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Configuration of antibodies for assay of urinary cortisol in dogs influences analytic specificity

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ABSTRACT

Whether the variation in the reported urinary corticoid-to-creatinine ratio in dogs is affected by the application of 2 commonly applied anticortisol antibodies was investigated. Free-catch morning urine samples of 50 healthy dogs were analyzed in duplicate with the use of 2 different polyclonal antibodies (antibody A and B) raised in different rabbits. Antibody A was raised against cortisol-3-carboxymethyl-oxime and antibody B against cortisol-21-hemisuccinate linked to BSA. Enzyme immunoassays were applied by using corresponding biotinylated labels. To examine possible cross-reactions with conjugated and nonconjugated cortisol metabolites, EIA measurements were performed with urine samples both before (directly assayed) and after diethyl-ether extraction, as well as after reversed-phase HPLC. Although the results correlated ($P < 0.001$), urinary corticoid concentrations and accordingly the urinary corticoid-to-creatinine ratios were 8 times higher when using antibody A than when using antibody B (mean ± SD corticoid concentrations, 223 ± 131 vs 29 ± 12 nmol/L; $P < 0.001$). Irrespective of the antibody used, extraction significantly decreased measured corticoid concentrations (antibody A, 158 ± 120 nmol/L; antibody B, 15 ± 8 nmol/L; $P < 0.001$), but the decrease was conspicuous when antibody A was used. Antibody A cross-reacted significantly with polar (eg, conjugated) metabolites, clearly depicted in the chromatogram by 3 additional peaks in earlier fractions well separated from cortisol. In contrast the assay that used antibody B was specific, showing only 1 major peak in the fractions eluting authentic cortisol. In summary, the study indicates that the configuration of the antibody considerably influences the analytic specificity of cortisol assays and underlines the pivotal importance of assay validation for each species and sample material.

1. Introduction

Canine hyperadrenocorticism (HAC) is a clinical syndrome caused by chronic exposure to excessive glucocorticoid concentrations. The key substance is cortisol, a steroid hormone produced mainly in the adrenal glands and controlled primarily by pituitary ACTH secretion. Although clinical signs are almost pathognomonic in many cases [1], laboratory findings are nonspecific, and endocrine tests are inevitable to establish the diagnosis. Currently recommended screening tests include the determination of the urinary corticoid-to-creatinine ratio (UCCR), and functional tests such as the ACTH-response test or the low-dose dexamethasone suppression test. Although the high negative predictive value of the UCCR and its usefulness to "rule out" HAC is widely acknowledged [2–4], the overall diagnostic efficacy is questioned

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[3,5]. Data on specificity of the test are controversial and range from 23% [3] to 95% [6]. Accordingly, some investigators interpret elevated UCCRs in a dog with physical and biochemical changes suspect for hypercortisolism as diagnostic for HAC [7], whereas this approach is declined by others [5]. Explanations for contradictory results focused on different modes of urine collection [8–11] and on criteria of patient selection [8,12]. Despite the generally recognized fact that assay methodology has a significant effect on canine urine cortisol measurement and can cause disagreement of results [6,13], this has not been further investigated in dogs.

Studies in humans already showed variable cross-reactivity of different anticortisol antibodies with other adrenal-derived secretory products such as cortisol-precursors and metabolites [14]. This can be explained by partial hapten inhibition during antibody production. Because cortisol is too small to elicit an immune response, it has to be linked to a macromolecule, for example, BSA (Fig. 1). Accordingly, functional groups at the coupling sites are masked, and steroids that differ directly at or nearby this position cannot be differentiated by the respective antibody. As a consequence, anticortisol antibodies are never completely specific for free authentic cortisol, and assay performance depends on the concentration of cross-reacting substances in the test medium. The composition and concentration of these substances are not only species dependent but also influenced by nonadrenal diseases [14–17].

