

ENDOCRINE TESTING IN THE LABORATORY: THE USE OF SERUM SEPARATOR TUBES FOR ROUTINE CORTISOL AND THYROID ASSAYS IN CANINE PATIENTS

by

CORRY K. YEUROUKIS

(Under the Direction of Bridget Garner)

ABSTRACT

The use of serum separator tubes has greatly increased efficiency in the laboratory and has reduced *in vitro* effects on biochemical results. Serum separator tubes have also been associated with interferences regarding endocrine assays and monitoring of therapeutic drug levels. This study measured canine serum cortisol and total thyroxine (TT4) values from both plain serum tubes and serum separator tubes to evaluate for any interference using the Immulite® 1000 chemistry analyzer. Linear regression and Bland-Altman analysis demonstrate good to excellent agreement between results from both tube types and no bias for the T4 samples. Cortisol results demonstrate good agreement and minimal negative bias that increased slightly over time. These results indicate that either tube can be used confidently to collect samples for T4 assays using the Immulite® 1000. Until canine specific bias levels can be established, SST should be used with caution for baseline cortisol testing in dogs.

INDEX WORDS: Endocrinology, Veterinary Medicine, Laboratory Medicine,
Hypothyroidism, Hyperadrenocorticism, Serum Separator Tube

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CHAPTER 1

HYPOTHYROIDISM

Hypothyroidism is a multisystemic disorder and is recognized as the most common endocrinopathy in dogs.¹ Acquired primary hypothyroidism is an auto-immune condition and accounts for approximately 95% of hypothyroid cases. Secondary hypothyroidism can result from impaired pituitary secretion of thyroid stimulating hormone (TSH), congenital malformation of the pituitary TSH deficiency or pituitary trauma/surgery.

Clinical signs leading to the suspicion of adult-onset hypothyroidism are non-specific and include lethargy, weakness, weight gain, mental dullness, and alopecia or recurrent skin infections. The most consistent clinicopathologic abnormalities include a nonregenerative anemia and increased serum cholesterol levels. Dogs with primary hypothyroidism typically have decreased serum total thyroxine (TT4) and free thyroxine (FT4). TSH is increased in 75% of dogs with primary hypothyroidism while 36-53% have circulating thyroglobulin autoantibodies.¹

Several tests are available for the diagnosis of hypothyroidism. Serum total thyroxine concentration measures bound and unbound circulating T4 and is the most widely used screening test for hypothyroidism. Relying on this test alone, however, can result in a misdiagnosis of hypothyroidism as non-thyroidal illness, time of day, and drug therapy can reduce serum TT4 and T3 concentrations.¹ Drugs that can decrease the TT4 concentration include glucocorticoids, phenobarbital, sulfonamides, clomipramine, aspirin, ketoprofen, and carprofen.¹

The effect of illness or drug therapy can decrease thyroid hormone binding to plasma carriers, increase metabolic clearance of thyroid hormones, decrease conversion of T4 to T3, or inhibit TSH secretion.² Free T4 is the unbound, biologically active portion of total T4. The measurement of FT4 by equilibrium dialysis can aid in differentiation of true thyroidal illness from non-thyroidal illness. FT4 will typically be reduced with true thyroidal illness but should be less affected by non-thyroidal illness. Additional diagnostic considerations include measurement of TSH values. The sensitivity of this assay has been brought into question however, as approximately 25% of hypothyroid dogs have TSH concentrations within the reference interval.² In general, it is recommended that TT4 values be assessed concurrently with FT4 and TSH. These three tests are often offered as a thyroid panel by many laboratories.

CHAPTER 2

HYPERADRENOCORTICISM

Hyperadrenocorticism (HAC) is another well-described, common endocrinopathy of older dogs and occurs due to prolonged exposure to inappropriately high plasma concentrations of cortisol. The condition can be either iatrogenic due to glucocorticoid therapy or spontaneous, occurring secondary to either a pituitary or adrenal tumor.³ Pituitary dependent HAC (PDH) results from increased ACTH production by a functional pituitary adenoma. Adrenal dependent HAC (AT) occurs secondary to the autonomous production of cortisol by a functional adrenocortical neoplasm. Diagnostic testing is only pursued when clinical suspicion is high, as indicated by a historical and clinical presentation consistent with HAC. Common clinical signs include polyuria, polydipsia, polyphagia, increased panting, abdominal distension, hepatomegaly, and alopecia. Common laboratory abnormalities include a leukocytosis characterized by a neutrophilia with a concurrent lymphopenia, increased alkaline phosphatase (ALP) concentration, increased alanine transaminase (ALT) concentration, hypercholesterolemia, hypertriglyceridemia, and hyperglycemia (mild). Urinalysis may reveal a urine specific gravity less than or equal to 1.020 with proteinuria and occasional concurrent urinary tract infection. A combination of clinical signs and concurrent laboratory abnormalities should warrant additional testing for HAC. Ideally, testing for HAC should occur in the absence of concurrent serious illness. Diagnosis of HAC involves the demonstration of increased production of cortisol and decreased sensitivity to the negative feedback loop for glucocorticoids.³

