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Effect of furosemide on comprehensive renin-angiotensin-aldosterone system activity of Thoroughbred horses

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Abstract

Background: Furosemide, a commonly used diuretic, activates the renin-angiotensin-aldosterone system (RAAS) in other species. Little is known about RAAS peptide activation in horses.

Hypothesis/Objectives: To evaluate equilibrium analysis as a practical method for RAAS quantification in horses and describe the RAAS response to a single dose of furosemide. We hypothesize that furosemide would cause transient increase in RAAS peptides in horses.

Animals: 14 healthy adult thoroughbreds from a university teaching herd.

Methods: Horses received either furosemide (1 mg/kg IV) or saline IV in a crossover study design. Protease-inhibited samples were compared with equilibrium analysis samples with Deming regression analysis. Renin-angiotensin-aldosterone system hormones were evaluated at 0, 0.25, 0.5, 4, and 24 hours postadministration, via equilibrium analysis. Values were compared with a mixed effects model.

Results: Correlation between protease inhibition and equilibrium analysis was high for angiotensin I peptide (AngI) and angiotensin II peptide (AngII) ($r = .92$ and .95, respectively). Baseline RAAS peptide concentrations were below the limit of detection except AngII (median, 7.5 [range, 3.5-14.0] pmol/L). Furosemide administration resulted in an increase in AngI (8.0 [0.5-15.5] pmol/L, $P = .03$), AngII (33.7 [9.6-57.9] pmol/L, $P = .0008$), angiotensin III peptide (AngIII) (2.9 [0.9-4.9] pmol/L, $P = .0005$), angiotensin IV peptide (AngIV) (2.0 [0.6-3.4] pmol/L, $P = .0005$), and angiotensin 1-5 peptide (Ang1-5) (5.6 [1.2-5.9] pmol/L, $P = .003$) at 4 hours. Differences are reported as difference in the mean (95% confidence interval [CI]).

Conclusions and Clinical Importance: Furosemide produced an increase in hormones associated with both the classical and alternative RAAS pathways. Serum equilibrium analysis is practical for RAAS analysis in horses.

Abbreviations: ACE, angiotensin-converting enzyme; Ald, aldosterone; Ang1-5, angtiotensin 1-5; Ang1-7, angiotensin 1-7; AngI, angiotensin I; AngII, angiotensin II; AngIII, angiotensin III; AngIV, angiotensin IV; aRAAS, alternative RAAS pathway; cRAAS, classical RAAS pathway; LLOQ, lower limit of quantitation; RAAS, renin-angiotensin-aldosterone system.

KEYWORDS
alternative RAAS, equine, loop diuretic, RAAS

1 | INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure and electrolytes in mammals. Reduced renal perfusion, inadequate delivery of sodium and chloride to the macula densa cells of the kidney, and sympathetic activation stimulate the release of renin from the juxtaglomerular apparatus. Renin converts angiotensinogen to angiotensin I (AngI), which contacts angiotensin-converting enzyme (ACE) on vascular endothelium and is converted to angiotensin II (AngII). AngII binds the angiotensin type-1 receptor (AT1R) eliciting key effects of the classical RAAS pathway (cRAAS) such as arteriolar vasoconstriction and sodium retention in the kidneys. Aldosterone (Ald) release by AngII further amplifies the effects of cRAAS. Metabolites of AngI and AngII degradation (angiotensin III [AngIII] and angiotensin IV [AngIV]) are also active and are thought to have cRAAS actions. Although short-term RAAS activation in response to fluid or electrolyte perturbations is compensatory, sustained activation of cRAAS promotes cardiovascular and renal remodeling and leads to dysfunction.[1-4] The counterbalancing alternative RAAS pathway (aRAAS) is comprised of angiotensin peptides (APs), such as angiotensin 1-9, angiotensin 1-7 (Ang1-7), and angtiotensin 1-5 (Ang1-5), enzymes, such as angiotensin-converting enzyme 2 (ACE2) and neprilysin, and the Mas receptor. Enzyme activity influences the balance between the cRAAS and aRAAS. Chronic activation of aRAAS has antioxidant, antifibrotic, and anti-inflammatory effects and is currently being evaluated as a therapeutic target.[5-8]

Select cRAAS peptides in the horse have been measured in neonates and adult horses in response to exercise and heart disease.[9-14] However, to the authors' knowledge, comprehensive AP profiles have only appeared in 1 other report.[15] Furosemide, a loop diuretic that activates RAAS,[16-19] is routinely used for treating exercise induced pulmonary hemorrhage in horses. Little is known about the activation of the cRAAS in horses in response to furosemide. Evaluation of the comprehensive AP profile in horses has been limited by assay availability and the requirement for immediate protease inhibition (PI), which is logistically challenging. The equilibrium analysis (EA) method is used to quantify APs in samples collected without PI and correlates significantly with PI samples in human and dog studies.[20,21] Confirming that AP concentrations measured via EA from routinely handled samples correlate to those measured from PI samples will make RAAS assessment more accessible to horses.