This study was performed to investigate the analytic specificity of 2 commonly applied polyclonal anticortisol antibodies for measurement of cortisol in canine urine. The hypothesis was that antibodies raised against cortisol-3-carboxymethyl-oxime (cortisol-3-CMO)-BSA cross-react with many conjugated cortisol metabolites because they cannot identify changes at the C-3 position of the molecule. Antibodies raised in rabbits against cortisol-3-CMO (antibody A [18,19]) and cortisol-21-hemisuccinate (cortisol-21-HS; antibody B [2,20]) linked to BSA (Fig. 1A) were compared using appropriate biotinylated labels. Analysis was performed from nonextracted as well as extracted urine samples and after reversed-phase (RP) HPLC. Further, fractions of pooled urine were analyzed with a widely used commercially available chemiluminometric immunoassay after separation with RP-HPLC.

2. Materials and methods

2.1. Animals and sample collection

All procedures were discussed and approved by the institutional ethics committee in accordance with good scientific practice guidelines and national legislation. Free-catch morning urine samples of 50 privately owned dogs were analyzed. Urine was collected with a special collection device by 1 of the authors that accompanied the owners and the dogs. The urine was stored in precooled glass tubes, stored in a Styrofoam box, and frozen at −20°C within 2 h to avoid microbial metabolism of cortisol. The dogs (19 different breeds [purebred], 23 mixed breed dogs; 23 male [7 intact], 27 female [10 intact]), ranged in age from 12 to 132 mo (median, 42 mo) and weighed 3.3 to 56 kg (median, 16.7 kg). They were considered to be healthy on the basis of a thorough physical examination and the absence of historical abnormalities. A BCS with a scale of 1 (very thin) to 5 (obese) was applied. Thirty-eight dogs had a BCS of 3 and 12 dogs a BCS of 4, respectively. Dogs with a BCS of 1 or 5 as well as animals with recurring gastrointestinal signs were excluded. To be able to include older dogs, dogs with mild heart murmurs, mild orthopedic problems, or dental problems were included. The urine was semiquantitatively analyzed with urine test strips (Combur 9 Test; Roche, Mannheim, Germany). Dogs with glucosuria and more than trace positive proteinuria, ketonuria, and hematuria were excluded.

2.2. Cortisol assays

Validation of assays A (antibody A) and B (antibody B) was performed similarly as described by Cekan [21] and additionally included linearity of dilution assessment. The cortisol standard curve of assay A ranged from 0.3 to 80 pg/well, that of assay B from 2 to 200 pg/well. The 50% intercept was 7.5 pg/well for assay A and 22 pg/well for assay B. The sensitivities of the tests were calculated as the least amount of standard that was different from a concentration of zero at the 95% confidence limit (0.3 and 2 pg/well). Intra-assay and interassay CVs were 7.8% and 8.7% for assay A, and 5.3% and 7.5% for assay B, respectively. Linearity was tested by serial dilution of pooled urine samples with assay buffer (dilution steps 1:5, 1:3, 1:2; with 3 measurements per dilution). The different sample volumes had no influence on the results ($P > 0.05$). Assay A and assay B showed cross-reactivity with the standards of corticosterone (6.2% vs 2.4%), allodihydrocortisol (4.6% vs 0.8%), and allotetrahydrocortisol (0.8% vs <0.1%), respectively [22].

The intrassay CV of the Immulite 1000 cortisol assay at a low (mean, 126.7 nmol/L), medium (mean, 266.2 nmol/L), and high (mean, 573.1 nmol/L) cortisol concentration was 5%, 8.1%, and 3.1%, respectively. The average recovery rate at different dilutions (100 μL of urine diluted with 25–300 μL of water) was 92%. Cross-reactivity is known for the endogenous steroids corticosterone (8.6%) and tetrahydrocortisol (0.9%; Siemens [23]).

2.3. EIA analysis from nonextracted and extracted samples

Urine samples were analyzed in duplicate as native specimens and after diethyl-ether extraction. The extraction was performed as follows: 200 μL of urine was extracted with 4 mL of diethyl-ether. After freezing the water phase at −20°C overnight, the organic phase was separated, evaporated under a stream of nitrogen, and reconstituted in assay buffer for the analysis.

