



Simultaneous steroids measurement in dogs with hyperadrenocorticism using a column-switching liquid chromatography-tandem mass spectrometry method

Noboru SASAKI^{1)†}, Thandar OO^{1)†}, Yoshikazu YASUDA¹⁾, Takahiro ICHISE²⁾, Noriyuki NAGATA³⁾, Nozomu YOKOYAMA³⁾, Kazuyoshi SASAOKA³⁾, Keitaro MORISHITA³⁾, Shouta MM NAKAYAMA²⁾, Mayumi ISHIZUKA²⁾, Kensuke NAKAMURA¹⁾, Mitsuyoshi TAKIGUCHI^{1)*} and Yoshinori IKENAKA²⁻⁵⁾

¹⁾Laboratory of Veterinary Internal Medicine, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18-jo Nishi 9-chome, Kita-ku, Sapporo, Hokkaido 060-0818, Japan

²⁾Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18-jo Nishi 9-chome, Kita-ku, Sapporo, Hokkaido 060-0818, Japan

³⁾Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Kita 19-jo Nishi 10-chome, Kita-ku, Sapporo, Hokkaido 060-0819, Japan

⁴⁾One Health Research Center, Hokkaido University, Kita 18-jo Nishi 9-chome, Kita-ku, Sapporo, Hokkaido 060-0818, Japan

⁵⁾Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom 2520, North West, South Africa

ABSTRACT. We developed an analytical method using an on-line column-switching liquid chromatography with triple quadrupole mass spectrometry (LC/MS/MS) for quantifying multiple steroids in serum. Using the developed method, we evaluated the serum concentration of nine steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, progesterone, 17 α -OH-progesterone and aldosterone) in dogs with hyperadrenocorticism (HAC). Serum was mixed with stable isotope internal standards and thereafter purified by the automated column-switching system. The limit of detection ranged 2–16 pg/ml for nine steroids. In the baseline samples, five steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, and 17 α -OH-progesterone) were detected in all dogs. The concentrations of cortisone, 11-deoxycortisol, and 17 α -OH-progesterone in dogs with HAC (n=19) were significantly higher those in dogs without HAC (n=15, $P<0.02$). After the adrenocorticotrophic hormone stimulation test, six steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, 17 α -OH-progesterone, and deoxycorticosterone) were above the limit of quantification in all dogs. Cortisol, corticosterone, cortisone, and deoxycorticosterone concentrations of dogs with HAC were significantly higher than those of dogs without HAC ($P<0.02$). In addition, 11-deoxycortisol and 17 α -OH-progesterone concentration was higher in dogs with HAC than in dogs without HAC ($P=0.044$ and $P=0.048$, respectively). The on-line column-switching LC/MS/MS would be feasible for measuring multiple steroids in dog serum. The results suggest that cortisone, 11-deoxycortisol, and 17 α -OH-progesterone would be related to HAC. Further studies are warranted to assess the clinical feasibility of steroid profile in dogs with HAC.

KEY WORDS: column-switching, dog, hyperadrenocorticism, liquid chromatography with triple quadrupole mass spectrometry (LC/MS/MS), steroid profile

J. Vet. Med. Sci.

83(11): 1634–1642, 2021

doi: 10.1292/jvms.21-0406

Received: 19 July 2021

Accepted: 1 September 2021

Advanced Epub:

17 September 2021

Hyperadrenocorticism (HAC), also known as Cushing's syndrome, is a common endocrine disorder in dogs [21]. Hyperadrenocorticism is suspected when clinical signs such as polyuria and polyphagia are consistent with the disease [3]. Results of a complete blood count, biochemistry, blood pressure, urinary analysis, and abdominal imaging may aid to diagnose HAC.

*Correspondence to: Takiguchi, M.: mtaki@vetmed.hokudai.ac.jp

†These authors equally contributed to this study

(Supplementary material: refer to PMC <https://www.ncbi.nlm.nih.gov/pmc/journals/2350/>)

©2021 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Measurement of serum cortisol concentration in adrenal function tests such as low dose dexamethasone suppression test (LDDST) and adrenocorticotrophic hormone stimulation tests (ACTH-ST) may confirm the diagnosis. Diagnosis of HAC has remained challenging because the results of adrenal function tests are sometimes false-positive and false-negative [5]. Non-adrenal illnesses (NAIs) may affect adrenocortical function, for example [5].

