IUCN-SSC Crocodile Specialist Group - Student Research Assistance Scheme

Progress Report

Steroid profiling in crocodilian urine and plasma by gas-chromatography/mass spectrometry









Lasya Christina Bekker

University of Pretoria

July 2009

Background of the Study

Steroid profiling by gas-chromatography/mass-spectrometry has been employed for extensive studies of urinary steroid metabolites in humans. However, nothing has been done in the crocodilians. The simultaneous investigation of plasma and urine hormones could provide valuable information on the endocrine axis of the crocodilians. This investigation will aid in the evaluation of the significance of this method and matrix (urine) as an indicator of endocrine disruption.

Steroid profiling in humans (urine) can be indicative of numerous endocrine dysfunctions, including reproductive and thyroid disorders, enzyme deficiencies/excesses, hypo-and hyper-cortisolism (including tumours of the adrenal), starvation, stress and hypertension.

Qualitative and quantitative investigation of steroids in crocodilians may serve as a valuable diagnostic tool in future. Work has already started on the Nile crocodile in South Africa and this investigation is to be continued on the American alligator. I was invited by Louis J. Guillette Jr., Professor, Howard Hughes Medical Institute, to visit the University of Florida in the summer of 2009, to work on our collaborative project that is part of my PhD studies.

Aquatic pollution is an important global concern and crocodilians, as top predators in aquatic ecosystems, are considered to be excellent sentinels of ecosystem health. Most crocodilians are listed by CITES (The Convention on International Trade in Endangered Species of Wild Fauna and Flora) as endangered or threatened, making it extremely difficult and in most cases inappropriate to kill them for research purposes.

Many aquatic pollutants (e.g. pesticides and pharmaceuticals) are bio-transformed by the liver and excreted in the urine, making urine a very useful diagnostic sample for scientists using crocodilians as sentinels. Likewise, the evaluation of a urine sample, from individual human or animal patients, is a well-established diagnostic approach to obtain clinical data for evaluation of health and confirmation of specific diseases (e.g. endocrine system abnormalities). This, in conjunction with plasma hormone evaluation, can provide useful information on the status of the animal.

The focus of this investigation is primarily on endogenous steroid hormones. Aquatic animals respond with great sensitivity to chemical changes in their environment and are considered to be important bio-indicators of pollution (EDC's). They are, therefore, widely used to monitor pollution.

Establishing reference values for healthy animals from non-polluted environments will also aid in future monitoring.

Study set-up

The aim of this study is to evaluate the plasma and urine of pre-selected groups of crocodilians with a standardized gas-chromatographic/mass spectrometric method for the detection of steroids and their metabolites. The expected outcome is the establishment of steroid profiling, using crocodilian plasma and urine, as a diagnostic tool in the investigation of the ECD effects.

Figure 1, 2 & 3 shows the laboratory at UFL where sample preparation was done：



Figure 1



Figure 2



Figure3

Approach and Methodology

The urine sample collection technique was relatively simple and a-traumatic. Crocodilian urine and blood samples were collected, simultaneously, for the investigation. The samples were frozen at -20°C until extraction.

Extraction of steroids from urine and plasma samples was achieved with a five-step sample preparation:

SPE (First extraction): The samples were centrifuged. For each sample, one C18 (octadecyl) solid phase extraction (SPE) cartridge was primed with methanol, and washed with de-ionized water. A volume of each centrifuged sample was measured out, to which the internal standard was added. Each sample was loaded onto its designated cartridge, which was then washed with de-ionized water. The conjugated steroids were eluted with methanol, and the eluates dried under nitrogen gas, in a heating block at 37˚C.

Hydrolysis: To each dried eluate, methanol, a sodium acetate/acetic acid buffer and enzyme was added. The tubes were stoppered and enzymatic hydrolysis allowed to take place overnight at 50 °C.

