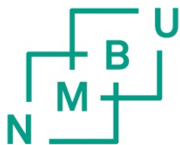

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Stability Study of Clinical Chemical Analytes in Canine Serum When Stored in Refrigerator

Stabilitetsstudie av klinisk kjemiske analytter i serum fra hunder ved lagring i kjøleskap

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Class of 2015

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Foreword

When interpreting the results from blood tests it is important that the results are accurate. Many veterinarians take the results literally without taking the factors affecting the analytes into account. Many factors, like storage time, temperature, centrifuge speed, and coagulation can affect the value of clinical chemical analytes measured in serum.
After talking to my supervisors, I have learnt how important it is to have an idea of how stable clinical chemical analytes in serum are and how little research has been done on this topic related to dogs. This intrigued me. I am mostly interested in small animal medicine where blood tests are one of the most important diagnostic tools. I also enjoy reading through and assessing the results of a blood test.

Summary

Title: Stability Study of Clinical Chemical Analytes in Canine Serum When Stored in Refrigerator

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The present study investigates the stability of clinical chemical analytes in serum stored for 14 days at +7°C. The study population included 10 dogs over 10 kg that came to the small animal teaching hospital NMBU between 30th of November till the 8th of December 2020. The dogs presented varied in breed, sex, age and clinical signs. After coagulation and centrifugation, the serum from each dog was divided into 4 aliquots, three were put into the refrigerator (+7°C) while one was analysed the same day. One aliquot from each dog was analysed after 0, 3, 7 and 14 days. The clinical chemical analytes measured were A:G, Alb, ALT, Amy, AP, AST, BA, Ca, Chol, CK, Cl, cortisol, Crea, CRP, Fruc, FT4, Glob, Glu, K, Lip, Na, Na:K, P, Tbili, Tprot, TSH, TT4 and urea. The results showed that most of the clinical chemical analytes in serum were stable at +7°C for 14 days. The most unstable clinical chemical analytes in the present study were CK, CRP, Fruc, FT4 and Tbili, all had over 10% concentration change after 14 days. This storage condition affected the clinical relevance of CK, Cl, cortisol, Crea, CRP, Fruc, FT4 and P.

Abbreviations and glossaries

Clinical chemical analytes

Alb: Albumin, one of two main proteins found in blood (1). High albumin levels indicate that a pet is dehydrated, and low levels may indicate decreased liver function, blood loss, gastrointestinal disease or kidney disease (2).

ALT: Alanine aminotransferase, an enzyme found mostly in the liver (3). An elevated serum level is caused by liver damage (4).

Amy: Amylase, pancreas enzyme. Increased amylase level in serum can indicate pancreatitis, chronic kidney disease or gastrointestinal disease (4).

AP: Alkaline phosphatase, an enzyme found in the liver. Elevated levels of AP in serum are most commonly caused by liver and bile diseases, can also be associated with Cushing's disease and bone growth (1).

AST: Aspartate aminotransferase, an enzyme found mostly in the liver and muscle (3). Elevated serum AST level is a result of injuries to liver or muscle cells (4).

BA: Bile acids, steroid acids derived from cholesterol and excreted into bile (4). Increased levels may indicate decreased liver function, abnormalities in blood flow to the liver, bile duct obstruction or pancreatitis (2).

Ca: Calcium, a mineral that is an important cation in the intra and extracellular fluid and plays part in the activity of the heart and neuromuscular activities (4).

Chol: Cholesterol, a steroid produced in the liver as a part of fat metabolism. Increase in cholesterol levels in serum can be associated with hormonal and metabolic diseases, liver diseases, and severe kidney disease (1).

CK: Creatine kinase, an enzyme found in the skeletal and cardiac muscle and the brain. Increased CK in serum is most commonly used as a marker of skeletal muscle injury in veterinary medicine (4).

Cl: Chloride, a mineral, an important anion in the extracellular fluid that plays a major role in the acid-base balance and in osmotic regulation (4).

Cortisol: A hormone, glucocorticoid produced in the adrenal cortex, or used as a medicament. It increases gluconeogenesis and has anti-inflammatory effect (4).

Crea: Creatinine, a waste product of muscle metabolism (4). Elevations may indicate renal disease, but an increase can also be due to prerenal or postrenal conditions. A decrease may be seen with overhydration, pregnancy, hyperthyroidism or cachexia but is usually not clinically relevant (2).

CRP: C-reactive protein, an acute phase protein. It is an important part of the immune system and is used as a biomarker of systemic inflammation (4).

Fruc: Fructosamine, glucose combined with protein. Since fructosamine has a longer half-life than glucose, it is an indicator of blood glucose concentration over longer period of time. Measured in dogs to e.g., manage insulin treatment (4).

FT4: Free thyroxine, T4 not bound to protein, the biologically active form of the thyroxine hormone. See TT4. Increase may indicate hyperthyroidism and decrease may indicate hypothyroidism (4).

GGT: Gamma-Glutamyl Transferase.

Glob: Globulin is derived by subtracting albumin from total proteins and includes several proteins such as acute phase proteins and immunoglobulins. Globulin transports substances involved in the inflammatory response and more (4). Increases may be seen with inflammation and chronic infection, decreases may occur through blood loss, gastrointestinal loss and immune deficiencies.

Glu: Glucose, monosaccharide, derivate from digestion of dietary carbohydrates and glycogen metabolism (4). Increased glucose level in blood may indicate diabetes mellitus, stress, fear, hyperthyroidism, pancreatitis, or hyperadrenocorticism and can be seen with some medicines. A decrease may be due to liver disease, juvenile dog, late pregnancy, some types of cancer or sepsis and may lead to collapse or coma (2).

K: Potassium, a mineral, the major intracellular cation that is important in the acid-base balance (4).

LD: Lactate dehydrogenase.

Lip: Lipase, pancreas enzyme. Lipase is also found in salvia and increase in lipase alone is unspecific (4).

M: Magnesium.

Na: Sodium, a mineral, the major extracellular cation of extracellular fluid (4).

P: Phosphorus inorganic, a mineral that plays important role in the mineral phase of bone and is involved in almost all metabolic processes (4).

Tbili: Total bilirubin in serum, bilirubin is a pigment produced primarily in the liver from breakdown of haemoglobin from red blood cells. Increase in total bilirubin indicates red blood cell destructions or decreased bile flow through the liver (1).

Tprot: Total protein, combined measurement of albumin and globulin (1). Increases may indicate dehydration, an inflammatory condition or some types of neoplasia and decreases with decreased liver function, blood loss, gastrointestinal loss and kidney loss (2).

TSH: Thyroid stimulating hormone, a hormone secreted from the anterior pituitary gland that stimulates the thyroid gland (4).

TT4: Total thyroxine, all thyroxine found in blood bound and not bound to protein. Thyroxine (T4) is a hormone secreted from the thyroid gland that influences metabolic rate (4). Increase may indicate hyperthyroidism and decrease may indicate hypothyroidism.

Urea: By-product produced from protein metabolism (4). Increased urea in serum indicates kidney disease and decreased can be seen with decreased protein intake, polyuria and liver disease (5).

Diagnoses

CKD: Chronic kidney disease.

Epilepsy: A central nervous system disorder.

Fibrosarcoma: Malignant mesenchymal tumour.

HypoT: Hypothyroidism, underactive thyroid.

IMM: Immune-mediated masticatory myositis, autoimmune disorder in the masticatory muscles.

IMPA: Immune-mediated polyarthritis, inflammation in multiple joints.

Insulinoma: Insulin-producing tumour, mostly located in the pancreas.

Meningitis: Inflammation of the meninges.

MG: Myasthenia gravis, neuromuscular disease.

Pseudocyesis: False pregnancy.

UTI: Urinary tract infection.

Key concepts

Clinical chemical analysis: Analysis of blood to determine the levels of various chemical compounds in blood for diagnostic and therapeutic purposes.

Electrolytes: Chemical elements that dissolve in water and disassociates into electrically charged particles. Positively charged electrolytes are called cations and negatively charged are called anions (4).

Haemolysis: Rupture of erythrocytes with release of haemoglobin (4).

Lipemia: An excess of lipids in the blood (4).

Metabolites: Substances produced during metabolism (4).

NMBU: Norwegian University of Life Science.

Plasma: The fluid portion of the blood (4).

RI: Reference interval.

Serum: The fluid and solute portion of the blood that does not take part in clotting (4).

y m: Year and month.

Introduction

Blood examination is an important part of diagnosis and evaluation of animal health. It is important that blood samples are treated in a standardized manner and that all important information is available when interpreting results.

Clinical Chemical Analytes in Serum

Abnormal clinical conditions may be associated with specific biochemical changes in the blood, and these changes can be detected by using specific analytical techniques. Blood consists of yellow, protein rich fluid called plasma, and cellular elements, including red blood cells, white blood cells, and platelets. Blood is essential to maintain fluid balance and transport elements like nutrients and oxygen (4). Clinical chemical analytes can be measured by using serum or plasma. Serum is preferable to the latter for most clinical chemical assays since it is not influenced by an anticoagulant as plasma is. Serum is obtained by collecting blood into tubes without anticoagulants and leaving them to clot for 30 – 45 minutes at room temperature. When blood coagulates, it is separated into two parts, a clear yellow fluid called serum, and a solid red part that settles to the bottom and contains red and white blood cells and platelets (1). After clotting, the tube is centrifuged to yield serum that can be analysed for clinical chemical analytes (3). Chemical analysis of serum is an important tool in determining the health status of animals. The results are used, e.g., to diagnose diseases, monitor the body´s response to medications, and to detect recurrences of diseases (6).

Total protein (Tprot) (combined total albumin (Alb) and globulin (Glob)) is an example of a clinical chemical analyte measured in serum. Increased Tprot in serum most often indicates dehydration or an inflammatory condition, while decreased Tprot in serum indicates loss of protein through the kidneys, the gastrointestinal tract, or a blood loss or reduced production of

albumin and/or globulin in the liver associated with decreased liver function (1).
Interpretation of results from analysis of a blood sample is based on assessing the values of several analytes together with clinical signs and other laboratory results such as haematology and urine analysis, to get an overview of the animal´s health. As an example, increase of liver enzymes (e.g., alanine aminotransferase (ALT), alkaline phosphatase (AP) and aspartate aminotransferase (AST)) in serum basically indicates liver injury. ALT is usually found in increased amounts in serum when liver cells are stressed or injured, while increased AP rather indicates affections of in the bile duct. Increased AST in serum can indicate both liver and muscle damage. A reduced value of total serum protein in addition to increased values of liver enzymes in serum may indicate a more severe liver damage (1).

To help the interpretation of the results from these tests, there exists a laboratory component called reference interval (RI), that represents estimated distributions of reference values from a healthy population of comparable individuals. Decision on diagnoses or start treatment are often based on values falling outside RI. The process of making a reference interval consists of defining the reference population and establishing selection, inclusion, and exclusion criteria. Selection criteria include biological, clinical, and geographical factors and can be dependent on age, sex, breed, vaccination status, and medicine use. A minimum of 120 individuals are recommended to establish a proper RI. The collection and handling of the samples should be done in a standardized manner and samples should be analysed using methods that are strictly monitored. Preanalytical factors can increase variation that is not due to biological or geographical factors. Sample type, e.g., serum or plasma, should be the same for all reference samples. Condition for analysis should be consistent with analysis of animal patient in everyday practice. Generally, RI covers the central 95% of reference values and are bounded by upper and lower reference limits. Generating RI often involves examination of

histograms that shows the distribution of the data and makes it easier to find values from samples that come from unhealthy or non-representative individuals. Reference intervals should be revalidated every 3-5 years (7). The reference interval used in animal practice has to be developed using the same analytical methods as the results it is compared to, since different analytical methods can have different RI for the same analyte. The present study used the RI developed at VetLab NMBU. The reference interval developed at VetLab NMBU applies to Norwegian population of healthy, adult dogs that have fasted. The laboratory results must be interpreted on the background of a RI that is used to distinguish between healthy and unhealthy individuals. To be able to interpret the results, clinicians must have knowledge of biological variation and the potential risk of false interpretation. Measurement of samples from different individuals will give different results because of the natural biological variation and the uncertainty of the measurement itself. The blood components fluctuate throughout the day and the concentration of the analytes measured in serum can be affected by food intake, biological cycles, exercise or the time of the day. Therefore, when comparing a patient's test results with a previous one the clinician must take the biological variation into account. Results not within RI does not always indicate a disease or a pathological condition, nor does results within RI always indicate the absence of a disease. However, the more abnormal the result is, the greater the probability that the result is related to a pathological condition. The interpretation of laboratory results starts with the clinician requesting the right test for the patient's problem. The expectation is that the result will provide the information that will support decision on a treatment (8). If results from a laboratory test are considered clinically relevant, they affect the action of the clinician and the decision on a treatment. Clinical relevance describes the importance of information to clinician and indicates whether the results of a study are meaningful or not to patient and/or clinician (9).

Factors affecting stability

The stability of a clinical chemical analyte is defined as the capability to maintain its concentration over time. Loss of stability is calculated as a function of concentration variation and time in a specific storage condition. Apart from storage time, there are other conditions that affect the stability of analytes. These conditions are e.g., container type, temperature, exposure to light, contact to air, centrifugation (10) site of blood collection, haemolysis, lipemia, and coagulation time (3).

Thus, both lipemia and haemolysis may interfere with the results of clinical chemical analytes. Lipemia is an excess of lipids in the blood, especially if the animal has recently eaten (4). Lipemia increases turbidity of the blood sample resulting in production of artificially high values of certain enzymes and can also interfere with the assays for glucose (Glu), total bilirubin (Tbili), Tprot, phosphorus (P), and urates (3). Haemolysis can have various causes related to blood sampling and is frequently caused by incorrectly filled tubes, incorrect needle sizes, and difficulty in collection of blood (11). Haemolysis causes decreased serum bile acids (BA) concentration but increased Alb, Tprot, CK (creatinine kinase) and potassium (K) (2). Increased K in serum caused by haemolysis occurs because of large amount of K leaks out of erythrocytes. Haemolysis affects serum K levels in dogs to a lower extent than in other animal species, except for Japanese dog breeds that have high K concentration in erythrocytes (12).

The type of tube selected is very important in blood collection. The stability of analytes can vary depending on the tube composition, type of additive or no additive, and what material the lid consists of.

Timing and duration of centrifugation can also interfere with the stability (10). Blood samples should not coagulate for longer than 45 minutes. Studies have shown that blood samples coagulated in room temperature for more than 45 minutes can have changes in concentration of many analytes, including Glu, P, lipids, and chlorides (Cl) (3). This can be related to cellular metabolism. The longer the blood is allowed to clot, the longer serum/plasma will be in contact with cell materials. Contact with cells can lead to an exchange of materials and affect the concentration of the clinical chemical analytes to be measured, especially electrolytes and small substances. This affects serum to a lesser extent than plasma, as the presence of cellular material after centrifugation is more frequent in plasma than in serum. Cellular material can therefore interfere with plasma analytes over a longer period of time.

