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### **Analytical validation of a new point-of-care assay for serum amyloid a in horses**

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**Running Title:** Equine SAA Point-Of-Care Assay Validation

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## Summary

**Background:** Serum amyloid A (SAA) is a major acute phase protein in horses. A new point-of-care (POC) test for SAA (Stablelab) is available, but studies evaluating its analytical accuracy are lacking.

**Objectives:** Evaluate the analytical performance of the SAA POC test by: (1) determining linearity and imprecision, (2) comparing results in whole blood with those in serum or plasma, and (3) comparing POC results with those obtained using a previously validated turbidimetric immunoassay (TIA).

**Study design:** Assay validation.

**Methods:** Analytical validation of the POC test was done in accordance with American Society of Veterinary Clinical Pathology guidelines using residual equine serum/plasma and whole blood samples from the Clinical Pathology Laboratory at the University of California-Davis. A TIA was used as the reference method. We also evaluated the effect of haematocrit (HCT).

**Results:** The POC test was linear for SAA concentrations of 0-1,000  $\mu\text{g/mL}$  ( $r = 0.991$ ). Intra-assay CVs were 13%, 18%, and 15% at high (782  $\mu\text{g/mL}$ ), intermediate (116  $\mu\text{g/mL}$ ), and low (64  $\mu\text{g/mL}$ ) concentrations. Inter-assay (inter-batch) CVs were 45%, 14%, and 15% at high (1,372  $\mu\text{g/mL}$ ), intermediate (140  $\mu\text{g/mL}$ ), and low (56  $\mu\text{g/mL}$ ) concentrations. SAA results in whole blood were significantly lower than those in serum/plasma ( $p = 0.0002$ ), but were positively correlated ( $r = 0.908$ ) and not affected by HCT ( $p = 0.261$ ); proportional

negative bias was observed in samples with SAA >500 µg/mL. The difference between methods exceeded the 95% confidence interval of the combined imprecision of both methods (15%).

**Main limitations:** Analytical validation could not be performed in whole blood, the sample most likely to be used stall side.

**Conclusion:** The POC test has acceptable accuracy and precision in equine serum/plasma with SAA concentrations of up to 1,000 µg/mL. Low inter-batch precision at high concentrations may affect serial measurements, and use of the same test batch and sample type (serum/plasma or whole blood) is recommended. Comparison of results between the POC test and the TIA is not recommended.

## **Introduction**

Serum amyloid A (SAA) is a major acute phase protein in horses, and its concentration increases rapidly and dramatically in response to inflammatory stimuli [1-5]. SAA in horses has been shown to have clinical utility for diagnosing the presence of inflammation, assessing response to therapy, and determining prognosis [1; 2; 4-6]. Development of SAA testing methodology in veterinary species has been hampered by the absence of species-specific standard material, and equine SAA has not yet been purified [7]. Cross-reactivity of human anti-SAA antibodies in equine serum and plasma has been well documented, allowing for validation of automated immunoassays [2; 8-10]. Despite its diagnostic accuracy and clinical usefulness, routine SAA testing in the United States has been limited by cost and stability of reagents. Thus, only a few laboratories offer SAA, requiring shipping of samples and longer turnaround time for results.

A new point-of-care (POC) test for equine SAA has been developed for use with whole blood, serum, and plasma samples (Stablelab)<sup>1</sup>. POC tests are useful for rapid, stall-side results. The POC test is currently being used by veterinary practitioners and in clinical research [11; 12]. However, to our knowledge, studies validating the analytical accuracy of the assay have not been reported. POC tests often use different methodology than standard immunoassays, and can be run on whole blood, such that HC can also affect results. An absence of published performance goals and data on the biological variation of SAA in horses complicates interpretation of analytical validation data. Nonetheless, analytical validation is critical for confirming that the test can accurately and reliably identify significant changes in SAA at blood concentrations that are important for making medical decisions.

The aim of the present study was to evaluate the analytical performance of the SAA POC test (Stablelab) by: (1) determining linearity and imprecision, (2) comparing results obtained in whole blood with those obtained using serum or plasma, and (3) comparing POC results with those obtained using a previously validated turbidimetric immunoassay (TIA)<sup>2</sup>. We hypothesised that the SAA POC test would have acceptable analytical performance in serum, plasma, and whole blood samples. Validation of the POC test will facilitate the appropriate use, interpretation, and monitoring of SAA results in equine patients in clinical settings.

## **Materials and Methods**

Study design was developed using guidelines for quality assurance and method comparison outlined by the American Society of Veterinary Clinical Pathology (ASVCP) [13; 14]. Total allowable error was not established a priori due to an absence of published data on analytical quality requirements for SAA in horses (see statistical analysis section for our approach to assessing method agreement). Sample collection and animal use was approved

by the Institutional Animal Care and Use Committee at the University of California-Davis. Owner consent was obtained for animals used in this study.

### **Index (POC) test**

The POC test<sup>1</sup> is a proprietary, lateral-flow, membrane-based immunoassay. Both semi-quantitative (visual colour indicator compared with a reference card) and quantitative (in  $\mu\text{g/mL}$ , using a handheld reader) SAA results can be obtained. For this study, only quantitative results were recorded and analysed. Serum is the sample material of choice, but the manufacturer reports that the assay also can be run on heparinised plasma, EDTA-anticoagulated whole blood, and fresh non-anticoagulated whole blood. The manufacturer reports “normal” values of  $<7.5 \mu\text{g/mL}$  and a working range of 0-3,000  $\mu\text{g/mL}$ .