Competitive EIAs were performed as described by Palme and Möstl [24] and Cekan [21] for antibody A (raised in rabbits against cortisol-3-CMO: BSA, working dilution of 1:250,000; Fig. 1). Polyclonal antibody B was also raised in rabbits, but in this case by injection of cortisol-21-HS linked with BSA (working dilution, 1:70,000) (Fig. 1). Biotinylated cortisol derivatives (DADOO-biotin = N-biotinyl-1,8-diamino-3,6-dioxaoctane) were used as competitive

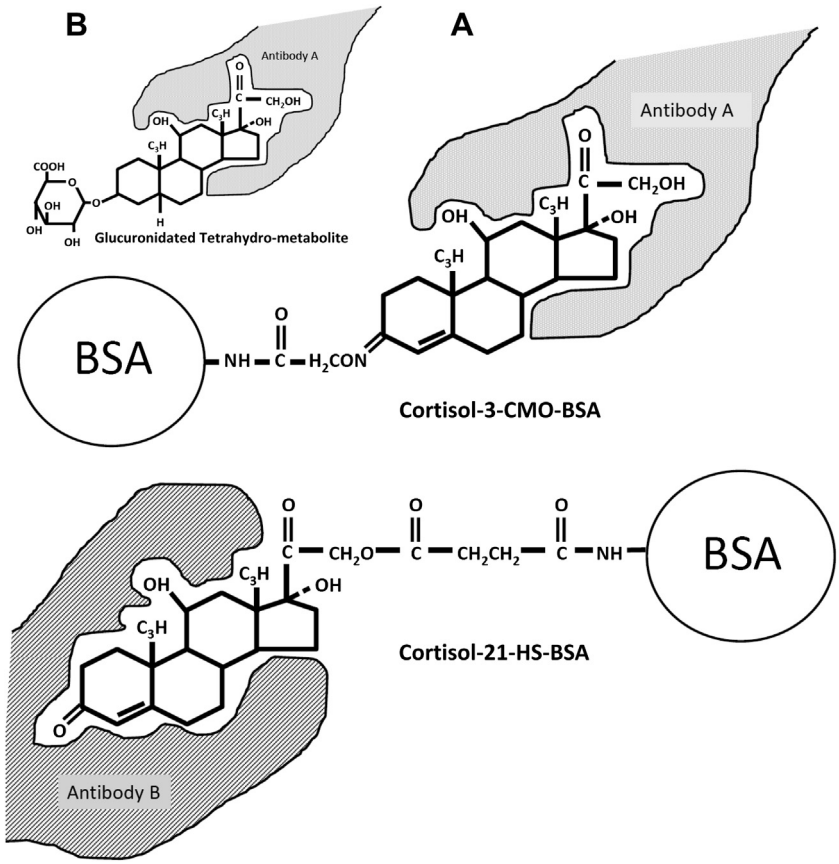

Fig. 1. (A) Polyclonal anticortisol antibodies were raised against cortisol-3-CMO–BSA (antibody A) and cortisol-21-HS–BSA (antibody B). These antibodies recognize only a specific part of the cortisol molecule. (B) Possible cross-reaction of antibody B with a common tetrahydro-metabolite of cortisol. Cortisol-3-CMO, cortisol-3-carboxymethyl-oxime; cortisol-21-HS, cortisol-21-hemisuccinate.

labels (working dilution, 1:160,000 and 1:100,000). Urine creatinine (measured in μmol/L) was determined by the enzymatic assay Creatinine plus (Roche Diagnostics, Vienna, Austria) on a Cobas 501 c (Roche Diagnostics), a fully selective chemistry analyzer. The test was applied according to the manufacturers' instructions. All ratios were expressed as $\times 10^{-6}$.

