Steroid Profiling for assessing Adrenal Hepatic Syndrome in Canine Hepatocellular Carcinoma

（ステロイドプロファイリングによる肝細胞癌発症メカニズムとしての副腎–肝臓連関の探索）

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Contents

Abbreviations

3α HSD	3α-hydroxysteroid dehydrogenase
3β HSD	3β-hydroxysteroid dehydrogenase
ACTH-ST	Adrenocorticotrophic hormone stimulation test
ACVIM	American College of Veterinary Internal Medicine
AHS	Adrenal hepatic syndrome
CKD	Chronic kidney disease
CYP11B1	11β-hydroxylase
CYP17	17α-hydroxylase
CYP21	21-hydroxylase
DDW	Double distilled water
ELISA	Enzyme-linked immunosorbent assay
ESI	Electron ionization spray
HAC	Hyperadrenocorticism
HCC	Hepatocellular carcinoma
HSD3B	3β-hydroxysteroid dehydrogenase
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
IDL	Instrumental detection limit
IS	Isotope-labelled internal standard
LC/MS/MS	Liquid chromatography triple quadrupole mass spectrometry
LDDST	Low dose dexamethasone suppression test
LOD	Limit of detection
LOQ	Limit of quantification
MCT	Mast cell tumor
MDL	Method detection limit
MMVD	Myxomatous mitral valve disease
MRM	Multiple reaction monitoring
NAFLD	Nonalcoholic fatty liver degeneration
NAIs	Non adrenal illness

PCA	Principal component analysis
STD	Standard
VH	Vacuolar hepatopathy
WASAVA	World Small Animal Veterinary Association

Notes

Contents of the present study were partially published in the following article list. The first article is corresponding to chapter 1. The second article is corresponding to chapter 2.

List of published articles

1. Sasaki N, Oo T, Yasuda Y, Ichise T, Nagata N, Yokoyama N, Sasaoka K, Morishita K, Nakayama SM, Ishizuka M, Nakamura K, Takiguchi M, Ikenaka Y. Simultaneous Steroids Measurement in Dogs with Hyperadrenocorticism Using A Column-Switching Liquid Chromatography-Tandem Mass Spectrometry Method. J Vet Med Sci, 83, 1634–1642, 2021.
2. Oo T, Sasaki N, Ikenaka Y, Ichise T, Nagata N, Yokoyama N, Sasaoka K, Morishita K, Nakamura K, Takiguchi M. Serum Steroid Profiling of Hepatocellular Carcinoma Associated with Hyperadrenocorticism in Dogs: A Preliminary Study. Front Vet Sci. 2022 Sep 28; 9:1014792. doi: 10.3389/fvets.2022.1014792. PMID: 36246328; PMCID: PMC9554308

General Introduction

Canine hyperadrenocorticism (HAC) is an endocrine adrenal disease with chronic overproduction of adrenocortical hormones. HAC was relatively common in the middle-aged to older dogs, one case per 100 dogs. Adrenocorticotropic hormone secreting pituitary tumors or cortisol-secreting functional adrenal tumors are the main causes of HAC in dogs[1]. The clinical suspicions of HAC are polyuria, polyphagia, polydipsia, panting, abdominal distension and endocrine alopecia. The diagnosis is supported by the results of complete blood count, serum biochemistry and imaging[2]. Cortisol is main glucocorticoid in dogs, and it was evaluated by the adrenal function test; low dose dexamethasone suppression test (LDDST) or adrenocorticotropic hormone stimulation test (ACTH-ST) to confirm the disease. The clinicopathologic abnormalities of liver such as elevated liver enzymes, steroid hepatopathy and glycogen accumulation in hepatic cells are frequently presented in dogs with HAC[3].

Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor which accounts for 50–70% of all hepatic tumors in dogs[4,5]. Morphologically, HCC is divided into massive, diffuse, or multifocal, however, the majority of canine HCCs are of the massive type with a large solitary single mass[6]. The prognosis of massive HCC is generally good if fully surgical resection of the mass can be attained[7]. Nevertheless, the etiopathogenesis of canine HCC have not been well elucidated yet. The major risks of HCC had been reported as aged over 10 years, some specific breeds and concurrent diseases[4,5]. In our previous study, HAC was established as the most common concurrent disease in dogs with HCC[8]. However, the mechanism on concurrent of HAC and HCC was unclear.

To clarify the involvement of HAC and HCC, I would like to propose the term adrenal-hepatic syndrome (AHS). That is simply defined that, AHS is a condition of chronic exposure to excess adrenal steroids and consequently makes the pathological changes in liver like increased value of hepatic enzyme activities, hepatomegaly, hepatic vacuolation and hepatocellular injury. As described above, the similar phenomenon was

occurring in dogs with HAC[2]. The alterations in cortisol precursors and other hormones are suggested to contribute consequence clinicopathogenesis on liver. Therefore, the measurement of multiple serum steroids may aid to elucidate AHS in HCC.

Furthermore, Scottish terriers with vacuolar hepatopathy (VH) show HAC clinical signs with high level of corticosteroid isoforms (alkaline phosphatase) and steroid imbalance[9-11]. Those dogs with vacuolar hepatopathy have high risks to proceed HCC[6,8,12,13]. In human, metabolic syndromes pretend to develop nonalcoholic fatty liver disease (NAFLD), and it is a leading cause of HCC currently[14]. Steroids induced liver pathology in dogs with HAC and Scottish terriers with VH is somewhat comparable to NAFLD-induced HCC in humans. Thus, assessing of steroid profile in AHS may be helpful to understand the mechanism of hepatocarcinogenesis in both dogs and humans.

In veterinary medicine, radioimmunoassay as well as immunoassays have been used as a gold standard to measure cortisol and its precursors because of their convenient to use and high detection sensitivity[15-18]. However, the methods could not be able to avoid the major downsides such as cross reactivity of target substances and limitation in sample numbers. Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) has taken the place to end those struggles in immunoassays by quantifying multiple steroid hormones at a single runtime with high specificity and sensitivity[19]. However, the utility of LC/MS/MS to the clinical samples is also challenging because there are many obstacles to overcome. The application involves complex preparation method, labor intensive workflow, ions suppression caused by matrix effects, considerable validation procedures with stable isotope labelled internal standards and even the high-cost reagents for making daily clinical diagnosis[19,20].

Thus, the objective of this study was to develop the reliable LC/MS/MS method to achieve the clinical motivation; steroid profile for accessing AHS in HCC. In the first part of the study, column switching LC/MS/MS was validated to perform comprehensive analysis of adrenal steroids in dogs with HAC and non-HAC dogs. In the second chapter, the sample preparation method was modified by combining conventional non-derivatization and keto-derivatization method, afterwards the study investigated the relation of HAC and HCC by investigating the steroid profile differences.

Chapter 1

Simultaneous Steroids Measurement in Dogs with Hyperadrenocorticism Using A Column-Switching Liquid Chromatography Triple Quadrupole Mass Spectrometry Method

1. Introduction

HAC is a typical endocrine condition in dogs, also called as Cushing's syndrome[1]. If the dogs show persistent clinical signs such as polyuria and polyphagia, HAC is the first consideration[3]. HAC can be diagnosed with the help of results from a complete blood count, serum biochemistry, urinary analysis, and abdominal imaging. Confirmative diagnosis is done by adrenal function tests such as LDDST and ACTH-ST by measuring serum cortisol concentration. Diagnosis of HAC is still challenging because none of the diagnostic method has 100% specificity and sensitivity. The adrenal function tests are sometimes present false-positive and false-negative by influence of other non-adrenal illnesses (NAIs)[2].

Figure 1 shows the pathway of steroid biosynthesis in adrenal glands. The major glucocorticoid is cortisol and is usually measured for the diagnosis of HAC in dogs. However, changes of precursors and other hormones are the clinical interest to investigate the diagnostic potential for HAC. Previous researches evaluated the serum concentrations of several adrenocortical hormones including cortisol, aldosterone, 17α-OH-progesterone, progesterone, and dehydroepiandrosterone-sulfate (DHEAS) in healthy dogs and dogs with HAC[15,21-23]. When serum cortisol concentration indicates ambiguous adrenal function, it is indicated that serum 17-OH-progesterone concentration has the ability to diagnose HAC[24-27].

Radioimmunoassay that has been approved for the measurement of multiple adrenocortical hormones in dogs[28]. Both radioimmunoassay and other immunoassays have good detection sensitivity and also feasible for clinical application. However, the major disadvantage of immunoassays is lack of specificity because the cross reaction among similar structural substances[29]. The simultaneous assessment of adrenocortical hormones and their metabolites is now increasingly done using LC/MS/MS[30-32]. In the principle of LC/MS/MS application, each analyte is characterized by its retention time on a chromatographic column, the mass to charge ratio (m/z) of each precursor ion, and specific fragment ions (product ions). Hence, several serum steroids and their metabolites

can be evaluated by avoiding cross reactivity during a single run time in LC/MS/MS. A few researches evaluated steroid profiles in dogs with pregnancy[33], in dogs with X-linked muscular dystrophy[34], and in dogs with gallbladder mucocele[35] using LC/MS/MS techniques. The sample preparation steps for LC/MS/MS analysis are cumbersome and time-consuming. Adrenocortical hormones are physiologically low in concentration; therefore, extraction and derivatization of targeted hormones are necessary to improve their sensitivity[33-35]. The higher throughput and simple sample preparation methods for the steroid measurement by LC/MS/MS have been attempted since the past decades[36]. The target analytes were separated by online column-switching using two-columns improve the selectivity and sensitivity of assay[37]. Samples are loaded on a first column and then eluted by the second column for analysis in the column-switching method. Drugs and metabolites in plasma and urine have been detected applying on column-switching LC/MS/MS[38-41].

Therefore, the aim of Chapter 1 was to develop and validate a simple analytical method using an automated column-switching LC/MS/MS. Then, we measured nine steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, progesterone, 17α-OH-progesterone and aldosterone) in dogs with HAC and dogs without HAC to assess the impact of HAC on steroid profiling.

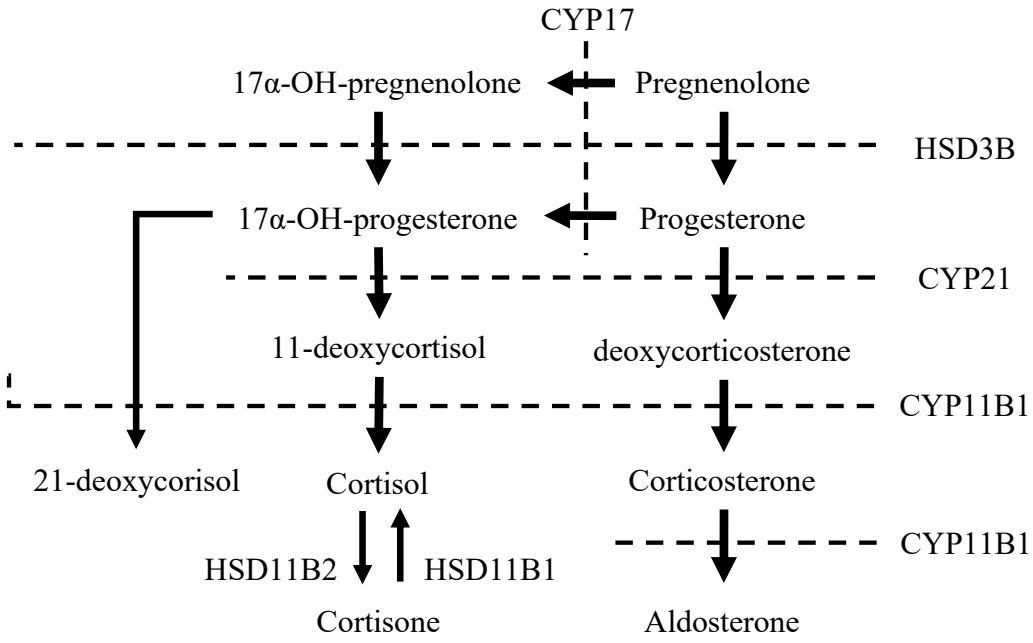


Figure 1. Biosynthesis pathway of steroids.
CYP17, 17α-hydroxylase; HSD3B, 3β-hydroxysteroid dehydrogenase; CYP21, 21-hydroxylase; CYP11B1, 11β-hydroxylase; HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; HSD11B2, 11β-hydroxysteroid dehydrogenase type 2.