### **Reference (TIA) test**

An SAA TIA<sup>2</sup> run on serum or plasma, was used as the reference test. The TIA was run at the University of Miami Comparative Pathology Laboratory (Florida, USA) according to manufacturer’s recommendations for use in human serum. Clustering of initial TIA results around 400  $\mu\text{g/mL}$  (Fig 1) indicated that the TIA was not performing as expected based on a previous validation study of equine serum [2]. In that study, it was found that additional dilution was required for equine samples, which have higher concentrations of SAA compared with human serum. Samples with  $>250 \mu\text{g/ml}$  in the present study were reanalysed using the recommended dilution for equine samples of 1:6 [2]. Once samples were appropriately diluted, the TIA performed as expected in equine serum, and results reported for the method comparison include only those obtained from the diluted samples. Results were reported by the laboratory in  $\text{mg/L}$ , and then converted to  $\mu\text{g/mL}$  for comparison with the POC test ( $1 \text{ mg/L} = 1 \mu\text{g/mL}$ ).

## Sample collection and processing

Residual paired EDTA whole blood and serum or heparinised plasma samples from horses were used in the study (n = 81). Samples were submitted on the same day for CBC and chemistry analysis to the Clinical Pathology Laboratory at the University of California-Davis between April 2015 and December 2015. Because no difference in TIA SAA results has been found between serum and heparinised plasma [15], either sample was considered as acceptable for inclusion in the study. When multiple samples from the same horse were submitted during this period, only the first set of samples was included. Samples from foals <1 week of age were excluded because elevations in SAA concentrations have been reported in foals in association with birth and colostrum intake [16; 17]. The goal of sample collection was to represent a wide range SAA concentrations within as broad a study population as possible, so no further inclusion criteria (e.g. breed, presenting complaint) were applied. For the method comparison, additional serum samples were obtained from a cohort of horses in an unrelated vaccination study (n = 20), 24 hours post-vaccination, to increase the number of samples with intermediate and high SAA concentrations [18]. The University of Miami Comparative Pathology laboratory donated an additional 25 frozen equine serum samples with high SAA concentrations.

EDTA whole blood samples were analysed using the POC test within 4 hours of collection by technicians in the Clinical Pathology Laboratory. The POC hand held reader does not provide quantitative results >3000 µg/ml, as such EDTA whole blood samples with SAA concentrations >3000 µg/ml were excluded from analysis. Serum and plasma samples with SAA concentrations >3000 µg/ml by the POC were diluted according to the recommendation of the manufacturer, using provided diluent vials, and re-analysed. HCT was determined on a haematology analyser (ADVIA 120)<sup>3</sup> as part of the CBC. Serum and heparinised plasma samples were stored at -20°C for up to 6 months until analysis.

## **Linearity**

Serum/plasma samples (n = 10) were pooled to create a high concentration pool with an average SAA concentration of 2,600 µg/mL based on the POC test. The concentration of the high serum pool was selected to remain within the working range of the POC test [1; 3; 5]. Seven dilutions (100%, 50%, 25%, 10%, 5%, 2.5%, 0%) were made using equine serum with an SAA concentration of 0 µg/mL by the index test, to span the reported working range of the assay. Each dilution was run in triplicate, and the mean ± s.d. was calculated.

## **Precision**

Serum/plasma samples (n = 9) were combined to create high, intermediate, and low concentration pools of approximately 1,000-1,500 µg/mL, 150-250 µg/mL, and 30-50 µg/mL. These concentrations were selected to span the working range of the assay and to reflect SAA concentrations around the medical decision point for an acute phase response (low pool), as well as higher concentrations encountered clinically (intermediate and high pools). Intra-assay variation (repeatability) was determined by running 20 replicates at each level on the same day, using the same batch of test cartridges (#EP1507). Inter-assay variation (reproducibility) was calculated by running 20 replicates at each level over 17 days, using 3 different batches of test cartridges (#EP1503, #EP1504, #EP1507). Mean, standard deviation (s.d.), and coefficient of variation (CV) were calculated for each pool.

## **Method comparison: POC vs. TIA**

Frozen serum/plasma samples (n = 125) were thawed at room temperature and analysed in two batches by one individual (D.S.) using the index test. A 0.5 mL aliquot of each sample was sent on ice by overnight shipping to the University of Miami Comparative Pathology



laboratory for TIA analysis. Residual serum or plasma was refrozen and used to make serum pools for linearity and precision experiments.

### **Data analysis**

Statistical analyses were performed using commercial software (Microsoft Excel 2011)<sup>4</sup>, (Analyse-it Method Validation Edition)<sup>5</sup>. Simple linear regression was used to compare expected and observed results for the linearity study. A paired t test was used to assess differences in SAA concentration measured by the POC between EDTA whole blood and serum/plasma, p values <0.05 were considered significant. Passing-Bablok regression analysis and Bland-Altman difference plots were used to assess accuracy and bias between POC results in serum and EDTA whole blood, and between POC results in serum and TIA results [14]. The combined imprecision was calculated using the inter-assay variation of the SAA POC test and the TIA and the formula  $\sqrt{CV^2_{\text{POC}} + CV^2_{\text{TIA}}}$  [10]. Agreement between the two methods was assessed using the 95% confidence interval around the calculated combined imprecision. The potential effect of HCT on POC SAA results was determined by comparing HCT with the difference in SAA concentration between whole blood and serum or plasma, using linear regression analysis [19].

