Veterinary Clinical Pathology ISSN 0275-6382

ORIGINAL RESEARCH

Initial analytic quality assessment and method comparison of an immunoassay for adrenocorticotropic hormone measurement in equine samples

Katherine L. Irvine[1,2], Kay Burt[1], Andrew J. Hill[3], Stephanie Shaw[4], Kostas Papasouliotis[1,2]

[1]School of Veterinary Sciences, University of Bristol, Langford, Bristol, UK; [2]Diagnostic Laboratories, [3]Equine Centre, Langford Veterinary Services, University of Bristol, Bristol, UK; and [4]TOSOH Bioscience Ltd, The Business Centre, Redditch, UK

Key Words
Endocrine diagnostic techniques, horses, pituitary diseases, validation studies

Correspondence
K. Papasouliotis, School of Veterinary Sciences, University of Bristol, Langford, Bristol BS40 5DU, UK
E-mail: kos.papasouliotis@bristol.ac.uk

DOI:10.1111/vcp.12326

Background: Equine pituitary pars intermedia dysfunction (PPID) may be diagnosed by measuring baseline plasma adrenocorticotrophic hormone (ACTH). The Immulite 1000 analyzer uses an automated chemiluminescence enzyme assay, previously validated for measuring equine ACTH. Recently, an automated bench-top immunoassay analyzer (AIA-360), designed for analytes in people, became available for veterinary use.
Objectives: Objectives were to evaluate analytic performance of the AIA immunoassay for measuring equine ACTH, and compare the results with those obtained by the Immulite.
Methods: Adrenocorticotrophic hormone was measured in plasma samples from 52 clinical cases. For the AIA, within- and between-run coefficients of variation (CV) were assessed, linearity and recovery studies performed, and observed total error (TE_{obs}) calculated. Correlation and agreement between the 2 analyzers were also evaluated.
Results: Within-run and between-run CV of the AIA ranged from 2.3% to 4% and 3.5% to 8%, respectively. ACTH recoveries ranged from 89.5% to 115.9%. TE_{obs} at 26.5 pg/mL ACTH was 4.1 pg/mL. The ACTH results (median: 25.9 pg/mL; range: 4.3–276.7 pg/mL) with AIA were significantly lower ($P < .0001$) than with the Immulite (median: 29.9 pg/mL; range: 10.3–639.0 pg/mL). Correlation between the 2 analyzers was $r = 0.882$ ($P < .0001$), with a significant bias for the AIA of −16 pg/mL. The 2 methods were not identical within inherent imprecision.
Conclusion: The AIA is precise for measuring ACTH in horses. Although correlation between the instruments is good, the values obtained by the immunoassays cannot be used interchangeably and should be interpreted using reference intervals established for each analyzer to avoid false negatives. Diagnostic sensitivity and specificity of the AIA-360 should be evaluated before clinical use.

Introduction

Major advances in diagnostic technology have allowed equine clinics to perform laboratory analyses in house. These are most commonly performed using bench-top hematology and biochemistry analyzers, which aid in the diagnosis of various diseases by generating results immediately.[1–6]

Pituitary pars intermedia dysfunction (PPID) is a common endocrinopathy in horses and ponies usually > 15 years old.[7] Affected horses generally show enlargement of the pituitary gland caused by macro- or microadenomas or hyperplasia, with excessive secretion of adrenocorticotrophic hormone (ACTH) from the pars intermedia caused by progressive loss of dopaminergic inhibition.[8–11] Clinical signs of PPID include recurrent laminitis, polyuria, polydipsia, lethargy, hirsutism, abnormal hair shedding, immune suppression, abnormal fat deposition, and muscle wasting.[8] Although this endocrinopathy is associated with various biochemical abnormalities, measurement of basal plasma ACTH is an accurate

test for diagnosis and for monitoring the response to treatment.[8,12–18]

One of the most common methodologies used for the measurement of ACTH is a nonradioactive chemiluminescent immunoassay.[15,17,19] The analyzers that employ chemiluminescence are expensive to purchase and maintain, however, require a high test volume for economic viability, and are technically demanding. For such reasons these instruments are used by commercial veterinary reference laboratories and are not suitable for in-practice use. Recently, an automated bench-top immunoassay analyzer (AIA-360), originally designed for measurement of various analytes in human blood, has become available for veterinary use.[20]

The aims of this study were (1) to evaluate the precision, accuracy and total observed error (TE_{obs}) of the AIA-360 immunoassay for measuring equine ACTH, and (2) to compare the results generated by the AIA-360 with those obtained by a chemiluminescent immunoassay which has been validated for use in horses.[15] This study provides the first step toward determining the clinical utility of the AIA-360 for equine ACTH measurement.

