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Evaluation of an Automated Fluorescence Enzyme Immunoassay for Quantification of Equine Insulin and Comparison to Five Other Immunoassays

Rose D. Nolen-Walston | Jeaneen C. Kulp | Darko Stefanovski | Andrew W. van Eps

Department of Clinical Studies, New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania, USA

Correspondence: Andrew W. van Eps (vaneps@vet.upenn.edu)

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ABSTRACT

Background: Hyperinsulinemia is an important and treatable risk factor for laminitis in horses.

Objectives: Evaluate the Tosoh AIA-360 automated fluorescence enzyme immunoassay for the measurement of serum insulin concentrations in horses, and compare it to five other immunoassays for insulin quantification.

Animals: One hundred serum samples from 83 horses were submitted for insulin measurement.

Methods: The Tosoh AIA-360 was assessed against a reference assay (radioactive immunoassay; RIA). Using the same samples, TOS-FEIA, ELISA, and three chemiluminescent immunoassays (CLIA) were assessed for correlation and agreement with RIA.

Results: The TOS-FEIA showed excellent correlation with RIA ($r^2 = 0.94$, $p < 0.0001$) and good agreement, with a Bland–Altman constant bias (limits of agreement) of −23.8 μIU/mL (−74.6 to 27.0) and Passing–Bablok fit of $y = -8.9 + 0.78x$. Mean coefficients of variation were 1.8% for intra-assay and 5.7% for inter-assay precision, with mean recovery upon dilution of 104.2%. The assay comparison yielded good or excellent agreement (constant bias, limits of agreement) with RIA in the < 100 μIU/mL cohort for the ELISA (−7.0, −21.4 to 7.4) and the Cobas e CLIA (−31.4, −60.9 to −1.6). Spuriously high results (2 to > 10-fold of RIA result) were obtained in approximately 10% of results from both Immulite 2000 and 2000XPi CLIA analyzers, rendering the agreement poor.

Conclusions and Clinical Importance: The TOS-FEIA had acceptable accuracy and precision for clinical use, including at concentrations of insulin < 100 μIU/mL. The ELISA and one CLIA (Cobas e) showed acceptable accuracy, but the Cobas e demonstrated marked bias compared with RIA. Both Immulite CLIA assays exhibited unacceptable accuracy.

1 | Introduction

Equine metabolic syndrome (EMS) is most commonly noted in obese predisposed horse breeds and has been shown to markedly increase the risk of laminitis. Although most fasted healthy horses have blood insulin concentrations < 20 μIU/mL using a variety of assays [1–6], hyperinsulinemia has been described as the most important clinicopathologic feature of EMS [7]. Hyperinsulinemia is assessed by basal testing or dynamic testing identifying the magnitude of insulin response to ingested non-structural carbohydrates. Most clinical decision points are < 100 μIU/mL. For dynamic insulin testing using the 0.15 and 0.45 mL/kg oral sugar tests, diagnostic cutoffs are > 45 and > 65 μIU/mL, respectively, obtained via RIA,

Abbreviations: CI, confidence interval; CLIA, chemiluminescent immunoassay; CV, coefficient of variation; EMS, equine metabolic syndrome; FEIA, automated fluorescence enzyme immunoassay; HBR, heterophilic blocking reagent; LC/MS, liquid chromatography and mass spectrometry; LOA, limits of agreement; RIA, radioimmunoassay; TE_a, acceptable total error; Te_o, observed total error.

according to recent expert consensus [8]. Results above this range warrant clinical intervention using diet or pharmacological treatment. In some instances, hyperinsulinemia can be considered a medical emergency, with experimental models of insulin infusion causing laminitis within hours [9, 10]. As a result, a rapid, accurate test that shows acceptable precision at concentrations < 100 μIU/mL has clinical utility for equine clinicians.

The quantification of insulin, both in horses and other species, can be problematic, with a variety of assay techniques that yield markedly different results with regard to both precision and constant as well as proportionate bias [11–13]. Equine insulin is measured using a variety of techniques, but assessment of accuracy is impeded by the lack of an international equine insulin reference standard. Insulin exhibits good homology across species, and radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) designed for samples from humans were validated in early studies of horses, although both yielded concentrations that were a fraction of those obtained using liquid chromatography and high-resolution/high-accuracy mass spectrometry (LC–MS), the gold standard [14, 15]. Although considered both accurate and precise, LC–MS historically has been cumbersome because it relies on antibody affinity extraction (which limits its clinical application for routine high-throughput analysis of larger peptides such as insulin). Subsequently, RIA (including Porcine Insulin RIA, Millipore, St. Charles, MO, USA; DSL-1600 insulin RIA, Diagnostic Systems Laboratories Inc., Webster, TX, USA; Coat-a-Count Insulin, Siemens Healthcare, Camberley, Surrey, UK [no longer available]) has been used in most method comparison studies as the de facto reference technique, although it is becoming less commonly used in clinical laboratories because of substantial regulatory burdens of radioisotope acquisition and use. Since then, a variety of modalities have been used to analyze insulin in samples from horses, including multiple RIA, chemiluminescent immunoassay (CLIA), ELISA, and lateral flow assay methods [11–13, 16–20]. However, these methods yield such divergent results that models to harmonize results among assays have been developed to allow clinicians to compare results across methodologies and analyzers.

We aimed to validate a novel automated fluorescence enzyme immunoassay (FEIA), the Tosoh AIA-360 (Tosoh Bioscience, Tokyo, Japan) for quantification of insulin in equine serum, and then compare its performance to 5 other assays of equine insulin, especially at clinically important concentrations of < 100 μIU/mL, using RIA as the reference assay.

2 | Methods

Study material consisted of 100 blood samples from 83 horses hospitalized at the University of Pennsylvania School of Veterinary Medicine's large animal hospital (New Bolton Center), submitted to the clinical laboratory for routine insulin measurement (either baseline or post-carbohydrate challenge). Blood was collected in plain evacuated tubes and allowed to clot at room temperature for 30 min, followed by centrifugation (1300 × *g*) for 10 min. Serum was harvested, transferred to 2 mL cryovials, and stored at −80°C until analysis. Each aliquot was used for a single assay.