The 1st aim of this study was to evaluate the correlation between the EA and PI methods of sample handling and analysis. We hypothesized that AP concentrations would be strongly correlated between EA and PI samples. The 2nd aim was to provide a baseline comprehensive RAAS profile in healthy horses and to characterize the response of RAAS to furosemide administration in healthy horses. We hypothesized that furosemide would cause transient increase in both cRAAS and aRAAS peptides in healthy horses.

2 | MATERIALS AND METHODS

2.1 | Animals

Fourteen adult Thoroughbred horses from the UC Davis Center for Equine Health Research herd were enrolled in the study between March and July 2022. Horses were determined to be healthy based on physical examination and a minimum database, which included a complete blood count and biochemistry panel (Siemens Advia 2120i, Siemens Medical Solutions, USA, Malvern, Pennsylvania; Roche Cobas6000 c501, Roche Diagnostics, Indianapolis, Indiana, respectively). Horses were also required to have an echocardiogram within 1 year of the study. Exclusion criteria included more than trace valvular regurgitation and evidence of structural cardiac abnormalities. Horses were acclimated to individual housing for 1 night before the study start and allowed continuous access to free choice water via an automatic waterer throughout the acclimatization and study period.

2.2 | Study design

A total of 14 adult thoroughbred horses (6-18 years of age, median 13 years) were enrolled in this study. All horses were deemed clinically healthy and euhydrated at the start of the study as indicated by their physical exams and minimum database. Eight of the 14 horses, 4 mares and 4 geldings aged 6 to 15 years old (median 10 years), underwent a single sampling via direct venipuncture. The goal of this group was to increase the sample size for assessing the baseline comprehensive RAAS in healthy horses to 14 horses. Blood sampling in this group was performed at 7:00 a.m. before feeding. The remaining 6 horses of equal mares and geldings, aged 12 to 18 years (median 14 years), were subsequently enrolled in a crossover study designed to evaluate the activation of RAAS peptides by furosemide. Investigators were not blinded to the treatment groups. The 6 horses in the crossover study were randomly allocated into 2 groups of 3 horses with a 2-week washout period between the 2 blocks of the crossover study (Microsoft Excel Version 2312). An IV jugular catheter was placed the night before the start of each block to facilitate multiple blood draws and minimize stress. Each group received a single administration of either 1 mg/kg furosemide IV or a placebo (volume-matched saline IV; 0.9%) at time zero

(7:00 a.m.). Treatments were reversed in the subsequent block. Blood sampling via a previously described 3-syringe-technique was performed before dosing (time zero), 15 minutes, 30 minutes, 1, 4, and 24 hours after dosing with waste samples being given back to each horse at each time point.[22] Horses were fed their typical grass hay meal after the 1-hour sampling point and then twice daily thereafter as was routine for the facility. This meal came 1.5 hours later than the horse's typical morning feeding regimen.

Samples were used to assess electrolytes and kidney values (the "Kidney Panel" included sodium [Na^+], chloride [Cl^-], potassium [K^+], bicarbonate [$HCO3^-$], total calcium [$Ca2^+$], phosphorus [P], blood urea nitrogen [BUN], creatinine [Crea], and serum albumin [Alb]). Samples were obtained at 1, 4, and 24 hours after dosing, as the baseline biochemistry values provided the predosing value (time zero). Red top tubes were allowed to clot for at least 30 to 60 minutes before centrifugation, and serum was separated in routine fashion. Kidney panels were run the day of sampling (Roche Cobas6000 c501, Roche Diagnostics, Indianapolis, Indiana).

2.3 | Renin-angiotensin system (RAS)-fingerprint and serum aldosterone analysis

Blood samples were allocated into lithium heparin tubes and additive free tubes (BD Vacutainer, Franklin Lakes, New Jersey) for plasma and serum, respectively. In the crossover study group, lithium heparin samples were placed on ice within 2 minutes and combined with commercial protease inhibitor (Attoquant Diagnostics, Vienna, Austria) as described in human, murine, and canine samples.[20,21,23,24] Protease-inhibited plasma samples were collected as all-time points except for the 24 hours because of limited PI availability. Additive free tubes were collected for all time points and allowed to clot for 30 to 60 minutes before centrifugation. All samples were centrifuged 15 minutes at 24°C and 3000 relative centrifugal force. Serum and plasma were separated in routine fashion and stored at −80°C before analysis. Angiotensin peptide concentrations were quantified from PI plasma at the 0, 15, and 30 minute and 1- and 4-hour time points in the furosemide treated horses and used for correlation analysis. Angiotensin peptides (AngI, II, III, IV, 1,7, and 1,5) were quantified from serum (EA method) for samples. The AP and Ald quantification was performed at a commercial laboratory (RAS-Fingerprint; Attoquant Diagnostics, Vienna, Austria) using liquid chromatography mass spectrometry (LC-MS/MS).[20] Briefly, for EA, serum was incubated for 1 hour at 37°C to establish equilibrium concentrations before stabilization with an inhibitor cocktail. The plasma samples stabilized with PI were thawed on ice. Both the serum and plasma samples were spiked with stable isotope-labeled internal standards for each angiotensin metabolite and with a deuterated internal standard for aldosterone at a concentration of 200 pg/mL. Angiotensin peptides and aldosterone then were quantified using LC-MS/MS. Details of the LC-MS/MS method are presented in Data S1. The LC-MS/MS methodology and EA was validated based on European Medicines Agency Guidelines.[25]