Materials and Methods

Animals

Equine blood samples submitted to the Diagnostic Laboratories during September 2012 and June 2013 were included in this prospective study. The samples were collected at the Equine Centre or at the owners' premises from horses and ponies following either a routine health check requested by the owners, monitoring the response to treatment for previously diagnosed PPID, or for a work up of a variety of clinical signs.

Samples

Blood was collected by jugular venipuncture into evacuated plastic tubes containing potassium-EDTA (Beckton Dickinson Co, Franklin Lakes, NJ, USA). Samples from animals referred to the Equine Centre were submitted to the Diagnostic Laboratories and refrigerated (4–8°C) immediately or within 1 h following hematologic analysis. Samples collected from animals at the owners' premises were placed immediately in a portable refrigerator and submitted chilled to the Diagnostic Laboratories no later than 3 h post collection. Plasma from all samples was then removed from the cellular fraction using a refrigerated centrifuge (4°C, 2000*g* for 5 min), aliquoted into 2–4 plastic vials and frozen at −20°C within 4 h following the initial blood collection. Samples that were grossly hemolyzed or icteric were excluded. Frozen plasma samples were stored for up to 1 year and used in this study with owners' consent. Two weeks before testing, all aliquoted samples were thawed at 4°C for up to 2 h, realiquoted into 150 μL or 1.5 mL single-use aliquots, and refrozen at −20°C.

On the day of testing, frozen samples were thawed at 4°C over a period of 1 h and kept at room temperature (23°C, range 22–25°C) for up to 10 min before the start of each assay run. All aliquot tubes (polypropylene) and assay cups (AIA-360 analyzer: polystyrene; Immulite: polypropylene) used were consistent.[15] Samples were handled in exactly the same way prior to analysis for both analyzers to ensure minimal introduction of analytic error.

Ideally, samples would have been separated within 1 h of venipuncture, frozen immediately, and analyzed within 14 days in accordance with published human ACTH guidelines.[21] While sample handling in this study was not in accordance with this protocol and therefore ACTH measurements might have been potentially lowered by an unknown magnitude, it was deemed sufficient for this paired sample analytical quality assessment.

Analyzers

The Immulite 1000 assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) employs a solid-phase, 2-site sequential chemiluminescent immunometric assay that uses mouse monoclonal and rabbit polyclonal anti-human ACTH antibodies for detecting ACTH. For each sample the instrument performs 12 measurements; the highest and lowest results are discarded and the remaining measurements are averaged to generate the final ACTH concentration. According to the manufacturer's data sheet, this immunoassay exhibits a small but potentially significant cross-reactivity with the region of human ACTH encompassing amino acids 18–39 (13% at 500 pg/mL; 15% at 5000 pg/mL).[22] This region corresponds to the corticotropin-like intermediate peptide (CLIP), which is a product of ACTH cleavage by prohormone convertase II.[23]

Routine maintenance, instrument preparation, setup, adjustment, assay, and quality control procedures were performed as defined in the Operator's Manual.[24] Two hundred microliters of the sample was placed in the test cup and the first results were available after 60 min. The lower and upper limits of ACTH detection set by the manufacturer are 9 and 1250 pg/mL, respectively. Within-run variation for our

laboratory was determined previously by measuring ACTH in the same sample 10 times sequentially. Three clinical equine samples were used containing low (mean ± standard deviation [SD]: 16.9 ± 1.1 pg/mL), medium (57 ± 3.1 pg/mL), or high (339 ± 14.5 pg/mL) concentration of ACTH. The within-run coefficient of variation (CV) values for these data are 6.5%, 5.4%, and 4.3%, respectively, with a mean of 5.4%.