Blood collection tubes are generally transparent, and the specimens will be exposed to light. Light can result in the loss of stability of some analytes depending on the intensity and type of light they are exposed to. Normally when samples are stored in refrigerators, they are stored in the dark and will not be affected by light.

When a tube is open during the blood analyse process, the solvents can evaporate to some extent. This leads to increase in concentration of most analytes and diffusion of dissolved gases. Even though the tube is closed, an evaporation can occur across the walls of the container, and if some oxygen has entered the container, an evaporation will continue. Temperature is an important catalyst of the chemical reactions leading to loss of stability of clinical chemical analytes. Time (storage time) is also important in relation to chemical reactions affecting analytes and influencing stability (10). Storage time is the agenda in the present study.

Previous studies

There is little data to be found on the stability of clinical chemical analytes in serum over time in veterinary medicine, and especially storage in the refrigerator for a longer time has not been adequately studied. There are however a few reports on the effects of storing serum samples from dogs and rats. These reports examine the stability for only a maximum of one week and some reports only examine a few analytes in the serum (13,14,15). In human medicine, stability analysis of serum has resulted in guidelines for serum storage (10). The few studies within veterinary medicine and studies in human medicine indicate that the stability of serum varies between species. Therefore, guidelines for storing human serum cannot be assumed to work for storage of serum from dogs (13).

In a study done by An & Park, 2014 (16), on the stability of serum in human medicine, 21 analytes where measured twice per day for 30 days. These analytes were calcium (Ca), P, Glu, blood urea nitrogen (BUN), creatinine (Crea), Uric acid, Cholesterol (Chol), Tprot, Alb, AP, Tbili, AST, ALT, lactate dehydrogenase (LD), gamma-glutamyl transferase (GGT), triglycerides, sodium (Na), potassium (K), Cl, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. The results showed that when serum was stored for 14 days at +4°C, five analytes had over 10% concentration difference after 14 days, ALT (-10%), AP (-13.8%), AST (-15%), Tbili (-11%) and LD (-18%).

In a study done by Thoresen et al., 1992 (14), of the stability of clinical chemical analytes in serum in clinically healthy dogs, 24 analytes were analysed after 3 days at +20°C. The analytes were: AP, ALT, AST, amylase (Amy), lipase (Lip), creatine kinase (CK), GGT, glutamate dehydrogenase, LD, Alb, Tprot, bile acids (BA), Tbili, Chol, Crea, fructosamine (Fruc), Glu, Ca, magnesium (M), P, Na, K, triglycerides, and urea. The results showed that storage of Fruc for 3 days at +20°C led to a mean of 8% decrease in the level of Fruc in the

serum. Storage of CK under the same conditions led to a mean of >10% decrease in CK levels in the serum. Of 24 analytes 4 analytes showed statistically significant changes ($p<0.05$) from day 0, (BA, Tbili, M, Na), but none had clinically relevant changes. In another study of serum stability in clinically healthy dogs, total thyroxine (TT4), free thyroxine (FT4) and cortisol were examined after storage for 5 days at +4°C. Only cortisol showed statistically significant difference (15). In the previously mentioned studies of serum in dogs, the serum was stored for 3 or 5 days (14,15), which means that even though all analytes were within the RI after 3 or 5 days, studies in human medicine have shown that values stable after 3 days can as well be unstable after 7 or 14 days (16).

In a study of the stability of serum in rats, where serum was stored for 7 days at +4°C, 17 analytes where analysed: Glu, BUN, Na, K, Cl, carbon dioxide (CO_2), Amy, Lip, Ca, P, Tprot, Alb, AST, ALT, AP, LD, and CK. The results showed that CK, LD and CO_2 had statistically significant change in serum level on day 7 ($p<0.05$), while the other analytes were stable (13).

Today´s practice

The central laboratory at the Norwegian University of Life Science (NMBU), VetLab NMBU, is a veterinary clinical pathology service and a research laboratory. It performs clinical chemical, haematological and endocrinological analyses of patient samples from the teaching hospitals at the Veterinary Faculty and private veterinary practices throughout the country. VetLab NMBU uses modern analysis equipment and a laboratory information system that helps to provide quality assurance and fast results on the same day as the sample is received for most analyses. It offers both single analyses for the various analytes in blood, and profiles that contain several analytes, for various species. There are small and large profiles, where frequently examined analytes are measured, and in addition there are special profiles that

measure analytes important in the examination of an organ or a specific disease. As for today, VetLab NMBU mostly examines large profiles sent from the clinics.

The serum that remains after the initial examination is routinely stored in a refrigerator (+7°C) at VetLab NMBU for 14 days. If veterinarians want to examine other serum analytes that were not part of the initial request, they can order additional analyses from these stored serum samples.

Aim of the present study

The aim of the present study is to examinate how stable the different clinical chemical analytes are in serum from dogs after storage in refrigerator for two weeks, and to find out if storage time influences the clinical relevance of the analytes.

Materials and methods

Materials

The recruitment process for this project took place through the small animal teaching hospital NMBU. Veterinarians and veterinary technicians at the hospital were informed about this project via an information sheet (see appendix 1), printed out and hung around the clinic. They were also informed orally. To be included in the study, dogs had to have an ordinary appointment at the animal clinic in the period from the 30th of November till the 8th of December 2020. A blood sampling was included in the routine diagnostics at these appointments and, as such, was already planned before the visit. Owners of the dogs were asked if they would allow the extra blood sampling and had to sign a consent form (see appendix 2) if they agreed.

Study population

The study population consisted of 10 dogs that arrived at the small animal teaching hospital NMBU on 30th of November, and 1st, 4th and, 7th of December 2020, before 12 o´clock. Dogs were included regardless of clinical signs, breed, age, and sex, but had to be over 10 kg. Three dogs were diagnosed with immune-mediated polyarthritis, two dogs were diagnosed with tumour (one with insulinoma, and one with fibrosarcoma), one dog was diagnosed with meningitis, and one with myasthenia gravis and immune-mediated masticatory myositis. One dog was diagnosed with urinary tract infection and epilepsy, one dog was diagnosed with chronic kidney disease and pseudocyesis, and one dog was diagnosed with hypothyroidism. The oldest dog was 12 years and 9 months old and the youngest was 1 year and 6 months old. Blood samples were taken from 4 male dogs and 6 bitches. A total of 8 breeds were represented in the study in addition to one dog of mixed breed. Two dogs were of the breed Flat-coated Retriever while the following breeds were included with one dog each: Border

Collie, Siberian Husky, Nova Scotia Duck Tolling Retriever, Boxer, Rottweiler, Staffordshire Bullterrier and Welsh Corgi Cardigan. Further information of individual dogs can be found in (Table 1).

Serum

At least 5 mL of whole blood was collected from each dog, so that at least 2 mL of serum could be expected. In general, 1.2 mL of blood yields 0.5 mL of serum after centrifugation and a minimum of 0.5 ml of serum is needed to analyse the analytes included in the present study once. To analyse four times, at least 2 mL of serum was needed from each dog, hence 5 mL of blood was considered enough for the analysis. Blood samples that showed signs of haemolysis were excluded.

Methods

Sampling

From each dog, 2 tubes without anticoagulant but containing clot activator (3 mL CAT Serum Clot Activator) were filled. All the blood samples from the same dog were delivered to VetLab NMBU before 12:00 o´clock the same day with a submission form. The samples were clearly marked with “PRØVE TIL STABILITETSFORSØK” (test for stability study).

Methods of analysis

At VetLab NMBU, the blood samples were centrifuged at 2000g for 10 minutes after clotting for 45 minutes. After centrifugation, the blood samples from each dog were divided into 4 equal aliquots of at least 0.5 mL of serum in each aliquot. One aliquot was examined the day the blood sample was taken, while the other three aliquots were marked according to how long they were to remain in the refrigerator (+7°C), they were marked: day 3, day 7, and day

14 respectively. The three aliquots were then stored in the refrigerator for 3, 7, or 14 days until they were analysed.

At VetLab NMBU, the clinical chemical analytes in the so called large profile, containing, Alb, albumin-globulin ratio (A:G), ALT, Amy, AP, AST, BA, Ca, Chol, CK, Cl, Crea, CRP, Fruc, Glob, Glu, K, Lip, Na, P, sodium-potassium ratio (Na:K), Tbili, Tprot and urea were analysed in addition to the analytes cortisol, Fruc, FT4, TSH and TT4. Clinical chemical analytes in large profile and fructosamine were analysed using Advia® 1800. The other clinical chemical analytes were analysed using IMMULITE® 2000. Further information on methods for the individual clinical chemical analytes can be found in (Table 2).

Statistical analysis

The study design was an experimental prospective study of clinical chemical analytes in serum. The percentage difference of each clinical chemical analyte from day 0 to day 3, day 7 and day 14 were calculated using the formula:

$$\frac{Tx - To}{To} \times 100$$

Where *To* denotes the value of the of the analyte on day 0 and *Tx* represents the value of the analyte at another time (day 3, 7, or 14). This formula was used to calculate the percentage difference between the three time periods from each dog separately. Then the mean and the median of the percentage difference of each clinical chemical analyte was calculated separately on day 3, 7, and 14.

A T-test was used to calculate if the percentage differences were statistically significant from day 0. A T-test is a statistical hypothesis test used to determine if there is a statistically significant difference between the means of two groups. A T-test is one of many statistical

tests which infer the validity of a null-hypothesis. The test is often used when sample sizes are relatively small. It is designed to see whether the means and standard deviations of two sets of distributions could potentially be subsamples of the same distribution. The null-hypothesis is that the two distributions originate from the same distribution. Multiple variations of the T-test exist, in the present study there is used the Correlated T-test, which is designed for experiments, where repeated measurements are compared. The T-test can be described like this,

$$T = \frac{\mu 1 - \mu 2}{\frac{\sigma(\mathrm{diff})}{\sqrt{n}}}$$

where μ1 and μ2 are the means of distribution 1 and 2, respectively. σ(diff) is the standard deviation of the differences between the paired values (e.g., the serum sample from Dog No. 1 on day 0 and day 14) and n is the number of samples. A high T-value indicates that the two distributions are different. An underlying assumption of the T-test is that the investigated values are randomly drawn from a normal distribution.

Statistically significant difference is a difference which cannot be attributed to chance. p value is the probability of the null hypothesis to be true. The null hypothesis in this case is that storage of serum samples in a refrigerator over 14 days does not affect the concentration of clinical chemical analytes in serum. The p value is often set to either <0.05 or <0.01. In the present study, $p < 0.05$ will be used which means that there is less than 5% probability that the null hypothesis is true. Statistical significance does not assure that the results are clinically relevant and can provide misleading results to the clinicians. Statistically significant difference can be found when the sample size is large and/or the intrasubject variability is low, even though the difference is too small to be considered clinically relevant by the clinicians. Therefore, it is relevant that results of laboratory studies are analysed having the clinical relevance of the results in mind (9). Reference intervals help assess if the results are

clinically relevant or not. Whether the increase/decrease in the analytes were clinically relevant after 3, 7 or 14 days in the refrigerator was assessed. In this study the results were considered clinically relevant if the median concentration change of an analyte indicates that concentration at the border of the RI will increase or decrease to levels that will affect the decision on a treatment.

To show the distribution of the percentage difference between days there was used box plots. To show change in concentration chance in serum sample between individual dogs there was used column chart.

Table 1. Sex, breed, age and diagnosis of each dog.[1]

Dog No.	*Sex*	*Breed*	*Age*	*Diagnosis*
1	Male	Flat-coated Retriever	5 y 7 m	Insulinoma
2	Female	Welsh Corgi Cardigan	3 y 6 m	MG + IMM
3	Female	Nova Scotia Duck Tolling Retriever	3 y 5 m	IMPA
4	Male	Boxer	1 y 6 m	Meningitis
5	Female	Staffordshire Bullterrier	2 y 7 m	Fibrosarcoma
6	Female	Rottweiler	4 y 9 m	IMPA
7	Male	Flat-coated Retriever	3 y 6 m	IMPA
8	Female	Siberian Husky	11 y 11 m	HypoT
9	Male	Border Collie	8 y 6 m	UTI + epilepsy
10	Female	Mixed breed	12 y 9 m	CKD + pseudocyesis

[1] y m = year and month, IMPA = immune-mediated polyarthritis, MG = myasthenia gravis, IMM = Immune-mediated masticatory myositis, UTI = urinary tract infection, HypoT = hypothyroidism, CKD = chronic kidney disease.

Table 2. Chemistry analyser, manufacturer and method used.[2]

Analyte	***Chemistry analyser***	***Manufacturer***	***Method***
Alb	Advia® 1800	Siemens Medical Solutions Diagnostics	BCG (bromocresol green)
ALT	Advia® 1800	Siemens Medical Solutions Diagnostics	Modified IFCC
Amy	Advia® 1800	Siemens Medical Solutions Diagnostics	Ethylidene Blocked-pNPG7
AP	Advia® 1800	Siemens Medical Solutions Diagnostics	Modified IFCC
AST	Advia® 1800	Siemens Medical Solutions Diagnostics	Modified IFCC
BA	Advia® 1800		Enzymatic amplification / Thio-NAD – Thio-NADH
Ca	Advia® 1800	Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA	Arsenazo III
Chol	Advia® 1800	Siemens Medical Solutions Diagnostics	Enzymatic/Trinder
CK	Advia® 1800	Siemens Medical Solutions Diagnostics	NAC activated (DGKC)
Cl	Advia® 1800	Siemens Medical Solutions Diagnostics	Ion Selective Electrode (ISE), diluted
Cortisol	IMMULITE® 2000	Siemens Medical Solutions Diagnostics	Solid-phase, competitive, chemiluminescent enzyme immunoassay
Crea	Advia® 1800	Siemens Medical Solutions Diagnostics	Jaffe, kinetic
CRP	Advia® 1800	Randox	Polyethylene glycol (PEG) enhanced immunoturbidimetric assay

[2] Alb = albumin, ALT = alanine transferase, Amy = amylase, AP = alkaline phosphatase, AST = aspartate alanine transferase, BA = bile acids, Ca = calcium, Chol = cholesterol, CK = creatine kinase, Cl = chloride, Crea = creatinine, CRP = C-reactive protein, Fruc = fructosamine, FT4 = free thyroxine, Glob = globulin, Glu = glucose, K = potassium, Lip = lipase, Na = sodium, P = phosphorus, Tbili = total bilirubin, Tprot = total protein, TSH = thyroid stimulating hormone, TT4 = total thyroxine.

