ADAPTATION AND ANALYTICAL VALIDATION OF A RADIOIMMUNOASSAY FOR THE MEASUREMENT OF HUMAN CHOLECYSTOKININ FOR USE IN DOGS

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2014

Major Subject: Biomedical Sciences

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ABSTRACT

Cholecystokinin (CCK) is an important neuroendocrine peptide in the gastrointestinal tract, being the major stimulant for exocrine pancreatic secretion and gall bladder contraction. As such, cholecystokinin release may be altered in many gastrointestinal diseases. Assays for the measurement of cholecystokinin in humans have previously been developed and validated. However, to our knowledge, no assay for the measurement of CCK in dogs has been analytically validated. Thus, the objectives of this study were to adapt a radioimmunoassay used for the measurement of plasma CCK in humans for use in dogs, perform the assay without human reagents, and to analytically validate this modified immunoassay for use with canine serum.

A human cholecystokinin radioimmunoassay protocol and antiserum were generously provided to us by the laboratory of Jens Rehfeld, Copenhagen, Denmark. Assay runs were set up to replace all human reagents that are part of the original protocol, followed by analytical validation of the adapted assay using canine serum samples by determination of sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability. A reference interval for cholecystokinin in canine serum was established using 90 serum samples from clinically healthy, fasted dogs (12 hrs), using the bottom 97.5th percentile.

The sensitivity of the assay was calculated to be 0.5 pmol/L. The lower limit of the working range of the assay was taken as the sensitivity at 0.5 pmol/L. For dilutional parallelism, observed-to-expected ratios ranged from 101.9 % to 253.6 % for 3 different

canine serum samples at dilutions of 1 in 2, 1 in 4, and, 1 in 8. For spiking recovery, observed to expected ratios ranged from 96.1 % to 68.2 % for 3 different canine serum samples at 4 different spiking concentrations. Coefficients of variation for intra-assay variability for 4 pooled serum samples were 3.8, 13.5, 7.9, and 3.9 %. Coefficients of variation for inter-assay variability of 4 pooled serum samples were 12.3, 11.6, 7.4, and 6.4 %. The reference interval for serum CCK concentration was established as 0.0 to 2.8 pmol/L.

All objectives outlined above were successfully met, analytically validating a radioimmunoassay for the measurement of cholecystokinin in canine serum. The radioimmunoassay for CCK described here is sufficiently accurate, precise, and reproducible, but has limited linearity in the lower end of the working range.

DEDICATION

To my loving family

ACKNOWLEDGEMENTS

I would first and foremost like to thank my parents for their endless encouragement, love, and support during my educational pursuits. I would also like to thank my committee members, Dr. Jan Suchodolski and Dr. Audrey Cook, for their guidance and support, and especially my committee chair, Dr. Jörg Steiner, for his encouragement, advice, and for giving me the opportunity to pursue a graduate degree with the Texas A&M University Gastrointestinal Laboratory. Special thanks to Dr. Jens Rehfeld of Copenhagen, Denmark, for generously allowing us to use his assay protocol and antibodies; we are very thankful for his essential contribution. I would also like to thank everyone in the GI Lab who has helped me along the way these past two years.

NOMENCLATURE

BSA	Bovine Serum Albumin
CCK	Cholecystokinin
NCS	Newborn Calf Serum
RIA	Radioimmunoassay

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CHAPTER I

INTRODUCTION

CHOLECYSTOKININ

In 1928, Ivy and Oldberg observed that canine intestinal extracts stimulated gallbladder contractions when infused into other dogs, and proposed the name cholecystokinin (CCK) for the causative agent.[1,2] In 1943, Harper and Raper found that similar intestinal extracts could also stimulate pancreatic enzyme secretion in cats, and proposed the name pancreozymin for the causative substance.[1] In 1971, Mutt and Jorpes purified the active substance in both of the intestinal extracts and determined their amino acid sequences. It was discovered then that CCK and pancreozymin were the same hormone, and the hormone is now exclusively referred to as cholecystokinin, regardless of the stimulatory function.[1,3] As such, CCK was among the first gastrointestinal hormones to be discovered.[4]

Although CCK is known to play a major role in the central nervous system (CNS), its vastly complex and diffuse effects in the CNS are unrelated to its function in the gastrointestinal tract.[2,3] The effects of CCK outside the gastrointestinal system are beyond the scope of this thesis.

In all species studied thus far, there is a single gene that encodes CCK.[1] In humans, the CCK gene is located on chromosome 3 in the 3q12-3pter region.[1,5] Mature CCK mRNA is about 750 bases long, exists in the brain and intestine in almost equivalent amounts, and it is most abundant in the cerebral cortex and the duodenum.[1]

Intestinal expression of CCK mRNA is modified by diet with levels declining in fasting animals and increasing after feeding.[1] CCK is synthesized from large peptide precursors and posttranslationally modified through sulfation of the seventh tyrosine residue of the COOH-terminus and amidation of the COOH-terminus.[3] Somatostatin has been shown to inhibit CCK gene expression, and exogenous bombesin can stimulate CCK secretion without affecting CCK gene expression.[1]

CCK exists in the body in many different forms; this is due to the peptide having a varying number of amino acid residues. CCK isoforms described include CCK 58, CCK 39, CCK 33, CCK 22, CCK 18, CCK 12, CCK8, CCK 7, CCK 5, and CCK 4.[1,6] The multiple forms of CCK are the result of differential processing by proteolytic enzymes of the main 115 amino acid precursor, preprocholecystokinin, which contains a single copy of cholecystokinin.[6] All of these N-terminally extended forms of CCK have in common an α-amidated carboxy-terminal end and require a sulfated tyrosine residue for full physiological activity.[1,6]

Sulfation is unusual for hormones, but in the case of CCK it is critical for biological potency.[1] In the pancreas and gallbladder, the unsulfated form of CCK is about 1,000-fold less active than the sulfated form.[1] CCK 8 is the major form of CCK found in the brain, and is the most biologically potent form of CCK that has been isolated.[1,5] In the intestine, brain, and blood of many species, the larger forms of CCK (CCK 33 and 58) are predominant, but are still less potent on a molar basis than CCK 8, and are less reactive with CCK antibodies.[1,5] It is thought that intracellular processing of

CCK occurs to produce the smaller, more biologically potent forms of CCK that are secreted into the blood.[1]

Gastrin, which has a similar structure to CCK, shares 5 carboxyl-terminal amino acids with CCK (Gly-Trp-Asp-Met-Phe-NH2).[1,5] As such, gastrin and CCK can interact with the receptors of each other and illicit vague responses.[1] Moreover, the close molecular similarity to gastrin has hampered earlier attempts to measure CCK concentrations by use of immunoassays, as almost all antiserum raised against CCK also cross-reacted with gastrin.[1] In order to bind specifically to CCK receptors, the CCK peptide must be extended to 7 amino acids.[1] Another problem that has hampered accurate quantification of CCK in serum is the fact that the average blood concentration of circulating gastrin is 10-100 times greater than that of CCK.[1]

CCK is able to exert its biological effects by binding to specific receptors on target cells. There are two forms of CCK receptors, the CCK-A receptor and the CCK-B receptor. In dogs, CCK-A receptors are found in the stomach and exocrine pancreas, and bind sulfated CCK approximately 1,000-fold more efficiently than unsulfated CCK or gastrin.[1,3] CCK-B receptors in dogs are found in the stomach, exocrine pancreas, endocrine pancreas, and brain, and bind both CCK and gastrin with equal affinity.[1] Recent cloning and expression of CCK receptor cDNAs has determined that the gastrin receptor in the brain and stomach and are identical to the CCK-B receptor in the brain.[1] Structurally, CCK receptors are composed of a seven transmembrane protein typical of a G-protein coupled receptor, and consist of 444 amino acids.[1] CCK binds to both receptors with three different affinities: very low, low, and high.[3] The dissociation

constant (K_d) values for the binding of CCK to the CCK-A receptor are: 10 μM (very low), 50-200 nM (low), and 50-300 pM (high).[3] The K_d values for the binding of CCK to the CCK-B receptor are: 10 μM (very low), 2-5 nM (low), and 100-300 pM (high).[3]

CCK receptors are activated through several pathways. Both CCK receptors activate phospholipase C (PLC), which causes the mobilization of intracellular calcium and the activation of protein kinase C (PKC), the mitogen-activated kinase (MAPK) cascade, and phosphatidylinositol 3 kinase (PI3K) pathway, causing the CCK-A receptor to increase pancreatic secretion and the CCK-B receptor to activate cell proliferation.[3] The CCK-A receptor also activates adenyl cyclase (AC), causing increased cyclic AMP concentration (cAMP), and the nitric oxide (NO)/cGMP pathway, causing cell proliferation and pancreatic secretion.[3] The CCK-B receptor is also responsible for mediating growth effects via activation of the JAK2/STAT3 pathway.[3]

CCK in the gastrointestinal tract is produced by endocrine cells of the intestinal mucosa.[1] A cell density gradient exists in the intestine, resulting in a greater number of CCK-producing cells in the proximal small intestine, and less CCK-producing cells in the distal small intestine.[1] Secretory granules containing CCK are concentrated around the basolateral surface of the cell.[1] Due to this orientation, food and other factors are allowed to interact with the apical surface of the cell, with the resulting signal cascade eventually leading to the release of CCK from the surface of the cell into the bloodstream.[1] Outside the gastrointestinal tract, CCK is synthesized in the pituitary gland and adrenal medulla of the central nervous system.[1] Although distributed in neurons throughout the brain, CCK is found in its highest concentrations in the cerebral

cortex; the only neuropeptide that is more predominant in the brain than CCK is neuropeptide Y (NPY).[1,5]

The exact mechanisms by which food causes CCK release in the gastrointestinal tract is still largely unknown, but food directly interacting with the exposed apical surface of the I cells (CCK cell) in the intestine as well as endogenously produced releasing factors secreted into the intestine are both thought to play a role.[1] The exact mechanisms regulating the production and secretion of CCK in the gastrointestinal system are also largely unknown, although evidence suggests that negative feedback is of great importance in the regulation of CCK secretion.[1]

The biological effect of CCK in the gastrointestinal tract, after being secreted locally or into the bloodstream, is to cause exocrine pancreatic secretion, pyloric contraction, gallbladder contraction, delay gastric emptying, and regulate satiety.[1,2] In the lower esophageal sphincter, sphincter of Oddi, and other sphincter regions, CCK produces relaxation as a result of its ability to stimulate nonadrenergic inhibitory neurons.[6] In regions like the gall bladder and small intestine, CCK produces a neurally mediated contraction due to its ability to release the contractile neurotransmitter acetylcholine.[6] CCK also acts on receptors in the stomach and various nerves to delay gastric emptying and regulate satiety.[1] Overall, CCK is responsible for coordinating ingestion, digestion, and disposal of nutrients in the gastrointestinal system.[1]

CHOLECYSTOKININ RADIOIMMUNOASSAYS

The technology of radioimmunoassays (RIA) was first developed by Yalow and Berson in the 1950s.[7] The development of a RIA for the measurement of insulin earned Dr. Yalow a Nobel Prize in Medicine in 1977.[8] This RIA for insulin allowed, for the first time, the accurate measurement of the concentration of peptide hormones in plasma.[7] It was the unmatched sensitivity and specificity of the RIA that allowed hormones that physiologically are present in only very low concentrations in the blood to be studied. By the 1970s, the methodology of the RIA had spread to other medical fields, including pharmacology, toxicology, infectious diseases, oncology, and hematology.[7]

An RIA is, in essence, a sensitive in vitro assay technique used to measure concentrations of antigens. An antigen can be defined as any substance that binds to an antibody. A hormone is an example of a frequently measured antigen. To perform an RIA, a known quantity of the antigen that is to be measured is linked to a radioactive molecule, for instance, by iodination with I^{125}. A known amount of the radiolabeled antigen is then mixed with a known concentration of antibody directed against said antigen. A plasma or serum sample containing an unknown amount of the same antigen is then added to the mixture. This results in competitive antibody binding between the radiolabeled antigen and the sample antigen, since the antigens compete for the same antibody binding sites. This mixture is allowed to incubate for varying amounts of time and at various temperatures. Then a separation solution is added to separate bound antigen-antibody complexes, and unbound antigen and antibody and the radioactivity of the antigen-antibody complexes is measured using a gammacounter.