### **Results**

The POC test was linear up to 3,000 µg/mL ( $R^2 = 0.991$ ) (Fig 2). Intra-assay CVs ranged from 13.3-17.8% and inter-assay (inter-batch) CVs ranged from 13.6-45.5% (Table 1). Although the same serum pools were used for both inter- and intra-assay experiments, one batch of test kits (#EP 1507) yielded consistently lower results, resulting in different mean SAA results for the high concentration pool. Inter-assay CVs for the high concentration serum pool were calculated independently for each of the three batches of test kits and were

38.6%, 42.5%, and 13.8% for pools having SAA concentrations of 1503 µg/mL, 1504 µg/mL, and 1507 µg/mL, respectively.

The Passing-Bablok correlation coefficient ( $r$ ) for SAA results in whole blood vs. serum/plasma was 0.908 (Fig 3A). There was a significant difference between SAA results in serum/plasma vs. whole blood samples ( $p = 0.002$ ), with a proportional negative bias (mean difference,  $-71.8 \pm 31.6$  µg/mL) observed in serum/plasma samples with SAA concentrations  $>500$  µg/mL (Fig 3B). HCT did not significantly affect SAA results ( $p = 0.261$ ), nor did it correlate with SAA concentrations ( $R^2 = 0.006$ ) (Fig 4).

The combined imprecision of the POC and TIA, using a CV of 15% and 5%, respectively, was 15.53%. The correlation coefficient for results obtained by the POC vs. TIA was 0.836 (Fig 5A). We found high correlation at SAA concentrations between 0 and 200 µg/mL (data not shown); however, the difference between methods exceeded the 95% confidence interval around the combined imprecision of both methods (Fig 6) [10; 14]. The POC test had a slight constant bias (mean difference =  $44.9 \pm 46.4$  µg/mL) that was not statistically significant ( $p = 0.12$ ) (Fig 5B). Dilution of samples with SAA concentrations  $>3000$  µg/mL ( $n = 15$ ) resulted in overestimation of SAA concentrations by the POC test when compared with TIA results. The correlation coefficient for the diluted  $>3000$  µg/mL samples vs. TIA was 0.654, with a strong positive proportional bias (mean difference,  $3596 \pm 836$  µg/mL). As such, samples with SAA concentrations  $>3000$  µg/mL by the POC were excluded from the statistical analyses for method comparison.

## Discussion

The POC test had acceptable analytical performance (linearity and precision) in equine serum/plasma for SAA concentrations up to 1,000 µg/mL. Thus, the POC test can be expected to readily distinguish healthy horses with low SAA concentrations ( $<20$  µg/mL) [20-22] from those with an acute phase response. Horses with severe systemic inflammation,

however, can have SAA concentrations  $>1,000 \mu\text{g/mL}$ , where results were less precise or exceeded the working range of the POC assay in our study. The POC test is therefore well suited for identifying the presence or absence of inflammation, but is less useful for monitoring changes in the severity of inflammation, for example, in response to treatment. Imprecision at high SAA concentration was attributable in part to differences in sample type and test batch; these variables should be kept constant when high accuracy is needed. Alternately, TIA can be used to quantify high SAA concentrations, but comparison of results with those obtained with the POC test is not recommended. Current dilution protocols recommended by the manufacturer of the POC test are not yet optimised for samples with very high SAA concentrations, and yielded variable results, often with marked overestimation compared to TIA results, in our study.

Although linearity assessment included samples with SAA concentrations approaching  $3,000 \mu\text{g/mL}$ , the lack of additional dilutions between the two highest concentration samples ( $2476 \mu\text{g/mL}$ ,  $1216 \mu\text{g/mL}$ ), limited our ability to fully assess linearity at these higher concentration ranges. High inter-assay imprecision at high SAA concentration was at least partially attributed to differences between batches of POC test cartridges. For this reason, serial POC results on the same patient should be obtained using the same test batch to accurately identify intra-individual changes in SAA concentration. It is not clear why one batch performed notably different from the other batches, as company quality control procedures should prevent release of production batches with suboptimal performance. As only 3 different batches were tested in the present study, it was not possible to estimate the extent of the batch variation, and further studies are needed to evaluate whether statistically significant batch variation exists. Nonetheless, inter-assay CVs for each of the other 2 batches indicated that differences in test batch did not entirely account for the poor inter-assay precision at high SAA concentrations.

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Another possible source of imprecision was variation in reading the results with the handheld reader. Although we did not specifically study this, we observed on occasion that the numerical result sometimes changed when a test cartridge was removed and then reinserted into the reader immediately after the initial reading. As the intensity of the test line was unlikely to have developed further in this short period of time, some variation in results might be attributable to the spectrophotometric reader. Per the POC manufacturer, the test cartridge needs to develop for 10 minutes once the diluted sample has been applied to the test well, after which the test result is read. This recommendation was followed meticulously in our study, and the reason for the disparity in results after reinsertion of the test cartridge into the reader was not identified.