Fruc	Advia® 1800	Horiba Medical (ABX Pentra)	Nitrotetrazolium-blue (NBT)
FT4	IMMULITE® 2000	Siemens Medical Solutions Diagnostics	Solid-phase, competitive chemiluminescent immunoassay
Glob			Tprot - Alb
Glu	Advia® 1800	Siemens Medical Solutions Diagnostics	Hexokinase
K	Advia® 1800	Siemens Medical Solutions Diagnostics	Ion Selective Electrode (ISE), diluted
Lip	Advia® 1800	Siemens Medical Solutions Diagnostics	Colorimetric rate
Na	Advia® 1800	Siemens Medical Solutions Diagnostics	Ion Selective Electrode (ISE), diluted
P	Advia® 1800	Siemens Medical Solutions Diagnostics	Phosphomolybdate/UV
Tbili	Advia® 1800	Siemens Medical Solutions Diagnostics	Vanadate oxidation
Tprot	Advia® 1800	Siemens Medical Solutions Diagnostics	Biuret
TSH	IMMULITE® 2000	Siemens Medical Solutions Diagnostics	Solid-phase, two-site, chemiluminescent immunometric assay
TT4	IMMULITE® 2000	Siemens Medical Solutions Diagnostics	Solid-phase, competitive chemiluminescent immunoassay
Urea	Advia® 1800	Siemens Medical Solutions Diagnostics	Enzymatic: urease with GLDH

Results

In (Table 3), the mean concentrations of the analytes on each day are shown along with the median. In (Table 4), both the median and the mean percentage difference is shown on day 3, 7, and 14. By using T-test, the significance $p<0.05$ for each analyte was calculated. In (Table 3 and 4), the analytes with statistically significant difference on each day are marked with *. In (Table 4) the analytes that have clinically relevant results after storage for 14 days or less are marked with **.

Change in the concentration in serum

Five analytes had >10% change in the serum concentration from first measurement to day 14. These were CRP (+29.5%), CK (-21.3%), Tbili, (-15.8%), Fruc (-12.5%), and FT4 (+12.2%). Of these CK, Fruc, and FT4, had a statistically significant difference. Four analytes had 5 – 10% change in the serum concentration from first measurement to day 14. These were P (+8.9%), Crea (+7.1%), Cl (+6.1%), and cortisol (+6.1%). Of these Crea, P and Cl had a statistically significant difference.

Enzymes

The enzymes measured in the present study were ALT, AP, AST, CK, Amy and Lip.

The liver enzymes (ALT, AP and AST) were all stable while stored at +7°C for 14 days. All of them had an increase in the serum concentration on day 3, and a decrease later on. AST was the most unstable liver enzyme measured, increased by 10.1% on day 3.

The concentration of CK decreased by 21% from day 0 to day 14, as seen in (Table 4). This percentage difference was statistically significant. Serum sample from dog No. 9, had the highest concentration difference (Δ conc) of CK from day 0 to day 14. The Δ conc of CK in serum sample from dog No. 9 was around double the Δ conc of the serum samples from the

other dogs. The distribution of the Δ conc of CK from day 0 to the measurement on day 3, 7 and 14 respectively is shown in (Figure 1).

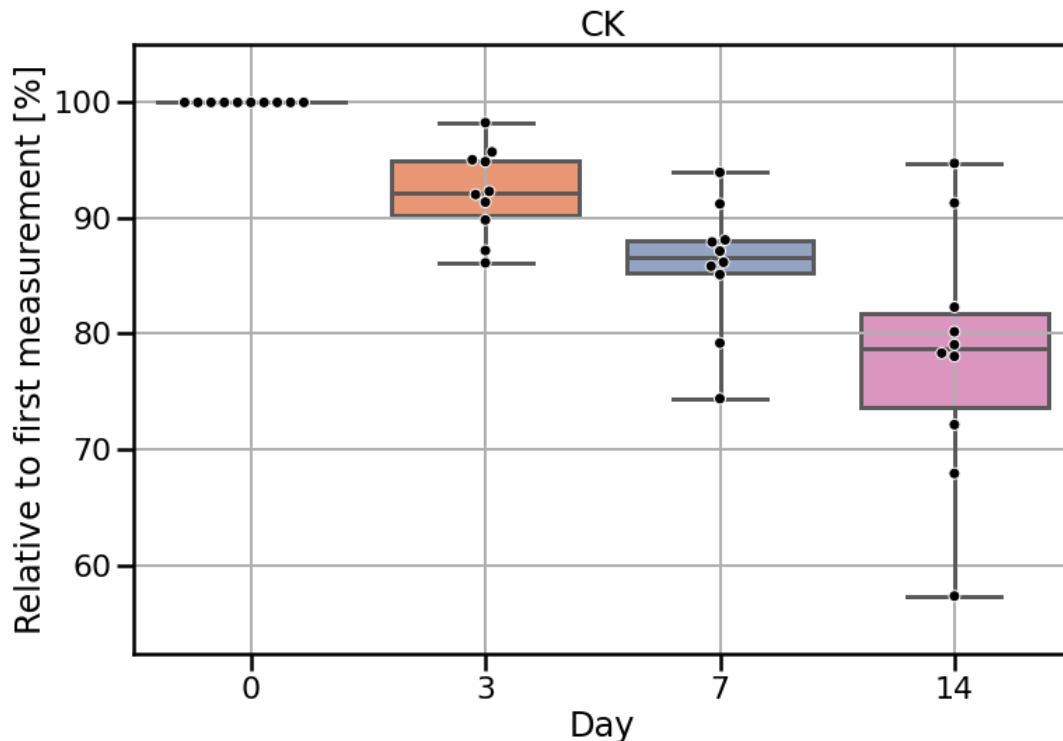

Figure 1. Distribution of the concentration difference of CK from day 0 to the measurement on day 3,7 and 14 respectively. The dots represent each serum sample, the grey lines in the middle represent the median, the coloured squares represent the values between the first and third quartile, 25% of scores fall below the first quartile value and 75% of the scores fall below the third quartile. The whiskers represent the maximum and minimum of the results. CK = Creatine kinase.

The concentration of Amy increased steadily over 14 days. Unlike Amy, the concentration of the other pancreas enzyme, Lip, decreased with time. The concentration of Amy increased by 2.4% from day 0 to day 14, and Lip decreased by 4.2% over the same period of time. Serum samples from dogs No. 1 and 3, had stable Lip concentration, 0% Δ conc through all 14 days. Serum sample from dog No. 6, had the highest Δ conc of Lip on every measurement day, (decrease by 10.3% from day 0 to day 3 and by 20.7% from day 0 to day 14). The Δ conc of Amy was similar in serum samples from all 10 dogs.

The distribution of the Δ conc of Amy from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 2), and in (Figure 3) the same thing is showed for Lip.

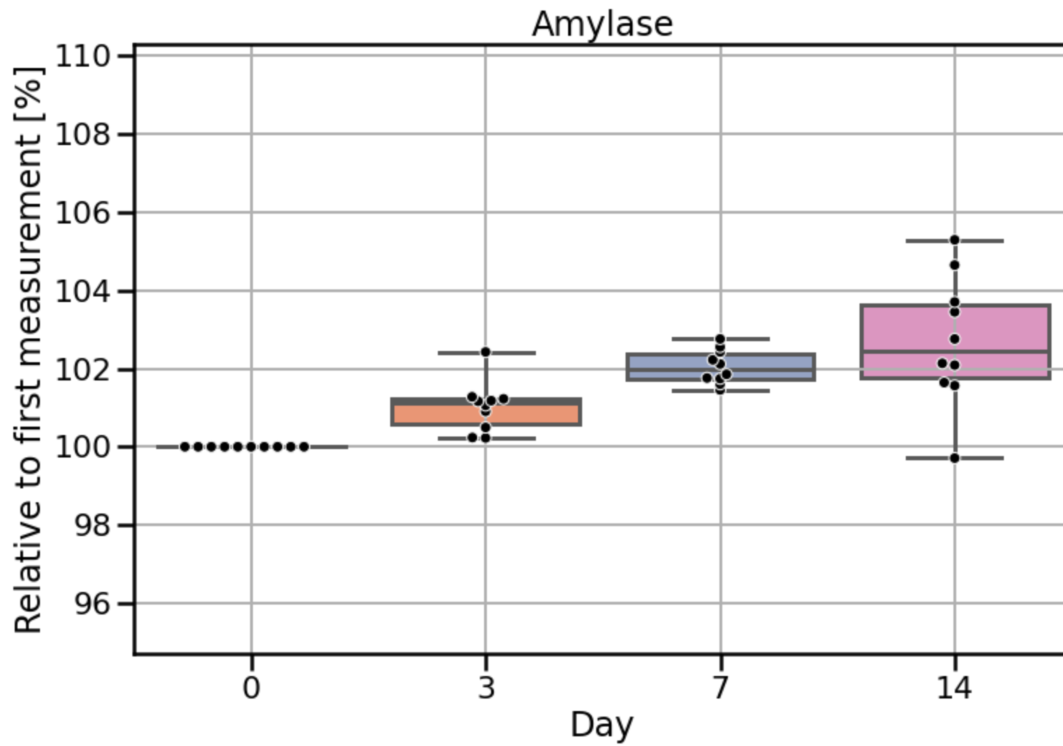

Figure 2. The distribution of the concentration difference of amylase from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

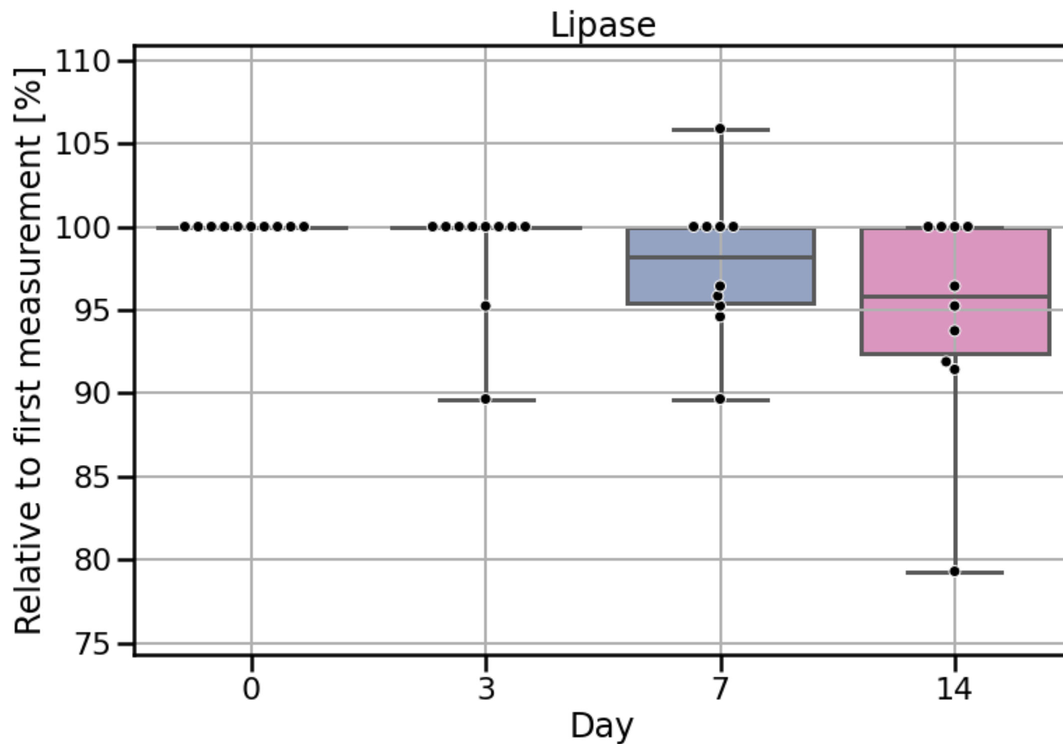

Figure 3. The distribution of the concentration difference of lipase from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

Hormones

The percentage difference of both TT4 and cortisol concentration in serum over a 14 days period varied a lot between serum samples. The Δ conc of TT4 varied between 15.1% decrease in serum sample from dog No. 1, to 28% increase in serum sample from dog No. 8 after 3 days, 14% decrease in serum sample from dog No. 1, and 46.5% increase in serum sample from dog No. 9, after 7 days and 7.8% decrease in serum sample from dog No. 6, and 15.8% increase in serum sample from dog No. 4, after 14 days in refrigerator. See (Figure 4) for how the serum concentration of TT4 changed over time in serum samples from each dog in relation to the upper and lower reference limit.

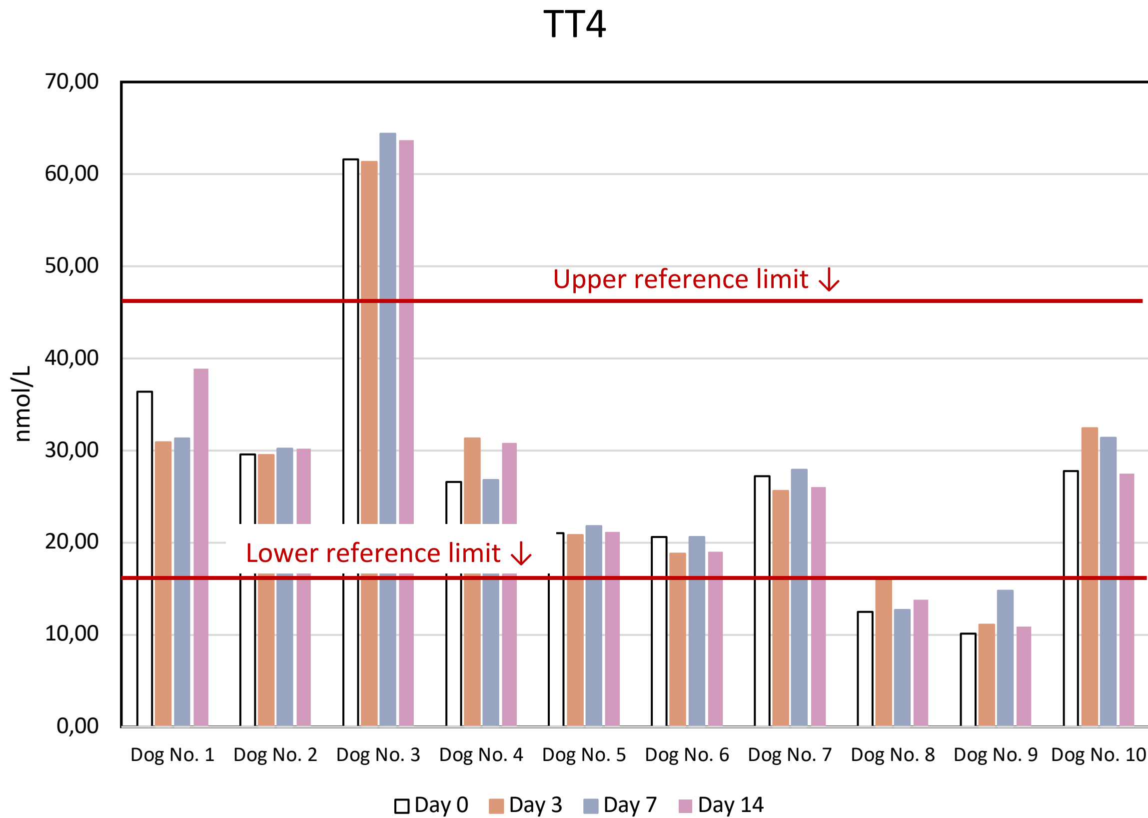

Figure 4. Concentration of TT4 in serum samples from each dog, before and after storage for 3, 7 and 14 days in relation to the upper and lower reference limit. The lower reference limit is 16 nmol/L, and the upper reference limit is 46 nmol/L. TT4 = total thyroxine.