2.1 | Immunoassay Comparison

All samples were assayed for insulin concentration using six different methods that currently are commercially available for the quantification of equine insulin, either for clinical or research application (Table 1). An insulin–specific RIA designed for use in humans using guinea pig anti-human insulin–specific antibody and validated for use in horses (Millipore RIA, Cornell Animal Health Diagnostic Center, Ithaca, NY) was assigned as the reference assay. Samples also were analyzed using the following methods: an automated FEIA (Tosoh 360-AIA) performed at New Bolton Center (University of Pennsylvania, Kennett Square, PA); two similar CLIA assays (Immulite 2000 and Immulite 2000xpi, Siemens, Washington DC), performed at New Bolton Center (University of Pennsylvania, Kennett Square, PA), and Cornell Animal Health Diagnostic Center (Ithaca, NY), respectively; a third CLIA (Cobas e, Roche Diagnostics, Indianapolis, IN) performed at The Diabetes Research Center, University of Pennsylvania, PA; and an ELISA (Mercodia, Uppsala, Sweden) performed at New Bolton Center (University of Pennsylvania, Kennett Square, PA). Because of limitations in sample volume, not all samples were analyzed using all assays.

2.2 | Tosoh AIA-360 Validation

The Tosoh AIA-360 analyzer (TOS-FEIA) was installed and maintained according to manufacturer instructions. Daily quality control was performed using commercial 3-level quality control materials (Lyphochek Immunoassay Plus Control, Bio Rad, Hercules CA). Intra-assay variability (TOS-FEIA) was assessed using three banked serum samples, with low (< 5 μIU/mL), medium (20–80 μIU/mL) and high (> 100 μIU/mL) insulin concentrations based on their original assay results measured using RIA. Ten replicates were run on each sample using the same test cup lot and operator. Inter-assay variability was assessed using five serum samples with insulin concentrations ranging from < 10 to > 250 μIU/mL. These were then assayed on five separate days using five different test cup lots, with the same operator. To assess recovery upon dilution and dilutional linearity, five banked serum samples with insulin concentrations ranging from 84.9 to 320 μIU/mL were aliquoted and then diluted to 66.7% (2:1), 50% (1:1), 25% (1:4), and 16.7% (1:6) concentrations. Dilution was performed using two diluents, using either Tosoh immunoassay reagent diluent (Tosoh America, Grove City, OH) or charcoal-stripped serum (CSS; Valley Biomedical, Winchester VA). Assays were performed on undiluted aliquots with each set of serial dilutions in the same batch (and test cup lot) and the same operator. Expected concentration was compared to the measured assay result, percentage recovery was calculated, and dilutional linearity assessed.

2.3 | Data Analysis

Continuous data were assessed using a Shapiro–Wilk test of normality, and non-parametric summary statistics (medians

TABLE 1 | Six assays used to compare the quantification of insulin in equine serum samples, their methodology (CLIA: chemiluminescent immunoassay; FEIA: automated fluorescence enzyme immunoassay; RIA: radioimmunoassay), the laboratory in which they are run, the assay design, and the number of samples analyzed by each method.

Method	Analyzer	Laboratory	Assay design	Number of samples assayed
RIA	MerckMillipore radioimmunoassay, Millipore Sigma, Burlington, MA	Animal Health and Diagnostic Lab, Cornell University, Ithaca, NY	Primary antibody: Guinea pig anti-human insulin Secondary antibody: Goat anti-guinea pig IgG Principle: Competitive assay	$N=100$
FEIA	Tosoh AIA-360, Tosoh Bioscience, Tokyo, Japan	New Bolton Center, University of Pennsylvania, Kennett Square, PA	Primary antibody: Mouse monoclonal anti-insulin Secondary antibody: Mouse monoclonal anti-insulin Principle: Sandwich assay	$N=99$
ELISA	Insulin ELISA, Mercodia, Uppsala, Sweden	New Bolton Center, University of Pennsylvania, Kennett Square, PA	Primary antibody: Mouse monoclonal anti-insulin Secondary antibody: Mouse monoclonal anti-insulin Principle: Sandwich assay	$N=94$
CLIA	Cobas e, Roche Diagnostics, Indianapolis, IN	Diabetes Research Center, University of Pennsylvania, Philadelphia PA	Primary antibody: Mouse monoclonal anti-insulin Secondary antibody: Mouse monoclonal anti-insulin Principle: sandwich assay	$N=100$
CLIA	Immulite 2000, Siemens, Washington DC	New Bolton Center, University of Pennsylvania, Kennett Square, PA	Primary antibody: Mouse monoclonal anti-insulin Secondary antibody: Polyclonal sheep anti-insulin conjugated to mouse monoclonal anti-insulin Principle: Sandwich assay	$N=86$
CLIA	Immulite 2000 XPi, Siemens, Washington DC	Animal Health and Diagnostic Lab, Cornell University, Ithaca, NY	Primary antibody: Mouse monoclonal anti-insulin Secondary antibody: Polyclonal sheep anti-insulin conjugated to mouse monoclonal anti-insulin Principle: Sandwich assay	$N=100$

and ranges) were used. Frequency counts and percentages were used for summarizing categorical variables. Coefficients of variance were calculated for the intra- and inter-assay precision studies and percent recovery for the dilution study. Dilutional linearity (expected versus measured concentration with serial dilution) was assessed using linear regression and goodness-of-fit (R^2) for both diluents. Agreement between the reference assay (RIA) and the TOS-FEIA assay was assessed using Bland–Altman plots to show bias and limits of agreement (LOA). Passing–Bablok regression [21] was selected as a robust, nonparametric method for fitting a straight line to data from two variables and does not make any assumptions about the distributions of the samples or their measurement error. Using published diagnostic cutoffs [8], the sensitivity and specificity of the TOS-FEIA were calculated using both the raw results as well as results standardized to RIA results for constant and proportional bias using the Passing–Bablok line of regression.

