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RADIOIMMUNOASSAY OF CANINE INSULIN, C-PEPTIDE AND GLUCAGON: A COMPARISON AND EVALUATION OF COMMERICAL RIA KITS

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Steven L. Stockham

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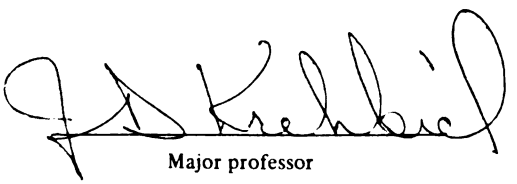

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RADIOIMMUNOASSAY OF CANINE INSULIN, C-PEPTIDE AND GLUCAGON: A COMPARISON AND EVALUATION OF COMMERCIAL RIA KITS

By

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Diplomate, A.C.V.P.

A THESIS

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ABSTRACT

RADIOIMMUNOASSAY OF CANINE INSULIN, C-PEPTIDE AND GLUCAGON:
A COMPARISON AND EVALUATION OF COMMERICAL RIA KITS

By

Steven L. Stockham, D.V.M.
Diplomate, A.C.V.P.

Evaluation criteria for five commercial insulin RIA kits included precision, dilutional parallelism, sensitivity, and comparison of canine IRI concentrations in four control sera. One insulin RIA kit (Corning Medical) had consistent acceptable precision, good dilutional parallelism, and adequate sensitivity. Other RIA kits had relatively poorer performance in either dilutional parallelism or precision. No two RIA kits quantitated the same IRI quantities in all four control sera.

Canine sera failed to adequately displace tracer from anti-synthetic human C-peptide antibody (Calbiochem-Behring Corporation). This finding suggested lack of species cross-immunoreactivity and was consistent with reported findings in other species.

Two commercial glucagon RIA kits had poor precision in quantitating low canine IRG concentrations. One RIA kit was modified and appeared to increase sensitivity, but precision remained inadequate.

To Marcia

Your love, understanding, and encouragement have made this possible.

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I. INTRODUCTION

Insulin and glucagon have been considered primary hormonal regulators of glucose homeostasis (Unger, 1971). Diabetes mellitus and functional beta cell islet neoplasms (insulinomas) have been the most frequently reported canine disorders relating to abnormal insulin activity (Capen and Martin, 1969; Kramer and Wilson, 1978; Njoku et al., 1972; Schall and Cornelius, 1977). Documentation of clinical disorders relating to abnormal glucagon activity in dogs has been limited.

Experimental evidence has indicated that diabetes mellitus is a syndrome associated with carbohydrate intolerance and with or without hypoinsulinemia. Hyperglucagonemia (Blazquez et al., 1977) hypersomatropinemia (Pierluissi and Campbell, 1980), hypersomatostatinemia (Krejs et al., 1979; Schusdziarra et al., 1977), and elevated glucocorticoids (Emmer et al., 1971) have been shown to be involved in diabetes mellitus. However, most of these reports involved human diseases or experiments with dogs. Spontaneous canine diabetes mellitus has been assumed to be a syndrome of hypoinsulinemia and roles of other hormones have not been adequately evaluated (Schall and Cornelius, 1977). In addition to above hormones, epinephrine (Levine and Haft, 1970) and somatomedins (Phillips and Vassilopoulou-Sellin, 1980) have roles in carbohydrate metabolism. Felig et al. (1976), Gerich et al. (1976), and Samols and Weir (1979) have reported relationships and regulations of these hormones.

Tentative diagnoses of canine insulinomas have been based on demonstraton of Whipple's triad (Caywood et al., 1979). Demonstration of concurrent hyperinsulinemia has been used to confirm diagnoses (Johnson, 1977). Connecting peptide (C-peptide) quantitations have been reported to be useful in evaluating hypoglycemic and hyperglycemic disorders of man (Horwitz et al., 1976; Rubenstein et al., 1977; Starr et al., 1979). Similar studies have not been reported in canine disorders.

Very few of above articles would have been written had it not been for radioimmunoassay (RIA) development. Canine immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) quantitations have generally utilized radioimmunoassays developed in research laboratories. These "private" assays have hindered RIA availability, created variations in inter-laboratory results, and decreased clinical applications of canine IRI and canine IRG quantitations.

Commercial RIA kits have proven useful for quantitating several canine hormones. However, most RIA kits have been developed to quantitate human hormones. It has been necessary to evaluate these assays for their ability to quantitate respective canine hormones.

The purpose of this project was to evaluate commercial insulin, glucagon, and C-peptide RIA kits. Those assays which reliably quantitate respective canine polypeptides could then be utilized for clinical and research investigations. Hormone quantitations could also be offered to veterinarians and other investigators on a reference laboratory basis. Complete hormonal evaluation of spontaneous canine endocrine disorders may lead to a better management of affected dogs, understanding of pathophysiologic mechanisms, and possibly identification of animal models for human diseases.

II. LITERATURE REVIEW

A. History

Radioimmunoassay (RIA) has become an important form of clinical chemistry in the last two decades. Classic investigations by Rosalyn S. Yalow, Ph.D. and the late Solomon A. Berson, M.D. led to RIA development. Their demonstration of insulin antibodies in insulin-treated humans (Berson et al., 1956) was not readily accepted by the scientific community. Yalow (1974) reported that the Journal of Clinical Investigation would not allow the word "antibody" in their original article but would accept the term "insulin binding globulin".

Berson and Yalow (1959) demonstrated that their human antibody to bovine and porcine insulins could react with preparations of bovine, porcine, equine, and ovine crystalline insulins. First RIA utilization for clinical investigations was with guinea pig antibody to bovine insulin (Yalow and Berson, 1959). Within the next year, Yalow and Berson (1960) reported insulin RIA utilization to quantitate immunoreactive insulin (IRI) during glucose tolerance tests and in subjects with functional islet cell tumors.

In the 1960's, most RIA development and utilization involved in-house reagent preparation. Today, over 200 biologically important substances can be quantitated with commercial RIA kits (Sweeny, 1979).

B. Radioimmunoassay Evaluation

As for any quantitative analytical procedure, an RIA should reliably quantitate substances of interest (analytes). Four basic criteria must

be evaluated for RIA validation: specificity, precision, sensitivity, and accuracy. (Kagan, 1975; Midgley et al., 1969; Moss et al., 1976; Skelly et al., 1973; Walsh, 1978; Zazuco Higa et al., 1974). However, acceptable limits and methods of evaluating these criteria varied among investigators.

1. Specificity

Optimal clinical RIA would measure only biologic active hormones and not closely related substances. Midgley et al. (1969) and Skelley et al. (1973) reported that antibody specificity for analyte was dependent on purity and species origin of immunogen, presence of immunologic similar substances (precursor hormones, degradation products), presence of unwanted radiolabelled substances, and differences in incubation medium composition.

Two methods have been recommended for assessing specificity: 1) demonstration of dilutional parallelism, 2) demonstration that related substances do not influence quantitation of analyte (Hafs et al., 1977; Witherspoon, 1979).

2. Precision

Witherspoon (1979) reported that precision is affected by technical steps (pipetting, incubation, vortexing, counting time) and slope of dose-response curve. He also recommended that precision be evaluated with intra-assay and inter-assay coefficients of variation (C.V.) with respective acceptable limits of 5-10 and 10-20. Other authors recommended another method referred to as the index of precision or precision coefficient (Aubert, 1970; Midgeley et al., 1969; Skelley et al., 1973). This method was defined as "the ratio between the standard deviation of

logit Y for each Y value and the slope of the regression line between values of logit Y and the corresponding log X".

3. Sensitivity

Definitions and methods of assessing sensitivity varied among authors. One group defined sensitivity as detection limits and assessed sensitivity by statistical calculations using zero concentration tubes (Ekins, 1975; Midgley et al., 1969; Skelley et al., 1973, Witherspoon, 1979; Zazuco Higa et al., 1974)

Another group defined sensitivity as the smallest quantity of antigen which can be measured reliably (Hafs et al., 1977; Kagan, 1975; Witherspoon, 1979). Using this definition, these investigators assessed sensitivity by determining analyte concentrations which displaced tracer by a fixed amount. These analyte concentrations corresponded to a 10% decrease from zero concentration in either the bound/free (B/F) or bound/bound-at-zero (B/B_0) partition indices.

4. Accuracy

Generally, two methods were recommended for assessing accuracy. Witherspoon (1979) stated that dilutions of a reference standard should give a parallel curve to kit standards. Longley (1976), Moss et al. (1976), Skelley et al. (1973), Walsh (1978), Witherspoon (1979), and Zazuco Higa et al. (1974) described a second method referred to as a recovery procedure. A "spiked" sample (addition of reference standard) was assayed and 100% recovery implied accuracy. In both methods, a reference standard was necessary. Bangham (1976) and Cotes (1974) discussed availability and utilization of immunoreactive reference

standards. World Health Organization[a] and associated institutions have provided many immunoreactive hormone standards, however canine standards were not included in their literature.

C. Canine Immunoreactive Insulin Quantitation

Canine IRI quantitations were determined by many laboratories and by a variety of methods. Evidence of RIA validation for canine serum or plasma was not found in the literature. Porcine insulin was used predominately for immunogens, tracers, and standards. Occasionally, bovine insulin was used for immunogen or tracer and human insulin for standards. Species sources of antibody were rarely reported.

Fasting canine IRI concentrations varied with methods of separating free and bound tracer. DeFronzo et al. (1978) reported 18-25 μU IRI/ml using a talc separation described by Rosselin et al. (1966). Juffe et al. (1977), Kaneko et al. (1977, 1978a, 1978b), and Manny et al. (1977) reported 2 to 18 μU IRI/ml using a dextran-coated charcoal method described by Herbert et al. (1965). Hommel and Fischer (1977) and Orosz (1974) utilized an alcohol separation technique and reported a mean 40 μU IRI/ml and 19-21 μU IRI/ml, respectively. Campbell et al. (1978) and Matsuyama et al. (1977) reported 38 $\pm$ 5 μU IRI/ml and 32 $\pm$ 4 μU IRI/ml, respectively, utilizing a pre-precipitation double antibody technique described by Hales and Randle (1963). Chen et al. (1977) used a commercial RIA[b] based on the same technique and reported a mean of 15 μU IRI/ml. Black et al. (1980) and Manns and Martin (1972) utilized

[a]WHO International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Holly Hill, Hampsted, London, NW3 6RB, England

[b]Amersham Insulin RIA Kit, Amersham Corporation, 2636 S. Clearbrook Dr., Arlington Heights, IL, 60005

post-precipitation double antibody methods and reported 8-22 μU IRI/ml and 37 ± 18 μU IRI/ml, respectively. Cherrington et al. (1978), Jennings et al. (1977), and Keller et al. (1977) utilized a commercial RIA[c] based on Sephadex® bound antibodies described by Wide and Parath (1966) and reported 13-23 μU IRI/ml.