The results for cortisol showed variation between 29.8% decrease and 21% increase from day 0 to day 14. Serum sample from dog No. 5 had the highest decrease in the serum cortisol concentration over all 14 days. See (Figure 5) for how the serum concentration of cortisol changed over time in serum samples from each dog in relation to the upper and lower reference limit.

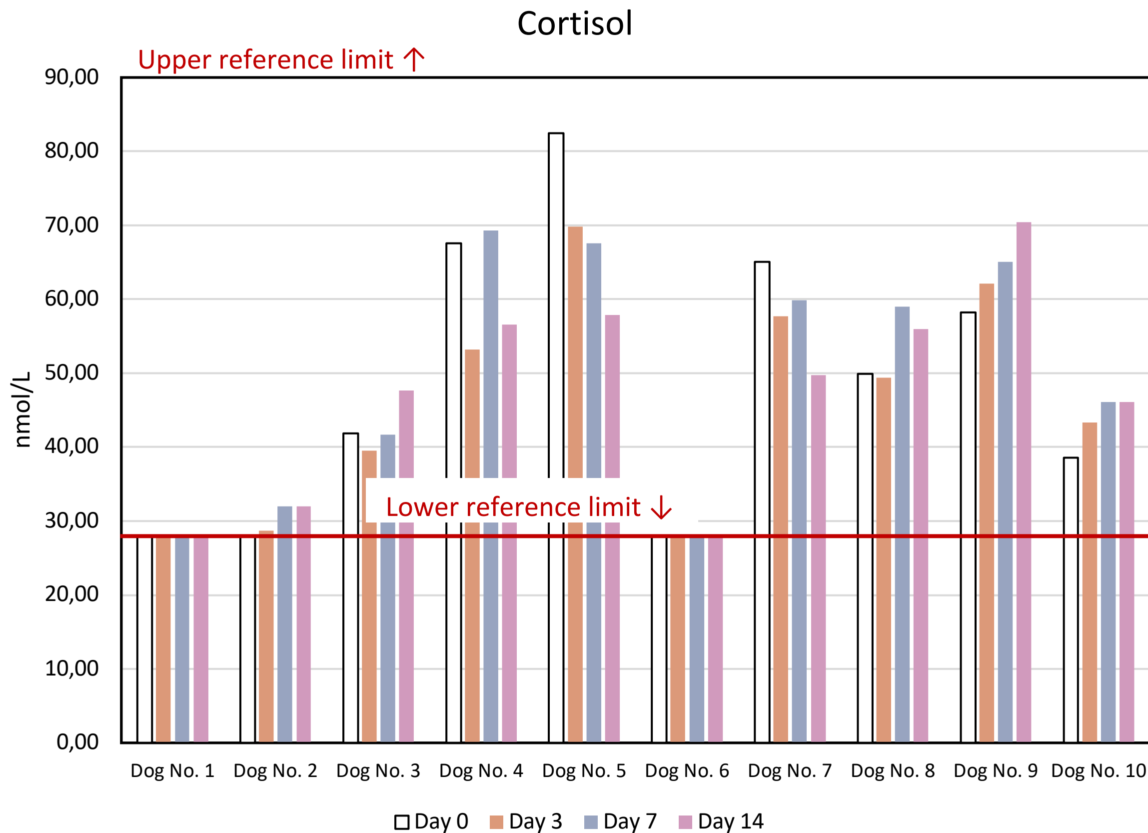

Figure 5. Concentration of cortisol in serum samples from each dog, before and after storage for 3, 7 and 14 days in relation to the upper and lower reference limit. The lower reference limit is 28 nmol/L, and the upper reference limit is 250 nmol/L.

The Δ conc of FT4 in serum was more concurrent between the serum samples than TT4 and the results were statistically significant. The median Δ conc of FT4 from first measurement increased by 8.8%, 6.8% and 12.2% on day 3, 7 and 14, respectively. The distribution of the Δ conc of FT4 from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 6).

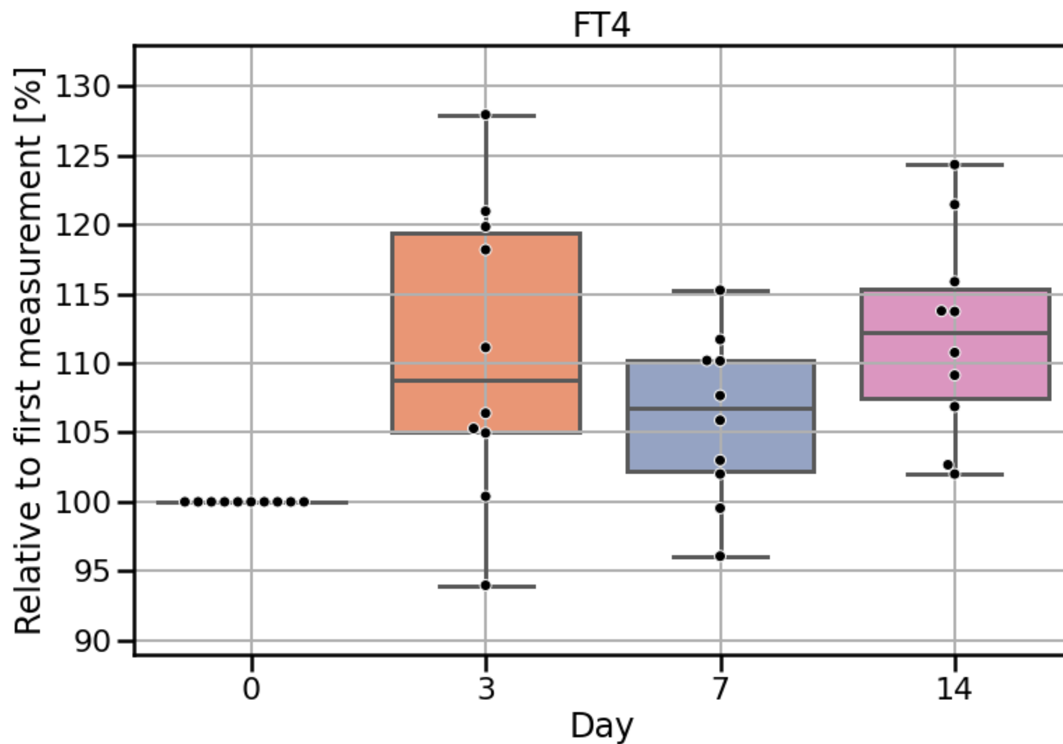

Figure 6. The distribution of the concentration difference of FT4 from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information. FT4 = free thyroxine.

Similar to the concentration change of the liver enzymes, the concentration of TSH increased at first and then decreased. The median concentration of TSH from day 0 increased by 5.7%, 6.5% and 2.2% on day 3, 7 and 14, respectively.

Cholesterol was stable while stored at +7°C for 14 days. All serum samples had little change in the concentration, most of them had increased concentration but one had a decrease. The median Chol concentration increased by 1.3% on day 14.

Metabolites

The metabolites measured in the present study were Alb, Glob, Tprot, Glu, Fruc, BA, urea, Crea and Tbili. The concentration of Tprot, Alb and Glob in serum were stable over 14 days. These proteins changed by less than 1% from first measurement to day 14.

Glucose was stable while stored at +7°C for 14 days. There was little change in the Glu concentration over 14 days, and the median difference was 0% on day 14.

Fructosamine concentration had decreased by 12.5% by day 14. The distribution of the Δ conc of Fruc from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 7).

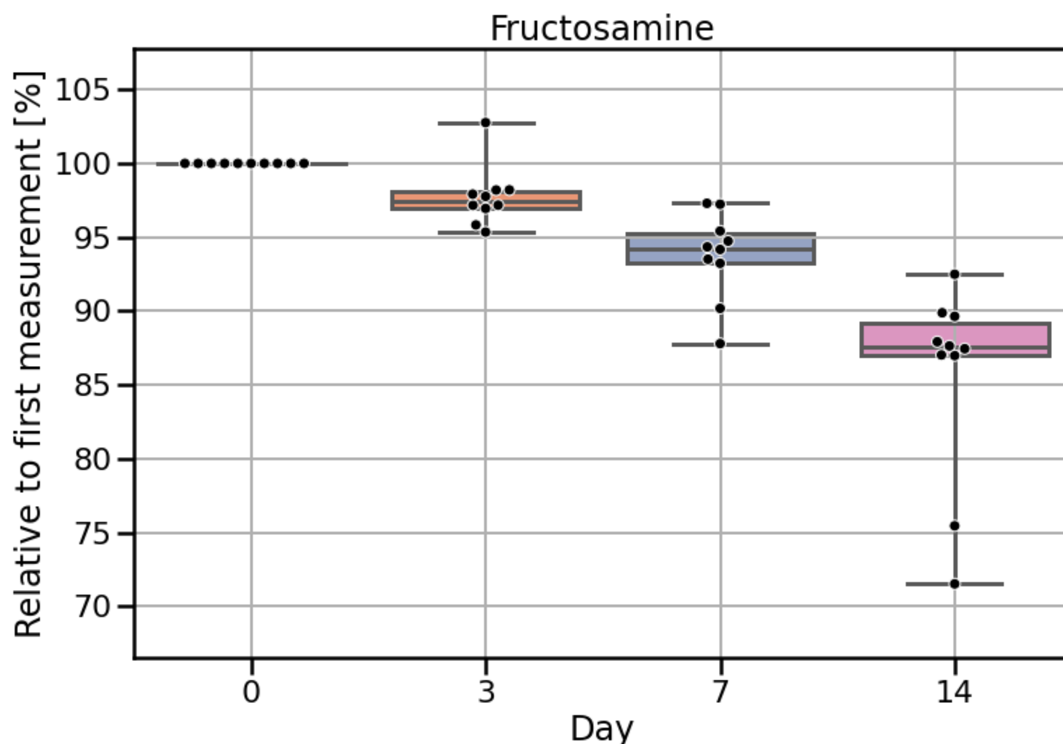

Figure 7. The distribution of the concentration difference of fructosamine from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

The concentration of BA in serum might have seemed stable with median Δ conc as 0% after 14 day. However, the mean change was 5%. The distribution of the Δ conc of BA from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 8). Serum samples from five of ten dogs were stable, had 0% Δ conc of BA after 14 days of storage, while the results for the others seemed random. See (Figure 9) for how the serum concentration of bile acids changed over time in serum samples from each dog in relation to the upper reference limit.

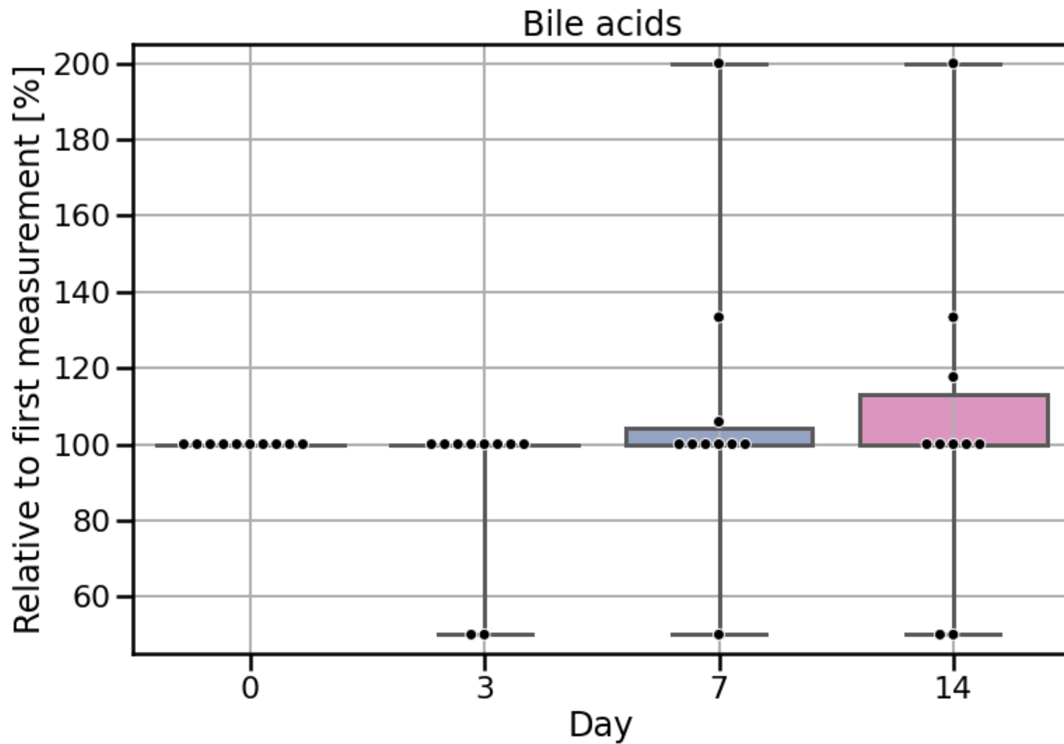

Figure 8. The distribution of the concentration difference of bile acids from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

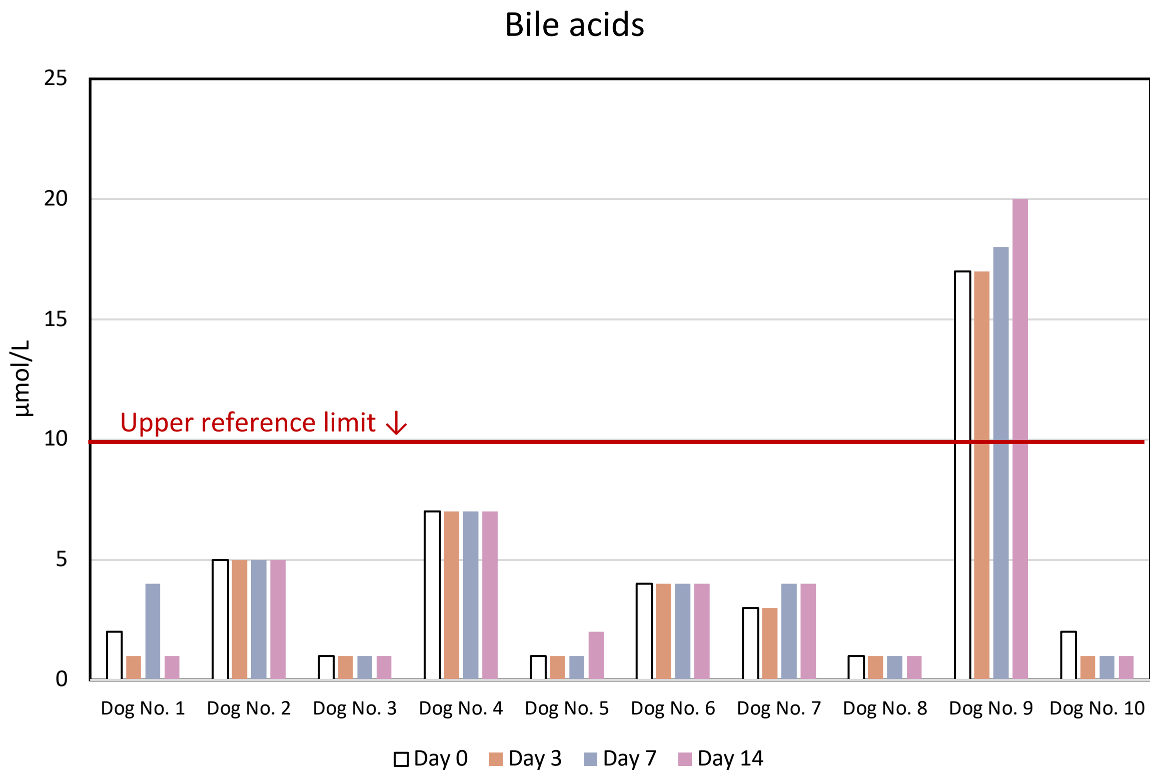

Figure 9. Concentration of bile acids in serum samples from each dog, before and after storage for 3, 7 and 14 days in relation to the upper reference limit. The upper reference limit is 10 µmol/L.