Proportionately higher SAA concentrations in serum/plasma as compared to whole blood samples indicated that serial SAA measurements for monitoring response to therapy or disease progression should be made using the same sample type. Presumably, differences in POC performance between serum and whole blood were related to a matrix effect. Additionally, precision data for serum and plasma may not be applicable to whole blood, which is the sample type most likely to be used stall-side. Because the POC methodology relies on a colour change, it is also possible that haemolysis, hyperbilirubinaemia, and lipaemia could affect results, and evaluation of these potential interferences is warranted. With these caveats, the analytical accuracy of the POC test, when run using serum/plasma samples, appears to be satisfactory for the detection of inflammation in horses. To more fully assess the stall-side usefulness of the POC test, validation of whole blood samples is indicated, but challenges in diluting, pooling, and storing whole blood complicates the practicality of such a study.

The TIA reference assay was designed for measurement of human SAA concentrations and optimised for lower SAA concentrations than those observed in some horses with severe systemic inflammation [23; 24]. Diluting equine samples is necessary to obtain correct readout at higher concentrations as shown previously [2]. Saturation of TIA

detection antibody-binding sites likely resulted in the falsely low results in non-diluted samples with high SAA concentrations, as a prozone effect has not been observed with SAA concentrations up to 6,000  $\mu\text{g/mL}$  in human serum [2; 24]. A similar discrepancy between the POC and TIA results was found in a recent study of horses with experimentally-induced septic arthritis [12]. In that study, none of the serum and synovial fluid samples analysed by TIA had SAA concentrations  $>400 \mu\text{g/mL}$ , while those measured using the POC had SAA concentrations between 400 and 1,400  $\mu\text{g/mL}$ . This discrepancy may not affect the ability to detect an acute phase response, but could affect the interpretation of serial measurements.

A limitation of our study was the variable (1-6 months) length of storage and repeated freeze-thaw cycles of serum/plasma samples. SAA is reported to be stable at  $-20^{\circ}\text{C}$  for up to 17 days [2; 8; 10], and samples stored at  $-20^{\circ}\text{C}$  for  $>1$  year were used in a previous TIA validation study [8]. Also, whole blood and serum samples ideally would have been analysed on the same day, but serum samples were batched in our study for ease of shipment to Miami.

In conclusion, the POC test may reliably be used to measure SAA concentrations of 0-1,000  $\mu\text{g/mL}$  in serum/plasma and detect the presence of an acute phase response in horses. At higher levels of SAA, such as can be observed in horses with severe systemic inflammation, high inter-batch imprecision may preclude accurate interpretation of serial results. When analysing SAA using whole blood, proportional negative bias was detected, suggesting that different results may be obtained with different sample types. Veterinarians using the POC test and investigators evaluating its diagnostic accuracy should be cognisant of these factors and use consistent sample type and test batches. The convenience of the POC test methodology will facilitate rapid identification of horses with elevated SAA concentrations in a variety of clinical settings.

### **Authors' declaration of interests**

The authors have declared no competing interests.

### **Ethical animal research**

Sample collection and animal use was approved by the Institutional Animal Care and Use Committee at the University of California at Davis. Owner consent was obtained for animals used in this study.

### **Sources of funding**

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### **Authorship**

All authors contributed to the study design, data analysis and interpretation, and preparation and approval of the manuscript. D. Schwartz contributed additionally to data collection and

study execution. N. Pusterla, S. Jacobsen, and M. Christopher contributed particularly to study design, data analysis, and interpretation.

#### **Manufacturers' addresses**

1. Stablelab, Epona Biotech Ltd. Sligo, Ireland.
2. LZ-SAA, Eiken Chemical Co., Japan.
3. Siemens, Malvern, Pennsylvania, USA.
4. Microsoft Corporation, Redmond, Washington, USA.
5. Analyse-it Software, Ltd., Leeds, Yorkshire, UK.

#### **Figure Legends**

**Fig 1:** Regression analysis of preliminary SAA results in equine serum/plasma in which sample dilution for the TIA method was according to manufacturer specifications. Samples with SAA concentrations  $>250 \mu\text{g/mL}$  by the POC test were underestimated by the TIA (yellow circle). Samples were subsequently re-analysed using a 1:6 dilution according to the recommendation for equine samples [2] prior to method comparison.

**Fig 2:** Linearity under dilution of the SAA POC test in equine serum/plasma.

**Fig 3:** Comparison of SAA POC test results in equine serum/plasma and whole blood ( $n = 88$ ). (A) Passing-Bablok regression ( $r = 0.908$ ). (B) Bland-Altman difference plot. Although correlation is high, there is a proportional bias, with increasingly lower results in serum/plasma at increasing concentrations beginning around  $500 \mu\text{g/mL}$ .

**Fig 4:** Bland-Altman bias plot comparing the difference in POC test results between serum/plasma and whole blood over the range of sample HCT.

**Fig 5:** Comparison of POC and TIA results for SAA (<3,000 µg/mL) in equine serum/plasma (n = 110). TIA was run based on recommendations for dilution in equine serum. (A) Passing-Bablok regression (r = 0.784) (B) Bland-Altman difference plot.