Specific reasons for IRI concentration variations reported in fasting dog serum or plasma were not found. However, comparisons of insulin radioimmunoassays using human sera or plasma have shown that different IRI concentrations could be quantitated in the same sample. Malvano et al. (1974) compared three RIA methods of quantitating human IRI. They reported lower concentrations in dextran-coated charcoal and resin methods (9.4 ± 3.6 and 8.7 ± 3.1 μU/ml) than in double antibody techniques (16.2 ± 4.5 μU/ml). Another comparison study with human sera and plasma resulted in different IRI concentrations in solid phase RIA[c], double antibody RIA[b], and ethanol precipitation RIA (Thorell and Lanner, 1973).

D. Canine Insulin Molecule Compared to Other Species

Smith (1972) reviewed insulin amino acid sequences determined for many species. Canine insulin has two polypeptide chains (A chain - 21 amino acids, B chain - 30 amino acids) connected by two disulfide bridges. Canine, porcine, fin whale, and sperm whale insulin molecules have identical amino acid sequences. Bovine insulin differs from canine insulin by having amino acid substitution at positions 8 and 10 of the A chain. Human insulin differs from canine insulin only at the carboxyl-terminal of the B chain by having one amino acid substitution.

[c]Phadebas Insulin Test, Pharmacia Diagnostics, 800 Centennial Avenue, Piscataway, New Jersey, 08854

Guinea pig, a common source of insulin antibodies, has an insulin molecule that differs from canine insulin at 8 positions on the A chain and 10 positions on the B chain.

Amino acid sequences (primary structure) have been shown to determine tertiary and quaternary structures (or molecular conformation) of proteins (Stryer, 1975). However, Berson and Yalow (1961) determined that some insulin antisera were able to distinguish between porcine and sperm whale insulins. Berson and Yalow (1963) reported that human antibodies against porcine insulin reacted with the region of porcine insulin that had identical amino acid sequences to human insulin. These findings led to the conclusion that other species specific factors determine insulin antigenic characteristics (Berson et al., 1964). Definitive evidence of cross-immunoreactivity between canine insulin and other insulins was not found. Cross-reactivity of canine proinsulin with insulin antibodies was not found in the literature; however, human proinsulin has been shown to cross-react with porcine insulin antibodies (Kubasik, 1978). Individual A and B chains of insulin did not cross react with insulin antibodies (Berson and Yalow, 1959; Starr et al., 1979).

E. C-peptide[d] Radioimmunoassays

Canine immunoreactive C-peptide (IRC-P) has been quantitated to a very limited extent. Kawanishi et al. (1977) reported quantitation of IRC-P in pancreatic venous blood. They indicated that the primary components of their assay consisted of synthetic canine C-peptide, rabbit antibody to synthetic canine C-peptide, and iodinated (^{125}I) tyrosine conjugated synthetic canine C-peptide.

[d]C-peptide - connecting peptide that links A and B chains of insulin in proinsulin molecule.

Work with bovine, porcine, and human C-peptides has indicated that species specific antisera is needed for radioimmunoassays (Chance, 1972; Kaneko et al., 1974; Kemmler et al., 1972; Rubenstein and Steiner, 1971). This requirement was attributed to differences in amino acid content or sequences in each respective species. Kemmler et al. (1972) and Peterson et al. (1972) reported that canine C-peptide has more differences in primary structure than other domestic species when compared to human C-peptide. Cross-immunoreactive studies with canine C-peptide were not found.

F. Glucagon Radioimmunoassays and Heterogeneity of Canine Glucagon

Unger et al. (1959) first described a RIA for glucagon. During the last twenty years, many controversies and contradictions have been reported in tissue sources of immunoreactive glucagon, glucagon action, biologic activity, and molecular forms of immunoreactive glucagon.

Tissue sources of canine immunoreactive glucagon (IRG), (synonyms: "true glucagon", pancreatic-like glucagon) have been determined by tissue extractions and isolated perfused organs. Highest concentrations were found in pancreas and gastric mucosa (Blazquez et al., 1976, 1977; Lefebvre and Luyckx, 1978; Morita et al., 1976). Smaller concentrations were found in jejunal, duodenal, ileal, and colonic mucosa (Morita et al., 1976; Muller et al.,1978), and brain (Conlon et al., 1979). Difficulties in obtaining purified IRG was exemplified by one report where 105 canine stomachs were used to obtain 1.5 µg of purified gastric IRG (Doi et al., 1979).

Tissue sources of canine glucagon-like immunoreactivity (GLI) were found in intestine (Muller et al., 1978; Unger et al., 1968), gastric

mucosa (Doi et al., 1979; Srikant et al., 1977), and brain (Conlon et al., 1979).

Classification of substances as IRG or GLI was established by dilutional parallelism studies using antisera generally accepted as pancreatic glucagon specific or non-specific antisera. Conlon et al. (1979), Faloona and Unger (1974), and Heding et al. (1976) concluded that antisera specific for IRG binds with C-terminal region of glucagon. Conlon et al. (1978, 1979) and Heding et al. (1976) reported that GLI lacked the C-terminal region of IRG and antiserum that reacts with GLI and IRG binds with N-terminal regions.

Harris et al. (1979) recommended that antisera for IRG have the following characteristics: 1) should react weakly or not at all with extracts of jejunum; 2) dilution of plasma should yield proportional glucagon immunoreactivity; 3) glucagon immunoreactivity should decrease after oral glucose administration; 4) depancreatized animals should have near zero concentrations of glucagon immunoreactivity.

The 29 amino acid sequences of human, bovine, and porcine glucagon molecules were reported to be identical (Bromer et al., 1971; Thomsen et al., 1972). Amino acid compositions of rat, rabbit, and porcine glucagon molecules were reported to be identical (Sundby and Markussen, 1971, 1972). Canine glucagon amino acid sequence or composition were not found in the literature nor did any author state that canine glucagon was identical to other species. Srikant et al. (1977) stated that canine and porcine glucagon dilution curves were parallel and thus implied immunoreactive unity.

Radioimmunoassay quantitation of canine glucagon has been limited to experimental studies to evaluate glucagon physiology. In 18 recent

articles reporting canine IRG concentrations in peripheral blood, only 2 reported quality control data or validation criteria. Buchanan et al. (1969) reported their assay had a precision of 216 pg/ml, detection limit of 310 pg/ml, and canine controls had IRG concentrations from 240 to 350 pg/ml. Nilsson and Uvnas-Wallenstein (1977) reported good recovery studies and a detection limit of 2 pg/ml. Reported concentrations of peripheral blood IRG (fasting or basal) ranged from 33 pg/ml (Eigler et al., 1979) to 278 $\pm$ 25 pg/ml (Buchanan et al., 1969). Considerably more variation has been reported in GLI with concentrations from 74 $\pm$ 15 pg/ml (Matsuyama et al., 1977) to 123 $\pm$ 56 mg/ml (Manns and Martin, 1972). The later was the only article that reported IRG or GLI concentrations above 1 ng/ml. Reports stating use of commerical glucagon radioimmunoassays were not found.

III. MATERIALS AND METHODS

A. Experiment 1 - Screening Evaluations of Commercial Insulin Radioimmunoassays

1. Commerical Insulin Radioimmunoassays Evaluated

Five commercial insulin radioimmunoassays developed for quantitation of human IRI were selected from 17 available kits (Sweeny, 1979) because of differences in their separation procedures or published reports of their use to quantitate canine IRI. (Species sources of RIA components and lot numbers of evaluated assays are listed in Table A1. Performance characteristics stated in each RIA kit package insert are listed in Table A2.) Each insulin RIA was run on two different days with reagents from one kit and using duplicate determinations. Existing laboratory instrumentation and computer programs for data reduction necessitated modifications of some manufacturers' assay protocols.

The following insulin RIA kits were evaluated:

a. Kit A - Insulin RIA Kit; Amersham Corporation

This immunoprecipitation assay utilized a double antibody modification where first and second antibody are complexed prior to their addition to incubation solutions as first described by Hales and Randle (1963). Modifications of the manufacturer's protocol were approved by an Amersham technical representative. Glass tubes were used as reaction and counting tubes instead of polystyrene tubes. A decanting method was used to separate supernatant and precipitate instead of a draining technique. Total count tubes and standards were run in duplicate.

b. Kit B - CIS Insulin Radioimmunoassay Kit, INSIK - 3-M; International CIS, Damon Diagnostics

This solid phase adsorption RIA utilized dextran-coated charcoal (DCC) to selectively adsorb free ligands as described by Herbert et al. (1965). Modifications of the manufacturer's protocol included decanting, counting free tracer, and insertion of a charcoal blank. Reconstituted phosphate buffer (pH 7.7) was titrated to pH 7.4 with 0.1 N HCl. Data reduction was based on bound tracer with the bound partition calculated as follows: Bound cpm = Total counts - Charcoal blank cpm - Free cpm

c. Kit C - Immophase™, Insulin ^{125}I Radioimmunoassay Test System; Corning Medical

This solid phase RIA utilized antibodies covalently bound to glass particles. Manufacturer's protocol was modified only by a quick vortexing of duplicate reaction tubes prior to incubation.

d. Kit D - Phadebas® Insulin Test; Pharmacia Diagnostics

This solid phase RIA utilized antibodies covalently bound to Sephadex® particles as described by Wide and Parath (1966). Manufacturer's protocol was followed.

e. Kit E - RSL Insulin Kit; Radioassay Systems Laboratories, Inc.

This immunoprecipitation RIA utilized double antibody principle as described by Soeldner and Slone (1965). Manufacturer's protocol was followed.