A urine cortisol: creatinine ratio can be used to screen for HAC, while common diagnostic tests include the ACTH stimulation test or low dose dexamethasone suppression test (LDDST). Following diagnostic test results suggestive of HAC, differentiation between PDH and AT can be accomplished with an abdominal ultrasound, LDDST, endogenous ACTH concentration, high dose dexamethasone suppression test (HDDST), and/or advanced cross-sectional imaging modalities.

CHAPTER 3

PREANALYTIC FACTORS AFFECTING LABORATORY TESTING

Serum is the preferred sample for the detection of the biochemical abnormalities associated with hypothyroidism and HAC, as well as for TT4 and cortisol assays. Blood is typically collected into a serum collection tube, which is either plastic or glass with a plain red rubber or plastic top. Once blood has completely clotted, the tube is centrifuged to separate the serum from the clot. The serum is then separated from the clot using a pipette and is placed in another red top or plastic storage tube.

Evacuated blood collection tubes were first introduced in the 1950s and have been the main blood collection devices utilized ever since.⁴ Serum separator tubes (SST) have introduced a level of convenience to the blood collection process including shorter clot activation time when compared to plain tubes as well as higher serum yield.⁴ Commercially available blood collection tubes contain a surfactant on the walls of the tube designed to minimize the adherence of blood components such as platelets and fibrin to the tube wall.⁴ Serum separator tubes also contain a separator gel which is meant to form a barrier between packed cells and serum.⁴ The addition of a separator gel has contributed to increased serum stability and has made the process of storing serum more efficient since there is no need to aliquot the serum to store it in a separate tube, as is the case with the plain collection tubes.^{4,5}

It was shown that several routine human analytes were stable in serum for at least 24 hours as long as the serum was immediately separated from the cells.⁶ When plasma or serum remained in contact with cells for a prolonged period serum was preferred for analysis because it

developed fewer changes than plasma.^{6,7} Endocrine testing was not evaluated as part of that study. In another study 33 common biochemical analytes, including cortisol, were shown to be stable in human serum samples stored at -80°C in the original serum separator tube for as long as 12 months.⁸

Though serum separator tubes have offered several levels of convenience, these serum separator tubes are not without drawbacks. Previous studies have shown that results from biochemical assays, including total triiodothyronine (TT3), using SST compared to plain red top blood collection tubes have yielded slight analytical, but perhaps not clinical, differences.^{9,10} Potential explanations focused on the gel barrier and the tube wall. The gel barrier may have the potential to absorb hydrophobic compounds which likely accounts for the falsely decreased serum levels of some therapeutic drugs including phenobarbital and phenytoin.^{9,10,11} The barrier gel may also release droplets into the serum that can interfere with sample analysis.^{9,10,11}

Meanwhile, tube wall surfactant, used to prevent adherence of blood components to the tube wall, has been reported to induce both statistically and clinically significant interferences in measured TT3 values compared to plain collection tubes utilizing the Immulite® 2500 analyzer.^{11,12} The difference in TT4 and cortisol was shown to vary more than 10% between tubes, but unlike the TT3 findings, these differences were determined to be of no clinical significance utilizing the Roche Modular, Abbott Architect, and Siemens Centaur analyzers.¹³ In response to this study, the manufacturer (Becton Dickinson and Company, Franklin Lakes, New Jersey) reformulated the serum separator tubes to include less surfactant.

Subsequent analyses of the reformulated SST on several endocrine assays, including cortisol, TT4 and TT3 using the Immulite® 1000 demonstrated close agreement with the standard glass tubes. Repeated experiments with the reformulated tubes resulted in no clinically significant differences among tube types for the same endocrine assays performed in the Immulite® 2000 analyzer as well.¹⁴

The Immulite® 1000 is a benchtop immunoassay analyzer used frequently in veterinary laboratory medicine due to readily available veterinary- specific assays such as canine FT4, canine TT4, and canine TSH. Many veterinary laboratories utilize the Immulite® 1000 for canine TT4 and cortisol testing, whereas fewer laboratories provide additional, less commonly utilized tests. Both assays (Siemens Healthcare, Erlangen, Germany) report a potential interference when blood is collected into a SST for analysis instead of the recommended plain serum collection tube. While the manufacturer mentions a brief study consisting of 8 dogs with no statistically significant difference in TT4 concentrations between SST and plain serum tubes, specific details are not provided. No studies are reported regarding the cortisol value. The lack of information regarding the clinical implications of the interference has led to confusion and frustration among general veterinary practitioners seeking reliable results for their patients.

