Validation of an automated enzyme immunoassay for the measurement of
serum total thyroxine in cats

Tim L. Williams MA VetMB PhD a#
Joy Archer VMD PhD FRCPath DECVP (Hon)FRCVS a

a University of Cambridge, Department of Veterinary Medicine, Madingley Road,
Cambridge, CB3 0ES.

# Corresponding author

Corresponding author email: timwilliams@cantab.net

Short title: Feline total T4 enzyme immunoassay validation
Abstract

Background: Hyperthyroidism is common in older cats, which necessitates frequent screening of serum total thyroxine (TT4) concentrations. Fast, cheap, and reliable ways to measure TT4 in cats are needed.

Objectives: Validation of a human TT4 enzyme immunoassay (EIA) for use with feline serum and derivation of a TT4 reference interval (RI) for cats aged 9 years and older.

Methods: Assay precision, reproducibility, and linearity were evaluated. Interference by hemolysis was also assessed. Method comparison studies between the EIA and previously validated radioimmunoassay (RIA) and chemiluminescent enzyme immunoassay (CEIA) were performed. Healthy cats (>9 years) were recruited from three UK first opinion practices.

Results: The human TT4 EIA demonstrated good precision and reproducibility and adequate linearity. Hemolysis did not significantly alter measured TT4 concentrations until hemoglobin concentration exceeded 8 g/L. Method comparison revealed proportional and constant error between EIA and RIA/CEIA. The TT4 RI for cats (>9 years) was calculated as 7.1-45.1 nmol/L (n=49).

Conclusions: The human TT4 EIA was successfully validated for use with feline serum and offers a rapid, cheap and reliable method for determination of serum TT4 concentrations in cats.

Keywords: hyperthyroidism, feline, reference interval
Introduction

Hyperthyroidism is the most common feline endocrinopathy, with a prevalence of 6% in cats aged over 9 years. Routine screening of senior and geriatric cats for hyperthyroidism is recommended, which has led to increasing demand for the measurement of serum total thyroxine (TT4) concentrations in cats. As a result, there is a need to identify methods of TT4 measurement which are rapid, cheap and reliable. Both radioimmunoassays (RIA) and chemiluminescent-enzyme immunoassays (CEIA) for TT4 have been validated for use in cats, however, both methods are costly to perform and require additional analysers which are expensive to purchase and maintain. An automated, homogeneous enzyme immunoassay (EIA) for the measurement of serum TT4 in cats, which can be run on automated biochemistry analysers, has been validated. However, the previously validated EIA is not commercially available at present. Although an alternative human EIA is available, this uses a different methodology to the previously validated EIA.

Diagnosis of feline hyperthyroidism is usually based on documentation of an elevated serum TT4 concentration, however some hyperthyroid cats can have a serum TT4 concentration in the high normal range, perhaps secondary to non thyroidal illness. It is also possible that the reference intervals (RI) currently utilised for TT4 in cats are inappropriate for senior and geriatric cats. The ASVCP guidelines for the determination of de novo reference intervals in veterinary species recommend that “the demographics of the reference population should be representative of the patient population for which the RI will be used in making clinical decisions.” Hence, a RI for TT4 should be derived exclusively from a population of senior and geriatric cats, since this is the population that is usually tested for hyperthyroidism.
The primary aim of the present study was to validate a commercially available human TT4 EIA\textsuperscript{a} for use with feline serum. The secondary aim was to compare the performance of a human TT4 EIA against the performance of both the radioimmunoassay (RIA) and chemiluminescent-enzyme immunoassay (CEIA) for TT4 in cats. The final aim was to establish a TT4 RI from a population of clinically healthy cats aged over 9 years in three UK based first opinion practices, since this should serve as a more appropriate RI for TT4 in this population. Utilisation of an appropriate RI for TT4 may also increase the sensitivity of TT4 for diagnosis of hyperthyroidism.