2.4. EIA analysis after RP-HPLC

As a clean-up procedure 2 mL of pooled morning urine was diluted with 8 mL of distilled water and loaded on activated solid-phase extraction columns (Sep-Pak Classic C18 cartridges; Waters, Milford, MA, USA). The cartridges were rinsed with 4 mL of distilled water, and the extracts were then eluted in 100% methanol. The organic solvent was evaporated under a stream of nitrogen. After reconstitution with 200 μL of methanol/water (30%/70%), 100 μL was injected into the HPLC system (RP column: Nova-Pac C 18; 3.8 × 150 mm; Waters; flow rate, 1 mL/min), using a linear methanol gradient from 30% to 50% and 40% to 60%. Fractions were collected at 20-s intervals and diluted in assay buffer (1:10), and sample volumes of 10 μL were analyzed in each individual fraction for immunoreactive substances (Immunoassay A [antibody A] vs B [antibody B] in the 30/50 chromatogram and Immunoassay A vs Immulite 1000 Cortisol assay in the 40/60 chromatogram). Immunoreactive cortisol concentrations were measured with the EIA as described in section 2.2. Cortisol assays, as well as with a competitive chemiluminescence immunoassay (Immulite 1000 cortisol; Diagnostic Products Corporation, Los Angeles, CA, USA [4]).

Retention times of the standards cortisol (4-pregnen-11β, 17α, 21-triol-3, 20dione), cortisone (4-pregnen-17α, 21-diol-3,11,20-trione), corticosterone (4-pregnen-11β, 21-diol-3,20-dione), 11-dehydrocorticosterone (4-pregnen-21-ol-3,11,20-trione), allodihydrocortisol (5α-dihydrocortisol), and allotetrahydrocortisol (5α-pregnan-3α,11β, 17α,21-tetrol-3,20-dione and 5α-pregnan-3 β,11β,17α,21-tetrol-20-one) were determined in separate runs. Steroid reference materials were purchased from Steraloids (Newport, RI, USA).

2.5. Statistical analysis

Data analysis was performed with the laboratory software package IBM SPSS version 21 (SPSS Inc; Chicago, IL, USA). All data were assessed for normality by the Kolmogorov–Smirnov test. Analysis of variance for repeated measurements and subsequently the Student

t test for paired samples with Bonferroni correction for multiple comparisons were applied. To assess the effects of age, sex, castrate status, and weight, these variables were entered into the analysis of variance as covariates and factors. Calculations were conducted with corticoid concentrations and UCCR as dependent variables. For correlation analysis Pearson correlation coefficient was used. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Competitive EIAs

Data were normally distributed and are expressed as mean ± SD. Analysis of variance showed significant differences ($P < 0.001$) between measurements with antibody A and antibody B (corticoid concentrations: $F = 159.4$; UCCR: $F = 201$; Fig. 2) and measurements from nonextracted and extracted urine (corticoid concentrations: $F = 96.2$; UCCR: $F = 103.3$; Fig. 2). The effect of extraction on the different assays was also significant ($P < 0.001$; corticoid concentration: $F = 76.4$; UCCR: $F = 75.3$). Differences were still significant ($P < 0.001$) after correction for age, weight, sex, and sexual status [corticoid concentrations $_{(\text{extraction; antibody})}$: $F = 30.8$, $F = 62.4$; UCCR $_{(\text{extraction; antibody})}$: $F = 25.3$, $F = 65.3$]. Only weight was found to have a significant effect on results [UCCR $_{(\text{extraction})}$: $F = 5.422$, $P = 0.024$; UCCR $_{(\text{antibody})}$: $F = 11.1$, $P = 0.002$].

Corticoid concentrations and the UCCRs measured in nonextracted urine with assay A were both significantly ($P < 0.001$) and on average 8 ± 3.5 times higher than the concentrations and ratios measured with assay B. For absolute concentrations, ratios, differences, and significance see Table 1. Box plots for urinary corticoid concentrations are depicted in Figure 2. Extraction significantly reduced immunoreactive corticoid concentrations and the UCCR in both assays ($P < 0.001$). Despite the stronger effect of extraction on the assay with antibody A, corticoid concentrations and the UCCRs measured with this assay were still at an average of 5.4 ± 3.7 times higher than the concentrations and ratios measured with assay B. For absolute concentrations, ratios, differences, and significance see Table 1. Box plots for urinary corticoid concentrations are depicted in Figure 2.

