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Influence of Collection and Storage Conditions on Adrenocorticotropic Hormone (ACTH) Measurements in Bottlenose Dolphins (*Tursiops truncatus*)

Lisa Lewis,[1] Stephen V. Lamb,[2] Adam M. Schaefer,[3] John S. Reif,[4] Gregory D. Bossart,[3, 4] and Patricia A. Fair[1]

[1]*NOAA's Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, 219 Fort Johnson Road, Charleston, SC 29412, USA*
E-mail: pat.fair@noaa.gov
[2]*Animal Health Diagnostic Center, Endocrinology Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*
[3]*Harbor Branch Oceanographic Institute, Florida Atlantic University, Fort Pierce, FL 39446, USA*
[4]*Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, USA*
[5]*Georgia Aquarium, 225 Baker Street, NW, Atlanta, GA 30313, USA*

Abstract

Identifying the effects of stressors on marine mammal health is critical for species conservation and collection. Adrenocorticotropic Hormone (ACTH) is a key indicator of peracute stress; however, handling methods may affect results. Thus, the aim of this study was to examine the effects of collection methods and storage temperature on the stability of bottlenose dolphin (*Tursiops truncatus*) ACTH measurements. Blood was collected from five managed-care bottlenose dolphins in four vacutainers™ in the following sequence: EDTA, sodium heparin, serum separator, and 2nd EDTA. The specimens were centrifuged, processed immediately, aliquoted into plasma and serum, and divided into four equal volumes. These aliquots were immediately stored at either 4º or -80º C for 24 h prior to the ACTH assay. ACTH concentrations were compared between the collection tube types and the storage temperatures. There was a significant difference in ACTH concentration across collection tube types. ACTH concentrations were highest in ethylenediaminetetraacetic acid (EDTA) tubes and lowest in serum separator tubes at both temperatures. Twenty-four hour storage temperature did not have a statistically significant impact on mean ACTH concentration. There was no significant difference in ACTH concentration between the first and last drawn EDTA tubes. Bottlenose dolphin whole blood should be collected in EDTA for ACTH measurement. Samples may be refrigerated and shipped on cold packs if assayed within 24 h of collection or immediately frozen and shipped. When multiple samples are collected in various collection tubes at the same time, the order in which tubes are filled does not affect the ACTH measurement.

Key Words: Adrenocorticotropic Hormone, ACTH, collection method, anticoagulant, EDTA, heparin, temperature, bottlenose dolphins, *Tursiops truncatus*

Introduction

Identifying the effects of stressors on marine mammal health is critical for species conservation as well as for evaluation of environmental health and ultimately human health (Fair & Becker, 2000; Bossart, 2011). Adrenocorticotropic Hormone (ACTH) and related hormones have been assessed in managed-care and free-ranging bottlenose dolphins (*Tursiops truncatus*) and other marine mammals as measurements of the stress response (Ortiz & Worthy, 2000; Forney et al., 2002; Schmitt et al., 2010; Tripp et al., 2010; Houser et al., 2011). The role ACTH plays in the adrenal mediated response in bottlenose dolphins is similar to that in other mammals (St. Aubin & Dierauf, 2001). The stress stimulus results in the secretion of Corticotropin Releasing Factor (CRF) from the hypothalamus and activation of the HPA axis. The subsequent secretion of ACTH from the pituitary gland acts on the adrenal gland, stimulating the secretion of cortisol, which, in turn, results in multiple metabolic and subsequent immunologic effects. Circulating ACTH levels rise within minutes of exposure to a peracute stressor, one with durations of 4 h or less followed by a return to baseline values; while cortisol levels do not peak for 1 to 4 h (Mostle & Palme, 2002; Reeder & Kramer, 2005). Therefore,

ACTH is expected to be preferred to cortisol as an indicator of peracute stress when sample collections occur immediately following exposure to the stress event. This has been confirmed in the Florida manatee (Tripp et al., 2010). Further, studies in marine mammals have shown that ACTH elicits a greater magnitude of the mineralocorticoid aldosterone than it does in terrestrial mammals (St. Aubin & Dierauf, 2001).