2. Materials and methods

2.1 Chemicals and reagents

Analytical grade of reagents including acetonitrile, methanol, formic acid, ammonium fluoride, and double distilled water (DDW) were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Standard (STD) and stable isotope labeled internal standard (IS) solutions of nine steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, progesterone, 17α-OH-progesterone and aldosterone) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Standards solutions

Stock solutions of all STDs and ISs were prepared at the concentration of 1 μg/ml in acetonitrile. The stock solutions of STD were mixed and diluted by acetonitrile to a working solution with the final concentration of 100 ng/ml. For a working solution of IS, stock solutions were mixed and diluted by acetonitrile.

2.3 Sample preparation

For protein precipitation, 50 μl of serum were mixed with 30 μl of the IS working solution and 170 μl of 1% formic acid in acetonitrile. The mixture was centrifuged at 10,000 × *g* for 10 min at 25°C. Thereafter, 100 μl of supernatant were applied to a Mono-Spin column (MonoSpin® Phospholipid, GL Sciences Co., Ltd., Tokyo, Japan) that was conditioned with 1% formic acid in acetonitrile. The spin column was then centrifuged at 2,000 × *g* for 1 min for removing phospholipids in the sample. The elution was mixed with 100 μl of 0.1% formic acid in DDW and was applied to the LC/MS/MS equipment.

2.4 LC/MS/MS analysis with column-switching online purification system

The online column-switching LC/MS/MS was equipped by an Agilent 6495B Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA) with an electrospray ionization (ESI) interface. The online column-switching system consisted of two binary pumps (1260 Infinity II, Agilent Technologies), a two position ten port switching valve (G4232B, Agilent Technologies), a trap column (Unison UK-C1 HT, φ3

μm, 10 × 4.6 mm, Imtakt, Kyoto, Japan), and an analytical column (Kinetex® Biphenyl, φ1.7 μm, 100 × 2.1 mm, Phenomenex Inc., Torrance, CA, USA) (Figure 2). During the first phase (0–0.5 min), the ten-port valve was at position 1>2, and 30 μl of sample were injected onto the trap column by the binary pump 1 with 90% DDW: 10% methanol at flow rate of 0.8 ml/min. During the second phase (0.5–10 min), the ten-port valve was switched to position 1>10, and steroids that retained on the trap column were eluted by the binary pump 2 with 50% ammonium fluoride in DDW: 50% ammonium fluoride in methanol at the flow rate 0.4 ml/min. Washing and equilibration of the trap column were also performed with 90% DDW: 10% methanol in the second phase. During the third phase (10–12 min), the valve was switch to position 1>2, and the analytical column was regenerated and equilibrated with 5% ammonium fluoride in DDW: 95% ammonium fluoride in methanol at the flow rate 0.4 ml/min.

The mass spectrometer was equipped in the positive ion mode. The target steroids were ionized by the ESI interface. The sheath gas temperature and flow rate were 350°C and 12 l/min respectively. The drying gas temperature, drying gas flow rate, and nebulizer pressure were set at 250°C, 14 l/min and 40 psi, respectively. The capillary voltage was set at 3500 V for the positive detection mode. The ion signals were acquired with multiple-reaction monitoring in the positive ionization mode. The mass spectrometry operating conditions were set in accordance with the manufacturer's instructions and the data acquisition and processing were conducted using the MassHunter® Workstation software (Agilent Technologies).

2.5 Assay performance

Quality control samples were prepared from pooled serum. Sera were collected from six intact female beagle dogs owned by the Animal Facility of Graduate School of Veterinary Medicine, Hokkaido University. The animal experiment was approved by the Experimental Animals Committee of Hokkaido University (No. 18-0142). Six dogs were defined healthy based on history, physical examination, complete blood count, blood biochemistry, urinary analysis, and abdominal sonography. The median age of dogs was 2.5 years (range 1–5 years), and the median body weight was 9.8 kg (range 9.4–11.5 kg).

Calibration curve was obtained in seven different measurements for evaluating linearity, the limit of detection (LOD), limit of quantification (LOQ). Calibration

standards of each STDs were prepared at nine concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 ng/ml) and mixed with the IS working solution. Calibration curves were obtained using the STD/IS peak area ratios versus STD/IS concentration ratios. Square of correlation coefficient (R^2), slope, and intercept of each calibration curve were calculated. The LOD and LOQ were calculated using the following formula respectively:

$$\text{LOD (pg/ml)} = \frac{\text{standard deviation of intercept}}{\text{average of slope}} \times 3.3$$

$$\text{LOQ (pg/ml)} = \text{LOD} \times 3$$

Removal and corrected recovery were calculated for assessing the method accuracy using the following formulae:

$$\text{Removal (\%)} = \frac{\text{peak area of IS in serum}}{\text{peak area of IS in DDW}} \times 100$$

$$\text{Recovery (\%)} = \frac{\text{peak area of IS in serum}}{\text{average peak area of IS in calibratiob standards}} \times 100$$

$$\text{Corrected recovery (\%)} = \frac{\text{recovery}}{\text{matrix effect}} \times 100$$

$$\text{Matrix effect (\%)} = (a - b/c) \times 100$$

a = peak area of analyte in serum spiked with STD

b = peak area of analyte in serum)

c = peak area of STD in DDW × 100

2.6 Patients

Serum obtained from 19 dogs with HAC and 15 dogs without HAC were retrospectively analyzed using the developed method. Since the ACTH stimulation test was performed in all dogs, 68 serum samples were used for this study. Dogs were represented to the Hokkaido University Veterinary Teaching Hospital during May 2013 to October 2020. Written informed consent was signed by all owners before blood collection. Serum was used for the measurement of cortisol concentration by an in-house enzyme-linked immunosorbent assay (ELISA) kit (IDEXX SNAPshot Dx Cortisol Test, IDEXX Laboratories, Westbrook, ME, USA). Thereafter, serum was stored at −80°C until the LC/MS/MS measurement.

The criteria of HAC were

• with one or more clinical signs that suggest HAC,

• and the results of ACTH-ST or LDDST (3 dogs) were consistent with HAC,

• and the clinical signs were improved by the trilostane treatment.

The patient criteria of 19 dogs with HAC and 15 dogs without HAC were presented in Table 1. Dogs without HAC were patients in whom HAC was excluded based on adrenal function tests (ACTH-ST in 15 dogs and LDDST in 2 dogs) and response to treatments for other diseases. The final diagnoses were hepatocellular carcinoma in four dogs, malassezia dermatitis in two dogs, and one dog each of myxomatous mitral valve disease, food responsive diarrhea, hepatic nodular hyperplasia, parathyroid gland adenoma, pulmonary thromboembolism, bronchiectasis, pancreatitis, megaesophagus, and incidental adrenal gland mass. There was not statistically significant difference in age or body weight between HAC dogs and dogs without HAC.

2.7 Method Comparison

Serum cortisol concentration by the LC/MS/MS method was compared with that measured by the ELISA method. Because cortisol concentration in 8 of 68 serum samples was above the detection limit of the ELISA method (*i.e.*, >30 μg/dl), cortisol concentrations of 60 samples were used for the comparison. Bland-Altman analysis was used to assess the agreement of two methods.

2.8 Statistical analysis

Statistical analyses were performed with a commercial software (JMP Pro version 14.0, SAS Institute Inc., Cary, NC, USA). Differences between HAC and non-HAC dogs were assessed using Wilcoxon rank sum test. Correlation of serum cortisol concentration between the in-house ELISA method and the LC/MS/MS method was determined by Pearson correlation test (r). A P-value below 0.05 was considered statistically significant.

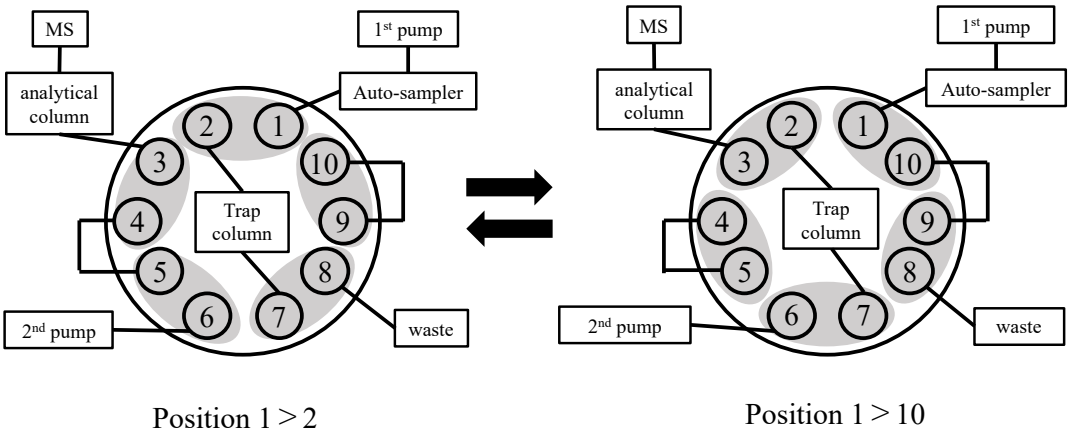


Figure 2. Schema of the on-line column-switching LC system.
At the position 1>2 (0-0.5 min), samples in the auto-sampler were injected onto the trap column via the port #1 and #2. At the position 1>10 (0.5-10 min), steroids which retained on the trap column were eluted by the 2nd binary pump and were transferred to the analytical column via the port #2 and #3.

Table 1. Demographic information of dogs with HAC and without HAC

	Dogs with HAC N=19	Dogs without HAC N=15
Breeds (n)	Miniature Dachshund (8)	Shiba Inus (3)
	Yorkshire terriers (2)	Pugs (2)
	West Highland White Terrier (2)	Chihuahuas (2)
	Pembroke Welsh Corgis (2)	Miniature Dachshund (1)
	Pomeranian (1)	Bulldogs (1)
	Toy poodle (1)	Toy poodle (1)
	Cocker spaniel (1)	Shih Tzu (1)
	Brussels Griffon (1)	Dalmatian (1)
	American cocker spaniel (1)	Siberian Huskey (1)
		Mixed breed (2)
Gender (n)	Male (0)	Male (2)
	Female (3)	Female (5)
	Neutered male (9)	Neutered male (7)
	Spay female (7)	Spay female (1)
Median age (range) (years)	11 (8–16)	12 (4 –14.0)
Median body weight (range) (kg)	7.0 (1.98–13.7)	8.24 (3.28–27)

3. Results

3.1 Method validation

Calibration curves were prepared at the concentration of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 ng/ml with every measurement. Square of correlation coefficient (R^2) was above 0.999 for each calibration curve of all hormones. Table 2 shows the LOD, LOQ, removal, and corrected recovery of each steroid. The LOD ranged 2–16 pg/ml for all steroids, and thus the LOQ was between 5 and 50 pg/ml. Corrected recoveries were above 68.9% for all steroids except for 21-deoxycortisol (53%).