The goal of this investigation is to evaluate the impact of the use of SST on TT4 and cortisol assay results using the Immulite® 1000. The results of this study will be used to provide a higher level of customer service at the diagnostic laboratory level and improve overall patient care.

CHAPTER 4

CANINE PATIENTS: INCLUSION AND EXCLUSION

A total of 127 dogs were included in this study; 40 control dogs, 41 TT4 patient group dogs, and 46 cortisol patient group dogs.

The control group consisted of adult dogs belonging to veterinary students or staff. For inclusion, control group dogs had to be free of previously diagnosed endocrinopathies and clinical signs of hypercortisolemia or hypothyroidism (polyuria, polydipsia, lethargy, and unexplained weight gain). They could not receive any current medications other than flea/heartworm preventative and had to be otherwise healthy. Health was determined by obtaining a medical history, physical exam, and a serum biochemistry profile. Dogs were excluded from the control group if any evidence of systemic illness was present on exam or biochemistry analysis. Dogs weighing under 4.0 kg were excluded from participation due to minimum blood sampling volume requirements.

For inclusion of the TT4 patient group, dogs had to demonstrate clinical or biochemical profile abnormalities suggestive of hypothyroidism, including but not limited to lethargy, weight gain, mental dullness, and hypercholesterolemia. The dogs were presenting for either initial diagnosis of hypothyroidism or for routine therapeutic monitoring. Patients currently receiving thyroid supplementation were included to increase enrollment due to the low number of undiagnosed hypothyroid patients presenting to a referral hospital setting.

For inclusion in the HAC patient group, dogs had to demonstrate clinical or biochemical profile abnormalities suggestive of HAC, including but not limited to polyuria/polydipsia,

alopecia, hepatic enlargement, elevated ALP concentration. The dogs were presenting for either initial diagnosis of HAC or for routine therapeutic monitoring with ACTH stimulation testing. The baseline cortisol values were used as data points. Three of the dogs included in the group had pre- and post-ACTH stimulated cortisol levels included in an effort to increase participant data points due to a low case load involving undiagnosed HAC patients presenting to a referral hospital setting.

CHAPTER 5

SAMPLE COLLECTION, PROCESSING, AND STORAGE

A blood volume of six milliliters was collected from each dog via the jugular or cephalic vein. The blood sample was divided equally into plain red top and SSTs. The time allotted for complete clot formation was 30-60 minutes per laboratory protocol.

The tubes were centrifuged at 1300 rcf for 10 minutes, per laboratory protocol. After serum separation, 250ul of serum was removed from each tube and analyzed for cortisol and/or TT4 concentrations using the Immulite® 1000. The remaining serum was left in the original collection tube, in contact with either the blood clot or separator gel. Plain red top and SSTs were stored with their serum at 4°C. An aliquot of 250 microliters was removed at both the 48 and 72-hour time points post-collection for additional analysis. Once removed, the serum was stored in plain plastic laboratory tubes at 4°C until analysis on the Immulite® 1000 could be completed, a point no longer than 72 hours after removal from the primary collection tube. At the end of 72 hours, the plain and serum collection tubes as well as any remaining serum sample were disposed of according to laboratory protocol.

CHAPTER 6

RESULTS

Statistical analysis was performed using a commercially available statistics program (GraphPad Software, Inc. La Jolla, California). Data were analyzed with a one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$. No significant differences were observed with comparison of plain tube and SST for both T4 and cortisol results at any of the time points for either the patient or control samples. Patient samples were also compared to control samples at each individual time point by tube type and, again, no significant differences were noted.

Results from the plain tubes and SSTs for both patient and control TT4 ($n=40$) data groups were graphically compared with a box and whisker plot (Figure 6.1A). For the affected dogs, $n=41$ for time 0, $n=38$ data points for time 48, and $n=29$ for time 72. The number of samples for analysis declined over time due to volume limitations of the sample. No significant differences were observed between the plain tubes and SSTs for either the patient or control group.

Figure 6.1B is used to graphically compare time points. No significant differences between time points were noted for either tube type. Not unexpectedly, the data distribution was narrower among the control group compared to the affected dogs.

