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Validation and application of an immunoradiometric assay for the determination of human parathyroid hormone fragment 1–34 in dog plasma following subcutaneous and intravenous administration

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Abstract

A method for the measurement of human parathyroid hormone fragment 1–34 (PTH_{1-34}) in dog plasma was developed by modification of a commercially available immunodiometric assay (IRMA) designed for the determination of rat PTH_{1-34} in serum. Major modifications were made to the assay in order to circumvent significant problems encountered during the validation of the IRMA. PTH_{1-34} was found to be highly unstable in both rat serum and dog serum and plasma at room temperature, in contrast to literature reports. The addition of a protease inhibitor cocktail to serum or plasma samples was necessary to prevent in-vitro proteolytic degradation of human PTH_{1-34} prior to analysis. Additionally, plasma was chosen over serum as the sample matrix to expedite the separation of samples from cells, minimizing proteolytic degradation prior to the addition of cocktail. Finally, the reported 100% cross-reactivity between rat and human PTH_{1-34} was found to be only 65%; therefore, a human PTH_{1-34} standard was substituted for the rat standard. These modifications allowed the accurate measurement of human PTH_{1-34} in plasma obtained from dogs dosed intrevenously and subcutaneously with human PTH_{1-34} using a commercially available kit.

Keywords: Immunoradiometric assay; Parathyroid hormone; Pharmacokinetics; Protease inhibitor cocktail; Stability

1. Introduction

Parathyroid hormone (PTH) is a naturally occuring polypeptide synthesized by the parathyroid glands. PTH regulates the homeostatic control of calcium and phosphate metabolism. The principal sites of activity are the skeleton, kidneys, and gastrointestinal tract. Subcutaneous (SC) administration of PTH increases cancellous bone mass in rats, dogs, and humans [1–6] and PTH represents a potential anabolic agent for restoration of bone mass in osteoporotic patients. Intact PTH contains 84 amino acids and has a molecular weight of 9425 Da [7]. The smallest fragment

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exhibiting full biological activity consists of residues 1–34 from the N-terminal region [8]. The smaller size of PTH_{1-34} makes it an attractive alternative to PTH for evaluation as a potential anabolic agent since direct synthesis is possible and characterization requirements are simplified. Although the pharmacological effects of SC and intravenous (IV) administration of PTH and PTH_{1-34} have been documented, little data exist on the pharmacokinetic profiles of either compound following SC or IV administration [9]. In order to estabilish the pharmacokinetic profile for human PTH_{1-34} a sensitive assay method is required to selectively measure it in serum or plasma. Historically, the measurement of PTH and its metabolites has been problematic due to the diversity of the circulating PTH metabolites, differences in the pharmacokinetic profiles of PTH and its metabolites and significant differences in specificity and sensitivity of PTH radioimmunoassay [10–12]. Several different PTH_{1-34} immunoassays are commercially available. The Nichols Institute Diagnostics Rat PTH immunoradiometric assay (IRMA) kit for the quantitative determination of PTH_{1-34} in rat serum was selected. Although sold for the analysis of rat PTH_{1-34}, the assay was reproted to have a 100% cross reactivity with human PTH_{1-34}. Additionally, since the Nichols assay is a two-site IRMA the chances for cross reaction with potential metabolite fragments of PTH_{1-34} should be reduced. The Nichols assay, however, required several modifications and an extensive validation in order to ensure the accurate and precise measurement of human PTH_{1-34}. The validated assay was used to generate pharmacokinetic profiles of human PTH_{1-34} in dogs following its administration by the SC and IV routes.

