**Unreliability of EDTA samples for measuring bioamine neurotransmitter levels in cats**

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**Abstract**

*Objective*: To assess the reliability of an EDTA-based method to measure cat blood bioamines

*Methods:* Test 1: Blood samples of 7 university laboratory cats were collected and transferred to EDTA, heparin and plain tubes to determine concentrations of four bioamines (serotonin, dopamine, epinephrine and norepinephrine). Correlation of measurements performed on EDTA plasma, with those on heparinised plasma or serum were assessed by intraclass correlation coefficients. Test 2: blood samples from 5 owned cats were collected and stored in EDTA tubes and divided between duplicate Eppendorf tubes labelled as different cats for blinding purposes and analysed independently for the same four bioamines as in Test 1. Reliability of concentration determination for these duplicates was assessed from intraclass correlation coefficients (ICC) and coefficients of variation (CV) values.

*Results:* In Test 1, there was no significant correlation between the EDTA plasma serotonin and serum serotonin concentrations. There were also no significant correlation between EDTA plasma and heparin plasma sample concentrations for either epinephrine or norepinephrine. There was a statistically significant but weak correlation between EDTA plasma and heparin plasma dopamine concentrations. In Test 2, there was no correlation for repeat-analysed serotonin and epinephrine concentrations. Although there were statistically significant correlations for dopamine and norepinephrine, coefficients of variation for each analyte were in excess of 30%.

*Conclusions and Relevance:* Before any further attempt is made to measure and report neurotransmitter concentrations in domestic cats, it is essential that the robustness of the methodology is carefully validated and these data presented at publication.

**Key-words:** feline, chromatography, catecholamines, serotonin, emotion

**1. INTRODUCTION**

Historically much research has focused on the use of glucocorticoids as physiological markers of stress in several biological animal matrices (1, 2). Measurement of bioamines such as serotonin and norepinephrine to assist in the evaluation of animals’ emotional states is a more recent phenomenon and the reliability of the available methods to evaluate them in non-human animals remains largely unknown; such methods are routinely validated for human samples. As part of a larger study into feline housesoiling and emotionality in which faecal glucocorticoids and bioamine were measured (3), cat blood samples were collected into EDTA, heparin and plain blood tubes for potential cross validation of blood bioamine level in the samples.

**2. METHOD**

Approximately 6 ml of blood was collected from the jugular vein of 12 cats who were fasted for 12 hours (7 university laboratory cats were used in Test 1 and 5 cats belonging to the research team were used in Test 2 – informed consents were gathered from all of them). The cats were very friendly to humans and thus did not impose difficulties during blood collection. Some of this blood was used for haematology and serum biochemistry in order to confirm general good health status of the participant cats. The remainder (approximately 4 ml) was transferred to EDTA, heparin and plain tubes for Test 1 to determine blood bioamine levels, or to EDTA tubes only for Test 2. Given some bioamines are photosensitive, tubes were protected from light by aluminium foil. Within about 15 minutes of collection, the tubes were centrifuged for 15 minutes under refrigeration (4 degrees C), at 3000 rpm. The supernatant was then transferred into amber Eppendorf tubes (approximately 1 mL in each). The Eppendorf tubes were then stored in a freezer (-80 degrees C) for a few days until analysis. The following two reliability tests were then performed:

Test 1 – *Effect of blood tube type* – Samples from the 7 university laboratory cats were stored in both EDTA and heparin tubes (for measuring dopamine, epinephrine, and norepinephrine) or EDTA and plain tubes (for measuring serotonin). Correlation between EDTA samples and either heparin or plain tube samples were assessed using intraclass correlation coefficients (ICC).

Test 2 – *Duplicate samples test* – Samples from 5 cats belonging to the research team were stored in a similar way in EDTA tubes and divided between two duplicate Eppendorf tubes which were then labelled as different cats for blinding purposes and analysed independently for the same four bioamines as Test 1. Researchers responsible for the laboratory analyses were not aware of the duplicate procedure. In order to test for reliability, concentrations from these duplicates were compared using intraclass correlation coefficients (ICC) and calculation of their coefficients of variation (CV).

Bioamine concentrations were determined by the quality assured Nephrology Department (Sao Paulo Federal University, Brazil) using high performance liquid chromatography (HPLC). In the case of catecholamines they were measured using ion-pair reverse phase chromatography coupled with electrochemical detection. Fast isocratic separation was obtained using an RP 18 Aquapore cation F micron, Brownlee Column (Applied Biosystems, San Jose, CA) (4.6-250mm) eluted with the following mobile phase: 0.02 M sodium dibasic phosphate, 0.02 M citric acid, pH 2.64, 10% methanol, 0.12 mM Na2EDTA, and 566 mg/L heptanesulfonic acid. The total time for sample analysis was 30 min. Plasma (500 μL) was previously submitted to the following purification steps: 50 mg Al2O5 were weighed out in centrifuge tubes and the samples were added in Tris-buffer, pH 8.8, plus 40 ml (8 ng) DHBA (internal standard, dihydroxybenzylamine). The suspension was vortex-mixed for 10 min. The precipitated alumina was washed three times and vortex-mixed with 1 ml water, and the catecholamines were eluted with 400 ml 0.1 M perchloric acid after 3 min of vortex mixing. After centrifugation for 3 min at 1500g, the supernatant was filtered and 100 ml were injected into the reverse phase column. The monoamine concentration was expressed as pg/mg cell protein.