ACTH in blood is inherently unstable due to its susceptibility to proteolytic degradation (Meakin et al., 1960; Ellis et al., 2003). The degradation and inactivation of ACTH has been well-known for many years, and care is needed in the collection of blood specimens for this assay (Meakin et al., 1960). However, systematic studies evaluating pre-analytical variability on ACTH assays in marine mammals is lacking. Currently, there are no data available on standardized specimen collection, processing, handling, and storage protocols for ACTH analysis in bottlenose dolphins. Information does exist on pre-analytical variability of ACTH in humans and several other species, including horses (Perkins et al., 2002), dogs (Hegstad et al., 1990), and fish (Sumpter & Donaldson, 1986). Studies comparing commonly used anticoagulants and serum on the stability of human ACTH found a significant negative effect on ACTH levels with heparin compared to EDTA. No significant difference was noted between serum and EDTA when samples were frozen immediately after collection (Evans et al., 2001). No difference in stability of ACTH was noted whether samples were collected in glass tubes, siliconized glass tubes, or plastic tubes (Preissner et al., 2004). However, plastic tubes decrease interference from hemolysis, and hemolysis has been shown to negatively affect ACTH (Preissner et al., 2004; Livesey & Dolamore, 2010). Knowledge of the effects of anticoagulants and serum on ACTH in bottlenose dolphins will aid in determining a recommended collection protocol. In addition, this information will allow a better understanding of previously analyzed samples and comparisons of data sets using differing protocols.

Temperature is another pre-analytical factor shown to affect ACTH measurements. When human EDTA samples incubated at 4º C for 24 h were compared to EDTA samples maintained at -20º C, the incubations at 4º C showed a significant negative effect on ACTH level (Evans et al., 2001). Currently, according to the UK website, www.assayfinder.com, 18 of 18 laboratories recommend freezing human plasma samples following collection and transporting samples in a frozen state for ACTH analysis. Collection of samples in the field requiring immediate freezing and frozen transport may be problematic logistically and adds expense and labor to testing. If collection occurs in a remote setting, these pre-analytical requirements may completely eliminate the ability to assess ACTH in some bottlenose dolphin populations. Therefore, it would be beneficial to determine the temperature requirements of samples collected for ACTH assay in bottlenose dolphins. In addition, if ACTH remains stable long-term in frozen samples, ACTH may be evaluated retrospectively through the use of frozen banked samples. In the present study, we examined the effects of commonly used anticoagulants and serum, as well as pre-analytical temperature, on ACTH measurement in bottlenose dolphins.

Methods

Blood Collection and Experimental Design

Samples were collected from five bottlenose dolphins at Sea Life Park by Dolphin Discovery, Waimanalo, Hawaii. Animals were trained to present their flukes to facilitate the collection of samples. Blood samples were drawn from the peri-arterial venous rete in the flukes using a 19-gauge needle and a 1.9-cm butterfly catheter with a vacutainer™ attachment (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA). Ten mL of whole blood was collected into the following collection tubes: sodium heparin, ethylenediaminetetraacetic acid (EDTA), and serum separator vacutainers™ (Becton, Dickinson, and Co.). The collection tubes were filled in the following order: EDTA-1, heparin, serum separator, and 2nd EDTA (EDTA-4). All tubes were gently inverted, and EDTA and heparin vacutainers™ were centrifuged immediately at 1,200 g for 15 min. Plasma from each vacutainer™ was transferred in 1 mL volumes to four cryovial transport tubes (Corning, Acton, MA, USA). Two cryovials from each EDTA and heparin sample were refrigerated following collection and shipped on cold packs. The remaining two cryovials from each EDTA and heparin sample were frozen to -80º C and shipped frozen on dry ice. The serum separator vacutainer™ tubes were allowed to clot for approximately 30 min and centrifuged at 1,200 g for 5 min. Serum from each vacutainer™ was transferred in 1 mL volumes to four cryovial transport tubes. Two of the cryovials were refrigerated and shipped on cold packs. The remaining two cryovials were frozen to -80º C and shipped frozen on dry ice. All samples were shipped overnight and analyzed approximately 24 h after collection.

ACTH Analysis

Samples were analyzed at the Animal Health Diagnostic Center (AHDC) Endocrinology Laboratory at the Cornell University College of Veterinary Medicine in Ithaca, New York. ACTH was measured using a solid-phase, two-site