SPE (Second extraction): The hydrolyzed mixtures were centrifuged. The previously used C18 cartridges were primed and washed as for the first extraction. Supernatants of the hydrolyzed mixtures were aspirated through the C18 cartridges, which were washed with de-ionized water. Each C18 cartridge was connected on top of a NH_2 (aminopropyl) cartridge and again connected onto the vacuum manifold. The free steroids were eluted with ethyl acetate into final tubes and the eluates dried under nitrogen gas.

Derivatization: Methoxylation (60 ºC, 15 minutes), followed by Silylation (100 ºC, 2 hours). The samples were transferred to vials which could be stored at -20°C until GC/MS-analysis could be performed.

Separation and detection: Gas Cromatographic/Mass Spectrometric (GC/MS) analyses, was achieved with a Thermo Polaris Q GC-MS, ion trap technology (Thermo Fisher Scientific, Texas, USA), using a TRACE TR5-MS capillary column (30m X 0.25 id X 0.25 film), (Thermo Fisher Scientific, Runcorn, UK). Data collection and qualtitative reporting was done using Xcalibur software.

GC conditions: Helium (5,5 N) was employed as carrier gas, with a flow rate of 1.2 milliliter per minute. The ion source of the mass spectrometer was set to an electron voltage of 70 eV.

The split method of injection with a split ratio of 30:1 was performed in the chromatographic separations. 1 µl sample was injected by auto injector onto the GC column. The carrier gas (helium UHP) was employed in the splitter-systems. The inlet temperature was set at 250 °C. To determine the split ratio, the measured purge flow was divided by the carrier gas flow rate.

The temperature program was as follows:

- initial isotherm 215 °C for 3 minutes;
- program rate: 1.6 °C per minute up to 226 °C,
- second isotherm at 226 °C for 3 minutes,
- second program rate: temperature ramped at 1.6 °C per minute up to 300 °C;
- final isotherm of 5 minutes.

Figures 4 & 5 show the Polaris Q instrument, and figure 6 shows the data system:



Figure 4: Thermo Polaris Q GC-MS in UFL laboratory



Figure 5: Thermo Polaris Q GC-MS : The Mass Spectrometer (MS) on the left, connected to the Gas Chromatograph(GC) on the right, with the autosampler on the top right corner of the GC.



Figure 6: Thermo Xcalibur data system, showing a chromatogram and mass spectrum of a selected peak.

Progress of the project

- Stored urine and plasma samples from American alligators (Alligator *Mississippienses*), were selected in the research laboratory of the Department of Zoology, University of Florida, Gainsville, FL.

- The selection of urine samples from the data-base in professor Lou Guillette's laboratory, was primarily based on those samples which had sufficient volumes to extract. Urine samples from 22 alligators: 7 from males, who had elevated testosterone levels, 7 from females with high progesterone, and 8 from females with abnormal estradiol levels, were selected, as well as five available matching plasma samples.

- Steroid metabolite standards, as well as testosterone and estradiol standards, were prepared at three concentration levels. Controls were planned (as shown in table 1) to support a smaller amount of enzyme than described in my original method – the amount of enzyme available was a limiting factor in the amount of samples that could be processed, and this was done in case the amount of enzyme added to the samples, had to be reduced.

- The head of the GC-MS laboratory, Dr. Timothy Garret, who was very helpful in getting my instrument set-up and the method optimized, unfortunately had to leave on Saturday, August 1. As the analyses proceeded, the detector sensitivety deacreased and the latter part of the samples require re-analyzing. I did not have the authority to open and clean the ion source, and with Dr. Garrett not present, I continued the injections of the samples without getting desirable results. Upon my return home, I contacted Dr Garret in this regard and he advised that it would not be possibleto re-analyze due to instrument demand.

- I have also contacted the laboratory assistants in Professor Lou Guilette's laboratory regarding creatinine values on the urine and plasma samples, which will enable me to quantify once all the steroid profiles are analyzed successfully.