Results from the plain tubes and SSTs for both patient and control cortisol ($n=40$) data groups were also graphically compared with box and whisker plots (Figure 6.2 A). For the affected dogs, $n=46$ for time 0, $n=38$ for time 48, and $n=32$ for time 72. As with the TT4 group,

the number of samples for analysis declined over time due to volume limitations of the sample. Similar to the TT4 data, there were no significant differences between the plain and SSTs for either the patient or control group. Figure 6.2B graphically compares time points. No significant differences were observed among time points. Similar to the TT4 group, the affected dogs showed a wider range than the control group. Linear regression analysis was also performed to evaluate the level of agreement between the two tube types for both assays. Linear regression for TT4 (Figure 6.3) showed good agreement between tube types for control ($R^2=0.936$) and excellent agreement for patient TT4 ($R^2=0.986$) groups. Analysis of the cortisol data (Figure 6.4) showed fair agreement between tube types for control ($R^2=0.813$) and good agreement for patient ($R^2=0.962$) groups.

Bland-Altman analysis was utilized to compare the plain serum and SST collection methods and to determine any bias between the two tube types. Figure 6.5 and Table 6.1 reveal a mean difference of zero and no bias between the two tube types for the TT4 assay. All data points are within two standard deviations from the mean difference. For the cortisol assay (Figure 6.6 and Table 6.2) there is a small negative bias between the plain tubes and SSTs which increases slightly over time in the patient group. The maximum bias of $-0.38\% \pm 0.75$ occurred at time 72. In a previous study, bias $> \pm 10\%$ was utilized to determine clinical significance for cortisol, TT3, TT4, and TSH results for human patients on the Immulite® 2500 analyzer.¹⁴ Using this decision threshold, and given that the bias for the cortisol group in this study did not exceed 2%, the degree of bias determined in this analysis may fall within a clinically acceptable range.

Three of the cortisol patient animals had both baseline and post-ACTH stimulation cortisol concentrations recorded whereas all other results consisted only of baseline cortisol data. The post-ACTH values were removed and analyzed with linear regression and Bland-Altman analysis, although there are only three total animals in this group. Linear regression shows good agreement between the plain and serum separator tubes (Figure 6.9). Bland-Altman analysis demonstrates minimal bias between plain serum tubes and SSTs, however the bias does increase with increased time of sample storage (Figure 6.10).

CHAPTER 7

DISCUSSION

Hyperadrenocorticism and hypothyroidism are two of the most common endocrinopathies seen in canine patients.^{1,3} These conditions present with a variety of clinical signs, many of which are non-specific but highly suggestive for each condition. When these signs are observed, especially when several signs are observed concurrently, veterinarians are alerted that additional diagnostic testing must be pursued. Both plasma and serum can be used for the assays, however serum is the preferred sample.

Veterinarians rely upon laboratories to provide reliable test results, as these assays can come with significant expense to owners and have treatment implications for patients. The serum collection tubes available are either plain red top serum collection tubes or SSTs that include a gel barrier between clotted blood and serum. Serum separator tubes reduce clot time and potentially eliminate the need for separation of serum from remaining blood prior to sample submission. In regard to some assays, specifically those measuring therapeutic drug levels and endocrine assays, the use of SSTs has been discouraged from use due to potential interferences from the surfactant, the gel barrier substance, or a combination of the two.^{9,10,11}

Endocrine assay methodology and sample requirements are specific for each analyzer used. Manufacturer recommendations for both the TT4 and cortisol assays discourage the use of serum separator tubes due to potential interference, yet they do not clarify the potential interference and how it affects results. This study sought to define the nature of the interference, if any.

The results of this investigation demonstrated excellent agreement and an absence of bias between plain serum tubes and SST for TT4 results in both control dogs and dogs presenting with clinical signs of hypothyroidism (Table 1). These results suggest that either a plain serum tube or a SST can be submitted for a TT4 assay using the Immulite® 1000. These results are consistent for up to 72 hours post-sample collection, which shows that the results are comparable between the two tubes in scenarios that mimic sample mail-in conditions.

