Original Article

Feline plasma adrenocorticotropic hormone: validation of a chemiluminescent assay and concentrations in cats with hypercortisolism, primary hypoadrenocorticism and other diseases

Antonio M Tardo[1], Claudia E Reusch[2], Sara Galac[3],
Sofia Fornetti[1], Alessandro Tirolo[1], Stefania Golinelli[1],
Dardan Shehdula[1] and Federico Fracassi[1] iD

Abstract

Objectives The aims of this study were to validate a commercially available chemiluminescent assay for measurement of feline plasma adrenocorticotropic hormone concentration (ACTH), to determine the normal reference interval (RI) of plasma ACTH in healthy cats, to assess plasma ACTH in cats with naturally occurring hypercortisolism (HC), primary hypoadrenocorticism (PH) and other diseases (OD), and to evaluate the effect of aprotinin on plasma ACTH degradation.

Methods Forty healthy cats, 10 with HC, 11 with PH and 30 with OD, were included. The chemiluminescent enzyme immunometric assay was evaluated by measurement of intra-assay precision, interassay precision and linearity. The RI for plasma ACTH in healthy cats was established using robust methods. Plasma ACTH of samples collected with and without aprotinin, stored at 4°C and assayed over a 6-day period, was measured.

Results The intra-assay coefficients of variance (CVs) ranged from 2.7% to 4.3% and interassay CVs from 3.3% to 10.7%. Dilution studies showed excellent accuracy ($R^2 > 0.99$). The RI for plasma ACTH in healthy cats was 32–370 pg/ml. Plasma ACTH was not significantly different between healthy cats and the OD group. Cats with pituitary-dependent hypercortisolism (PDH) and PH had significantly higher plasma ACTH than the other groups. Plasma ACTH did not show significant differences when samples collected with and without aprotinin were compared.

Conclusions and relevance The Immulite chemiluminescent assay is a valid technique for measuring plasma ACTH in cats and the RI of plasma ACTH is quite wide. Owing to the low overlap between healthy or OD cats and cats with HC or PH, the measurement of plasma ACTH appears to be useful and should be included in the diagnostic work-up when HC or PH are suspected. Furthermore, the measurement of plasma ACTH may be an accurate test for differentiating PDH from adrenal-dependent hypercortisolism.

[1]Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, Bologna, Italy
[2]Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland
[3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Corresponding author:
Federico Fracassi DVM, PhD, DECVIM-CA,
Department of Veterinary Medical Sciences,
University of Bologna, Via Tolara di Sopra 50,
Ozzano dell'Emilia, Bologna, Italy
Email: federico.fracassi@unibo.it

Introduction

Naturally occurring hypercortisolism (HC) and primary hypoadrenocorticism (PH) are rare conditions in cats,[1–9] and their diagnosis can be challenging. However, adrenocortical axis disorders in cats are increasingly recognised and often included in the list of differential diagnoses. Once a diagnosis of HC is established, it is crucial to differentiate between pituitary-dependent HC (PDH) and adrenal-dependent HC (ADH) so that appropriate treatment can be instituted. Evaluation of plasma adrenocorticotropic hormone concentration (ACTH) can be useful when one of these conditions is suspected and can assist in determining the cause of feline HC. Although studies[10–22] have been undertaken to evaluate the hypothalamic–pituitary–adrenal (HPA) axis in healthy cats, cats with pituitary or adrenal disorders, and cats with other diseases (OD), only a few have mentioned the measurement of plasma ACTH.[10,15,16,18,21,22] This may be due, at least partly, to the lack of a current validated chemiluminescent assay for measurement of feline plasma ACTH and of a reference interval (RI) for plasma ACTH in healthy cats.

Immunoradiometric assays (IRMAs) showed good performance in cats;[18,22] however, the advantages of chemiluminescent methods over IRMA include lack of exposure to radioisotopes, easy integration into laboratory processes and rapid turnaround times. Currently, the chemiluminescent assay has been validated for the measurement of plasma ACTH in dogs,[23] but there are no studies that have evaluated its use in cats. A validated commercially available chemiluminescent assay may be used as a reference for future clinical studies investigating feline adrenocortical axis diseases. Moreover, the identification of an RI for feline plasma ACTH would help veterinary practitioners in the diagnosis of differential HPA disorders. Hence, the objectives of this study were to validate a chemiluminescent enzyme immunometric assay to measure feline plasma ACTH, determine the normal RI of plasma ACTH in healthy cats and assess plasma ACTH in cats with HC and PH, and to determine if OD may influence plasma ACTH.