Urea was stable the first 7 days, had however on day 14 increased 2.5% in the median concentration and the results were statistically significant. Distribution of the Δ conc of urea from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 10).

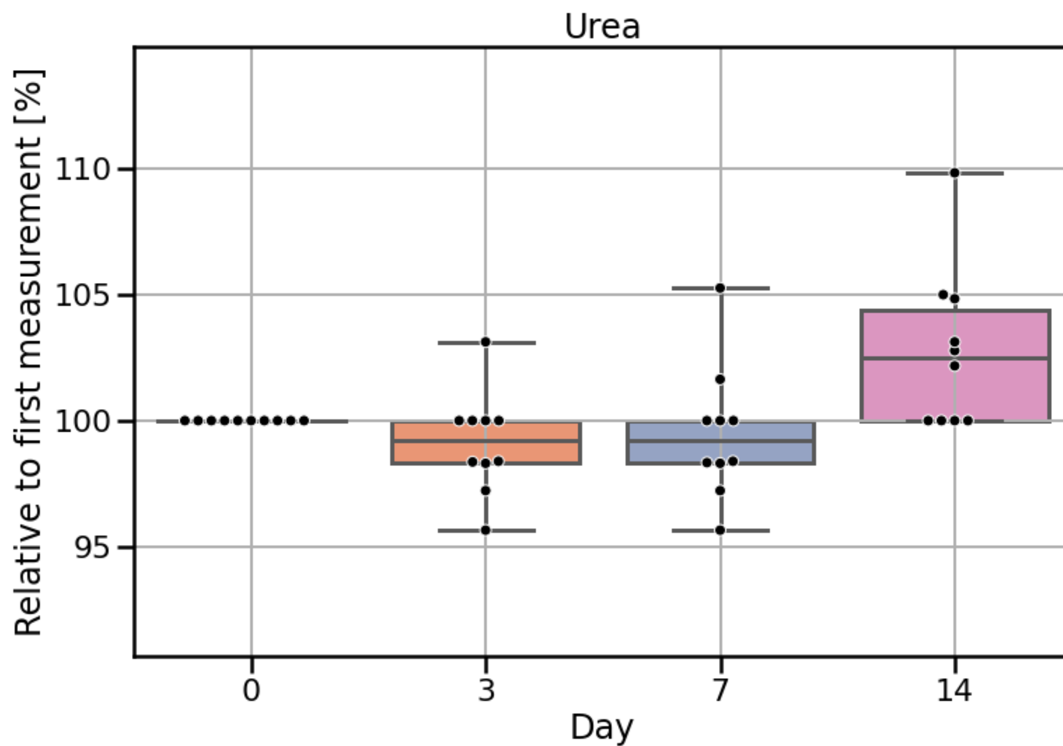

Figure 10. The distribution of the concentration difference of urea from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

The median Crea concentration increased by 7% from first measurement to day 14 and the results were statistically significant. The distribution of the Δ conc of Crea from day 0 to the measurement on day 3, 7 and 14 respectively can be seen in (Figure 11).

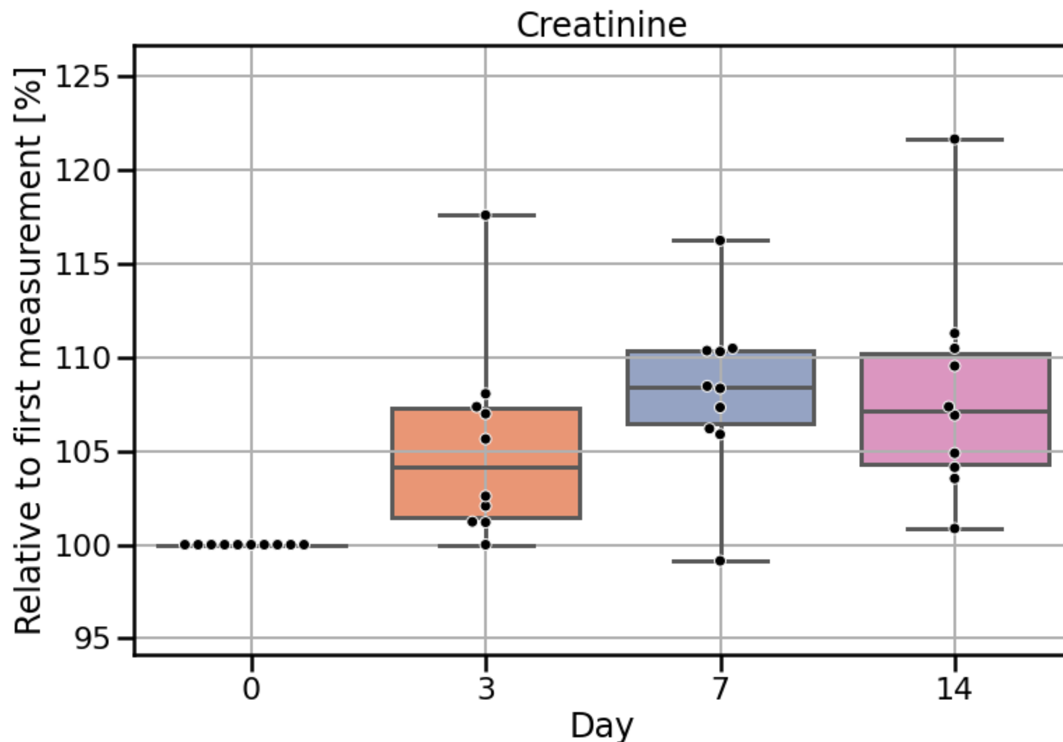

Figure 11. The distribution of the concentration difference of creatinine from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

The median concentration of Tbili decreased by 0% on day 3, and on day 7 and 14 by 15% and 15.8%, respectively. There was high variance in the concentration change of Tbili between the serum samples from the different dogs. Total bilirubin concentration in serum sample from dog No. 7 remained stable with 0% change from day 0 to day 14. The concentration of Tbili had the highest increase in serum sample from dog No. 2 from day 0 to day 7 (50%), and from day 0 to day 14 (100%).

Minerals

The minerals measured were Ca, P, Na, K and Cl. The electrolytes, Ca, Na, K and Cl had little to no change in the concentration on day 3, <1% difference. On day 7 the concentration of these electrolytes had increased slightly in most dogs, the median difference for Ca was 1.4%, for Na 1.6%, for K 2.1%, and for Cl 2.8%. On day 14, the concentration of the

electrolytes had increased even more, especially the concentration of Cl. The median difference was 1.5%, 3.2%, 3.4% and 6.1% for Ca, Na, K and Cl, respectively. Phosphorus increased steadily over time. The median P concentration had increased by 3%, 5.3% and 8.9% on day 3, 7 and 14, respectively. Figure 12, 13, 14, 15, and 16 show distribution of the Δ conc from day 0 to the measurement on day 3, 7 and 14 for Ca, Na, K, Cl and P, respectively.

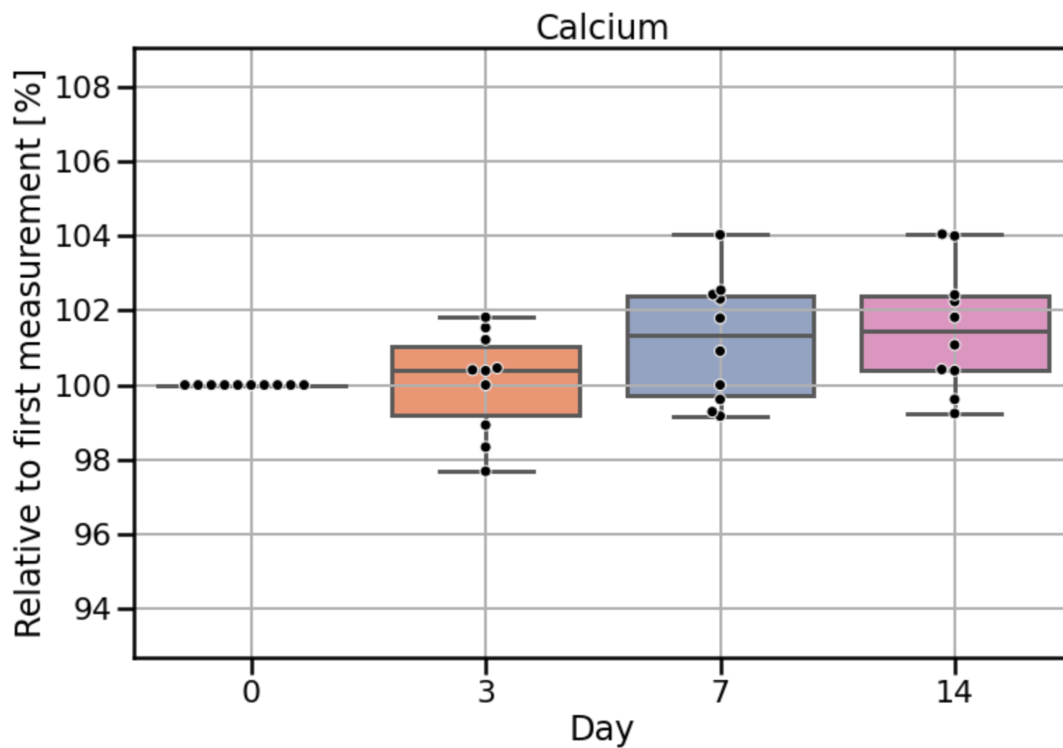

Figure 12. The distribution of the concentration difference of calcium from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

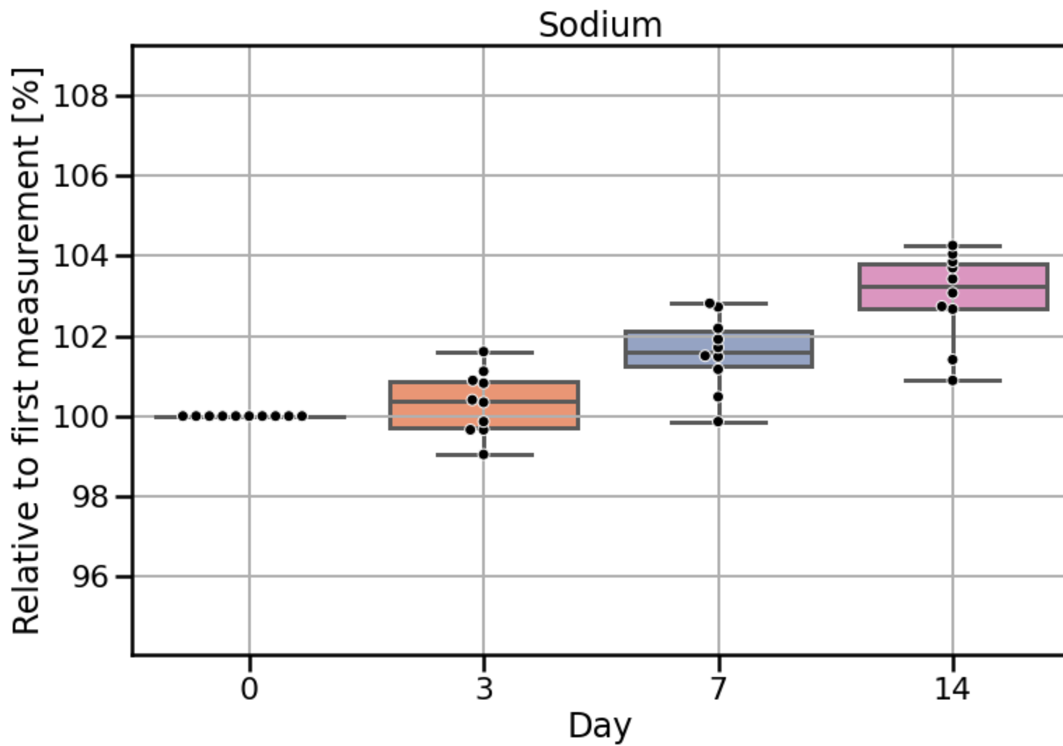

Figure 13. The distribution of the concentration difference of sodium from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