Results for the cortisol assay also demonstrate fair to good agreement between plain serum and SST samples for baseline cortisol testing from control dogs and dogs presenting with clinical signs suggestive of HAC. There is a slight negative bias on Bland-Altman analysis, indicating the plain serum tube measures lower than the SST (Table 2). Although small, the bias does increase over time, suggesting that a component of the SST may be positively interfering with the cortisol assay. The change over time also suggests samples that are not processed and analyzed immediately, such as those mailed to a reference laboratory, are more likely to have noticeable changes. In a previous study, bias $> \pm 10\%$ was utilized to determine clinical significance for cortisol, TT3, TT4, and TSH results for human patients on the Immulite® 2500 analyzer.¹⁴ Using this decision threshold and, given that the bias for the cortisol group did not exceed 2%, the bias determined in this analysis may fall within a clinically acceptable range. To the authors' knowledge, clinically acceptable bias limits for cortisol testing have not been established in canines. In order to more definitively determine whether or not this level of bias is clinically significant, additional work to establish these limits is needed.

The results for the post-ACTH stimulation cortisol patients also demonstrate good agreement and minimal bias between the two collection tube types, however only three animals are available in this group. The assay results and bias increase steadily over time. Additional

investigations with a larger sample size into the clinical significance of this finding will be required before serum separator tubes can be used with confidence in testing other than baseline cortisol testing. Complete ACTH stimulation, low-dose dexamethasone and high-dose dexamethasone suppression tests were not evaluated in this study and provide yet another avenue for further investigation.

Limitations of this study include a small sample size and the absence of age- and gender-matched control and affected animals. Additionally, 40 data points were not available for all time points due to specimen volume limitations. Repeating this study with a larger sample size will serve to reinforce these reported results. In order to apply these findings in a clinical setting, clinically significant thresholds for bias in canine cortisol testing will also need to be developed.

In summary, these findings suggest that both plain serum tubes and SSTs can be used for TT4 testing in dogs using the Immulite® 1000. Submission of a sample in a serum separator tube should not be a cause of rejection for TT4 samples. Bias, while small, was present in canine cortisol testing. This difference is minimal and smaller than the acceptable bias limits in human patients, but canine limits have not yet been established. As a result, further investigation on the use of SSTs in canine cortisol testing is needed. Until then, SSTs should be used with continued caution for canine cortisol testing. These results and conclusions are specific for the Immulite® 1000 analyzer and should not be extrapolated to other chemistry analyzers as the methodology for the endocrine assays may be different. The Immulite® 2000 is now available for use, which utilizes the same methodology as the Immulite® 1000. The main differences between the two models are updated computer software and automation of sample loading into the Immulite® 2000. These findings should be applicable to the Immulite® 2000 as well, however a repeat of this study using the Immulite® 2000 could also be considered.

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TABLES

Table 6.1. Bland-Altman bias data for control and patient TT4 groups.

Parameter	TT4 Control 0	TT4 Control 48	TT4 Control 72
Bias	0.00	-0.01	0.02
SD of Bias	0.12	0.13	0.12
95% Limits of Agreement	-0.23 to 0.24	-0.25 to 0.24	-0.23 to 0.26
	TT4 Patient 0	TT4 Patient 48	TT4 Patient 72
Bias	-0.04	-0.01	-0.01
SD of Bias	0.19	0.16	0.14
95% Limits of Agreement	-0.34 to 0.41	-0.33 to 0.31	-0.28 to 0.29

Table 6.2. Bland Altman bias data for control and patient cortisol groups

Parameter	Cortisol Control 0	Cortisol Control 48	Cortisol Control 72
Bias	-0.06	-0.42	-0.38
SD of Bias	0.26	0.50	0.75
95% Limits of Agreement	-0.58 to 0.45	-1.40 to 0.56	-1.86 to 1.10
	Cortisol Patient 0	Cortisol Patient 48	Cortisol Patient 72
Bias	-0.30	-0.59	-0.70
SD of Bias	0.19	0.16	0.14
95% Limits of Agreement	-2.81 to 2.21	-3.93 to 2.75	-2.92 to 1.51

