The Veterinary Journal 306 (2024) 106151

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Urinary steroid profiling using liquid chromatography-tandem mass spectrometry for the diagnosis of canine Cushing's syndrome

N. Nagata [a,b,1], H. Sawamura [b,1], Y. Ikenaka [c,d,e,f], K. Morishita [b], K. Hosoya [b], N. Sasaki [b], K. Nakamura [g], M. Takiguchi [g,*]

[a] Joint Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan
[c] Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan
[d] Water Research Group, Unit for Environmental Sciences and Management, North-West University, 11 Hoffman Street, Potchefstroom 2531, South Africa
[e] One Health Research Center, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan
[f] Translational Research Unit, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan
[g] Laboratory of Veterinary Internal Medicine, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan

ARTICLE INFO

Keywords:
Canine Cushing's syndrome
Diagnosis
Hyperadrenocorticism
LC/MS/MS
Urinary steroids

ABSTRACT

Serum cortisol measurements by chemiluminescence enzyme immunoassay (CLEIA) are widely used to diagnose hypercortisolism (HC) or Cushing's syndrome in dogs. However, they are associated with problems such as the need for multiple blood collections under stressful conditions or cross-reactivity between hormones. Therefore, a less invasive and more accurate diagnostic method is required. This study aimed to develop a urinary steroid profile analysis method using liquid chromatography-tandem mass spectrometry (LC/MS/MS) and to evaluate its clinical usefulness. Sixty-five healthy dogs and 38 dogs with suspected HC were included in the study. Using LC/MS/MS, the levels of 11 steroid hormones in the urine were determined. We established the upper limit of the reference interval for each urinary steroid-to-creatinine ratio and evaluated their diagnostic performances. The levels of the five steroid hormones were significantly higher in the 14 dogs with HC than in the 24 dogs with mimicking HC and 65 healthy dogs. The urinary corticosterone-to-creatinine ratio showed the highest diagnostic accuracy (area under the curve, 0.96). A significant correlation was seen between urinary cortisol concentrations measured by LC/MS/MS and CLEIA ($rs = 0.88$, $P < 0.001$), although the CLEIA measurements were significantly higher than the LC/MS/MS measurements ($P < 0.001$). LC/MS/MS-based urinary steroid profiles are a promising tool for diagnosing canine HC.

1. Introduction

Naturally occurring canine hypercortisolism (HC), or Cushing's syndrome, is an endocrine disorder characterized by excessive cortisol secretion caused by pituitary tumors and, less commonly, by adrenocortical tumors (Kooistra and Galac, 2012). This disease is characterized by various clinical signs, including polyuria/polydipsia, polyphagia, skin manifestations, and abdominal enlargement, with a wide range of severities (Behrend et al., 2013), making diagnosis challenging. Therefore, the diagnosis of canine HC is based on clinical signs and clinicopathological findings consistent with the syndrome, as well as imaging and endocrinological tests (Kooistra and Galac, 2012; Behrend et al., 2013; Bennaim et al., 2019). The commonly used endocrine tests to diagnose canine HC include the ACTH stimulation test, low-dose dexamethasone suppression test (LDDST), and urinary corticoid-to-creatinine ratio (UCCR).

The UCCR provides the concentration of urinary corticoids, predominantly cortisol, indexed with the creatinine levels in the urine (Kooistra and Galac, 2012; Behrend et al., 2013). Compared to the ACTH stimulation test and LDDST, which require multiple blood collections and drug injections in a veterinary hospital, UCCR can evaluate adrenal cortex function noninvasively and simply using urine collected at home,

* Corresponding author.
E-mail address: mtaki@vetmed.hokudai.ac.jp (M. Takiguchi).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.tvjl.2024.106151
Received 7 February 2024; Received in revised form 26 May 2024; Accepted 27 May 2024
Available online 29 May 2024

Table 1
Detection rates of urinary steroid hormones in healthy controls and clinical cases.