The AIA-360 analyzer (TOSOH Bioscience GmbH, Griesheim, Germany) utilizes a 2-site immunoenzymometric assay, which is performed entirely within small, single-use plastic test cups containing 12 lyophilized magnetic beads coated with a polyclonal goat anti-human ACTH antibody and 100 μL of a polyclonal goat anti-human ACTH antibody conjugated to bovine alkaline phosphatase. The ACTH within the plasma sample is bound by both the antibody on the magnetic beads and the enzyme-labeled polyclonal antibody. The beads are washed to remove unbound enzyme-labeled polyclonal antibody and are then incubated with the fluorogenic substrate, 4-methylumbelliferyl phosphate. The amount of enzyme-labeled antibody that binds to the beads is directly proportional to the ACTH concentration in the test sample. For each sample the instrument measures the reaction rate 4 times at 20, 60, 130, and 295 s after initiation and before the calculation of the final ACTH concentration. According to the manufacturer's data sheet, this immunoassay exhibits no cross-reactivity with the region of human ACTH encompassing amino acids 18–39 (CLIP).[25] Calibration and daily check and maintenance procedures were carried out as described in the System Operator's Manual.[26] Daily checks included running of manufacturer-supplied human quality control materials (QCM) twice at 2 concentrations (50 and 300 pg/mL). A sample volume of 150 μL was required, and the time to generate the first result was 20 min, with another result every 100 s thereafter. The lower and upper limits of detection set by the manufacturer are 2 and 2000 pg/mL, respectively.

Method comparison

Clinical plasma samples were split into 2 aliquots and run once on each instrument simultaneously and with identical handling, utilizing reagents from the same batch and according to the manufacturer's instructions.[24,26]

Precision

Precision was assessed by within- and between-run repeatability. Within-run repeatability was determined by measuring ACTH in the same sample 10 times sequentially within a single assay run. Three clinical equine samples containing a low, medium, or high concentration of ACTH as measured by the AIA were used. Between-run repeatability was determined by analyzing the same sample once on 5 consecutive working days using the same samples with low, medium, and high concentrations of ACTH.

Accuracy

Accuracy was assessed by performing linearity (reportable range) and recovery studies. Linearity was determined by serial dilution (neat, 1/2, 1/4, 1/8, 1/16, 1/32) of the sample with high ACTH concentration using 0.9% saline as a diluent.[27,28] Neat and diluted samples were then measured once sequentially within the same assay run. A curve representing measured vs expected ACTH concentration was then constructed and linearity assessed over the full range of clinical samples used in this study. Three or 4 replicate samples for each dilution are recommended in the ASVCP guidelines to avoid false rejection of a method, however, if linearity can be demonstrated using single measurements, it is sufficient for analysis.[29,30]

The recovery experiment was performed by diluting the high concentration sample with the low concentration sample (high:low 75:25, 50:50, 25:75, 10:90) and measuring these mixed samples sequentially within the same assay run. Measured and expected ACTH concentrations for each diluted sample were then compared and the recovery percentages calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA). A D'Agostino and Pearson omnibus test for normality showed that both AIA and Immulite data were not normally distributed, therefore data were analyzed using nonparametric testing.

Within- and between-run repeatabilities were expressed as coefficient of variation (CV) following calculation of the mean and SD for each set of results. Linearity was evaluated by plotting measured against expected ACTH concentration, and determining the slope and intercept using simple linear regression. Deviation from linearity was assessed using a Runs test. Absolute and percentage TE_{obs} were calculated by the following equations: TE_{obs} = 2*SD + bias (pg/mL), and TE_{obs} (%) = 2*CV + bias (%).[31]

Bias for the AIA-360 analyzer was determined using manufacturer-supplied QCM containing human ACTH (within a bovine albumin matrix) according to the following equation: bias = (target-measured)/target; "target" was the manufacturer-specified mean value for the QCM and "measured" was the mean QCM ACTH concentration measured by the AIA over a 3.5-month period.[31,32] This method of bias calculation typically produces the lowest bias. Between-run SD and CV for clinical samples were used for the TE_{obs} calculations.[32]