Spearman's rank correlation was assessed between the reference assay (RIA) and each additional assay. Agreement was assessed using Bland–Altman plots to show bias and 95% LOA. Passing–Bablok regression was performed to evaluate overall agreement and quantify systematic bias (intercept) and proportional bias (slope) of the experimental assay. This linear equation was then utilized to create a conversion factor between assays. All tests were performed both with the entire data set and additionally only for samples falling in the most clinically important range of $<100\,\mu$IU/mL, as measured by RIA. Analyses were conducted using Stata 18MP (StataCorp, College Station TX) and Prism 10 (GraphPad Software, Boston, MA), with two-sided tests of hypotheses and a p value <0.05 as the criterion for statistical significance.

3 | Results

3.1 | Tosoh AIA-360 Validation

Intra-assay precision testing generated an overall mean intra-assay coefficient of variation (CV) of 1.8% (Table 2); inter-assay precision testing produced a mean CV of 5.7% across five different runs on different days (Table 3). The mean recovery of samples diluted with CSS was 111.9% (range, 98.9%–139.3%), with a dilutional linearity R^2 of 0.9956 ($p<0.0001$; Figure 1). With Tosoh diluent, the mean recovery was 104.2% (range, 92.0%–117.7%) with R^2 of 0.9971 ($p<0.0001$).

The TOS-FEIA showed good correlation ($r_s=0.96$, $p<0.0001$) with RIA across a range of sample insulin concentrations of 11.7–318 μIU/mL as well as in the subset of samples with insulin concentrations $<100\,\mu$IU/mL ($r_s=0.92$, $p<0.0001$; Table 4). Bland–Altman plots of the full dataset indicated a constant bias (SD) of −23.79 (25.91) and 95% LOA of −74 to 27. For samples $<100\,\mu$IU/mL, the constant bias (SD) was lower, at −16.66 (9.41) with 95% LOA of −35.11 to 1.80 (Figures 2 and 3, Table 5). Tests of agreement using Passing–Bablok regression indicated a constant bias of −8.9 (95% confidence interval [CI], −11.7 to −5.6) and proportional bias of 0.78 (95% CI, 0.71, 0.88) for the full dataset (Figure 4). When limited to samples with insulin concentrations $<100\,\mu$IU/mL, constant and proportional bias were −10.1 (95% CI, −13.1 to −7.2) and 0.85 (95% CI, 0.75–0.95), respectively (Figure 5).

TABLE 2 | Intra-assay precision for the Tosoh 360-AIA Fluorescence Enzyme assay was assessed using three banked equine serum samples of varied insulin concentration (as measured with radioimmunoassay), including low (< 5 μIU/mL), medium (20–80 μIU/mL), high (> 100 μIU/mL).

Replicate #	Low	Medium	High
1	3.7	32.2	198.2
2	3.7	31.9	202.0
3	3.9	31.6	197.6
4	3.9	32.1	193.4
5	3.8	31.7	199.8
6	3.9	31.2	194.8
7	3.8	31.3	200.9
8	3.7	31.1	197.9
9	3.9	32.2	199.7
10	4.0	31.4	197.4
Mean	3.8	31.7	198.2
SD	0.1059	0.4165	2.6293
CV%	2.8%	1.3%	1.3%

Note: Ten consecutive replicates were run on each sample using the same plate and operator. Mean, standard deviation (SD) and coefficient of variation (CV%) were calculated for each concentration.

Using the inter-assay CV and the y-intercept (constant bias) from the Passing–Bablok regression analysis, the total observable error ($TE_o=|bias|\ (\%)+2CV$) [22] for the TOS-FEIA was 20.3% for all samples and 21.5% for the samples with insulin concentrations $<100\,\mu$IU/mL. Sensitivity and specificity of the TOS-FEIA at two commonly used cutoffs for the diagnosis of insulin dysregulation after the oral sugar test (45, 65 μIU/mL) were assessed using both raw values and values equivalent to the RIA using the fit yielded by Passing–Bablok regression ($y=-8.9+0.78x$; Table 6).

3.2 | Immunoassay Comparison

Results from the RIA reference assay were plotted against each of the five comparators, and Spearman rank correlation coefficients (r_s) were reported for the complete dataset as well as the cohort of samples with insulin concentrations $<100\,\mu$IU/mL. This analysis showed good to excellent correlation for TOS-FEIA, ELISA, and Cobas e, and poor correlation between RIA and both the Immulite 2000 and immulite 2000 XPi (Table 4). Agreement was assessed using Bland–Altman analysis (Figures 2 and 3, Table 5) and Passing–Bablok regression (Figures 4 and 5), both for all samples and samples with insulin concentrations $<100\,\mu$IU/mL. The poor correlation and agreement results noted for the Immulite were associated with

TABLE 3 | Inter-assay precision for the Tosoh 360-AIA Fluorescence Enzyme assay was assessed using five banked equine serum samples with insulin concentrations of < 10 to > 250 μIU/mL (RIA).

Sample#	Day 0	Day 2	Day 67	Day 95	Day 186	Mean	SD	CV%
1	9.7	9.8	9.0	8.1	9.7	9.3	0.723	7.8%
2	20.9	19.2	17.0	18.5	20.3	19.2	1.535	8.0%
3	70.5	65.3	65.6	59.4	63.9	64.9	3.977	6.1%
4	175.5	174.6	168.2	167.9	170.0	171.2	3.584	2.1%
5	304.9	291.6	322.1	290.7	316.1	305.1	14.139	4.6%

Note: Frozen aliquots were thawed and assayed on 5 separate days, using 5 test cup lots with the same operator. Mean, standard deviation (SD) and coefficient of variation (CV%) were calculated for each sample.