Steroid	HC ($n = 14$)	Mimicking HC ($n = 24$)	Healthy controls ($n = 65$)
Cortisol	100 %	100 %	94 %
Corticosterone	100 %	100 %	58 %
Cortisone	100 %	100 %	100 %
Progesterone	64 %	50 %	89 %
17-hydroxyprogesterone	79 %	67 %	40 %
11-deoxycortisol	93 %	96 %	78 %
11-deoxycorticosterone	64 %	50 %	51 %
21-deoxycortisol	0 %	0 %	0 %
Aldosterone	36 %	50 %	14 %
Androstenedione	79 %	67 %	77 %
Testosterone	0 %	13 %	0 %

HC, Hypercortisolism.

which is a stress-free environment for dogs. However, as with other endocrinological tests, the diagnostic accuracy of UCCR is not 100 % (Behrend et al., 2013). The reported sensitivities and specificities of UCCR in two recent studies using urine collected at home ranged from 91.7 %–92.4 % and 81.9 %–100 %, respectively (Zeugswetter et al., 2010; Nagata et al., 2022).

In veterinary medicine, immunological assays are typically used to measure serum or urinary cortisol levels (Behrend et al., 2013). In general, cross-reactivity among steroid hormones results in inaccurate values in immunological assays (Galeandro et al., 2014; Holst et al., 2015; Sasaki et al., 2021b). In addition, because specific antibodies are used for each hormone, the simultaneous measurement of multiple hormones is not possible. In contrast, liquid chromatography-tandem mass spectrometry (LC/MS/MS) can simultaneously measure multiple steroid hormones without cross-reactivity-related errors. Therefore, the LC/MS/MS method for urinary steroid hormone calculations may provide better diagnostic performance.

In this study, we developed and validated an analytical method for measuring 11 steroid hormones in urine using LC/MS/MS. We also established the upper limit of reference intervals for each urinary steroid hormone-to-creatinine ratio in healthy dogs and evaluated their diagnostic performance for canine HC.

2. Materials and methods

2.1. Animals

This study was approved by the Ethics Committee of the Hokkaido University Veterinary Teaching Hospital (Approval number, 2021–06; Approval date, 26 July 2021).

Sixty-five dogs that visited a small animal primary care facility between February 2021 and February 2022 for health checks were included in this study. These dogs had no apparent disease and were considered healthy based on history, physical examination, blood tests, and urinalysis. The urine for routine urinalysis was collected at home by the owners, and residual urine samples were used for this study. Informed consent was obtained from dog owners to collect residual urine samples. Urine was centrifuged at 1500 rpm for 5 min, and the supernatant was stored at −20 °C until analysis.

Of the dogs that presented to our hospital between November 2020 and November 2022, 38 with suspected HC based on clinical signs, blood and biochemical findings, and ultrasonographic findings were included in this study. In all the cases, the urine for routine urinalysis was collected at home by the owners, and residual urine samples were used for this study. Dogs treated with glucocorticoids at initial presentation were excluded. Informed consent was obtained from dog owners to collect residual urine samples. Urine was centrifuged at 1500 rpm for 5 min at 4 °C, and the supernatant was stored at −80 °C until analysis. The criteria for HC diagnosis were: (1) at least one common clinical sign

Table 2
The upper limit of reference intervals for urinary steroid hormone-to-creatinine ratios in 65 healthy dogs.

Steroid	Initial number of dogs	Number of removed outliers	*P*-value for symmetry test (untransformed)	*P*-value for symmetry test (Box-Cox transformed)	Upper limit of reference interval (90 % confidence interval)	Methods
Cortisol	65	0	0.084	NA	2.46 (2.19–2.69)	Robust (untransformed)
Corticosterone	65	0	0	0	0.134 (0.087–0.143)	Nonparametric
Cortisone	65	0	0.191	NA	3.75 (3.32–4.21)	Robust (untransformed)
Progesterone	65	1	0.065	NA	0.0162 (0.0139–0.0184)	Robust (untransformed)
17-hydroxyprogesterone	65	0	0	0	0.0153 (0.0087–0.0168)	Nonparametric
11-deoxycortisol	65	3	0.001	0.10	0.0451 (0.0380–0.0542)	Robust (Box-Cox transformed)
11-deoxycorticosterone	65	0	0	0	0.0380 (0.0253–0.0466)	Nonparametric
21-deoxycortisol	65	NA	NA	NA	NA	NA
Aldosterone	65	9	NA	NA	NA	NA
Androstenedione	65	1	0.065	NA	0.0178 (0.0160–0.0200)	Robust (untransformed)
Testosterone	65	NA	NA	NA	NA	NA

NA, Not applicable.