2. Sources of Canine and Commercial Control Sera

Canine insulin high sera (K-9 Ins Hi) and canine insulin low sera (K-9 Ins Lo) controls were collected from four dogs. Two dogs were fasted 30 hours prior to blood collection to produce a control serum with

low insulin concentration (K-9 Ins Lo). Two dogs were given 50% Dextrose (1 gm/kg) intravenously 20 minutes prior to initiation of blood collection to produce a control serum with high insulin concentration (K-9 Ins Hi). All dogs were atropinized (atropine sulfate, 0.2 mg/pound body weight) and anesthetized (sodium thiamylal, methoxyfluorane, to effect) prior to catheterization of right carotid arteries. Blood was collected in plain glass tubes and allowed to clot for 1-2 hours at room temperature. Sera from each pair of dogs was pooled, mixed, and aliquoted into appropriately labelled plastic vials. Control sera were frozen (-25°C) until the day of analysis.

Two levels of commercial lyophilized control sera (Corning Medical) were used in all assays of this experiment. These control sera contained porcine insulin with reference values of 44 $\pm$ 12 μU/ml (CS-1) and 137 $\pm$ 22 μU/ml (CS-2). Reconstituted sera were analyzed on preparation days or within shelf life recommended by manufacturer.

3. Criteria for Evaluation and Comparison of RIA Kits

a. Dilutional Parallelism

Dilutional parallelism was evaluated by two methods: linear regression of expected versus measured concentration and comparison of undiluted concentration to corrected concentration. Zero standards of each respective RIA kit were used for diluents. Duplicate determinations of each dilution were made in each replicated assay. Dilutions of 4/5, 3/5, 2/5, 1/5, and 1/10 were made the day of assay according to the following procedure:

1. 4/5 dilution: 800 µl K-9 Ins Hi + 200 µl diluent, vortex
2. 3/5 dilution: 600 µl K-9 Ins Hi + 400 µl diluent, vortex
3. 2/5 dilution: 800 µl K-9 Ins Hi + 1200 µl diluent, vortex
4. 1/5 dilution: 800 µl 2/5 dilution + 800 µl diluent, vortex
5. 1/10 dilution: 800 µl 1/5 dilution + 800 µl diluent, vortex

Linear regression (y=mx + b) of expected versus measured concentrations were calculated on a programmed calculator[e]. Linear regression figures for replicated assays were averaged for comparison of RIA kits.

Mean concentration of K-9 Ins Hi was compared to corrected concentration of dilutions as per cent of original concentration.

b. Precision

Duplicate determinations of each control were made at the beginning and end of each set of assayed samples. Intra-assay coefficient of variation (C.V.) for each control was calculated from mean concentrations of duplicates. Mean intra-assay C.V. for each RIA kit was calculated using mean intra-assay C.V. of each control. Inter-assay C.V. for each control was calculated from mean IRI concentration of replicated assays. Mean inter-assay C.V. for each RIA kit was calculated using inter-assay C.V. of each control. Intra-assay C.V. and inter-assay C.V. of less than 10 were selected as acceptable limits, 10-15 as questionable and greater than 15 as unacceptable.

c. Sensitivity

Ability of each insulin RIA kit to detect small quantities of IRI was assessed by three methods.

[e]Model 1930, Electronic Display Calculator for Statistics; Moore, The Calculator Company, Orange, N.J.

1. Sensitivity limit of each assay was defined as twice the standard deviations of zero binding. Transformations of zero tube cpm to μU/ml were done using logit-log transformation formulas. The following equations were to calculate sensitivity limits: y=mx + b where:

$$y = \text{logit} = \ln\left(\frac{U}{100-U}\right),$$

where $U = \frac{\text{lowest cpm of zero concentration duplicate}}{\text{mean cpm of zero concentration duplicate}} \times 100$

or $U = B/B_0 \times 100$

b= y-intercept, extrapolated from logit-log transformation of standard curve

m = slope, regression coefficient of logit-log transformation of standard curve

x = log [μU IRI/ml] represented by cpm difference from mean cpm of zero concentration duplicates.

Antilog x equals μU IRI/ml above "0" concentration representing cpm difference from mean cpm of zero concentration duplicates. Corresponding μU IRI/ml in replicate assay was also calculated. Each μU IRI/ml and corresponding negative μU IRI/ml were utilized as variables in calculating standard deviation; therefore, mean μU IRI/ml would equal zero.

2. The amount of IRI required to give 10% displacement in B/F dose-response curve was estimated by mathematical interpolation of B/F versus concentration standard curve. Each standard tube of replicated assays was considered as an individual point. Dose response was assumed to be linear between zero concentration and the standard which gave at least, or close to, 10% decrease in tracer binding. Mean μU/ml and standard deviation were calculated from four interpolated concentrations.

3. The amount of IRI required to give 10% displacement in B/B_0 dose-response curve was estimated by graph interpolation or extrapolation of logit B/B_0 versus logarithm of dose response curve. Mean and standard deviation of IRI concentrations were calculated.

d. Comparison of Immunoreactive Insulin Concentrations in Four Control Sera

Eight determinations of each control concentration of IRI were made. These concentrations represented duplicate tubes at beginning and end of replicated assays. For each RIA kit, mean concentrations and standard deviations were calculated for each control serum.

One way analysis of variance was used to calculate f values. Differences in means among kits were analyzed with Tukey's Honest Significant Difference (HSD) test (Gill, 1978).

4. Data Reduction

Scintillation data (cpm) were reduced to μU IRI/ml via computer programs[f] using logit-log transformation.

B. Experiment 2 - Screening Evaluation of Commercial C-peptide Radioimmunoassay

1. RIA Kit Evaluated: Kit F - Radioimmunoassay of Human C-peptide; Calbiochem-Behring Corp.

This immunoprecipitation assay utilized double antibody principle. Synthetic human C-peptide was used as immunogen and for standards. Tyrosylated synthetic human C-peptide was iodinated with ^{125}Iodine for use as a tracer. Manufacturer's instructions were followed except total counts were determined in individual duplicate tubes. (Component Lot #'s are listed in Table A1.)

[f]Animal Health Diagnostic Laboratory, Endocrine Diagnostic Section, Lansing, Michigan 48909

2. Sources of Samples

Canine insulin high serum (K-9 Ins Hi) and canine insulin low serum (K-9 Ins Lo) were used for canine samples. Collection of these sera was outlined in Materials and Methods, Experiment 1, page 13.

Human sera was collected from a volunteer to serve as a source of human C-peptide. A clinically healthy male, 31 years old, ingested 50% Dextrose (2 g/kg) orally after an 18 hour fast. Fasting, 30 minute, and 60 minute blood samples were collected and sera harvested. Sera were frozen (-20°C) until the day of analysis.

3. Criteria for Evaluation of RIA Kit

Based on previous reports of lack of species cross-reactivity (Chance, 1972; Kemmler et al., 1972; Rubenstein and Steiner, 1971) screening evaluation of this assay was limited to a predicted clinical correlation and dilutional parallelism. Sera obtained from fasting and glucose challenged normal humans should have low and high C-peptide concentrations, respectively (Block et al., 1972). Similar results would be expected in the dog based on pancreatic vein concentrations of C-peptide in response to glucose (Kawanishi et al., 1977). Multiple dilutions of K-9 Ins Hi were used to assess dilutional parallelism.

4. Data Reduction

Scintillation data cpm were reduced to ng IRC-P/ml via computer programs using logit-log transformations.[f]

C. Experiment 3 - Screening Evaluation of Commercial Glucagon Radioimmunoassays

1. Commercial Glucagon Radioimmunoassays Evaluated

a. Kit G - Radioimmunoassay of Pancreatic Glucagon; Cambridge Nuclear Radiopharmaceutical Corporation

This immunoprecipitation RIA utilized double antibody principle. No modification of the manufacturer's protocol was made in this experiment.

b. Kit H - RSL Glucagon Kit; Radioassay Systems Laboratories, Inc.

This RIA utilized double antibody principle. Addition of duplicate total count tubes was the only modification to the manufacturer's procedure. (Lot numbers of Kit G and Kit H and corresponding reagents used in this experiment are listed in Table A1.)

2. Sources of Control Plasma

Canine glucagon plasma #1 (K-9 Glcn-1) and canine glucagon plasma #2 (K-9 Glcn-2) were collected from four dogs as described in Materials and Methods, Experiment 1, page 13. Plasma from fasted dogs was designated as K-9 Glcn-1 and glucose challenged dogs as K-9 Glcn-2.

Blood collected in glass tubes containing K_3EDTA was immediately mixed with 0.55 Trypsin Inhibiting Units (TIU)[g] per milliliter blood and placed in an ice bath. These tubes remained in ice until centrifugation at 4°C. Plasma was pooled in a chilled glass container and aliquoted into appropriately labelled glass vials and frozen (-25°C). Two commerical control plasmas[h] designated as CP-Lo (850 $\pm$ 450 pg/ml) and as CP-Hi (5,250 $\pm$ 2000 pg/ml) were assayed in duplicate in replicated runs of Kit G and once in Kit H.

3. Criteria for Evaluation of RIA Kits

Evaluation criteria of two glucagon RIA kits included precision, dilutional parallelism, sensitivity, and comparison of immunoreactive glucagon (IRG) concentration in four plasma controls. Criteria were

[g]Aprotinin - Sigman Chemical Company, Box 14508, St. Louis, Mo., 63178

[h]Cambridge Nuclear Pharmaceutical Corporation, 575 Middlesex Turnpike, Billerica, Ma., 01865, (Lot #'s 20 NCLC 1 and Lot #21 HCLC1)

calculated and compared as outlined for the insulin RIA kit experiment (Materials and Methods, Experiment 1, page 14).

4. Data Reduction

Scintillation data cpm were reduced to pg IRG/ml via computer programs using logit-log transformation.[f]

D. Experiment 4 - Modifications of Commercial Glucagon Radioimmunoassay

1. Commercial Glucagon RIA Kit Evaluated

Kit G[i] of Experiment 3 was selected because of two factors: 1) Kit G had higher percent B_0 than Kit H[j] (33% versus 27%). 2) Kit G had a 1 day incubation prior to addition of second antibody where Kit H had a 5 day incubation. One day incubation would be a better clinical assay with faster "turn-around time".