2.4 | Statistical analysis

Data were analyzed using commercially available software (GraphPad Prism 9.5.1, GraphPad Software by Dotmatics, Boston, Massachusetts; G*power, Heinrich Heine University, Düsseldorf; and JMP Pro16, Buckinghamshire, England). Values below the lower limit of quantitation (LLOQ) were represented as LLOQ/2. Comparisons between PI and EA values were evaluated using Deming regression analysis. Because aldosterone is produced directly by the adrenal and no enzymatically cleaved from angiotensin precursors like the other AP a change in concentration of aldosterone between the 2 methods was not anticipated; thus, aldosterone concentrations were compared with a Bland-Altman analysis. Continuous data were evaluated for normality with Shapiro-Wilk tests. For evaluation of AP and kidney panels over time, a repeated measures mixed effects model was used where applicable. Fixed effects included individual horse and treatment block, whereas random effects included treatment and time point. Post hoc testing was performed with Tukey's multiple comparisons tests. Data are presented as mean, difference in mean (95% confidence interval [CI]), or median and range where applicable. Significance was set at $P \leq .05$.

The sample size for correlation between PI and EA was calculated based on the use of Spearman's correlation analysis. Based on pervious comparison performed in dogs, a Spearman's r value of 0.7 was used to estimate a sample size with 13 pairs.[16] This was estimated based on an 80% power and 0.05 allowable error based on calculations in G*power (Heinrich Heine University, Düsseldorf).

The sample size for the crossover study design was calculated using a Student's t-test for paired comparison of serum ACE activity. Based on published evaluation of ACE activity in racehorses administered furosemide, a difference in ACE activity of 10 nmol/mL/min and a standard deviation of 3 were used to calculate the appropriate sample size.[12] A sample size of 4 horses per group was predicted to reach statistical significance with 95% power and 0.05 allowable error based on calculations in JMP Pro16 (Buckinghamshire, England). Six horses were used in each group to allow for dropout because of unforeseen circumstances.

3 | RESULTS

3.1 | Correlation of EA with PI samples

Angiotensin peptides were not measurable in baseline (before furosemide) PI samples. After furosemide stimulation of RAAS, only AngI, AngII, and Ald had detectable concentrations when PI was used. There was a high correlation between PI and EA methods for AngI ($r = .92$, Slope 1.28 [CI, 1.03-1.61], n = 19 pairs) and AngII ($r = .95$, Slope 4.79 [CI, 4.22-5.51], n = 30 pairs) (Figure 1). Aldosterone showed strong correlation ($r = 1.0$, Slope 1.01 [CI, 0.99-1.04] n = 10 pairs). Compared with the PI method, EA method of aldosterone quantitation resulted in a systemic bias of −9.5 pmol/L (95% limits of agreement, −31.3 to 12.4) (Figure 1C).

3.2 | Baseline concentrations of comprehensive RAAS hormones

Baseline comprehensive RAAS hormone concentrations were measured via EA. Only AngII (median, 7.5 pmol/L [range, 3.5-14.0]) was detectable and found in all baseline samples. All other APs were below the LLOQ, except for 1 horse with an AngI concentration of 4.4 pmol/L and a different horse with an aldosterone concentration of 29.9 pmol/L. Aldosterone concentration at baseline (26.6 pmol/L) was also detectable in the paired PI sample from this horse.

3.3 | Classical RAAS pathway response to furosemide

The EA analysis results were used to evaluate the effect of furosemide administration on RAAS (Figure 3). Significant differences between groups are reported as difference in mean [95% CI]. The furosemide group showed significant increase in AngI at 4 hours postdosing as compared with the control group at the same time point (8.0 [0.5-15.5] pmol/L, $P = .03$ Figure 2). AngII showed significant increases in the furosemide group versus control group at 4 hours (33.7 [9.6-57.9] pmol/L, $P < .001$, Figure 2B), as did AngIII (2.9 [0.9-4.9] pmol/L, $P < .001$, Figure 2C), and AngIV (2.0 [0.6-3.4] pmol/L, $P < .001$, Figure 2D).

In the furosemide treated group, AngI concentrations were significantly increased from baseline at both 30 minutes and 4 hours (7.75 [0.2-15.3] pmol/L, $P = .04$, and 14.8 [7.3-22.3] pmol/L, $P < .0001$, respectively, Figure 2A). Several AP concentrations were significantly increased at the 4 hour time point: AngII (59.4 [35.2-83.6] pmol/L, $P < .0001$, Figure 2B), AngIII (4.0 [2.0-6.1] pmol/L, $P < .0001$, Figure 2C), and AngIV (2.3 [0.9-3.7] pmol/L, $P < .0001$, Figure 2D). Aldosterone concentrations were also significantly increased in the furosemide group at this time point (460.5 [686.2-234.7] pmol/L, $P < .0001$, Figure 2E). A significant increase from baseline was noted in the control group at the 4-hour time point for AngII and aldosterone (25.9 [1.7-50.1] pmol/L, $P = .03$ and 283.48 [509.22-57.75] pmol/L, $P < .01$, respectively Figure 2B,E). There were no significant alterations from baseline at 24 hours in both treatment and control groups. A single furosemide-treated horse had detectable AngIV at the 24-hour time point, and 2 furosemide-treated horses had measurable aldosterone at the 24-hour time point.