**Materials and methods**

Blood and urine samples were obtained from cats (n=134) at three UK first opinion practices as part of a free of charge screening programme for cats aged >9 years. The Ethics and Welfare Committee of the Department of Veterinary Medicine, University of Cambridge approved the use of residual patient samples for research purposes (Project approval CR56 and CR77). Complete blood count, serum biochemistry (including total thyroxine concentration (TT4) by EIA) and urinalysis (including urine protein: creatinine ratio (UPC)) were performed. In addition, serum samples from cats which presented to the Queen’s Veterinary School Hospital, University of Cambridge or the RSPCA Clinic, Cambridge were used. All samples had TT4 determined by RIA or CEIA at the time of submission, with excess serum sample stored for up to 18 months at -80°C until batch analysis of TT4 by EIA.

The EIA,\textsuperscript{a} which is primarily designed for the measurement of TT4 in human serum, was performed by an automated biochemistry analyser\textsuperscript{c} using standard programming data and instructions provided by the manufacturer. The EIA uses 8-anilino-1-naphthalene sulfonic acid to dissociate thyroxine from binding proteins. The dissociated thyroxine in the sample is
then allowed to compete with glucose-6-phosphate dehydrogenase (G6PDH) labelled thyroxine for a fixed amount of anti-thyroxine specific antibody sites in the solution. In the absence of thyroxine in the sample, the G6PDH labelled thyroxine is bound by the specific antibody and the enzyme activity is inhibited, thus this creates a direct relationship between enzyme activity in the sample and the TT4 concentration. Enzymatic activity of G6PDH is determined spectrophotometrically at 340nm by measuring the ability of G6PDH to convert nicotinamide adenine dinucleotide (NAD) to NADH. The human TT4 calibrators provided with the assay kit were used for assay calibration, with an additional calibrator (12.9 nmol/L) also added, which was made by dilution of the highest calibrator provided with the kit (258 nmol/L).

Precision and repeatability of the EIA was assessed by evaluating intra- and inter-assay coefficients of variation for serum samples with low, medium and high serum TT4 concentrations. For intra-assay precision three replicates of each sample were evaluated within the same run. For assessment of inter-assay variability, pooled feline serum samples were evaluated in duplicate on three consecutive working days. The limit of blank was determined by measurement of the TT4 in deionised water (diH₂O), which was evaluated in triplicate on three consecutive working days. The limit of blank was calculated as the mean interpolated TT4 concentration in diH₂O + 2*standard deviation of TT4 in diH₂O. The lower limit of quantitation was calculated as the lowest concentration at which TT4 could be detected with a CV <20% when samples were analysed in triplicate. Linearity was evaluated using feline serum pools of high, medium and low TT4 concentrations (151.1 nmol/L, 96.6 nmol/L, 86.4 nmol/L, 11.6 nmol/L and 3.9 nmol/L), with dilution samples prepared by mixing the high or medium, and low pooled samples. The linearity was determined by comparing the observed TT4 concentrations following dilution with the expected (calculated) TT4 concentrations. Interference by hemolysis was determined by addition of feline blood
hemolysate to feline serum samples. The hemolysate was prepared by washing of feline erythrocytes three times in saline, before hemolysis of the erythrocytes by addition of diH$_2$O. The hemolysate was sequentially added to the serum samples, with the final hemoglobin concentration of the serum determined spectrophotometrically. Expected TT4 concentrations were calculated in order to correct for dilution of the sample following addition of the hemolysate.

For the method comparison studies, a mixed population of hyperthyroid and euthyroid cats was used. Diagnosis of hyperthyroidism was based on a TT4 >55 nmol/L (by CEIA) or >65 nmol/L (by RIA). TT4 measurements were made by RIA$^e$ and CEIA$^f$ at two commercial laboratories.$^g,h$ Method comparison was performed between the EIA and RIA, and the EIA and CEIA by Deming regression analysis using commercially available software,$^i$ and by construction of Bland-Altman plots.$^9$ Samples with a TT4 >154 nmol/L by EIA (upper limit of dynamic range of assay) were excluded from the method comparison studies.