2. Modifications of Glucagon RIA Kit

A technical representative of the RIA kit manufacturer recommended several modifications to increase sensitivity. These recommendations included the following: 1) increase analyte reagent volume; 2) dilute tracer by 50%; 3) dilute first antibody (Ab_1) (sheep anti-porcine glucagon); 4) combinations of 1 through 3.

a. Stage 1 Modifications

1. Reagent Preparations

Normal kit serum was reconstituted with 4.5 ml of kit buffer instead of 10 ml. Tracer was reconstituted with 1.0 ml double distilled water, then 0.5 ml of reconstituted tracer was added to the 4.5 ml of

[i] "Radioimmunoassay of Pancreatic Glucagon" Cambridge Nuclear Radiopharmaceutical Corporation, 575 Middlesex Turnpike, Billerica, MA, 01865

[j] RSL Glucagon Kit, Radioassay Systems Laboratories, Inc., Carson, CA, 90746

reconstituted normal serum. Additional standards (50 pg/ml, 35 pg/ml, and 70 pg/ml) were prepared by 1/200 dilution of 10,000 pg/ml, 1/10 and 1/5 dilution of 350 pg/ml, respectively, using kit buffer. First antibody (Ab_1) was reconstituted according to the manufacturer's guidelines and then diluted 8/10, 7/10, and 6/10 with kit buffer. The remaining assay components were reconstituted according to directions.

2. Assay procedure

Tracer volume was 50 µl instead of 100 µl. All other reagent volumes were as directed by the manufacturer. A set of standards were analyzed with each dilution of Ab_1. All time and handling conditions were constant for each set of standards. Determination of bound cpm was by time required to reach 10,000 counts for each tube.

b. Stage 2 Modifications

1. Reagent preparation

Two vials of kit normal serum were reconstituted. One vial was used to reconstitute tracer, then reconstituted tracer and second vial were combined and mixed. One 100 pg/ml standard was diluted 1/2 and 1/4 to obtain 50 pg/ml and 25 pg/ml standards. First antibody (Ab_1) was reconstituted with 14 ml kit buffer to obtain a 5/7 (71%) dilution of Ab_1. Other reagents were reconstituted as directed by the manufacturer.

2. Assay Procedure

All volumes and procedures were followed as directed by the manufacturer. Determinations of bound cpm were made from time required to reach 10,000 counts in each tube. The first two assays were conducted in duplicate and the third in triplicate.

3. Source of Samples

Four canine control plasmas and one commercial control plasma were used. The fasting control plasma (K-9 Glcn-1) and post-dextrose control

plasma (K-9 Glcn-2) of Experiment 3 were included. Another fasting control plasma was collected from a dog after a 3 day fast and designated as pre-arginine control plasma (Pre-Arg). A post-arginine control plasma (Post-Arg) was collected from this dog after intravenous infusion of 10% L-Arginine solution[k] at a dosage of 1 gm/kg over 20 minutes. Aprotinin-treated plasma was frozen (-20°C) until analysis. Serum was also collected before and after arginine infusion for glucose and IRI quantitation. Commercial control plasma[h] (850 $\pm$ 450 pg/ml) was used in Stage 1 and parts of Stage 2.

4. Criteria for Evaluation of Modified Glucagon RIA Kit

a. Stage 1

Binding parameters of original RIA (Experiment 3) were compared with results obtained after modifications. Displacement of tracer from B_0 was assessed with standards and with canine control plasmas. Control plasma IRG concentrations and duplicate C.V.'s were compared.

b. Stage 2

Reproducibility of standard curves was compared by tracer binding parameters and by linear regression data of logit-log transformation. Duplicate and inter-assay C.V.'s were assessed for the five control plasmas.

[k] L-Arginine-HCl (25 gm) (United States Biomedical Corporation, Cleveland, Ohio) dissolved in 250 ml of 0.9% NaCl(aq), (resulting pH = 5.5)

IV. RESULTS

A. Experiment 1 - Screening Evaluations of Commercial Insulin Radioimmunoassays

1. Specificity

By both methods of assessment (Tables 1 and 2), Kit C had the best dilutional parallelism. Kit A had good parallelism except for 1/5 dilution. Measured concentration of IRI in Kit B was consistently lower than the expected concentration. Kit D had good parallelism at 4/5 and 3/5 dilutions, but greater dilutions resulted in increasing ratios of measured versus expected concentrations. Kit E had good parallelism at 4/5 dilution, but greater dilutions yielded decreasing ratios of measured versus expected concentration.

Table 1. Dilutional parallelism assessment of insulin RIA kits by linear regression

Kit	Correlation Coefficient (r)	Slope (m)	Y-Intercept
A	0.976	1.088	-2.04
B	0.999	1.250	-1.15
C	0.999	1.006	0.61
D	0.978	1.282	-10.43
E	0.990	0.976	4.92

Table 2. Dilutional parallelism assessment of insulin RIA kits by comparison of corrected concentration to undiluted concentration

Kit	Undiluted	4/5	3/5	2/5	1/5	1/10
A	100	95.7	94.8	96.6	145.6	96.0
B	100	82.0	84.4	83.9	90.2	92.4
C	100	98.4	98.3	96.8	90.8	102.0
D	100	96.0	98.0	120.3	148.9	221.2
E	100	98.2	85.7	75.1	70.2	72.5

2. Precision

All RIA kits gave acceptable precision if mean intra-assay C.V. and mean inter-assay C.V. are used as the criteria (Table 3). Kit C consistently had the best precision for all controls. Reproducible quantitation of IRI in K9 Ins Lo was questionable in Kits A, B, and D (intra-assay) and Kits A, B, and E (inter-assay). Intra-assay precision was questionable in Kit D with CS-1 sera. Reproducible quantitation of IRI in CS-2 was questionable in Kit B (intra-assay), in Kit D (intra-assay), and in Kit A (inter-assay). Precision in all RIA kits was considered acceptable in quantitating IRI in K-9 Ins Hi.

3. Sensitivity

Sensitivity limits ranged from 0.8 μU IRI/ml in Kit D to 4.4 μU IRI/ml in Kit A (Table 4). Amount of IRI required to displace tracer by 10% on

Table 3. Intra-assay and inter-assay precision of insulin RIA kits

Kit	Control	IRI (μU/ml) Mean (n=8)	Intra-assay C.V. Mean Each Control n=2	Intra-assay C.V. Mean all Controls n=4	Inter-assay C.V. Each Control n=2	Inter-assay C.V. Mean All Controls n=4
A	K-9 Ins Lo	14	12.8	8.3	17.3	10.2
	CS-1	37	8.2		7.9	
	K-9 Ins Hi	53	8.2		4.1	
	CS-2	115	3.9		11.6	
B	K-9 Ins Lo	17	13.0	10.0	16.5	7.8
	CS-1	51	5.4		2.4	
	K-9 Ins Hi	60	7.1		2.3	
	CS-2	133	14.3		9.9	
C	K-9 Ins Lo	20	5.7	5.3	1.3	3.8
	CS-1	57	4.2		0.2	
	K-9 Ins Hi	71	4.7		5.0	
	CS-2	138	6.5		8.5	
D	K-9 Ins Lo	20	13.4	10.1	2.3	4 6
	CS-1	31	10.2		5.3	
	K-9 Ins Hi	56	5.9		7.3	
	CS-2	95	10.8		3.4	
E	K-9 Ins Lo	17	5.6	7.5	18.9	8.9
	CS-1	44	9.1		6.4	
	K-9 Ins Hi	75	5.6		10.1	
	CS-2	146	9.7		0.1	

Table 4. Sensitivity assessment of insulin radioimmunoassay kits

Kit	Sensitivity Limit (0.0 + 2 S.D.) (μU/ml) (n=4)	10% Decrease in B/F from B_O (μU/ml) (mean) (n=4)	10% Decrease in B/B_O from B_O (μU/ml) (mean) (n=2)
A	4.4	4.8	5.8
B	2.0	3.1	4.1
C	1.0	4.3	8.0
D	0.8	10.6	10.1
E	1.6	1.9	3.3

B/F curve ranged from 1.9 μU IRI/ml in Kit E to 10.6 μU IRI/ml in Kit D (Table 4). Amount of IRI required to displace tracer by 10% on logit B/B_O curve ranged from 3.3 μU IRI/ml in Kit E to 10.1 μU IRI/ml in Kit D. Comparing methods of assessing RIA sensitivity, Kit D had the best calculated sensitivity limit, but required the most IRI to displace tracer 10% in the two partition indices.

4. Comparison of Immunoreactive Insulin Concentrations in Four Control Sera

Variations in IRI quantitated in each control were mild in the low canine control sera but marked in the high commercial control sera (Table 5). Compared to other assays, Kit A quantitated the lowest concentrations in canine sera (K-9 Ins Lo and K-9 Ins Hi). Highest IRI concentrations in canine sera were quantitated in Kit C and Kit D (K-9 Ins Lo) and Kit C and Kit E (K-9 Ins Hi). Lowest IRI concentrations in commercial controls (CS-1, CS-2) were quantitated by Kit D. Kit C quantitated highest IRI concentrations in CS-1 and Kit E quantitated

Table 5. Control sera immunoreactive insulin (IRI) concentrations

Kit	K-9 Ins Lo* µU/ml (N=8) Mean	S.D.	K-9 Ins Hi* µU/ml (n=8) Mean	S.D.	CS-1* µU/ml (n=8) Mean	S.D.	CS-2* µU/ml (n=8) Mean	S.D.
A	14	2.4	53	4.6	37	3.7	115	11.2
B	17	2.8	60	4.3	51	2.9	133	20.8
C	20	1.3	71	4.1	57	2.3	138	13.3
D	20	2.6	56	4.4	31	3.2	95	10.1
E	17	2.5	75	7.0	44	4.4	146	13.2

*Fasting canine sera (K-9 Ins Lo), post-dextrose canine sera (K-9 Ins Hi), low commercial control sera (CS-1), high commercial control sera (CS-2)

highest concentration in CS-2. No consistent pattern was seen in results, but generally Kit A and Kit D gave lower results and Kits C and E gave higher results. Using Tukey's test to compare means, significant differences were found among most RIA kits in each control (Table 6).

Table 6. Comparison of mean immunoreactive insulin (IRI) concentrations by Tukey's test (Gill, 1978)

Control	HSD Interval (µU IRI/ml) (q, 0.05, 4, 30)	Insulin RIA Kit Comparison
K-9 Ins Lo*	2.6	A B E C D
K-9 Ins Hi*	5.5	A D B C E
CS-1*	4.4	D A E B C
CS-2*	17.7	D A B C E

*Fasting canine sera (K-9 Ins Lo), post-dextrose canine sera (K-9 Ins Hi), low commercial control sera (CS-1), high commercial control sera (CS-2)

B. Experiment 2 - Screening Evaluation of Commercial C-peptide Radioimmunoassay

1. Predicted Clinical Correlation

Based on logit-log transformation of scintillation counts, lower concentrations of IRC-P were found in K-9 Ins Hi than K-9 Ins Lo (Table 7). Concentrations of IRC-P in canine sera did not parallel corresponding increase in IRI concentrations. Concentration of IRC-P in human sera approximated reference values in the package insert and paralleled the increase in IRI concentrations (Table 7).