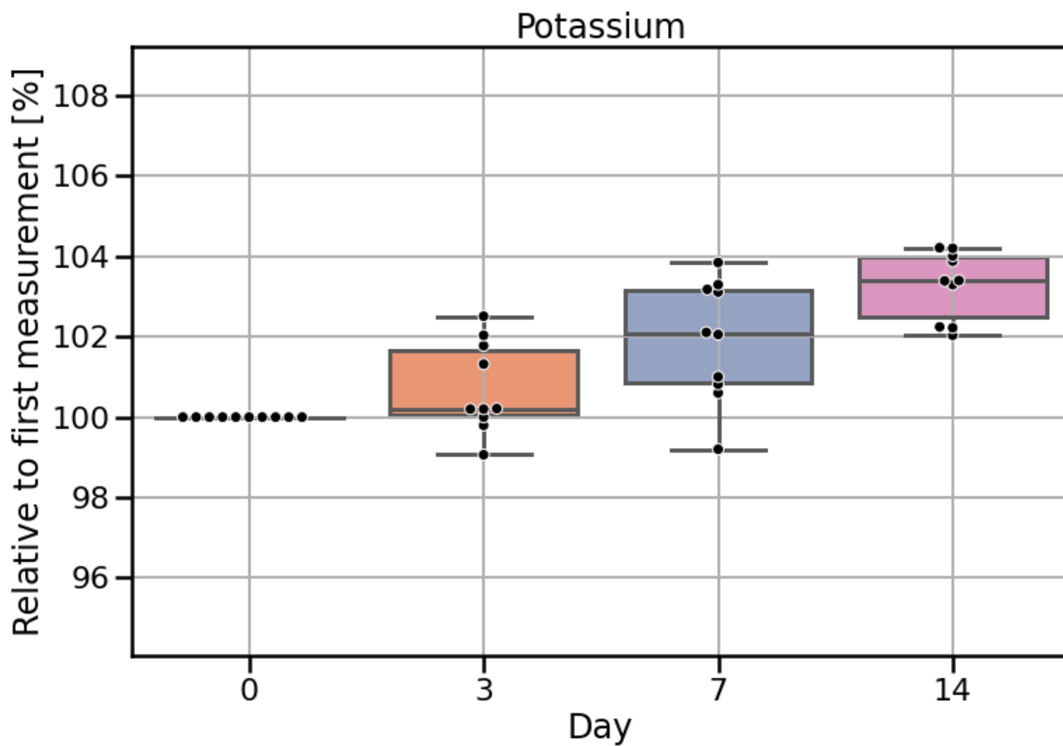

Figure 14. The distribution of the concentration difference of potassium from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

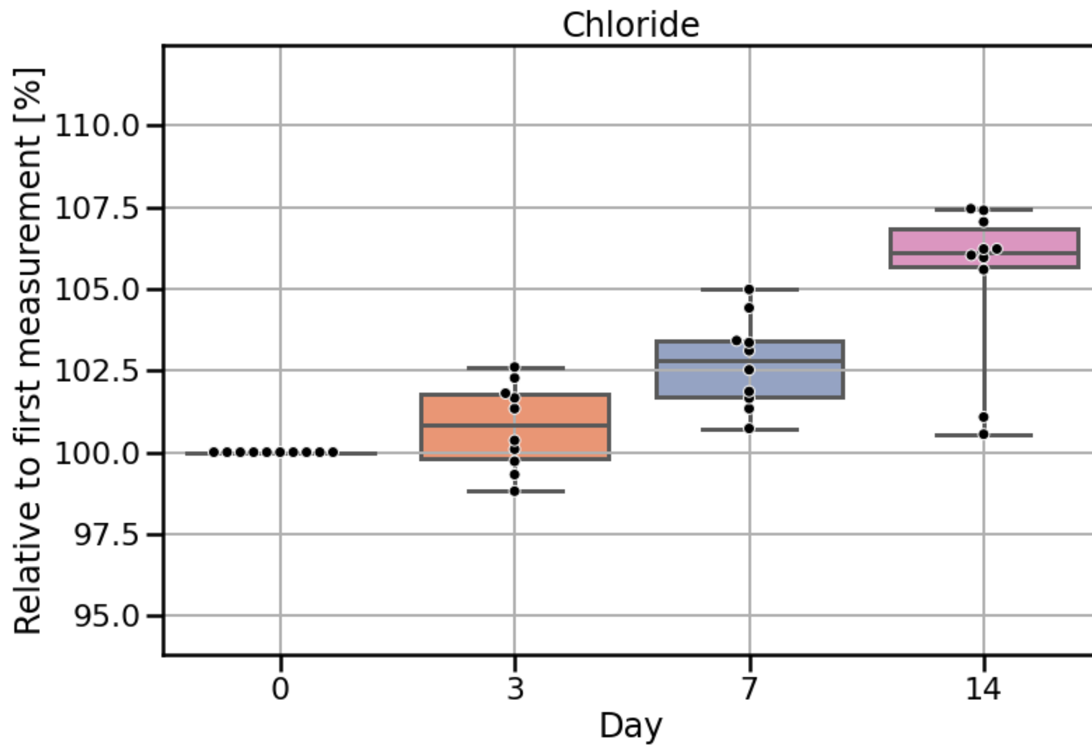

Figure 15. The distribution of the concentration difference of chloride from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

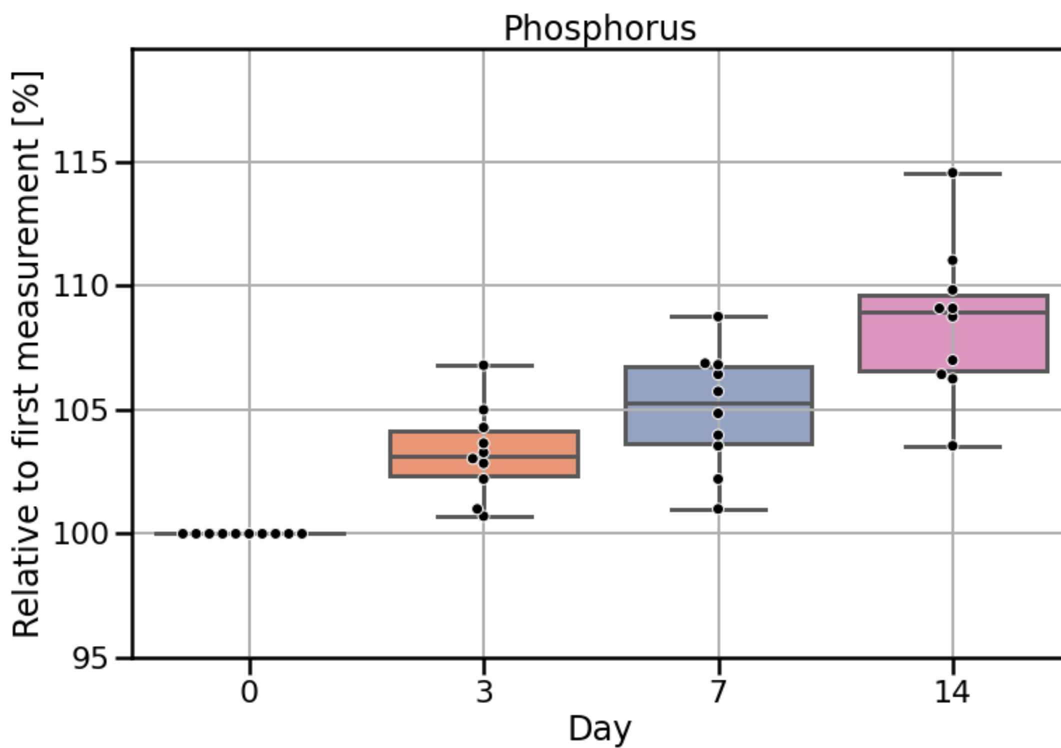

Figure 16. The distribution of the concentration difference of phosphorus from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information.

Others

C-Reactive Protein was one of the most unstable analytes measured in serum. Eight of ten serum samples had >10% Δ conc on day 3 and the majority of the serum samples showed >20% difference in the concentration on day 14. The distribution of the Δ conc of CRP from day 0 to the measurement on day 3, 7 and 14 respectively is shown in (Figure 17). The concentration difference of CRP varied between 65.6% decrease in serum sample from one dog to 115% increase in serum sample from another after 3 days storage, 54.8% decrease and 175% increase after 7 days storage and 46.2% decrease and 220% increase after 14 days storage. A serum sample from dog No. 1 had the highest increase in the CRP concentration over all 14 days and serum sample from dog No. 7 had the highest decrease in the CRP concentration over the same period of time. See (Figure 18) for how the serum concentration of CRP changed over time in serum samples from each dog in relation to the upper reference limit.

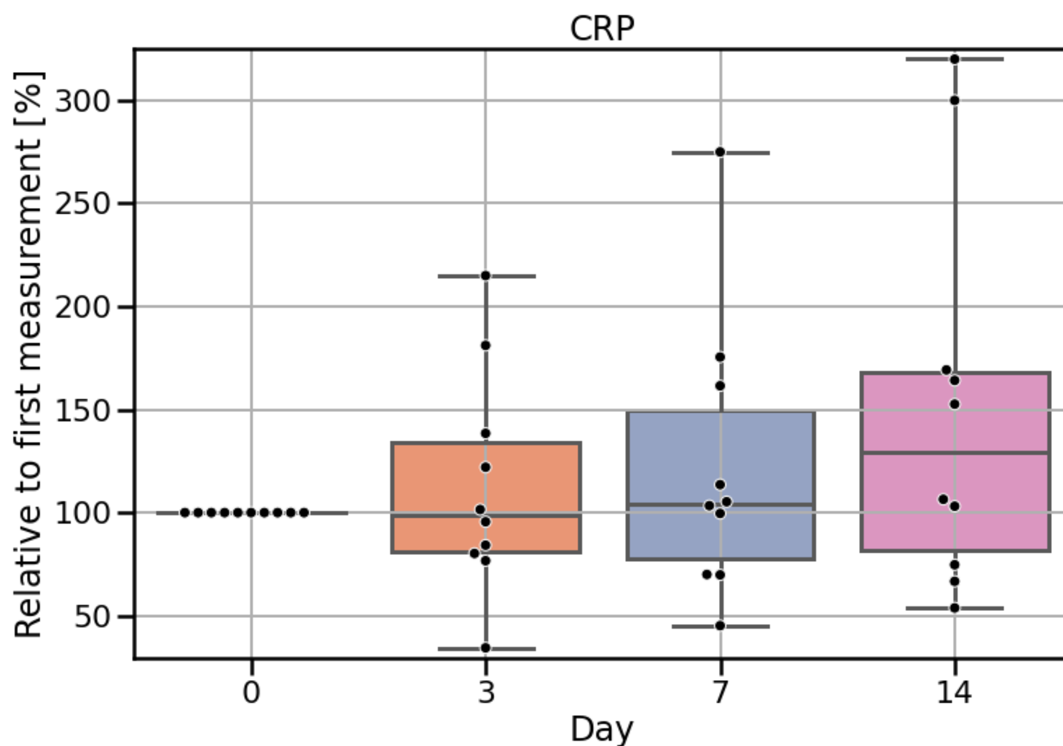

Figure 17. The distribution of the concentration difference of CRP from day 0 to the measurement on day 3, 7 and 14 respectively. See (Figure 1) for more information. CRP = C-reactive protein.

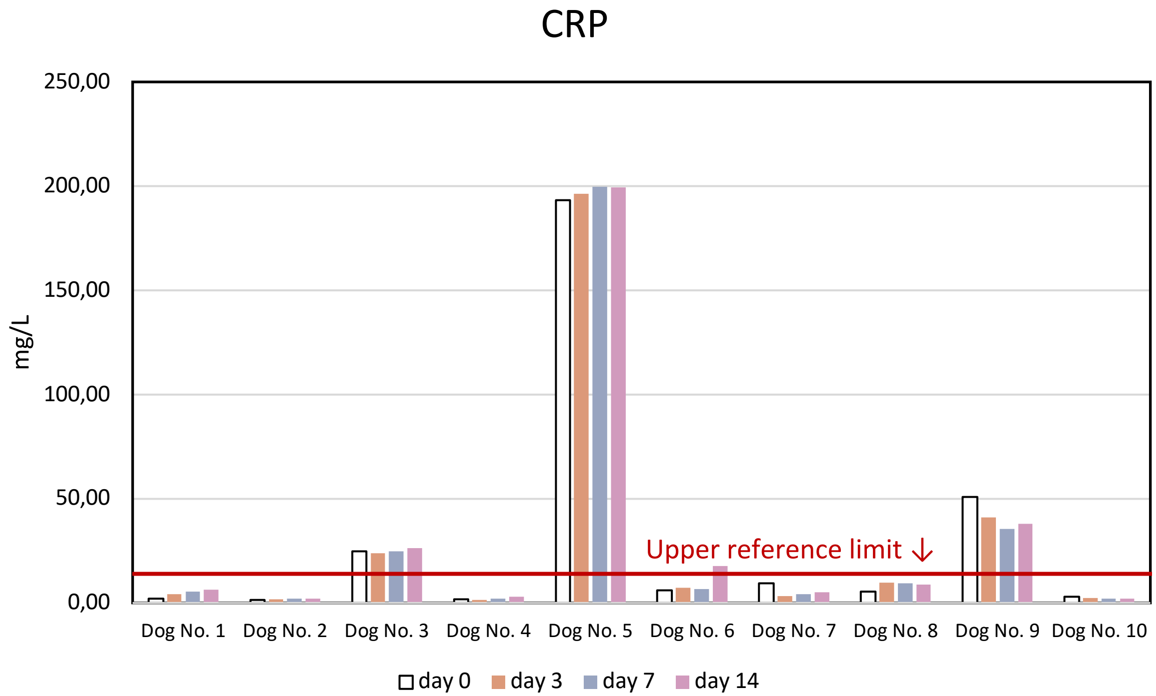

Figure 18. Concentration of CRP in serum samples from each dog, before and after storage for 3, 7 and 14 days in relation to the upper reference limit. The upper reference limit is 15 mg/L. CRP = C-reactive protein.