Cats recruited to the healthy control group had blood and urine samples taken as part of a free of charge screening programme for cats aged over 9 years. Haematology, routine biochemistry, urinalysis (including urine sediment examination) and TT4 (by RIA or CEIA) were performed on all cats. To be included in the healthy control group for derivation of the RI for TT4, cats had to have no clinical history of disease, and have no significant abnormalities on clinical examination reported by the attending veterinarian, other than dental disease, entropion or presence of a systolic heart murmur (without evident congestive heart failure). Cats with evidence of renal azotemia (serum creatinine concentration >153 µmol/L with urine specific gravity <1.035), TT4 >55 nmol/L or >65 nmol/L (by CEIA or RIA respectively), or with evidence of pyuria or bacteriuria, were excluded from the healthy control group. The TT4 RI was determined from the cats that were included in the healthy control group using computerised software,$^j,10$ which calculated the lower and upper limits of
the TT4 reference interval by the robust method using Box-Cox transformed data. The 90% confidence intervals (CI) for the upper and lower limits of the reference interval were also reported.

Results

Inter- and intra-assay precision were acceptable (CV <12%) at all levels tested (Table 1). The assay was linear in the range of 5.6-151 nmol/L (r²=0.997, Figure 1) with acceptable analyte recovery (<18% deviation from calculated value at any point) throughout the range of TT4 concentrations tested. The limit of blank was calculated to be 6.3 nmol/L and the lower limit of quantitation was calculated to be 6.9 nmol/L. Hemolysis of the sample up to 8 g/L (consistent with 3+ hemolysis grossly) did not result in significant changes to the TT4 concentration, although hemolysis at 15 g/L did result in a 27% increase in the measured TT4 concentration when compared with the calculated TT4 concentration (Table 2).

For the method comparison studies, a population of 56 hyperthyroid cats was used, which had a median age of 14.8 years (interquartile range 12.6-16.1 years). The population of hyperthyroid cats consisted of 31 female neutered cats and 25 male neutered cats, and all were domestic short or long haired cats. When comparing the EIA with the RIA (n=36, 11/36 were hyperthyroid cats) revealed proportional error (slope 1.27, 95% CI 1.02-1.53) but no significant constant error (intercept 1.40, 95% CI -8.34 - 11.13). Correlation between the EIA and RIA was good (r=0.945, Figure 2) and the Bland Altman plots demonstrated that in the majority of cases, the difference between the EIA and RIA TT4 concentrations was within 2 standard deviations of the mean difference between the methods (Figure 3). Method comparison between the EIA and CEIA (n=81, 43/81 were hyperthyroid cats) revealed proportional (slope 1.16, 95% CI 1.12 - 1.19) and constant error (intercept -4.04, 95% CI -
Correlation between the EIA and CEIA was good \((r=0.987, \text{ Figure 4})\) and the Bland Altman plots demonstrated that in the majority of cases, the difference between the EIA and RIA TT4 concentrations was within 2 standard deviations of the mean difference between the methods (Figure 5).

Within the healthy reference population, there were 26 female neutered cats and 23 male neutered cats with a median age of 12 years (interquartile range 11-14 years). The majority of cats \((n=43)\) were domestic short or long haired cats, and six other breeds (Burmese, Bengal, Devon Rex, Persian, Russian Blue and Siamese) were also represented \((n=1\) for each breed).

Six cats were diagnosed with pre-renal azotemia (serum creatinine concentration >153 \(\mu\text{mol/L}\) with concurrent urine specific gravity \(\geq 1.035\)), and 13 cats had an elevated serum ALT activity (>62 IU/L, range 66-193 IU/L), however no cats had an increased serum ALP activity (>93 IU/L). The TT4 of the healthy cats aged over 9 years ranged between <6.9 nmol/L and 50.3 nmol/L (Figure 6) and the RI was calculated to be 7.1-45.1 nmol/L \((n=49)\).

The 90% CI for the lower and upper limits of the TT4 RI were 4.3-10.6 nmol/L and 40.1-50.3 nmol/L respectively.