FIGURES

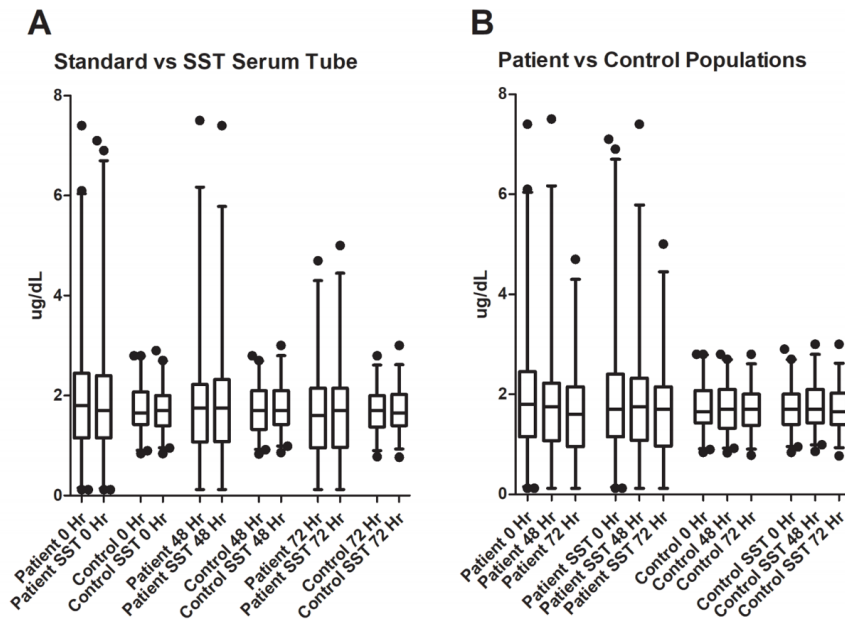


Figure 6.1 TT4 data, Box and Whisker Plots

There were no observed between tube types for either the control or patient group (A). There were also no significant differences between time points (B). Control group $n=40$. Patient group time 0 $n=41$, time 48 $n=38$ and time 72 $n=29$.

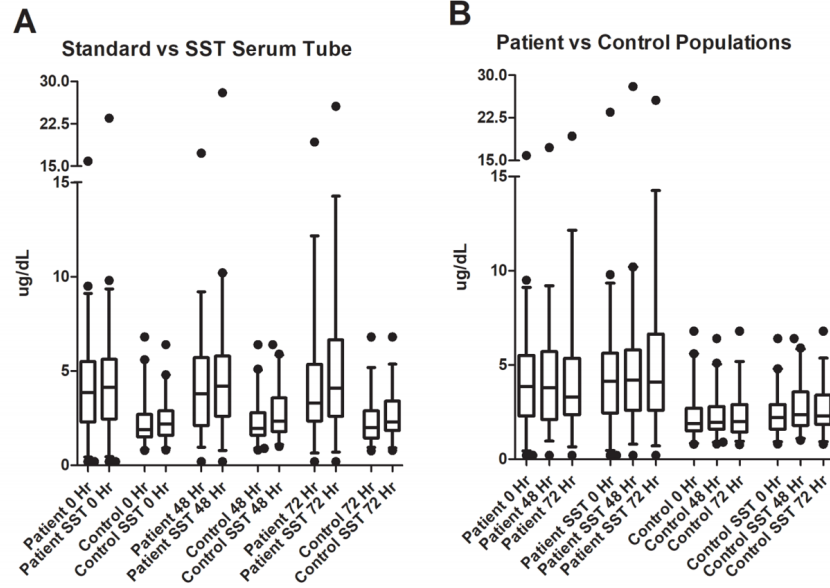


Figure 6.2 Cortisol Data, Box and Whisker

There were no observed differences between tube types for either the control or patient group (A). There were also no significant differences between time points (B). Control group $n=40$. Patient group time 0 $n=46$, time 48 $n=38$ and time 72 $n=32$.

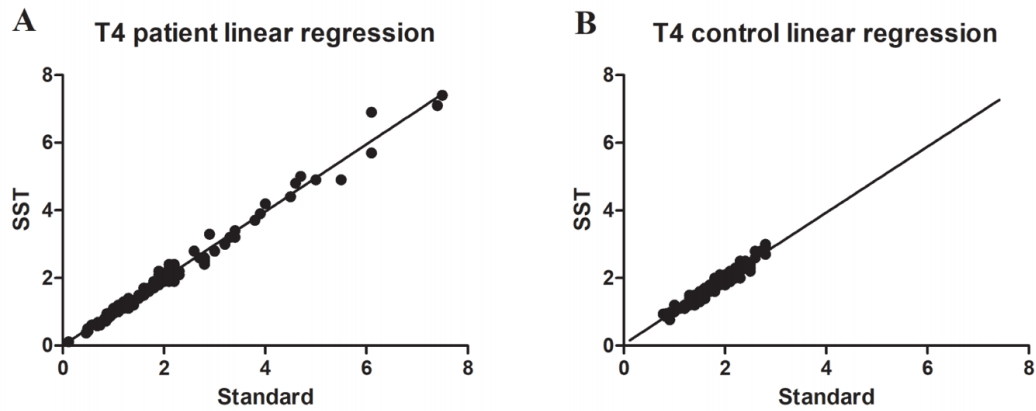


Figure 6.3. TT4 Linear Regression

Linear regression for TT4. There is excellent agreement between tube types for patients ($R^2=0.986$) (A) and good agreement in control dogs ($R^2=0.936$) (B). Control group $n=40$. Patient group time 0 $n=41$, time 48 $n=38$ and time 72 $n=29$.