Moderate-to-high correlations were found between measurements with assay A and assay B [corticoid concentrations$_{(\text{unextracted})}$: $r = 0.712$, $P < 0.001$; corticoid concentrations$_{(\text{extracted})}$: $r = 0.72$, $P < 0.001$; UCCR$_{(\text{unextracted})}$: $r = 0.565$, $P < 0.001$; UCCR$_{(\text{extracted})}$: $r = 0.588$, $P < 0.001$]. With the exception of the UCCR measured with assay A, measurements from extracted urine correlated significantly with measurements from nonextracted urine [corticoid concentrations$_{(\text{antibody A})}$: $r = 0.399$, $P = 0.004$; corticoid concentrations$_{(\text{antibody B})}$: $r = 0.799$, $P < 0.001$; UCCR$_{(\text{antibody A})}$: $r = 0.234$, $P = 0.102$; UCCR$_{(\text{antibody B})}$: $r = 0.763$, $P < 0.001$].

3.2. RP-HPLC

In agreement with the elution of the cortisol standard, an immunoreactive peak was also measured in fractions 49 to 50 with the use of assay A and B (methanol gradient, 30%–50%). The chromatogram of assay A showed additional 3 large peaks in the less polar fractions 1 to 8 and 41 to 47 (2 peaks), whereas assay B displayed only 2 small peaks in fractions 3 and 40 (Fig. 3). The standards cortisone, 5α-pregnan-3β,11β,17α,21-tetrol-20-one, 11-dehydrocorticosterone, allodihydrocortisol, and corticosterone eluted in the fractions 44 to 45, 45 to 47, 52 to 53, 59 to 61, and 69 to 70, respectively. The chemiluminometric assay showed immunoreactive metabolites in fractions 5 to 10 and only a small peak in fractions 76 to 80 (methanol gradient from 40% to 60%, authentic cortisol

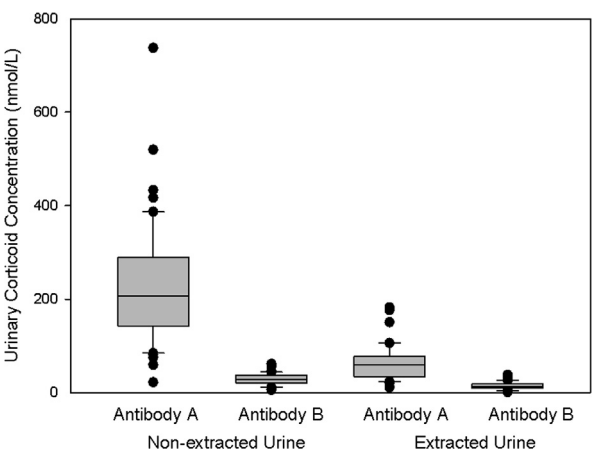

Fig. 2. Box-whiskers plots of urinary corticoid-to-creatinine ratios of 50 healthy dogs measured directly and after extraction with diethyl-ether. Immunoreactive cortisol was measured with polyclonal antibodies raised against cortisol-3-carboxymethyl-oxime (antibody A) and cortisol-21-hemisuccinate (antibody B) linked to BSA. The lines within the boxes represent the median, and the upper and lower boundaries of the boxes represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are shown as circles. All groups differed significantly ($P < 0.001$).