3.2 Application the method to the clinical samples

The concentrations of cortisol, corticosterone, cortisone, 11-deoxycortisol, and 17α-OH-progesterone were above the LOQ in the baseline samples of all dogs. Serum progesterone concentration was detected in only 3 of 19 HAC dogs and 2 of 15 dogs without HAC, respectively. In the baseline samples, 21-deoxycortisol was not detected in all dogs. Serum aldosterone was measurable in 14 of 19 HAC dogs and 9 of 15 dogs without HAC. Figure 3 shows the concentrations of nine steroids in the baseline samples. The median cortisone concentration of dogs with HAC (9.22 ng/ml, range 5.50–17.67) was significantly higher than that of dogs without HAC (7.54 ng/ml, range 3.06–15.27, P=0.015). In dogs with HAC, the median 11-deoxycortisol concentration was 4.42 ng/ml (range 1.79–33.69) and was significantly higher than in dogs without HAC (1.9 ng/ml, range 0.31–15.21, P=0.02). In addition, 17α-OH-progesterone concentration of dogs with HAC (median 0.94 ng/ml, range 0.33–11.33) was significantly higher than that of dogs without HAC (median 0.24 ng/ml, range 0.06–15.69, P=0.018). However, the median cortisol concentration of dogs with HAC was 4.5 μg/dl (range 1.48–10.1) and was not significantly different from that of dogs without HAC (2.54 μg/dl, range 0.46–7.68, P=0.09).

In addition to five steroids that were present in all dogs before the ACTH-ST, deoxycorticosterone concentration was above the LOQ in post-ACTH serum of all dogs. Progesterone concentration was quantified in 15 of 19 HAC dogs and 10 of 15 dogs without HAC after the ACTH stimulation. Although 21-deoxycortisol was present in

some dogs (8 of 19 HAC and 2 of 15 dogs without HAC), the detection rate was lower than 50% after the ACTH administration. Serum aldosterone concentration was above the LOQ in 17 of 19 HAC dogs and 13 of 15 dogs without HAC. Cortisol concentration (median 12.52 μg/dl, range 9.52–29.42), corticosterone concentration (median 30.75 μg/dl, range 15.36–123.03), cortisone concentration (median 11.78 ng/ml, range 7.39–22.71), and deoxycorticosterone concentration (median 1.54 ng/ml, range 0.35–5.16) of dogs with HAC were significantly higher than those of dogs without HAC (median 6.56 μg/dl, range 2.59–12.69, $P<0.0001$; median 18.22 μg/dl, range 6.48–32.94, $P<0.001$; median 8.82 ng/ml, range 4.28–15.12, $P=0.01$; median 0.74 ng/ml, range 0.09–3.71, $P=0.02$), respectively (Figure 4). The median concentration of 11-deoxycortisol was 22.51 ng/ml (range 7.19–332.17) and that of 17α-OH-progesterone was 3.42 ng/ml (range 1.29–11.87) in dogs with HAC, while they were 15.41 ng/ml (range 2.02–96.35, $P=0.044$) and 2.21 ng/ml (range 0.28–22.03, $P=0.048$) in dogs without HAC, respectively.

3.3 Method comparison

Figure 5A shows the correlation of serum cortisol concentration in 60 samples measured by both the ELISA and LC/MS/MS methods. Significant correlation was observed between the ELISA and LC/MS/MS methods ($r=0.74$, $P<0.0001$). Bland-Altman plot shows that most differences (52/60) are above the line of equality (zero) and that the difference increases as the mean concentration increases (Fig. 5B).

Table 2. The limit of detection, limit of quantification, removal, and corrected recovery of each steroid

	LOD (pg/ml)	LOQ (pg/ml)	removal (%)		corrected recovery (%)	
			Average	cv (%)	Average	cv (%)
Cortisol	5	16	106.1 ± 8.5	8.1	74.9 ± 3.9	5.3
Corticosterone	12	36	108.3 ± 10.2	9.4	75.3 ± 3.4	4.5
Cortisone	4	13	108.5 ± 6.3	5.8	78.8 ± 3.0	3.8
11-deoxycortisol	7	22	106.5 ± 10.2	9.6	70.5 ± 4.8	6.8
21-deoxycortisol	16	50	111.0 ± 14.0	12	53.2 ± 8.5	16
Deoxycorticosterone	12	36	111.1 ± 9.2	8.2	76.1 ± 3.2	4.2
Progesterone	2	7	108.9 ± 4.1	3.7	69.5 ± 1.6	2.4
17α-OH-progesterone	2	5	107.8 ± 10.9	10.1	68.9 ± 5.6	8.1
Aldosterone	9	28	110.2 ± 14.0	13	74.0 ± 0.8	1.1

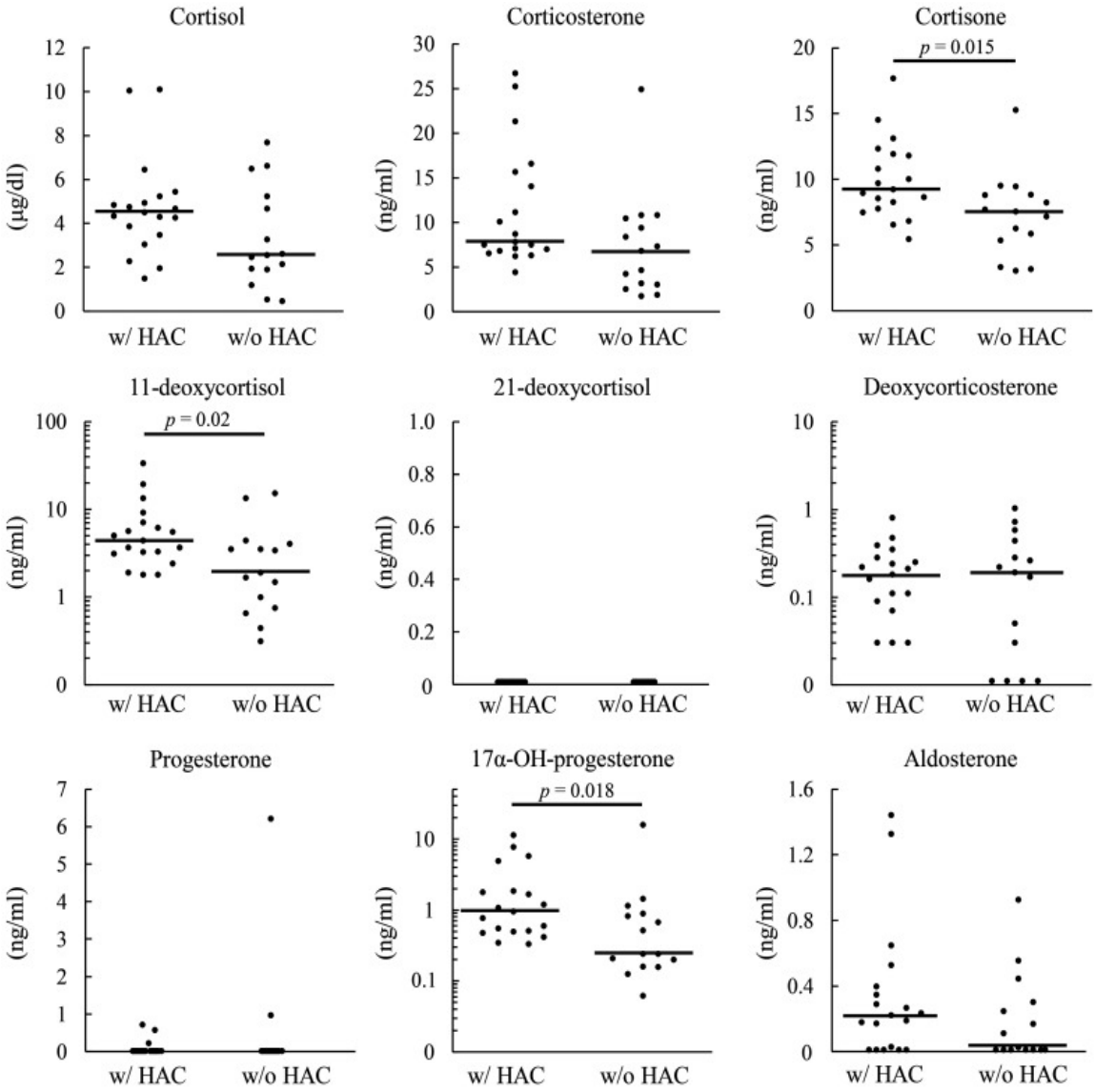


Figure 3. Concentration of nine steroids in baseline serum.
Cortisone, 11-deoxycortisol, and 17α-OH-progesterone of HAC dogs (n = 19) was higher than that of dogs without HAC (n = 15). Bars, the median concentration in each group.

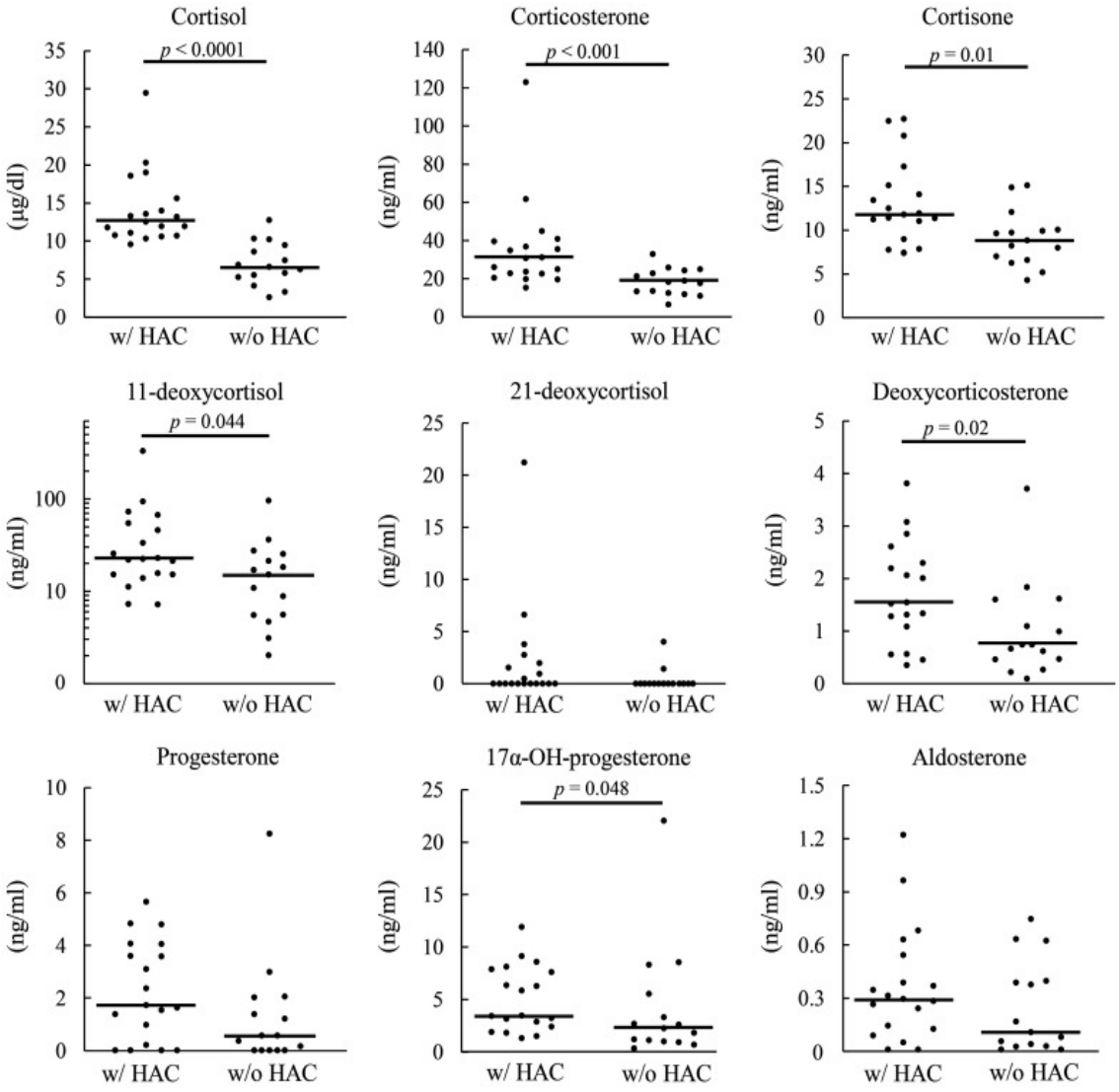


Figure 4. Concentration of nine steroids in post-ACTH serum.
Cortisol, Corticosterone, Cortisone, 11-deoxycortisol, deoxycorticosterone, and 17α-OH-progesterone of HAC dogs (n = 19) was higher than that of dogs without HAC (n = 15). Bars, the median concentration in each group. ACTH, adrenocorticotropic hormone.