Table 3. Median (mean) concentration of each analyte from the present study measured on day 0, 3, 7, and 14.[3]

Analytes	*RI*	*Day 0*	*Day 3*	*Day 7*	*Day 14*
Alb	32 – 44 g/L	35 (35)	35 (35)	35 (35)	36 (35)
ALT	0 – 80 U/L	53 (58)	53* (60)	53 (59)	52 (58)
Amy	0 – 1050 U/L	733 (780)	739* (786)	748* (786)	756* (799)
AP	0 – 90 U/L	109 (217)	109 (219)	108 (218)	108 (216)
AST	0 – 40 U/L	39 (45)	40 (47)	40 (45)	37 (43)
BA	0 – 10 µmol/L	3 (4)	2 (4)	4 (5)	3 (5)
Ca	2.2 – 2.9 mmol/L	2.5 (2.5)	2.5 (2.5)	2.6 (2.5)	2.6* (2.6)
Chol	3.4 – 10 mmol/L	6.1 (6.3)	6.1* (6.3)	6.1* (6.4)	6.1* (6.4)
CK	0 – 200 U/L	187 (230)	172* (212)	155* (200)	139* (182)
Cl	99 – 115 mmol/L	108 (108)	108 (109)	111* (111)	113* (114)
Cortisol	28 – 250 nmol/L	46 (49)	46 (46)	53 (50)	49 (47)
Crea	65 – 110 µmol/L	85 (85)	86* (89)	91* (92)	91* (91)
CRP	0 – 15 mg/L	6 (30)	6 (29)	6 (29)	8 (31)
Fruc	250 – 315 µmol/L	232 (232)	227* (227)	223* (218)	205* (200)
FT4	7 – 45 pmol/L	18 (21)	21* (22)	19* (22)	19* (23)
Glob	22 – 31 g/L	24 (24)	24 (24)	24 (24)	24 (24)
Glu	3.6 – 6.6 mmol/L	5.00 (5.03)	5.00 (5.01)	5.00 (5.03)	5.00 (5.01)
K	3.7 – 5.8 mmol/L	4.5 (4.6)	4.6 (4.6)	4.7* (4,7)	4.7* (4.7)
Lip	0 – 150 U/L	23 (24)	22 (23)	22 (23)	22* (22)
Na	140 – 154 mmol/L	146 (146)	146 (147)	148* (148)	151* (150)
P	0.9 – 2 mmol/L	1.37 (1.35)	1.41* (1.39)	1.44* (1.42)	1.48* (1.46)
Tbili	0 – 7 µmol/L	0.75 (1.01)	0.65 (0.95)	0.55 (0.86)	0.50 (0.75)
Tprot	54 – 75 g/L	60 (59)	60 (59)	60* (60)	60 (59)
TT4	16 – 46 nmol/L	27 (27)	28 (28)	27 (28)	27 (28)
TSH	0 – 0.045 µg/L	0.11 (0.13)	0.11* (0.14)	0.11* (0.14)	0.11 (0.13)
Urea	3.5 – 7.2 mmol/L	5.8 (5.4)	5.8 (5.4)	5.9 (5.4)	5.8* (5.6)

[3]The underlined values are outside the reference interval.
*Values are statistically significant ($p<0.05$) different from day 0 value.
Alb = albumin, ALT = alanine transferase, Amy = amylase, AP = alkaline phosphatase, AST = aspartate alanine transferase, BA = bile acids, Ca = calcium, Chol = cholesterol, CK = creatine kinase, Cl = chloride, Crea = creatinine, CRP = C-reactive protein, Fruc = fructosamine, FT4 = free thyroxine, Glob = globulin, Glu = glucose, K = potassium, Lip = lipase, Na = sodium, P = phosphorus, RI = reference interval, Tbili = total bilirubin, Tprot = total protein, TSH = thyroid stimulating hormone, TT4 = total thyroxine

Table 4. Mean and median percentage difference on day 3, 7 and 14.[4]

	Day 3 (%)		*Day 7 (%)*		*Day 14 (%)*	
Analytes	*Mean*	*Median*	*Mean*	*Median*	*Mean*	*Median*
Alb	-0.6	-0.4	+0.07	+0.5	-0.1	-0.2
ALT	+3.2*	+2.9*	+1.5	+0.8	-0.3	0
Amy	+1*	+1.1	+2*	+2	+2.7*	+2.4*
AP	+0.06	+0.2	+0.96	0	+0.08	0
AST	+8.1*	+10.1*	+3.4	+4	-1.4	0
BA	-10	0	+8.9	0	+5.1	0
Ca	+0.1	+0.4	+1.2*	+1.4*	+1.5*	+1.5*
Chol	+0.8*	+0.6*	+1.4*	+1.5*	+1.3	+1.3
CK**	-7.7*	-7.8*	-14.1*	-13.3*	-21.9*	-21.3*
Cl**	+0.8	+0.8	+2.7*	+2.8*	+5.4*	+6.1*
Cortisol**	-3.3	-0.5	+4	+1.3	+1.1	+6.1
Crea**	+5.3*	+4.1*	+8.3*	+8.4*	+8.1*	+7.1*
CRP**	+13	-1.5	+22	+4.3	+51	+29.5
Fruc**	-2.3*	-2.5*	-6.2*	-5.7*	-14.4*	-12.5*
FT4**	+10.9*	+8.8*	+6.2*	+6.8*	+12.1*	+12.2*
Glob	+0.7	0	+0.8	0	+0.5	0
Glu	-0.3	0	+0.1	0	-0.4	0
K	+0.7	+0.2	+1.9*	+2.1*	+3.3*	+3.4*
Lip	-1.5	0	-2.2	-1.8	-5.2*	-4.2*
Na	+0.3	+0.4	+1.6*	+1.6*	+3*	+3.2*
P**	+3*	+3*	+5*	+5.3*	+8.6*	+8.9*
Tbili	-1.9	0	-10.7	-15	-18.2	-15.8
Tprot	+0.3	+0.23	+0.7*	+0.17*	+0.1	+0.01
TSH	+7.3*	+5.7*	+7.6*	+6.5*	+3.3	+2.2
TT4	+4.1	-0.4	+6.1	+2.3	+3.4	+2.7
Urea	-0.9	-0.8	-0.5	-0.8	+2.8	+2.5*

[2]+ represent an increase and – represents a decrease from day 0 value.
*Values are statistically significantly ($p<0.05$) different from day 0 value.
**Values are clinically relevant after 14 days of storage.
Alb = albumin, ALT = alanine transferase, Amy = amylase, AP = alkaline phosphatase, AST = aspartate alanine transferase, BA = bile acids, Ca = calcium, Chol = cholesterol, CK = creatine kinase, Cl = chloride, Crea = creatinine, CRP = C-reactive protein, Fruc = fructosamine, FT4 = free thyroxine, Glob = globulin, Glu = glucose, K = potassium, Lip = lipase, Na = sodium, P = phosphorus, Tbili = total bilirubin, Tprot = total protein, TSH = thyroid stimulating hormone, TT4 = total thyroxine.

Discussion

Results from analytes with high variation

The results show that most clinical chemical analytes in serum are stable for 14 days at +7°C. However, some of these analytes had a high variation in the Δ conc between serum samples from the ten dogs studied. One cannot trust that the concentration of these analytes remains stable during storage. These analytes were BA, cortisol, CRP and TT4.

Hormones

The Δ conc of TT4 varied a lot between serum samples in dogs and no conclusion on the stability of TT4 concentration in serum could be made**.** Serum samples from dogs No. 8 and 9 had TT4 concentration under the RI on day 0, the serum samples from these two dogs had a high increase in the serum concentration of TT4. Serum sample from dog No. 3 had TT4 concentration over the RI on day 0, the concentration of TT4 remained stable over 14 days in serum sample from this dog. Hypothyroidism might affect the stability of TT4 in serum according to this result, but since the Δ conc varied so much between the dogs, independent on diagnosis no conclusion could be made on this matter.

Along with TT4 concentration in serum, the Δ conc of cortisol over 14 days varied between serum samples from the different dogs and the results were not significant. Serum sample from dog No. 5 had the highest decrease in the serum cortisol concentration over all 14 days. Serum samples from dogs No. 1 and 6 had a stable cortisol concentration, 0% Δ conc, over all 14 days. This might not be true since the cortisol concentration in the serum samples from these dogs were <28 nmol/L all 14 days and the analyser used in this study does not register measurement of cortisol under 28 nmol/L. If the serum samples from dogs No. 1 and 6 would not have been included in the median Δ conc, the median Δ conc would be 3.4% decrease from day 0 to day 3, 7.2% increase from day 0 to day 7, and 13% increase from day 0 to day

14. This might be a better estimation of how storage over time affects cortisol concentration in serum. Serum samples from five dogs had an increased serum cortisol concentration over time, while serum samples from three dogs had a decrease of similar size.

Metabolites

The concentration difference of BA concentration in serum varied a lot between the serum samples from the ten dogs studied. Dog No. 9, had BA concentration over the RI, the Δ conc of BA in serum sample of this particular dog increased by 5.9% from day 0 to day 7 and by 17.7% from day 0 to day 14. The increased concentration of BA in serum samples after storage might therefore be attributed to a liver disease, or other diseases related to high BA concentration in serum. Because of the differences in the concentration change between samples it is hard to make any conclusions about the stability of BA in serum, and the results were not significant.

Others

The concentration difference of CRP varied a lot, the serum sample from dog No. 1 had the highest increase in the CRP concentration over all 14 days and serum sample from dog No. 7 had the highest decrease in the CRP concentration over the same period of time. Both dog No. 1 and No. 2 had healthy CRP levels in serum. Because of a broad variation in the results between the serum samples, no proper conclusion on the stability could be made. Since the majority of the serum samples showed >20% difference in the concentration on day 14, one can assume that CRP in serum is unstable when stored over 14 days at +7°C.

Individual variations

The most unstable serum sample came from dog No. 1, which had the highest Δ conc when looking at every clinical chemical analyte together. The instability of the serum sample from dog No. 1 is mostly attributed to the high Δ conc of CRP. The most stable serum sample from

day 0 to day 7 came from dog No. 6. However, the serum sample that came from dog No. 6, was unstable when stored for 14 days. This is attributed to the high Δ conc of CRP in serum sample from dog No. 6 after 14 days of storage.

Metabolites

Serum sample from dog No. 9 had the highest Δ conc of Alb, Fruc and Crea on all measurement days. Some of these analytes had decreased concentration in serum while other had increased concentration in serum. The serum sample from dog No. 3 had the highest increase in metabolites concentration from day 0 to day 3, serum sample from dog No. 1 and 2 had the highest increase in metabolites concentration from day 0 to day 7 and serum sample from dog No. 2 had the highest increase in metabolites concentration from day 0 to day 14.

Minerals

Serum samples from dogs No. 7 and 8, had the highest Δ conc after 14 days of storage in all of the minerals. The serum samples from these two dogs did not have higher Δ conc of minerals after 3 and 7 days of storage.

Clinical relevance

One important aspect of studying the stability of clinical chemical analytes in serum is finding out how storage over time will affect the clinical relevance. The results from the present study show that the clinical relevance can be affected in some clinical chemical analytes in serum when stored at +7°C, up to 14 days. When reading the blood test results, one has to be aware of the fact that a clinical chemical analyte not within the RI are not always abnormal. Reference interval is only a guideline used by veterinarians and laboratorians. When one analyte is outside of the RI in a serum sample that has been stored over time, an advice is to look at the results from the other analytes that are connected to the same disease or organ. For example, the results for CK should have some correlation with the results for AST, the results

for Fruc should have some correlation with the results for Glu, and the results for Cl should more or less correlate with the results for Na and K. Clinical relevance of an analyte is also less likely to be affected in nonspecific analytes that are connected to more than one organ or disease. Small increases in the Δ conc over time can be related to the precision of the analysis and is not clinically relevant. Therefore, analytes with <6% Δ conc will not be considered to have clinically relevant results in the present study. In the present study the results showed that the clinical relevance may be affected in the concentration of CK, Cl, cortisol, Crea, CRP, Fruc, FT4 and P when stored at 7+°C for 14 days.

Enzymes

The results in the present study of the Δ conc of CK is clinically relevant. Serum levels of CK increases rapidly after muscle damage and the magnitude of the increase is somewhat proportional to the degree of muscle damage (2). Serum CK can be evaluated in many diverse muscle diseases. The most marked increase in serum CK activity (>20000 U/L) are associated with necrotizing myopathies or muscular dystrophies. Generalized inflammatory myopathies usually show moderate increase in serum CK concentration (2000-20000 U/L), while focal inflammatory myopathies such as masticatory muscle myositis, endocrine myopathies, neuropathies and other congenital muscle diseases are normal or only mildly increased (0-2000 U/L) (17). If serum is stored over 14 days at +7°C serum concentration slightly over 20000 U/L would decrease to around 16000 U/L, this might lead to false diagnoses, rather to diagnose the animal with necrotizing myopathies or muscular dystrophies a veterinarian might diagnose it with generalized inflammatory myopathies. This is still very high serum concentration of CK, and most veterinarians would run more diagnostic tests before making a final decision. The clinical relevance of the serum concentration of CK would mostly affect the congenital muscle diseases while the storage of serum might also mask the severity of a muscle injury.

Hormones

While the results for FT4 might be clinically relevant the serum concentration of FT4 is usually compared to the serum concentration of TT4, TSH and Chol. The median Δ conc of TT4, TSH and Chol remained stable in serum over 14 days at +7°C. However, in some situations this storage condition would affect the clinical relevance of these hormones and hypothyroidism could be ruled out in hypothyroid dogs or more tests could be considered necessary. Reduced concentration of FT4, TT4 and TSH and increased concentration of Chol in serum indicates hypothyroidism (12).

Cortisol increased by 6.1% after storage for 14 days and this result is clinically relevant when a low-dose dexamethasone suppression test (LDDS test) is performed. Low-dose dexamethasone suppression test is done on dogs suspected with hyperadrenocorticism and it can detect the cause of the disease. The test involves injecting 0.01 mg/kg dexamethasone intravenous and take a blood sample after 4 hours and 8 hours. In a healthy dog low-dose of dexamethasone inhibits pituitary secretion of adrenocorticotropic hormone which causes prolonged decline in circulating cortisol. In dogs with pituitary dependent hyperadrenocorticism, low-dose dexamethasone causes small suppression of circulating cortisol, but it is no longer suppressed 8 hours after the injection. In dogs with adrenocortical tumour low-dose dexamethasone does not cause reduced cortisol concentration in serum. In some situations, storing serum for 14 days could lead to a healthy dog being diagnosed with hyperadrenocorticism. Additionally, a dog with pituitary dependent hyperadrenocorticism could be diagnosed with adrenocortical tumour (18). However, storing serum over 14 days period is not normally practised when taken LDDS test.

Metabolites

Seven of ten serum samples had Fruc concentration below the RI (250-315 µmol/L) on day 0. The three serum samples that did have Fruc concentration within the RI initially, all fell

below the RI on day 3, or on day 7. This could mean that the RI for Fruc in serum at VetLab NMBU is set too high. The serum samples that were within the RI were all from bitches. Nothing else could be linked between the serum samples inside or outside of the RI. The population is too small to conclude about the difference in the serum concentration of Fruc between bitches and male dogs, especially since there were more bitches than male dogs in the present study. Because RI cannot be established using less than 20 dogs, I cannot state that the RI VetLab NMBU uses is wrong. However, if we look at the RI for other methods used at other laboratories to measure the Fruc concentration in serum the RI seems to be lower, e.g., Idexx (19,20). Normal concentrations of Fruc can differ between different populations according to breed, geography, and more (21). It would be interesting to do a further study on this matter related to the method used in the VetLab NMBU in Norway. Fructosamine concentration in serum is an indicator of blood glucose over longer period of time than glucose concentration in serum and it is used to manage e.g., an insulin treatment (4). If a blood test from an undiagnosed diabetic patient shows high glucose concentration in serum, veterinarian might order a fructosamine concentration test from the remaining serum. This might lead to decreased fructosamine concentration in the serum sample and the veterinarian might believe the high glucose concentration would be attributed to stress and not diabetes. If a blood test is stored from a diabetic animal, that is not responding to an insulin treatment, the fructosamine concentration in serum might be reduced when the serum is analysed. This could lead to false interpretation by the veterinarian. Serum Fruc at the lower half of the RI or below should raise concern for periods of hypoglycaemia in a diabetic dog or reversion to noninsulin-requiring diabetic state (22).