Plasma ACTH is considered to be highly unstable in blood because of proteolytic degradation.[24–27] For this reason, care must be taken during the pre-analytical phase and it is recommended that a constant low temperature be maintained from the point of blood sampling until analysis.[28] In clinical practice, however, this procedure may present logistical problems because samples for plasma ACTH measurement are frequently shipped to an external laboratory. Therefore, an additional aim of the present study was to determine if aprotinin, a protease inhibitor previously investigated for use in human and canine samples,[23,29–31] may help prevent ACTH degradation in feline plasma samples.

Materials and methods

Validation of the chemiluminescent assay

Plasma ACTH was measured using a chemiluminescent enzyme immunometric assay (Immulite 2000 ACTH; Diagnostic Product Corporation), which measures the intact ACTH molecule (amino acids 1–39). The method uses a combination of a monoclonal murine anti-ACTH antibody and an alkaline phosphatase-labelled polyclonal rabbit anti-ACTH antibody directed against the C-terminal region and the N-terminal region, respectively. The manufacturer's instructions indicated an analytical sensitivity for the assay of 5 pg/ml and a calibration range of up to 1250 pg/ml. There is some cross-reactivity (16.6–17.3%) with ACTH fragment 18–39 (corticotropin-like intermediate lobe peptide). There is no reported interference with alpha-melanocyte-stimulating hormone (α-MSH).[32]

Intra-assay variability was determined by calculating the coefficients of variance (CVs) for eight replicates of three plasma samples having different concentrations of ACTH; analyses were repeated eight times within the same run. Interassay variability was determined by calculating the CVs for eight replicates of three plasma samples, having different concentrations of ACTH, analysed eight times on eight different days. Linearity was evaluated by measurement of two samples, one containing moderate concentrations of ACTH and one containing high concentrations of ACTH, before and after dilution (1:2, 1:4 and 1:8, respectively), using the diluent provided by the manufacturer. To assess linearity, the measured ACTH concentrations were plotted against expected values in a linear regression analysis. Accuracy after spiking was determined by adding 100 µl samples containing moderate and high concentrations of ACTH to samples containing a low concentration of ACTH. The measured ACTH concentrations were then compared with predicted ACTH concentrations.

Animals

Healthy cats In order to establish the RI of feline plasma ACTH, 40 healthy client-owned cats, including 20 males (10 castrated) and 20 females (17 spayed) with a median age of 4.0 years (range 1.0–15.6 years), were included. Breeds comprised 32 domestic shorthairs (DSHs), four Maine Coons, two Kurilian Bobtails, one British Shorthair and one Siberian. The cats were considered healthy based on history and results of physical examination, complete blood count (CBC), biochemistry profile and urinalysis. None of the cats had received any medications for at least 3 months before enrolment in the study except for routine vaccination and ectoparasite or endoparasite prophylaxis. All cats were prospectively included according to the study protocol, which was

approved by the Scientific Ethics Committee of the University of Bologna (no. 747/2016).

Cats with naturally occurring hypercortisolism and primary hypoadrenocorticism Ten cats with HC and 11 with PH were retrospectively identified from the medical records of three referral institutions – (1) Veterinary Teaching Hospital, University of Bologna, Bologna, Italy; (2) Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; and (3) Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands – which use the same method for measuring plasma ACTH (Immulite 2000 ACTH).

Cats with HC included six castrated males and four spayed females with a median age of 12.4 years (range 6.10–15.0 years). Breeds comprised eight DSHs and two Persians. Cats were considered to have been appropriately diagnosed with HC if clinical signs and laboratory findings were consistent with the disease and at least one of the following endocrine tests yielded a positive result: (1) low-dose dexamethasone suppression test (LDDST); (2) ACTH stimulation test (ACTHST); or (3) urinary cortisol:creatinine ratio. Differentiation of PDH from ADH was based on the results of the LDDST and the appearance of the adrenal and pituitary glands determined by ultrasonography, CT or both methods. On the basis of the above, PDH was diagnosed in eight cats and ADH in two cats.