Cortisol is the main adrenal glucocorticoid hormone in dogs. Several precursors and other hormones are in the biosynthesis pathway of steroids (Fig. 1). It is of clinical interest whether the measurement of other adrenocortical hormone concentrations is useful for diagnosis of HAC. Previous studies investigated the serum concentrations of several adrenocortical hormones including cortisol, aldosterone, 17 α -OH-progesterone, progesterone, and dehydroepiandrosterone-sulfate (DHEAS) in healthy dogs and dogs with HAC [9, 12, 20, 29]. It is suggested that serum 17 α -OH-progesterone concentration has potential for diagnosing HAC when serum cortisol concentration suggests equivocal adrenal function [2, 4, 6, 23]. Multiple adrenocortical hormones measurements have been commonly conducted using radioimmunoassay validated for use in dogs [10]. Radioimmunoassay, as well as other immunoassay, is convenient for the clinical use and has high detection sensitivity. Meanwhile, the major limitation of immunoassay is lack of the specificity due to cross reactivity with structurally similar compounds [16]. Liquid chromatography with triple quadrupole mass spectrometry (LC/MS/MS) has become more popular in simultaneous measurement of adrenocortical hormones and their metabolites [11, 18, 26]. Within LC/MS/MS, each hormone is characterized by the retention time on a chromatographic column, the mass to charge ratio (m/z) of each precursor ion, and specific fragment ions (product ions). Therefore, one advantage of a LC/MS/MS-based method is to measure multiple adrenocortical hormones and metabolites in a single run with high specificity. A few researches assessed steroid profiles in pregnant dogs [14], in dogs with X-linked muscular dystrophy [19], and in dogs with gallbladder mucocele [1] using LC/MS/MS techniques. Since the physiological concentrations of some adrenocortical hormones are relatively low, extraction and derivatization of targeted hormones are needed to achieve a better assay sensitivity [1, 14, 19]. The extraction and/or derivatization steps before LC/MS/MS analysis are cumbersome and time-consuming. In the past decades, extensive efforts have been conducted to develop higher throughput and simpler sample preparation methods in steroid measurements by LC/MS/MS [28]. A column-switching technique is a two-column approach for separating analytes to increase selectivity and sensitivity of assay [13]. Samples are loaded on a first column and are eluted directly onto a second column for analysis in the column-switching method. Column-switching LC/MS/MS has been utilized for the detection of drugs and metabolites in plasma and urine [17, 32, 33]. Herein we developed and validated a simple analytical method using an automated column-switching LC/MS/MS for measuring multiple steroids in serum. We measured nine steroids (cortisol, corticosterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, progesterone, 17 α -OH-progesterone and aldosterone) in dogs with HAC and dogs without HAC to assess the impact of HAC on steroid profiling.

MATERIALS AND METHODS

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, 17 α -OH-progesterone and aldosterone) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

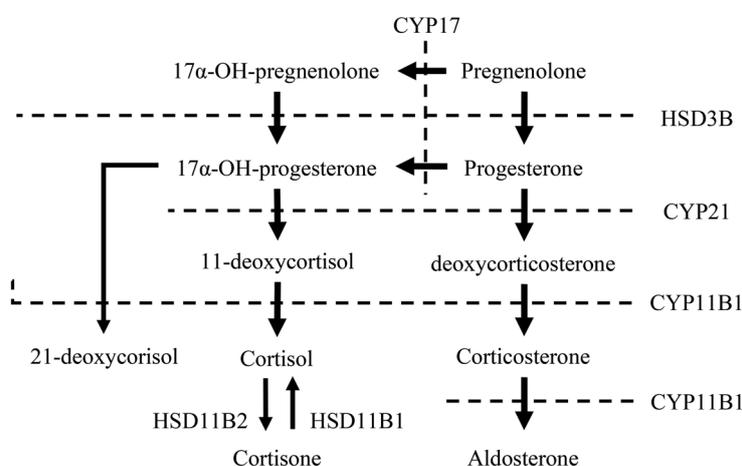


Fig. 1. Biosynthesis pathway of steroids. CYP17, 17 α -hydroxylase; HSD3B, 3 β -hydroxysteroid dehydrogenase; CYP21, 21-hydroxylase; CYP11B1, 11 β -hydroxylase; HSD11B1, 11 β -hydroxysteroid dehydrogenase type 1; HSD11B2, 11 β -hydroxysteroid dehydrogenase type 2.

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. The final concentration of each IS in the IS working solution is shown in [Supplementary Table 1](#).

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.

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) ([Fig. 2](#)). The procedures for automated extraction of nine steroids from serum involved three phases ([Supplementary Table 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 selected m/z ions for nine steroids and their stable isotope labeled ISs are listed in [Supplementary Table 3](#). 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).