Absolute and relative TE_{obs} at 2 ACTH concentrations ($TE_{obs\text{-}med}$, $TE_{obs\text{-}high}$) were calculated using the bias for "level 1" (mean 50 pg/mL) and "level 2" (mean 300 pg/mL) QCM. Since the 2 QCM samples contained ACTH concentrations similar to those in 2 of the clinical samples (26.5 pg/mL, 278 pg/mL) employed for the repeatability studies, the between-run SD and CV values for these clinical samples were then used for the final calculation of $TE_{obs\text{-}med}$ and $TE_{obs\text{-}high}$, representing TE_{obs} at and above the upper reference limit (29 or 47 pg/mL, dependent on time of year), respectively.[17] TE_{obs} was not calculated for low ACTH concentration since this was not deemed clinically relevant.

Plasma ACTH distributions for the AIA and Immulite were compared using a Wilcoxon rank-sum test. Correlation between the 2 instruments was assessed using Spearman's correlation. Deming regression analysis was used to determine constant (intercept) and proportional (slope) error between the analyzers; error SD for equine ACTH were not known for the Immulite and therefore assumed to be the same for both methods.

A Bland–Altman plot was generated to assess the degree of agreement between the 2 analyzers.[33] Agreement was considered good when there was no real bias or the bias (mean of the differences, AIA-Immulite) was subjectively small, the 95% limits of agreement (mean of differences ± 2SD) were subjectively narrow, and 95% points fell within the limits of agreement. Agreement was also evaluated using acceptance limits that were based on the inherent imprecision of the 2 methods and calculated by applying the interval formula: 0 ± 1.96*combined CV% of the 2 methods. Methods were judged identical when more than 95% of the measurements fell within the acceptance limits.[34]

Results

Horses

Blood samples were collected from 52 horses and ponies (age: 3–27 years) following a routine health check requested by the owners (n = 22), monitoring response to treatment for previously diagnosed PPID (n = 3), or for exhibiting a variety of clinical signs (n = 27). Of the 27 clinical cases, 9 presented with signs indicative of PPID, 4 presented only with lameness, 3 with signs indicative of intestinal colic, 6 with chronic gastrointestinal signs (anorexia, diarrhea, weight loss), 2 with bilateral conjunctivitis, 2 with signs indicative of airway disease, and one with a toe abscess.

Precision and accuracy of AIA-360

The mean within- and between-run CV values with low, medium and high equine samples were within acceptable limits (< 10%) for an immunassay (Table 1). Dilution of the clinical sample with high ACTH concentration resulted in a linear regression equation with R^2 = .997, nonsignificant deviation from linearity over the full range of diluted samples used in this study (Figure 1), and acceptable recovery rates for an immunoassay (Table 2).

Table 1. Within-run and between-run precision using equine clinical samples with high, medium and low concentrations of ACTH measured using the AIA-360.

	Within-Run			Between-Run		
Samples	Mean (pg/mL)	SD (pg/mL)	CV (%)	Mean (pg/mL)	SD (pg/mL)	CV (%)
High	283.4	9.2	3.3	278.3	12.3	4.4
Medium	24.4	0.6	2.3	26.5	0.9	3.5
Low	6.2	0.2	4.0	6.8	0.5	8.0

CV indicates coefficient of variation.

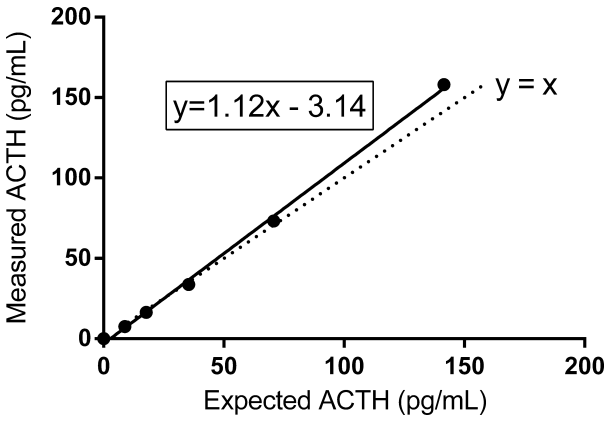

Figure 1. Linear regression for measured vs. expected ACTH concentration with the AIA-360 demonstrated a proportional error of 1.12 (95% CI 1.06–1.19) and constant error of −3.14 pg/mL (95% CI −7.62 to 1.33 pg/mL). The line of identity $y = x$ is shown as a dotted line. CI indicates confidence interval.