2. Dilutional Parallelism

No parallelism was seen in dilutions of K-9 Ins Hi serum.

3. Displacement of Tracer in Undiluted and Diluted Canine Sera

Tracer displacement in B/F index ranged from 0.8% to 7.9% and in B/B_0 index from 0.5% to 5.2%. These displacements were considered below levels where quantitation can be reliable.

C. Experiment 3 - Screening Evaluation of Commercial Glucagon Radioimmunoassays

1. Specificity

Assessment of dilutional parallelism was not possible as the experiment was designed. Kit G had poor precision in dilutions of K-9 Glcn-1. Duplicate C.V.'s ranged from 32.3 to 126.2.

Kit H had poor precision and inadequate sensitivity to quantitate IRG in dilutions. Counts per minute (cpm) were higher than zero tubes in thirteen of twenty tubes. Duplicate C.V.'s for those with "quantitated" IRG ranged from 11.2 to 81.6.

Table 7. Human C-peptide RIA evaluation

Sample	% Decrease in B/F Index[a] Mean	% Decrease in B/B_O[b] Mean	IRC-P ng/ml Mean	IRI[c] µU/ml
K-9 Ins Lo (n=6)	7.4	5.0	0.44	20
K-9 Ins Hi (n=8)	3.2	2.1	0.22	71
K-9 Ins Hi Dilutions				
80% (n=2)	3.4	2.2	0.20	---
60% (n=2)	0.8	0.5	0.04	---
40% (n=2)	7.9	5.2	0.47	---
20% (n=2)	6.8	4.5	0.40	---
10% (n=2)	3.1	2.0	0.18	---
Human				
-Fasting	26.4	19.0	1.8	13
-30 min.[d]	53.6	42.9	5.4	68
-60 min.[d]	63.7	53.3	8.1	140

[a] % decrease $B/F = (1 - \frac{\text{mean } B/F}{B/F \text{ @ } B_O}) \times 100$

[b] % decrease $B/B_O = \%B_O - \text{mean } \%B/B_O$

[c] Kit C - Experiment 1 (Corning Medical)

[d] Time after 2 g dextrose/kg orally

2. Precision

Neither assay (Kit G nor Kit H) had acceptable precision at IRG concentrations present in controls K-9 Glcn-1 or K-9 Glcn-2 (Table 8). Inter-assay precision of CP Lo and CP Hi were greater than acceptable limits in Kit G.

Table 8. Intra-assay and inter-assay precision of glucagon RIA kits

Kit	Control	IRG (pg/ml) mean	Intra-assay Mean each Control n=2	C.V. Mean Canine Controls n=2	Inter-Assay Mean each Control n=2	C.V. Mean Canine Controls n=2
G	K-9 Glcn-1*	46	60.7	48.5	129.0	84.8
	K-9 Glcn-2*	80	36.3		40.7	
	CP Lo*	969	----		18.2	
	CP Hi*	6383	----		22.2	
H	K-9 Glcn-1*	7	98	55.5	76.0	67.4
	K-9 Glcn-2*	33	13		58.7	
	CP Lo*	67	---		----	
	CP Hi*	81	---		----	

*Fasting canine plasma (K-9 Glcn-1), post-dextrose canine plasma (K-9 Glcn-2), low commercial control plasma (CP-Lo), high commercial control plasma (CP-Hi)

3. Sensitivity

In three methods of assessing sensitivity, Kit H appeared to have better sensitivity than Kit G (Table 9). However, sensitivity limits of each kit approached mean IRG concentrations estimated in K9 Glcn-1 (Table 8). Calculated concentration of IRG required to give 10% displacement of

Table 9. Sensitivity assessment of commercial glucagon RIA kits

Kit	Sensitivity Limit 0 + 2 S.D. (pg/ml) (n=4)	10% Decrease in B/F from B_0 (pg/ml) (mean) (n=4)	10% Decrease in B/B_0 from B_0 (pg/ml) (mean) (n=2)
G	31	73	105
H	6	10	14

tracer in B/F and B/B_0 dose response curves (Table 8) exceeded the IRG concentration estimated in K-9 Glcn-1.

4. Comparison of Immunoreactive Glucagon in Four Control Plasmas

Lack of precison (Table 8) prevented valid comparison of IRG concentrations. Kit G appeared to quantitate higher concentrations than Kit H. This observation was pronounced in IRG concentrations measured in commercial control plasmas (CP Lo and CP Hi).

D. Experiment 4 - Modifications of Commercial Glucagon Radioimmunoassay

1. Stage 1

Binding parameters of modified RIA were compared with original RIA (Table 10). Total counts decreased 31% when 50% tracer was used. Little change was seen in non-specific binding. Percent bound at zero (% B_0) decreased with greater dilutions of first antibody (Ab_1). The % B/B_0 in standards also decreased with Ab_1 dilutions except for an apparent reversal of % B/B_0 at 70 and 100 pg/ml. Greater than 10% displacement (% B/B_0 partition) occurred at 100 pg/ml in 70% Ab_1 assay and 50 pg/ml in

Table 10. Binding parameters of original and modified glucagon RIA

	Original Assay (mean) (n=4)	Modified Assay - 50% Tracer & 80% Ab_1 (mean) (n=2)	70% Ab_1 (mean) (n=2)	60% Ab_1 (mean) (n=2)
Total counts (cpm)	8594	5970	5970	5970
$\%B_{NSB}$[a]	2.6	2.7	2.6	3.2
$\%B_0$	33.1	30.9	28.3	26.6
$\%B/B_0$ @ 35 pg/ml	----	99.1	96.0	91.4
$\%B/B_0$ @ 50 pg/ml	----	96.6[c]	95.0	88.9
$\%B/B_0$ @ 70 pg/ml	----	87.6	93.9	84.6
$\%B/B_0$ @ 100 pg/ml	90.4	95.2[c]	86.7[c]	85.3[c]
$\%B/B_0$ @ 350 pg/ml	74.5	67.9	66.4	62.9
$\%B/B_0$ @ 1000 pg/ml	51.7	46.3	42.0	41.4
$\%B/B_0$ @ 3000 pg/ml	39.7	31.4	31.3	29.9
$\%B/B_0$ @ K-9 Glcn-1	95.6	82.0	85.4	78.7
$\%B/B_0$ @ K-9 Glcn-2	91.5	76.1	80.9	76.5
$\%B/B_0$ @ CP-Lo	58.6	54.8[c]	53.5	51.3
$\%B/B_0$ @ CP-Hi	27.4	26.0[c]	20.6	22.0
Y-Intercept[b]	5.84	6.55	6.50	5.17
Regression Coefficient[b]	-0.800	-0.939	-0.951	-0.785
Correlation Coefficient[b]	-0.994	-0.945	-0.986	-0.992

[a] - percent bound due to non-specific binding
[b] - from linear regression of logit vs log transformation
[c] - single determinations

60% Ab_1 assay. Erroneous binding at 70 and 100 pg/ml standards in 80% Ab_1 assay prevented assessment of sensitivity. Both canine control plasmas (K-9 Glcn-1, K-9 Glcn-2) displaced tracer greater than 10% at all Ab_1 dilutions, where neither had in the original assay. The Y-intercept and regression coefficients of original assay were between 70% Ab_1 and 60% Ab_1 standard curves. Best correlation coefficient was seen in 60% Ab_1 standard curve. Mean concentration of IRG in K9 Glcn-1 were 3, 3.2, and 4.7 times higher (60%, 70%, and 80% Ab_1 respectively) than IRG estimated in Experiment 3 (Table 11). A similar trend was seen in K-9 Glcn-2 with IRG concentration of 2, 2.5, and 3.9 times higher (60%, 70%, and 80%, respectively) than IRG estimated in Experiment 3. Lower IRG

Table 11. Immunoreactive glucagon quantitated in original and modified glugagon RIA

	K-9 Glcn-1[a] (pg/ml)[b]	K-9 Glcn-2[a] (pg/ml)[b]	CP-Lo[a] (pg/ml)[c]	CP-Hi[a] (pg/ml)[c]
Original Assay	46(26.1)[d]	80 (36.4)[d]	969 (4.7)[d]	6383 (5.4)[d]
Modified Assays				
80% Ab_1	213 (3.3)	311 (4.7)	869 (---)	3244 (---)
70% Ab_1	147 (25.0)	205 (25.3)	804 (3.1)	3859 (0.4)
60% Ab_1	137 (5.8)	160 (10.4)	679 (17.2)	3672 (10.4)

[a] - fasting canine plasma (K-9 Glcn-1), post-dextrose canine plasma (K-9 Glcn-2), low commercial control plasma (CP-Lo), high commercial control plasma (CP-Hi)
[b] - mean, n=8, from Experiment #3
[c] - mean, n=4, from Experiment #3
[d] - numbers in paranthesis represents duplicate C.V.

concentrations were quantitated in both low commercial control (CP-Lo) and high commercial control (CP-Hi). Only half of the duplicate C.V.'s were less than 10.0 in both original RIA and modified assays. Single determinations of CP-Lo and CP-Hi were made in 80% Ab_1 assay because of insufficient control plasma volume.

2. Stage 2

Best correlation coefficent in replicated assays was -0.980 (Table 12). Within this limit of poor correlation, displacement of tracer in standard curves appeared to be reproducible. Percent bound at zero concentration (% B_0) ranged from 33.3% to 40.1%. At 25 pg/ml standard, bound tracer was higher than at zero concentration (102.1%). "Analyte" in zero tubes was kit buffer. Nine of fourteen duplicates had C.V.'s of less than 10.0 (Table 13). Only one of five control plasmas had an inter-assay C.V. of less than 10 (K-9 Glcn-1). Two controls had greater than 20 inter-assay C.V. Concentration of IRG estimated in a fasting dog (Pre-Arg) and post-arginine infusion (Post-Arg) appeared to be approximately the same.