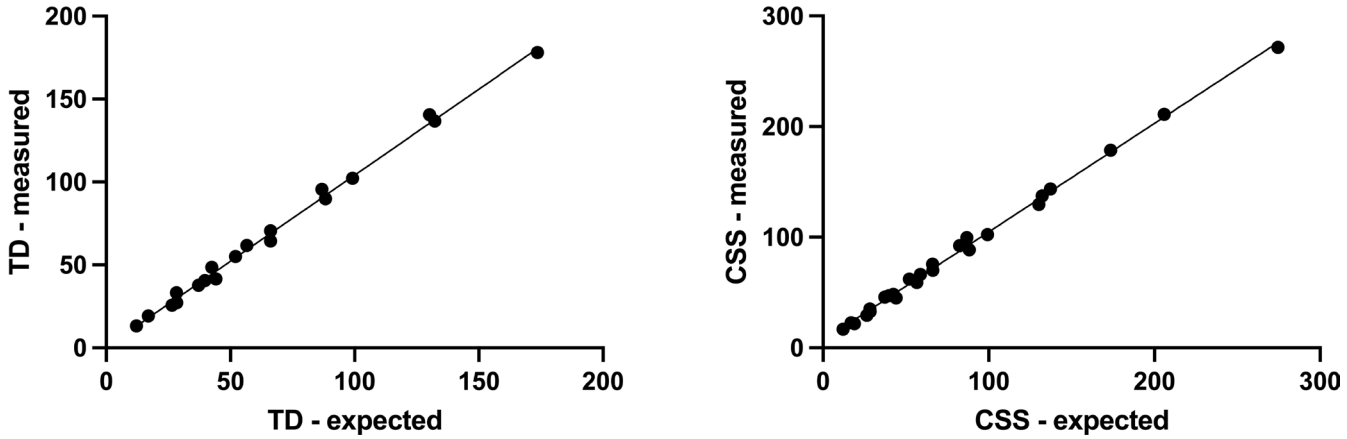

FIGURE 1 | Linearity of insulin concentrations of five equine serum samples measured with the Tosoh 360-AIA Fluorescence Enzyme Immunoassay, serially diluted with either charcoal-stripped serum (CSS) or Tosoh immunoassay reagent diluent (TD). Measured values versus expected insulin concentrations reveal Goodness-of-fit of R^2 of 0.9956 ($p < 0.0001$) for dilution with TD, and R^2 of 0.9971 ($p < 0.0001$) with CSS.

TABLE 4 | Spearman correlation coefficients between radioimmunoassay and five other immunoassays for the quantification of insulin in equine serum samples. Results are displayed for the entire dataset ($n = 100$; r_s all) and samples with insulin < 100 μIU/mL by RIA ($n = 72$; $r_s < 100$).

	r_s (all)	p	r_s (<100)	p
Tosoh AIA-360	0.96	< 0.0001	0.92	< 0.0001
Mercodia ELISA	0.94	< 0.0001	0.94	< 0.0001
Cobas e	0.99	< 0.0001	0.98	< 0.0001
Immulite 2000	0.67	< 0.0001	0.40	0.0011
Immulite 2000 XPi	0.69	< 0.0001	0.45	0.0001

a subset of samples that had markedly higher insulin concentrations using both Immulite analyzers compared with RIA. In these cases, insulin concentrations that were 2 to > 10 times the RIA results were noted in 10.9% (7/64) of Immulite 2000 results and 10.5% (8/76) of Immulite 2000 XPi samples in samples with insulin concentrations < 100 μIU/mL (Figure 5).

4 | Discussion

In our method validation experiment, we found that the Tosoh 360-AIA FEIA equine insulin immunoassay showed clinically acceptable agreement and accuracy when compared to RIA. Although banked frozen samples were used for the study, equine insulin shows good stability for short periods at room temperature and with refrigeration [16] and after freezing and thawing [11, 16]. The TOS-FEIA uses a light-emitting diode illuminant, non-flow cell photometry method, with a maximum throughput of 36 tests per hour, yielding results in approximately 20 min. Additionally, the analyzer has a small footprint, is cost-effective even for small batches, and is technically simple to maintain and run. It performed with acceptable accuracy for the diagnosis of hyperinsulinemia at clinically important insulin concentrations of < 100 μIU/mL, which encompasses the commonly used decision cutoffs that are used to designate insulin dysregulation both in baseline samples and after carbohydrate challenge tests. The results showed a constant bias of approximately −17 to −24 μIU/mL compared to the RIA, but correlation with RIA was high, suggesting that either instrument- or method-specific reference intervals should be used for results generated by this analyzer, or that these results should be mathematically harmonized to be congruent with RIA results, as suggested previously [23].

The measurement of hormones is complicated by several intrinsic factors, the most important of which is that they are present