Table 2. Recovery of ACTH from equine samples measured using the AIA-360.

% Sample				
High	Low	Expected (pg/mL)	Measured (pg/mL)	Recovery (%)
100	0	283	283	–
75	25	214.5	192	89.5
50	50	146	137.8	94.4
25	75	77.4	76.9	99.3
10	90	36.3	42.1	115.9
0	100	8.9	8.9	–

High = sample with high concentration of adrenocorticotrophic hormone (ACTH); Low = sample with low concentration of ACTH.

The absolute $TE_{obs\text{-}med}$ was below the recommended absolute total allowable error (TE_A) for human samples, whereas the relative $TE_{obs\text{-}high}$ was above the relative recommended TE_A (Table 3).

Method comparison of AIA with immulite using clinical samples

On the Immulite, ACTH results ranged from 4.3 to 276.7 pg/mL (median: 25.9 pg/mL), and on the AIA from 10.3 to 639 pg/mL (median: 29.9 pg/mL). These were statistically highly significantly different ($P < .0001$), although the overall correlation between the results from the 2 analyzers was moderate ($r = .882$; $P < .0001$). Exclusion of the highest ACTH result (AIA: 276.7 pg/mL; Immulite: 639 pg/mL) did not significantly alter the correlation ($r = .875$; $P < .0001$). Deming regression analysis generated a slope (proportional error) of 0.49 (95% CI 0.42–0.56), which did not differ significantly from linearity, and an intercept (constant error) of 12.82 pg/mL (95% CI 5.70–19.95 pg/mL; Figure 2A).

The evaluation of the Bland–Altman difference plot (AIA – Immulite), however, revealed an important mean bias of −16.03 pg/mL (95% CI 118–86 pg/mL), and one value each outside the upper and lower limits of agreement (Figure 2B). Acceptability limits were calculated using the mean within-run CV for the AIA (measured in this study as 3.2%) and Immulite (measured previously as 5.4%) analyzers. Both CV were calculated using single sample methods, therefore the combined CV was 6.3% (formula: $\sqrt{[5.4^2 + 3.2^2]}$).[34] The calculated acceptability range was −12.3% to 12.3%, and only 15/52 or 28.8% of all measurements fell within these limits.

Discussion

To the authors' knowledge, this is the first study in which a bench-top (AIA-360) and a reference (Immulite 1000) analyzer were compared for ACTH measurements in equine clinical samples. The within-run precision of the AIA (3.2%) was comparable to that reported by the AIA manufacturer for measuring ACTH in human patients (2.5%), and even lower than in the Immulite in our laboratory (5.4%), and also than the ones reported in previous equine studies (6% and 9.3%).[15,19] In contrast, the mean between-run precision of the AIA (5.3%) was higher than that reported using human samples (3%), but still lower than the precision previously reported for equine ACTH measurement by the Immulite 1000 (8.1%).[15] Nevertheless, direct comparisons between the various precision studies are difficult due to the use of different protocols.

Because there is no gold standard method for quantifying ACTH in equine samples, and no commercially available equine ACTH reference material, the accuracy of the AIA was assessed indirectly using linearity and recovery experiments as reported in earlier studies.[35,36] A high coefficient of determination ($R^2 = .997$) was generated for ACTH concentrations in a serially diluted sample, with a mean recovery of 99.8%. This was not only within the recommended range of 90–110% but also almost identical with the mean recoveries reported by the AIA manufacturer using human samples (100.1%), and by others (101%) using the Immulite 1000 for measuring ACTH in equine

Table 3. Total observed error (TE_{obs}) for 2 levels of human quality control material (QCM) and equine samples measured using the AIA-360, and published total allowable error (TE_A) for human samples.