sequential, chemiluminescent enzyme immunometric assay (Immunlite ACTH, Siemens Healthcare Diagnostics, Los Angeles, CA, USA). The reagents are specifically designed for use with the Immulite 1000® automated analyzer. This assay is used regularly for clinical diagnostic quantification of ACTH in unextracted equine (Perkins et al., 2002) and canine (Scott-Moncrieff et al., 2003; Galac et al., 2005) plasma samples by the AHDC Endocrinology Laboratory and has been used in research applications for other species primarily with equine samples (Donaldson et al., 2005; Gold et al., 2007). The analytical sensitivity of the assay is 9 pg/mL, and the calibration range is up to 1,250 pg/mL. Immunological specificity was evaluated by diluting three separate bottlenose dolphin EDTA plasma pools 3:4, 1:2, 1:4, 1:8, and 1:16. The percent observed over expected for each diluted pool with initial concentrations of 110.0, 78.3, and 68.2 pg/mL was 95, 94, and 92% with an overall mean of 94%. Intra-assay was evaluated using three bottlenose dolphin EDTA plasma pools each pipetted separately from ten separate sample cups by the instrument. Also, one of these pools was also pipetted five times each from two separate sample cups. The percent coefficient of variation (% CV) of the bottlenose dolphin plasma pools with mean values of 126.6, 73.3, and 31.4 pg/mL was 4.8, 5.0, and 6.1%. The one pool retested a total of ten times from two sample cups had a mean value of 72.7 pg/mL and a % CV of 5.1%. Inter-assay precision at the time of this study for two canine (n = 103 each) and one equine (n = 51) control samples tested on separate days with mean values of 52.0, 294.2, and 115.1 pg/mL was 6% for each, respectively.

Statistical Analysis

A repeated measures analysis of variance (ANOVA) was used to compare ACTH concentration for each collection tube type and storage temperature to determine the potential effect of these variables on ACTH concentration. The sphericity assumption for the repeated measures test was not met, so the Greenhouse-Geisser correction was used. All analysis was conducted using IBM *SPSS Statistics 20* for Windows (IBM Corporation, Armonk, NY, USA). A p value of < 0.05 was considered significant.

Results

There was a significant difference in ACTH concentration across collection tubes (p = 0.04) and for tubes held at 4º and -70º C (Table 1). However, there was little intra-individual variability among individual bottlenose dolphins across temperature conditions for EDTA collected samples (Figure 1). Mean EDTA-1 concentrations (25.85 pg/mL) were significantly higher than those for heparin (18.62 pg/mL) and serum (16.76 pg/mL) collected samples. The mean EDTA-4 results (25.24 pg/mL) were also significantly higher than heparin and serum collection tubes. Storage temperature at 4º or -70º C did not have a statistically significant impact on mean ACTH concentration (p = 0.12) (Table 1).

Discussion

Among hormone analytes, ACTH has been the most problematic with stability (Diver et al., 1994). Results from our study indicate that the pre-analytical stability of ACTH in bottlenose dolphins varies with the type of collection tube. Similar to human studies, ACTH levels found in bottlenose dolphin plasma samples obtained in sodium heparin tubes are significantly lower compared to plasma samples obtained in EDTA. Degradation by blood enzymes is one cause for instability of ACTH, and EDTA is known to protect peptides from proteolysis through its chelating properties. Whereas no difference was identified between plasma EDTA and serum in human ACTH assays, results from our study found ACTH in serum to be significantly lower compared to EDTA plasma samples in the bottlenose dolphin. Differences in protein content and enzyme activity between serum and plasma may explain this result. Serum

Table 1. Mean and standard deviation of ACTH concentrations (pg/mL) by blood collection tube type and temperature (4º and -70º C)

	Storage temperature	
Collection tube	4º C	-70º C
EDTA-1	25.85 ± 6.88	25.98 + 7.45
Heparin	18.62* + 4.88	20.94* + 6.81
Serum	17.76* + 4.42	16.10* + 4.62
EDTA-4	25.24 + 8.82	27.78 + 8.11

*Indicates significant differences between tubes held at 4º and -70º C ($p < 0.05$)