In general, the greater the concentration of sample antigen, the less radiolabeled antigen is bound, and thus a lower count of radioactivity is measured. If the sample antigen concentration is low, the opposite is true; a greater amount of radioactivity will be measured. Using a set of known antigen standards, a binding curve is generated and the concentration of the unknown antigen is estimated based on its binding in relation to this curve. RIAs have many advantages, such as their generally low cost, consistency of results, and simplicity.

Early attempts at developing an RIA for the measurement of CCK were met with great difficulty. This was due to a variety of factors: conventional isotope labeling techniques involved oxidation, which had deleterious effects on the structure and thus biological function of CCK, there was a shortage of peptides for immunization, and the COOH-terminal sequence of CCK is shared with gastrin, and is strongly immunogenic, making antisera raised against CCK non-specific.[9,10] Typical isotopic labeling techniques led to oxidation of the two methionyl residues on CCK 8; this was overcome using nonoxidative labeling as previously mentioned.[9] Currently, peptides are no longer in short supply; sulfated CCK 8 and iodinated sulfated CCK 8 are commercially available for use as standard and tracer, respectively.

Radioimmunoassays were first described in the 1950s, but it was not until 1977 that the first reliable CCK RIA was developed by Jens Rehfeld, Copenhagen, Denmark.[9] This assay measured porcine CCK 33, sulfated CCK 8, and a portion of CCK 39. The hallmark of this assay was the use of a nonoxidative radiolabeling technique to create a suitable I^{125} tracer, which was found to have the same immunoreactivity as native CCK

33.[9] This non-oxidative radiolabeling technique involves conjugation of an I^{125} ester to two of the free NH_2 groups on the lysyl side chains of CCK.[9] An antiserum was raised against CCK 33 in guinea pigs and rabbits through injection of purified porcine CCK 33. The result was the development of the first sensitive and reliable RIA for CCK.[9]

In 1983, Jansen and Lamers described one of the first assays with adequate specificity and sensitivity to accurately measure CCK in human plasma.[11] Similar to the Rehfeld assay; they used porcine CCK 33 as a standard, and also as a stimulus for antibody production in both guinea pigs and rabbits. A major flaw of this assay was that their antibody did not reliably bind to CCK peptides with less than 14 amino acid residues.[11] This limitation is of great significance as we know today that CCK 8 has a major biological role in the gastrointestinal tract. However, this assay was highly sensitive for CCK, showed no cross-reactivity with gastrin, and was shown to be capable of accurately measuring CCK in human tissue and plasma.[11] This report was also among the first to describe that the infusion of exogenous CCK results in pancreatic enzyme secretion and gallbladder contraction in humans.[11]

Around the same time as the previous assays, other assays for CCK were under development.[12] Like the previous assays they also measured CCK 33, both in humans and also dogs. However, similar to the Jansen and Lamers assay, these RIAs did not reliably measure CCK 8.[12]

In 1988, yet another CCK RIA was described by Ohgo et al.; this assay was the first to use an antiserum produced from sulfated CCK 8 injected into rabbits.[13] This assay was used to measure CCK in human plasma before and after a meal. It was found

that CCK increased as much as 4 fold following ingestion of a test meal.[13] They also showed that their antibody bound CCK 39, CCK 33, and CCK 8 with equimolar potency.[13]

In 1998, the early CCK RIAs were improved by the production of a highly specific CCK antiserum, antibody 92128. This antibody proved to bind sulfated CCK 8, CCK 22, CCK 33, and CCK 58 with equimolar potency, and showed no cross-reactivity with gastrin, a problem that had plagued all previous CCK RIA antisera to some degree.[10] Binding with equimolar potency allows the measurement of the major biologically active forms of CCK simultaneously, instead of individually, allowing for a more accurate measurement and estimation of total plasma CCK concentration. Thus, a single assay for the accurate measurement of cholecystokinin in plasma had been described using a highly specific antibody.[10] This same antibody was generously supplied to the Gastrointestinal Laboratory at Texas A&M University by Jens Rehfeld, Copenhagen, Denmark, for use in the development of a CCK radioimmunoassay for dogs.

As of 2013, there are several commercial RIA and ELISA kits available for the measurement of CCK. However, to our knowledge, none of these assays have been analytically validated for use with canine serum. Thus, the objectives of this study were to modify the RIA for measurement of serum CCK in humans described by Rehfeld for use in dogs without using human reagents, and to analytically validate this modified immunoassay for use with canine serum.[10] This work will allow future studies on the

role and biological function of CCK in the dog, and in dogs with suspected alterations in CCK secretion.

CLINICAL APPLICATION

To date, there are no assays that have been analytically validated for use with canine serum. Being able to accurately measure CCK in dog serum is important to determine alterations in CCK secretion in dogs with gastrointestinal disease. In humans, alterations in CCK secretion have been described in patients with celiac disease, chronic pancreatitis, hepatic cirrhosis, Nelson's disease, and Cushing's disease[11,14,15,16,17,18] Increased plasma CCK concentrations in patients with chronic pancreatitis have been reported in the literature.[16] Increased plasma CCK concentrations in patients with hepatic cirrhosis have also been reported in the literature.[17] Large amounts of CCK have been found in pituitary adenomas of patients with Nelson´s syndrome and some patients with Cushing´s disease.[18]

OBJECTIVE

While there is a wealth of information on CCK and alterations of CCK secretion in humans, there is a distinct lack of information on similar abnormalities in dogs. This is likely due to the complexity of the commonly used RIAs, their antiserum, and their inclusion of human reagents, which require special permits, safety precautions, and clearance by the governing bodies of the laboratory. In order to measure CCK in dogs, an analytically validated assay for this species and sample type (serum, plasma, whole

blood) to be used should exist before reliable and reproducible measurements can be made. This will then allow studies to be conducted in order to determine if a clinical disorder might be a result of an abnormal CCK secretion. The assay proposed here would allow for continued research without the issues associated with using human reagents, or in using an assay protocol that has not been analytically validated for use with canine serum.

The research plan was to establish the original protocol as described by Rehfeld at the Gastrointestinal Laboratory at Texas A&M University, changing steps sequentially in order to modify the assay to remove all human reagents.[10] The human reagents to be removed are human plasma and 5% human serum albumin. Changes made to the original protocol included: the transition from plasma samples to serum samples, a change of incubation time from 7 days to 3 days, the use of canine plasma instead of human plasma, and the use of 5% newborn calf serum in place of 5% human albumin.

CHAPTER II

MODIFICATION OF RADIOIMMUNOASSAY

INTRODUCTION

The first step in the process of developing a canine CCK radioimmunoassay was the modification of the assay for use in humans described by Jens F. Rehfeld, Dept. of Clinical Biochemistry, Rigshospitalet, DK-2100 Copenhagen, Denmark. After several successful runs using the original protocol, all human reagents were sequentially replaced with reagents of either canine or bovine origin. The resulting sets of reagents were tested for agreement with the original assay. The original radioimmunoassay uses human plasma and human albumin, both of which require special handling and disposal procedures, as well as special permits, which were among the main reasons these reagents were selected for replacement. The following sections detail the process of assay modification from the original protocol up until the working protocol was established prior to analytical validation.

MATERIALS AND METHODS

Materials - All commonly used research materials and supplies were obtained from VWR Scientific, West Chester, PA. Radiolabeled I^{125}CCK-8 was purchased from Perkin Elmer, Waltham, MA (NEX203010UC) in 10 μCi amounts and used until its expiration date. Synthetic sulfated CCK-8 used for making standards was purchased from Sigma-Aldrich, St. Louis, MO (C2175) and immediately reconstituted, diluted to a

8 nM stock solution, aliquoted, and stored at -20°C. Antibody 92128-5 was obtained by generous donation from Professor Jens F. Rehfeld, Dept. of Clinical Biochemistry, Rigshospitalet, DK-2100 Copenhagen, Denmark. Anti-CCK-8 was purchased from Sigma-Aldrich, St. Louis, MO (C2581). Activated charcoal used in the separation solution was purchased from Sigma-Aldrich, St. Louis, MO (C7606) and stored at room temperature until use. Thimerosal, used in Barbital Buffer I, was purchased from Sigma-Aldrich, St. Louis, MO (T4687) and stored at room temperature until further use. Sodium azide, used in barbital buffer II, was purchased from Sigma-Aldrich, St. Louis, MO (S8032) and stored at room temperature until use. Barbital for use in the RIA buffers was purchased from Sigma-Aldrich, St. Louis, MO (B0375) via usage certification from the Large Animal Pharmacy at the Texas A&M University College of Veterinary Medicine. Canine serum albumin was purchased from Equitech-Bio, Inc., Kerrville, TX (CASA62) or purified from canine plasma using an ÄKTA Fast Protein Liquid Chromatography purification system with Hi-Trap Blue HP columns, all purchased from GE Life Sciences, Pittsburgh, PA. Canine fresh frozen plasma was purchased from Animal Blood Resources International, Dixon, CA, or obtained through use of outdated bags from the Veterinary Medical Teaching Hospital at Texas A&M University. Bovine serum albumin was purchased from Milipore, Billerica, MA (Cat # 126593). Newborn calf serum was purchased from Sigma-Aldrich, St. Louis, MO (N4637). Human fresh frozen plasma was purchased from SeraCare Life Sciences, Milford, MA (23D00). Human serum albumin was purchased from CSL Behring, King of Prussia, PA (44206-251-05). Amicon Ultra-15 50 kDa centrifugal filters were

purchased from Milipore, Billerica, MA. The gamma counter used (1470 Wallac Wizard) was purchased from Perkin Elmer, Waltham, MA. The gel electrophoresis system used for SDS-PAGE was purchased from Life Technologies, Grand Island, NY. The gel used for estimation of purity and molecular weight was a NuPAGE Bis-Tris 10% MiniGel purchased from Life Technologies, Grand Island, NY.