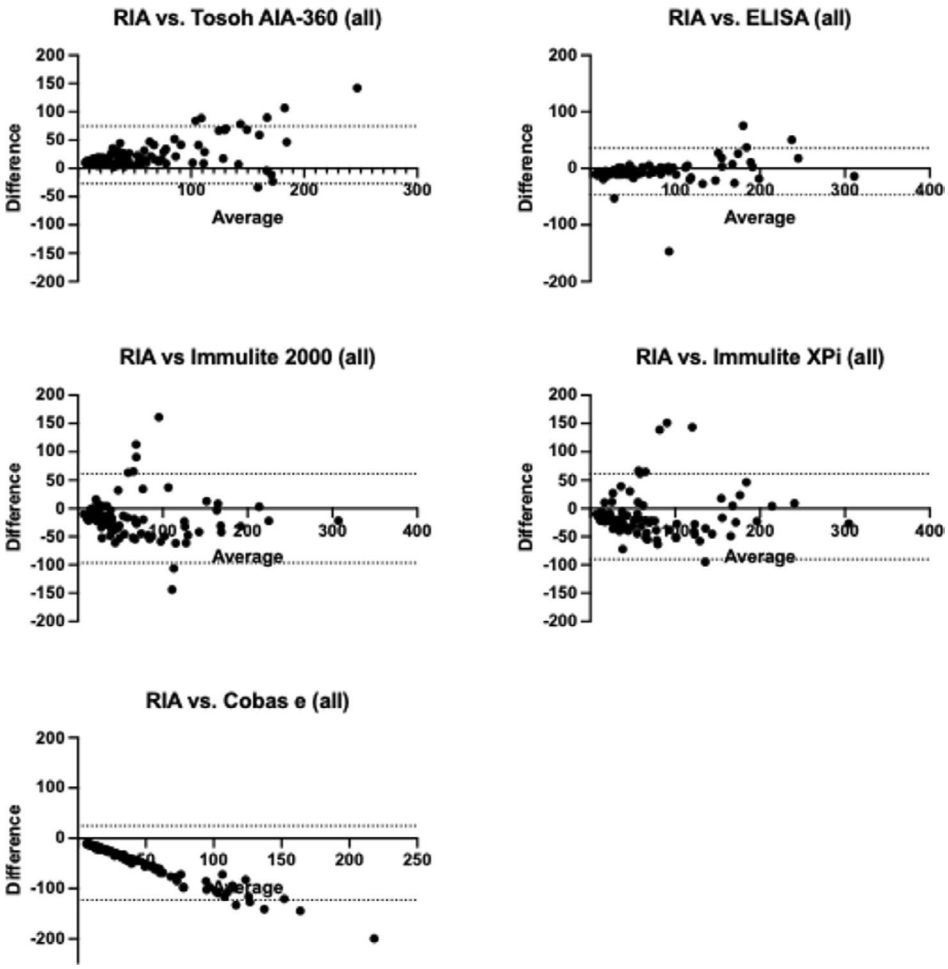

FIGURE 2 | Bland–Altman plots of the absolute difference between insulin concentrations of equine serum ($n = 100$) analyzed via radioimmunoassay (RIA) with values of 11.7–318 μIU/mL, versus automated fluorescence enzyme immunoassay (Tosoh AIA-360; $n = 99$), Mercodia ELISA ($n = 94$), and three chemiluminescent immunoassay methods: Cobas e ($n = 100$), Immulite 2000 ($n = 86$), and Immulite 2000 XPi ($n = 100$).

in the blood in very low concentrations. For example, most peptide hormones are found in the serum of healthy humans at concentrations of 1–50 picomoles per liter, which is 10–100 million times lower than the concentration of albumin, approximately 0.7 mol per liter [24]. Despite this challenge, for an assay to be clinically valid, it must generate results that are precise and accurate enough to allow effective clinical decision making. This goal is described by the assigned value of total allowable error (TE_a), defined as a "quality goal that sets a limit for combined imprecision (random error) and bias (inaccuracy, or systemic error) that is tolerable in a single measurement" [22]. For any test, TE_a is based ideally on clinical outcomes but in most situations such data are lacking, and the value is derived from clinician opinion, professional recommendations, or regulatory and proficiency testing. Ideally, biologic variation also is considered (i.e., how much the measurement varies within a subject around a homeostatic set point) [25], and this factor is considered along with analytical variation, as described by the total observable error (TE_o) of the assay itself, calculated as ($|\text{bias}|\ [\%] + 2\text{CV}$) [22]. For an assay to be clinically useful, the total observable error must be less than the total allowable error. In laboratories measuring insulin in humans, TE_a for insulin ranges from 6% to 32% [22, 26], which suggest the values of Te_o of approximately 20% obtained in our study may fall within acceptable limits. However, the bias used in this calculation is compared to RIA, which is in itself markedly biased when compared to LC/MS. These factors make the interpretation of TE_o and TE_a difficult.

As recognition of the importance of insulin measurement in horses grows, the need for fast, accurate testing and standardization of results increases. Hyperinsulinemia can be considered a medical emergency, and rapid results (ideally < 6 h) can facilitate interventions that may be lifesaving if the development of laminitis is to be averted. The ideal test should be accurate, with prompt turnaround, technically simple to run, and inexpensive even for small batches. Because of the lack of an international insulin standard or true gold standard for horses, the absolute accuracy of the TOS-FEIA (or any of the other assays) was not assessed. Weighing the qualities of the tests evaluated, RIA offers the advantage of being functionally considered the reference assay, and many published diagnostic ranges are derived from this methodology. However, in an early study, RIA produced results that were approximately one-third of the result produced by the gold standard of LC/MS analysis [12]. Although it is considered overall reliable, RIA requires specialized equipment, its radioactive reagents impose a regulatory burden, and it is therefore often not run on a daily basis in laboratories that offer it, increasing turnaround time.