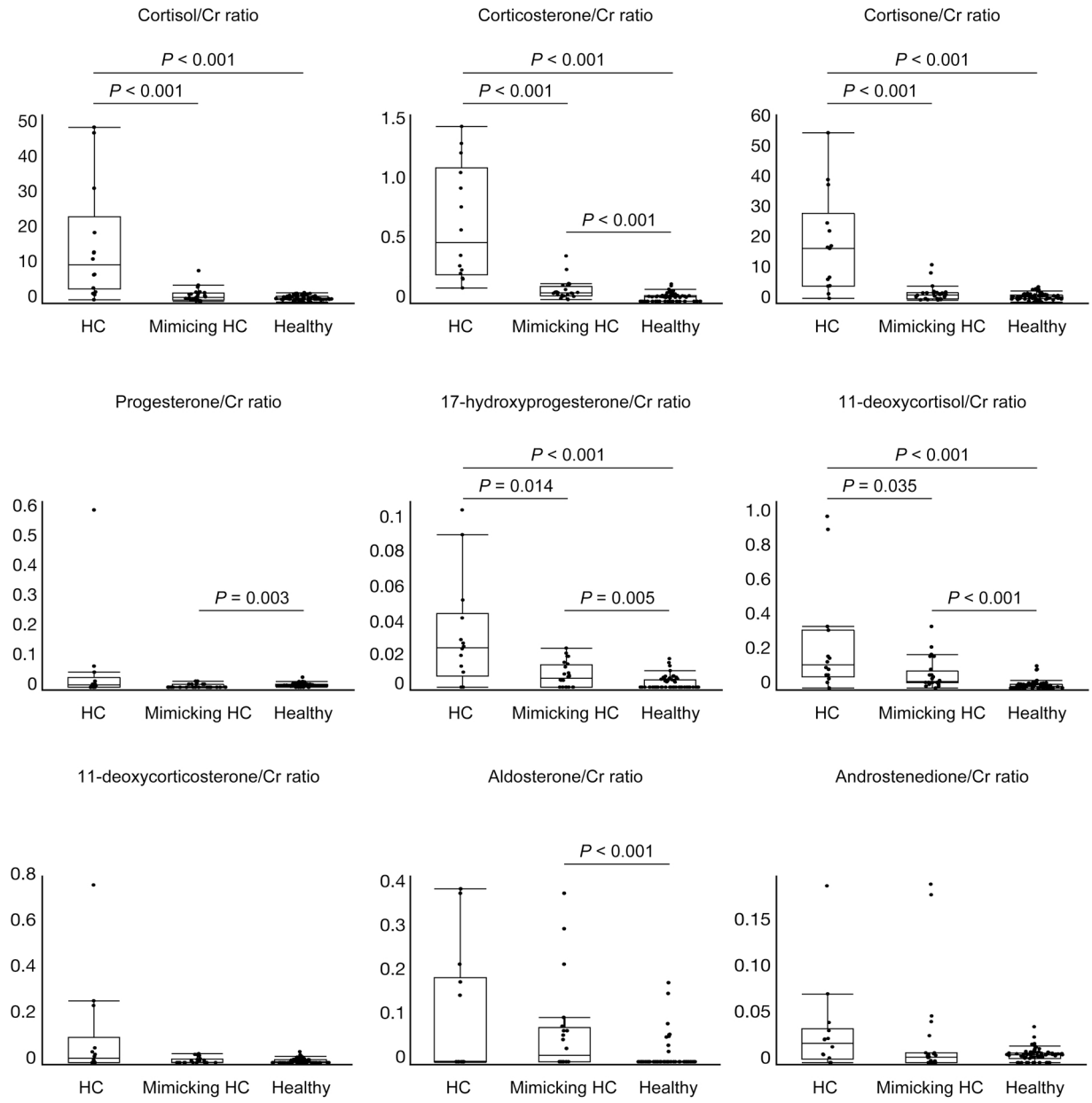

Fig. 1. Urinary steroid hormone-to-creatinine ratios. Box and whisker plots show nine steroid hormone-to-creatinine (Cr) ratios in dogs with hypercortisolism (HC) ($n = 14$), mimicking HC ($n = 24$), and healthy controls ($n = 65$).

of canine HC, such as polydipsia, polyuria, polyphagia, panting, abdominal distension, alopecia, and thin skin; (2) at least one clinicopathological abnormality consistent with canine HC, which included increased alkaline phosphatase (ALP), increased alanine aminotransferase (ALT), and decreased urine specific gravity (<1.020); (3) results of standard endocrine tests such as LDDST, ACTH stimulation test, and UCCR, and (4) confirmed response to trilostane treatment. Dogs were classified as mimicking HC group when the criteria above were not met, and definitive diagnoses other than HC were confirmed. Serum cortisol concentration was determined using an enzyme-linked immunosorbent assay (SNAP Cortisol Test Kit, IDEXX Laboratories) or a chemiluminescence enzyme immunoassay (CLEIA) (IMMULITE 1000 or 2000, Siemens Healthineers), and urinary cortisol concentration was determined using CLEIA.