**Fig 6:** Comparison of POC and TIA results for SAA (<200 µg/mL) in equine serum/plasma (n = 53). The dashed lines represent the 95% confidence intervals around the combined inherent imprecision of the assays.

**Table 1:** Imprecision of the POC test for serum amyloid A in equine samples.

Experiment	Serum/plasma pool	No. of replicates	SAA (µg/mL)	Coefficient of variation (%)
Intra-assay	High	20	782.3 ± 103.9*	13.3
	Intermediate	20	115.8 ± 20.6	17.8
	Low	20	63.9 ± 9.3	14.5
Inter-assay	High	20	1372.4 ± 624.9*	45.5
	Intermediate	20	140.1 ± 19.0	13.6
	Low	20	55.9 ± 8.5	15.2



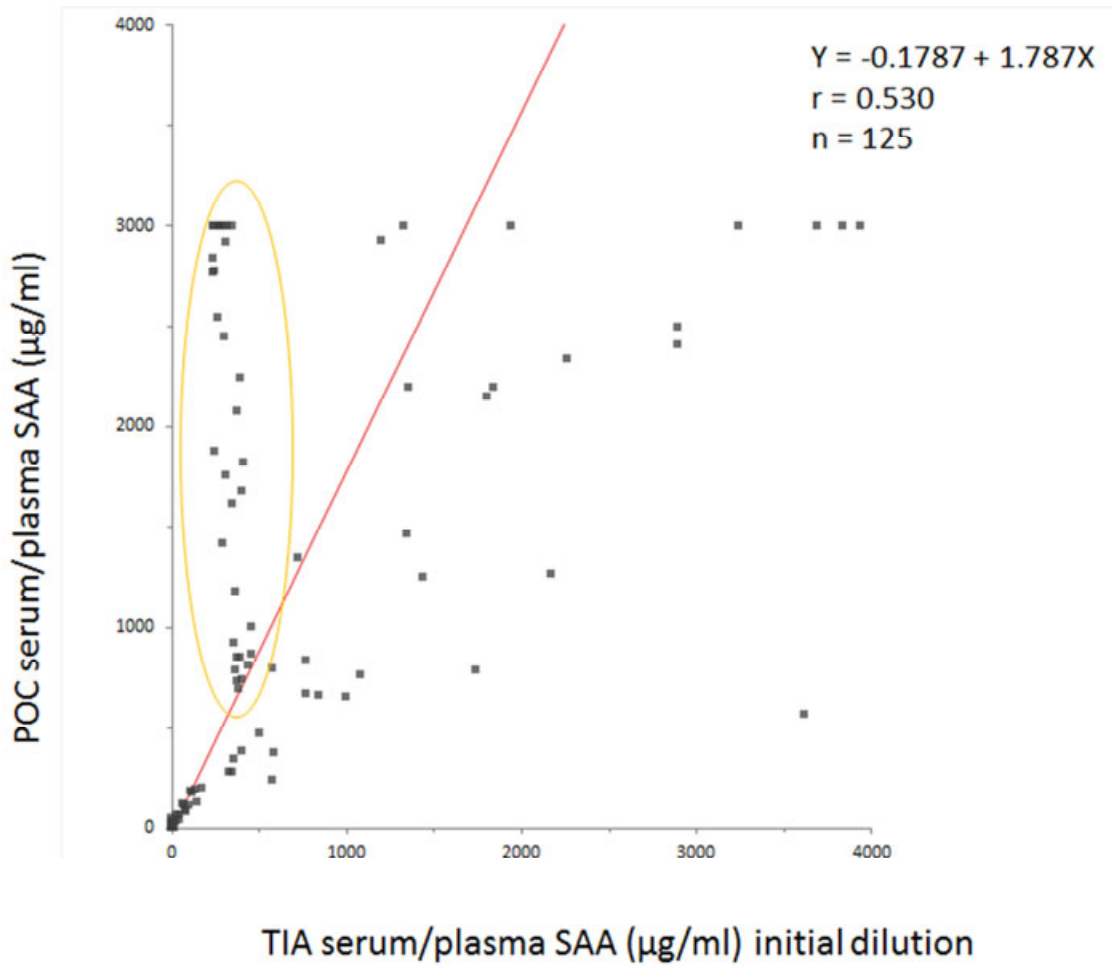
\*One POC test batch (EP 1507) yielded consistently lower SAA concentrations than other batches, resulting in different mean SAA values for the high concentration pool in intra-assay and inter-assay experiments.