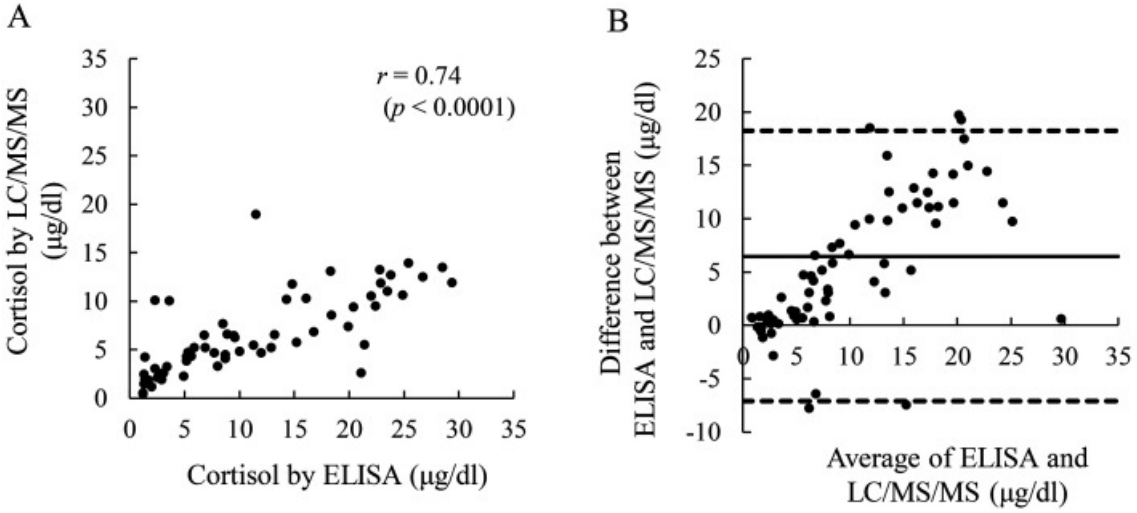


Figure 5. Method comparison.

(A) Correlation of the two methods (n = 60). Significant correlation was observed between the methods. (B) Bland-Altman plot of cortisol (n = 60). The solid line is the mean difference, and the dot lines are 95% limit of agreement (LOA).

4. Discussion

We developed a simple analytical method for quantification of multiple steroids in dog serum. The pre-treatment steps in this study were protein precipitation and removal of phospholipids by centrifugation, followed by the programed solid phase extraction on the trap and analytical columns. The trap column (Unison UK-C1) is superior to the conventional reversed phase columns in separating hydrophilic compounds under formic acid conditions with the high flow pressure. The analytical column (Biphenyl) has high selectivity for aromatic compounds and allows better separation of isomers and analogs. The combination of the two columns in the on-line column switching system might contribute to high-resolution peak separation of nine steroids in serum. Both the LOD and LOQ of our method were comparable with previous studies using manually solid phase extraction on single column[33,34] and derivatization[35]. In addition, good, corrected recoveries (68.9–78.8%)[39,40] were obtained for all steroids except 21-deoxycortisol. Although the corrected recovery of 21-deoxycortisol was low (50%), the sensitivity (LOD=16 pg/ml) and the accuracy (removal 110 ± 14%) were comparable with those in the previous study[39]. The results of this study suggest that the on-line column switching LC/MS/MS method is feasible for measuring multiple steroids in dog serum.

Serum progesterone and 21-deoxycortisol concentration were below the LOQ in many dogs. Sanders *et al.* reported that the high expression level of *CYP17* in the zona fasciculata of canine adrenal glands and suggested that the zone-specific *CYP17* expression contributed to the zone-specific cortisol production[41]. It may be likely that progesterone is rapidly converted to 17α-OH-progesterone and thus the serum progesterone concentration may not be enough high to be detected in the physiological condition of nonpregnant dogs. A previous study showed that the serum 21-deoxycortisol concentration ranged in the order of pmol/l[42]. The LOQ of 21-deoxycortisol in this study was 16 pg/ml, which corresponded to 3 pmol/l. It may be necessary for the development of LC/MS/MS methods to improve the sensitivity of quantifying the serum 21-deoxycortisol concentration in dogs. However, 21-deoxycortisol may not be more useful than other steroids in veterinary medicine.

Measurement of 21-deoxycortisol is used for the diagnosis of congenital adrenal hyperplasia due to *CYP21* deficiency in human medicine[39]. To our knowledge, mutations in *CYP21* have not been reported in dogs[43,44]. Three steroids (17α-OH-progesterone, 11-deoxycortisol, and cortisone) in the baseline samples and six steroids (cortisone, cortisol, corticosterone, 17α-OH-progesterone, deoxycorticosterone and 11-deoxycortisol) in the post-ACTH samples were higher in dogs with HAC than in dogs without HAC, although there were considerable overlaps of the concentrations between the two groups. The previous study using radioimmunoassay reported that high concentration of both 17α-OH-progesterone and 11-deoxycortisol in HAC dogs[42]. It may be possible that 17α-OH-progesterone, 11-deoxycortisol, and cortisone contribute to the clinicopathology of HAC. Serum 11-deoxycorisol concentration is a possible biomarker for human adrenocortical cancer[45,46]. Plasma concentration of 11-deoxycortiol, 21-deoxycortisol, deoxycorticosterone, corticosterone and cortisol increased in human Cushing syndrome[47], though steroid profile of human Cushing syndrome has not been fully understood[48].

Comparison of the methods showed the considerable discrepancy between the in-house ELISA and our LC/MS/MS method for cortisol quantification. The ELISA method had proportional and fixed biases and tended to show higher cortisol concentration compared with our LC/MS/MS method. The discrepancy in the cortisol concentrations was consistent with the previous report[33]. Higher cortisol concentration in the ELISA method may be due to the cross-reactivity of other steroids or endogenous compounds. It is obscure whether drugs (except prednisolone) and endogenous compounds interact with the particular ELISA assay according to the manufacturer. The ACVIM consensus suggests that cortisol concentrations vary among assay and thus does not recommend any particular assay for cortisol[3]. The LC/MS/MS method in this study could be a reliable tool for quantifying cortisol in clinical samples because our method specifically measured cortisol as well as other steroids with relatively simple preparation of the samples.

The diagnostic feasibility of steroid profiling by LC/MS/MS was not elucidated in the current study. As HAC dogs of this study had significant higher post-ACTH cortisol concentration than dogs without HAC, it is unclear whether the measurements of precursors and metabolites have potential for diagnosing canine HAC. It must be noted

that assessment of the clinical feasibility of steroid profile is not the primary objective of this study, but that we intend to illustrate in this study that the developed method used here can be useful for characterizing steroid profile in HAC dogs. Some HAC dogs might be classified as dogs without HAC due to the low sensitivity of the ACTH-ST[2]. Even though HAC was excluded by the response to treatments for other diseases, the LDDST was performed in only three dogs with HAC and two dogs without HAC. More in-depth studies with appropriate control groups including healthy dogs should assess the feasibility of steroid profile for diagnosing HAC in dogs.

In conclusion, we developed the on-line column switching LC/MS/MS method with simple preparation and high accuracy for quantifying multiple steroids in serum. The findings showed that HAC impacted on steroid profile with elevated concentration of cortisone, 11-deoxycortisol and 17α-OH-progesterone in the baseline serum and, cortisol, cortisone, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone and 17α-OH progesterone in post-ACTH serum. Beside the cortisol, the additional five steroids were suggested to contribute the clinicopathogenesis of HAC. Future studies would evaluate the diagnostic feasibility of steroid profile by the LC/MS/MS measurement.

5. Summary

Nine serum steroids could be accurately and precisely measured using the established online Column Switching LC/MS/MS techniques. Comparison of the two techniques, ELISA and LC/MS/MS revealed a significant discrepancy. HAC impacted on steroid profile with elevated concentration of cortisone, 11-deoxycortisol and 17α-OH-progesterone in the baseline serum and, cortisol, cortisone, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone and 17α-OH-progesterone in post-ACTH serum. Beside the cortisol, the additional five steroids were suggested to contribute the clinicopathogenesis of HAC.

Chapter 2

Serum Steroid Profiling of Hepatocellular Carcinoma Associated with Hyperadrenocorticism in Dogs

1. Introduction

The most prevalent hepatobiliary disease in dogs is hepatocellular tumor. HCC is the sixth most common cancer worldwide in humans and 50–70% of all hepatic tumors in dogs[4,5 49,50]. Viral infections and metabolic disorders are the two main etiopathologies of HCC in humans[51]. HCC frequently manifests in dogs aged over 10 years, and over-presented in male dogs were also anecdotally reported. However, gender predisposition, specific risk factors, and the precise mechanisms of hepatocarcinogenesis are not well characterized in dogs[52,53]. Some dog breeds, such as Miniature Schnauzers, Shih Tzus, Welsh Corgis, and Beagles, have high risks of developing HCC, and concurrent HAC is outwardly linked to the development of HCC. Scottish Terriers with vacuolar hepatopathy were also found to have a higher risk of developing HCC[6,8,12,13].

Elevation of corticosteroid isoforms of alkaline phosphatase and hepatocellular vacuolation on histology are very common in Scottish Terriers[9-11]. 34% of Scottish terriers with vacuolar hepatopathy were progressed to HCC[12]. Furthermore, Scottish terriers with vacuolar hepatopathy disease show HAC clinical signs with steroid imbalance. According to the recent study, changes of a single nucleotide polymorphism in HSD17B2 causes an increase in adrenal sex steroids were suggested[41,44]. Leela-Arporn *et al*. investigated the concurrent rate of massive HCC and hypercortisolemia with HAC suggestive clinical signs[8]. As a result, 10 dogs with HAC were concurrent in 44 dogs with HCC. It follows that excess adrenal steroids impact to the liver and contributes to HCC is not surprising.

In humans, metabolic syndrome has the potential to increase the risk of NAFLD, which can lead to HCC. The relationship between hepatic lipid accumulation and progressive of HCC is still required to clarify. The clinicopathologic abnormalities of HAC, steroid hepatopathy caused by chronic exposure of excess adrenal hormones are similar to NAFLD-induced HCC[2,14]. An association between HAC and HCC in dogs may contribute to understanding HCC development from NAFLD in humans. In dogs with HAC, cortisol is the most common and powerful glucocorticoid for diagnosis. Other

adrenal hormones, such as 11-deoxycortisol, corticosterone, or 17-OH progesterone, have been thought to contribute to HAC[17,24,54]. In order to understand the relationship between HAC and HCC, it may be useful to assess large number of steroids so called steroid profile in dogs (Figure 6).