2.2. Urinary steroid hormone and creatinine analysis using LC/MS/MS

Standard solutions (STDs) and stable isotope-labeled internal standard solutions (ISs) of 11 steroids, including cortisol, corticosterone, cortisone, progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, 11-deoxycorticosterone, 21-deoxycortisol, aldosterone, androstenedione, and testosterone were used for the analysis (see Appendix A: Supplementary Table 1).

Urine (100 μL) was mixed with 10 μL of IS working solution and 500 μL of acetonitrile, and 500 μL of acetonitrile-saturated sodium acetate buffer was added and mixed. The mixture was centrifuged at 12000 × g for 10 min at 5 °C. The supernatant (400 μL) was applied to a bilayer column (AL-N/VRA-PR, GL Science) conditioned with acetonitrile. After the solid phase extraction, 800 μL of the solution was concentrated and dried using a centrifugal evaporator. Then, 50 % methanol containing 0.1 % formic acid (100 μL) was added to the dried sample, and the resulting solution was used for LC/MS/MS.

Urinary steroid hormones were analyzed using a 6495B Triple Quad LC/MS (Agilent Technologies) with an HPLC system of a 1290 Infinity II LC system (Agilent Technologies). A Kinetex® Biphenyl (φ 2.6 μm, 3.0 × 100 mm, Phenomenex) analytical column was used. Electrospray

Table 3
Diagnostic accuracy of urinary steroid hormone-to-creatinine ratios for differentiating between HC and mimicking HC.

Steroid	AUC	Cutoff (derived from Youden index)	Sensitivity	Specificity	Cutoff (derived from healthy controls)	Sensitivity	Specificity
Cortisol	0.88	2.78	0.79	0.88	2.46	0.79	0.75
	(95 % CI: 0.77–1)		(95 % CI: 0.56–1)	(95 % CI: 0.75–1)		(95 % CI: 0.53–1)	(95 % CI: 0.57–0.91)
Corticosterone	0.96	0.183	0.93	0.92	0.134	0.93	0.79
	(95 % CI: 0.90–1)		(95 % CI: 0.77–1)	(95 % CI: 0.79–1)		(95 % CI: 0.76–1)	(95 % CI: 0.62–0.95)
Cortisone	0.90	5.14	0.86	0.88	3.75	0.86	0.88
	(95 % CI: 0.78–1)		(95 % CI: 0.64–1)	(95 % CI: 0.74–1)		(95 % CI: 0.62–1)	(95 % CI: 0.73–1)
17-hydroxyprogesterone	0.77	0.0187	0.64	0.92	0.0153	0.64	0.83
	(95 % CI: 0.59–0.96)		(95 % CI: 0.40–0.88)	(95 % CI: 0.77–1)		(95 % CI: 0.38–0.91)	(95 % CI: 0.65–0.96)
11-deoxycortisol	0.75	0.0664	0.79	0.71	0.0451	0.86	0.54
	(95 % CI: 0.57–0.92)		(95 % CI: 0.54–1)	(95 % CI: 0.50–0.89)		(95 % CI: 0.60–1)	(95 % CI: 0.35–0.74)

AUC, Area under the curve; CI, Confidence interval; HC, Hypercortisolism

ionization was used as the ionization method. The mobile phase, flow rate, temperature, mobile phase gradient, and MS conditions are shown (see Appendix A: Supplementary Table 2). Each clinical sample was analyzed individually and not duplicated.

The analyte calibration curves were obtained by measuring STD working solutions (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 ng/mL mixed with the IS working solution (final concentration of 1.0 ng/mL). The STD/IS peak area ratios versus STD/IS concentration ratios were used for the calibration curves.

The limit of detection (LOD), instrumental detection limit (IDL), method detection limit (MDL), practical quantitation limit (PQL), absolute recovery, matrix effect, and the corrected recovery for each steroid hormone were calculated using distilled water containing IS or pooled free-catch urine samples obtained from a volunteer intact female dog (see Appendix A: Supplementary Data).