Assay performance

Quality control samples were prepared from pooled serum. Serum 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

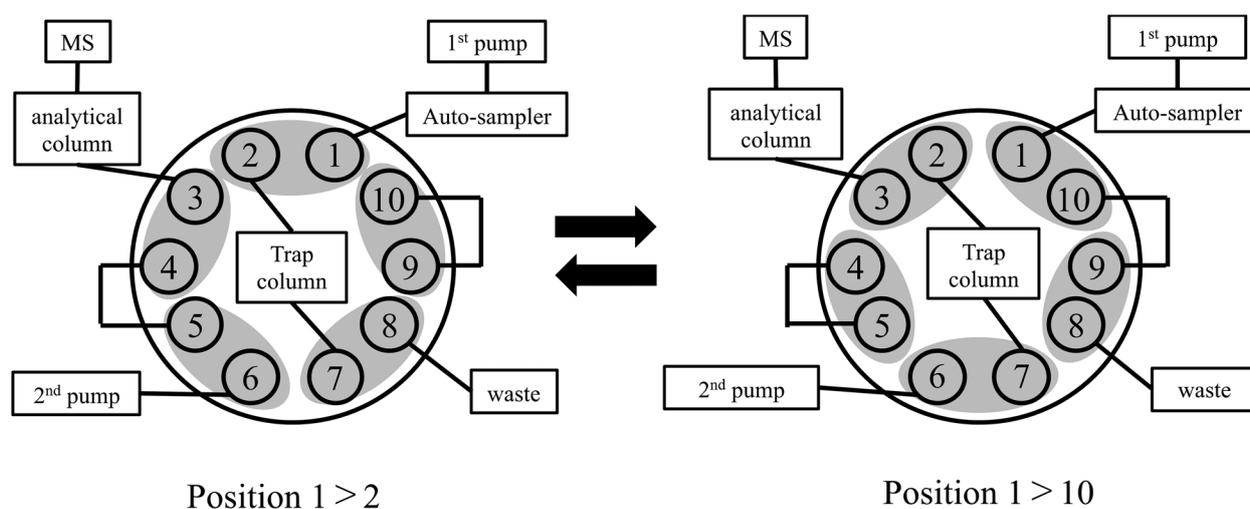


Fig. 2. Schematic illustration of the on-line column-switching system. At the position 1>2 (0–0.5 min), samples at the auto-sampler were injected onto the trap column via the port #1 and #2. At the position 1>10 (0.5–10 min), steroids which retained on the trap column were eluted by the 2nd binary pump and were transferred to the analytical column via the port #2 and #3. MS, mass spectrometer.

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 limit of detection (LOD) and LOQ were calculated using the following formula respectively:

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

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

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

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

$$\text{recovery (\%)} = (\text{peak area of IS in serum}) / (\text{average peak area of IS in calibration standards}) \times 100$$

$$\text{matrix effect (\%)} = (\text{peak area of analyte in serum spiked with STD} - \text{peak area of analyte in serum}) / (\text{peak area of STD in DDW}) \times 100$$

$$\text{corrected recovery (\%)} = \text{recovery} / \text{matrix effect} \times 100$$

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 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 population of 19 dogs with HAC consisted of 8 Miniature Dachshunds, 2 Yorkshire Terriers, 2 West Highland White Terriers, 2 Pembroke Welsh Corgis, 1 Pomeranian, 1 Toy Poodle, 1 Cocker Spaniel, and 1 Brussels Griffon. There were 9 neutered males, 10 females (3 intact, 7 spayed). The median age was 11 years (range, 8–16 years) and the median body weight was 7 kg (range, 1.98–13.7 kg).

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 were 3 Shiba Inus, 2 Pugs, 2 Chihuahuas, 1 Bulldog, 1 Miniature Dachshund, 1 Siberian Husky, 1 Toy Poodle, 1 Shih Tzu, 1 Dalmatian, and 1 mixed breed dog. Nine were males (2 intact, 7 neutered) and six were females (5 intact, 1 spayed). The median age was 12 years (range, 4–14 years) and the median body weight was 8.24 kg (range, 3.28–27 kg). There was not statistically significant difference in age or body weight between HAC dogs and dogs without HAC.

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.*, $>30 \mu\text{g/dl}$), cortisol concentrations of 60 samples were used for the comparison. Bland-Altman analysis was used to assess the agreement of two methods.

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.

RESULTS

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 1 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%).

Table 1. 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	12.0	53.2 ± 8.5	16.0
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
17 α -OH-progesterone	2	5	107.8 ± 10.9	10.1	68.9 ± 5.6	8.1
Aldosterone	9	28	110.2 ± 14.0	13.0	74.0 ± 0.8	1.1

LOD: Limit of detection, LOQ: Limit of quantification, CV: Coefficient variation.