Original Protocol - To establish the assay protocol received from Dr. Jens Rehfeld, Copenhagen, Denmark, a 25% human albumin solution was combined with deionized water in a 1:5 ratio creating a 5% human albumin solution. Four-hundred µL aliquots of the 5% human albumin solution were transferred to 2 mL sample tubes. Eight-hundred µL of 96% (vol/vol) ethanol were pipetted into each 400 µL albumin aliquot. The aliquots were intermittently vortexed for 10 seconds each. After vortexing, all tubes were centrifuged for 15 minutes at 4,000 rpm and room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were then evaporated at room temperature in a vacuum concentrator for 5 hours. These extracts were then reconstituted to their original 400 µL volume with either standards or controls. For each assay run 13 human albumin extracts were used: one each for the nonspecific binding tubes and the total binding tubes, one each for the 8 standards, and one each for the 3 controls. Barbital buffers I (20 mM barbital, 0.6 mM thimerosal, 0.11% human albumin, pH 8.4) and II (20 mM barbital, 0.13 g/L sodium azide, pH 8.4) were used as buffers for the assay: both buffers were filtered through a 0.2 µm filter and degassed. Barbital buffer I was used as an RIA buffer, and for dilutions and reconstitutions. Barbital buffer II was used as a separation solution for the measurement

of bound vs. unbound CCK concentration. After the buffers were prepared, standards were made using commercially available synthetic sulfated CCK-8. One mg of CCK-8 was reconstituted in 10 mL of barbital buffer I. The resulting 8.8 μM solution was diluted to $8x10^{-10}$ M using barbital buffer I to make a working solution. Standards of 1, 2.5, 5, 10, 20, 30, and 50 pM were made from the standard working solution. Four, 7.5, and 15 pM controls were also made from this standard working solution. Each standard was raised to the desired volume using barbital buffer I as the diluent. Antibodies were diluted using barbital buffer I from 10 μL aliquots of antibody 92128-5, which was originally reconstituted into 250 μL of pure water. To carry out the assay, the RIA was set up in duplicate fashion with two tubes for each preparation (total count, non-specific binding, total binding, standards, controls, and samples). On the day of the assay, antibody 92128-5, albumin extracts, standards, and controls were taken out of the freezer and thawed at room temperature. Barbital buffer I was taken out of the 4°C refrigerator and allowed to warm to room temperature (approximately 22°C). RIA tubes were arranged and labeled. RIA tracer was taken from the freezer and diluted with barbital buffer I to 1,000 cpm +/- 100 cpm based on gammacounter readings for 60 seconds. Barbital buffer I was placed in each tube in the respective amounts as described in Figure 1. Albumin extracts were reconstituted to 400 μL with standard and control solutions, and then added to each tube in an amount as described in Figure 1. Antibody 92128-5 was diluted to the desired concentration using barbital buffer I, and then added to each tube in an amount as described in Figure 1. All tubes were then taken to a separate radiation room where 100 μL of tracer were added to each tube. All tubes were

Figure 1 – RIA setup. This figure shows reagent volumes required for the RIA setup as described in the original protocol. All tubes are set-up and assayed in duplicate fashion. The 0 sample is one of the extract tubes filled with buffer.

Reference:

TC:	1,300 µL Barbital Buffer I
	100 µL Tracer
NSB:	1,150 µL Barbital Buffer I
	150 µL 0 Sample
	100 µL Tracer
TB:	1,000 µL Barbital Buffer I
	150 µL 0 Sample
	150 µL Antibody
	100 µL Tracer

Standards/Controls/Samples:

Std/Control:	1,000 µL Barbital Buffer I
	150 µL Standard/Control
	150 µL Antibody
	100 µL Tracer
Sample:	1,000 µL Barbital Buffer I
	150 µL Sample
	150 µL Antibody
	100 µL Tracer

vortexed and allowed to incubate for 7 days at 4°C. On day 7, a separation solution was prepared using 80 mL barbital buffer II, 20 mL of human plasma, and 6 g of activated charcoal. The solution was kept on ice while being vigorously mixed with a magnetic stirrer for 30 minutes. After 30 minutes, 500 µL of separation solution was added to each tube. All tubes were vortexed and allowed to incubate at room temperature for 15 to 30 minutes. All tubes were then centrifuged for 10 minutes at 4,000 rpm and 4°C. The supernatants from all tubes (except TC tubes) were transferred from the original tube to a new tube, tapping once. The supernatant tubes were counted in an automatic gammacounter for 5 minutes. Counts per minute (cpm) were measured for all tubes. The cpm data was entered into an Excel® spreadsheet where average, standard deviation, correlation of variance, and B/B_0 were calculated. B/B_0 was calculated using the formula: $B/B_0 = (B - NSB) / (B_0 - NSB)$, where B is the count of the supernatant and B_0 the count of the total binding (TB). A four-parameter log-logistic (4PL) standard curve was generated in Prism 6® by plotting the logarithm of the concentration against B/B_0. All unknown values were extrapolated based on the standard curve.

After the original protocol was established, the following changes were made to the protocol to facilitate the measurement of CCK in canine serum: human plasma in the separation solution was replaced with canine fresh frozen plasma, human serum albumin in the albumin extracts and barbital buffer I were replaced with bovine serum albumin, canine albumin, or newborn calf serum, and the incubation time of the assay was decreased from 7 days to 3 days. Success was determined through comparison of an

assay run with and without the proposed changes. The materials and methods used to modify the assay are outlined as follows:

Sigma Anti-CCK-8 - After establishing the original protocol, a commercial antibody for CCK was purchased from Sigma Aldrich. Antibody 92128-5 that was received from Jens Rehfeld is only available in a limited amount. As a proactive step to avoid running out of the original antibodies, the commercial antibody against CCK was purchased and used in an attempt to replace the original antibody. The assay was carried out using the protocol described above, with the addition of the following steps: Sigma anti-CCK-8 was reconstituted and diluted to the desired concentrations for testing binding percentage, and run in comparison to antibody 92128-5 in the same assay runs.

Canine Plasma - After finding suitable antibody dilutions for antibody 92128-5 and Sigma anti-CCK-8, the next step in modifying the original assay was to try to replace all human reagents with non-human reagents. The assay was carried out using the protocol described above, with the addition of the following steps: human plasma in the separation solution was replaced with canine fresh frozen plasma. Also, Sigma anti-CCK-8 was used instead of antibody 92128-5.

Canine Albumin Purification - Due to an indefinite backorder of canine albumin, the expense of the limited quantities that were available, and the need to replace all human reagents with canine reagents, the decision was made to purify canine albumin using FPLC. Canine fresh frozen plasma was thawed at room temperature. After thawing, 5 mL plasma aliquots were filtered through 0.8 μm, 0.45 μm, and 0.2 μm syringe filters, and then frozen at -20°C for future use. A binding buffer (50 mM

KH_2PO_4, pH 7.0) and an elution buffer (50 mM KH_2PO_4, 1.5 M KCl, pH 7.0) were prepared, filtered at 0.2 µm, and degassed. A storage solution consisting of 0.1 M KH_2PO_4 and 20% ethanol was made and used to store columns, when not in use, at 4°C. Five mL of 0.2 µm filtered canine plasma was thawed and diluted 1:2 with binding buffer immediately before use. The FPLC ÄKTA Purifier was set up with three 5 mL Hi Trap Blue HP columns connected in series to scale up operation. Binding buffer was introduced to the columns at 5 mL per minute to wash out the preservative and storage solution. The columns were then equilibrated with 5 column volumes of binding buffer (75 mL) at a rate of 5 mL per minute. Ten mL of diluted and filtered canine plasma was injected onto the columns at a rate of 5 mL per minute. The columns were then eluted with 5 column volumes (75 mL) of elution buffer at a rate of 5 mL per minute and collected as 5 mL fractions until no more material appeared in the effluent. The elution buffer was washed out with storage solution and all columns were stored at 4°C until use. All secondary peak fractions were collected and combined. Secondary peak fractions were divided among 4 Amicon Ultra-15 50 kDa centrifugal filters and placed in swinging bucket rotors and centrifuged at 4,000 x g and 25°C for 15 minutes. The eluent beneath the filter was discarded and 1X PBS, pH 7.4 was added to raise the concentrated solution remaining in the filter to its original volume. Three more centrifugations were carried out in this fashion and the remaining 4 100-500 µL canine albumin concentrates were combined and frozen at -80°C. The combined frozen concentrate was placed in a lyophilizer and allowed to freeze-dry until no moisture remained. Approximate albumin concentrations were determined using Nanodrop, Bradford, and Ultrospec 2000. SDS-

PAGE with a NuPAGE 10% Bis-Tris 10 well mini-gel was used with 1X MOPS and SeeBlue +2 and Mark 12 standards to determine the presence of canine albumin and estimate its approximate purity. FPLC runs and purification were continued until enough canine albumin was obtained to test assay compatibility. The freeze-dried canine albumin was reconstituted with 1X PBS, pH 7.4 before use.

Barbital Buffer I with Canine Albumin - The next step in modifying the original assay was replacing human albumin in barbital buffer I with canine albumin obtained from FPLC. The assay was carried out using the protocol described above, with the addition of the following steps: Sigma anti-CCK-8 was used in conjunction with antibody 92128-5, and 0.55 g of canine albumin was used instead of human albumin in a fresh 500 mL batch of barbital buffer I. Ten canine samples were obtained and extracted using the methods in the original protocol. The samples were used to compare the effectiveness of both antibodies, and to verify that canine albumin was a viable replacement for human albumin. Four-hundred µL aliquots of each sample were transferred into 2 mL sample tubes. Eight hundred µL of 96% (vol/vol) ethanol was then pipetted into each 400 µL albumin aliquot. The aliquots were then intermittently vortexed for 10 seconds each. After vortexing, the tubes were centrifuged for 15 minutes at 4,000 rpm and room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were evaporated at room temperature in a vacuum concentrator for 5 hours. Another 800 µL of 96% (vol/vol) ethanol was pipetted into each dried 400 µL albumin aliquot and dried for another 5 hours. These sample extracts were then reconstituted to their original 400 µL volume

with barbital buffer I or stored at -20°C until use. Several assay runs were performed using the original protocol to compare the two antibodies and canine albumin versus human albumin in barbital buffer I.

7 Versus 3 Day Incubation - Seven day RIA incubations were becoming a hindrance to the project, so steps were taken to decrease the incubation time. The assay was carried out using the protocol described above, with the addition of the following steps: canine albumin was used instead of human albumin in barbital buffer I, canine plasma was used instead of human plasma in the separation solution, both 3 and 7 day incubations were used, and a 1:20,000 antibody 92128-5 dilution was used.

New Barbital Buffer I - The canine albumin that had been purified using FPLC was determined to be produced in an amount that was insufficient for continued use based on the time required for purification. The decision was made to replace canine albumin in barbital buffer I with either newborn calf serum (NCS) or bovine serum albumin (BSA). The assay was carried out using the protocol described above, with the addition of the following steps: canine albumin, BSA, and NCS were used in separate solutions of barbital buffer I, canine plasma was used instead of human plasma in the separation solution, a 3 day incubation time was used instead of a 7 day incubation time, and a 1:20,000 antibody 92128-5 dilution was used. The BSA barbital buffer I was prepared using 1.1 g/L of bovine serum albumin. A NCS barbital buffer I was prepared using 0.11 % newborn calf serum.