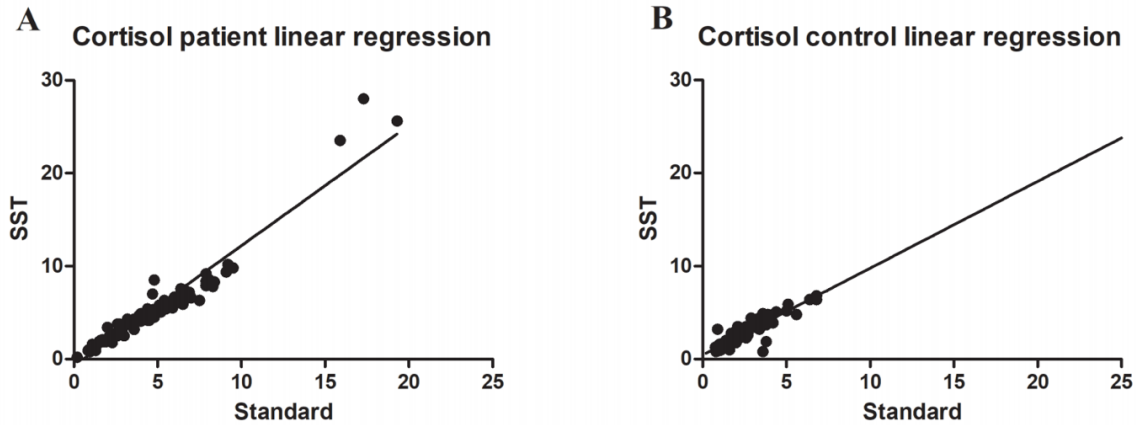


Figure 6.4 Cortisol Linear Regression

Linear regression for cortisol. There is good agreement between tube types for patients ($R^2=0.962$) (A) and fair agreement in control dogs ($R^2=0.813$) (B). Control group $n=40$. Patient group time 0 $n=46$, time 48 $n=38$ and time 72 $n=32$.

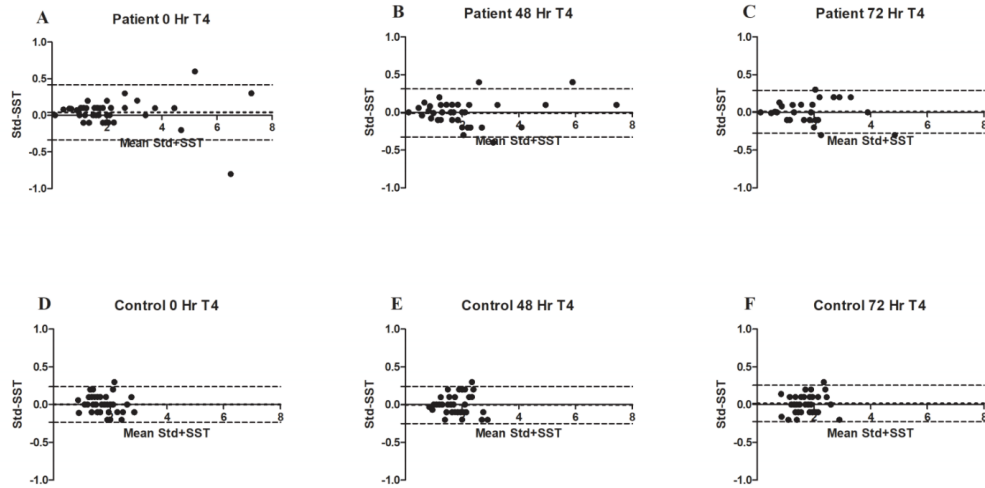


Figure 6.5 Bland-Altman analysis for TT4 group

These Bland-Altman plots show that there is a mean difference of zero and no bias between tube types. All data points are within two standard deviations from the mean difference. The central dashed line represents the mean difference while the outer dashed lines represent the 95% limits of agreement. Control group $n=40$. Patient group time 0 $n=41$, time 48 $n=38$ and time 72 $n=29$.