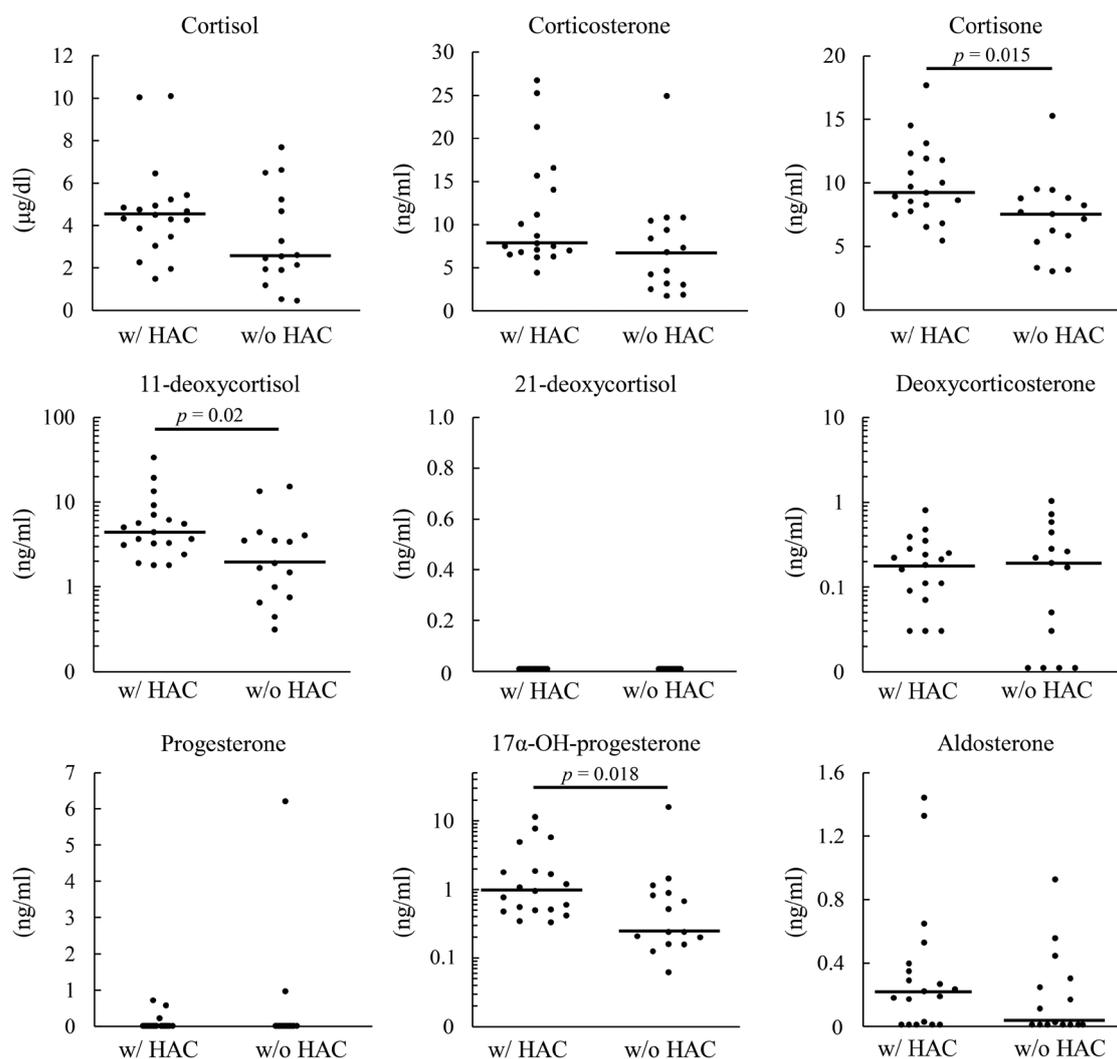


Fig. 3. Serum concentration of nine steroids in baseline sample. Cortisone, 11-deoxycortisol, and 17 α -OH-progesterone concentration in hyperadrenocorticism (HAC) dogs (n=19) was higher than in dogs without HAC (n=15). Bars, the median concentration in each group.

Application the method to clinical samples

The concentrations of cortisol, corticosterone, cortisone, 11-deoxycortisol, and 17 α -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 $\mu\text{g/dl}$ (range 1.48–10.1) and was not significantly different from that of dogs without HAC (2.54 $\mu\text{g/dl}$, range 0.46–7.68, $P=0.09$).

In addition to five steroids that were present in all dogs before the ACTH stimulation, 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 $\mu\text{g/dl}$, range 9.52–29.42), corticosterone concentration (median 30.75 $\mu\text{g/dl}$, 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 $\mu\text{g/dl}$, range 2.59–12.69, $P<0.0001$; median 18.22 $\mu\text{g/dl}$, 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 (Fig. 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.