Albumin Extracts - The final step in converting the original assay protocol was replacement of human albumin in the albumin extracts. BSA extracts were made by

adding 1 g of bovine serum albumin in 20 mL deionized water and then taking 400 µL aliquots of the 5% BSA solution and adding 800 µL of 96% (vol/vol) ethanol into each 400 µL albumin aliquot. The aliquots were then intermittently vortexed for 10 seconds each. After vortexing, the tubes were centrifuged for 15 minutes at 4,000 rpm and room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were evaporated at room temperature in a vacuum concentrator for 5 hours. These albumin extracts were then reconstituted to their original 400 µL volume with barbital buffer I, standards, or controls, or stored at -20°C until use. Newborn calf serum extracts were obtained by adding 1 mL of NCS in 20 mL deionized water and then taking 400 µL aliquots of the 5% NCS solution and adding 800 µL of 96% (vol/vol) ethanol into each 400 µL NCS aliquot. The aliquots were then intermittently vortexed for 10 seconds each. After vortexing, the tubes were centrifuged for 15 minutes at 4,000 rpm and room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were then evaporated at room temperature into a vacuum concentrator for 5 hours. These NCS extracts were then reconstituted to their original 400 µL volume with barbital buffer I, standards, or controls, or stored at -20°C until further use. The assay was carried out as described above, with the addition of the following steps: barbital buffer I was made with canine albumin, NCS, or BSA as three separate barbital buffers, extracts used were human serum albumin extracts, NCS extracts, or BSA extracts, canine plasma was used in the separation solution, a 3 day incubation time was used, and a 1:20,000 antibody 92128-5 dilution was used.

RESULTS

The optimal antibody dilution was determined for antibody 92128-5 using the original protocol (Table 1). Although the results of an antibody dilution assay run originally showed desired binding when using Sigma anti-CCK-8 (Table 2), antibody 92128-5 was unsuccessfully replaced by Sigma anti-CCK-8 when the standard curve was generated and the samples were measured (Table 3). Human plasma in the separation solution was successfully replaced by canine fresh frozen plasma (Table 4). Canine albumin was purified by FPLC (Figs 2 and 3) and used in barbital buffer I to replace human serum albumin (Tables 5 and 6). Antibody 92128-5 was successfully used to measure canine samples with canine albumin in barbital buffer I and canine plasma in the separation solution (Table 6). A 3-day assay incubation time was determined to be equivalent to a 7-day incubation time (Table 7). Newborn calf serum was used to replace canine albumin in barbital buffer I (Table 8). Newborn calf serum was used to replace human albumin in the albumin extracts (Table 9). A typical standard curve is shown in Figure 4.

Table 1 – Determination of optimal antibody dilution. This table shows the results of an assay run using antibody 92128-5 at different dilutions, barbital buffer I with human albumin, human albumin extracts, and human plasma in the separation solution. This table shows the results for determining an antibody dilution that would result in approximately 40% binding. Binding percent (B%) is calculated by dividing the average cpm (counts per minute) of the antibody dilution by the average cpm of the TC (total count) and multiplying by 100. (SD = standard deviation; %CV = coefficient of variation).

Tube ID	Tube 1	Tube 2	Mean	SD	%CV	B%
	cpm	cpm	cpm	cpm		
TC	1029.7	1009.9	1019.8	14.0	1.4	
NSB	22.1	24.1	23.1	1.4	6.1	
1:10,000	591.8	574.2	583.0	12.4	2.1	57.2
1:15,000	449.2	457.7	453.5	6.0	1.3	44.5
1:20,000	357.6	382.4	370.0	17.5	4.7	36.3
1:25,000	311.5	311.9	311.7	0.3	0.1	30.6

Table 2 – Determination of optimal commercial antibody dilution. This table shows the results of an assay run using Sigma anti-CCK-8 at different dilutions, barbital buffer I with human albumin, human albumin extracts, and human plasma in the separation solution. This figure shows the results for determining an antibody dilution that would result in approximately 75% binding. Binding percent (B%) is calculated by dividing the average cpm (counts per minute) of the antibody dilution by the average cpm of the TC (total count) and multiplying by 100. (SD = standard deviation; %CV = coefficient of variation).

Tube ID	Tube 1	Tube 2	Mean	SD	%CV	B%
	cpm	cpm	cpm	cpm		
TC	968.9	963.7	966.3	3.7	0.4	
NSB	25.6	27.8	26.7	1.6	5.8	
1:5,000	804.3	774.4	789.4	21.1	2.7	81.7
1:10,000	789.5	780.7	785.1	6.2	0.8	81.2
1:20,000	769.0	813.0	791.0	31.1	3.9	81.9
1:40,000	754.7	755.3	755.0	0.4	0.1	78.1
1:80,000	672.5	704.4	688.5	22.6	3.3	71.2
1:160,000	511.4	512.6	512.0	0.8	0.2	53.0
1:320,000	359.1	331.0	345.1	19.9	5.8	35.7
1:640,000	192.2	185.6	188.9	4.7	2.5	19.5

Table 3 – Use of Sigma anti-CCK-8 antibody for generation of a standard curve. This table shows the results of an assay run using Sigma anti-CCK-8 at a 1:60,000 dilution with standards and controls, barbital buffer I with human albumin, human albumin extracts, and canine plasma in the separation solution. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		1007.5	1065.8	1036.7	41.2	4.0			
NSB		28.1	29.0	28.6	0.6	2.2			
TB		820.4	806.3	813.4	10.0	1.2	1.0	100.0	
Std 1	1.0	799.4	799.4	799.4	0.0	0.0	1.0	98.2	
Std 2	2.5	778.2	766.0	772.1	8.6	1.1	0.9	94.7	
Std 3	5.0	781.7	773.6	777.7	5.7	0.7	1.0	95.5	
Std 4	10.0	760.6	750.5	755.6	7.1	0.9	0.9	92.6	
Std 5	20.0	675.9	649.3	662.6	18.8	2.8	0.8	80.8	
Std 6	30.0	625.1	686.2	655.7	43.2	6.6	0.8	79.9	
Std 7	50.0	568.7	556.5	562.6	8.6	1.5	0.7	68.0	
Ctrl 1	4.0	760.8	738.8	749.8	15.6	2.1	0.9	91.9	8.2
Ctrl 2	7.5	737.8	732.6	735.2	3.7	0.5	0.9	90.0	10.7
Ctrl 3	15.0	724.9	695.0	710.0	21.1	3.0	0.9	86.8	15.4

Table 4 – Removal of human plasma from the separation solution. This table shows the results of an assay run using Sigma anti-CCK-8 at different dilutions, barbital buffer I with human albumin, human albumin extracts, and canine plasma in the separation solution. Binding percent (B%) is calculated by dividing the average cpm (counts per minute) of the dilution by the average cpm of the TC (total count) and multiplying by 100. (SD = standard deviation; %CV = coefficient of variation).

Tube ID	Tube 1	Tube 2	Mean	SD	%CV	B%
	cpm	cpm	cpm	cpm		
TC	1139.9	1092.5	1116.2	33.5	3.0	
NSB	28.3	30.5	29.4	1.6	5.3	
1:40,000	738.4	771.1	754.8	23.1	3.1	67.6
1:60,000	772.2	776.7	774.5	3.2	0.4	69.4
1:80,000	688.6	703.5	696.1	10.5	1.5	62.4

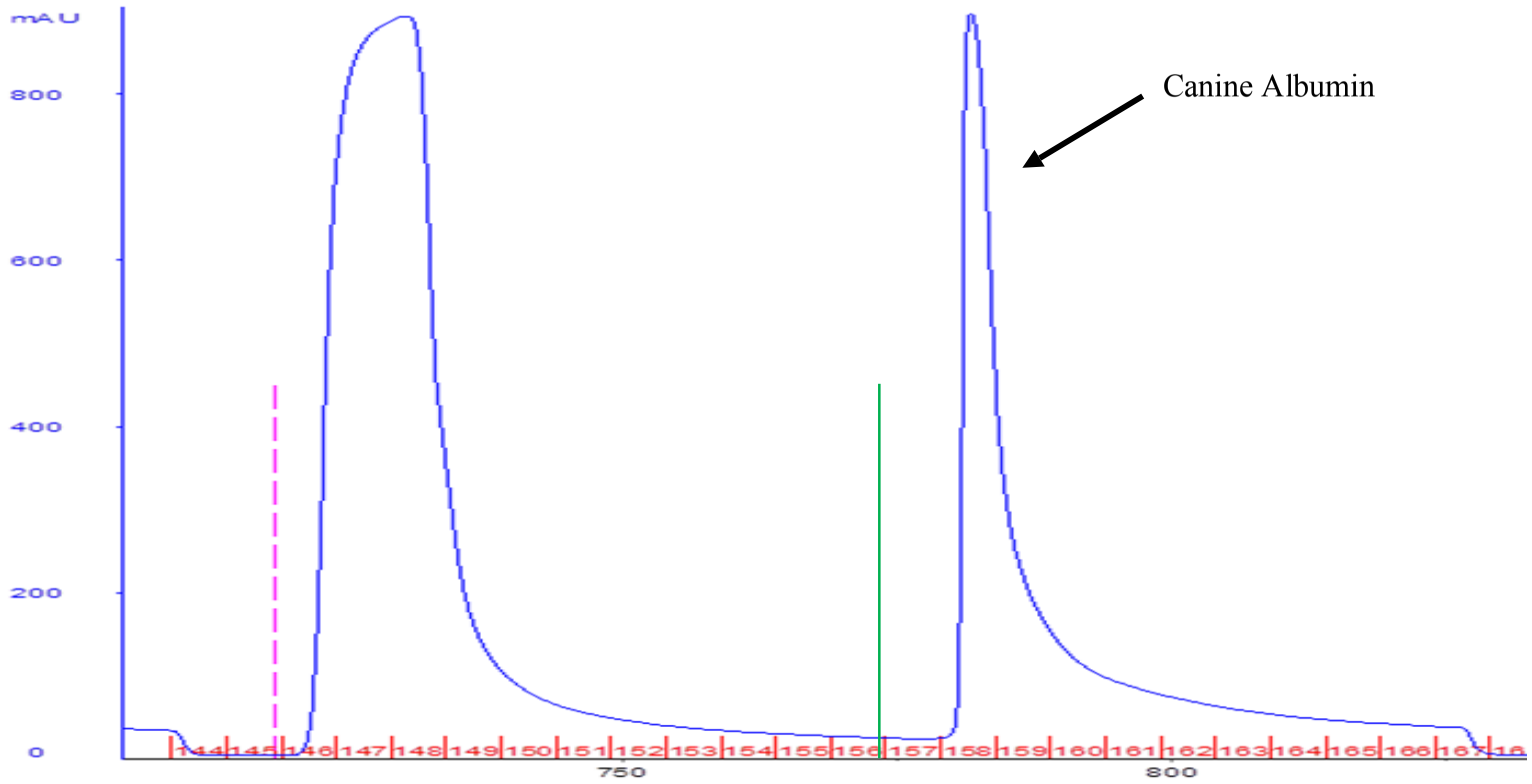


Figure 2 – Affinity chromatography of partially purified canine albumin. This figure shows an example of an FPLC affinity chromatography curve generated from applying buffers to the FPLC column containing canine plasma and measuring absorbance at 280 nm. Peak 1 contains binding buffer and flow-through material that is not bound to the column. Peak 2 contains material that was eluted from the column using elution buffer and contains canine albumin. Red numbers indicate the beginning of each fraction collected. The blue line depicts the absorbance at a wavelength of 280 nm during chromatography. The solid green line indicates a change from binding buffer to elution buffer. The pink dotted line indicates the injection point of canine plasma. The arrow points to the column peak containing canine albumin. Column: 3 Hi-Trap Blue HP® (5 mL) connected in series; flow rate: 5 mL/min; elution buffer: 50 mM KH_2PO_4, 1.5 M KCl, pH 7.0.