3.4 | Alternative RAAS pathway response to furosemide

Significant differences between groups are reported as difference in mean [95% CI]. Ang1-5 concentration in the furosemide-treated horses was significantly increased as compared to the control group at the 4-hour time point (5.6 [1.2-9.9] pmol/L, $P = .003$, Figure 2F). Angiotensin1-5 was significantly increased compared to the baseline in the furosemide group at

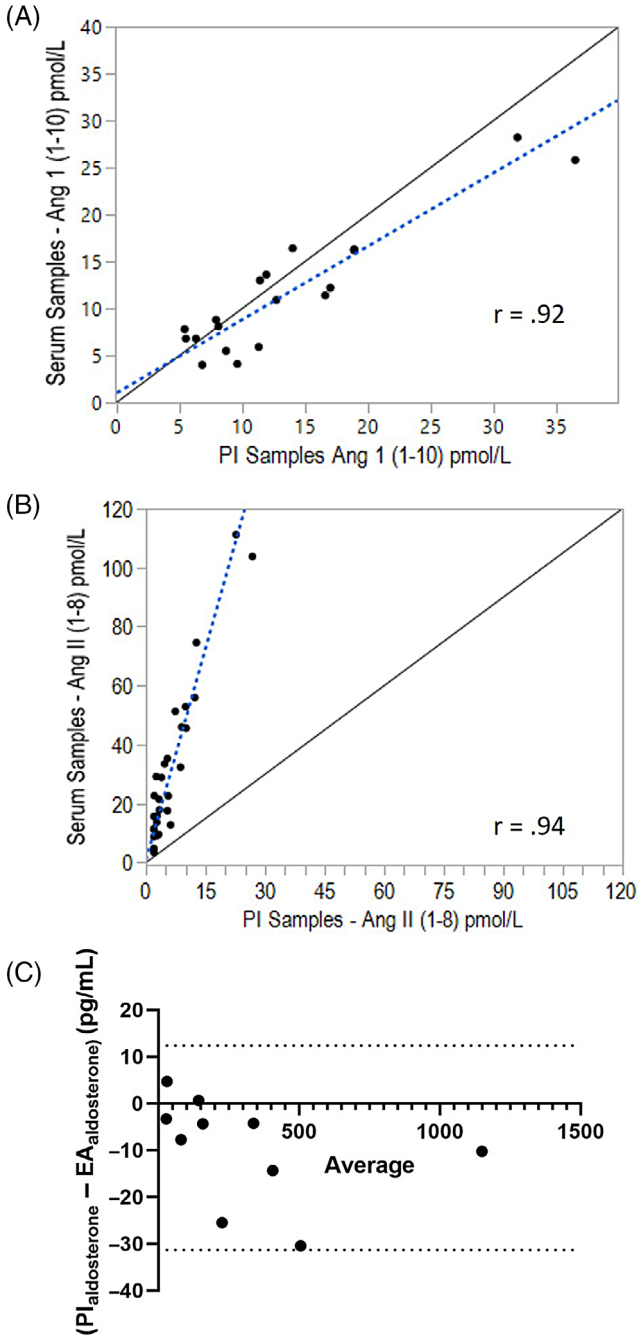

FIGURE 1 Correlation between the angiotensin peptide concentrations in protease inhibited (PI) versus equilibrium analysis (EA) samples in healthy adult horses as measured by liquid chromatography mass spectrometry (LC-MS/MS). Angiotensin I peptide, considered portion of classical RAAS pathway (cRAAS) pathway (AngI, A) and angiotensin II peptide, considered portion of cRAAS pathway (AngII, B) were the only angiotensin peptides detectible in circulation (PI samples) and were, therefore, selected for evaluation by Deming regression. Strong correlation was noted for AngI (19 pairs available for correlation) and AngII (30 pairs available for correlation). $P < .05$ was considered statistically significant. Band-Altman was selected for aldosterone as the concentration should not be affected by sample preparation method. Solid line indicates correlation of best fit, with 95% confidence interval (CI) indicated by dotted line.