To ensure adequate precision and accuracy of the analysis, one quality control (QC) sample was added for every 10 clinical samples, and each was analyzed accordingly. In each analysis, it was confirmed that the inter-day error of the QC samples was within 10 %.

The detection rate was calculated as the proportion of dogs with non-zero measurements for each steroid hormone in the HC group, mimicking HC group, and healthy control group, respectively.

The urinary creatinine concentration was measured as previously described (Sasaki et al., 2021a). The ratio of urinary steroid hormones (nmol/l) to creatinine (mmol/l) was calculated.

2.3. Statistical methods

JMP Pro version 17.0 (SAS Institute) was used for statistical analysis. The groups were compared for clinical characteristics and urinary steroid hormone-to-creatinine ratios using the Kruskal–Wallis and Steel–Dwass tests. Spearman's rank correlation test was used for correlation analysis between urinary cortisol concentrations measured by LC/MS/MS and those measured by CLEIA. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was used to evaluate the urinary steroid hormone-to-creatinine ratios to diagnose HC. The optimal cut-off value was determined using the Youden index. The 95 % confidence intervals for the ROC analyses were determined using RStudio (version 2024.04.1+748). The reference interval for the urinary steroid hormone-to-creatinine ratio was determined by using a robust method with a software program (Reference Value Advisor V2.1, downloaded from http://www.biostat.envt.fr/reference-value-advisor/) (Geffré et al., 2011; Friedrichs et al., 2012). After removing the outliers, robust methods using untransformed data or Box-Cox transformed data were performed to establish the upper limit of reference intervals. Nonparametric methods were used when both untransformed and Box-Cox transformed distributions were not Gaussian. When steroid hormones were not detected, the value was considered as 0. Statistical significance was set at 0.05.

3. Results

The median age and body weight of 65 healthy dogs was 2.6 years (range, 5 months–14.7 years) and 5.5 kg (range, 2.1–27.0 kg). Thirty-eight dogs were female (37 spayed), and 27 were male (25 neutered). Twenty-one breeds were included in the study: mixed breeds ($n = 11$), Chihuahua ($n = 9$), Toy poodle ($n = 9$), Miniature dachshund ($n = 5$), Bichon frise ($n = 4$), Miniature schnauzer ($n = 4$), Shiba ($n = 4$), Yorkshire terrier ($n = 3$), Cavalier King Charles spaniel ($n = 2$), Labrador retriever ($n = 2$), Papillon ($n = 2$), Beagle ($n = 1$), Bernese mountain dog ($n = 1$), Boston terrier ($n = 1$), Golden retriever ($n = 1$), Jack Russel terrier ($n = 1$), Maltese ($n = 1$), Pomeranian ($n = 1$), Shih Zhu ($n = 1$), Welsh corgi ($n = 1$), and West Highland white terrier ($n = 1$).

The 14 dogs with HC and 24 dogs mimicking HC showed no significant differences in their median age [12.8 years (range, 9.9–15.1 years) vs. 11.2 years (range, 2.3–15.8 years); $P = 0.173$] and median body

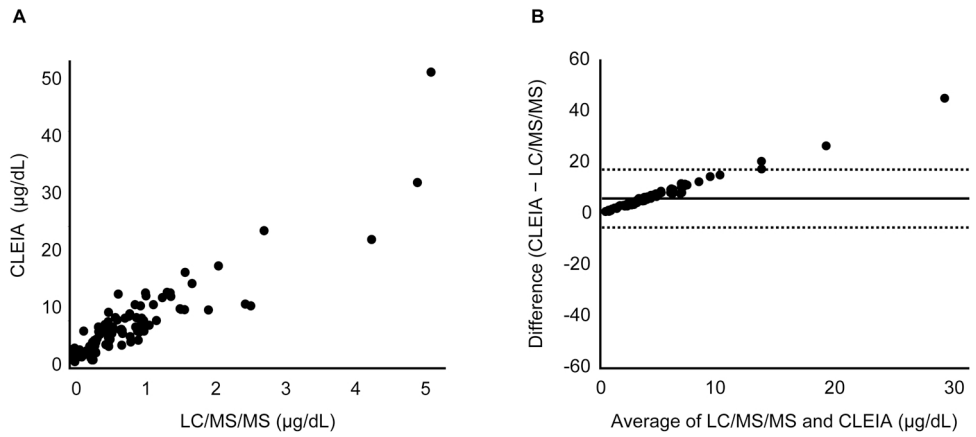

Fig. 2. Comparison of urinary cortisol concentrations measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS) and chemiluminescence enzyme immunoassay (CLEIA). (A) A significant correlation is seen between LC/MS/MS and CLEIA ($n = 99$, $rs = 0.88$, $P < 0.001$). (B) Bland–Altman plots show significantly higher urinary cortisol concentration measured by CLEIA than by LC/MS/MS ($n = 99$, mean difference 6.34 μg/dL, 95 % confidence interval: 5.19–7.49 μg/dL, $P < 0.001$). The solid line shows the mean difference. Dashed lines show the 95 % limit of agreement.