Figure 3 – SDS-PAGE of partially purified canine albumin. This NuPAGE 10% Bis-Tris gel was stained with Coomassie blue. A molecular weight standard was loaded into lane 1. An FPLC flow-through peak was loaded into lane 2. An FPLC eluent peak was loaded into lane 3. The molecular weight of canine albumin is around 66 kDa.

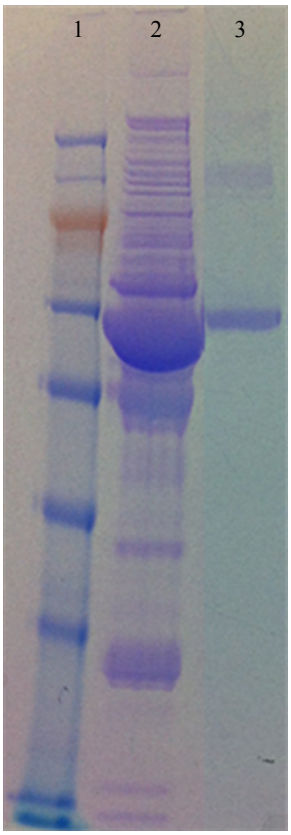


Table 5 – Sigma anti-CCK-8 with standards and samples. This table shows the results of an assay run using Sigma anti-CCK-8 at a 1:60,000 dilution with standards/controls and samples, barbital buffer I with canine albumin, human albumin extracts, and canine plasma in the separation solution. Several serum samples from healthy dogs were included in this assay run. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		1134.3	1132.8	1133.6	1.1	0.1			
NSB		25.1	25.1	25.1	0.0	0.0			
TB		734.2	803.4	768.8	48.9	6.4	1.0	100.0	
Std 1	1.0	748.7	767.9	758.3	13.6	1.8	1.0	98.6	
Std 2	2.5	792.3	766.4	779.4	18.3	2.3	1.0	101.4	
Std 3	5.0	760.6	733.9	747.3	18.9	2.5	1.0	97.1	
Std 4	10.0	720.4	687.9	704.2	23.0	3.3	0.9	91.3	
Std 5	20.0	648.7	654.4	651.6	4.0	0.6	0.8	84.2	
Std 6	30.0	627.7	625.0	626.4	1.9	0.3	0.8	80.8	
Std 7	50.0	580.5	566.1	573.3	10.2	1.8	0.7	73.7	
Ctrl 1	4.0	704.2	759.5	731.9	39.1	5.3	1.0	95.0	7.1
Ctrl 2	7.5	738.1	729.7	733.9	5.9	0.8	1.0	95.3	6.8
Ctrl 3	15.0	706.0	736.5	721.3	21.6	3.0	0.9	93.6	8.6
Sample 1		804.8	797.8	801.3	4.9	0.6	1.0	104.4	
Sample 2		824.4	819.5	822.0	3.5	0.4	1.1	107.1	
Sample 3		789.8	799.4	794.6	6.8	0.9	1.0	103.5	
Sample 4		776.9	795.0	786.0	12.8	1.6	1.0	102.3	
Sample 5		821.3	830.1	825.7	6.2	0.8	1.1	107.7	
Sample 6		748.2	764.8	756.5	11.7	1.6	1.0	98.3	3.4
Sample 7		820.5	808.9	814.7	8.2	1.0	1.1	106.2	
Sample 8		826.7	850.5	838.6	16.8	2.0	1.1	109.4	
Sample 9		765.5	803.7	784.6	27.0	3.4	1.0	102.1	
Sample 10		796.8	829.3	813.1	23.0	2.8	1.1	105.9	

Table 6 – Antibody 92128-5 with standards and samples. This table shows the results of an assay run using antibody 92128-5 at a 1:20,000 dilution with standards/controls and samples, barbital buffer I with canine albumin, human albumin extracts, and canine plasma in the separation solution. Several serum samples from healthy dogs were included in this assay run. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		1095.5	1101.8	1098.7	4.5	0.4			
NSB		27.7	31.8	29.8	2.9	9.7			
TB		455.2	443.5	449.4	8.3	1.8	1.0	100.0	
Std 1	1.0	411.0	391.0	401.0	14.1	3.5	0.9	88.5	
Std 2	2.5	346.4	373.3	359.9	19.0	5.3	0.8	78.7	
Std 3	5.0	291.5	287.0	289.3	3.2	1.1	0.6	61.8	
Std 4	10.0	217.2	222.9	220.1	4.0	1.8	0.5	45.4	
Std 5	20.0	153.9	160.4	157.2	4.6	2.9	0.3	30.4	
Std 6	30.0	119.6	131.5	125.6	8.4	6.7	0.2	22.8	
Std 7	50.0	88.2	97.0	92.6	6.2	6.7	0.1	15.0	
Ctrl 1	4.0	303.2	323.1	313.2	14.1	4.5	0.7	67.5	4.0
Ctrl 2	7.5	243.3	238.2	240.8	3.6	1.5	0.5	50.3	8.3
Ctrl 3	15.0	197.6	182.0	189.8	11.0	5.8	0.4	38.1	13.7
Sample 1		391.8	384.5	388.2	5.2	1.3	0.9	85.4	1.4
Sample 2		381.2	405.8	393.5	17.4	4.4	0.9	86.7	1.3
Sample 3		392.3	432.2	412.3	28.2	6.8	0.9	91.2	0.8
Sample 4		336.5	320.5	328.5	11.3	3.4	0.7	71.2	3.4
Sample 5		399.1	395.2	397.2	2.8	0.7	0.9	87.6	1.2
Sample 6		330.7	330.5	330.6	0.1	0.0	0.7	71.7	3.3
Sample 7		387.9	386.2	387.1	1.2	0.3	0.9	85.2	1.5
Sample 8		406.1	403.3	404.7	2.0	0.5	0.9	89.4	1.0
Sample 9		359.8	356.1	358.0	2.6	0.7	0.8	78.2	2.3
Sample 10		346.1	365.7	355.9	13.9	3.9	0.8	77.7	2.4

Table 7 – Assay run with 3 day incubation. This table shows the results of an assay run using antibody 92128-5 at a 1:20,000 dilution with standards/controls and samples, barbital buffer I with canine albumin, human albumin extracts, canine plasma in the separation solution, and a 3 day incubation time. Several serum samples from healthy dogs were included in this assay run. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		951.3	970.0	960.7	13.2	1.4			
NSB		18.1	24.1	21.1	4.2	20.1			
TB		427.8	401.2	414.5	18.8	4.5	1.0	100.0	
Std 1	1.0	374.1	379.8	377.0	4.0	1.1	0.9	90.5	
Std 2	2.5	312.4	325.9	319.2	9.5	3.0	0.8	75.8	
Std 3	5.0	247.1	262.6	254.9	11.0	4.3	0.6	59.4	
Std 4	10.0	203.4	204.9	204.2	1.1	0.5	0.5	46.5	
Std 5	20.0	153.1	147.5	150.3	4.0	2.6	0.3	32.8	
Std 6	30.0	110.7	112.5	111.6	1.3	1.1	0.2	23.0	
Std 7	50.0	75.4	83.9	79.7	6.0	7.5	0.1	14.9	
Ctrl 1	4.0	287.4	286.2	286.8	0.8	0.3	0.7	67.5	3.9
Ctrl 2	7.5	218.2	239.2	228.7	14.8	6.5	0.5	52.8	7.5
Ctrl 3	15.0	167.8	164.3	166.1	2.5	1.5	0.4	36.8	15.0
Sample 1		287.3	284.6	286.0	1.9	0.7	0.7	67.3	3.9
Sample 2		365.6	353.8	359.7	8.3	2.3	0.9	86.1	1.2
Sample 3		335.3	364.2	349.8	20.4	5.8	0.8	83.5	1.5
Sample 4		379.5	383.7	381.6	3.0	0.8	0.9	91.6	0.7
Sample 5		390.3	364.8	377.6	18.0	4.8	0.9	90.6	0.8
Sample 6		353.3	348.0	350.7	3.7	1.1	0.8	83.8	1.5
Sample 7		360.9	361.5	361.2	0.4	0.1	0.9	86.5	1.2
Sample 8		320.1	353.0	336.6	23.3	6.9	0.8	80.2	1.9
Sample 9		272.7	283.3	278.0	7.5	2.7	0.7	65.3	4.3
Sample 10		360.0	364.5	362.3	3.2	0.9	0.9	86.7	1.2