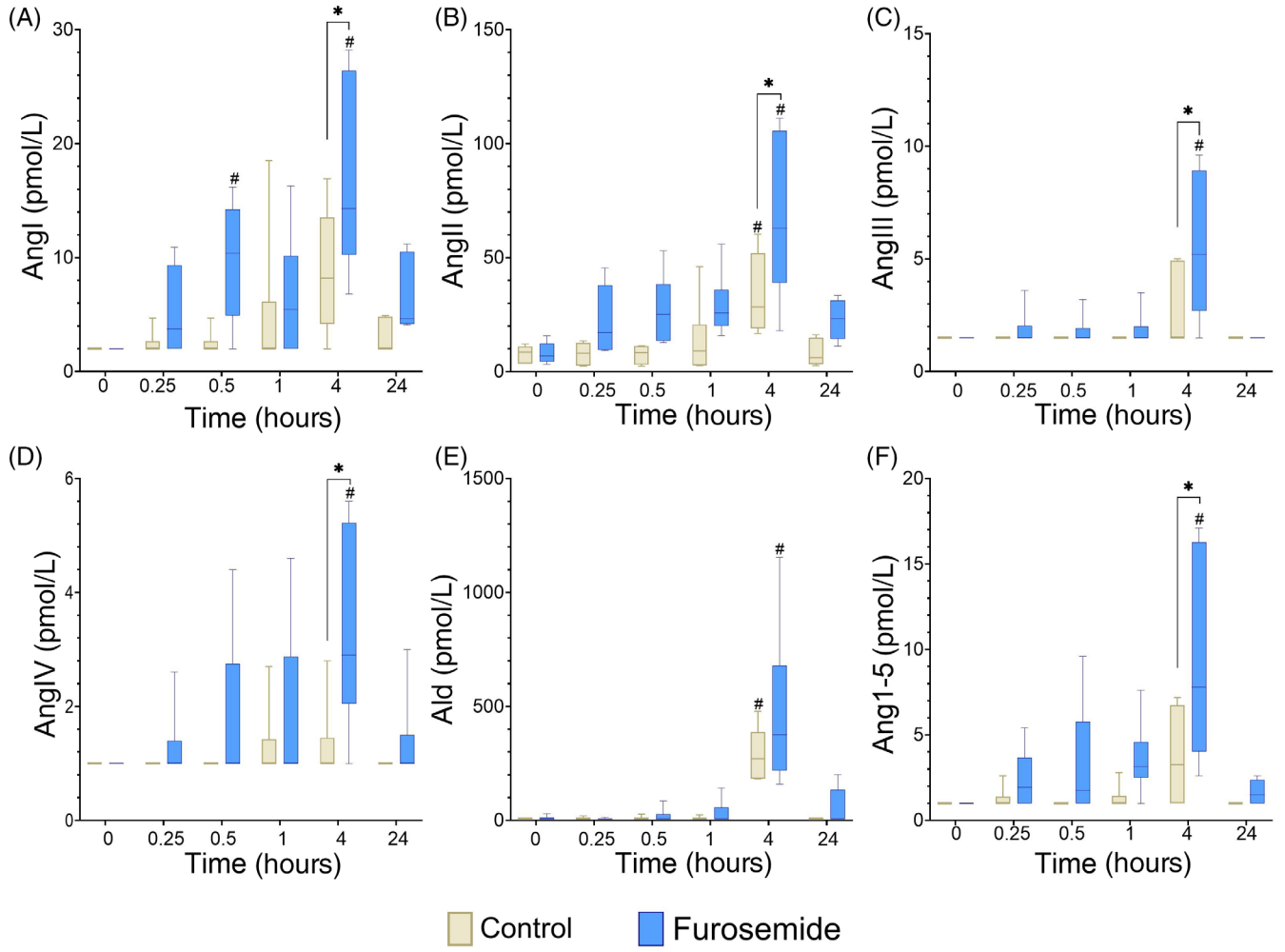

FIGURE 2 Changes in angiotensin peptide and aldosterone concentrations following a single dose of IV furosemide or saline control in healthy horses (n = 6). Measurable variables of the classical RAAS pathway were: angiotensin I peptide, considered portion of classical RAAS pathway (cRAAS) pathway (AngI, A), angiotensin II peptide, considered portion of cRAAS pathway (AngII, B), angiotensin III peptide, considered portion of cRAAS pathway (AngIII, C), angiotensin IV peptide, considered portion of cRAAS pathway (AngIV, D), aldosterone, considered portion of cRAAS pathway (Ald, E). The single measureable peptide of the alternative RAAS pathway was angtiotensin 1-5 peptide, considered portion of alternative RAAS pathway (aRAAS) (Ang1-5, F). Values represented include median, interquartile range, and range. Lower end of range represents values below the lower limit of quantitation. Horses were fed their regular morning meal after the 1-hour time point. Significant difference between furosemide and control groups at given time point are indicated by *$P < .05$. #A significant difference from baseline (time 0) in the respective group (furosemide or control).

4 hours (8.3 [4.0-12.6] pmol/L, $P < .0001$; Figure 2F). Three horses from the furosemide group had detectable Ang1-5 concentrations at 24 hours; otherwise, the remaining samples were below the LLOQ. Angiotensin 1,7 was not detectable in any of the control horses at any time but was detected in 2 furosemide-treated horses at the 4-hour time point (3.4 and 4.7 pmol/L, LLOQ 3.0 pmol/L, respectively) (Figure 3).