2. Materials and methods

2.1. Reagents

Rat PTH IRMA kits were obtained from Nichols Institute Diagnostics (San Juan Capistrano, CA). Human PTH_{1-34}, rat PTH_{1-34} and human PTH fragment 13–34 were synthesized by Bachem (Torrance, CA). Human PTH fragments 1–13, 1–26 and 21–34 were obtained from Advanced ChemTech (Louisville, KY). Pepstatin, phenylmethylsulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) leupeptin, benzamidine and aprotinin were obtained from Sigma (St. Louis, MO). *Z*-Phe–Ala-fluoromethylketone (*Z*-Phe–Ala-CH_2F) was obtained from Enzyme Systems Products (Dublin, CA). Ethyl alcohol was obtained from Aaper Alcohol and Chemical Company (Shelbyville, KY). Ultrapure water was 18.2 MΩ-cm obtained from a Millipore Milli-Q Plus system (Bedford, MA). A 0.9% sodium chloride (saline) solution was obtained from Baxter (Valencia, CA). Rat serum, dog serum and EDTA dog plasma were obtained from Pel-Freez (Rogers, AR).

2.2. Preparation of human PTH_{1-34} standards

A 1 mg ml^{-1} stock solution of human PTH_{1-34} in saline was prepared and stored in 0.5 ml aliquots at −80°C. Standards were prepared by further diluting this solution with sample diluent from the IRMA kit. For the assay procedure, 10 concentrations of human PTH_{1-34} standards ranging from 0 to 2000 pg ml^{-1} were prepared.

2.3. Preparation of protease inhibitor cocktail

PMSF (100 mmol l^{-1}), pepstatin (1 mmol l^{-1}) and *Z*-Phe–Ala-CH_2F (10 mmol l^{-1}) were prepared as separate ethyl alcohol stock solutions. EDTA (500 mmol l^{-1}), leupeptin (10 mmol l^{-1}), benzamidine (1 mol l^{-1}) and aprotinin (2 mg ml^{-1}) were prepared as separate aqueous solutions. Stock solutions of each inhibitor were stored in aliquots at −20°C for up to 1 month. An aliquot of each inhibitor stock was thawed and the cocktail prepared fresh on the day of use. The cocktail was prepared by adding each inhibitor stock as follows to obtain the final working concentrations shown in Table 1: 50 μl PMSF, 200 μl EDTA, 10 μl pepstatin, 100 μl leupeptin, 100 μl benzamidine, 10 μl aprotinin and 10 μl *Z*-Phe–Ala-CH_2F (total volume 480 μl per 10.0 ml plasma).

2.4. Preparation of spiked serum or plasma samples

Spiked serum or plasma samples were prepared by diluting a 1 mg ml^{-1} human PTH_{1-34} stock solution in saline with rat serum, dog serum or dog plasma to the desired concentrations. Spiked samples were aliquoted into sterile polypropylene cryovials and stored at −80°C.

2.5. Preparation of spiked plasma samples containing protease inhibitor cocktail

Dog plasma containing protease inhibitor cocktail was prepared by adding the appropriate volume of cocktail to plasma to obtain the working concentrations shown in Table 1 (48 μl cocktail per 1.0 ml of plasma). Spiked plasma samples were then prepared by diluting appropriate aliquots of a 1 mg ml^{-1} human PTH_{1-34} stock solution with dog plasma containing protease inhibitor cocktail to obtain the desired concentration of human PTH_{1-34}. Spiked plasma plus cocktail samples were aliquoted (0.5 ml) into polypropylene cryovials and stored at −80°C.

2.6. Assay procedure

The Nichols rat PTH assay is a two-site IRMA utilizing two different polyclonal goat antibodies to the N-terminal region (1–34) of rat PTH purified by affinity chromatography. One of the antibodies is immobilized onto a solid support to capture the PTH molecule and the other antibody is radiolabeled for detection. A sample containing PTH is incubated simultaneously with an antibody-coated bead and the ^{125}I-labeled antibody. The PTH contained in the sample is bound by both the immobilized and labeled antibodies to form a sandwich complex. At the end of the incubation period, the bead is washed to remove any unbound antibody. The detected radioactivity of the antibody complex bound to the bead is directly proportional to the amount of PTH in the sample.