In veterinary medicine, immunoassay is used as a gold standard method because of its convenience and high sensitivity[16,18,55]. However, the main disadvantage is less precise due to the cross-reactivity of structurally related steroids[18]. Since LC/MS/MS can detect many steroid hormones with high specificity and sensitivity. It has become trended to use in clinical laboratories[19,20]. Although different preparation methods for LC/MS/MS have been described for quantifying multiple steroid hormones in biofluids, some of them are highly sensitive for a small number of steroids, while others can detect dozens but have inadequate sensitivity[56]. The chemical derivative reagent reacts with the functional groups of the target analytes (amine, hydroxyl, or carbonyl groups) as an additional sample pretreatment technique to improve ionization[57].

Thus, the purpose of Chapter 2 was to prepare sample pretreatment procedure for successful measurement of multiple steroid hormones, and investigate the relationship between HAC and HCC in dogs using serum steroid profiling.

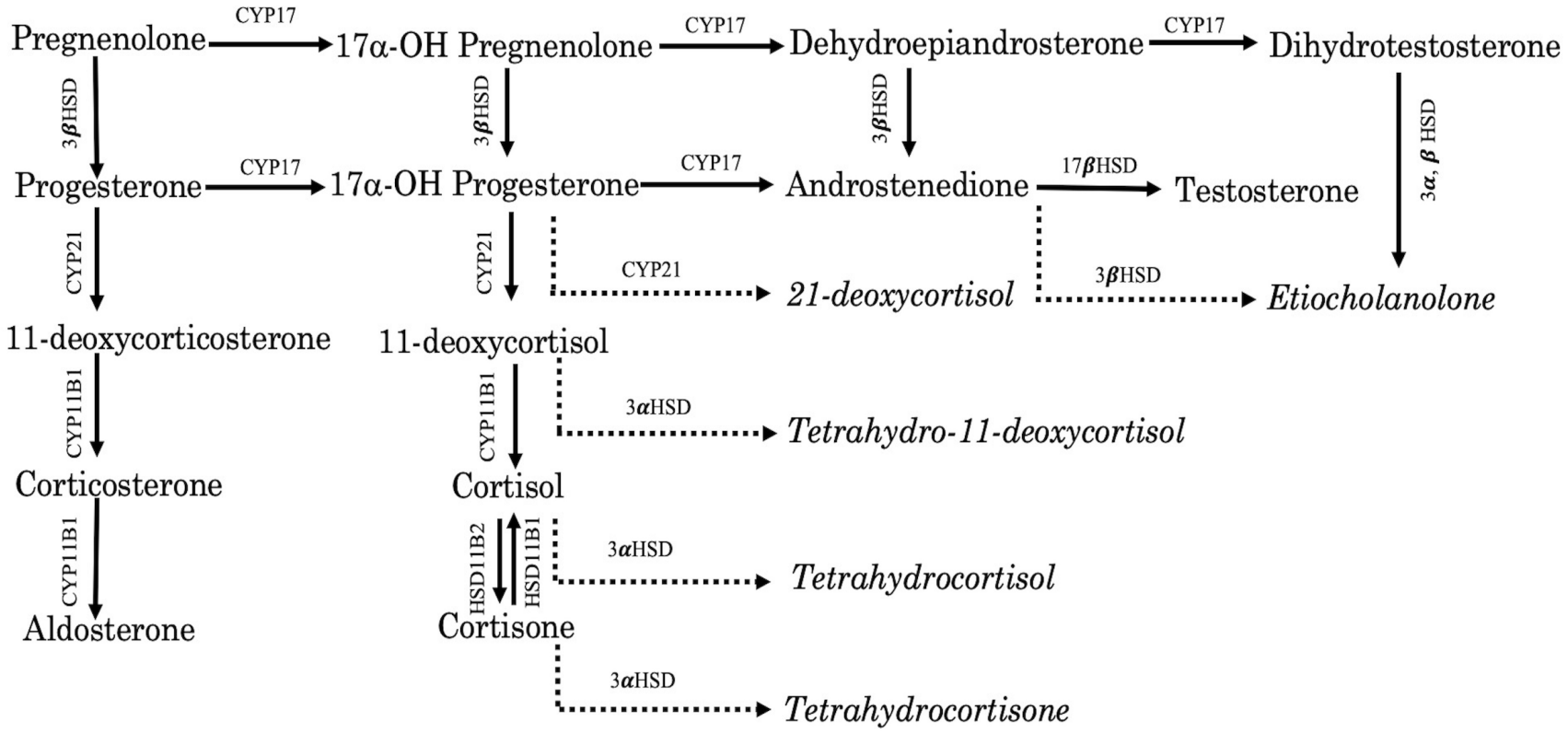


Figure 6. Schematic pathway of steroids and metabolites. CYP17, 17α-hydroxylase, CYP21, 21-hydroxylase, CYP11 B1, 11β hydroxylase, 3α HSD, 3α-hydroxysteroid dehydrogenase, 3β HSD, 3β-hydroxysteroid dehydrogenase. Italic: Metabolites

2. Materials and methods

2.1 Study population

From September 2019 to December 2021, a retrospective study was conducted at the Hokkaido University Veterinary Teaching Hospital (HUVTH). A total of 46 dogs, 31 massive HCC and 15 HAC cases were included in this study. Among the 31 massive HCC cases, 10 dogs had concurrent HAC, while 21 were HAC-free. Of the 15 dogs with HAC, abdominal ultrasonography revealed no hepatic lesion. The other endocrine disorders were 2 dogs with hypothyroidism and 1 dog with hyperparathyroidism. Myxomatous mitral valve disease (MMVD) in 5 dogs, biliary sludge in 3 dogs, chronic kidney disease (CKD) in 2 dogs, bladder stones in 2 dogs, atopic dermatitis in 2 dogs, and one each had pyoderma, mast cell tumor (MCT), hypercalcemia, and breast cancer were presented as other medical complications. Informed consent was obtained from all owners of the dogs involved in this study.

Liver tumors were collected when HCC-suspected dogs were undergoing surgery at HUVTH. Formalin-fixed liver tissues were sent to private laboratory and HCC was histologically confirmed by a board certificated pathologist. The pathological diagnosis of HCC was defined according to the guidelines of the World Small Animal Veterinary Association (WSAVA) Liver Standardization Group[58]. Diagnostic criteria of HAC were the presence of one or more HAC-suggestive clinical signs, a positive result of the ACTH-ST, and clinical response to the trilostane treatment following 3 months[3]. Six HCC dogs without HAC were received corticosteroid treatment more than 2 weeks. Those dogs were excluded from the further analysis, and serum profiling was performed in 15 HAC, 15 HCC and 10 dogs with both diseases.

Blood of 2 ml volume was collected at their first visit to our hospital and serum was obtained by centrifuging at 3,000 rpm for 10 minutes. Serum was stored at -80°C until analysis of steroid profile. The breed, age, body weight, gender distribution, and neutered status of all dogs are described in Table 3.

2.2 Chemicals and reagents

A total of 19 steroids in the serum were quantified using LC/MS/MS. The standard (STD) and stable isotope-labeled internal standard (IS) of 19 steroids were purchased from Toronto Research Chemicals (Toronto, Canada), Cerilliant (Round Rock, TX, USA) or Sigma-Aldrich (St. Louis, MO, USA). Double-distilled water (DDW) and liquid chromatography grade-analytical reagents (methanol and acetonitrile) were bought from Kanto Chemical Co., Ltd. (Tokyo, Japan). Formic acid (abt.99%) was obtained from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan). The phospholipid removal MonoSpin® column was purchased from GL Sciences Co., Ltd. (Tokyo, Japan). Amplifex^{TM} keto reagent kits were acquired from AB SCIEX Pte. Ltd (Framingham, MA, USA). Working solutions for STD and IS mixtures were dissolved in methanol at 10 ng/mL and 100 ng/mL concentrations, respectively.

2.3 Sample preparation

In total, 19 steroids (14 adrenal steroids and 5 metabolites) in the baseline serum of 40 dogs were quantitatively measured using two sample preparation methods. The preparation procedure is described below.

2.3.1. Non-derivatization method

For the protein precipitation, 25 µL of serum was thoroughly mixed with 10 µL IS-mixture working solution and 90 µL of 1% formic acid containing acetonitrile. The concentration of each steroid in the IS-mixture was 10 ng/mL. The mixture was centrifuged at 10,000 × g for 10 min at 25°C. Afterward, 100 µL of the supernatant was applied to the Mono-spin column (MonoSpin® Phospholipid, GL Sciences Co., Ltd., Tokyo, Japan) that was conditioned with 1% formic acid acetonitrile. The spin column was centrifuged at 3,000 × g for 2 min to remove phospholipids in serum. The eluate was evaporated at 60 °C using a centrifugal vaporizer CVE-2000D (EYELA, Bohemia, NY, USA). Then, 100 µL of 50% methanol-DDW containing 0.1% formic acid was added to reconstitute the dried tube.

2.3.2. Keto-derivatization method

For the protein precipitation, 25 µL of serum was mixed with 10 µL of the IS-mixture working solution and 90 µL of 1% formic acid in acetonitrile. The mixture was vortexed and centrifuged at 10,000 × g for 10 min. Afterward, 100 µL of the organic layer was loaded into the Mono-spin column (MonoSpin® Phospholipid, GL Sciences Co., Ltd., Tokyo, Japan) which conditioned with 1% formic acid acetonitrile. The phospholipids in the samples were removed by centrifuging the spin column at 3,000 × g for 2 min. The eluate was dried at 60°C using the speed vacuum evaporation. The samples were derivatized with keto-derivatization reagent (Amplifex™ Keto Reagent Kit, AB SCIEX Pte. Ltd., Framingham, MA, USA). The dried eluate was reconstituted with 50 µL of the keto-derivatization reagent and incubated for 60 min on a vortex mixer. The derivatization reaction was stopped by combining with 50 µL DDW. The final 100 µL solution was transferred to the LC/MS/MS system.

2.4 LC/MS/MS/ analytical conditions

LC/MS/MS was performed using an Agilent 6495B Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA) coupled with a high-performance liquid chromatography system (1260 Infinity II, Agilent Technologies). The 50 µL solution was injected into the LC/MS/MS system. Chromatographic separation was carried out on the Shimpack Biphenyl column (φ2.6 µm, 100 × 3 mm, Shimadzu, Kyoto, Japan) for the non-derivatization method and Poroshell 120/ EC-C18 column (φ2.7 µm, 100 × 3 mm, Santa Clara, US) for the keto-derivatization method, respectively. The mass spectrometry was run in the positive electrospray ionization (ESI) multiple reaction monitoring (MRM) mode. The data acquisition and processing were operated using the Agilent Mass Hunter Workstation software (Agilent technologies). A gradient program and column settings are described in Table 4A and 4B for the non-derivatization and keto-derivatization methods, respectively. The mass to charge transition of 19 steroids and internal standards in positive MRM mode are presented in Table 5A and 5B for the non-derivatization and keto-derivatization methods, respectively.

2.5 Validation of the method

Serum samples required for method validation were collected from 6 healthy, intact female beagle dogs owned by the animal facility of the Graduate School of Veterinary Medicine, Hokkaido University. The method performance was determined by calculating the instrumental detection limit (IDL) and method detection limit (MDL) of the steroid hormones.