3.5 | Kidney values and electrolyte panels

No significant differences across treatment or time point were noted for BUN, phosphorus, sodium, or bicarbonate. Significant differences for biochemistry variables are reported as difference in the mean [95% CI]. Creatinine was significantly increased when comparing the furosemide to the control group at 4 hours after dosing (0.2 [0.02-0.4] mg/dL, $P = .02$) and was also significant comparing the furosemide group at 4 hours to baseline values (0.3 [0.1-0.5] mg/dL, $P < .001$). Albumin was increased in the furosemide group when compared with the control group at 1 hour after dosing (0.5 [0.3-0.7] g/dL, $P < .0001$). Albumin was also significantly increased compared to baseline in the furosemide group at 1 and 4 hours (0.5 [0.3-0.7] mg/dL, $P < .001$, 0.4 [0.2-0.6] mg/dL, $P < .001$, respectively) as well as in the control group at 4 hours (0.2 [0.04-0.4 g/dL], $P < .001$). Potassium increased in the furosemide group at 4 hours compared with baseline (0.6 [0.03-1.1] mMol/L, $P = .03$). Chloride was significantly lower than baseline in the furosemide-treated group at 1 hour and 24 hour time points (−3.2 [−0.7 to −5.7] mMol/L, $P < .01$ and −2.7 [−0.2 to −5.2],

$P = .03$, respectively). Calcium was increased at 4 hours as compared with baseline in both the control and furosemide-treated group (0.8 [0.3-1.3] mg/dL, $P < .001$; 0.9 [0.4-1.5] mg/dL, $P < .0001$, respectively).

A single horse experienced acute colic signs with tachycardia (80 bpm) after the 1 hour time point after furosemide dosing and was treated with a single half-label dose of flunixin meglumine IV (0.5 mg/kg, Merck & Co., Inc., Rahway, New Jersey) and was sedated with 0.3 mg/kg xylazine hydrochloride (Rompun, Dechra Veterinary Products, Overland, Kansas) to facilitate nasogastric tubing with a single dose of enteral fluids (10 mL/kg) with an added standard electrolyte packet (ReSorb, Zoetis Inc., Parsippany, New Jersey). This horse's 4 hour Crea concentration was increased (2.2 mg/dL) above her baseline value (1.6 mg/dL), but returned to 1.7 mg/dL by 24 hours. This horse's 4 hour Crea value is the only value that exceeded the upper reference value of 2.0 mg/dL. Electrolyte values over time were not considered clinically important in this horse. Analysis was repeated with exclusion of this horse and the results were not affected; thus, these results were retained in the final analysis.

3.6 | Discussion

This study demonstrates that EA with LC-MS/MS is a practical method for quantifying angiotensin peptide concentrations in healthy horses. The horses in this study had low circulating AP concentrations yet experienced transient activation of both the classical and alternative RAAS pathways after a single dose of furosemide. Previous methodology for measurement of RAAS hormones in horses has been limited by the labile nature of AP, sample handling techniques, and a lack of comprehensive AP quantification assays. High sensitivity LC-MS/MS allows for measurement of low concentrations of circulating