Eleven cats with PH, including nine castrated males and two spayed females with a median age of 8.0 years (range 2.0–11.0 years), were retrospectively enrolled. Breeds comprised six British Shorthairs, three DSHs, one Bengal and one Chartreux. Hypoadrenocorticism was diagnosed by means of an ACTHST; the disease was considered primary owing to electrolyte abnormalities (hyponatremia and/or hyperkalaemia) and high plasma ACTH.

The LDDST was performed by injecting dexamethasone (Dexadreson; Intervet) at 0.1 mg/kg IV and collecting blood at baseline and after 4 and 8 h. The ACTHST was performed by collecting blood samples at 0 and 60 mins after the intravenous administration of 0.125 mg tetracosactide esacetate – synthetic ACTH (Synacthen; Novartis).

Cats with OD Thirty cats with OD, including 20 males (18 castrated) and 10 females (seven spayed) with a median age of 10.2 years (range 1.0–19.0 years), were prospectively enrolled in the study. Breeds included 26 DSHs, and one each of Exotic Shorthair, Chartreux, Don Sphynx and Norwegian Forest Cat. The cats had been diagnosed with diabetes mellitus (n = 10), hyperthyroidism (n = 5), gastrointestinal disease (n = 6), chronic kidney disease (n = 2), acute kidney injury (n = 2), hyperaldosteronism (n = 2), central diabetes insipidus (n = 1), hypovolemic

shock (n = 1) and septic shock (n = 1). All cats underwent a complete work-up, including physical examination, CBC, biochemistry profile and urinalysis. The decision on whether to carry out additional diagnostics (ie, diagnostic imaging, tests for concurrent diseases) was the responsibility of the clinician managing the case.

Sample collection and processing
Blood samples for the determination of plasma ACTH were collected from the jugular or cephalic vein into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500 g for 8 mins, and plasma was immediately transferred to plastic tubes, stored at 4°C and analysed within 8h or, alternatively, stored at −80°C and thawed at room temperature immediately before analysis. All analyses, except for retrospectively collected data (ie, cats with HC and PH), were performed in duplicate and the results averaged.

To determine the effect of aprotinin on ACTH degradation, three sets of blood samples with different ACTH concentrations collected with and without aprotinin (150 kallikrein inhibitor units aprotinin/ml whole blood) were assayed over a 6-day period. The plasma from each sample was divided into six aliquots, stored at 4°C and measured on days 0, 1, 2, 3, 4 and 5 after blood collection.

Statistical analysis
Descriptive statistics were generated to characterise the study population. Continuous variables were presented as mean ± SD or median and range (minimum and maximum value), depending on whether the data were normally or not normally distributed, respectively. Categorical variables were described with frequencies, proportions or percentages. Plasma ACTH values above the upper limit of quantification of the assay were reported as >1250 pg/ml and values below the analytical sensitivity of the assay were reported as <5 pg/ml. The RI of plasma ACTH was calculated using robust methods.[33] The Kruskal–Wallis test with Dunn's post-test was used for comparison of plasma ACTH among groups (healthy, PDH, PH, OD). The two cats diagnosed with ADH were excluded from the analysis. Differences between samples at a given time point with or without aprotinin were compared using a repeated measures ANOVA. Values of $P < 0.05$ were considered significant.