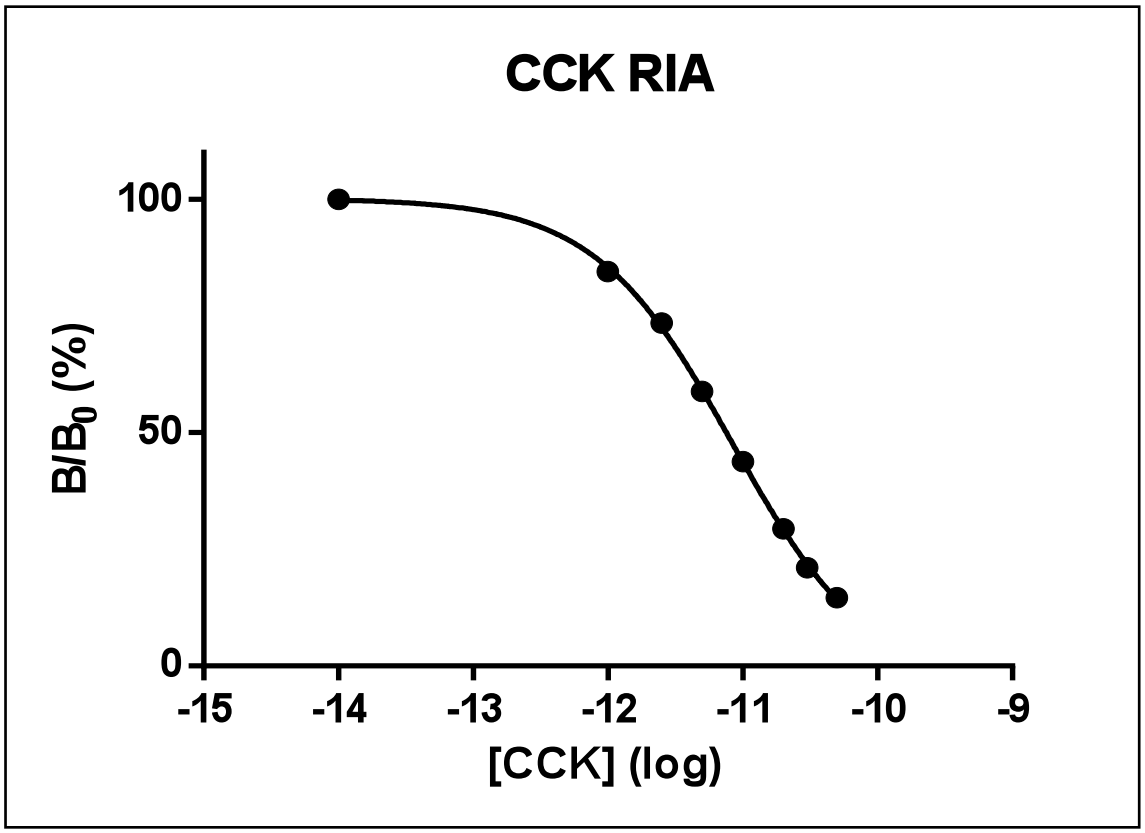
Table 8 – Barbital buffer I with newborn calf serum. This table shows the results of an assay run using antibody 92128-5 at a 1:20,000 dilution with standards/controls, barbital buffer I with newborn calf serum, human albumin extracts, canine plasma in the separation solution, and a 3 day incubation time. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		1067.8	1053.1	1060.5	10.4	1.0			
NSB		22.0	22.9	22.5	0.6	2.8			
TB		448.2	434.3	441.3	9.8	2.2	1.0	100.0	
Std 1	1.0	404.8	395.9	400.4	6.3	1.6	0.9	90.2	
Std 2	2.5	327.3	337.2	332.3	7.0	2.1	0.7	74.0	
Std 3	5.0	274.8	296.2	285.5	15.1	5.3	0.6	62.8	
Std 4	10.0	208.5	204.3	206.4	3.0	1.4	0.4	43.9	
Std 5	20.0	134.8	141.0	137.9	4.4	3.2	0.3	27.6	
Std 6	30.0	107.0	119.5	113.3	8.8	7.8	0.2	21.7	
Std 7	50.0	73.4	71.8	72.6	1.1	1.6	0.1	12.0	
Ctrl 1	4.0	313.2	306.0	309.6	5.1	1.6	0.7	68.6	3.7
Ctrl 2	7.5	230.2	217.7	224.0	8.8	3.9	0.5	48.1	8.6
Ctrl 3	15.0	169.2	167.7	168.5	1.1	0.6	0.3	34.9	14.8

Table 9 – Assay run with newborn calf serum extracts. This table shows the results of an assay run using antibody 92128-5 at a 1:20,000 dilution with standards/controls, barbital buffer I with newborn calf serum, newborn calf serum extracts, canine plasma in the separation solution, and a 3 day incubation time. B/B_0 is calculated by subtracting the average non-specific binding (NSB) cpm (counts per minute) from the average cpm of the standards, and dividing by the average cpm of the TB (total binding) minus the NSB cpm. This number is multiplied by 100 to get the percent binding (B%; which is the binding percent relative to TB). (Std = standard; Ctrl = control; SD = standard deviation; %CV = coefficient of variation; CCK = CCK concentration).

Tube ID	Nominal	Tube 1	Tube 2	Mean	SD	%CV	B/B_0	B%	CCK
	pM	cpm	cpm	cpm	cpm				pM
TC		1043.4	1048.7	1046.1	3.7	0.4			
NSB		29.0	28.6	28.8	0.3	1.0			
TB		428.0	450.4	439.2	15.8	3.6	1.0	100.0	
Std 1	1.0	382.3	368.7	375.5	9.6	2.6	0.8	84.5	
Std 2	2.5	313.7	347.1	330.4	23.6	7.1	0.7	73.5	
Std 3	5.0	263.5	276.4	270.0	9.1	3.4	0.6	58.8	
Std 4	10.0	210.7	206.1	208.4	3.3	1.6	0.4	43.8	
Std 5	20.0	151.2	147.7	149.5	2.5	1.7	0.3	29.4	
Std 6	30.0	115.1	115.4	115.3	0.2	0.2	0.2	21.1	
Std 7	50.0	85.8	92.1	89.0	4.5	5.0	0.1	14.7	
Ctrl 1	4.0	290.0	305.0	297.5	10.6	3.6	0.7	65.5	3.7
Ctrl 2	7.5	244.8	230.1	237.5	10.4	4.4	0.5	50.8	7.3
Ctrl 3	15.0	172.6	176.0	174.3	2.4	1.4	0.4	35.5	14.7

Figure 4 – A typical standard curve of the CCK RIA. This figure shows a typical four-parameter log-logistic (4PL) standard curve generated in Prism 6®. The standard concentrations are: 1, 2.5, 5, 10, 20, 30 and 50 pM, which are plotted on the x-axis in a logarithmic scale. Zero pM was plotted as $1x10^{-14}$, the smallest non-zero value capable of being plotted by Prism 6. B% values are plotted on the y-axis and are calculated from $B/B_0 = (B - NSB) / (B_0 - NSB)$, where B is the supernatant cpm, and B_0 is the total binding (TB) cpm. All unknown values are extrapolated based on where their B/B_0 falls along the standard curve. All unknown values were multiplied by a factor of 10,000 to obtain the actual results.



DISCUSSION

The original radioimmunoassay protocol was used to successfully determine the optimal dilution of antibody 92128-5 to an approximate tracer-antibody binding of 40%. Due to the limited supply of antibodies used in the original protocol, a commercial antibody against CCK (Sigma anti-CCK-8) was purchased and compared to the original antibody. A 1:60,000 Sigma anti-CCK-8 dilution was determined to be ideal with a tracer-antibody binding of approximately 70%. The antibody dilutions were repeated and used to test the replacement of human plasma with canine plasma in the separation solution. No obvious differences were observed between the binding percentages of the assay runs with human plasma and canine plasma. Due to an indefinite backorder of canine albumin, canine albumin was purified from canine plasma using affinity-chromatography FPLC columns, molecular weight centrifugal filters, and lyophilization. The purified canine albumin was used to replace human albumin in barbital buffer I. After testing the purified canine albumin in barbital buffer I with the Sigma anti-CCK-8 antibodies, it was noted that the binding percentages among standards were non-sensible in that similar binding percentages were observed with standards of very different concentrations. Thus, the commercial antibodies (Sigma anti-CCK-8) were not useful to establish a standard curve and a decision was made to progress with the original antibodies for all subsequent assay runs due to desired binding and separation in the standards and samples. The results of the 7 versus 3 day incubation time showed no obvious differences between the two incubation times. Due to the dramatic time-savings, the 3 day incubation time was used for all subsequent assay runs. The results of the

albumin extract assay run showed no obvious difference between human albumin or NCS in the albumin extracts. Thus, newborn calf serum was used to produce the albumin extracts for all subsequent assay runs. The assay protocol, now in its final form, was used to validate the radioimmunoassay for measurement of CCK concentrations in canine serum.

CHAPTER III

VALIDATION OF A CANINE CHOLECYSTOKININ RADIOIMMUNOASSAY

INTRODUCTION

After successfully replacing all human reagents in the original CCK assay protocol with reagents of either canine or bovine origin, the adapted protocol had to be validated. The assay was validated by determination of sensitivity, linearity, accuracy, precision, and reproducibility by testing assay sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability and by establishing a reference interval and assessing analyte stability.

MATERIALS AND METHODS

Materials - All commonly used research materials were purchased from VWR Scientific, West Chester, PA. Radiolabeled ^{I125}CCK-8 was purchased from Perkin Elmer, Waltham, MA (NEX203010UC) in 10 μCi amounts and used until its one-month expiration date. Synthetic sulfated CCK-8 used for making standards was purchased from Sigma-Aldrich, St. Louis, MO (C2175) and immediately reconstituted, diluted to a 2.18 nM stock solution, aliquoted, and stored at -20°C. Antibody 92128-5 was obtained by generous donation from the lab of Jens Rehfeld, Copenhagen, Denmark. Activated charcoal used in the separation solution was purchased from Sigma-Aldrich, St. Louis, MO (C7606) and stored at room temperature until use. Thimerosal, used in Barbital Buffer I, was purchased from Sigma-Aldrich, St. Louis, MO (T4687) and stored at room

temperature until use. Sodium azide, used in Barbital Buffer II, was purchased from Sigma-Aldrich, St. Louis, MO (S8032) and stored at room temperature until use. Barbital for use in the RIA buffers was purchased from Sigma-Aldrich, St. Louis, MO (B0375) via usage certification from the Large Animal Pharmacy at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University. Canine fresh frozen plasma was purchased from Animal Blood Resources International, Dixon, CA, or obtained from outdated bags within the laboratory or the Veterinary Medical Teaching Hospital at Texas A&M University. Newborn calf serum was purchased from Sigma-Aldrich, St. Louis, MO (N4637). The gamma counter used (1470 Wallac Wizard) was purchased from Perkin Elmer, Waltham, MA. Polypropylene RIA tubes were purchased from VWR Scientific, West Chester, PA.

Adapted Protocol - After multiple assay runs as discussed in Chapter II, the final protocol, having been established, was used to validate the CCK radioimmunoassay for use in dogs. The adapted protocol used for validation is outlined as follows: One mL of NCS was thawed and diluted 1:20 with deionized water to create a 5% NCS solution. Four hundred µL aliquots of 5% NCS were dispensed into 2 mL sample tubes. Eight hundred µL of 96% (vol/vol) ethanol was then pipetted into each 400 µL NCS aliquot. The aliquots were then intermittently vortexed for 10 seconds each. After vortexing, the tubes were centrifuged for 15 minutes at 4,000 rpm at room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were evaporated at room temperature in a vacuum concentrator for 5 hours. These extracts were then reconstituted to their original 400 µL volume with standards or

controls for the assay run, or stored at -20°C until use. Canine samples were obtained through external jugular venipuncture and placed in either red whole blood tubes or serum separator tubes and centrifuged for 18 minutes at 3,500 rpm. Serum was pipetted from the remaining blood components and 400 µL aliquots were placed into 2 mL sample tubes. Eight hundred µL of 96% (vol/vol) ethanol was then pipetted into each 400 µL sample aliquot. The aliquots were then intermittently vortexed for 10 seconds each. After vortexing, the tubes were centrifuged for 15 minutes at 4,000 rpm at room temperature. The resulting clear supernatant was decanted from each tube into a fresh sample tube. The supernatants were evaporated at room temperature in a vacuum concentrator for 5 hours. After drying, another 800 µL of 96% (vol/vol) ethanol was pipetted into each dried sample aliquot. The solution was evaporated at room temperature in a vacuum concentrator for another 5 hours. The sample extracts were then reconstituted to their original 400 µL volume with barbital buffer I (20 mM barbital, 0.6 mM thimerosal, 0.11 % NCS, pH 8.4) for the assay run, or stored at -20°C until use. Barbital buffer II contained 20 mM barbital and 0.13 g/L sodium azide, also pH 8.4. After achieving proper pH, both buffers were filtered through a 0.2 µm filter and degassed. Standards were made using commercially available synthetic sulfated CCK-8. Two hundred-fifty µg of CCK-8 was reconstituted in 10 mL of barbital buffer I. The resulting 2.19×10^{-5} M solution was diluted to 2.19×10^{-10} M using barbital buffer I to create a working solution. One, 2.5, 5, 10, 20, 30, and 50 pM standards were prepared from the standard working solution. Four, 7.5, and 15 pM controls were also prepared from the standard working solution. Each standard was raised to the desired volume