Serotonin was also determined by using ion-pair reverse phase chromatography coupled with electrochemical detection. The separation was obtained using a Chromolith SpeedROD 50-4.6mm reverse phase column (Merck). The HPLC system consisted of a pump (model LC-10 ADVP-Shimadzu), electrochemical detector (model L-ECD-6A-Shimadzu), automatic injector (SIL-20AC-Shimadzu) mm). Serotonin was separated by isocratic elution, flow of 1.0 ml / min for approximately 10 minutes, the mobile phase being composed of 0.02M sodium phosphate (Merck), 0.02M sodium citrate (Merck), EDTA 40 mg (Merck), 5 mg (Sigma) heptane sulfonate and 10% methanol (Licrosolv) with pH adjusted to 2.53 by the addition of 50% perchloric acid (Merck). Plasma (100 μL) was deproteinized with 50 μL ice cold 0.34 M perchloric acid, vortexed for 1 minute, followed by freezing (liquid nitrogen) for 5 minutes and centrifuged at 10000 rpm at 4 degrees C for 40 minutes. 894 μL of sodium disulfite and 66 μL of internal standard (DHBA diluted 100-fold from the 1 mg / mL stock in perchloric acid) were then added to 40 μL of the supernatant. 100 μL of the sample was injected into the reverse phase column. The serotonin concentration was expressed as pg/mg cell protein.

Both sample extraction and HPLC separation were performed on the same day, thus avoiding any interassay variation associated with differences in time to analysis. Furthermore, DHBA constitutes an internal standard for calculation and control purposes in relation to HPLC.

This method has been reported to be both reliable and valid for human blood samples (4,5).

**3. RESULTS**

*Effects of blood tube type* – there was no correlation between plasma EDTA and serum concentrations of serotonin (ICC=0.351, p=0.180). EDTA samples had a reported median concentration of serotonin of 731.93 ng/ml, compared to a value of 549.93 ng/ml for samples stored in plain tubes. There was no correlation between EDTA and heparin plasma measurements of epinephrine (ICC=0.147, p=0.351) and norepinephrine (ICC=0.039, p=0.454). The median concentration of epinephrine was 303.23 pg/ml for EDTA samples and 393.69 pg/ml for heparin samples; and for norepinephrine median concentrations were 366.79 pg/ml versus 350.89 pg/ml. Although there was a significant correlation between EDTA and heparin plasma measurements for dopamine (ICC=0.781, p=0.012), the correlation was considered weak. EDTA samples had a median reported concentration of dopamine of 235.03 pg/ml compared to a value of 193.29 pg/ml for heparinised samples.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Cat ID | **Sero plain**  **(ng/ml)** | **Sero EDTA**  **(ng/ml)** | **Dopa\* heparin**  **(pg/ml)** | **Dopa\* EDTA**  **(pg/ml)** | **Epi heparin**  **(pg/ml)** | **Epi EDTA**  **(pg/ml)** | **Nor herapin**  **(pg/ml)** | **Nor EDTA**  **(pg/ml)** |
| ***CAT 1*** | 433,14 | 731,93 | 548,08 | 412,32 | 430,71 | 336,86 | 329,02 | 501,49 |
| ***CAT 2*** | 805,56 | 534,19 | 120,26 | XXX | 404,89 | 442,16 | 356,26 | 466,76 |
| ***CAT 3*** | 843,66 | 1167,81 | 137,18 | 125,49 | 244,11 | 303,23 | 350,89 | 348,06 |
| ***CAT 4*** | 373,73 | 1175,79 | 92,85 | 121,46 | 331,59 | 151,85 | 300,92 | 384,11 |
| ***CAT 5*** | 549,93 | 453,49 | 249,41 | 260,57 | 388,24 | 177,99 | 399,05 | 366,79 |
| ***CAT 6*** | 380,71 | 567,83 | 302,75 | 209,50 | 393,69 | 259,96 | 289,13 | 215,81 |
| ***CAT 7*** | 1105,04 | 1300,58 | 123,13 | 279,30 | 453,1 | 384,76 | 399,23 | 218,60 |

Table 1. Results of the Effects of Blood tube type Test. Samples from the 7 university laboratory cats were stored in both EDTA and heparin tubes (for measuring dopamine, epinephrine, and norepinephrine) or EDTA and plain tubes (for measuring serotonin). Sero: serotonin, Dopa: dopamine, Epi: epinephrine, Nor: norepinephrine. \*represents a significant positive correlation between dosages in the two samples. XXX represents undetermined concentration for this sample.