When cats with an EIA TT4 >45.1 nmol/L were compared with cats with a TT4 >65 nmol/L by RIA, there was diagnostic agreement in 31/36 cases \((86\%, \text{ Figure 2})\), and all discordant cases had a TT4 concentration close to the upper reference limit for the EIA or RIA. When cats with an EIA TT4 >45.1 nmol/L were compared with cats with a TT4 >55 nmol/L by CEIA, there was diagnostic agreement in all 81 cases (Figure 4).

**Discussion**

The EIA for TT4 demonstrated excellent precision and reproducibility at medium and high concentrations of TT4, and good precision and reproducibility at low TT4 concentrations.
The critical decision limit for TT4 is approximately 50-60 nmol/L in cats (for the diagnosis of hyperthyroidism) and at these concentrations the assay performance was excellent. Linearity of the assay was also demonstrated with feline serum. Hemolysis (at clinically relevant concentrations) did not appear to significantly alter the measured serum TT4 concentrations, although very marked hemolysis did artefactually increase the TT4 concentration. Unfortunately the effect of lipemia on the measured serum TT4 concentration was not assessed as part of the present study, and should be investigated in future studies.

The method comparison study demonstrated proportional and/or constant error between the methods, with the EIA generally underestimating the TT4 concentration compared with the RIA and CEIA. This indicated that reference intervals could not be transferred between methods, and thus a new reference interval for the EIA was required. It is possible that storage of serum at -80°C for up to 18 months might have resulted in a decrease in the measured TT4, which could account for the tendency for the EIA to underestimate the TT4 when compared with the RIA and CEIA. Storage of serum at -20°C for up to 35 days does not significantly alter the measured TT4 concentration, however further studies to investigate the effect of prolonged storage at -80°C on the measured serum TT4 concentration are warranted. The method comparison studies did, however, demonstrate that there was good diagnostic agreement between the EIA and RIA/CEIA. In the present study, it was impossible to determine if the EIA was better at diagnosing hyperthyroidism than the RIA or CEIA, as this would require another gold standard method to be used for the diagnosis of hyperthyroidism, such as scintigraphy.

The calculated upper reference limit for TT4 of 45.1 nmol/L (by EIA), was lower than the upper limit of the reference interval reported for the RIA and CEIA methods (65 and 55 nmol/L respectively). Samples included in the reference interval were not stored at -80°C and therefore will not have been subject to pre-analytical error. This lower value may partly
reflect the relatively small number of animals that were included in the reference population in the present study (n=49), or may reflect a more appropriate upper reference interval limit for cats aged over 9 years. Older cats are more likely to have concurrent non thyroidal illness (such as dental disease) which might decrease serum TT4 concentrations, therefore a reference interval for TT4 derived exclusively from older cats might be expected to be lower than a reference interval generated from a more heterogeneous population which included younger, healthier cats.

Cats with an isolated high ALT without other clinical evidence of hepatic disease were not excluded from the healthy control group because; the cats demonstrated no clinical signs of hepatic disease, the elevation in ALT was a relatively frequent finding (occurring in 13 cats), and the elevations in ALT that were observed were relatively mild (<2x upper limit of reference interval) in the majority of cases. Based on this, it seems unlikely that many of these cats had significant hepatocellular disease, however the presence of hepatic disease, which might have suppressed TT4 concentrations, could not be excluded fully without further invasive investigations such as liver biopsy and histopathology.