2.6 Calibration curves and QC samples

The calibration curve was created using 9 concentrations of the STD mixture (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 ng/mL) dissolved with 1 ng/mL of the IS-mixed in methanol. Peak areas of steroid hormones were manually identified with the corresponding IS peak area in each sample. The analyte peak area was less than the peak area of the lowest calibration point (0.001 ng/mL) and the concentration less than the MDL value was set as the zero concentration. The concentration of steroid hormone was calculated using the following equation.

$$Cs = Css \times \left(\frac{As}{Ass} - b\right) / a$$

Cs = amount (ng) of target steroids
Css = amount (ng) of IS
As = Peak area of target steroids
Ass = Peak area of IS
a = slope of the calibration curve

2.7 Statistical analysis

Statistical analyses were performed using commercial software (JMP Pro®16, version 16.0.0, SAS Institute Inc, Cary, NC, USA). Principle component analysis (PCA) was performed to see the steroid pattern difference between the disease groups. The Steel-Dwass test was used for non-parametric multiple comparison of median steroid concentration between HCC, HAC, and HCC with HAC dogs. p (<0.05) was considered statistically significant for all comparisons.

Table 3. Demographic information of dogs in HCC, HAC, and HCC with HAC

	HCC N=15	HAC N=15	HCC with HAC N=10
Breeds (n)	Miniature Dachshund (2) Shiba (1) Miniature Schnauzer (1) Chihuahua (1) Beagle (1) Shih Tzu (1) Siberia Huskey (1) Japanese Spitx (1) Samyoed (1) Bichon Frise (1) Mix breed (4)	Miniature Dachshund (2) Shiba (2) Miniature Schnauzer (1) Chihuahua (4) West Highland White Terrier (2) Miniature Pinscher (1) American Cocker Spaniel (1) Yorkshire Terrier (1) Mix breed (1)	Miniature Dachshund (1) Shiba (1) Miniature Schnauzer (1) Chihuahua (1) Shih Tzu (1) Boston Terrier (1) Beagle (1) Mix breed (3)
Gender (n)	Male (1) Female (2) Neutered male (6) Spay female (6)	Male (1) Female (1) Neutered male (6) Spay female (7)	Male (0) Female (2) Neutered male (4) Spay female (4)
Median age (range) (years)	10.7 (7.2–14.8)	12.5 (5.5 –14.0)	10.8 (7.7–14.3)
Median body weight (range) (kg)	7.0 (2.2–27.0)	8.7 (2.7–17.7)	9.8 (3.9–21.6)

Table 4A. Gradient program and column temperature in the non-derivatization method

Pump system	Mobile phase A	0.1% formic acid in DDW	
	Mobile phase B	0.1% formic acid in methanol	
Temperature		50℃	
Gradient condition pump system	Mobile phase A (%)	Mobile phase B (%)	Flow rate (ml/min)
0 min	60.00	40.00	0.4
0.50 min	60.00	40.00	0.4
0.51 min	60.00	40.00	0.8
3.00 min	60.00	40.00	0.8
19.00 min	15.00	85.00	0.8
19. 50 min	0.00	100.00	0.8
21.00 min	0.00	100.00	0.8
21.01 min	60.00	40.00	0.4

Ion source	Sheath gas temp (℃)	Sheath gas flow rate (L/min)	Drying gas temperature (℃)	Drying gas flow rate (L/min)	Nebulizer (psi)	Capillary (V)
Electrospray Ionization	350.00	12.00	290	19	40	3500

Table 4B. Gradient program and column temperature in the keto-derivatization method

Pump system	Mobile phase A	0.1% formic acid in DDW	
	Mobile phase B	0.1% formic acid in methanol	
Temperature		50℃	
Gradient condition pump system	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0 min	65.00	35.00	0.4
1.50 min	65.00	35.00	0.4
1.51 min	65.00	35.00	0.8
17.00 min	40.00	60.00	0.8
17.01 min	0.00	100.00	0.8
18. 50 min	0.00	100.00	0.8
18.51 min	65.00	35.00	0.4

Ion source	Sheath gas temp (℃)	Sheath gas flow rate (L/min)	Drying gas temperature (℃)	Drying gas flow rate (L/min)	Nebulizer (psi)	Capillary (V)
Electrospray Ionization	350.00	12.00	290	17	220	3500

Table 5A. Multiple reaction monitoring condition of steroids and their internal standards in the non-derivatization method

Native STD/ Stable isotope labeled IS	Precursor ion (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Collision Energy (V) Quantifier	Collision Energy (V) Qualifier	Polarity
Progesterone	315.2	97.0	108.9	24	28	+
Progesterone-d9	325.0	100.1	113.2	20	32	+
17α -OH progesterone	331.2	109.1	97.2	28	44	+
17α -OH progesterone-13C3	334.0	100.2	81.2	32	68	+
Cortisol	363.2	121.1	91.0	32	68	+
Cortisol-d4	367.4	121.0	97.1	24	52	+
Cortisone	361.2	163.0	91.1	25	70	+
Cortisone-d8	369.2	168.2	93.4	20	72	+
Corticosterone	347.2	121.2	91.0	28	68	+
Corticosterone-d4	351.4	121.0	97.1	44	40	+
11-deoxycorticosterone	331.5	109.2	97.0	32	20	+
11-deoxycorticosterone-13C3	334.1	100.0	112.1	24	28	+
11-deoxycortisol	347.2	97.1	109.0	32	28	+
11-deoxycortisol-d5	352.0	100.3	113.1	28	28	+
21-deoxycortisol	347.5	91.1	311.6	72	16	+
21-deoxycortisol-d8	355.3	319.3	125.2	16	24	+
Aldosterone	361.2	343.3	91.1	20	80	+
Aldosterone-d4	365.3	347.2	97.0	20	36	+
Androstenedione	287.2	97.1	109.1	20	24	+
Androstenedione -13C3	290.0	100.0	112.0	28	28	+
Testosterone	289.2	97.1	109.0	20	28	+
Testosterone-13C3	292.0	100.1	111.9	28	20	+

Table 5B. Multiple reaction monitoring condition of steroids and their internal standards in the keto-derivatization method

Native STD/ Stable isotope labeled IS	Precursor ion (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Collision Energy (V) Quantifier	Collision Energy (V) Qualifier	Polarity
Pregnenolone	431.4	372.2	126.1	24	44	+
Pregnenolone-d4	435.5	376.3	130.1	28	48	+
17α -OH pregnenolone	447.3	370.2	388.2	28	20	+
17α -OH pregnenolone-13C3d2	451.4	374.2	392.4	28	24	+
Etiocholanolone	405.5	346.2	91.1	28	72	+
Etiocholanolone-d5	410.3	351.2	105.0	16	72	+
Dehydroepiandrosterone	403.4	344.2	105.1	28	68	+
Dehydroepiandrosterone-d5	408.0	349.2	162.2	28	48	+
Dihydrotestosterone	405.4	346.2	91.1	32	80	+
Dihydrotestosterone-d3	408.5	349.2	81.1	24	60	+
Tetrahydrocortisol	481.0	118.1	116.0	56	44	+
Tetrahydrocortisol-d5	486.0	118.2	60.1	48	68	+
Tetrahydrocortisone	479.0	118.0	59.1	40	64	+
Tetrahydrocortisone-d5	484.0	118.0	116.1	52	40	+
Tetrahydro-11-deoxycortisol	465.0	118.1	59.1	48	80	+
Tetrahydro-11-deoxycortisol-d5	470.0	118.1	115.9	44	44	+

3. Results

The assay performance (IDL and MDL) values of each steroid are described in Table 6.

The detection rate of steroid hormones in the non-derivatization and keto derivatization methods in dogs with HCC, HAC, and both diseases are described in Table 7. 21-deoxycortisol and aldosterone were detected in one-fourth of the total samples. Androstenedione and dehydroepiandrosterone were hardly detected around 10%. Etiocholanolone was detected only in one HAC dog while testosterone and dihydrotestosterone were detected in each dog of the HCC with HAC group. The detection rate of 19 steroids was not significantly different between groups ($p>0.05$). Steroid hormones with a detection rate of higher than 50% were selected for further statistical analysis.

PCA explains none of serum 19 steroids made steroid pattern difference among the groups (Figure 7). Figures 8 and 9 compare the median concentrations of 9 steroids and 2 metabolites, respectively. None of the 11 steroid concentrations significantly differed between dogs with HCC, HAC, and both diseases ($p>0.05$).

Table 6. Assay performance of the non-derivatization and keto-derivatization methods

Detection Method	Steroid Hormones	IDL (pg)	MDL (ng/ mL)
Non-derivatization method	Cortisol	0.02	0.13
	Cortisone	0.02	0.18
	11-deoxycortisol	0.01	0.06
	11-deoxycorticosterone	0.02	0.02
	Corticosterone	0.02	0.04
	Progesterone	0.01	0.01
	17α-OH progesterone	0.01	0.09
	Androstenedione	0.03	0.09
	Testosterone	0.01	0.19
	21-deoxycortisol	0.01	0.03
	Aldosterone	0.06	0.06
Keto-derivatization method	Pregnenolone	0.02	0.03
	17α-OH pregnenolone	0.02	0.02
	Tetrahydrocortisol	0.02	0.03
	Tetrahydrocortisone	0.04	0.02
	Tetrahydro-11-deoxycortisol	0.01	0.02
	Dehydroepiandrosterone	0.03	0.04
	Dihydrotestosterone	0.04	0.01
	Etiocholanolone	0.02	0.06

Table 7. Detection rate (%) of steroid hormones in the non-derivatization and keto-derivatization methods

Detection Method	Steroid Hormones	Overall dogs (n = 40)	HCC (n = 15)	HAC (n= 15)	Both diseases (n = 10)	Chi Square ($p < 0.05$)
Non-derivatization method	Progesterone (%)	95 (38/40)	93 (14/15)	100 (15/15)	90 (9/10)	NS
	17α-OH progesterone (%)	68 (27/40)	67 (10/15)	67 (10/15)	70 (7/10)	NS
	11-deoxycorticosterone (%)	86 (34/40)	80 (12/15)	93 (14/15)	80 (8/10)	NS
	11-deoxyortisol (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	Corticosterone (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	Cortisol (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	Cortisone (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	Aldosterone (%)	25 (10/40)	27 (4/15)	27 (4/15)	20 (2/10)	NS
	21-deoxycortisol (%)	35 (14/40)	20 (3/15)	47 (7/15)	40 (4/10)	NS
	Androstenedione (%)	13 (5/40)	13 (2/15)	20 (3/15)	0	-
	Testosterone (%)	5 (2/40)	7 (1/15)	7 (1/15)	0	-
Keto-derivatization method	Pregnenolone (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	17α-OH pregnenolone (%)	78 (31/40)	80 (12/15)	73 (11/15)	80 (8/10)	NS
	Tetrahydrocortisol (%)	93 (37/40)	87 (13/15)	93 (14/15)	100 (10/10)	NS
	Tetrahydrocortisone (%)	100 (40/40)	100 (15/15)	100 (15/15)	100 (10/10)	NS
	Tetrahydro-11-deoxycortisol (%)	48 (19/40)	33 (5/15)	40 (6/15)	80 (8/10)	NS
	Dehydroepiandrosterone (%)	10 (4/40)	13 (2/15)	13 (2/15)	0	-
	Dihydrotestosterone (%)	5 (2/40)	7 (1/15)	7 (1/15)	0	-
	Etiocholanolone (%)	3 (1/40)	0	7 (1/15)	0	-