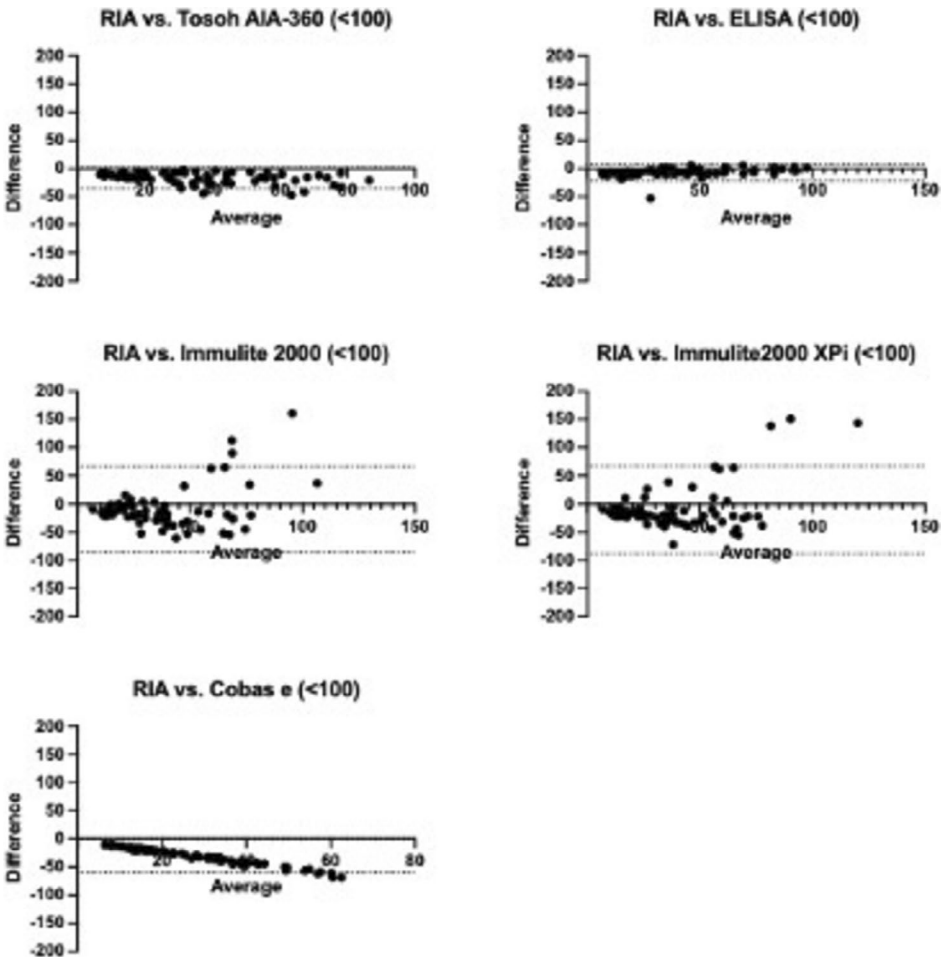

FIGURE 3 | Bland–Altman plots of the absolute difference of a cohort of equine serum samples with insulin concentrations of $<100\,\mu$IU/mL ($n=72$) analyzed via radioimmunoassay (RIA), versus automated fluorescence enzyme immunoassay (Tosoh AIA-360), Mercodia ELISA, and three chemiluminescent immunoassay methods: Cobas e, Immulite 2000, and Immulite 2000 XPi.

TABLE 5 | Bland Altman bias (standard deviation) and 95% limits of agreement (LOA) between radioimmunoassay (RIA) and five other immunoassays for the quantification of insulin in equine serum samples. Results are displayed for the entire dataset ($n=100$; (all)) and samples with insulin concentration $<100\,\mu$IU/mL by RIA ($n=72$; (<100)).

	Bland–Altman bias (SD; all)	Bland–Altman 95% LOA (all)	Bland–Altman bias (SD; <100)	Bland–Altman 95% LOA (<100)
Tosoh AIA-360	23.8 (25.9)	−74.6, 27.0	−16.7 (9.4)	−35.1, 1.8
Mercodia ELISA	−5.5 (21.0)	−46.7, 35.7	−7.0 (7.3)	−21.4, 7.4
Cobas e	−49.4 (37.5)	−122.9, 24.2	−31.3 (15.1)	−60.9, −1.6
Immulite 2000	−17.7 (40.1)	−96.4, 61.0	−9.6 (38.7)	−85.5, 66.3
Immulite 2000 XPi	−14.6 (38.6)	−90.2, 60.9	−10.4 (39.6)	−88.1, 67.3

The Mercodia ELISA showed a small, negative constant bias and narrow LOA when compared with RIA, with rare outliers. It takes approximately 3 h to complete the assay, and therefore samples usually have to be submitted by noon for same-day results in our laboratory. Additionally, it is expensive for small numbers of samples because of the need to dedicate wells for the measurement of standards and requires specific expertise to perform. However, given its excellent accuracy, this assay is used for confirmation of unexpected results derived from more rapid, less expensive assays such as the TOS-FEIA and can be used as an internal standard, especially when results from other testing modalities are discrepant. The Cobas e CLIA showed the most precise correlation with the RIA, but also showed marked constant and proportional bias, often returning values 50% lower than RIA on the same sample. Although the data it produces appear reliable, the results would either need to be standardized to standard RIA units or assay-specific reference values derived. Immulite analyzers are used in many commercial laboratories