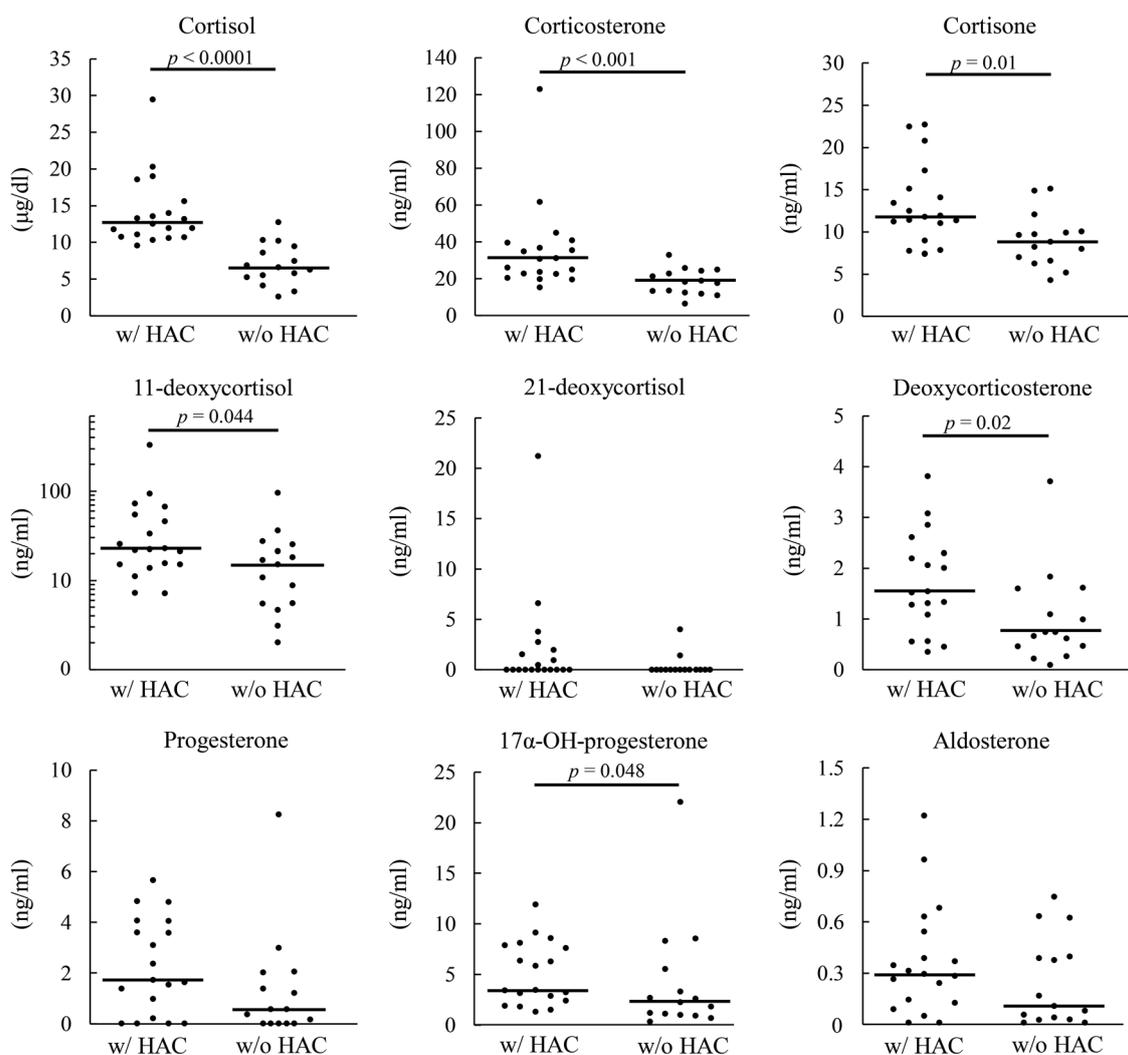


Fig. 4. Serum concentration of nine steroids after the adrenocorticotropic hormone (ACTH) stimulation. Cortisol, Corticosterone, Cortisone, 11-deoxycortisol, deoxycorticosterone, and 17α -OH-progesterone concentration in hyperadrenocorticism (HAC) dogs ($n=19$) was higher than in dogs without HAC ($n=15$). Bars, the median concentration in each group.