In conclusion, a human TT4 EIA was successfully validated for use with feline serum and offers a rapid, cheap and reliable method for determination of serum TT4 concentrations in cats. The EIA appears to underestimate the TT4 concentration compared with the RIA and CEIA, and the RI for TT4 in cats aged over 9 years was also lower than the reference range reported for the RIA and CEIA. Further studies are warranted to investigate if the age specific RI for cats reported in this study increases the sensitivity of TT4 for the diagnosis of hyperthyroidism.
Footnotes

a DRI Thyroxine Assay, Microgenics Corporation, Freemont, CA, USA.
b CEDIA Total T4 Assay, Boehringer Mannheim Corp., Indianapolis, IN, USA.
c Olympus AU400, Beckman Coulter, High Wycombe, UK.
d Sysmex XT-2000iV, Sysmex Corporation, Hyogo, Japan.
e Gamma Coat M total T4 radioimmunoassay, DiaSorin Inc, Stillwater, Minn.
f IMMULITE Total T4, Siemens Healthcare, Camberley, UK.
g Nationwide Specialist Laboratories, Stapleford, Cambridge, UK.
h IDEXX Laboratories, Wetherby, UK.
i MedCalc Statistical Software version 14.8.1, MedCalc Software bvba, Ostend, Belgium.

j Reference Value Advisor version 2.1 (http://www.biostat.envt.fr/spip/spip.php?article63)
References


Figure 1. Linearity of the human enzyme immunoassay (EIA) for serum total thyroxine concentration (TT4) using pooled feline serum with low and high TT4 concentrations. The line of equality is shown.

Figure 2. Method comparison of radioimmunoassay (RIA) and enzyme immunoassay (EIA) for feline serum total thyroxine concentration (TT4). The solid line represents the line of equality. The dotted lines represent the upper limits of the reference intervals for the RIA and EIA.

Figure 3. Bland Altman plot showing the difference between the measured serum total thyroxine concentration (TT4) by the radioimmunoassay (RIA) and enzyme immunoassay (EIA) against the average TT4 measured by the RIA and EIA. The solid line represents the mean difference between the methods and the dotted lines present the mean ± 2 x standard deviation difference between the methods.

Figure 4. Method comparison of enzyme immunoassay (EIA) and chemiluminescent-enzyme immunoassay (CEIA) for feline serum total thyroxine concentration (TT4). The solid line represents the line of equality. The dotted lines represent the upper limits of the reference intervals for the EIA and CEIA.

Figure 5. Bland Altman plot showing the difference between the measured serum total thyroxine concentration (TT4) by the chemiluminescent-enzyme immunoassay (CEIA) and enzyme immunoassay (EIA) against the average TT4 measured by the RIA and CEIA. The solid line represents the mean difference between the methods and the dotted lines present the mean ± 2 x standard deviation difference between the methods.
Figure 6. Scatter plot showing the serum total thyroxine (TT4) concentrations of 49 healthy cats aged over 9 years. The dotted lines indicate the lower and upper limits of the calculated reference interval for TT4 in cats aged over 9 years (7.1-45.1 nmol/L).
Table 1. Intra- and inter-assay coefficients of variation (CV) at low, medium and high serum concentrations of feline total thyroxine (TT4) calculated using a human TT4 enzyme immunoassay.

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay variability (n=3)</th>
<th>Inter-assay variability (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT4 concentration (nmol/L)</td>
<td>Mean TT4 concentration (nmol/L)</td>
</tr>
<tr>
<td>Low</td>
<td>11.2</td>
<td>10.2</td>
</tr>
<tr>
<td>Medium</td>
<td>54.0</td>
<td>56.0</td>
</tr>
<tr>
<td>High</td>
<td>137.5</td>
<td>139.6</td>
</tr>
</tbody>
</table>
Table 2. Effect of hemoglobin concentration on feline serum total thyroxine (TT4) concentrations measured by a human TT4 enzyme immunoassay.

<table>
<thead>
<tr>
<th>Hemoglobin concentration of serum (g/L)</th>
<th>Calculated serum TT4 concentration (nmol/L)</th>
<th>Observed serum TT4 concentration (nmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>62.2</td>
<td>66.3</td>
<td>107</td>
</tr>
<tr>
<td>8</td>
<td>48.4</td>
<td>47.8</td>
<td>99</td>
</tr>
<tr>
<td>15</td>
<td>26.9</td>
<td>34.1</td>
<td>127</td>
</tr>
</tbody>
</table>