Table 12. Reproducibility of standard curves in modified glucagon RIA (50% Tracer & 71% Ab_1)

	Run 1	Run 2	Run 3	Mean	S.D.
Total Counts (cpm)	5071	5374	5024	5156	190
$\%B_{NSB}$[a]	5.1	4.0	6.2	5.1	1.1
$\%B_0$	40.1	34.4	33.3	35.9	3.7
$\%B/B_0$ @ 25 pg/ml	101.4	102.7	----	102.1	---
$\%B/B_0$ @ 50 pg/ml	93.2	92.5	91.7	92.5	0.8
$\%B/B_0$ @ 100 pg/ml	84.8	83.4	84.1	84.1	0.7
$\%B/B_0$ @ 350 pg/ml	63.7	69.8	69.7	67.7	3.5
$\%B/B_0$ @ 1000 pg/ml	39.6	41.2	36.6	39.1	2.3
Y-Intercept[b]	6.42	6.02	6.13	6.19	0.2
Regression Coef.[b]	-0.996	-0.912	-0.945	-0.951	0.042
Correlation Coef.[b]	-0.974	-0.980	-0.970	-0.975	0.005

[a] - percent bound due to non-specific binding

[b] - from linear regression of logit vs. log transformation

Table 13. Immunoreactive glucagon quantitated in modified glucagon RIA (50% Tracer & 71% Ab_1)

Sample[a]	Run 1 (pg/ml)	Run 2 (pg/ml)	Run 3 (pg/ml)	Mean (pg/ml)	Inter-assay C.V.
K-9 Glcn-1	180 (1.7)[b]	125 (0.2)[b]	185 (19.3)[c]	163	20.4
K-9 Glcn-2	270 (0.2)	242 (17.4)	274 (14.1)	262	6.7
Pre-Arg	156 (2.2)	136 (8.7)	187 (11.1)	160	16.1
Post-Arg	193 (5.8)	148 (12.3)	194 (9.1)	178	14.7
CP-Lo	1056 (4.3)	584 (3.3)	---	820	40.7

[a] - fasing canine plasma (K-9 Glcn-1), post-dextrose canine plasma (K-9 Glcn-2), fasting canine plasma before arginine infusion (Pre-Arg), canine plasma after arginine infusion (Post-Arg), low commercial control plasma (CP-Lo).

[b] - number in paranthesis represents duplicate C.V.

[c] - number in paranthesis represents triplicate C.V.

V. DISCUSSION

The purpose of clinical chemistry procedures is to identify normal and abnormal values that can be used to establish presence or absence of disease. If abnormal, data may help define type, severity, and location of lesion. However, accurate clinical chemistry interpretation is dependent on the reliability of the procedure used to quantitate substances of interest. Radioimmunoassays cannot be assumed to be valid for a particular species. One must evaluate assays and establish that they can quantitate analytes reliably before medical decisions are made.

A. Experiment 1 - Screening Evaluation of Commercial Insulin Radioimmunoassays

Kit C had the best dilutional parallelism (specificity), best precision (reproducibility), and good sensitivity. These findings suggested that Kit C would be the best among the RIA kits tested for further evaluation, for establishment of reference canine IRI concentrations, and for clinical and research investigations of diseases related to insulin. Precision and sensitivity data obtained in experiment were similar to performance characteristics listed in package insert (Table A2). Accuracy of IRI quantitation was not assessed because canine IRI standards were not available.

Kit A had good dilutional parallelism except for one duplicate at 1/5 dilution (189% of expected concentration). Assuming procedural error, dilutional parallelism was similar to Kit C. Precision assessment did

not meet precision limits set for screening evaluations. Intra-assay and inter-assay C.V.'s were above 10 at 14 μU/ml. Inter-assay C.V. at 115 μU/ml was also unacceptable. This experiment's precision data was equal to or better than performance characteristics stated in package insert (Table A2). Sensitivity assessment was considered adequate.

Kit B quantitated consistently lower concentrations in dilutional parallelism evaluation (82-92.4% of expected). Dilutions were made with kit buffer consisting of a phosphate buffered saline (PBS) with bovine serum albumin. This buffer may have altered binding properties of antibody (affinity) or altered canine IRI (antigenicity). Precision was not acceptable at 17 μU/ml and at 133 μU/ml. Variability at low concentrations exceeded manufacturer's stated performance characteristics (Table A2). Sensitivity was adequate. Lack of dilutional parallelism must be considered as a major deficiency and thus specificity of Kit B must be considered questionable. Evaluation of parallelism with insulin-deficient canine serum would be necessary to further evaluate specificity.

Kit D had poor dilutional parallelism at 2/5, 1/5, and 1/10 dilutions of K-9 Ins Hi. Diluent was kit buffer consisting of PBS with protein. Sensitivity limit (0.8 μU/ml) suggested adequate sensitivity to quantitate IRI in respective dilutions (22.4, 11.2, 5.6 μU/ml). Partition index assessment of sensitivity (B/F = 10.6 μU/ml, B/B_0 = 10.1 μU/ml) indicated that at 1/5 and 1/10 dilutions, quantitation problems may have been due to sensitivity. Individual determinations at 2/5 dilutions were 107.2% and 133.4% of expected. Assuming procedural error in second determination, it may be concluded that Kit D has dilutional parallelism within its range of sensitivity. Intra-assay precision was questionable

in three or four controls; however inter-assay precision was good. Assessment of intra-assay precision in this experiment exceeded performance characteristics stated in package insert (Table A2). However, inter-assay precision was better. In this experiment, Kit D had relatively poor performance and could not be recommended for further evaluation.

Kit E quantitated progressively lower IRI concentrations with increasing dilutions. Diluent was kit buffer consisting of PBS with guinea pig serum. Based on this observation, Kit E specificity must be questioned even though its package insert stated 100% cross-reactivity with canine insulin. Intra-assay precision was good, but inter-assay precision at low concentrations (17 μU/ml) was unacceptable. Sensitivity was adequate. Performance characteristics were not stated in Kit E package inserts. Kit E could not be recommended for quantitation of canine IRI unless other assessment methods demonstrated specificity.

According to statistical comparison of IRI concentrations in four control sera, evaluated radioimmunoassays did not quantitate equal IRI concentrations at all four levels. This finding dictates that reference values for clinical and research investigations must be established for each RIA utilized. Utilization of published reference values obtained with another RIA must not be encouraged. Inter-laboratory comparison of RIA's would be valuable to establish inter-laboratory reproducibility.

Even though statistically different, IRI concentrations in low control (14-20 μU/ml) may not be clinically significant. Differences were more apparent at higher concentrations (95-146 μU/ml) and clinically could be significant. Reference values involving elevated IRI concentration should be established for each investigator's RIA. These

reference values may include IRI levels during glucose tolerance tests (intravenous or oral), glucagon tolerance tests, and expected concentrations found in functional beta cell neoplasms (insulinomas). Indices used to interpret pancreatic release of insulin (insulin-glucose ratios, insulinogenic index) would also require reference values (reference values for Kit C are summarized in Appendix B). If IRI quantitation is offered by a reference laboratory, information supporting storage and handling of samples should be provided (preliminary studies on stability of canine IRI are found in Appendix C.)

Reasons for differences in mean IRI concentrations quantitated among kits may be multiple. These reasons may include different anti-insulin antibodies, efficiency in separation techniques, and variations in calibration of standards. Package insert in Kit C stated that its standards were calibrated with a World Health Organization immunoreactive reference standard. Supplier of Kit B reported[1] that its standards were also calibrated against the same WHO immunoreactive standard. Other manufacturers did not supply calibration information. Differences in RIA kit standards could be evaluated by assaying standards as unknowns in other RIA's.

B. Experiment 2 - Screening Evaluation of Commerical C-peptide Radioimmunoassays

No evidence was found to indicate that Kit F antibody (Ab_1) had cross-immunoreactivity with canine C-peptide. Small displacement of tracer in canine controls and in dilutions of canine sera could have two explanations. First, this RIA may not be designed to quantitate very low levels of canine C-peptide, i.e., below sensitivity of assay.

[1](Personal Communication)

Sensitivity limit calculated from one assay was 0.28 ng/ml. Sensitivity stated in package insert was 0.8 ng/ml (90% B/B_0). As reports of circulating peripheral canine C-peptide were not found, this possibility cannot be ruled out.

The second and more likely explanation for the minimal tracer displacement is lack of cross-immunoreactivity. Binding of tracer to first antibody (anti-C-peptide) was less in canine sera than in zero concentration standards. This may be due to greater non-specific binding rather than true competition for antibody binding sites. This explanation is consistent with reports stating requirements of species specific antisera (Chance, 1972; Kaneko et al., 1974; Kemmler et al., 1972; Rubenstein and Steiner, 1971). Dr. Altszuler[m] has obtained synthetic canine C-peptide from Japan but has not attempted to develop a canine C-peptide assay.

C. Experiment 3 - Screening Evaluation of Commercial Glucagon Radioimmunoassays

As the experiment was designed, it is difficult to reliably evaluate these RIA's. Neither Kit G or Kit H had reproducible quantitation of IRG present in canine controls (K-9 Glcn-1, K-9 Glcn-2). This lack of precision may have been due to inadequate sensitivity. Kit G precision in commercial controls (CP-Lo and CP-Hi) was inadequate. Concentrations of IRG in four controls appeared to differ markedly. Kit G quantitated 14 times more IRG in CP-Lo and 79 times more IRG in CP-Hi than did Kit H. This degree of variation may be due to differences in standards,

[m] (Personal Communication) Dr. Norman Altxzuler, Department of Pharmacology, School of Medicine, 550 First Ave., New York, N.Y. 10016.

differences in calibration of standards, or procedural inaccuracies. To my knowledge, a reference immunoreactive standard is not available for calibrating standards and thus variations in IRG quantities among RIA's must be expected. Based on this experiment, neither Kit G nor H could be recommended for clinical or research IRG quantitation.

D. Experiment 4 - Modifications of Commercial Glucagon Radioimmunoassay

This experiment was performed in two stages. Stage 1 involved three simultaneous assays with diluted tracer and different dilutions of first antibody (Ab_1). Stage 2 involved three replicated assays with diluted tracer and diluted first antibody.

First stage modifications appeared to increase the assay's sensitivity by increasing initial slope of dose-response curve. With greater first antibody dilutions, the initial decrease in tracer binding became larger. This finding would be an improvement over the apparent inadequate sensitivity found in Experiment 3.

Apparent reversal of expected binding in 80% Ab_1 at 50 pg/ml and 70 pg/ml cannot be explained to author's satisfaction. Technician error was not a primary factor as counting data and assay set-up were thoroughly checked. Problems may have been related to lack of precision at low dose range.