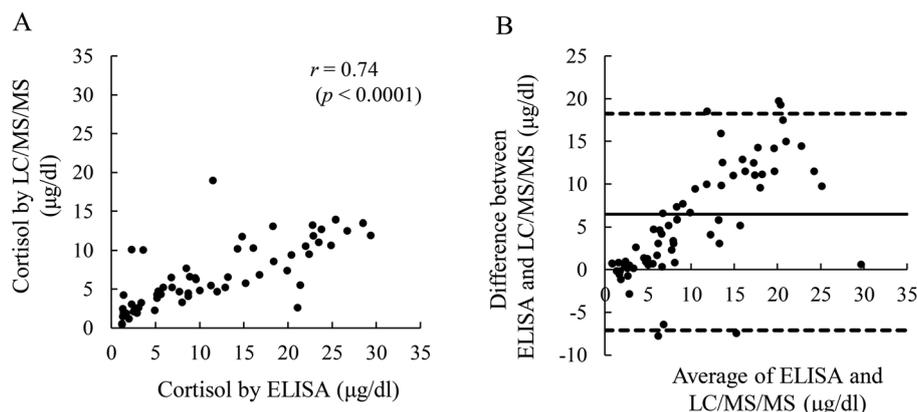


Fig. 5. Method comparison. **(A)** Correlation of the two methods ($n=60$). Significant correlation was observed between the methods. **(B)** Bland-Altman plot of cortisol ($n=60$). The solid line is the mean difference, and the dot lines are 95% limit of agreement. LC/MS/MS, liquid chromatography with triple quadrupole mass spectrometry.

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).

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 programmed solid phase extraction (SPE) 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 SPE on single column [14, 19] and derivatization [1]. In addition, good corrected recoveries (68.9–78.8%) [8, 15] 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 $110 \pm 14\%$) were comparable with those in the previous study [8]. 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.* [24] 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. 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 [27]. 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 [8]. To our knowledge, mutations in *CYP21* have not been reported in dogs [30, 34]. 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 RIA assay reported that high concentration of both 17α -OH-progesterone and 11-deoxycortisol in HAC dogs [27]. It may be possible that 17α -OH-progesterone, 11-deoxycortisol, and cortisone contribute to the clinicopathology of HAC. Serum 11-deoxycortisol concentration is a possible biomarker for human adrenocortical cancer [25, 31]. Plasma concentration of 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, corticosterone and cortisol increased in human Cushing syndrome [7], though steroid profile of human Cushing syndrome has not been fully understood [22].

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 [14].

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 [5]. 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. In addition to cortisol, three steroids (17 α -OH-progesterone, 11-deoxycortisol, and cortisone) were suggested to be related to HAC. Future studies would evaluate the diagnostic feasibility of steroid profile by the LC/MS/MS measurement.

CONFLICT OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENTS. This work was the result of using research equipment shared in MEXT Project for promoting public utilization of advanced research infrastructure (Program for supporting introduction of the new sharing system, Grant Number JPMXS0420100619). This study was supported by Grants in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (YI, 18H0413208; MT, 20H03139). This study was partly supported by Grant for Japan-related Research Project from The Sumitomo Foundation (YI). TO was supported by the World-leading Innovative and Smart Education (WISE) Program (1801) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