Table 1
Protease inhibitor cocktail

Inhibitor	Protease	Working concentration
PMSF	Serine	0.5 mmol l^{-1}
EDTA	Metallo	10 mmol l^{-1}
Pepstatin	Aspartic	1 μmol l^{-1}
Leupeptin	Serine/cysteine	100 μmol l^{-1}
Benzamidine	Broad spectrum	10 mmol l^{-1}
Aprotinin	Serine	2 μg ml^{-1}
Z-Phe-Ala-CH_2F	Cysteine	10 μmol l^{-1}

Standard (rat or human), control, or unknown sample (200 μl) was pipetted into a polypropylene, round-bottom tube (Falcon 12 mm × 75 mm; Becton Dickinson, Lincoln Park, NJ). Each determination was performed in duplicate. Next, the ^{125}I-labeled goat anti-rat PTH_{1-34} (100 μl) containing less than 7.4 kBq of radioactivity was added to each tube. A Biomeck 1000 Automated Workstation (Beckman Instruments, Inc., Fullerton, CA) was used to dispense samples and antiserum to the tubes. The tubes were then gently vortexed. A polystyrene bead coated with the second polyclonal antibody specific for a different epitope of rat PTH_{1-34} was added to each tube using a bead dispenser. The tubes were covered with Parafilm (American National Can, Greenwich, CT) and incubated at room temperature for 18–24 hours. Following incubation, the beads were washed three times by adding 2.0 ml of diluted kit wash solution to each tube and aspirating the contents to remove any unbound antibody. The radioactivity bound to each bead was counted in a gamma counter for 1 min (Packard MINAXI 5530 Gamma Counter, Packard Instrument Company, Meriden, CT). The bound radioactivity (background corrected counts per minute, y axis) was plotted against the concentration of the corresponding standard (pg ml^{-1}, x axis). A four-parameter logistics fit (Immunofit EIA/RIA software, Version 3.0, Beckman Instruments) was used to generate the standard curve and determine human PTH_{1-34} levels in samples. Samples with values > 1500 pg ml^{-1} were diluted in sample diluent and reassayed.

2.7. Assay development

2.7.1. Accuracy of assay

Two sets of standards were prepared to examine the accuracy of the assay. The Nichols IRMA kit contained six rat PTH_{1-34} standards ranging from 0 to 1900 pg ml^{-1}. Each standard vial was reconstituted as per kit instructions. A separate set of human PTH_{1-34} standards, consisting of 10 concentrations ranging from 0 to 2000 pg ml^{-1}, were prepared as described above. Samples spiked with rat PTH_{1-34} were prepared by diluting a 100 μg ml^{-1} aqueous stock solution of rat PTH_{1-34} with kit sample diluent to obtain a concentration of 500 pg ml^{-1}. Samples spiked with human PTH_{1-34} were prepared by diluting the 1 mg ml^{-1} stock solution of human PTH_{1-34} in IRMA kit sample diluent to obtain a concentration of 500 pg ml^{-1}. Samples were assayed and concentrations determined from standard curves generated by both rat PTH_{1-34} and human PTH_{1-34} standards.

2.7.2. Stability at room temperature

The stability of human PTH_{1-34} was studied in IRMA kit sample diluent, rat serum, dog serum, dog plasma and dog plasma containing protease inhibitor cocktail. Each matrix was spiked to contain 500 pg ml^{-1} human PTH_{1-34} and was then allowed to remain at room temperature for 0, 2 and 4 h before assaying.

2.8. Assay validation

2.8.1. Assay precision

Intra-assay precision was determined by analyzing multiple replicates ($n = 10$) of dog plasma plus inhibitor cocktail spiked with human PTH_{1-34} at 50 pg ml^{-1}, 500 pg ml^{-1}, and 1000 pg ml^{-1} in the same assay. Inter-assay precision was detemined by measuring these same spiked samples in 20 determinations on different days.

2.8.1. Parallelism

Parallelism was assessed by taking a sample of dog plasma plus protease inhibitor cocktail containing 100 000 pg ml^{-1} human PTH_{1-34} and diluting it with kit sample diluent. Dilutions of 1:100, 1:200, 1:500, 1:1000, and 1:2000 were assayed.