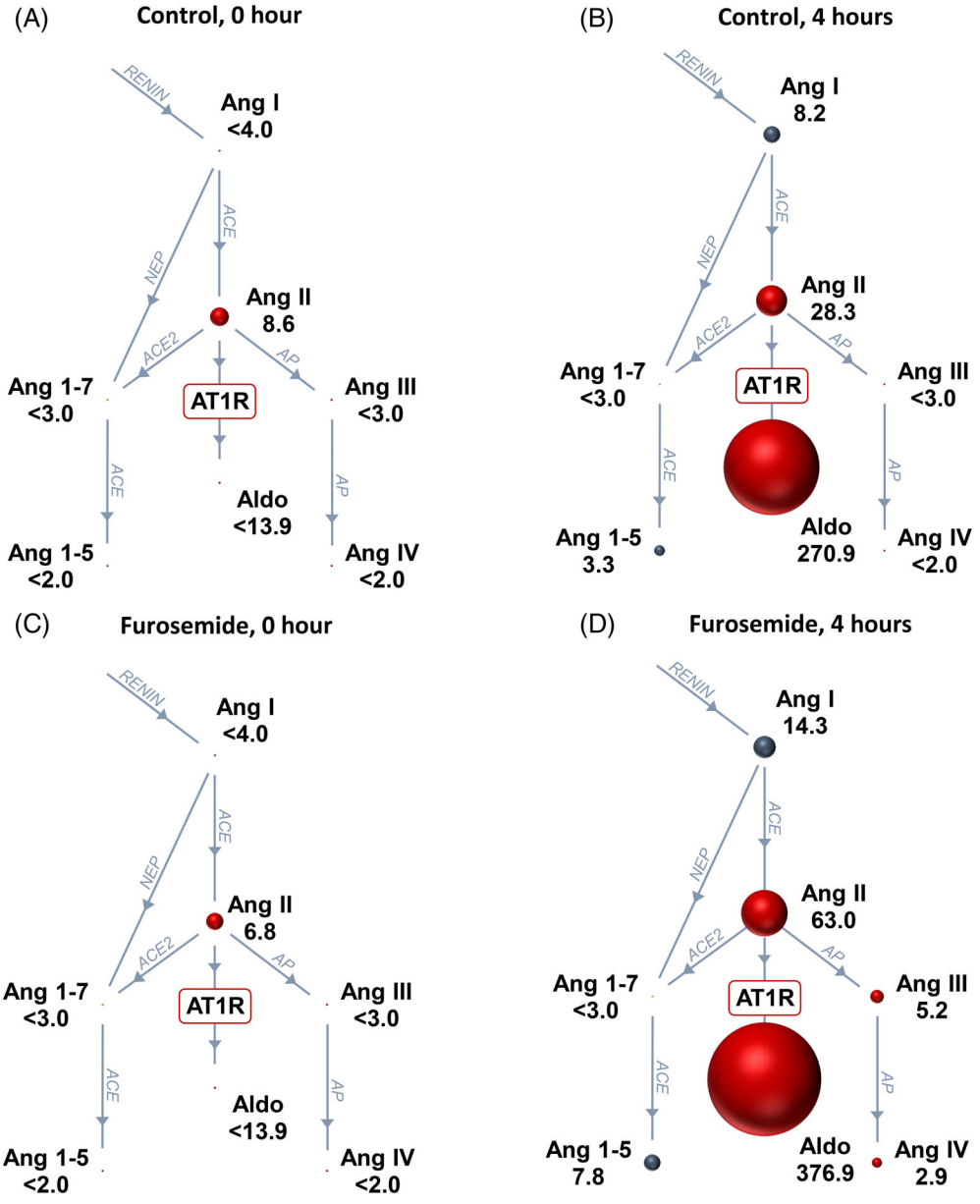

FIGURE 3 (A–D): Serum equilibrium concentrations of angiotensin peptides (RAS-Fingerprint) and aldosterone concentrations in 6 healthy horses receiving furosemide or saline control at baseline (A and C) and 4 hours postdosing (B and D). The sphere sizes are quantitative and represent the amount of peptide determined by liquid chromatography mass spectrometry (LC-MS/MS) with median concentrations (pmol/L) shown next to the spheres. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Aldo, aldosterone; Ang, angiotensin; AP, aminopeptidase; AT1R, angiotensin type-1 receptor; NEP, neutral endopeptidase (neprilysin); RAS, renin-angiotensin system.

hormones which, based on the current results, appears to be relevant in the equine species. Another advantage of LC-MS/MS for AP quantification is its selectivity to identify subtle differences in mass. The amino acid sequences of AP only differ from 1 another slightly and relying on recognition of an epitope in immunoassays is less specific than discriminating APs by mass using LC-MS/MS. This study documents a strong correlation between the EA and PI methods of AngI and AngII quantification. Aldosterone concentration should not be affected by sample handling technique as it is not metabolized by circulating proteases. Compared with the PI methodology, aldosterone concentrations were slightly higher however these calculations are based on a relatively small sample size (10) and given the size of bias (9.5 pmol/L) are likely clinically insignificant. The easier sample handling with the EA approach makes this a practical method for analyzing AP in animals in the clinical and field setting.

Baseline AP concentrations in these horses were low compared with humans and dogs under similar conditions.[7,21] Baseline cRAAS peptide concentrations quantified by analytical techniques such as radioimmunoassay and competitive ELISA have been reported in horses.[9,13,26,27] Previous data in horses only included AngI and AngII and Ald with little documentation on the full angiotensin peptide profile. Studies utilized radioimmunoassay and competitive ELISA for generalized Ang II and Ald quantification in healthy treadmill horses, pulling horses, and endurance horses.[9,13,26] These studies all found higher Ald concentrations when compared with our cohort, and AngII concentrations were orders of magnitude higher in endurance models and pulling models.[9,13,26] As these assays rely on identification of epitopes and kits use different antibodies, it is difficult to directly compare AP and Ald concentrations among these studies. The lower values with LC-MS/MS methods as compared with other assays may reflect its greater specificity. In addition, these studies used fit horses in regular work in contrast to the current horses, which were sedentary and not fit, which may have affected baseline AP concentrations. These studies also used privately owned horses thus activity and feed withholding before sampling was inconsistent. Data in horses suggests exercise induces RAAS activation during exercise and this response is augmented by furosemide administration.[28,29] However, effects of exercise training over time are not reported in horses and data in humans suggests that AP concentrations could decrease with exercise training.[30]