REFERENCES

1. Aicher, K. M., Cullen, J. M., Seiler, G. S., Lunn, K. F., Mathews, K. G. and Gookin, J. L. 2019. Investigation of adrenal and thyroid gland dysfunction in dogs with ultrasonographic diagnosis of gallbladder mucocele formation. *PLoS One* **14**: e0212638. [Medline] [CrossRef]
2. Behrend, E. N., Kempainen, R. J., Boozer, A. L., Whitley, E. M., Smith, A. N. and Busch, K. A. 2005. Serum 17-alpha-hydroxyprogesterone and corticosterone concentrations in dogs with nonadrenal neoplasia and dogs with suspected hyperadrenocorticism. *J. Am. Vet. Med. Assoc.* **227**: 1762–1767. [Medline] [CrossRef]
3. Behrend, E. N., Kooistra, H. S., Nelson, R., Reusch, C. E. and Scott-Moncrieff, J. C. 2013. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). *J. Vet. Intern. Med.* **27**: 1292–1304. [Medline] [CrossRef]
4. Benitah, N., Feldman, E. C., Kass, P. H. and Nelson, R. W. 2005. Evaluation of serum 17-hydroxyprogesterone concentration after administration of ACTH in dogs with hyperadrenocorticism. *J. Am. Vet. Med. Assoc.* **227**: 1095–1101. [Medline] [CrossRef]
5. Bennaim, M., Shiel, R. E. and Mooney, C. T. 2019. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 2: Adrenal function testing and differentiating tests. *Vet. J.* **252**: 105343. [Medline] [CrossRef]
6. Chapman, P. S., Mooney, C. T., Ede, J., Evans, H., O'Connor, J., Pfeiffer, D. U. and Neiger, R. 2003. Evaluation of the basal and post-adrenocorticotrophic hormone serum concentrations of 17-hydroxyprogesterone for the diagnosis of hyperadrenocorticism in dogs. *Vet. Rec.* **153**: 771–775. [Medline]
7. Eisenhofer, G., Masjkur, J., Peitzsch, M., Di Dalmazi, G., Bidlingmaier, M., Grüber, M., Fazel, J., Osswald, A., Beuschlein, F. and Reincke, M. 2018. Plasma steroid metabolome profiling for diagnosis and subtyping patients with Cushing syndrome. *Clin. Chem.* **64**: 586–596. [Medline] [CrossRef]
8. Fiet, J., Le Bouc, Y., Guéchet, J., Hélin, N., Maubert, M. A., Farabos, D. and Lamazière, A. 2017. A liquid chromatography/tandem mass spectrometry profile of 16 serum steroids, including 21-deoxycortisol and 21-deoxycorticosterone, for management of congenital adrenal hyperplasia. *J. Endocr. Soc.* **1**: 186–201. [Medline]
9. Frank, L. A., Schmeitzel, L. P. and Oliver, J. W. 2001. Steroidogenic response of adrenal tissues after administration of ACTH to dogs with hypercortisolemia. *J. Am. Vet. Med. Assoc.* **218**: 214–216. [Medline] [CrossRef]
10. Frank, L. A., Rohrbach, B. W., Bailey, E. M., West, J. R. and Oliver, J. W. 2003. Steroid hormone concentration profiles in healthy intact and neutered dogs before and after cosyntropin administration. *Domest. Anim. Endocrinol.* **24**: 43–57. [Medline] [CrossRef]
11. Gaudl, A., Kratzsch, J. and Ceglarek, U. 2019. Advancement in steroid hormone analysis by LC-MS/MS in clinical routine diagnostics - A three year recap from serum cortisol to dried blood 17 α -hydroxyprogesterone. *J. Steroid Biochem. Mol. Biol.* **192**: 105389. [Medline] [CrossRef]
12. Hill, K. E., Scott-Moncrieff, J. C. R., Koshko, M. A., Glickman, L. T., Glickman, N. W., Nelson, R. W., Blevins, W. E. and Oliver, J. W. 2005. Secretion of sex hormones in dogs with adrenal dysfunction. *J. Am. Vet. Med. Assoc.* **226**: 556–561. [Medline] [CrossRef]
13. Hilton, J., Yokoi, F., Dannals, R. F., Ravert, H. T., Szabo, Z. and Wong, D. F. 2000. Column-switching HPLC for the analysis of plasma in PET imaging studies. *Nucl. Med. Biol.* **27**: 627–630. [Medline] [CrossRef]
14. Holst, B. S., Kushnir, M. M. and Bergquist, J. 2015. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for analysis of endogenous steroids in the luteal phase and early pregnancy in dogs: a pilot study. *Vet. Clin. Pathol.* **44**: 552–558. [Medline] [CrossRef]
15. 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. [Medline] [CrossRef]