Results
Assay validation and RI
CVs for intra-assay precision for plasma sample 1, plasma sample 2 and plasma sample 3, having different concentrations of ACTH (sample 1: 98.9 ± 4.0 pg/ml; sample 2: 334.2 ± 9.1 pg/ml; sample 3: 539.2 ± 23.2 pg/ml), were 4.1%, 2.7% and 4.3%, respectively. CVs for interassay precision of plasma sample 1, plasma sample 2 and
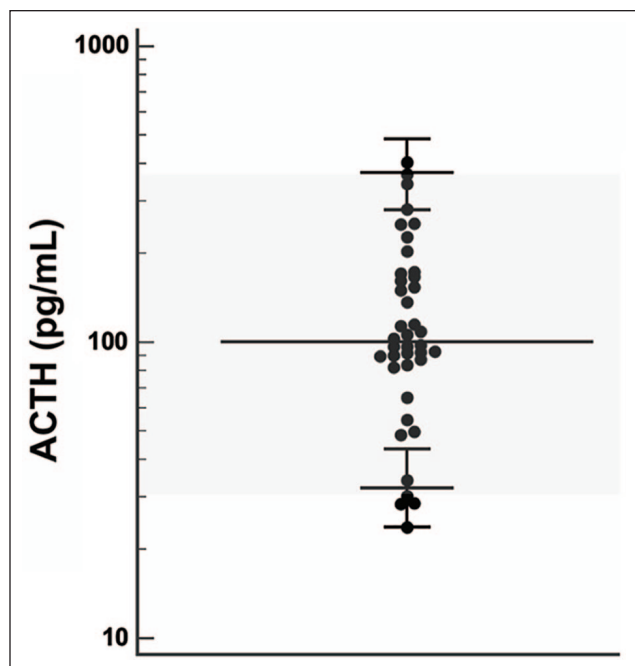
Figure 1 Reference interval for plasma adrenocorticotropic hormone (ACTH) in 40 healthy cats. Horizontal bars represent median value and 90% confidence interval (calculated using robust methods)

plasma sample 3 (sample 1: 58.8 ± 6.3 pg/ml; sample 2: 302.1 ± 21.5 pg/ml; sample 3: 900.2 ± 30.1 pg/ml) were 10.7%, 7.1% and 3.3%, respectively. Linear regression analysis of ACTH assay provided R^2 values of 0.994 ($P = 0.002$) for plasma samples containing moderate concentrations of ACTH and 0.997 ($P = 0.001$) for plasma samples containing high concentrations of ACTH. Recovery of ACTH after spiking ranged from 88.1% to 91.4% of the predicted concentration.

The RI for plasma ACTH was 32–370 pg/ml (median 99 pg/ml; range 24–400 pg/ml) (Figure 1). The 90% confidence interval (CI) of the lower limit was 24–43 pg/ml and the 90% CI of the upper limit was 277–478 pg/ml.

Plasma ACTH
Plasma ACTH results of healthy cats and cats with PDH, PH and OD are reported in Table 1. Plasma ACTH did not show significant differences between healthy and OD groups ($P > 0.99$), and in cats with PDH compared with PH cats ($P > 0.99$). Cats with PDH and PH had significantly higher plasma ACTH values compared with cats in the other two groups ($P < 0.001$). Plasma ACTH in only one of the eight PDH cats (≥ 331, minimum) overlapped with concentrations found in healthy (≤ 400, maximum) and OD cats (≤ 355, maximum). Nevertheless, there were overlapping results between two cats with PDH (> 1250, maximum) and PH cats (> 1250, minimum) (Figure 2). The two cats diagnosed with ADH both showed plasma ACTH values < 5 pg/ml (ie, below the detection limit of the assay).

The plasma ACTH values in the presence and absence of aprotinin were not significantly different in the three sets of samples analysed (cat 1, $P = 0.12$; cat 2, $P = 0.14$; cat 3, $P = 0.07$). As expected, for each set of samples there was a decreasing trend of measured plasma ACTH with time. After only 1 day of storage at 4°C, the decline in measured plasma ACTH ranged from 0.7% to 29% (median 16%). The change over time in plasma ACTH of samples collected with and without the addition of aprotinin is shown in Table 2.

Discussion

The chemiluminescent enzyme immunometric assay used in the present study is precise and accurate for the measurement of ACTH concentration in feline plasma samples. This assay showed acceptable intra- (< 5%) and interassay (< 15%) CVs. Results of dilution studies demonstrated excellent accuracy of the method ($R^2 > 0.99$). The performance of the assay used in the current study compared favourably with the data reported in the literature for the IRMA.[22] It is also worth mentioning that the chemiluminescent assay does not require exposure to radioisotopes and appears to be easy to integrate into laboratory functions. Based on these considerations, it should be recommended to laboratories performing endocrine tests.