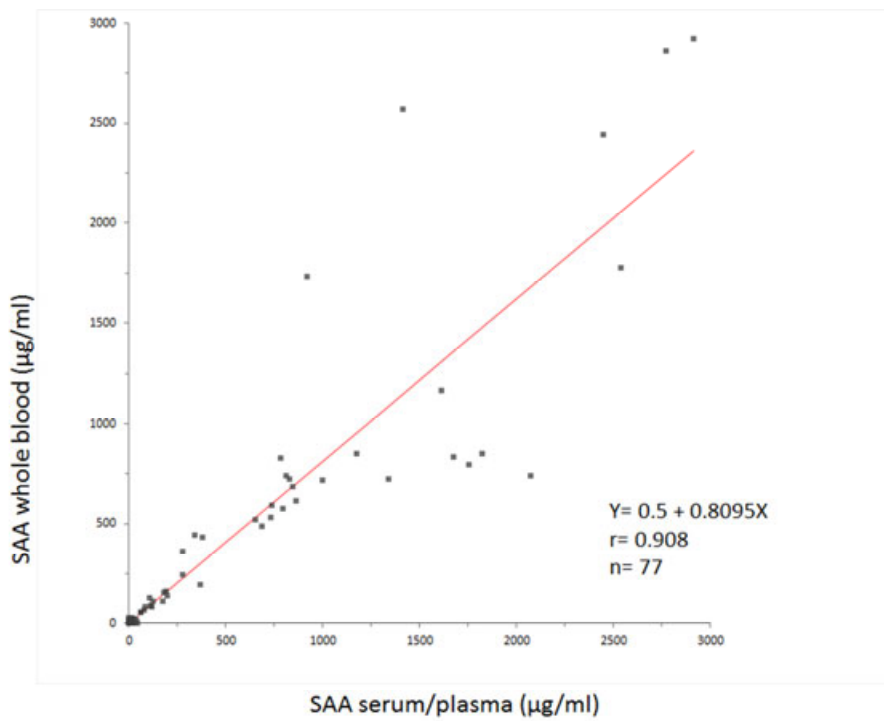
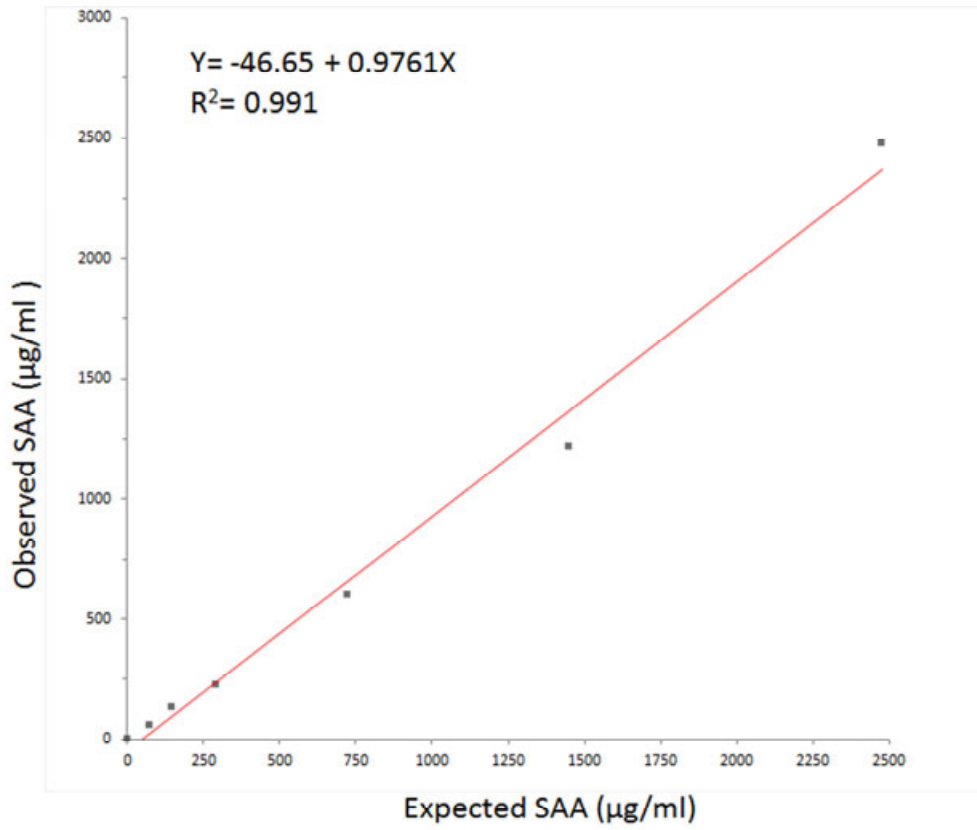
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