QCM Level	Target QCM ACTH (pg/mL)	Measured QCM ACTH (pg/mL)	Clinical Sample ACTH level	TE_{obs} for Equine Samples (pg/mL)	TE_{obs} for Equine Samples (%)	TE_A for Human Samples*
Level 1	50.0	52.2	Low–Medium	4.1	11.3	9.1 pg/mL
Level 2	300.0	324.1	Medium–High	48.8	16.8	10%

*The Royal College of Pathologists of Australasia (RCPA) published values appropriate for the sample ACTH level.[39]

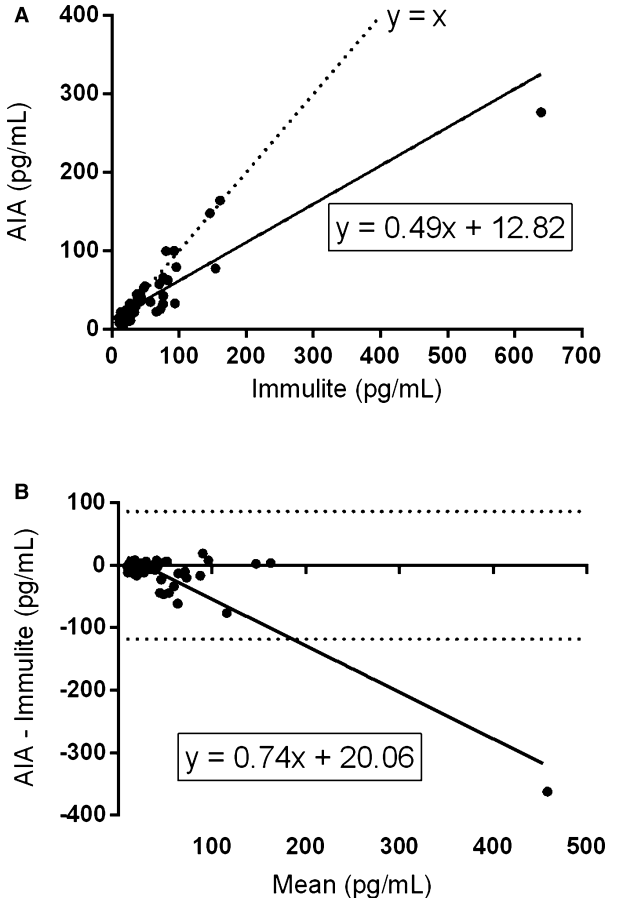

Figure 2. (**A**) Deming regression (solid line) demonstrated a proportional error of 0.49 and constant error of 12.82 pg/mL equine ACTH for the AIA-360 in comparison to the Immulite. The line of identity $y = x$ is shown as a dotted line. (**B**) The Bland–Altman plot revealed a mean bias for the AIA-360 of −16.03 pg/mL and showed a proportional bias of 0.74 for measured equine ACTH in comparison with the Immulite. The 95% confidence interval for upper (mean +2SD) and lower (mean −2SD) limits of agreement are shown as dotted lines.

samples.[15,37] The minimum individual recovery was 89.5%, which is close to 90% and likely of no significance. However, the maximum recovery was 115.9%, which is outside the 90–110% range, the most widely cited range for recovery experiments. Recovery percentages are a reflection of TE_{obs}, and as such must also be compared to TE_A to facilitate interpretation.[30] A recovery of 115.9% equates to 5.8 pg/mL, which may be considered acceptable on the basis of the total allowable error for this concentration of analyte (see below). It is also of note that there was an apparent trend for decreasing recovery as ACTH concentration increased. Linear regression of measured vs expected concentrations with a Runs test for linearity showed that this trend was not significant (data not shown).

Linearity was confirmed throughout the range of ACTH concentrations measured in the clinical samples (AIA: 4.3–276.7 pg/mL; Immulite: 10.3–639 pg/mL), including the range of baseline ACTH values potentially used as cutoff points for the diagnosis of PPID. However, the actual cutoff level (29, 47 or 50 pg/mL) used to diagnose PPID varies with season, reflecting circumannual differences in baseline ACTH level in horses, and also with the desired diagnostic sensitivity.[17,38] This range also included the cutoff concentration of 100 pg/mL used for the thyrotropin-releasing hormone (TRH) stimulation test (see below).