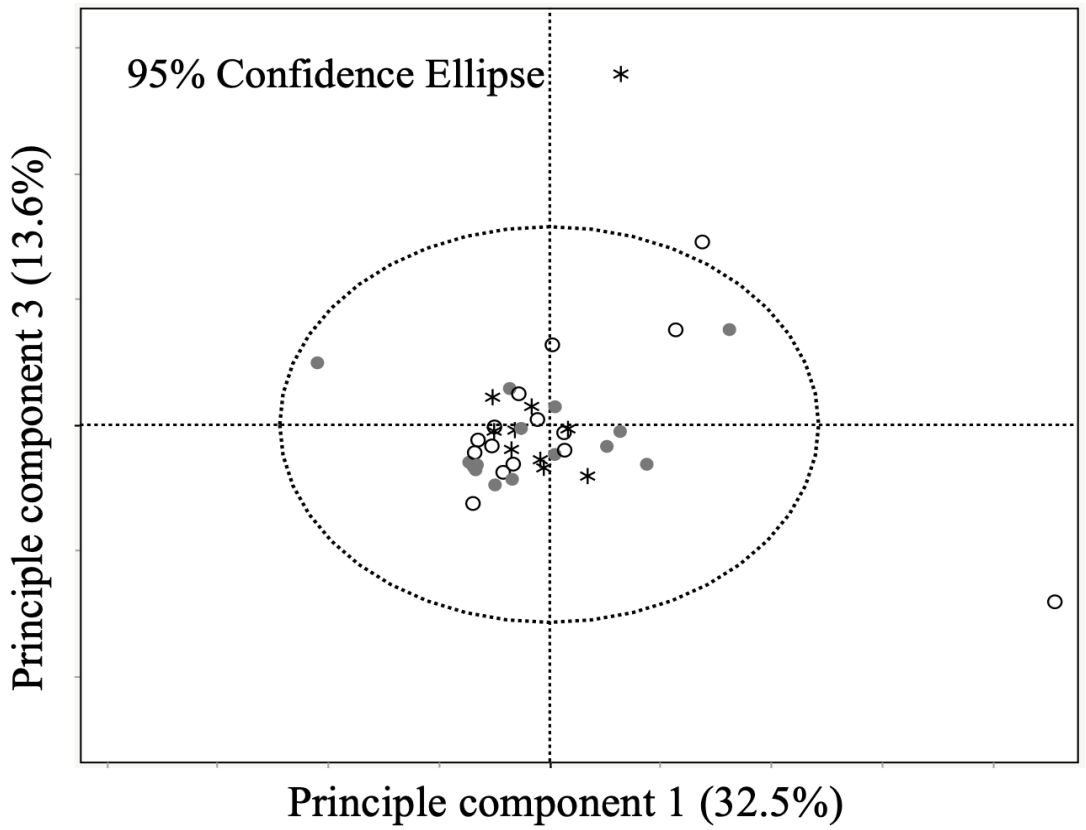
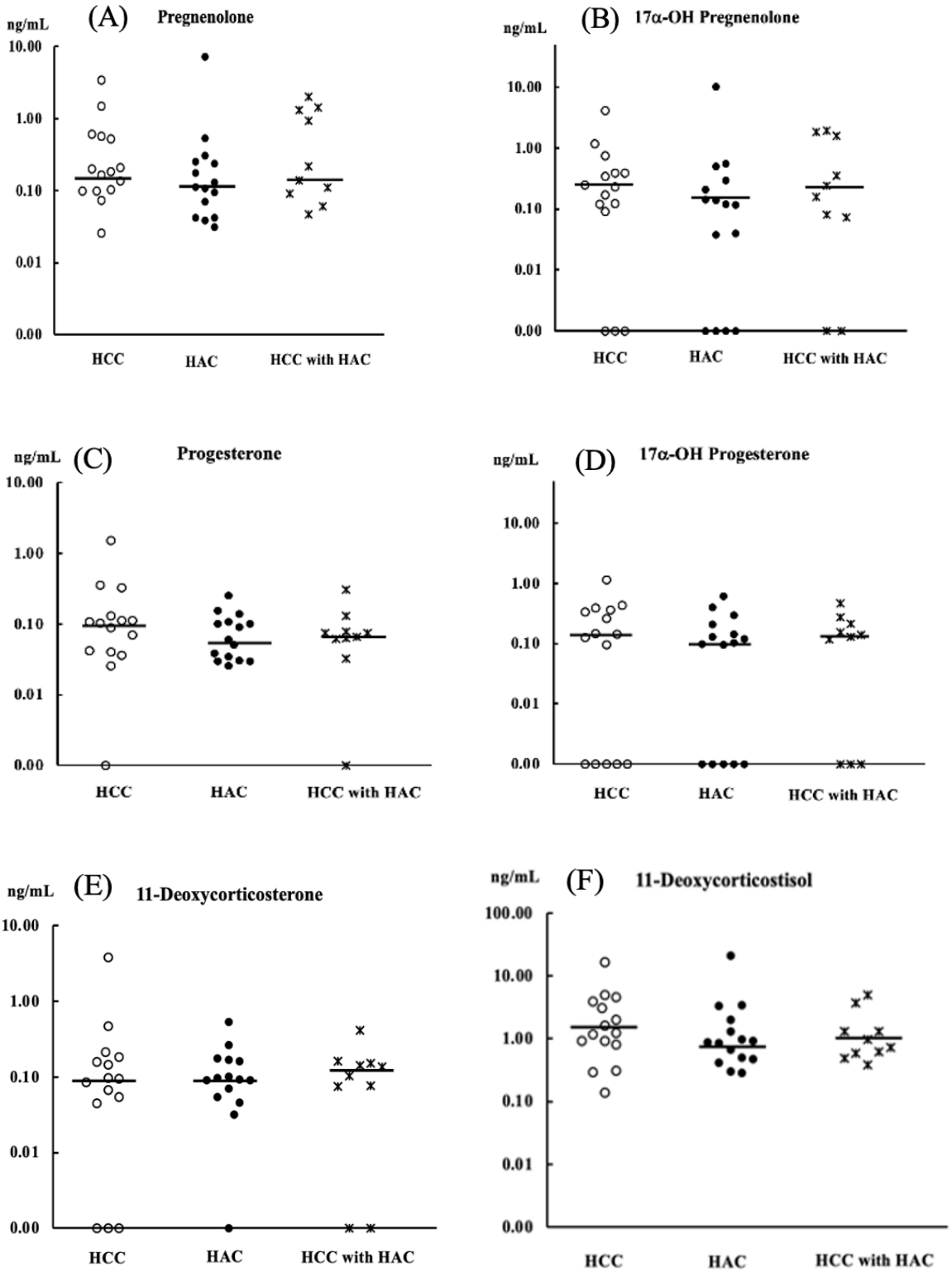


Figure 7. Principal component analysis (PCA) of the concentration of 19 steroids. Points labeled in white circles are HCC dogs, in grey circles are HAC dogs, and in asterisk are dogs with both diseases


(A) Pregnenolone
ng/mL
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC
(B) 17α-OH Pregnenolone
ng/mL
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC
(C) Progesterone
ng/mL
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC
(D) 17α-OH Progesterone
ng/mL
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC
(E) 11-Deoxycorticosterone
ng/mL
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC
(F) 11-Deoxycorticostisol
ng/mL
100.00
10.00
1.00
0.10
0.01
0.00
HCC
HAC
HCC with HAC

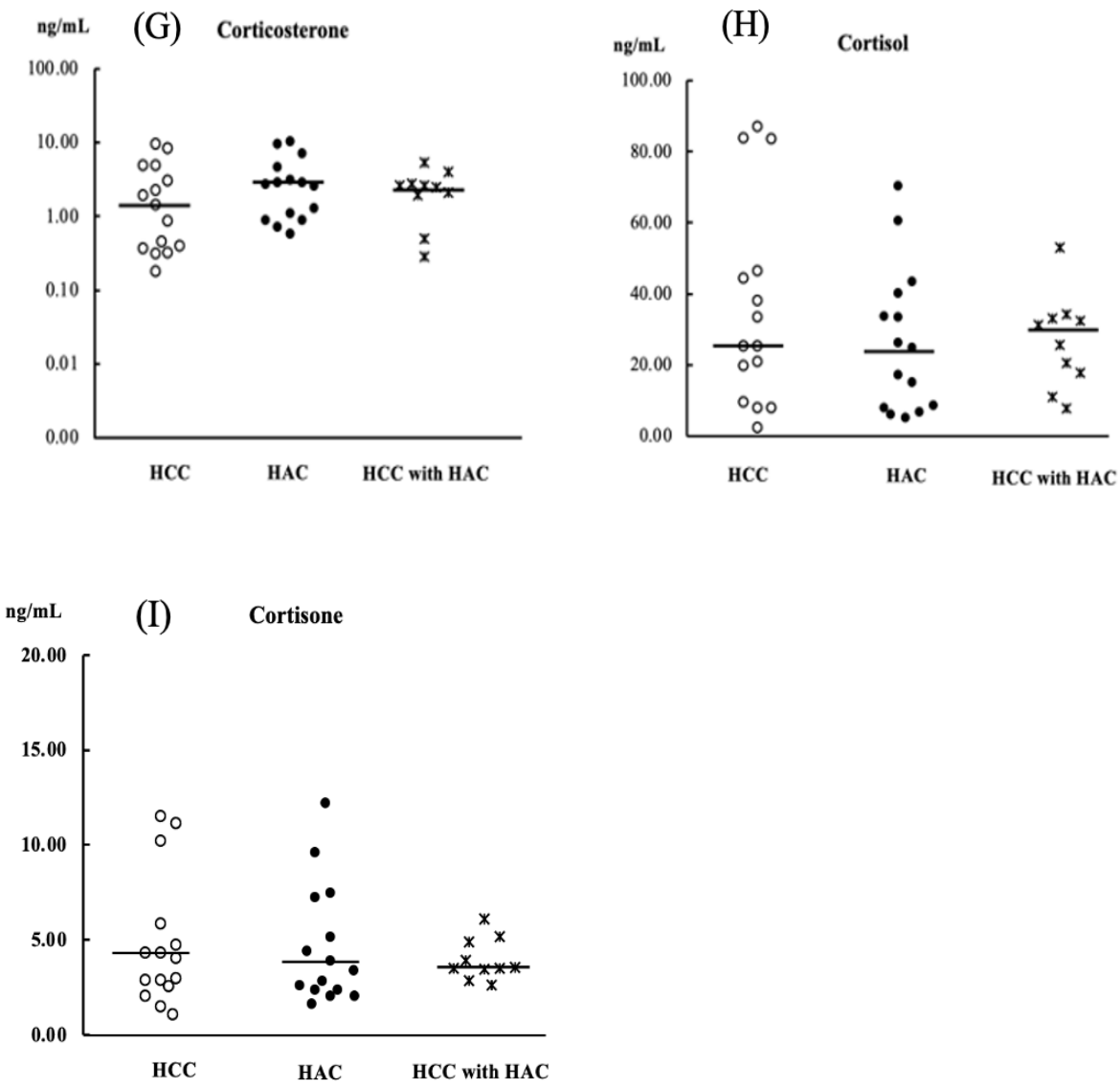


Figure 8. Comparison of 9 steroids concentration (A, B, C, D, E, F, G, H, I) in baseline serum of HCC (n = 15), HAC (n = 15) and HCC with HAC dogs (n = 10). Bars; the median concentration of steroids in each group

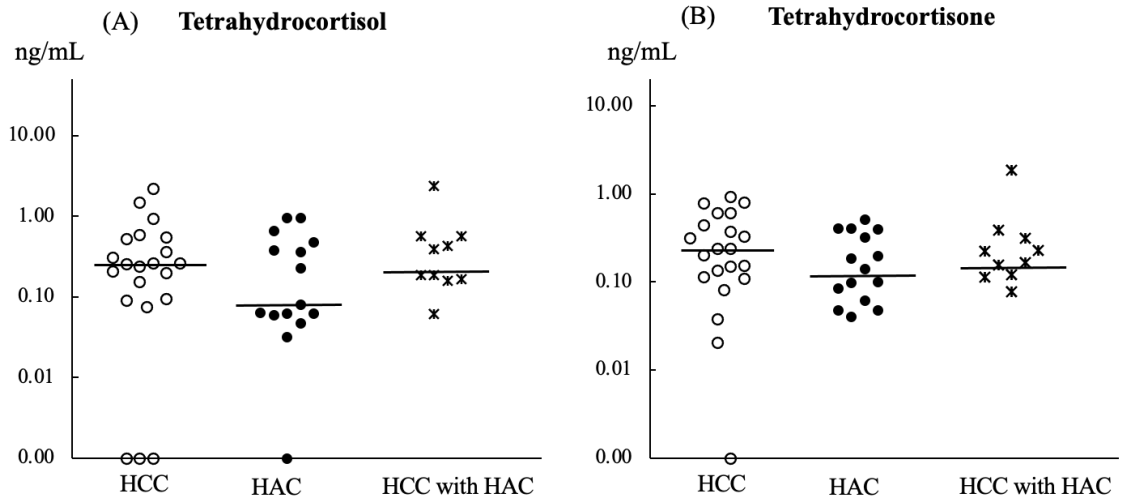


Figure 9. Comparison of 2 metabolites concentration (A and B) in baseline serum of HCC (n = 15), HAC (n = 15), and HCC with HAC dogs (n = 10). Bars; the median concentration of steroids in each group

4. Discussion

The co-occurrence of massive HCC and HAC was comparable, 32% (10/31) in this study and 23% (10/44) in previous study [8]. However, serum steroid profiling at the time of diagnosis failed to find the association between HAC and HCC.