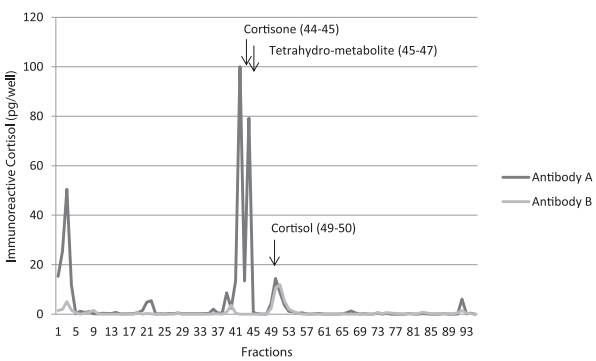

Fig. 3. Chromatographic profiles of canine urine determined by RP-HPLC after solid-phase extraction. With a nonpolar stationary phase and an aqueous mobile phase RP-HPLC separates steroids according to their polarity. More polar molecules (eg, conjugated metabolites) are readily integrated into the aqueous mobile phase and appear in earlier fractions on the left side of the chromatogram. Fractions were analyzed by 2 different EIAs. Arrows indicate the position of standards chromatographed in separate runs. Numbers in parentheses refer to the specific elution fractions of these standards. PR, reversed-phase.

Table 1
Urinary immunoreactive cortisol concentrations and urinary corticoid:creatinine ratios.

	Antibody A, mean ± SD	Antibody B, mean ± SD	Difference, mean ± SD	*P* value[a]
Corticoid concentration				
Nonextracted urine (nmol/L)	223 ± 131	29 ± 12	194 ± 122	<0.001
Extracted urine (nmol/L)	65 ± 40	14 ± 8	51 ± 35	<0.001
Difference (nmol/L)	158 ± 120	15 ± 8		
Difference (%)	61 ± 53	53 ± 19		
P value[a]	<0.001	<0.001		
Corticoid-to-creatinine ratio ($\times 10^{-6}$)				
Nonextracted urine	39.4 ± 20.6	5.2 ± 2.1	34.2 ± 19.5	<0.001
Extracted urine	12.1 ± 8.3	2.5 ± 1.4	9.7 ± 7.6	<0.001
Difference	27.3 ± 20.4	2.7 ± 1.4		
P value[a]	<0.001	<0.001		

[a] After Bonferroni adjustment for multiple comparisons.

eluting in fractions 75 to 78). A comparable elution pattern was observed with assay A (data not shown).

4. Discussion

The data reported herein confirm our hypothesis that the analytic specificity of different anticortisol antibodies has a significant effect on the results of canine urinary cortisol measurements and that antisera raised against the cortisol-3-CMO derivate (antibody A) are less specific. Results suggest that assays with this antibody cross-react with conjugated metabolites abundant in canine urine and accordingly overestimate urinary cortisol concentrations. In contrast assays that use the anti–cortisol-21-HS antibody (antibody B), which is currently applied at Utrecht University, are highly specific. This is a likely explanation for the high positive predictive value of the UCCR to diagnose canine Cushing's syndrome in the Utrecht studies, which could not be achieved by other groups that used different assays.

The fact that antisera raised against cortisol are not functionally equivalent can be explained by peculiarities of antibody production. Because the cortisol molecule is too small to induce antibody formation, it has to be linked to a macromolecule- for example, BSA- to elicit an immune response. Possible linking sites include the positions C-3 and C-21 of the steroid (Fig. 1A). Antibodies are raised against these different steroid–protein conjugates. The drawback of this approach is that functional groups at the coupling site are hidden. The consequence is partial hapten inhibition, that means that steroids that differ close by or directly at the position of the binding site cannot be differentiated (Fig. 1B) [25]. Thus, anticortisol antibodies are never completely specific for free authentic cortisol, and the possible interferences with cortisol precursors and metabolites, so called "corticoids," are numerous [26]. For serum cortisol measurements these cross-reactivities are likely to be of minor importance because the amount of conjugated metabolites in plasma is assumed to be low.

Under the assumption that cortisol metabolites are correlated with free cortisol and reference ranges are appropriately adapted, the use of a highly specific antibody might not be necessary. However, a metabolic shift with a greater percentage of interfering metabolites that distort the results is observed in pregnant women, in the late luteal phase of the menstrual cycle, and in various non-adrenal diseases, including hypothyroidism, obesity, collagen disease, and decreased liver function [14–16]. Increased urinary cortisol metabolites in the face of normal urinary cortisol concentrations have been reported in patients with obesity-related metabolic syndrome [16] and non–alcoholic fatty liver disease [17].