In our study, the RI for plasma ACTH in cats was wide (32–370 pg/ml) and different from that observed in dogs (6–58 pg/ml).[34] The reference values reported here appear similar to those reported by Javadi et al,[18] who used an immunoradiometric assay in 130 healthy house cats (ACTH 14–359 pg/ml). The wide distribution of plasma ACTH may be due, at least partly, to the episodic secretion of this hormone from the pituitary gland.[35] Samples may have been collected during a pulse of

Table 1 Range and median values of plasma adrenocorticotropic hormone (ACTH) in healthy cats, and in cats with pituitary-dependent hypercortisolism (PDH), primary hypoadrenocorticism (PH) and other diseases (OD)

Variable (unit)	Healthy	PDH	PH	OD
ACTH (pg/ml)	24–400 (99)*	331–>1250 (690)†	>1250†	22–355 (86)*

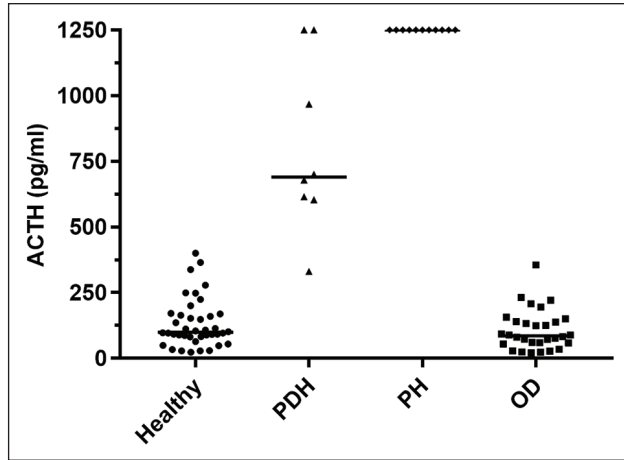Different symbols indicate statistically significant differences between the groups ($P < 0.05$)

Figure 2 Plasma adrenocorticotropic hormone (ACTH) in healthy cats (n = 40), and cats with pituitary-dependent hypercortisolism (PDH, n = 8), primary hypoadrenocorticism (PH, n = 11) and other diseases (OD, n = 30). Horizontal bars represent median values

hormone release. Another important aspect concerns the sensitivity of cats to stress. It has been demonstrated that even relatively mild stresses, such as handling or intradermal skin testing, may cause a sharp increase in plasma concentrations of cortisol, ACTH and α-MSH.[36] For this reason, the stress of transport, handling and blood sampling may have caused high plasma ACTH in some cats. However, for the analysis of the RI, we decided not to remove the outliers, in order to have a result that reflects the real clinical setting.

ACTH is a polypeptide tropic hormone synthesised and secreted by the anterior pituitary gland. ACTH is derived from a high-molecular-weight precursor molecule called pro-opiomelanocortin (POMC). POMC is first processed to pro-ACTH which is, in turn, cleaved into ACTH by the prohormone convertase 1.[37] A recent study in human medicine, involving 87 clinical laboratories, reported a POMC interference in the measurement of

plasma ACTH by immunometric methods.[38] The mean cross-reactivity reported by Monaghan et al[38] for the chemiluminescent assay used in the present study was 2.2% (range 1.1–3.5%). This cross-reactivity was reflected in a large percentage increase in ACTH measured of up to 261% (range 52–261%), depending on the concentration of POMC and the method used. Several studies reported that plasma ACTH precursors (POMC and pro-ACTH) are increased in large and aggressive pituitary tumours in both humans and dogs.[34,39–41] Moreover, a recent investigation demonstrated high plasma ACTH precursors in feline patients with PDH.[21] In the same study, the concentration of plasma ACTH precursors in healthy cats was up to 450 pg/ml. Unfortunately, in our study, the concentration of plasma ACTH precursors was not evaluated. However, considering the above, it is possible that POMC-derived peptides might have interfered with the ACTH measurement by the assay.

In the present study, we were able to demonstrate that the measurement of plasma ACTH can help in differentiating cats with HC and PH from those with mimicking diseases and healthy cats. Plasma ACTH did not show significant differences between healthy cats and the OD group, suggesting that the influence of disorders other than HC or PH on the plasma concentration of ACTH might be considered marginal. Cats with PDH and PH showed plasma ACTH significantly higher than the other two groups and only one PDH cat had a plasma ACTH within the RI.