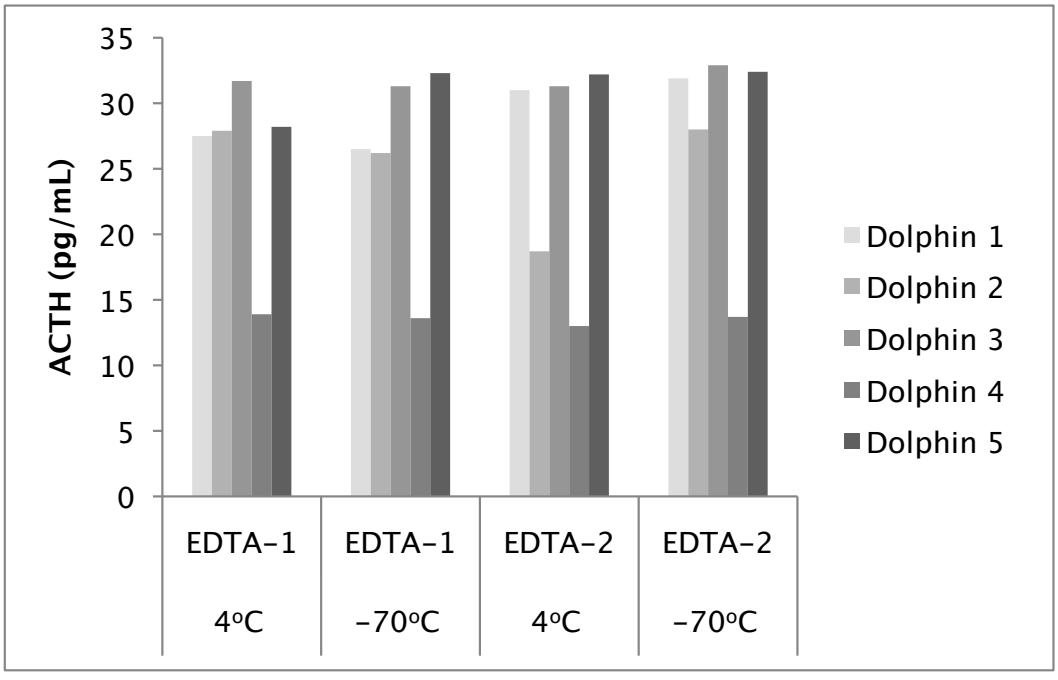

Figure 1. Mean ACTH concentrations (pg/mL) by EDTA-1 and EDTA-4 collection tube and temperature (4º and -70º C)

lacks the coagulation factors found in plasma. Therefore, serum has lower total protein content than plasma. Differing protein contents have been shown to result in differences in immunoassay results (Bielohuby et al., 2012). In addition, enzyme activity is lower in plasma than serum. Therefore, hormones affected by enzymatic degradation would be less stable in serum due to the higher enzyme activity (Bielohuby et al., 2012). EDTA has been shown widely to be the preferred anticoagulant for ACTH measurement in humans (Evans et al., 2001), dogs (Hegstad et al., 1990), and horses (Perkins et al., 2002), and also fish (Sumpter & Donaldson, 1986). In our study, there was no difference in ACTH levels between the EDTA tubes collected first vs the EDTA tubes collected following collection in heparin and serum separator tubes. Therefore, there was no effect on ACTH levels as a result of inter-tube contamination. We can also conclude there was no significant physiological change in the study of bottlenose dolphins' ACTH levels across the brief collection period.

In addition to the type of collection tube, several studies have demonstrated that both temperature and time are important variables that have an impact on ACTH measurements. The levels of several hormones are compromised by a delay in the plasma separation from blood, and ACTH is one of those most influenced (Ellis et al., 2003). One study examined multiple hormones and found that collection of human samples into EDTA, and storage and transport at 0º to 4º C was suitable for all hormones except for ACTH and that the other hormones were stable for greater than 120 h at 4º C (Evans et al., 2001). Our results show no difference in ACTH levels between bottlenose dolphin samples maintained at 4º C for 24 h prior to testing compared to samples maintained at -80º C for 24 h. These findings were consistent for EDTA, sodium heparin, and serum samples. Our results differ from those found in humans for which ACTH remained stable for 18 h in EDTA at 4º C and for 3 h in serum at 4º C with immediate centrifugation (Preissner et al., 2004). Significant changes were observed for ACTH in the plasma fraction of human blood that is held at 4º or 24º C for up to 24 h before separation (Ellis et al., 2003). However, other human studies have shown that immediate centrifugation of plasma samples prolongs the stability of ACTH to 24 h even when stored at room temperature (Reisch et al., 2007). ACTH was found to be stable in whole blood from horses when held in plastic tubes for 8 h prior to separating the plasma (Perkins et al., 2002). Optimal storage handling for ACTH measurement in dogs is EDTA anticoagulant and blood collected followed by centrifugation within 15 to 90 min and plasma stored in plastic containers for no longer than 1 mo at -20º C (Hegstad

et al., 1990). Similar recommendations were made for horses with collection in an EDTA tube but plasma separated within 2 h and stored in a plastic tube with storage at -70º C (Perkins et al., 2002). Further studies are needed to determine the effect on ACTH levels of bottlenose dolphin samples stored at 4º C for periods longer than 24 h. In addition, the length of time ACTH remains stable in frozen samples would be valuable information but was outside the scope of this study.

Based on our results, we make the following recommendations on ACTH assay sample collection in bottlenose dolphins. Whole blood should be collected in EDTA and centrifuged immediately following collection. Samples may be refrigerated and shipped on cold packs if assayed within 24 h of collection. If time to assay is longer than 24 h, freezing and shipping frozen samples would be a suitable alternative. When multiple samples are collected in various collection tubes at the same time, the order in which tubes are filled does not affect the ACTH assay.

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