Another problem was an apparent increase in IRG in canine controls and a decrease in commercial controls as compared to original assay quantitations (Experiment 3). Dilutions of tracer and first antibody should allow analytes greater opportunity to bind to antibody and thus displace tracer. However, the effect should be equal for both standard IRG and unknown IRG. Canine controls had been frozen (-25°C) for approximately 3 months between Experiment 3 and 4. Thus, dehydration may have

concentrated plasma components. If storage conditions do not explain observed differences, this assay's specificity for canine IRG must be questioned.

For a RIA to reliably quantitate, standards and unknown IRG competition for antibody binding sites must be constant. Observed charges in the modified assay would suggest that antibody-standard analyte interactions were altered differently than antibody - unknown analyte interaction.

Modifications may have decreased antibody affinity for analytes in standards and thus canine IRG concentrations appeared higher. On the other hand, modifications may have increased antibody affinity for canine IRG and thus appeared to displace more tracer than in the original assays. Apparent decreasing concentrations of canine IRG with greater dilutions of the first antibody also suggest altered kinetics in analyte-antibody interactions.

A major problem in the original assays was lack of precision. Duplicate C.V.'s for canine controls were better in 80% Ab_1 and 60% Ab_1 assays. However, mean duplicate C.V.'s for standards were 1.43, 4.59, 2.13, and 4.59 in original, 80% Ab_1, 70% Ab_1, and 60% Ab_1 assays, respectively. This lack of duplicate reproducibility in standards made construction and interpretation of standard curves difficult.

In the second stage of glucagon RIA modification, precision remained a major problem. Duplicate (or triplicate) C.V.'s for canine controls ranged from 0.2 to 19.3 with only seven of twelve C.V.'s less than 10.0. Only one of four canine controls had inter-assay C.V. of less than 10.0. Mean C.V. for standards in replicated assays with 71% Ab_1 were 4.5, 3.6, and 3.2. This degree of variation in standards made construction of

standard curves difficult. This problem was demonstrated by relatively poor correlation coefficients. Removal of standards that were out-liers from best-fit curve could improve linearity, but should not be encouraged.

This consistent lack of precision in original and modified RIA's suggests that reliable quantitations of canine IRG would be difficult. If there are a large differences in IRG concentrations between normal dogs and those dogs with expected elevated IRG concentrations, perhaps this commerical glucagon RIA could be used as a semi-quantitative assay and have clinical applications. Another problem arises from the lack of precision, i.e., evaluation of dilutional parallelism. Results from this study indicate that a markedly elevated canine IRG pool would be needed before dilutional parallelism could be re-evaluated. Another problem identified in the second stage (71% Ab_1 modification) was an apparent greater tracer binding at 25 pg/ml than at B_0. This "hook effect" has been described by Cresto and Yalow (1974), who suggested that excess antibody gives antigen-antibody complexes at low analyte concentrations.

An attempt to produce a high canine IRG control plasma by arginine infusion was unsuccessful. Post-arginine infusion concentration of IRG was approximately 18 pg/ml higher than the fasting level.

Clinical investigations of glucagon's role in a variety of canine disorders is needed. This commercial glucagon radioimmunoassay or modifications cannot be recommended at this time for clinical investigations. Results of this study dictate that additional modifications or investigations may be necessary or that another glucagon RIA should be evaluated. If additional evaluation of this RIA is desired, several factors must be considered. Due to lack of precision, triplicates will probably be

required to give best semi-quantitation. This will necessitate increased expense in canine IRG quantitation and increased sample volume. Another approach to assay modification may be to increase analyte volume. This would dictate purchasing increased volumes of standards and require larger sample volumes. Abnormal (elevated) concentrations of canine IRG should be demonstrated to be at least 100% higher than normal concentrations. A control plasma with IRG concentrations at least 300% higher than normal concentrations will probably be needed for assessment of dilutional parallelism. Based on results of one arginine infusion, another method of stimulating glucagon production and release will be needed. It is recommended that a reference laboratory be used to quantitate canine IRG concentration in clinical cases and future control plasma before any further investigations are considered.

VI. SUMMARY AND CONCLUSION

Commercial radioimmunoassay (RIA) kits developed for quantitation of human immunoreactive insulin (IRI), immunoreactive C-peptide (IRC-P), and immunoreactive glucagon (IRG) were evaluated for their ability to quantitate corresponding canine immunoreactive hormones.

Evaluation criteria of five insulin RIA kits included precision, dilutional parallelism, sensitivity, and comparison of IRI concentrations in control sera. One RIA kit (Corning Medical) had good dilutional parallelism, consistently good precision, and adequate sensitivity. Other RIA kits evaluated had poorer performance in dilutional parallelism or precision. No two RIA kits quantitated the same IRI concentrations in all four control sera. Reference values for fasting canine IRI, canine IRI response to High Dose Intravenous Glucose Tolerance Test (HD-IVGTT), and stability of canine IRI were determined with Corning's insulin RIA kit.

Canine sera caused minor tracer displacement from anti-C-peptide antibody in the human C-peptide RIA kit (Calbiochem-Behring Corporation). This finding would be consistent with a lack of species cross-immunoreactivity.

Two commercial glucagon RIA kits had poor precision in quantitating low concentrations of canine IRG. Modifications of one RIA kit (Cambridge Nuclear Radiopharmaceutical Corporation) appeared to increase sensitivity, but precision remained inadequate. Neither glucagon RIA kit can be recommended for quantitation of canine IRG until additional evaluations are conducted.

Additional evaluation or utilization of commercial RIA kits are needed. Reference values for IRI during glucagon tolerance tests are needed. A RIA for canine C-peptide would be an asset in evaluation of canine disorders involving insulin. Immunoreactive glucagon determinations will be required before glucagon's role in a variety of clinical disorders in dogs is understood.

APPENDICES

APPENDICES

Appendix A - Component, Performance Characteristics, and Binding Parameters

The components and lot numbers of commercial radioimmunoassays evaluated in Experiments 1, 2, and 3 are listed in Table A1. Performance characteristics stated in insulin RIA kit package inserts are listed in Table A2. Binding parameters of commercial radioimmunoassays in Experiments 1, 2, and 3 are listed in Table A3.

Table A1. Components and lot numbers of evaluated commercial radioimmunoassays

RIA Kit	Tracer	Primary Antibody Immunogen; Antibody	Standards
A - Species Source	bovine	bovine, guinea pig porcine	human
Lot #	228	228	228
B - Species Source	porcine	porcine; guinea pig	human
Lot #	I/117#3	IA/45, SCO1 B	1K519#7
C - Species Source	porcine	porcine; guinea pig	porcine
Lot #	20040	03109	01639A
D - Species Source	porcine	porcine; guinea pig	porcine
Lot #	2505	1266 U	2919 U
E - Species Source	porcine	porcine; guinea pig	porcine
Lot #	46	45	43
F - Species Source	syn. human	syn. human; rabbit	syn. human
Lot #	02A010	025005	X-92X096
G - Species Source	porcine	porcine; rabbit	porcine
Lot #	1209512L	20AFLA4	20AGLC1
H - Species Source	porcine	porcine; rabbit	porcine
Lot #	102L	95	104

Table A2. Performance characteristics stated in insulin RIA kit package inserts

	Specificity		Precision (C.V.)*			Sensitivity
	Substance - Cross reactivity		@ µU/ml	@ µU/ml	@ µU/ml	
Kit A	----	----	@ 32	@ 60	@ 140	---
		intra-assay	6.5	10.5	12.0	
		inter-assay	≤ 20.0	≤ 20.0	≤ 20.0	
Kit B	insulin (human)	100%	@ 9.4	@ 26.1	@ 73.9	2.5 µU/ml (a)
	insulin (bovine)	100%	intra-assay 8.1	5.0	6.6	
	insulin (procine)	100%	inter-assay 10.5	9.1	9.8	
	insulin (rat)	90%				
	proinsulin	7.0%				
	glucagon	0.2%				
	C-Peptide	< 0.01%				
Kit C	insulin	100%	@31.0	@139.0	---	1.3 µU/ml (b)
	proinsulin	3.9%	intra-assay 4.6	5.2		
	glucagon	0%	inter-assay 8.4	6.9		
	human growth hormone	0%				
Kit D	proinsulin	< 28%	@16.9	@ 48.8	@ 79.8	< 2.3 µU/ml (c)
			intra-assay 11.0	6.6	4.9	
Kit E	insulin (human)	100%	----	----	----	---
	insulin (dog)	100%				
	insulin (porcine)	100%				
	proinsulin	19%				
	glucagon	0.09%				
	C-Peptide	0.003%				

(a) amount of insulin that causes 5% decrease of initial binding
(b) amount of insulin reflected by 2 standard deviations form 0.0 µU/ml
(c) not defined
(*) C.V. reported is the mean C.V. for each control

Table A3. RIA kit binding parameters in replicated assays[a]

Kit	Total Counts (cpm) Mean (n=4)	$\%B_O$ Mean (n=2)	$\%B_{NSB}$ Mean (n=2)	Range of Standards[b]	% B/B_O Range of Standards (mean)
A	26,665	29.5	3.2	10-160	83.3-20.7
B	11,611	41.9	6.0	9-200	81.5-19.3
C	9,681	52.5	---	6.25-400	92.3-25.5
D	15,634	11.0	---	5-320	96.0-37.8
E	9,161	36.8	5.9	2.5-200	91.6-17.3
F	8,769	34.3	1.8	1-50	88.2-11.2
G	8,594	33.1	3.7	100-10,000	90.4-18.0
H	12,904	26.7	6.2	10-1,000	92.7-5.5

[a] except Kit F - single assay

(b) Kits A,B,C,D,&E - μU IRI/ml
Kit F - ng IRC-P/ml
Kits G & H - pg IRG/ml

Appendix B - Immunoreactive Insulin Reference Values: Fasting and High Dose Intravenous Glucose Tolerance Test (HD-IVGTT)

1. Insulin Radioimmunoassay Kit Utilized

"Immophase"™, Insulin ^{125}I Radioimmunoassay Test System (Corning Medical) was used for IRI determinations.

Insulin RIA quality control was monitored during HD-IVGTT sample analysis by total counts, $\%B_0$, linear regression values of logit-log transformation of standard curve, and duplicate, intra-assay, and inter-assay coefficients of variation. Commercial control sera* were assayed in duplicate before and after unknown samples. Canine control sera (K-9 Ins Lo, K-9 Ins Hi) were assayed in duplicate before, after, and between each set of HD-IVGTT samples.