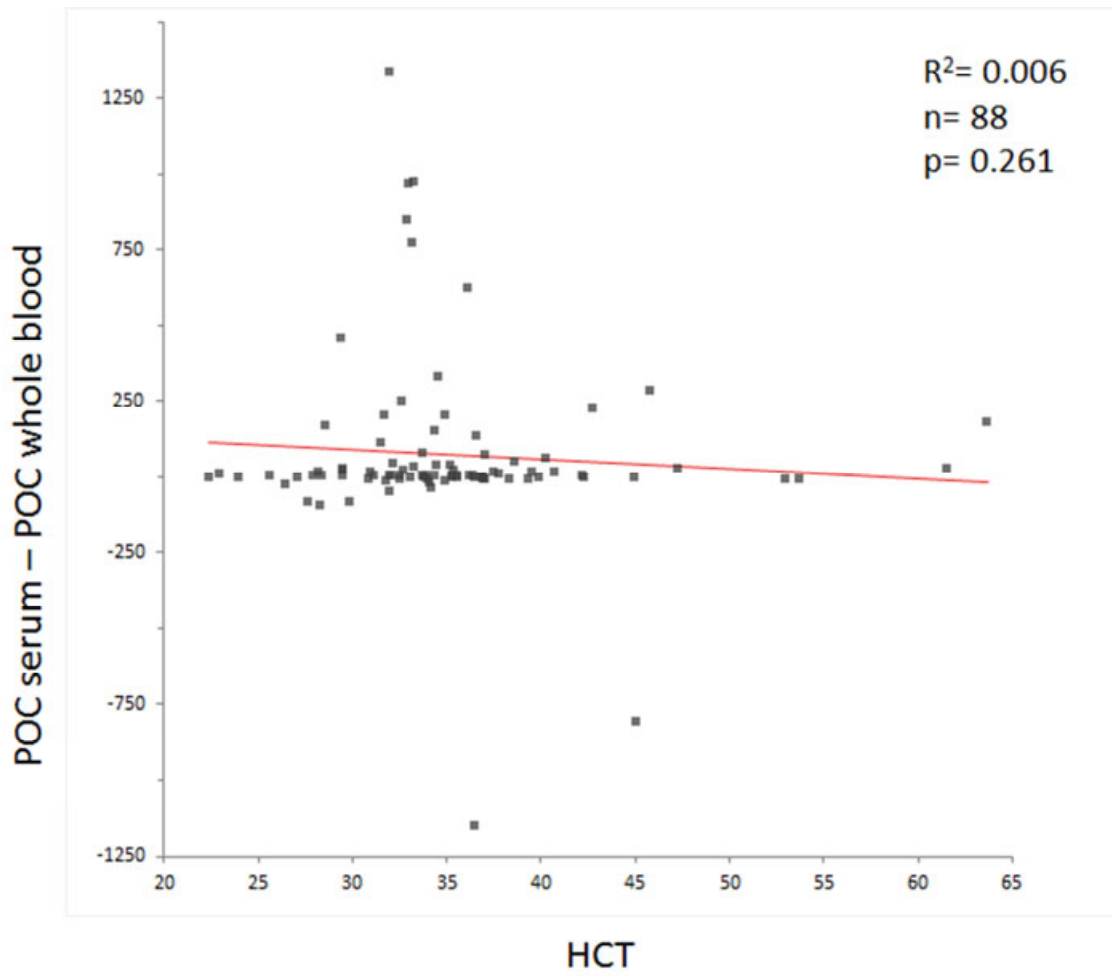
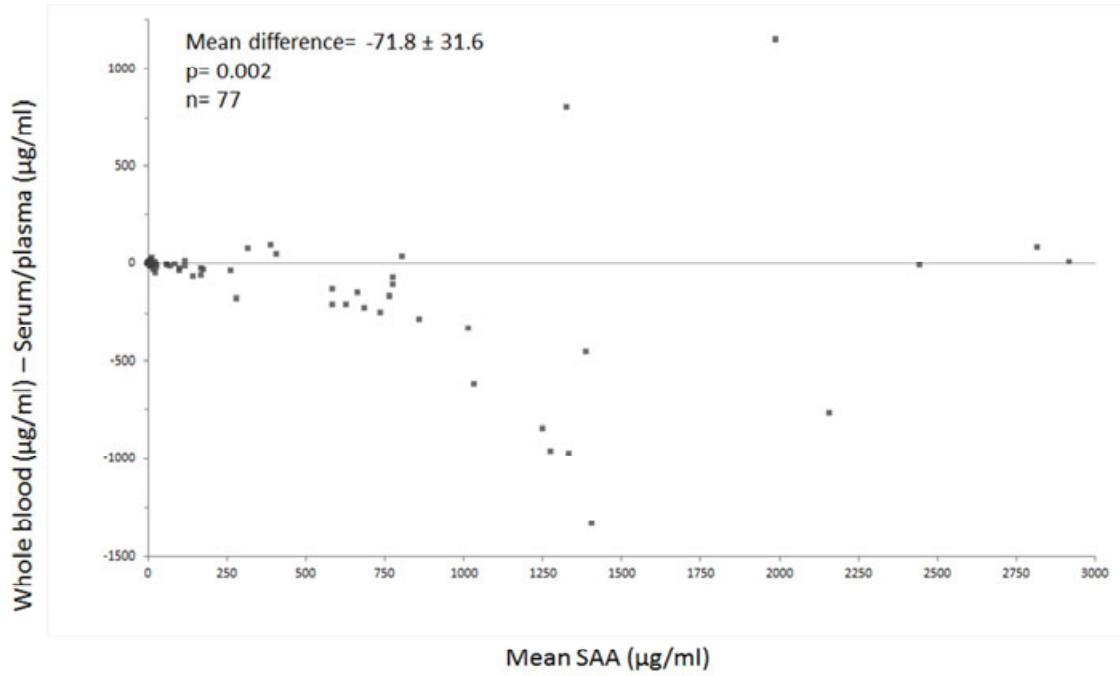