Activation of both arms of the comprehensive RAAS pathway was noted in the current study with the most notable effect occurring at the 4-hour time point. Activation of RAAS was transient which is logical given the relatively short half-life (2.27 hours in healthy horses) of a single IV dose of furosemide.[31] In racehorses, it is recommended to administer furosemide 4 hours before racing activity and the recommended dosage is 0.5 to 1 mg/kg IV for management of exercise-induced pulmonary hemorrhage.[32] Given the current findings, we expect furosemide-induced RAAS activation to occur approximately at the time of race-start. In the racetrack setting, the activation could be more profound given the warm-up exercise period before racing and water withholding, consistent with RAAS activation noted in an incremental exercise model.[9] Activation of RAAS in this study was mild. Whether diuretic administration combined with exercise or higher dosages of diuretic would lead to greater RAAS activation is unknown, yet these could be methods to achieve greater RAAS activation in the horse. There is activation of RAAS in dogs with pharmacologic activation and during naturally occurring cardiovascular diseases such as valvular heart disease.[16,21,33] In people, study of comprehensive RAAS profiles has refined the treatment and prognostic approach for diseases such as hypertension, chronic cardiac disease, and kidney disease.[2-4,6]

Increased cRAAS concentrations were seen in the untreated control group at 4 hours after dosing. Diurnal variations in RAAS hormones occur in many species including horses.[1,20,34] Timing and frequency of feeding also affects the RAAS system where multiple small feedings throughout the day do not cause fluctuations in RAAS activity, and twice daily feedings stimulate RAAS activity for up to 3 hours after each feeding.[34] In the current study, the samples up through 1-hour were fasted samples, and the 4-hour sampling time point occurred approximately 3 hours after feeding, so diurnal variation in association with feed schedule may explain the activation seen in control horses. Efforts were taken to minimize external variables affecting the RAAS system including housing acclimation, catheter placement the evening before study design, and lack of forced exercise in horses during the study period.

Furosemide blocks the resorption of sodium, chloride, and potassium in the thick ascending loop of Henle of the nephron. Furosemide administration in horses decreases serum potassium, chloride and calcium concentrations but not sodium.[35,36] Serum sodium concentration was unchanged in the current study and chloride concentrations were mildly lower in all samples after baseline. Serum potassium was significantly increased as compared to baseline in the furosemide treated group and calcium concentrations were significantly increased as compared to baseline in both the control and furosemide treated groups, although the increases were not considered a clinically relevant change. This difference could reflect the feeding time and lack of water deprivation following furosemide dosing in this study. Significant increases in sodium and decreased potassium are known to occur during a meal, with potassium rising in the postprandial period and likely stimulating aldosterone release.[34] The lack of rise in sodium after the meal may be reflective of total water balance as water was not restricted in the current study, but objective measurements of water intake were not done. Total solids can increase in the 1st hour after administration of IV furosemide in horses withheld from water consumption.[31,36] Total solids were not measured in this study; however albumin was increased in the furosemide group at 1 hour. These changes could have been affected by the lack of water deprivation and free access to water in this study. Creatinine concentration at 4 hours was significantly higher than baseline in the furosemide group. This rise fits or approaches the definition of acute kidney injury in horses (an increase of 0.3 mg/dL within a 48-hour interval).[37,38] Only the horse treated for an acute colic episode had a Crea concentration above of the reference interval. Baseline values before treatment

could be critical to early detection of kidney injury. This finding warrants careful consideration when administering furosemide to dehydrated horses or horses with renal insufficiency.

The reported colic episode in 1 horse after receiving furosemide was unexpected and an uncommon adverse effect secondary to furosemide administration. This horse also had acute kidney injury evidenced by an increase in Crea concentration of 0.6 mg/dL between 1 and 4 hours. The author acknowledges that the increase in Crea concentration could be influenced by the administration of alpha-2 agonist and nonsteroidal anti-inflammatory medications deemed necessary at the time of colic episode.[37] The rise in Crea appeared to be transient with return to baseline Crea by 24 hours postdosing and clinical signs resolved by the 4-hour time point. Per product label, gastrointestinal adverse effects with furosemide may be associated with electrolyte imbalance, which were not considered to be clinically important across time points in the affected mare. Other causes of colic could be considered such as coincidental timing of ovulation, mild acute gastrointestinal vascular event, or other gastrointestinal dysmotility event.

Limitations to this study include small sample size. The availability of water for horses for the duration of the study could have allowed them to compensate for the diuretic-induced fluid loss, thus affecting RAAS activation. Additional monitoring of water intake, urine outputs and urinary concentrations of RAAS peptides could provide additional global information on RAAS activation. Twenty-four–hour time weighted averages from multiple blood concentrations or possibly urine concentrations of aldosterone or AP normalized to Crea could allow for a more wholistic evaluation of RAAS activity. The timing of feeding, occurring after the 1-hour sampling time point, could have influenced RAAS activity. The single-dose furosemide diuretic model does not mimic chronic activation secondary to naturally occurring cardiovascular or kidney disease. This experiment does not replicate the conditions of a racehorse being treated with furosemide before racing.

Baseline AP concentrations are low in healthy horses. Activation of the cRAAS (AngI, AngII, Ang III, and AngIV), and aRAAS (Ang1-5) occurred after a single dose of furosemide. The EA approach was highly correlated to the protease-inhibited approach to AP quantification and the EA approach is a logistically feasible method for further RAAS peptide profile analysis.

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CONFLICT OF INTEREST DECLARATION

Oliver Domenig is a founding member and employee of Attoquant Diagnostics. No other authors declare a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of California, Davis IACUC.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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