis, giving insight into the underlying mechanisms.

In conclusion, this study showed that in cynomolgus monkeys as well as in rats, simultaneous measurement of blood levels of adrenal steroids, including precursors, can be a valuable method to sensitively evaluate drug effects on adrenal steroidogenesis and to investigate the underlying mechanisms. To the best of our knowledge, this is the first report that comprehensively analyzed physiological and drug-induced changes in the blood profile of adrenal

steroids in cynomolgus monkeys.

ACKNOWLEDGMENTS

We wish to thank Yasuhiro Sasaki for conducting the animal experiments, Yumi Tateishi for her histotechnical work, Chikako Horike for her contract management work, and members of Sumika Chemical Analysis Service, Ltd. (Osaka, Japan) and LSI Medience Corporation (Tokyo, Japan) for the measurement of steroid concentrations.

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

REFERENCES

- Briggs, K., Barber, C., Cases, M., Marc, P. and Steger-Hartmann, T. (2015): Value of shared preclinical safety studies - The eTOX database. *Toxicol. Rep.*, **2**, 210-221.
- Chung, S., Son, G.H. and Kim, K. (2011): Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. *Biochim. Biophys. Acta*, **1812**, 581-591.
- Cooke, C.R., Whelton, P.K., Moore, M.A., Caputo, R.A., Bledsoe, T. and Walker, W.G. (1979): Dissociation of the diurnal variation of aldosterone and cortisol in anephric subjects. *Kidney Int.*, **15**, 669-675.
- Engelhardt, D., Dörr, G., Jaspers, C. and Knorr, D. (1985): Ketconazole blocks cortisol secretion in man by inhibition of adrenal 11 beta-hydroxylase. *Klin. Wochenschr.*, **63**, 607-612.
- Engelhardt, D., Weber, M.M., Miksch, T., Abedinpour, F. and Jaspers, C. (1991): The influence of ketoconazole on human

- adrenal steroidogenesis: incubation studies with tissue slices. *Clin. Endocrinol. (Oxf.)*, **35**, 163-168.
- Hanukoglu, I. (1992): Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. *J. Steroid Biochem. Mol. Biol.*, **43**, 779-804.
- Harvey, P.W. and Everett, D.J. (2003): The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: critical omissions from regulatory endocrine disrupter screening strategies for human health? *J. Appl. Toxicol.*, **23**, 81-87.
- Hille, U.E., Zimmer, C., Vock, C.A. and Hartmann, R.W. (2011): First Selective CYP11B1 Inhibitors for the Treatment of Cortisol-Dependent Diseases. *ACS Med. Chem. Lett.*, **2**, 2-6.
- Hinson, J.P. and Raven, P.W. (2006): Effects of endocrine-disrupting chemicals on adrenal function. *Best Pract. Res. Clin. Endocrinol. Metab.*, **20**, 111-120.
- Inomata, A. and Sasano, H. (2015): Practical approaches for evaluating adrenal toxicity in nonclinical safety assessment. *J. Toxicol. Pathol.*, **28**, 125-132.
- Johansson, M.K., Sanderson, J.T. and Lund, B.O. (2002): Effects of 3-MeSO₂-DDE and some CYP inhibitors on glucocorticoid steroidogenesis in the H295R human adrenocortical carcinoma cell line. *Toxicol. In Vitro*, **16**, 113-121.
- Koal, T., Schmiederer, D., Pham-Tuan, H., Röhring, C. and Rauh, M. (2012): Standardized LC-MS/MS based steroid hormone profile-analysis. *J. Steroid Biochem. Mol. Biol.*, **129**, 129-138.
- Kushnir, M.M., Rockwood, A.L. and Bergquist, J. (2010): Liquid chromatography-tandem mass spectrometry applications in endocrinology. *Mass Spectrom. Rev.*, **29**, 480-502.
- Maeda, N., Tanaka, E., Suzuki, T., Okumura, K., Nomura, S., Miyasho, T., Haeno, S. and Yokota, H. (2013): Accurate determination of tissue steroid hormones, precursors and conjugates in adult male rat. *J. Biochem.*, **153**, 63-71.
- Miller, W.L. and Bose, H.S. (2011): Early steps in steroidogenesis: intracellular cholesterol trafficking. *J. Lipid Res.*, **52**, 2111-2135.
- Nimkarn, S. and New, M.I. (2008): Steroid 11 β -hydroxylase deficiency congenital adrenal hyperplasia. *Trends Endocrinol. Metab.*, **19**, 96-99.
- Ribelin, W.E. (1984): The effects of drugs and chemicals upon the structure of the adrenal gland. *Fundam. Appl. Toxicol.*, **4**, 105-119.
- Rosol, T.J., Yarrington, J.T., Latendresse, J. and Capen, C.C. (2001): Adrenal gland: structure, function, and mechanisms of toxicity. *Toxicol. Pathol.*, **29**, 41-48.
- Schöneshöfer, M., Schefzig, B. and Arabin, S. (1980): Short-term kinetics of serum adrenal steroids and plasma ACTH after a single dose of metyrapone in man. *J. Endocrinol. Invest.*, **3**, 229-236.
- Shackleton, C. (2010): Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis. *J. Steroid Biochem. Mol. Biol.*, **121**, 481-490.
- Tochitani, T., Yamashita, A., Kouchi, M., Fujii, Y., Matsumoto, I., Miyawaki, I., Yamada, T. and Funabashi, H. (2016): Changes in plasma concentrations of corticosterone and its precursors after ketoconazole administration in rats: an application of simultaneous measurement of multiple steroids using LC-MS/MS. *Exp. Toxicol. Pathol.*, **68**, 125-131.
- Tochitani, T., Yamashita, A., Matsumoto, I., Kouchi, M., Fujii, Y., Miyawaki, I., Yamada, T. and Bando, K. (2017): Usefulness of Simultaneous Measurement of Plasma Steroids, Including Precursors, for the Evaluation of Drug Effects on Adrenal Steroidogenesis in Rats. *Toxicol. Pathol.*, **45**, 756-763.
- Umberkoman-Wiita, B., Hansen, S., Herbert, J. and Moore, G.F. (1981): Circadian rhythms in serum and CSF cortisol of rhesus monkeys, and their modulation by timed injections of L-5-hydroxytryptophan. *Brain Res.*, **222**, 235-252.
- White, P.C., Curnow, K.M. and Pascoe, L. (1994): Disorders of steroid 11 β -hydroxylase isozymes. *Endocr. Rev.*, **15**, 421-438.
- Yan, Z., Rafferty, B., Caldwell, G.W. and Masucci, J.A. (2002): Rapidly distinguishing reversible and irreversible CYP450 inhibitors by using fluorometric kinetic analyses. *Eur. J. Drug Metab. Pharmacokinet.*, **27**, 281-287.
- Yarrington, J.T. and Reindel, J.F. (1996): Chemically induced adrenocortical degenerative lesions. In: *Monographs on pathology of laboratory animals, endocrine system*. 2nd ed. (Jones, T.C., Capen, C.C. and Mohr, U., ed.), pp. 467-476. Springer, New York.