Acceptability of a method during analytical performance assessment is ideally determined using preset analytical quality specifications, such as the TE_A, and a method is considered acceptable when $TE_{obs} < TE_A$.[31] To the authors' knowledge, there is no published information regarding TE_A for ACTH measurement in animals. For human ACTH detection, The Royal College of Pathologists of Australasia (RCPA) has published TE_A values of 2 pmol/L (9.1 pg/mL) and 10% for samples containing low to medium (< 20 pmol/L [91 pg/mL]), and medium to high (≥ 20 pmol/L) concentrations of ACTH, respectively.[39] In our study, the calculated absolute $TE_{obs\text{-}med}$ of 4.1 pg/mL was lower than the TE_A of 9.1 pg/mL reported by RCPA for samples with low to medium concentrations of ACTH. To further assess the acceptability of the AIA method, we calculated TE_{obs} for 2 ACTH concentrations (29 and 50 pg/mL) potentially used as cutoff points for clinical diagnosis of PPID using the Immulite method.[17,38] For interpreting TE_{obs} (and not defining diagnostic cutoff values), inserting these concentrations into the Deming regression equation, which expresses the mathematical relationship between the 2 methodologies ($y = 0.49x + 12.82$; Figure 2A), we estimated that the cutoff values for clinical diagnosis using the AIA system were likely to range from approximately 27 to 38 pg/mL. Application of the $TE_{obs\text{-}med}$ of 11.3% to these values resulted in an absolute TE_{obs} range of 3.1–4.3 pg/mL, which we propose is an acceptable margin of error for the diagnosis of PPID.

An alternative method for PPID diagnosis is a TRH stimulation test, where a post-TRH stimulation ACTH of < 100 pg/mL is considered not diagnostic for PPID. Using this value in the Deming regression equation above generates an estimated post-TRH stimulation decision point for the AIA analyzer of approximately 62 pg/mL. In this study, the calculated $TE_{obs\text{-}high}$ was 16.8%, which is above the TE_A of 10% reported by the RCPA for samples with medium to high concentrations of ACTH. Nevertheless, 16.8% results in an TE_A of 10 pg/mL, which the authors pro-

pose is also an acceptable margin of error. Further analyses defining accurate reference intervals and diagnostic cutoffs for the AIA-360 in PPID diagnosis are needed to calculate diagnostic TE_{obs}, but these results are promising.

Correlation between the 2 analyzers was good (r = .882) but the slope value generated by regression analysis showed that, overall, the AIA results were lower than those obtained by the Immulite for 36/52 (69%) samples analyzed. This observation was confirmed by examination of the Bland–Altman plots, which revealed a significant mean bias of −16 pg/mL with a very wide 95% CI and the presence of a trend for the difference between the 2 results to become more marked as ACTH concentration increased in the samples. Although only one sample (2%) fell outside the 95% limits of agreement on the Bland–Altman plot, the limits of agreement were considered too wide to be clinically relevant (Figure 2B). This sample had a AIA-determined ACTH of 276.7 pg/mL, which was markedly lower than the Immulite-determined ACTH of 639 pg/mL. As instrument or operator error could not be identified, we propose that this marked discrepancy could potentially be explained by detection of CLIP by the Immulite but not the AIA. A study into the distribution of various pituitary peptides in the plasma of normal horses and one horse with PPID demonstrated a 13-fold increase in ACTH in the affected horse, yet a more marked (117-fold) increase in CLIP.[40] This resulted in an approximate ACTH concentration of 420 pg/mL and a combined ACTH+CLIP concentration of 7000 pg/mL. Further investigation is needed into the relative concentrations of ACTH and CLIP in horses with PPID, especially as this may affect the interpretation of data in future studies evaluating various assays for the detection of equine ACTH. Although the sample falling outside the Bland–Altman upper and lower limits of agreement was neither grossly hemolyzed nor lipemic, information on the effect of hemolysis or lipemia on the performance of the AIA analyzer in equine samples is not available. Interference studies are therefore required to investigate this particular aspect further.

We assessed the interchangeability of the AIA and Immulite methodologies using the inherent combined imprecision of the 2 instruments and showed that the 2 methods cannot be considered identical, since 71.2% of the generated measurements fell outside the acceptance limits. This finding may be explained by the 2 fundamental differences between the 2 immunoassays: (1) the Immulite uses one monoclonal and one polyclonal antibody, whereas the AIA employs 2 polyclonal antibodies for measuring ACTH. It is possible that different epitopes between the human and equine ACTH may affect how efficiently antibodies from the 2 analyzers detect equine ACTH. (2) The Immulite measures human CLIP, and therefore potentially equine CLIP, whereas the AIA does not. Although only one sample fell outside the 95% limits of agreement on the Bland–Altman plot, the limits of agreement were considered too wide to be clinically relevant.