16. Krasowski, M. D., Drees, D., Morris, C. S., Maakestad, J., Blau, J. L. and Ekins, S. 2014. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. *BMC Clin. Pathol.* **14**: 33. [[Medline](#)] [[CrossRef](#)]
17. Kushnir, M. M., Rockwood, A. L., Bergquist, J., Varshavsky, M., Roberts, W. L., Yue, B., Bunker, A. M. and Meikle, A. W. 2008. High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. *Am. J. Clin. Pathol.* **129**: 530–539. [[Medline](#)] [[CrossRef](#)]
18. Kushnir, M. M., Rockwood, A. L., Roberts, W. L., Yue, B., Bergquist, J. and Meikle, A. W. 2011. Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clin. Biochem.* **44**: 77–88. [[Medline](#)] [[CrossRef](#)]
19. Martins-Júnior, H. A., Simas, R. C., Brolio, M. P., Ferreira, C. R., Perecin, F., Nogueira, G. P., Miglino, M. A., Martins, D. S., Eberlin, M. N. and Ambrósio, C. E. 2015. Profiles of steroid hormones in canine X-linked muscular dystrophy via stable isotope dilution LC-MS/MS. *PLoS One* **10**: e0126585 [[CrossRef](#)]. [[Medline](#)]
20. Monroe, W. E., Panciera, D. L. and Zimmerman, K. L. 2012. Concentrations of noncortisol adrenal steroids in response to ACTH in dogs with adrenal-dependent hyperadrenocorticism, pituitary-dependent hyperadrenocorticism, and nonadrenal illness. *J. Vet. Intern. Med.* **26**: 945–952. [[Medline](#)] [[CrossRef](#)]
21. Perez-Alenza, D. and Melian, C. 2017. Hyperadrenocorticism in dogs. pp 4345–4389. In: Textbook of Veterinary Internal Medicine: Diseases of dog and cat, 8th ed. (Ettinger, S. J., Feldman, E. C. and Cote, E. eds.), Elsevier, St. Louis.
22. Rege, J., Turcu, A. F., Else, T., Auchus, R. J. and Rainey, W. E. 2019. Steroid biomarkers in human adrenal disease. *J. Steroid Biochem. Mol. Biol.* **190**: 273–280. [[Medline](#)] [[CrossRef](#)]
23. Ristic, J. M., Ramsey, I. K., Heath, E. M., Evans, H. J. and Herrtage, M. E. 2002. The use of 17-hydroxyprogesterone in the diagnosis of canine hyperadrenocorticism. *J. Vet. Intern. Med.* **16**: 433–439. [[Medline](#)] [[CrossRef](#)]
24. Sanders, K., Mol, J. A., Kooistra, H. S., Slob, A. and Galac, S. 2016. New insights in the functional zonation of canine adrenal cortex. *J. Vet. Intern. Med.* **30**: 741–750. [[Medline](#)] [[CrossRef](#)]
25. Schweitzer, S., Kunz, M., Kurlbaum, M., Vey, J., Kendl, S., Deutschbein, T., Hahner, S., Fassnacht, M., Dandekar, T. and Kroiss, M. 2019. Plasma steroid metabolome profiling for the diagnosis of adrenocortical carcinoma. *Eur. J. Endocrinol.* **180**: 117–125. [[Medline](#)] [[CrossRef](#)]
26. Sciffer, L., Barnard, L., Baranowski, E. S., Gilligan, L. C., Taylor, A. E., Arlt, W., Shackleton, C. H. L. and Storbek, K. H. 2019. Human steroid biosynthesis, metabolism and excretion are differently reflected by serum and urine steroid metabolomes: A comprehensive review. *J. Steroid Biochem. Mol. Biol.* **194**: 1–25.
27. Sieber-Ruckstuhl, N. S., Boretti, F. S., Wenger, M., Maser-Gluth, C. and Reusch, C. E. 2008. Evaluation of cortisol precursors for the diagnosis of pituitary-dependent hypercortisolism in dogs. *Vet. Rec.* **162**: 673–678. [[Medline](#)] [[CrossRef](#)]
28. Stanczyk, F. Z. and Clarke, N. J. 2010. Advantages and challenges of mass spectrometry assays for steroid hormones. *J. Steroid Biochem. Mol. Biol.* **121**: 491–495. [[Medline](#)] [[CrossRef](#)]
29. Syme, H. M., Scott-Moncrieff, J. C., Treadwell, N. G., Thompson, M. F., Snyder, P. W., White, M. R. and Oliver, J. W. 2001. Hyperadrenocorticism associated with excessive sex hormone production by an adrenocortical tumor in two dogs. *J. Am. Vet. Med. Assoc.* **219**: 1725–1728, 1707–1708. [[Medline](#)] [[CrossRef](#)]
30. Takada, K., Kitamura, H., Takiguchi, M., Saito, M. and Hashimoto, A. 2002. Cloning of canine 21-hydroxylase gene and its polymorphic analysis as a candidate gene for congenital adrenal hyperplasia-like syndrome in Pomeranians. *Res. Vet. Sci.* **73**: 159–163. [[Medline](#)] [[CrossRef](#)]
31. Taylor, D. R., Ghataore, L., Couchman, L., Vincent, R. P., Whitelaw, B., Lewis, D., Diaz-Cano, S., Galata, G., Schulte, K. M., Aylwin, S. and Taylor, N. F. 2017. A 13-steroid serum panel based on LC-MS/MS: Use in detection of adrenocortical carcinoma. *Clin. Chem.* **63**: 1836–1846. [[Medline](#)] [[CrossRef](#)]
32. Wang, Y., Lehmann, R., Lu, X., Zhao, X. and Xu, G. 2008. Novel, fully automatic hydrophilic interaction/reversed-phase column-switching high-performance liquid chromatographic system for the complementary analysis of polar and apolar compounds in complex samples. *J. Chromatogr. A* **1204**: 28–34. [[Medline](#)] [[CrossRef](#)]
33. Ye, X., Tao, L. J., Needham, L. L. and Calafat, A. M. 2008. Automated on-line column-switching HPLC-MS/MS method for measuring environmental phenols and parabens in serum. *Talanta* **76**: 865–871. [[Medline](#)] [[CrossRef](#)]
34. Zimmerman, K. L., Panciera, D. L., Hoeschele, I., Monroe, W. E., Todd, S. M., Werre, S. R., LeRoith, T., Fecteau, K. and Lake, B. B. 2018. Adrenocortical challenge response and genomic analyses in Scottish Terriers with increased alkaline phosphatase activity. *Front. Vet. Sci.* **5**: 231. [[Medline](#)] [[CrossRef](#)]