In dogs with suspected HC, the most common problem with the ACTH assay is poor sensitivity,[42] as often the concentrations of ACTH do not differ between healthy dogs and those with PDH.[43,44] Interestingly in cats, unlike the canine species, the ACTH assay exhibited a greater sensitivity. In fact, in the present study, 87% of cats with PDH (n = 7/8) showed a plasma ACTH above the normal RI. It is also noteworthy that, in the current study, all cats with PH (n = 11) showed a plasma ACTH greater than the upper limit of quantification of the method

Table 2 Change in plasma adrenocorticotropic hormone (ACTH) over time in three sets of samples collected with and without the addition of aprotinin (150 KIU/ml), stored at 4°C and assayed over a 6-day period

Time interval (day)	ACTH in cat 1, which had PDH (pg/ml)		ACTH in cat 2, which was healthy (pg/ml)		ACTH in cat 3, which had PDH (pg/ml)	
	Aprotinin	No aprotinin	Aprotinin	No aprotinin	Aprotinin	No aprotinin
T0	720	765	63	58	955	941
T1	659 (−8.5%)	638 (−16%)	53 (−16%)	57 (−1%)	677 (−29%)	668 (−29%)
T2	564 (−21%)	533 (−30%)	54 (−15%)	44 (−23%)	592 (−38%)	495 (−47%)
T3	519 (−28%)	419 (−45%)	43 (−32%)	32 (−45%)	514 (−46%)	417 (−56%)
T4	473 (−34%)	361 (−53%)	41 (−35%)	30 (−48%)	317 (−67%)	376 (−60%)
T5	393 (−45%)	299 (−61%)	32 (−49%)	25 (−57%)	271 (−72%)	241 (−74%)

Data in parentheses represent the percentage of change in hormone concentration compared with T0
KIU = kallikrein inhibitor units; PDH = pituitary-dependent hypercortisolism

(>1250 pg/ml). However, such clear differentiation between healthy cats and those with adrenocortical-axis disorders may, at least partly, reflect the low number of cases included. It is not possible to exclude the possibility that more cats with PDH or PH would have shown a plasma ACTH concentration within the normal RI if a greater number of cases had been included.

Data from the present study support the proposal that the measurement of plasma ACTH in cats may be regarded as a useful test and should always be part of the diagnostic work-up when HC or PH are suspected. Furthermore, our results suggest that measurement of plasma ACTH may be an accurate test, as in dogs,[44] for differentiating PDH from ADH. The advantages of measuring plasma ACTH include the requirement of a single sample and the test can definitively diagnose ADH. Otherwise, pulsatile ACTH secretion, the sensitivity of cats to stress and inappropriate sample handling allowing ACTH degradation increases the likelihood of a false-positive result in healthy cats. For this reason, it is strongly suggested that a positive result is confirmed through the use of further diagnostic tests (ie, LDDST, ACTHST or diagnostic imaging). However, further prospective investigations involving a large cohort of cats are necessary to determine the exact performance of the ACTH assay and its clinical utility.

Previous investigations in humans[30,31] and dogs[23,29] have suggested that the use of aprotinin, a protease inhibitor, may help prevent ACTH proteolytic degradation after sample collection. In our study, plasma ACTH values in the presence and absence of aprotinin were not significantly different in the three sets of samples analysed. These results might be explained by the fact that the proteolytic properties of plasma may vary among different species and, conceivably, the concentration of aprotinin used in the present study was not an adequate volume to prevent the enzymatic degradation of ACTH in the feline patient. Based on our findings, if plasma samples are handled appropriately (ie, collected and maintained under cold conditions until analysis), the presence of aprotinin might not be necessary for avoiding ACTH degradation. However, the results described here need to be confirmed in a prospective study including a greater number of samples for analysis.

The main limitation of the current study is the small number of cases included. This is partly due to the fact that diseases affecting the adrenocortical axis are rarer in cats compared with dogs. A further limitation is that the performance of the chemiluminescent assay was not directly compared with a previously validated IRMA method.

Conclusions

The results of the present study demonstrate that the Immulite chemiluminescent assay is a valid technique for measuring ACTH concentration in feline plasma samples. The RI of feline plasma ACTH is wide and different from that of the dog. Owing to the low overlap between healthy cats or cats with OD and those with PH or HC, the measurement of plasma ACTH appears to be useful and should be included in the diagnostic work-up when PH or HC are suspected. In addition, plasma ACTH measurement appears to be an accurate test for determining the aetiology of feline HC.

ORCID iD Federico Fracassi https://orcid.org/0000-0003-3121-2199

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