weights [6.8 kg (3.7–32.7 kg) vs. 4.6 kg (1.7–16.7 kg); $P = 0.099$]. Twenty-two dogs were female (20 spayed), and 16 were male (13 neutered). Fourteen breeds were included: Toy poodle ($n = 7$), Chihuahua ($n = 6$), mixed breeds ($n = 6$), Miniature dachshunds ($n = 4$), Pomeranian ($n = 3$), French bulldog ($n = 2$), Shiba ($n = 2$), Shih Zhu ($n = 2$), Bichon frise ($n = 1$), Cavalier King Charles spaniel ($n = 1$), English springer spaniel ($n = 1$), Pug ($n = 1$), Welsh corgi ($n = 1$), and Yorkshire terrier ($n = 1$).

The 14 dogs with HC included 11 with pituitary-dependent HC and 3 with adrenocortical tumors. The final diagnoses of mimicking HC included nonfunctional adrenal masses with or without chronic kidney disease ($n = 8$), pheochromocytoma ($n = 5$), chronic kidney disease ($n = 3$), primary polydipsia ($n = 2$), alopecia X ($n = 1$), dietary hyperlipidemia ($n = 1$), hypothyroidism ($n = 1$), obesity ($n = 1$), sudden acquired retinal degeneration syndrome ($n = 1$), and urethral sphincter mechanism incompetence ($n = 1$).

The LOD, IDL, and MDL for cortisol were 5 pg/mL, 53 fg, and 210 pg/mL, respectively. Details of the LOD, IDL, MDL, PQL, absolute recovery, matrix effect, and corrected recovery for each hormone are provided in Appendix A: Supplementary Table 3.

The detection rates for each urinary steroid hormone in healthy controls and clinical cases are shown in Table 1. The upper limit of the reference interval for each urinary steroid hormone-to-creatinine ratio derived from 65 healthy controls is shown in Table 2.

Fig. 1 compares the urinary steroid hormone-to-creatinine ratios among the three groups. The levels of five steroid hormones (cortisol, corticosterone, cortisone, 17-hydroxyprogesterone, and 11-deoxycortisol) were significantly higher in the HC group than in the mimicking HC and healthy groups. The diagnostic accuracy of these five steroid hormones in differentiating HC from mimicking HC is shown in Table 3. The urinary corticosterone-to-creatinine ratio had the highest diagnostic accuracy, with an AUC of 0.96.

The correlation between urinary cortisol concentrations measured using LC/MS/MS and CLEIA in the 99 dogs is shown in Fig. 2A. There was a significant correlation between the LC/MS/MS and CLEIA values ($rs = 0.88$, $P < 0.001$). Urinary cortisol measured by CLEIA was significantly higher than that measured by LC/MS/MS (mean difference 6.34 μg/dL, 95 % confidence interval: 5.19–7.49 μg/dL, $P < 0.001$) (Fig. 2B). As the urinary cortisol concentration increased, the difference increased.

4. Discussion

In this study, the median urinary cortisol-to-creatinine ratio in the 65 dogs measured by LC/MS/MS was 1.07 nmol/mmol (range, 0–2.58 nmol/mmol), which is comparable to 1.0 nmol/mmol (range, 0.3–2.6 nmol/mmol) reported in a previous study of 20 healthy dogs measured by Gas chromatography-mass spectrometry (Galeandro et al. 2014). The age of the healthy dogs in our study (median, 2.6 years) and the previous study (median, 5.4 years) were relatively young. Because basal blood cortisol levels significantly increase with increasing age (Reul et al. 1991), further studies to establish age-related reference intervals for urinary cortisol-to-creatinine ratio are needed. We found a significant correlation between measurements using LC/MS/MS and CLEIA. However, the urinary cortisol concentrations measured by CLEIA were higher than those measured by LC/MS/MS in all dogs, suggesting false-increased measurements with CLEIA due to cross-reactivity. Further, this difference between LC/MS/MS and CLEIA measurements increased as the urinary cortisol concentration increased. Measuring cortisol using immunological assays, such as CLEIA, generally shows cross-reactivity among steroid hormones (Galeandro et al., 2014; Holst et al., 2015; Sasaki et al., 2021b), resulting in false-increased measurements.