2. Reference Canine Population

Twenty clinically healthy dogs, 6 males, 12 females, 2 spayed females ranging from 8 months to 4 years of age, and 10.3 to 36.0 kilogram body weight were used. Breeds represented in this population included Golden Retriever (4), Black Labrador (8), Retriever-Beagle cross (4), Springer Spaniel (2), and mixed breed (2). Fifteen dogs were housed in a research kennel, five dogs were privately owned pets.

Reference population qualifications included fasting hematologic and selected serum chemistry values within normal reference ranges for adult dogs established by the Clinical Pathology Laboratory, Veterinary Clinical Center, Michigan State University. Reference population hematologic and chemistry values listed in Table B1. No dog had histories of

*Corning Medical, Corning Glass Works, Medfield, MA, 02052

disorders that would have potential for affecting carbohydrate tolerance, i.e., pancreatic, adrenal, or hepatic disease.

3. High-Dose IV-GTT Procedure and Sample Handling

Dogs were fasted overnight (15-18 hours). An intravenous Teflon® catheter* was inserted and secured in the right cephalic vein. Pre-IV-GTT, 5, 10, 15, 30, and 60 minute samples were collected via catheter. Ninety and 120 minute samples were collected from either right or left jugular veins via venipuncture. Heparinized saline (1000 USP Na Heparin in 250 ml 0.9% saline) was used for catheter flush both before and after catheter-obtained samples. Approximately one ml of blood was discarded prior to catheter collection of samples. Catheter collected samples were collected within one minute of schedule time. Ninety and 120 minute samples were collected within 1-5 minutes of scheduled time.

After collection of pre-IV-GTT samples, 50% dextrose was administered at a dosage of 1 gm/kg body weight (30-60 secs) in the left cephalic vein. Timing of subsequent samples began at the end of dextrose administration. Blood samples for glucose and IRI determination were allowed to clot for 45-75 minutes and centrifuged at 2760 RPM (1000 g) for 10-15 minutes. Harvested sera was divided into two parts, 300 µl for serum glucose and the remainder (greater than 1 ml) for IRI. Serum for glucose determination was assayed the day of collection or stored overnight (4°C). Serum for IRI assays was frozen (-25°C) in appropriately labelled plastic vials until the day of analysis.

*I.V. Cath, #6768 or #6769; Becton-Dickinson and Company, Rutherford, N.J. 07070

4. Results

Results are summarized in Tables B1, B2, and B3. Quality control data are summarized in Table B4. Dog 13 was removed from reference values because of marked IRI response during HD-IVGTT. Dog 13's IRI concentrations (from 0 to 120 min) were 32, 137, 168, 208, 104, 43, 31, and 36 μU/ml. Values from 10 minutes to 120 minutes were greater than two standard deviations above respective means (n=20). Glucose disappearance constant was 3.04, well within reference range.

5. Calculations

1. Insulin-Glucose Ratio (IRI/G) (Atkins et al., 1979)

$$\frac{IRI}{G} = \frac{[IRI]}{[glucose]} \times 100$$

2. Insulinogenic Index ($\Delta IRI/\Delta G$) (Kaneko et al., 1977)

$$\frac{\Delta IRI}{\Delta G} = \frac{[IRI]\ (5\ min.) - [IRI]\ (0\ min.)}{[Glucose]\ (5\ min.) - [Glucose]\ (0\ min.)} \times 100$$

3. Glucose Disappearance Constants (k) (%/min.)

(a) $k = \frac{\ln [Glucose]\ 15\ min. - \ln [Glucose]\ 45\ min.}{45\ min. - 15\ min.}$

(Chen et al., 1977)

(b) k = -230 b, where b equals slope of linear regression of glucose concentration at 15, 30, and 45 min. (Dyck and Moorhouse, 1966; Moorhouse et al., 1964)

(c) k = -69.3/t ½, where t½ equals time interval for glucose concentration to decrease from 300 to 150 mg/dl on a line drawn between 15 min and 45 min on a log [glucose] versus time graphic plot. (Greve et al., 1973)

Table B1. Reference population laboratory data summary[a]

	$\bar{X}$	S.D.	Range
HEMATOLOGIC			
Total Protein (g/dl)[b]	6.4	0.5	5.2-7.1
PCV (%)	45	4	36-50
Hemoglobin (g/dl)	16	1	13-18
RBC (x 10^6/µl)	6.68	0.6	5.63-7.86
MCV (fl)	67	2	62-71
MCHC (g/dl)	35.7	0.6	34.8-36.9
Total White Blood Cell Count(/µl)	10,470	3,257	4,600-19,400
Segmented Neutrophils (/µl)	7,332	2,892	3,128-13,968
Non-segmented Neutrophils (/µl)	15	38	0-134
Lymphocytes (/µl)	2,149	652	1,157-3,290
Monocytes (/µl)	353	248	0-837
Eosinophils (/µl)	615	605	0-2,328
Basophils (/µl)	6	25	0-111
Platelets (subjective)	-	-	Adequate
CHEMISTRIES			
BUN (mg/dl)	15	4	9-27
Glucose (mg/dl)	89	6	79-101
Total Protein (g/dl)[b]	6.0	0.4	5.1-6.9
Albumin (g/dl)	3.6	0.3	3.2-4.1
Globulin (g/dl)	2.4	0.4	1.8-3.4
Sodium (mEq/L)	146	3	140-150
Potassium (mEq/L)	4.5	0.3	4.2-5.1
Alanine Aminotransferase (IU/L)	24	9	14-45
Alkaline Phosphatase (IU/L)[b]	39	21	10-95
Cortisol (ng/ml)	40	21	12-90

[a] All determinations by Clinical Pathology Laboratory, Veterinary Clinical Center, Michigan State University: (J.D. Krehbiel, D.V.M., Ph.D., Diplomate ACVP, Director) except cortisol (Endocrinology Laboratory, AHDL, Michigan State University (R.F. Nachreiner, D.V.M., Ph.D., Director)

(b) Two dogs were immature (8 month Golden Retriever, 13 month Great Dane) and had lower total proteins and higher alkaline phosphatases than remainder of population.

Table B2. HD-IVGTT reference values

Assessment Method		Time (Minutes)								
		0	5	10	15	30	45	60	90	120
Glucose	Mean	89	388	337	281	160	99	85	78	87
(mg/dl)	S.D.	6	36	36	40	38	24	13	7	9
(n=19)	lowest	79	356	294	225	95	63	54	63	65
	highest	101	472	426	383	220	155	114	89	102
IRI	Mean	24	80	79	74	53	23	21	19	20
μU/ml	S.D.	9	28	30	30	23	6	4	4	4
(n=19)	lowest	13	42	34	26	20	16	15	12	12
	highest	43	132	132	119	102	38	31	25	28
IRI / glucose	Mean	27	21	24	27	33	24	25	24	23
	S.D.	11	8	10	13	12	7	6	5	6
(n=19)	lowest	13	10	9	9	13	14	17	16	13
	highest	49	36	42	53	51	38	37	39	34

Table B3. HD-IVGTT reference values

Assessment Method	Mean	S.D.	Lowest	Highest
Insulinogenic Index ($\Delta IRI/\Delta G$)	19.2	8.0	7.6	33.0
Glucose Disappearance Constants (%/min)				
$k = \frac{\ln C_{15} - \ln C_{45}}{45 - 15}$	3.54	0.76	2.27	5.14
$k = -230\ b$	3.53	0.76	2.27	5.13
$k = -69.3/t\frac{1}{2}$	3.57	0.79	2.31	4.95
Glucose $t\frac{1}{2}$ (graphic) (min)	20.3	4.5	14	30

Table B4. Quality control data; IRI quantiation for HD-IVGTT reference values

	Standard Curve			
	Mean n=8	S.D.	Inter-assay C.V.	Range
Total Counts (cpm)	9260	799	8.6	7994-10,467
$\%B_0$	54.5	1.4	2.5	52.3-56.5
Y-Intercept	3.80	0.08	2.2	3.65-3.89
Regression Coefficient	-0.883	0.017	1.9	-0.903 - -0.858
Correlation Coefficient	-0.998	0.001	0.1	-0.998 - -0.996
r^2	0.996	0.001	0.1	0.992 - 0.996

		Precision				
Control	Mean μU/ml	Duplicate C.V.		Intra-Assay C.V.		Inter-Assay C.V.
		mean (n=310)	range	mean (n=8)	range	mean (n=8)
All Samples	---	4.0	0.1-23.2	--	--	----
K-9 Ins Lo (n=64)	23			6.3	4.7-7.7	4.1
K-9 Ins Hi (n=64)	69			3.8	2.2-5.5	3.4
CS-1* (n=64)	36			5.0	3.1-7.1	3.8
CS-2* (n=64)	132			4.2	0.9-6.7	4.2

*Commercial Control Sera, Corning Medical

Appendix C - Stability of Canine Immunoreactive Insulin

To assess the stability of IRI in different environmental temperatures, aliquots of two canine sera and one canine plasma were placed in four different temperatures for times ranging one day to ten days. Aliquots of canine sera were also thawed and refrozen from one to five times. Results are summarized in Table C1.

Immunoreactive insulin appears to be stable up to ten days at 4°C and 12°C. The IRI at low concentration (serum-1) appears to be stable at 24°C. However, high concentrations of IRI (serum-2, plasma) appeared to deteriorate after one day at 24°C. Immunoreactive insulin at 37°C was not stable.

No changes in IRI concentrations were found after thawing and refreezing serum up to 5 times.

Table C1. Stability of canine immunoreactive insulin (time and temperature)

Sample	IRI on Day of Analysis* (μU/ml)	Storage Temperature (°C)	Days Stored at Given Temperature (μU IRI/ml) 1	2	3	5	10
serum-1	22	4	22	21	22	21	22
serum-2	66	4	65	65	67	65	64
plasma	95	4	93	96	93	95	91
serum-1	22	12	23	22	22	22	24
serum-2	68	12	68	68	65	64	62
plasma	89	12	93	90	86	90	89
serum-1	22	24	23	20	21	18	20
serum-2	66	24	62	60	57	51	39
plasma	95	24	92	89	87	79	79
serum-1	22	37	18	16	14	12	10
serum-2	69	37	45	35	31	25	16
plasma	95	37	70	54	45	35	26
			Times Thawed and Refrozen before Analysis 1	2	3	4	5
serum-2	66	-25 to 24 and return	62	62	63	64	64

* - samples thawed day of analysis of storage or handling samples

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