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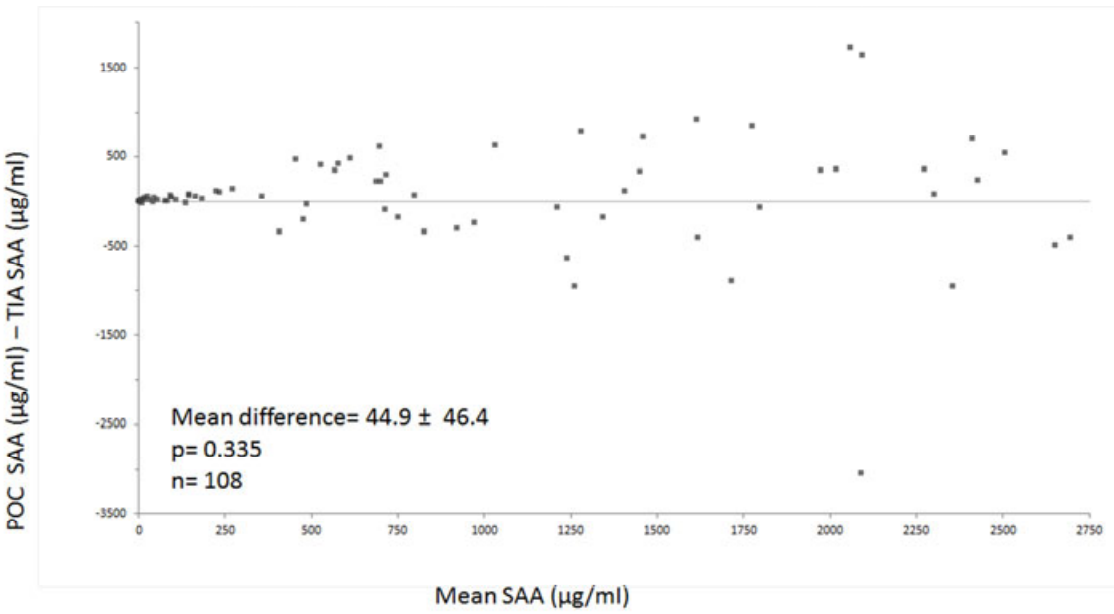
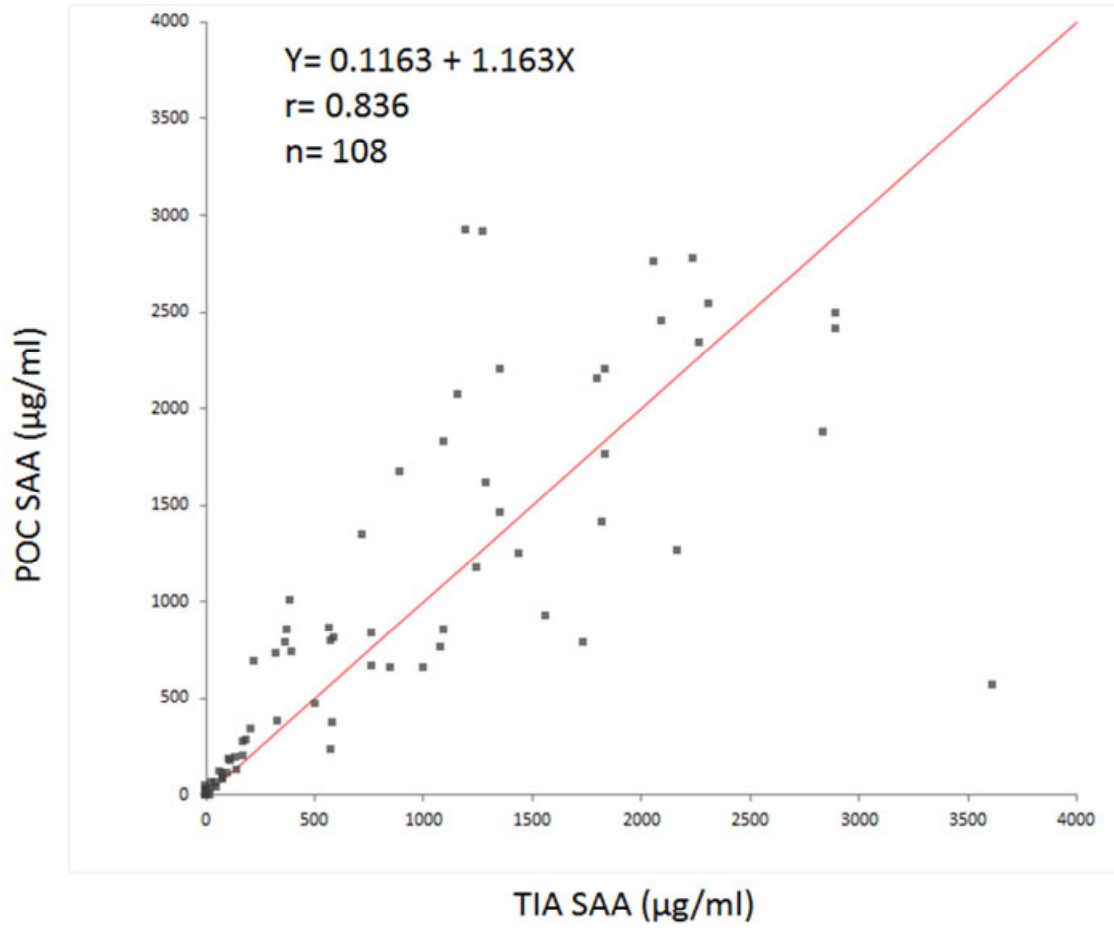


Fig. 6