Comparison of urinary steroid hormone-to-creatinine ratios in 14 dogs with HC and 24 dogs mimicking HC showed significant differences in five steroid hormones: cortisol, corticosterone, cortisone, 17-hydroxyprogesterone, and 11-deoxycortisol. The AUC for urinary cortisol-to-creatinine ratio was 0.88, which was not superior to those previously reported using CLEIA or ELISA (AUC: 0.97, 0.94, and 0.93) (Jensen et al., 1997; Zeugswetter et al., 2010; Nagata et al., 2022). The sensitivity of urinary cortisol-to-creatinine ratio in this study was relatively low (0.79) when the cut-off value derived from healthy controls (2.46 nmol/mmol) was used. However, the specificity (0.75) in this study was higher than what has been previously reported using CLEIA (0.65) using a similar cut-off value (Zeugswetter et al., 2010). This high specificity can be explained by the use of LC/MS/MS, which accurately measures urinary cortisol with a low rate of false-high values in the mimicking HC group.

The corticosterone-to-creatine ratio showed the best diagnostic performance for canine HC in this study. It is unclear why corticosterone was superior to cortisol as a diagnostic indicator, but possibly it was the primary secreted steroid hormone in our study population. Corticosterone- and aldosterone-secreting adrenocortical tumors have been reported in a dog (Behrend et al., 2005). In this study, some dogs diagnosed with adrenocortical tumors might have had more corticosterone secretions with relatively low cortisol levels. Some dogs diagnosed with pituitary-dependent HC also showed relatively high corticosterone and low cortisol levels, indicative of variations in steroid hormone profiles in dogs with HC.

In clinical situations, urinary steroid profiles can be exceedingly

valuable for the diagnosis of functional adrenocortical tumors that do not predominantly secrete cortisol. This study included a dog with polyuria and polydipsia with a unilateral adrenal tumor accompanied by contralateral adrenal atrophy. An ACTH stimulation test showed subnormal cortisol concentrations before (1.5 μg/dl) and after (5.9 μg/dl) stimulation. In LDDST, cortisol concentrations were not suppressed but remained consistently low at baseline (1.4 μg/dl) and 4 h and 8 h after dexamethasone administration (0.9 μg/dl and 1.1 μg/dl, respectively). These results are consistent with those from non-cortisol-secreting functional adrenocortical tumors. The urinary steroid profile showed that while the urinary cortisol-to-creatinine ratio was within the reference range (2.1 nmol/mmol), the corticosterone-to-creatine ratio was remarkably increased (1.23 nmol/mmol), indicative of a corticosterone-secreting adrenocortical tumor. Other possible applications include the diagnosis of subclinical Cushing's syndrome (Athimulam et al., 2021) or the differentiation between pituitary- and adrenal-dependent hypercortisolism (Gao et al., 2023). Further studies using urinary steroid profile analyses may be helpful in clinical applications in small-animal practice.

5. Conclusions

We developed an accurate and sensitive system for measuring urinary steroid hormone levels using LC/MS/MS. Using this system, we established the upper limits of the reference intervals for urinary steroid-to-creatinine ratios in healthy controls and confirmed that some were clinically useful. Urinary cortisol concentrations measured by LC/MS/MS and CLEIA showed a significant correlation, although the latter produced false-increased values due to cross-reactivity. Further studies with larger study populations are necessary to validate the clinical usefulness of urinary steroid profile analyses.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work was supported, in part, by JSPS KAKENHI, awarded to N. Nagata (Grant Number 22K05980), Y. Ikenaka (Grant Numbers 17K2003807, 18H0413208, 22K18425, 23H00512) and M. Takiguchi (Grant Numbers 20H03139, 23H02374), and Clinical Research Promotion Fund 2021-2022 by Hokkaido University Veterinary Teaching Hospital. We wish to thank all staff at the Poplar Animal Hospital (Sapporo, Japan) and Hokkaido University Veterinary Teaching Hospital for generously collecting the urine samples. Preliminary results were presented as an Abstract at the 33rd European Congress of Veterinary Internal Medicine for Companion Animals, Barcelona, 21–23 September 2023.

Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: …

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2024.106151.

References

Athimulam, S., Grebe, S., Bancos, I., 2021. Steroid profiling in the diagnosis of mild and overt Cushing's syndrome. Best Practice Research Clinical Endocrinology Metabolism 35, 101488.

Behrend, E.N., Kooistra, H.S., Nelson, R., Reusch, C.E., Scott-Moncrieff, J.C., 2013. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). Journal of Veterinary Internal Medicine 27, 1292–1304.

Behrend, E.N., Weigand, C.M., Whitley, E.M., Refsal, K.R., Young, D.W., Kemppainen, R. J., 2005. Corticosterone- and aldosterone-secreting adrenocortical tumor in a dog. Journal of American Veterinary Medical Association 226, 1662–1666.

Bennaim, M., Shiel, R.E., Mooney, C.T., 2019. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 2: Adrenal function testing and differentiating tests. The Veterinary Journal 252, 105343.

Friedrichs, K.R., Harr, K.E., Freeman, K.P., Szladovits, B., Walton, R.M., Barnhart, K.F., Blanco-Chavez, J., American Society for Veterinary Clinical Pathology, 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Veterinary Clinical Pathology 41, 441–453.

Galeandro, L., Sieber-Ruckstuhl, N.S., Riond, B., Hartnack, S., Hofmann-Lehmann, R., Reusch, C.E., Boretti, F.S., 2014. Urinary corticoid concentrations measured by 5 different immunoassays and gas chromatography-mass spectrometry in healthy dogs and dogs with hypercortisolism at home and in the hospital. Journal of Veterinary Internal Medicine 28, 1433–1441.

Gao, C., Ding, L., Zhang, X., Yuan, M., Tang, S., Li, W., Ye, Y., Liu, M., He, Q., 2023. Distinct serum steroid profiles between adrenal Cushing syndrome and Cushing disease. Frontiers in Endocrinology 14, 1158573.

Geffré, A., Concordet, D., Braun, J.P., Trumel, C., 2011. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Veterinary Clinical Pathology 40, 107–112.

Holst, B.S., Kushnir, M.M., 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. Veterinary Clinical Pathology 44, 552–558.

Jensen, A.L., Iversen, L., Koch, J., Høier, R., Petersen, T.K., 1997. Evaluation of the urinary cortisol: creatinine ratio in the diagnosis of hyperadrenocorticism in dogs. Journal of Small Animal Practice 38, 99–102.

Kooistra, H.S., Galac, S., 2012. Recent advances in the diagnosis of Cushing's syndrome in dogs. Topics in Companion Animal Medicine 27, 21–24.

Nagata, N., Sawamura, H., Morishita, K., Hosoya, K., Yokoyama, N., Sasaoka, K., Sasaki, N., Nakamura, K., Ikenaka, Y., Takiguchi, M., 2022. Urinary corticoid to creatinine ratios using IMMULITE 2000 XPi for diagnosis of canine hypercortisolism. The Journal of Veterinary Medical Science 84, 954–959.

Reul, J.M., Rothuizen, J., de Kloet, E.R., 1991. Age-related changes in the dog hypothalamic-pituitary-adrenocortical system: neuroendocrine activity and corticosteroid receptors. The Journal of Steroid Biochemistry and Molecular Biology 40, 63–69.

Sasaki, N., Ikenaka, Y., Inoue, Y., Ichise, T., Nagata, N., Ishizuka, M., Nakayama, S.M., Nakamura, K., Takiguchi, M., 2021a. Urinary free metanephrines measurement in dogs with adrenal gland diseases using a new simple liquid chromatography tandem mass spectrometry method. The Journal of Veterinary Medical Science 83, 648–655.

Sasaki, N., Oo, T., Yasuda, Y., Ichise, T., Nagata, N., Yokoyama, N., Sasaoka, K., Morishita, K., Nakayama, S.M., Ishizuka, M., et al., 2021b. Simultaneous steroids measurement in dogs with hyperadrenocorticism using a column-switching liquid chromatography-tandem mass spectrometry method. The Journal of Veterinary Medical Science 83, 1634–1642.

Zeugswetter, F., Bydzovsky, N., Kampner, D., Schwendenwein, I., 2010. Tailored reference limits for urine corticoid:creatinine ratio in dogs to answer distinct clinical questions. Veterinary Record 167, 997–1001.