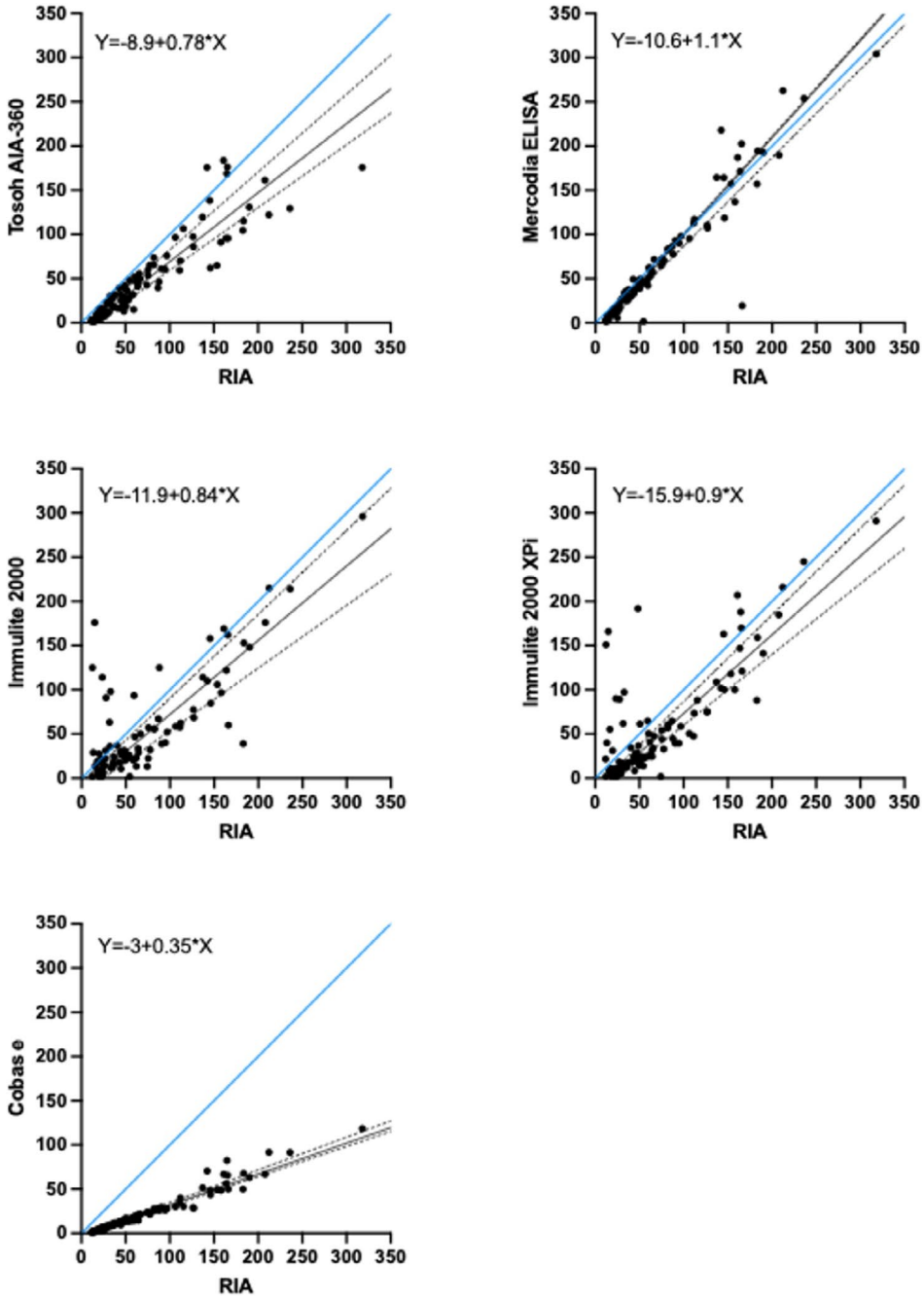

FIGURE 4 | Passing–Bablok regression of insulin concentrations of equine serum ($n = 100$) analyzed via radioimmunoassay (RIA) with values of 11.7–318 μIU/mL, versus automated fluorescence enzyme immunoassay (Tosoh AIA-360; $n = 99$), Mercodia ELISA ($n = 94$), and three chemiluminescent immunoassay methods: Cobas e ($n = 100$), Immulite 2000 ($n = 86$), and Immulite 2000 XPi ($n = 100$). The solid black line represents the line of best fit (dashed lines, 95% confidence interval), and the blue line identifying perfect agreement. In this figure, the *x*-intercept indicates constant bias, and the slope, proportional bias.

because they can run a wide variety of endocrine tests and have a low cost per sample with a throughput of up to 200 tests per hour. However, neither of the Immulite CLIA analyzers performed acceptably for clinical use. The low correlation and agreement between these assays and RIA can be explained by a certain amount of general imprecision, but mostly by occasional inappropriately high results. As noted, results 2 to > 10 times the concentration obtained by RIA were noted in approximately 10% of samples with insulin concentrations < 100 μIU/mL. For example, a sample with an insulin concentration of 14.74 μIU/mL on RIA was reported as 176 μIU/mL by the Immulite 2000 and 166 μIU/mL by the Immulite 2000 XPi, an almost 12-fold difference. Incidentally, each of the other three analyzers reported values < 5 μIU/mL for this sample.

The explanation for the occasionally aberrantly high results generated by both Immulite CLIA analyzers is unknown. This finding has not been reported in other insulin assay comparison studies for horses using the Immulite 2000 [27, 28] or Immulite 2000XPi [28, 29]. Immunoassays are at risk of