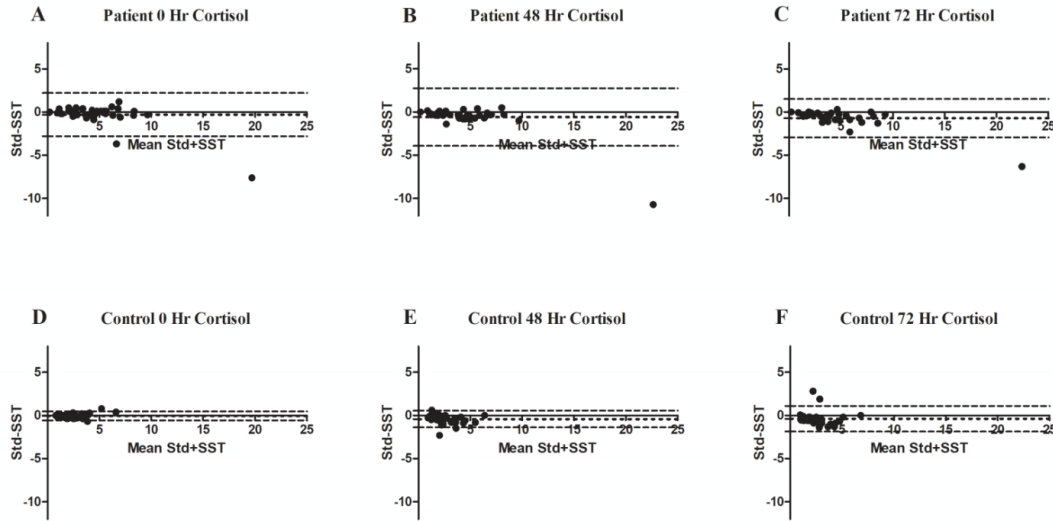


Figure 6.6 Bland-Altman analysis for cortisol group

Bland-Altman demonstrates minimal negative bias between the plain and SST collection methods. The majority of data points are within two standard deviations of the mean difference, with the exception of one outlier animal in the patient group. The central dashed line represents the mean difference, while the outer dashed lines represent the 95% limits of agreement. Control group $n=40$ animals for each time point. Patient group time 0 $n=46$, time 48 $n=38$, time 72 $n=32$.

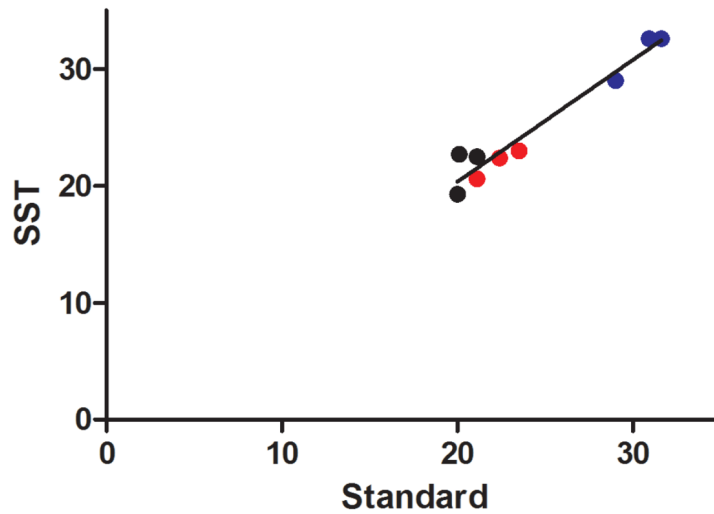


Figure 6.7 Linear Regression, post-ACTH stimulation cortisol patients

Linear regression demonstrates good agreement between tube types among all time points for each patient ($R^2=0.910$). The black dots represent Patient 1, red dots represent Patient 2, and the blue dots represent Patient 3.

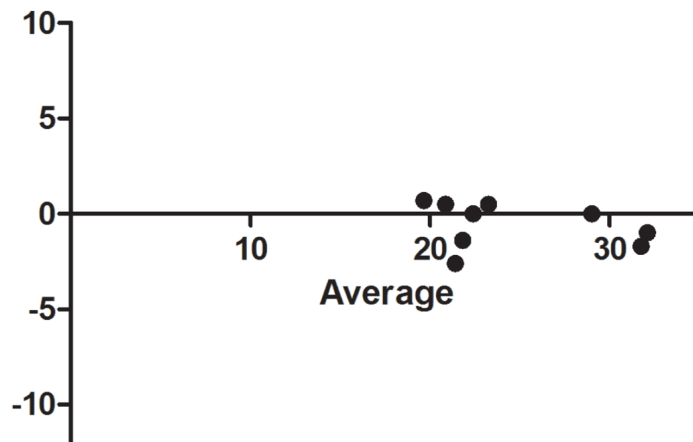


Figure 6.8 Bland-Altman analysis of Post-ACTH stimulation cortisol patient dogs

Bland-Altman analysis demonstrates a mean difference of zero and minimal negative bias between tube types.