Only a few studies, not including sick dogs, have focused on the characterization of canine cortisol metabolism and excretion [27–31]. After the injection of titrated cortisol into healthy dogs, 77% of the radioactivity was rapidly excreted into the urine [31]. Seven percent of urinary radioactivity was extractable with diethyl-ether, suggesting that as in humans [32] only a small portion is excreted as unconjugated free cortisol. Because conjugation to glucuronic or sulfuric acid takes place primarily at the 3-hydroxyl position of the molecule, a position masked when working with antibody A, significant cross-reactivity and, as a consequence, higher urinary corticoid concentrations can be expected in canine urine.

In line with this, 8 times higher "corticoid" concentrations and UCCRs were measured with antibody A compared with antibody B. To test the hypothesis that this was in fact a consequence of interfering polar metabolites, analyses were repeated after liquid/liquid extraction with diethyl-ether. This extraction procedure separates hydrophobic steroids from aqueous media and eliminates many hydroxylated and conjugated more hydrophilic compounds (eg, sulfates and glucuronides). As expected, extraction considerably reduced corticoid concentrations. The conclusion that results are grossly distorted by polar, likely conjugated cortisol metabolites, was additionally substantiated by RP-HPLC with the use of the organic solvent methanol, showing 3 large peaks in earlier polar fractions of the chromatogram. Whereas 1 of these peaks presumably represented the conjugated (eg, glucuronidated) or sulfated metabolites, the other peaks approximately corresponded with the elution profile of cortisone and 5α-pregnan-3β,11β,17α,21-tetrol-20-one (similar to tetrahydrocortisol). The fact that only 5α-pregnen-3β,11β,17α,21-tetrol-20-one but not cortisone is extracted by diethyl-ether highlights the fact that organic solvent extraction can reduce but not eliminate the problem of antibody cross-reactivity. Although solvent extraction is strongly recommended in the human literature, this

expensive and time-consuming procedure can introduce additional errors and does not automatically improve the diagnostic performance of an assay [33].

The Immulite 1000 Cortisol assay, which is currently used in many commercial laboratories worldwide, also showed large peaks in early polar fractions of the chromatogram. These peaks corresponded to the early peaks obtained with antibody A and likely represented conjugated compounds abundant in canine urine. This cross-reactivity and accordingly low antibody specificity could explain the conspicuously high UCCRs and the large overlap of results observed between dogs with and without HAC in a recent study [4]. The importance of antibody specificity for measuring urine cortisol concentrations in dogs does not automatically apply to canine serum in which the concentrations of cross-reacting conjugated metabolites are low. Furthermore, the composition of urinary cortisol metabolites is highly species specific.

In contrast to assay A and the Immulite 1000 Cortisol assay, the results obtained with assay B were barely influenced by conjugated cortisol metabolites. Although organic solvent extraction significantly reduced measured corticoid concentrations, the reduction was small and likely a consequence of losses due to the extraction process. Accordingly, extraction with dichloromethane had no effect on the standard curve or the corticoid concentrations in an earlier study [2]. The RP-HPLC showed 1 main peak in the fractions eluting authentic cortisol. The lower concentrations obtained with this assay correspond to results from studies that used a comparable antibody [11,34,35].

5. Conclusion

This study shows that the use of different anticortisol antibodies has a major effect on urinary immunoreactive cortisol measurements and consequently the UCCRs. Although the antibody raised against cortisol-21-HS–BSA was highly specific for cortisol, the antibody raised against cortisol-3-CMO–BSA showed significant cross-reactivity with numerous metabolites and overestimated authentic cortisol in urine. The results of this study reinforce the principle of assay validation for every species and specimen under investigation and provide another plausible explanation for contradictory results about the utility of the UCCR to diagnose canine HAC.

Acknowledgments

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