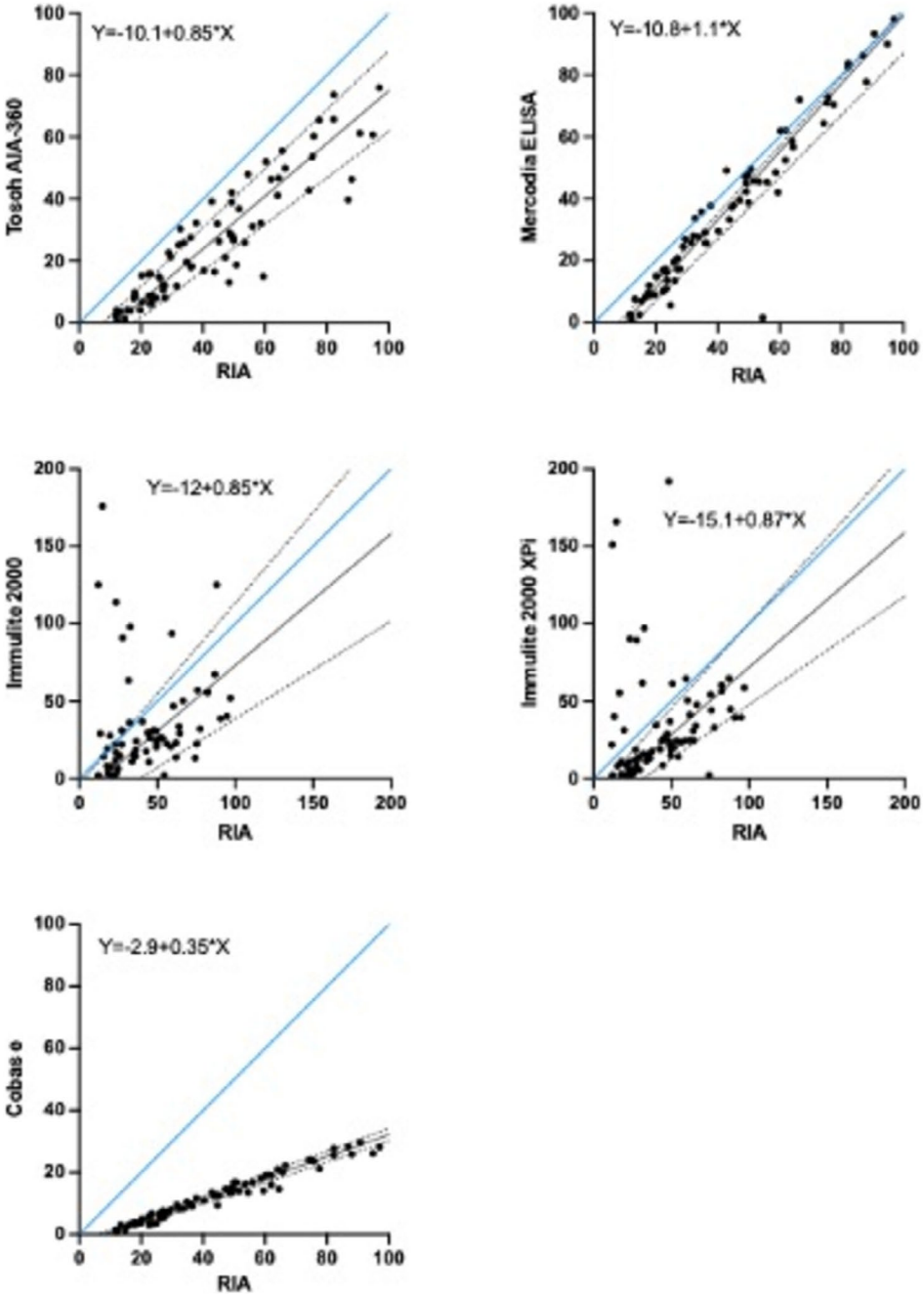

FIGURE 5 | Passing–Bablok regression of a cohort of equine serum samples with insulin concentrations of $<100\,\mu IU/mL$ ($n=72$) analyzed via radioimmunoassay (RIA), versus automated fluorescence enzyme immunoassay (Tosoh AIA-360), Mercodia ELISA, and three chemiluminescent immunoassay methods: Cobas e, Immulite 2000, and Immulite 2000 XPi. The solid black line represents the line of best fit (dashed lines, 95% confidence interval), and the blue line identifying perfect agreement. In this figure, the *x*-intercept indicates constant bias, and the slope, proportional bias.

interference from related hormones or hormone-binding proteins. In this case, we believe these are unlikely to be related to insulin (e.g., proinsulin or C-peptide) or isometrically similar molecules from a different family, although this possibility cannot be excluded. Another possibility is that these patients have formed heterophilic antibodies only recognized by the Immulite assays. Heterophilic antibodies are formed by exposure to external antigens that bind immunoglobulins of other species and have been implicated in interference in a wide range of immunometric tests [30]. One case report described an erroneously increased immunoassay insulin concentration in a child. The aberrant result eventually was found to be caused by interference from a human antimouse antibody (HAMA 181 ng/mL) [31]. Remarkably, heterophilic (antimouse) antibodies are detected in 11.7% of human hospital inpatients [32], but also in 8.7% of samples from a hospital population of horses [33], and in 4.7% of healthy horses [34]. These proportions are somewhat similar to the percentage of samples we noted with spuriously high values (approximately 10%) in this study. Heterophilic blocking reagents (HBR) composed of murine immunoglobulin are routinely used in human medical laboratories to negate the effect of these antibodies and have been noted to be variably effective in both studies of horses, with chicken immunoglobulin Y [33] and rabbit or

TABLE 6 | Sensitivity and specificity of the Tosoh 360-AIA Fluorescence Enzyme assay at two clinically important decision cutoffs, 45 and 65 μIU/mL, for dynamic insulin testing using the 0.15 and 0.45 mL/kg oral sugar tests, respectively.

Cutoff > 45 μIU/mL		
Raw	Sensitivity	66.7%
	Specificity	100.0%
Corrected	Sensitivity	82.5%
	Specificity	88.1%
Cutoff > 65 μIU/mL		
Raw	Sensitivity	71.4%
	Specificity	100.0%
Corrected	Sensitivity	94.6%
	Specificity	93.5%

Note: Values are calculated against results from reference radioimmunoassay (RIA) using equine serum samples ($n = 72$) with insulin < 100 μIU/mL. Sensitivity and specificity were calculated with both unmodified values (raw), as well as those values standardized to the RIA units (corrected) using the Passing–Bablok regression formula $y = -8.9 + 0.78$ derived from the same data.

mouse immunoglobulin [34] used as a HBR in anti-Müllerian hormone and equine growth factor ELISA assays, respectively.

In conclusion, we found that the Tosoh AIA-360 FEIA fulfills important desired characteristics for the measurement of equine insulin samples. It is rapid, inexpensive, simple to run, adequately precise, and shows good correlation with RIA, but its constant bias of approximately −10 to −20 μIU/mL should be considered when assessing samples with concentrations near important clinical decision thresholds. The Mercodia ELISA shows superior precision but is limited as a routine clinical testing modality by both expense and slower turnaround time. Likewise, the Cobas e CLIA shows excellent precision and correlation with RIA, but marked constant and proportional bias mandates that results are either harmonized to the RIA output scale or separate reference ranges are generated. Both Immulite 2000 and 2000 XPi assays produced occasional spuriously high results of a magnitude that would substantially alter clinical diagnosis and could lead to unnecessary treatment. Based on this finding, and their poor correlation and agreement with RIA, we do not recommend them for insulin quantification in horses. Further investigation into the application of HBR in the immunoassay of samples from horses may lead to improvements in accuracy for the subset of horses with heterophilic antibodies and provide insight into a possible cause for the discordant results generated by the Immulite CLIA analyzers.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Authors declare no Institutional Animal Care and Use Committee or other approval was needed. Authors declare human ethics approval was not needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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