2.8.3. Hook effect

Human PTH_{1-34} was spiked into dog plasma containing protease inhibitor cocktail at levels ranging from 100 pg ml^{-1} to 10 μg ml^{-1} and assayed undiluted to determine high dose hook effect.

2.8.4. Freeze/thaw stability

The stability of human PTH_{1-34} to repeated freeze/thaw cycles was examined using dog plasma plus inhibitor cocktail containing 500 pg ml^{-1} human PTH_{1-34}. The stability was examined after one, two, or three freeze/thaw cycles. In each treatment cycle, samples were thawed rapidly at 37°C in a water bath until only a small piece of frozen material remained. The samples were then removed from the water bath and allowed to thaw further at ambient temperature. Samples were then refrozen at −80°C for at least 1 h.

2.8.5. Stability at −80°C

A sample was prepared containing 500 pg ml^{-1} human PTH_{1-34} in dog plasma plus inhibitor cocktail and stored in 0.6 ml aliquots at −80°C. Aliquots were removed from storage and assayed over a period of time ranging from 1 day to 3 months.

2.8.6. Cross reactivity with PTH fragments

Cross reactivity of human PTH fragments smaller than fragment 1–34 was investigated. 1 mg ml^{-1} stock solutions of human PTH fragments 1–13, 1–26, 21–34 and 13–34 were prepared as separate solutions in saline. Samples were prepared by further diluting each stock solution with kit sample diluent to obtain a final concentration of 1000 pg ml^{-1}.

2.9. Dog pharmacokinetic study

Twelve mature beagle dogs (2–4 years) were dosed in IV and SC fashions at a 5 μg kg^{-1} dose level using a cross-over design. Dogs were dosed

once weekly. Doses of human PTH_{1-34} were prepared in sterile saline and administered through a 22 gauge needle into the cephalic or jugular vein (IV) or in the dorsal cervical region (SC). Total volume was less than 0.5 ml kg^{-1} body weight. Post-dosing measurement of the aliquot confirmed the concentration of the solution as a check against degradation or absorption to the glass. Blood samples for human PTH_{1-34} analysis were collected at 0, 5, 10, 15, 30, 45, and 60 min and 2, 4, 8, and 24 h post-dosing. Whole blood was collected in a 7 ml purple top tube containing 0.07 ml of 15% EDTA (K_3) solution (10.5 mg) and kept on ice. Plasma was separated from cells within 20 min of collection by centrifugation (3600 rev min^{-1}, 20 min) at 4°C. Protease inhibitor cocktail was added to the dog plasma to obtain the working concentrations shown in Table 1 (24 μl cocktail was added to 0.5 ml plasma). Samples were stored at −80°C until analysis. The pharmacokinetic study was performed under the approval of the Institutional Animal Care and Use Committee. All animals were cared for in accordance with the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

3. Results

3.1. Assay modifications

3.1.1. Selection of standards

The IRMA kit was designed to measure rat PTH_{1-34} but was reported to cross react 100% with human PTH_{1-34}. To determine cross reactivity, rat and human PTH_{1-34} spiked in kit sample diluent (500 pg ml^{-1} each) were analyzed against standard curves generated using both rat and human PTH_{1-34}. The rat PTH_{1-34} sample gave the expected recovery (109%) on the rat standard curve (data not shown). Similarly, the human PTH_{1-34} sample gave the expected revovery (102%) on the human standard curve (data not shown). However, the human PTH_{1-34} sample gave only a 65% recovery when determined using the rat standard curve (data not shown). Human PTH_{1-34} standards were therefore selected to replace the rat standards contained in the Nichols kit to ensure accurate measurement of human PTH_{1-34} in samples.