using barbital buffer I as the diluent. To carry out the assay, RIA tubes were used in duplicate, with tubes for total count (TC), non-specific binding (NSB), total binding (TB), and each standard or control. On the day of the assay, the antibody, albumin extracts, standards, and controls were taken out of the freezer and thawed at room temperature. Barbital buffer I was taken out of the refrigerator and allowed warm to room temperature. RIA tubes were arranged and labeled in duplicate fashion. Tracer was taken out of the freezer in the GI Lab radiation room and then diluted with barbital buffer I to 1,000 cpm +/- 100 cpm based on gammacounter readings for 60 seconds. Barbital buffer I was placed in each tube in the respective amounts as described in Figure 1. Newborn calf serum extract tubes were reconstituted to 400 µL with standard and controls, and then added to each tube in an amount as described in Figure 1. Sample extract tubes were reconstituted to 400 µL with barbital buffer I and added to each tube in an amount as described in Figure 1. Antibody 92128-5 was diluted to a dilution of 1:20,000 using barbital buffer I and added to each tube in an amount as described in Figure 1. All tubes were then taken to a separate radiation room where 100 µL of tracer were added to each tube. All tubes were vortexed and allowed to incubate for 3 days at 4°C. On day 3, a separation solution was prepared using 80 mL barbital buffer II, 20 mL canine plasma, and 6 g of activated charcoal. The solution was kept on ice and vigorously mixed on a magnetic stirrer for 30 minutes. After 30 minutes, 500 µL of separation solution was added to each tube. All tubes were vortexed and allowed to incubate at room temperature for 25 minutes. All tubes were then centrifuged for 10 minutes at 4,000 rpm at 4°C. The supernatant from all tubes (except TC tubes) was

transferred from the original tube to a new tube, tapping once. The supernatant tubes were counted in an automatic gammacounter for 5 minutes and results were expressed as counts per minute. The cpm data was entered into an Excel® spreadsheet where average, standard deviation, correlation of variance, and B/B_0 were calculated. B/B_0 was calculated using the formula: $B/B_0 = (B - NSB) / (B_0 - NSB)$, where NSB is the non-specific binding cpm, B is the supernatant cpm, and B_0 is the total binding (TB) cpm. A four-parameter log-logistic (4PL) standard curve was generated in Prism 6® by plotting the logarithm of the concentration against B/B_0. All unknown values were extrapolated based on where their B/B_0 falls along the standard curve.

Validation - Assay sensitivity was determined using 10 duplicates of the total binding tube. Each duplicate received 1,000 µL barbital buffer I, 150 µL reconstituted NCS extract, 150 µL diluted antibody 92128-5, and 100 µL ^{I125}CCK-8 tracer. The modified canine RIA protocol was run with standards, and the resulting cpm data was evaluated by calculating the mean and standard deviation of the 10 duplicates. Three standard deviations were subtracted from the mean count and the value plotted on the standard curve to determine the corresponding CCK concentration. This calculated sensitivity was also used as the lower limit of the working range. To determine dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability, 300 leftover serum samples from dogs for which serum had been submitted for diagnostic testing to the Gastrointestinal Laboratory at Texas A&M University were evaluated using the new protocol to find samples with CCK concentrations that fell into different areas of the working range of the assay. For dilutional parallelism, 3 serum samples were

selected and tested at full strength, and at dilutions of 1 in 2, 1 in 4, and 1 in 8. For spiking recovery, 3 pools of serum were generated from the 300 serum samples to obtain sufficient serum volume to carry out the validation experiments. Serum pools were spiked with a known concentration of CCK-8 such that they would fall into different areas of the working range of the assay. Spiking recovery was determined by taking samples from the 3 serum pools and spiking them with 0, 1.25, 2.5, and 5 pM of CCK in barbital buffer I. For intra-assay variability and inter-assay variability, 4 pools of serum were generated from the 300 serum samples to obtain sufficient serum volumes to carry out the assay. Serum pools were spiked with a known concentration of CCK-8 such that they would fall into different areas of the working range of the assay. Intra-assay variability was determined by evaluating 4 different serum samples 6 times within the same assay run. Inter-assay variability was determined by evaluating 4 different serum samples in 6 consecutive assay runs. A reference interval for CCK in canine serum was established using 90 serum samples from clinically healthy, fasted (12 hrs) dogs using the lower 97.5th percentile. A stability study was conducted to determine the stability of CCK in canine serum under different storage conditions. Nine canine serum samples were obtained from healthy, fasted dogs (12 hrs). The samples were aliquoted into 5 different 2 mL sample tubes using a volume of 400 µL each. An aliquot from each dog was measured for CCK at the following time points: immediately (baseline); after 24 hours at 4°C or -80°C; after 1 week at -80°C; and after 4 weeks at -80°C. Prism 6® was used for all analyses. Each data set was tested for normality by use of a Kolmogoro-

Smirnov normality test. For stability evaluation, serum CCK concentrations were compared by use of repeated-measures ANOVA with multiple comparisons.

RESULTS

A typical standard curve for the assay is shown in Figure 4. The sensitivity of the assay was calculated to be 0.5 pM using three standard deviations from the mean of 10 assay runs of a sample containing no CCK (Table 10). Thus, the lower limit of the working range of the assay was also 0.5 pM. For dilutional parallelism, observed-to-expected ratios ranged from 101.9 % to 253.6 % (162.4 % ± 54.4 %) for 3 different canine serum samples at dilutions of 1 in 2, 1 in 4, and 1 in 8 (Table 11). For spiking recovery, observed-to-expected ratios ranged from 81.4 % to 68.2 % (73.2 % ± 4.0 %) for 3 different canine serum samples at 4 different spiking concentrations (Table 12). Coefficients of variation for intra-assay variability of 4 pooled serum samples were 3.8, 13.5, 7.9, and 3.9 % (Table 13). Coefficients of variation for inter-assay variability of 4 pooled serum samples were 12.3, 11.6, 7.4, and 6.4 % (Table 14). The reference interval for serum CCK concentration was established as ≤ 2.8 pM (Fig 5). The serum concentrations for each of the 9 serum samples did not vary significantly in samples stored at 4°C for 24 hours or at -80°C for 24 hours, 1 week, or 4 weeks (Fig 6 and Table 15).

Table 10 – Sensitivity of CCK RIA. This table shows the results for the determination of the sensitivity of the assay using 10 duplicates of the total binding (TB) tube. The mean minus 3 standard deviations was plotted on a standard curve to obtain the sensitivity. (SD = standard deviation).

Tube ID	Tube 1	Tube 2	Mean
	cpm	cpm	cpm
TB 1	433.0	428.9	431.0
TB 2	428.1	453.2	440.7
TB 3	427.7	425.1	426.4
TB 4	423.2	442.1	432.7
TB 5	422.0	437.8	429.9
TB 6	431.7	441.8	436.8
TB 7	428.1	442.2	435.2
TB 8	443.0	429.8	436.4
TB 9	445.0	431.8	438.4
TB 10	397.6	438.2	417.9
Mean			432.5
SD			6.3
Mean - 3(SD) = 413.5 ⇨ 0.5 pM			

Table 11 – Dilutional parallelism of CCK RIA. This table shows the data from serial dilutions of 3 serum samples throughout the working range of the assay. All 3 serum samples were measured at dilutions of 1 in 1, 1 in 2, 1 in 4, and 1 in 8.

Sample A	Observed	Expected	O/E ratio
Dilution	pm	pm	%
1 in 1	14.2		
1 in 2	7.3	7.1	101.9
1 in 4	5.2	3.6	146.7
1 in 8	2.6	1.8	143.9

Sample B	Observed	Expected	O/E ratio
Dilution	pm	pm	%
1 in 1	3.9		
1 in 2	2.8	1.9	146.5
1 in 4	1.4	1.0	140.3
1 in 8	1.1	0.4	247.8

Sample C	Observed	Expected	O/E ratio
Dilution	pm	pm	%
1 in 1	3.9		
1 in 2	2.1	1.9	107.7
1 in 4	1.7	1.0	173.2
1 in 8	1.2	0.5	253.6

Table 12 – Spiking recovery of CCK RIA. This table shows the data obtained from the spiking recovery performed on 3 different serum sample pools in the lower end of the control range. All 3 serum pools received CCK-8 additions of 0, 1.25, 2.5, and 5 pM.

Pool A			
Added	Observed	Expected	O/E ratio
pM	pM	pM	%
0	2.9		
1.25	3.0	4.1	72.3
2.5	3.7	5.4	68.2
5	6.1	7.9	77.8

Pool B			
Added	Observed	Expected	O/E ratio
pM	pM	pM	%
0	1.6		
1.25	2.1	2.8	72.9
2.5	3.3	4.1	81.4
5	4.7	6.6	70.7

Pool C			
Added	Observed	Expected	O/E ratio
pM	pM	pM	%
0	2.4		
1.25	2.6	3.7	70.6
2.5	3.5	4.9	71.5
5	5.5	7.4	73.5

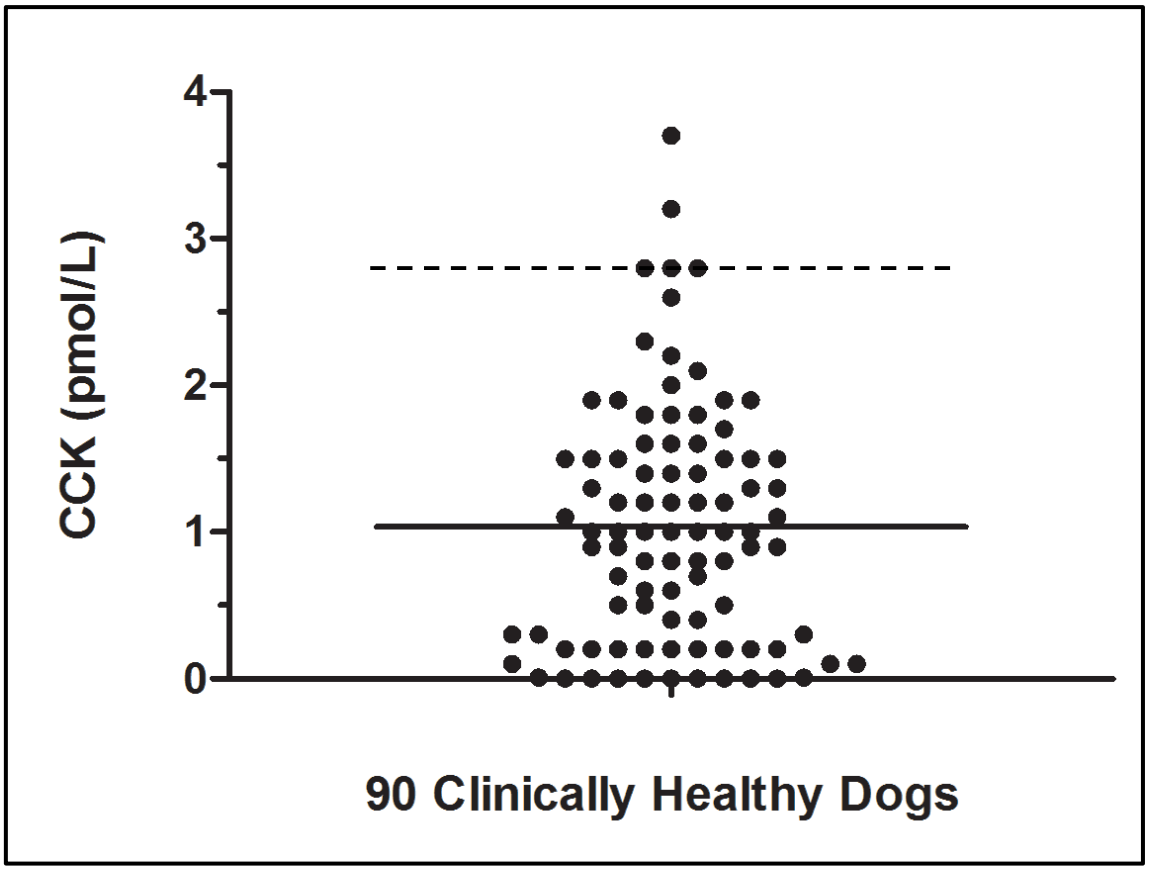
Table 13 – Intra-assay variability of CCK RIA. This table shows the results obtained from the determination of intra-assay variability for CCK RIA using 4 different serum pools. (SD = standard deviation; %CV = coefficient of variation).