The reliable measurement of steroid hormones is a powerful technique to investigate their hormonal status in endocrine-related diseases. By combining the elution effect and a biphenyl column, the technique provided sufficient column separation and symmetric peaks for isobaric steroids like 21-deoxycortisol, 11-deoxycortisol, and corticosterone[59]. Following the non-derivatization preparation steps, derivatization with the keto-reagent is simple, fast, and sensitive compared with other different derivatization strategies[60-62.] The MDL values were acceptable, and the present LC/MS/MS method could be used to measure multi-steroid hormones (Table 6).

Steroid profiling was unable to establish a connection between HAC and HCC since there was no steroid profile differences on PCA (Figure 7) and none of the 11 steroids were significantly differ between the groups (Figures 8 and 9). Chronic exposure to exogenous or endogenous glucocorticoid hormones is considered to cause vacuolar hepatopathy, and risk of HCC[63]. Study of the Sepesy *et al.* support our findings that is 45% of vacuolar hepatopathy was occurred without exposure to endogenous or exogenous glucocorticoids in dogs[11]. Some vacuolar hepatopathy dogs developed HCC with or without changes of non-cortisol hormones and HAC clinical signs[12]. Even in the Scottish terriers, the exact mechanism of steroidogenesis disorders in the progression of vacuolar hepatopathy to HCC has been unclear.

In human, NAFLD occurs in 20% of people with Cushing's disease and 5% of it precedes to HCC development[64]. Excess glucocorticoids stimulate gluconeogenesis in the liver and consequently cause hepatic steatosis, steatohepatitis, and fibrosis in human[63]. In the medical record of current study, only 4 dogs were presented with glycogen accumulation in the histopathological result of HCC. Based on those findings,

hepatopathy with glycogen accumulation may be occurred in HAC dogs but there has no steroid-induced hepatic inflammation and hepatic progressive changes like human HCC.

The current study quantified five androgens (testosterone, androstenedione, dehydroepiandrosterone, dihydrotestosterone and etiocholanolone), however, the concentration of those steroids in many dogs were detected lower the MDL value. That findings show that androgens are physiologically low concentration in dogs. Androgens may not be major role for HCC development even though androgens can activate the oncoprotein transcription in human HCC and neuter status might be a risk factor for some malignancies in dogs[35,65,66].

While our results do not support the idea that steroidogenesis changes may impact on the development of hepatocarcinogenesis, the coincidence of HAC in HCC is a concern. Other confounding factors like changes in expression of steroidogenic enzymes, genetic or epigenetic changes are also thought to influence the occurrence of HAC in dogs with HCC. Additionally, HCC may also play a role in the steroid imbalance as an alternative to our hypothesis. Hepatocarcinogenesis may disturb steroidogenesis or steroid metabolism in the liver. Steroids and metabolites in liver tissues and 24-hour urine samples may be useful to confirm metabolic changes inside the liver in future analysis. This study only evaluated serum steroids at single time point and was unable to determine when HCC or HAC first started in dogs. Therefore, follow-up case studies were warranted to prove the alternative theory.

To conclude our study, we developed the LC/MS/MS method for measuring multiple steroid hormones in a small amount of serum. The concentration of 19 serum steroids was not different in dogs with HCC, HAC, and both diseases. We concluded that HAC with respect to serum steroid profiling may not be associated with the development of HCC, and steroidogenesis may not contribute to the hepatocarcinogenesis. Additional measurement of steroids inside the liver and metabolites from the liver should be used to conduct more in-depth research on steroid imbalance in hepatocarcinogenesis.

5. Summary

The differences in steroid profiles between HCC, HAC, and dogs with both diseases were examined in this chapter. Serum 11 steroids were quantified using the traditional non-derivatization method, and 8 steroids using the keto-derivatization method. Eleven of the 19 steroids had a detection rate more than 50% in each study group. PCA shows no steroid profile difference among the study groups and none of the 11 steroids significantly differed between dogs with HCC, HAC or both diseases. Although we had hypothesized that steroidogenesis might contribute to the hepatocarcinogenesis, the result proved steroids may not be involved in development of hepatocellular carcinoma. Other confounding effects or factors may influence on concurrence of HAC in dogs with HCC.

General Conclusion

The goal of this study was to clarify AHS in dogs with HCC by analyzing the steroid profile. To prove our clinical motivation, the sample treatment procedure and LC/MS/MS method was validated for the multi-measurements of steroids in serum samples of dogs with or without HAC, dogs with HCC and dogs with both diseases.

In chapter 1, the clinical feasibility of steroid profile were illustrated by using the developed online column switching LC/MS/MS. The method precisely measured the cortisol as well as other steroids with simple sample preparation, hence it may be a useful tool to replace the immunoassay in clinical laboratories. Current findings showed that the concentration of 17α-OH progesterone, 11-deoxycortisol and cortisone in baseline sera and 17α-OH progesterone, cortisol, cortisone, corticosterone, 11-deoxycortisol and 11-deoxycorticosterone in post-ACTH sera were significantly high in dogs with HAC compared with dogs without HAC. In addition to the cortisol, the other adrenal steroids would be suggested to contribute clinicopathogenesis of HAC in dogs.

In chapter 2, the steroid profile differences in dogs with HCC, HAC and both diseases have been investigated. The concurrent rate of HAC in dogs with HCC were comparable between present and previous report. However, 19 serum steroids failed to demonstrate the relationship of AHS in HCC. Even though chronic exposure to exogenous or endogenous glucocorticoid hormones is thought to promote vacuolar hepatopathy and may risk to hepatocarcinogenesis, only 4 hypercortisolemic dogs had hepatic glycogen accumulation in the HCC histology of the current work. Androgen hormones play a significant role in human HCC, however androgen concentrations found in this investigation were below the method detection limit. Therefore, it became obvious that androgen hormones could not be used as a risk factor for developing HCC in dogs.

This work made it clear that steroidogenesis may not involve in the process of hepatocarcinogenesis, however, the concurrent rate of HAC and HCC is noticeable. In such case, other contributing factors are thought to affect the AHS in HCC. The present study could be able to know only the steroid changes in serum. Therefore, quantification

of steroids and metabolites inside liver tissues and 24 hours urine might be helpful to understand steroid imbalance in hepatocarcinogenesis. Additionally, current study is a type of retrospective study and could not be able to confirm HCC or HAC was resented first with steroidogenic disorder. In future, follow up or prospective measurement of steroids of those dogs are warranted to clarify AHS in dogs with HCC.

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Summary in Japanese（和文要旨）

Steroid Profiling for assessing Adrenal Hepatic Syndrome in Canine Hepatocellular Carcinoma

（ステロイドプロファイリングによる肝細胞癌発症メカニズムとしての副腎–肝臓連関の探索）

この論文では、副腎皮質ホルモンが慢性的に過剰に分泌されることにより、肝臓に病的な変化が生じる状況を副腎肝臓症候群（AHS）と呼ぶ。犬の副腎皮質機能亢進症（HAC）は、副腎皮質ホルモンの慢性的な過剰産生を伴う副腎内分泌疾患である。HAC の犬では、肝酵素活性の上昇、ステロイド肝症、肝細胞のグリコーゲン蓄積などの肝臓の臨床病理学的異常が頻繁に認められる。従って、HAC の犬では AHS と同様の現象が起こっていると考えられる。肝細胞癌（HCC）は、犬の肝腫瘍の 50-70％を占める一般的な原発性肝腫瘍である。これまで、HAC は犬の巨大 HCC の最も同時発生率の高い疾患として知られている。しかし、犬における HAC と肝発癌の正確なメカニズムはよく分かっていない。さらに、空胞性肝症（VH）とステロイドの不均衡を有するスコティッシュ・テリアは、HCC を発症するリスクが高いことも明らかにされた。したがって、コルチゾール前駆体や他のホルモンの変化は、肝臓の臨床病理学的変化に寄与していることが示唆される。

メタボリックシンドロームによる非アルコール性脂肪性肝疾患（NAFLD）は、現在、ヒトの HCC の主要な原因となっている。ステロイドによって引き起こされる犬の HAC やスコティッシュテリアの VH の肝病態は、ヒトの NAFLD による HCC に似ていると考えられる。従って、AHS におけるステロイドプロファイルを評価することは、イヌおよびヒトの肝発癌のメカニズムを理解する上で有用であると思われる。そこで、本研究では、Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) を用いて複数の血清ステロイドホルモンを測定することで、AHS が HCC に関与することを明らかにすることを目的とした。

第 1 章では、自動カラムスイッチング LC/MS/MS を用いた簡易分析法を開発し、HAC がステロイドプロファイルに与える影響について検討した。ELISA 法で測定したコルチゾール濃度は LC/MS/MS よりも高かった。2 つの方法の比較では、ELISA と LC/MS/MS の間に矛盾があることがわかった。その結果、HAC はステロイドプロファイルに影響を与え、ベースライン血清ではコルチゾン、11-デオキシコルチゾール、17α-OH-プロゲステロンが高濃度となり、ACTH 刺激後血清ではコルチゾール、コルチゾン、11-デオキシコルチゾール、コルチコステロン、11-デオキシコルチコステロン、17α-OH プロゲステロンが高

値となることが明らかになった。 コルチゾールに加えて、さらに 5 種類のステロイドが HAC の臨床病態に寄与していることが示唆された。

第 2 章では、HCC、HAC、および両疾患を有する犬における血清ステロイドプロファイルの違いについて検討した。血清中の 19 種類のステロイドは、従来の非誘導体化法およびケト誘導体化法で測定可能であった。いずれのステロイドも HCC、HAC、両疾患の犬で有意な差は認められなかった。この結果は、ステロイド生成は HCC の発生に関与していない可能性を示唆するものである。

本研究では、ステロイドプロファイルの違いにより HCC における AHS を証明することはできなかったが、HAC と HCC の同時性は明らかである。このことから、HAC と HCC の相互作用は他の交絡因子の影響を受けている可能性があることが示唆された。本研究では、血清中のステロイドの情報のみであったため、今後、肝組織や 24 時間尿中のステロイドならびに代謝物を分析することで、ステロイドの不均衡と肝発癌の関係を明らかにすることが期待される。さらに、ステロイドの不均衡と肝発癌のどちらが先行するかを明らかにするためには、これらの犬におけるステロイドの追跡測定が有用であろう。