Table 2
Room temperature stability of human PTH_{1-34}

Sample matrix	Percent of expected		
	Time (h)		
	0	2	4
Diluent	110	107	108
Dog serum	53	40	28
Dog plasma	86	79	72
Dog plasma +cocktail	100	93	86

3.1.2. Stability of human PTH_{1-34} at room temperature

The stability of human PTH_{1-34} was examined initially in IRMA kit sample diluent and serum (kit recommended sample matrix) at room temperature for up to 4 h. The results are summarized in Table 2. No decrease in measured PTH_{1-34} concentration was observed in diluent samples over time. However, a substantial decrease was observed in dog serum. At time 0 h (samples assayed immediately), only 53% of the spiked human PTH_{1-34} was recovered and after 4 h the recovery had dropped to 28%. This trend was observed in rat serum as well as in dog serum (data not shown). Several different sources of both rat and dog serum were tested to confirm this finding (data not shown). Stability of human PTH_{1-34} was then investigated in EDTA plasma. The PTH_{1-34} loss was less in plasma but degradation still occurred as shown in Table 2. Addition of a protease inhibitor cocktail to dog plasma stabilized human PTH_{1-34} for up to 2 h at room temperature (Table 2). This time period (2 h) is sufficient to set up a 200 tube assay. For all remaining studies, EDTA plasma containing protease inhibitor cocktail was used as the sample matrix for the determination of human PTH_{1-34} levels. It has since been demonstrated that the proteolytic degradation of human PTH_{1-34} can be

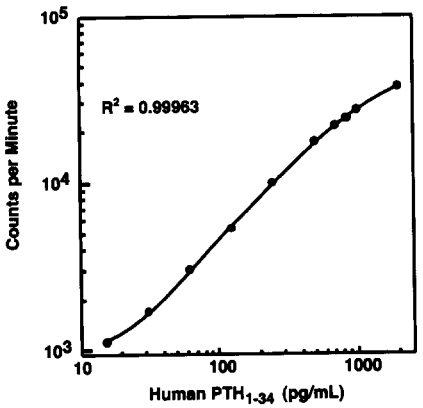

Fig. 1. Log–log plot of four-parameter logistics fit of human PTH_{1-34} standards.

completely stopped by acidifying the standards and samples to pH 4.0 (data not shown).

3.2. Assay characteristics

3.2.1. Data analysis and detection limit

Several different curve-fitting methods (linear regression, cubic spline, quadratic, and four-parameter logistics fit) were examined to determine the best fit for the standard curve. Fig. 1 shows a typical standard curve for human PTH_{1-34} using a four-parameter logistics fit. The mean ED_{50} was 730 pg ml^{-1} over six assays. The lower limit of detection calculated from three standard deviations (SDs) of the zero standard (20 determinations) at the 95% confidence limit was 5.8 pg ml^{-1}. The lower limit of quantitation calculated from 10 SDs of the zero standard (20 determinations) was 15.8 pg ml^{-1}. Back-calculated concentrations of the standards using the four-parameter logistics fit produced the lowest deviation from expected values, generally within 2% of expected, while standards fit by linear regression gave deviations of up to 30% (data not shown).

Table 4
Parallelism of PTH IRMA

Sample dilution	Expected PTH (pg ml^{-1})	Percent recovery	%RSD
1:100	1000	97	3.3
1:200	500	96	4.6
1:500	200	93	6.2
1:1000	100	94	3.9
1:2000	50	99	5.0

3.2.2. Assay accuracy and precision

Intra-assay accuracy and precision were determined by assaying dog plasma samples containing low, medium, and high concentrations of human PTH_{1-34} multiple times ($n = 10$) in the same assay. Inter-assay precision was determined by measuring these samples multiple times ($n = 20$) in assays on different days. Intra-assay variability ranged from 2.5–5.7% relative standard deviation (RSD) and inter-assay variability ranged from 4.0–7.2% RSD (Table 3).

3.2.3. Parallelism

Parallelism was assessed by assaying serial dilutions of plasma sample containing 100 000 pg ml^{-1} human PTH_{1-34}. Excellent parallelism was demonstrated in samples diluted within the range of the standard curve (Table 4).