	Pool 1	Pool 2	Pool 3	Pool 4
Number of repeats	6	6	6	6
Mean (pM)	2.2	3.0	10.6	21.1
SD (pM)	0.1	0.4	0.8	0.8
%CV	3.8	13.5	7.9	3.9

Table 14 – Inter-assay variability of CCK RIA. This table shows the results obtained from the determination of inter-assay variability for CCK RIA using 4 different serum pools. (SD = standard deviation; %CV = coefficient of variation).

	Pool 1	Pool 2	Pool 3	Pool 4
Number of repeats	6	5	6	6
Mean (pM)	2.1	3.5	10.2	19.9
SD (pM)	0.3	0.4	0.7	1.3
%CV	12.3	11.6	7.4	6.4

Figure 5 – Reference interval for CCK RIA. The mean serum concentration for CCK as measured in 90 clinically healthy dogs was 1.03 ± 0.83 pmol/L. The reference interval was determined by the bottom 97.5th percentile and was ≤ 2.8 pmol/L. Several serum samples from healthy dogs were included in this assay run. The mean CCK concentration is depicted by the solid line. The reference interval is shown by the dotted line.



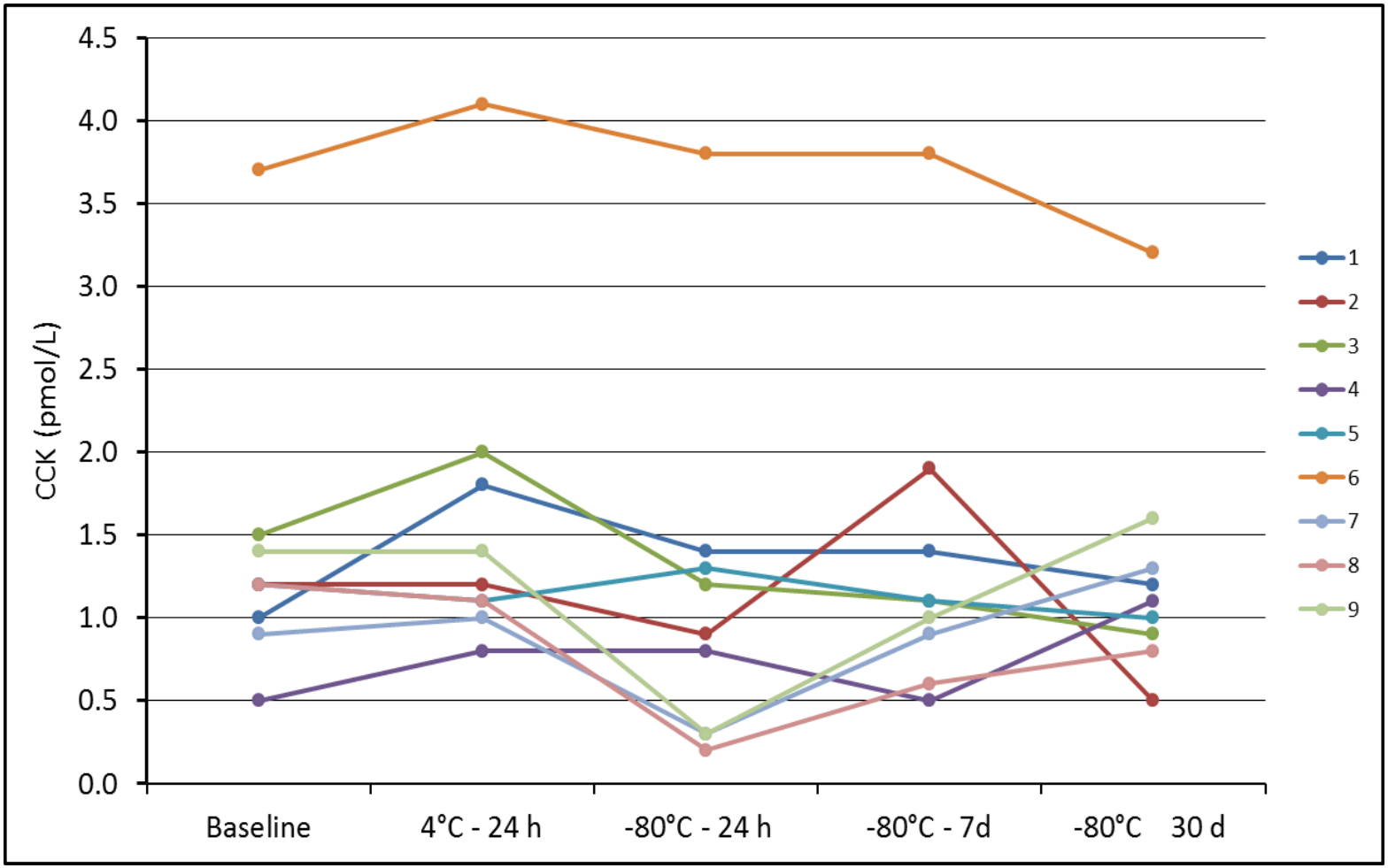


Figure 6 – Stability of CCK in serum. This figure shows CCK concentrations of 9 different canine serum samples at 3 different time points and 2 different storage conditions. (h = hours; d = days).

Table 15 – ANOVA of stability study. This table shows the results of an ANOVA for assessing the stability of serum CCK concentrations at different storage conditions.

Repeated-measures ANOVA with multiple comparisons			
Number of families	1		
Number of comparisons per family	4		
α (alpha)	0.05		
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?
Baseline vs. 4°C for 24 h	-0.21	-0.52 to 0.10	No
Baseline vs. -80°C for 24 h	0.27	-0.28 to 0.82	No
Baseline vs. -80°C for 7 d	0.03	-0.38 to 0.45	No
Baseline vs. -80°C for 30 d	0.11	-0.37 to 0.59	No

DISCUSSION

A radioimmunoassay for the measurement of CCK in canine serum was successfully analytically validated. The observed-to-expected ratios for dilutional parallelism ranged from 101.9% to 253.6%. All 3 pools had adequate dilution ratios at 1 in 2; however, all 3 pools went above 140% at the 1 in 4 and 1 in 8 dilutions. This suggests that the assay has limited linearity in the lower end of the working range. Inferences for the higher end of the working range cannot be determined from this data. The observed to expected ratios for spiking recovery ranged from 81.4% to 68.2%. While no specific criteria exist for acceptable values of spiking recovery, all samples measured had a low recovery rate (81% to 68%). However, spiking led to increased concentrations suggesting, that while some of the spiked material was lost to measurement the assay does indeed measure the analyte in question.

Coefficients of variation for intra-assay variability of 4 pooled serum samples were 3.8, 13.5, 7.9, and 3.9%. All 4 pools had CVs below 15%, which suggests that the assay is sufficiently precise. Coefficients of variation for inter-assay variability of 4 pooled serum samples were 12.3, 11.6, 7.4, and 6.4%. Pool 2 only had 5 repeats due to an error encountered during the assay. All 4 pools had CVs below 15%, which suggests that the assay is sufficiently reproducible. The reference interval for the assay was calculated to be ≤ 2.8 pM from the lower 97.5th percentile of serum CCK concentrations in 90 clinically healthy dogs.

The results of the stability study indicate that CCK is stable in serum when stored for no more than 30 days at temperatures that samples are routinely exposed to during

shipping and storage until extraction. Stability was not evaluated for samples at room temperature, thus samples obtained for analysis should be shipped at a maximum of 4°C. There was some variation of results between the different time points, but the sample with the highest CCK concentration showed the lowest variability.

The radioimmunoassay for CCK described here is sufficiently accurate, precise, and reproducible, but has limited linearity in the lower end of the working range. To our knowledge, this is the first validation of a CCK RIA specific for canine serum, and without human reagents. Target values for the purposes of assay validation were taken from previously validated radioimmunoassays, although no specific standards for validation have been established.[10,19] The purpose of this project was to successfully modify a CCK RIA for use in humans for the measurement of canine serum CCK. Clinical studies are necessary to determine the clinical usefulness of measuring CCK concentrations in dogs with gastrointestinal disease.

CHAPTER IV

CONCLUSIONS

Cholecystokinin is an important neuroendocrine peptide in the gastrointestinal tract, being the major stimulant for exocrine pancreatic secretion and gall bladder contraction. Current research focuses on CCK outside the gastrointestinal tract in humans. While there is a wealth of information on CCK and disorders associated with alterations in CCK release in humans, there is a distinct lack of information on similar disorders in dogs. This is likely due to the complexity of current CCK RIAs and the use of human reagents.

Thus, the objectives of this study were to modify a radioimmunoassay used for the measurement of plasma CCK in humans for use in dogs, to perform said assay without human reagents, and to analytically validate this modified immunoassay for use with canine serum. To accomplish the objectives, assay runs were set up to replace all human reagents that are part of the original protocol, followed by analytical validation of the adapted assay using canine serum samples by determination of sensitivity, dilutional parallelism, spiking recovery, intra-assay variability, and inter-assay variability. All objectives outlined above were successfully met, analytically validating a radioimmunoassay for the measurement of cholecystokinin in canine serum. An assay now exists that has been analytically validated for canine serum and is free of human reagents, allowing for accurate measurements to be made from routinely drawn canine

serum samples. This will allow future studies to determine the role of altered CCK secretion in dogs with GI disease.

The radioimmunoassay for CCK described here is sufficiently accurate, precise, and reproducible, but has limited linearity in the lower end of the working range. After evaluating 90 serum samples from clinically healthy dogs with the validated RIA, the reference interval was established as 0.0 to 2.8 pM. Further research is needed to determine the usefulness of this assay in research animals or clinical patients.

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