There were some limitations in this study. In our study population, only one horse had a very high ACTH result, and 4 samples were > 100 pg/mL. More samples with very high ACTH are therefore needed to fully compare the 2 immunoassays. Twenty-seven of the 52 samples (50%) had ACTH measured by Immulite that were above the current, seasonally adjusted (November to July) decision point of 29 pg/mL for PPID in the UK, while 18/52 (35%) samples generated results > 47 pg/mL (reference value for the remainder of the year). Although these decision points have not been defined in our own laboratory, a sufficient number of samples with increased ACTH have been included in the study for meaningful analysis of results in PPID horses diagnosed using baseline ACTH measurement. There were, however, insufficient clinical samples for method comparison at the cutoff concentration of 100 pg/mL, therefore further analysis is required before the AIA is used for ACTH measurement in the TRH stimulation test.

Although the analyzers measure each sample multiple times to generate an average result, each sample was measured only once on each instrument. While duplicate or triplicate readings would have been preferred, single measurements are acceptable when assessing agreement between analyzers providing this is taken into account when determining acceptability based on combined inherent imprecision.[34] Our $TE_{obs\text{-}med}$ calculation was used to represent TE_{obs} at the likely cutoff level, however, the bias measurement was determined at 50 pg/mL, slightly above the main Immulite cutoff of 29 pg/mL. Ideally a bias at around 29 pg/mL, more in line with the equine CV, should have been used for this calculation to represent TE_{obs} truly at the "cutoff" level. Further analysis using diluted QCM and/or equine specific QCM may be useful to determine this, although it is unlikely this would have any significant effect.

There were also several potential preanalytic limitations in this study. The interference of bilirubin on the immunoassays was not assessed, which may be of particular relevance in a study as this one given the higher bilirubin concentration in normal equine plasma relative to other species including people. Secondly, although human ACTH is sufficiently stable if

plasma is separated within 4 h of collection, data regarding equine ACTH stability are not readily available, and the equine reference interval is much lower than in people.[41,42] Published human protocols also recommend separation of plasma from the cellular fraction within one hour of venipuncture, followed by immediate freezing, and analysis within 14 days.[21] The extended sample handling time and freeze–thaw of the samples in this study may therefore have lowered the measured ACTH concentration. For the specific purposes of the study, this effect was of no clear significance unless a differential effect of sample handling on CLIP relative to ACTH contributed to the disparity between the 2 analyzers. Nevertheless, it is of note that sample handling is a frequent issue in equine ACTH testing, due in part to the often ambulatory nature of equine clinical practice. Yard facilities may limit sample separation at the time of sampling, and veterinary practices performing PPID diagnosis infrequently may follow inconsistent standard procedures in sample handling.

We conclude that, following a short training and familiarization period, the AIA-360 is easy to use, provides results quickly and is simple to maintain. We propose that the instrument is suitable for general practice because its size is similar to that of an in-house hematology analyzer and it employs a wasteless individual test cup technology. Our analytic evaluation studies indicate that the AIA method is precise for measuring ACTH in horses, with an acceptable TE_{obs} in the likely clinical decision point range. The correlation between the 2 instruments is good, although overall the ACTH results are lower with the AIA than with the Immulite. The agreement studies indicate that the values obtained by the 2 immunoassays cannot be used interchangeably and should therefore be interpreted using reference intervals established for each individual analyzer. This is of particular importance for avoiding false negatives since the mean AIA bias of −16 pg/mL is significant. Further studies are required for the establishment of AIA-specific reference intervals, which will allow the evaluation of the diagnostic performance (including sensitivity and specificity) and clinical utility of the AIA for equine ACTH measurement.

Acknowledgments

The study was supported by TOSOH Bioscience Ltd. Part of KLI's junior clinical training scholarship (internship) was funded by TOSOH Bioscience Ltd.

Disclosure: The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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