Table 3
Precision of PTH IRMA

Expected PTH (pg ml^{-1})	Intra-assay		Inter-assay	
	Percent recovery	%RSD	Percent recovery	%RSD
50	96	2.5	97	4.0
500	98	3.5	99	4.8
1000	94	5.7	99	7.2

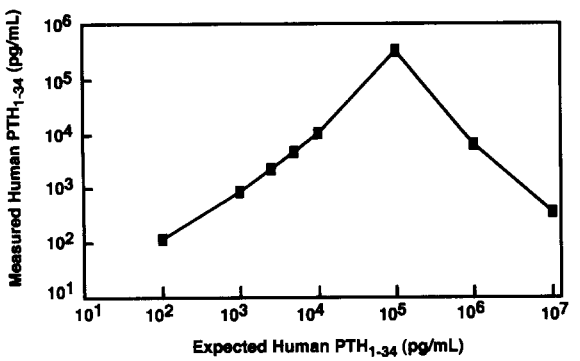

Fig. 2. Log–log plot of measured human PTH_{1-34} concentrations versus expected human PTH_{1-34} concentrations in spiked dog plasma samples.

3.2.4. Hook effect

A high dose hook effect was observed at approximately 100 000 pg ml^{-1} human PTH_{1-34} as seen in Fig. 2. The bound radioactivity increased steadily up to this concentration and then steadily decreased to 10 μg ml^{-1}, the highest cncentration tested.

3.2.5. Freeze/thaw and storage stability

Quantitative recovery of human PTH_{1-34} was obtained for up to two freeze/thaw cycles with recoveries ranging from 96–100% (data not shown). Recovery dropped to 90% in the third cycle, indicating a potential stability problem (data not shown). No change in the concentration was observed for a 500 pg ml^{-1} human PTH_{1-34} dog plasma sample containing inhibitor cocktail when stored at −80°C over a 3 month period (data not shown).

3.2.6. Cross reactivity of smaller human PTH fragments

Specificity of the PTH IRMA was assessed by testing 1000 pg ml^{-1} samples of several smaller PTH fragments in the assay. Apparent human PTH_{1-34} values of 349 pg ml^{-1} and 35 pg ml^{-1} were obtained for fragments 1–26 and 13–34 respectively (data not shown). Fragments 1–13 and 21–34 showed no detectable binding (data not shown).

3.3. Dog pharmacokinetic study

The average pharmacokinetic profiles obtained for the 12 dogs following IV and SC administration of human PTH_{1-34} are shown in Fig. 3. A full account of the pharmacokinetic profiles obtained for the 12 dogs will be published separately.

4. Discussion

The Nichols PTH IRMA assay required several major modifications in order to provide accurate

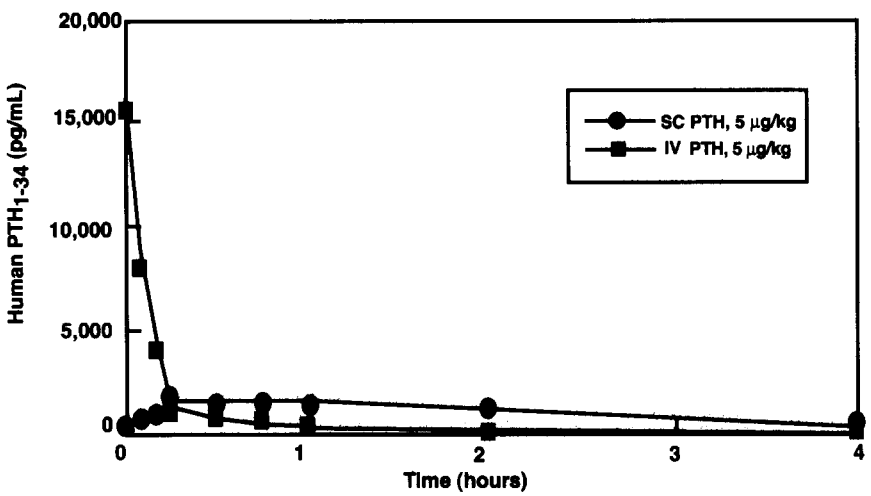

Fig. 3. Plot of average human PTH_{1-34} plasma levels versus time obtained for 12 dogs following SC and IV administration of 5 µg ml^{-1} human PTH_{1-34}.