*Duplicate samples test* – Serotonin (ICC=0.00, p=0.915) and epinephrine (ICC=0.00, p=0.664) showed no correlation, with CV of around 55% and 67% respectively (Table1). Although there was a significant correlation for the concentrations of dopamine (ICC=0.714, p=0.038) and norepinephrine (ICC=0.962, p< 0.001), the CV for both was in excess of 30% (see Table 1 for full data).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Cat ID | **Sero 1**  **(ng/ml)** | **Sero 2**  **(ng/ml)** | **Dopa 1\***  **(pg/ml)** | **Dopa 2\***  **(pg/ml)** | **Epi 1**  **(pg/ml)** | **Epi 2**  **(pg/ml)** | **Nor 1\***  **(pg/ml)** | **Nor 2\***  **(pg/ml)** |
| ***F*** | 896.62 | 1634.95 | 367.14 | 615.97 | 30.19 | 377.28 | 1417.30 | 1297.60 |
| ***G*** | 216.15 | 1916.51 | 271.65 | 204.79 | 232.39 | 133.88 | 693.76 | 812.26 |
| ***E*** | 1289.75 | 1749.23 | 166.82 | 74.39 | 77.68 | 76.34 | 145.65 | 40.32 |
| ***N*** | 1527.56 | 1305.72 | 205.52 | 138.27 | 286.07 | 258.15 | 811.79 | 644.39 |
| ***C*** | 1505.08 | 1617.95 | 133.14 | 107.74 | 242.39 | 67.25 | 275.21 | 424.09 |
| ***CV (%)*** | 54.81 | | 33.46 | | 66.95 | | 39.37 | |

Table 2. Results of the Duplicate Samples Test. A single sample was taken from each cat and split into two (indicated by the numbers 1 and 2). Sero: serotonin, Dopa: dopamine, Epi: epinephrine, Nor: norepinephrine. \*represents a significant positive correlation between dosages in the two samples. CV(%) = Coefficient of Variation calculated as a percentage based on the root mean square approach.

**4. DISCUSSION**

Using HPLC to measure feline bioamines in samples stored in EDTA tubes appears to be unreliable, with little correlation and very large CV detected in even the same sample when analysed twice. Thus it should not be assumed that a method used to assay human samples, will necessarily be valid for non-human animals. Although our results have focused on EDTA samples, without undertaking a similar duplicate test procedure, it cannot be assumed that the same problem is not present in either lithium heparinised plasma or serum. The poor correlation between samples cannot be explained by intra-cat variability as compared values in both tests came from the same cat samples transferred to different tubes; great inter-cat variability particularly for serotonin is present, though.

We hypothesize that a problem just affecting the EDTA samples exists, but it is possible that it might be a wider problem than is currently recognised with sample storage and the HPLC method used. This is of concern as previous studies correlating behaviour problems with emotionality in pet dogs have used this method for measuring serotonin and catecholamines (6,7) We also note a widespread absence of the CV in published studies examining the relationship between blood or other sources of bioamines and problem behaviour (8). This is a standard metric used to assess the reliability of the analysis and should be considered essential in any such report.

Although it might seem most likely that the source of the problem relates to the antioxidative properties of EDTA being insufficient to prevent oxidative degradation (9), this should not be assumed. Several studies on the stability of catecholamines (including one testing human and cat blood samples) recommend the use of heparinised tubes containing an antioxidant and immediate centrifugation (9, 10, 11), however our results indicate that this should not be assumed to be a reliable method unless appropriate reliability assessments have been made together with a careful evaluation on the influence of pre-analytical procedures on measured bioamines. As noted by Alberghina et al (12) in their study of ELISA kits for measuring serum serotonin in dogs, several pre-analytical factors such as handling, storage time and temperature also need to be considered and standardised in reporting before any method is recommended for use in practice, or comparisons made between studies.

**5. CONCLUSIONS**

Before any further attempt is made to measure and report on neurotransmitter levels in species for which an established method has not been developed, it is essential that the reliability of the methods used are evaluated and reported, rather than assumed from the human data.

ACKNOWLEDGEMENTS - We would like to thank Carine Savalli Redigolo for the statistical analysis. We are also thankful for FAPESP Research Foundation for funding the project and giving a doctorate scholarship to D. Ramos.

CONFLICT OF INTEREST - Authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING – FAPESP Research Foundation - 08/55385-6

ETHICAL APPROVAL – Approval received from the FMVZ-USP Ethical committee.

INFORMED CONSENT – Cat owners provided informed consents when accepted to take part in this research.

INFORMED CONSENT FOR PUBLICATIONS - Cat owners provided informed consents for publication when accepted to take part in this research.

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