Creatinine production is relatively stable and increased serum Crea is connected to decreased glomerular filtration rate. The cut off value to diagnose a dog with azotaemia can be 115, 126 or 150 µmol/L depending on the clinic (23,24). 115µmol/L is lower than 7.1% increase of 110

μmol/L which is the upper reference limit of Crea in serum. Azotaemia is a medical condition where there is a high level of nitrogen-containing compounds such as Crea and urea in blood. Azotaemia can have prerenal, renal or postrenal causes. Azotaemia can lead to kidney failure if not controlled (4). Therefore, the results for Crea are clinically relevant after storage for 7 or 14 days, when the cut off at 115 μmol/L is used. But since the cut off level varies between clinics the clinical relevance of the results for Crea can be debated.

Minerals

Serum concentration of minerals must be maintained at narrow limits. Small changes in the concentration of electrolytes may indicate an acid-base imbalance and lead to clinicians to decide on iv-fluid injection. Serum concentration of Na and Cl are often measured in relation to each other, the results from the present study showed that Cl increases disproportional to Na when serum is stored over time. If the degree of hypochloraemia is proportional to the degree of hyponatraemia the clinicians will come with the following differential diagnoses: loss through vomiting or secretory diarrhoea. If chloride is decreased to a greater degree than sodium, differential diagnoses related to metabolic alkalosis must be considered (12). This disproportional Δ conc of Na:Cl is therefore clinically relevant.

Calcium was the most stable mineral measured in the present study and does not show clinically relevant results since 1.5% increase from 2.9 mmol/l is 2.94 mmol/L. However, the results for P showed that storing serum for 14 days can lead to an increase by 0.2 mmol/L in the serum concentration of P. When Ca is within the RI and there is a mild to moderate hyperphosphatemia clinicians could diagnose the animal with nutritional secondary hyperparathyroidism. Calcium is carefully regulated and need only change by 0.01 mmol/L to stimulate production and release of parathyroid hormones that will normalize the serum calcium levels. Therefore, when serum Ca is measured it is usually within the RI but at the lower end. Phosphorus, on the other hand is not regulated as well, therefore increased serum P

is a key to diagnose nutritional secondary hyperparathyroidism (12). The results of Δ conc of P from the present study are therefore clinically relevant.

Others

The median concentration of CRP increased by 29.5% which is considered to be clinically relevant. High serum CRP concentration indicates inflammation and/or infection and will lead to a treatment with anti-inflammatory medicaments.

This shows how measurements of serum samples stored over time are not necessarily a stable indicator of the healthiness of the individual from which the sample was taken. It might be wise to take another blood sample when serum has been stored over time, if the analytes that had clinically relevant results after storage are important for the diagnosis. In the future it might be possible to introduce some tolerances in relation to storage time, widen, raise or lower the RI for the different analytes in serum when it is stored over time, accordingly to the results in this and other similar studies. For some analytes it could be possible to define when measurements are not reliable after storage over defined period of time.

Comparison with previous studies

Most of the results were pretty similar to results from previous studies (Table 5). The comparative studies are, a study done by Thoresen et al., 1992, on the stability of analytes in canine serum for 3 days at +20°C, a study done by An & Park, 2014, on the stability of analytes in human serum for 14 days at +4°C, and a study done by Cray et al., 2009, on the stability of analytes in rat serum for 7 days at +4°C.
Glucose was one of the most stable analytes measured in serum in all of the studies including the present one. The other studies also showed similar results to the present study for the stability of CK, Crea and Tbili.

The greatest difference between studies were the results for the liver enzymes. In a study done by An & Park, 2014, on human serum, the serum concentration of AST, ALT and AP decreased by >10% after 14 days storage, while these enzymes were more stable in the present study and the other two studies mentioned earlier (13,14). This is most likely attributed to the difference between species.

Other factors than storage time affecting stability

As mentioned in the introduction, there are several factors that can affect the stability of clinical chemical analytes in serum. There is a possibility that some of these factors have affected some serum samples in the present study but not others since the stability of some analytes differed between the serum samples from the 10 dogs to a large extent.

Changes in enzymes like Amy, ALT, AP, AST, CK and Lip are hypothesized to occur due to instability of enzymes isoforms (13). Since CK had the highest percentage difference on day 14 it is possible that CK has the most unstable isoforms of these enzymes in dogs. It is also possible that human serum has more unstable isoforms of the liver enzymes than canine serum.

Increased serum concentration of minerals over time may be connected to cellular remnants in the serum samples (10). Since there was a higher increase in the serum concentration of all minerals in serum samples from dogs No. 7 and 8 after 14 days, there is a possibility that higher concentration of cellular remnants entered these two serum samples, possibly by longer coagulation period.

Increased serum concentration of many analytes over time can be connected to evaporation. This is especially true for metabolites (16). Serum sample from dog No. 2 had the highest increase in metabolite concentration in serum from day 0 to day 7 and day 14. Therefore, there might have happened evaporation in the aliquots marked day 7 and day 14 in serum sample from dog No 2, which lead to this increase. High increase in the concentration of

metabolites might have also happened in serum sample from dog No. 1 in aliquot marked with day 7 and serum sample from dog No. 3 in aliquot marked with day 3.

Bias and variability

The present study consists of a small population that makes it hard to make a statistically relevant result. Most similar studies use a population of healthy dogs, and often use the same breed but the present study used dogs with different diagnoses and of different breeds. It is possible that the stability of clinical chemical analytes differs between different dog breeds like it differs between species, and that some diseases affect the stability of clinical chemical analytes in serum. However, using a varied population of dogs makes the present study more relevant in general practice were blood samples are taken from clinically ill individuals of different breeds. Because of this, median was used in the present study rather than mean. Median is a more stable estimator, since an abnormal concentration of an analyte in one serum sample will skew the mean concentration.

The blood samples were standardized as much as possible, the same kind of blood tube was used for all, the same centrifuge technique, the same refrigerator for all serum samples, and the same analyse technique used for each analyte at each time. There might have been variation between blood sampling techniques, how long it took each blood sample to arrive to VetLab NMBU after blood sampling, and therefore how long it was allowed to coagulate.

In every laboratory procedure there is some uncertainty, and the present study is not an exception. The Δ conc varied to a large extent between serum samples in some clinical chemical analytes measured, and this variation can be linked to measurement uncertainty of the analyser or the laboratory procedure done by humans. The measurement uncertainties were not available for the analysers used in the present study, IMMULITE 2000 and Advia 1800. It is unknown why some analytes were significant on day 3 and/or 7 but not on day 14 but it might be related to measurement uncertainty or preanalytical factors.

Table 5. Comparing results from previous studies to the results from the present study, the median percentage difference from day 0 value from each study.[5]

	Day 3 (%)			***Day 7 (%)***			***Day 14 (%)***	
Analytes	*The present study*	*Thoresen et al., 1992*	*An & Park, 2014*	*The present study*	*Cray et al., 2009*	*An & Park, 2014*	*The present study*	*An & Park, 2014*
Alb	-0.4	<±3	0	+0.5	-2.7	-2.5	-0.2	0
ALT	+2.9*	<±3	-6	+0.8	+0.4	-5	0	-10
Amy	+1.1	+4*		+2	+1.4		+2.4*	
AP	+0.2	+6	-2	0	-2.5	-7.5	0	-13.8
AST	+10.1*	<±3	-4.5	+4	-3.8	-7.5	0	
BA	0	+54*		0			0	
Ca	+0.4	<±3	+3	+1.4*	+0.8	+3	+1.5*	+5
Chol	+0.6*	<±3	0	+1.5*		+5.5	+1.3	+4.5
CK	-7.8*	-20*		-13.3*	-11*		-21.3*	
Cl	+0.8		-1	+2.8*	-0.2	-1	+6.1*	+1.3
Crea	+4.1*	+7	+4.5	+8.4*		+2	+7.1*	+4.5
Fruc	-2.5*	-8		-5.7			-12.5*	
Gluc	0	<±3	0	0	+1.6	+1.3	0	-1.3
K	+0.2	+0.4	-2.5	+2.1*	-0.7	-2.5	+3.4*	0
Lip	0	+8		-1.8	-0.9		-4.2*	-15
Na	+0.4	<±3	0	+1.6*	-1	0	+3.2*	+1
P	+3*	<±3	-1	+5.3*	-0.5	0	+8.9*	+2.5
Tbili	0	-11.5	-11	-15		-11	-15.8	-11
Tprot	+0.23	<±3	-1.3	+0.17*	-4.1	-1.3	+0.01	-3
Urea	-0.8	+4		-0.8			+2.5*	

[5] + represent an increase and – represents a decrease from the day 0 value, <± represent increase or decrease under the mentioned value.
*Values are statistically significantly ($p<0.05$) different from the day 0 value.
Alb = albumin, ALT = alanine transferase, Amy = amylase, AP = alkaline phosphatase, AST = aspartate alanine transferase, BA = bile acids, Ca = calcium, Chol = cholesterol, CK = creatine kinase, Cl = chloride, Crea = creatinine, Fruc = fructosamine, Glu = glucose, K = potassium, Lip = lipase, Na = sodium, P = phosphorus, Tbili = total bilirubin, Tprot = total protein.

Conclusions

According to the present study most clinical chemical analytes in serum were stable for 14 days at +7°C. However, this storage condition did affect the clinical relevance of a few analytes. The analytes that were most unstable in the present study were CK, CRP, Fruc, FT4 and Tbili, all had over 10% change in the concentration after 14 days. The clinical relevance was affected in the concentration of CK, Cl, cortisol, Crea, CRP, Fruc, FT4 and P after 14 days of storage at +7°C. The RI for Fruc might be too high since most serum samples had Fruc concentration below the RI on day 0. Sometimes it can be wise to use other, more stable analytes in serum that are linked to the same diseases or organs to compare with the results, e.g., the results for CK should have some correlation with the results for AST, the results for Fruc should have some correlation with the results for Glu, and the results for Cl should more or less correlate with the results for Na and K. Reference intervals are only a guideline that 95% of serum samples from healthy animals fall within, and one has to be careful when using it.

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Sammendrag

Tittel: Stabilitetsstudie av klinisk kjemiske analytter i serum fra hunder ved lagring i kjøleskap

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Veileder: Hege Brun-Hansen og Karin Hultin Jäderlund, Institutt for sports- og familiedyrmedisin

Denne studien undersøker stabiliteten av kliniske kjemiske analytter i serum lagret i 14 dager ved +7° C. Studiepopulasjonen inkluderte 10 hunder over 10 kg som ankom til Dyresykehuset-smådyr fra 30. november til 8. desember 2020. Hundene varierte i rase, kjønn, alder og kliniske tegn. Etter koagulering og sentrifugering ble serumet fra hver hund delt i 4 alikvoter, tre ble satt inn i kjøleskap mens en ble analysert samme dag. En alikvot fra hver hund ble analysert etter 0, 3, 7, og 14 dager. De kliniske kjemiske analyttene som ble målt, var A:G, Alb, ALT, Amy, AP, AST, BA, Ca, Chol, CK, Cl, cortisol, Crea, CRP, Fruc, FT4, Glob, Glu, K, Lip, Na, Na:K, P, Tbili, Tprot, TSH, TT4 og urea. Resultatene viste at de fleste kliniske kjemiske analyttene i serum var stabile etter lagring ved +7°C i 14 dager. De mest ustabile kliniske kjemiske analyttene i denne studien var CK, CRP, Fruc, FT4 og Tbili, som alle hadde over 10% median konsentrasjonsendring etter 14 dager. Denne lagringen påvirket den kliniske relevans av CK, Cl, cortisol, Crea, CRP, Fruc, FT4 and P.

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Appendix

Appendix 1

Til Dyrepleiere og Veterinærer ved Dyresykehuset smådyr.

Jeg trenger hjelp til innsamling av materiale til mitt fordypningsarbeid:

STABILITETSSTUDIE AV KLINISK KJEMISKE ANALYTTER I SERUM VED LAGRING I KJØLESKAP.

Fordypningsoppgaven min går ut på å måle stabiliteten av ulike klinisk kjemiske analytter i serum over tid.

I dag rekvireres vanligvis stor profil på Sentrallaboratoriet. Restserum lagres i kjøleskap på Sentrallaboratoriet i 14 dager. Det er mulig å etterbestille analyser (for eksempel TT4, fruktosamin og kortisol) fra disse lagrede serumprøver i opptil 14 dager. Bakgrunnen til fordypningsoppgaven min er å undersøke om man kan stole på analyseresultatene etter lagring av serum i kjøleskap i 3, 7 og 14 dager .

Prøvene må tas enten **mandag, tirsdag eller fredag**, for å unngå at noen av analysedagene kommer i helgen.
Blodprøver skal tas fra 10 inneliggende hunder over 10 kg – som det allerede skal tas blodprøve av på klinikken.
Fra hver hund fylles 2 fullblodsrør helt fullt (3ml CAT serum clot activator, rød kork med sort ring).
Blodprøvene leveres før kl 12 samme dag på Sentrallaboratoriet med en papir-rekvisisjon som i **tillegg til de vanlige opplysningene er tydelig merket «PRØVE TIL STABILITETSFORSØK».**

Jeg håper dere er villige til å bidra til undersøkelsen min ved å ta ett ekstra serumrør og merke papirrekvisisjonen som angitt. Dersom det er noen spørsmål vennligst ta kontakt med meg eller mine veiledere.

Vennlig hilsen,
Runa Thrastardottir
Veiledere: Karin Hultin Jäderlund, Hege Brun-Hansen

Appendix 2

SAMTYKKE FRA EIER

Jag (eier/ansvarlig person) ………………………………………………………… gir herved tillatelse til at det fra hunden………………………………………………….. blir tatt blod i ett ekstra blodprovsrør (3 ml blod) vid blodprovstagning.

Det ekstra blodprovsrøret skall lagras i kjøleskap och dærefter skall blodet analyseras 3, 7 och 14 dagar efter blodprovstagningen. Formålet med analyserna ær att undersøka stabiliteten av blodprovsverdier i kjøleskapslagrade blodprover. Undersøkelsen kommer att ge svært nyttig information till kliniskt verksamma veterinærer.

Resultater vill bli offentliggjort uten eiers eller hundens identitet.

Dato:………………………… Signatur eier/ansvarlig person:………………………………………………………………

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