and precise data for the mesurement of human PTH_{1-34} in dog plasma samples. Assay results for the analysis of human PTH_{1-34} in assay buffer were consistently low using a rat PTH_{1-34} standard curve but were accurate when human PTH_{1-34} was used to construct the standard curve. Although the assay was reported to have a 100% cross reactivity with human PTH_{1-34}, the current results indicated that the cross reactivity with human PTH_{1-34} was roughly 65%. Therefore human PTH_{1-34} was used to generate the standard curve used to determine unknown concentrations of human PTH_{1-34}. Initial attempts to analyze spiked serum samples, dog or rat, using human PTH_{1-34} standards again resulted in consistently low recoveries. Serum spiked with human PTH_{1-34} and assayed immediately repeatedly yielded a recovery of only about 53% and recovery continued to drop over time as the sample remained at room temperature. A literature survey, as well as various vendor kit instructions, made no reference to stabiltiy problem for PTH_{1-34} in serum, in fact serum is the preferred sample medium for the clinical measurement of human PTH [13]. However, a protein of similar structure, parathyroid hormone related protein, was known from the literature to be unstable toward proteolytic degradation [14,15]. The possibility of proteolytic degradation of human PTH_{1-34} was supported by the finding that human PTH_{1-34} was more stable in EDTA plasma than serum. Serine proteases, metalloproteases, aspartic acid proteases and cathepsins are the four major families of proteases. The addition of an inhibitor cocktail, containing at least one inhibitor for each protease class, to plasma stabilized human PTH_{1-34} for at least a 2 h period. For this work, a cocktail (Table 1) containing seven different protease inhibitors was used. It is likely that not all seven inhibitors are needed to prevent the proteolytic degradation of human PTH_{1-34}; however, this was not investigated during this study. Therefore, although serum was the recommended medium for the analysis of PTH_{1-34}, plasma containing the protease inhibitor cocktail was found to be preferable for all human PTH_{1-34} measurements.

The modifications to the Nichols PTH IRMA kit allowed the accurate and precise determination of human PTH_{1-34} in dog plasma over a concentration range of 15–1500 pg ml^{-1}. The assay was free of nonspecific matrix interferences as demonstrated by the parallelism observed for the dilutions of dog plasma samples. A hook effect was observed for the human PTH_{1-34} analysis only at extremely high PTH_{1-34} concentrations (> 100 ng ml^{-1}). Although a hook effect at such a high analyte concentration was not a major concern, the initial analysis of IV and SC dog samples included a serial dilution study to ensure that the plasma levels were not in the range of a potential hook effect. Human PTH_{1-34} was shown to be stable in plasma containing a protease inhibitor cocktail for at least 3 months when stored at − 80°C and was stable for up to two freeze/thaw cycles.

To determine the pharmacokinetic profile for a compound the analytical methodology must selectively measure only the compound of interest. Human PTH and human PTH_{1-34} have been shown to undergo extensive proteolytic metabolism by the liver and therefore the potential for cross reaction with smaller PTH fragments was of concern [16]. PTH fragments 1–13, 1–26, 21–34 and 13–34 were evaluated for potential cross reaction in the assay. These fragments were selected for evaluation based on their potential formation by trypsin cleavage of human PTH_{1-34}. Only fragment 1–26 had any significant cross reaction, indicating that the assay possessed a high degree of specificity for the intact PTH_{1-34} molecule. The assay was applied to the analysis of plasma samples obtained from dogs dosed with human PTH_{1-34} by the IV and SC routes. The pharmacokinetic profiles obtained from the dosed animals exhibited the typical elimination and distribution/elimination phases expected for compounds administered by IV and SC means.

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