- The profiles that were successful show very promising information, but it is desirable to have a combination of the results to formulate a conclusion. Arrangements have been made to courier the samples to South Africa for re-analysis. I have recently started collaborating with a facility in South Africa where the analysis can be continued on the same equipment that was used at UFL.

- I am positive that he final results can lead to a publication.

Results

Steroid profiles from the group of 7 male alligators were analyzed successfully. Noisy backgrounds were eliminated by doing both background extraction of the whole total ion chromatogram (TIC), subtracting the blank TIC, as well as the noise adjacent to each peak.

Retention times and spectra of the standards were used to locate and identify the steroid metabolites in the samples.

List of abbreviations:

An	androsterone
DHEA	dehydroepiandrosterone
Et	etiocholanolone
HAn	11-hydroxy androsterone
HEt	11-hydroxy etiocholanolone
KAn	11-keto androsterone
m/z	mass/charge ratio
Pd	pregnanediol
Pt	pregnanetriol
THB	tetrahydrocorticosterone
THE	tetrahydrocortisone
THF	tetrahydrocortisol
α-THF	allotetrahydrocortisol
THS	tetrahydro compound S

Sample preparation data and qualitative results are shown in table 1. Urine samples from the group of male alligators were qualitatively investigated and identified steroid metabolites are indicated.

Table 1: Data on sample preparation and qualitative results

Smpl name	Sample ID	Date	amt extracted	u-creat	smpl pos	steroids identified
test 08	Steroid L1	7/29/2009	1 ug each		4	An, DHEA, Corticosterone
test 09	Steroid L2	7/29/2009	2ug each		5	An, DHEA, Corticosterone
test 10	Steroid L3	7/29/2009	10 ug each		6	An, DHEA, Corticosterone
test 11	S1 ID	7/29/2009	various **		7	Et, Kan, HAn, Pd, a-THF
test 12	S2 ID	7/29/2009	various **		8	THS, Pt, THB, THE, THF
test 13	Blank	7/29/2009	0		9	n/a
test 14	NASA5143	4/18/2008	10 mL	nd	10	HAn
test 15	NASA3092	4/15/2009	32 mL	nd	11	Et , HAn
test 16	NASA3108	4/20/2009	37 mL	nd	12	HAn, Het
test 17	NASA3083	4/13/2009	39 ml	nd	13	HAn, THS
test 18	NASA5310	11/13/2008	11 mL	nd	14	Het
test 19	NASA5152R	5/16/2008	10 mL	nd	15	none
not analyzed	NASA5087	1/29/2008	10 mL	nd	not anlzd	-
test20	NASA5345	12/30/2008	10 mL	nd	16	none
test21	NASA5008R	1/20/2009	10 mL	nd	17	
test22	NASA5316	11/21/2008	10 mL	nd	18	-
test23	NASA5106	2/22/2008	22 mL	nd	19	-
test24	Stigmasterol	7/29/2009	20 ug	nd	20	Stigmasterol
test25	NASA5234	7/18/2008	10 mL	nd	21	-
test26	NASA5314	11/21/2008	9 mL	nd	22	-
test27	NASA5053	4/18/2008	11 mL	nd	23	-
test28	NASA5319	11/21/2008	10 mL	nd	24	-
test29	NASA5155	4/30/2008	11 mL	nd	25	-
test30	NASA3121	4/9/2009	36 mL	nd	26	-
test31	NASA5058	12/01/2007	8.5 ml	nd	27	-
test32	NASA5055	10/24/2007	10 mL	nd	28	-
test33	NASA5053	10/18/2007	10 mL	nd	29	-
test34	NASA5182	5/23/2008	9 mL	nd	30	-
test35	NASA5150	4/25/2008	9 mL	nd	31	-
test36	NASA5365R	no date	19 mL	nd	32	-
test37	NASA5365R	no date	19 mL	nd	33	-
test38	NASA5147	4/23/2008	8.5 mL	nd	34	-
test39/40/41	KtrlZa/b/c	7/29/2009	14 mL	nd	35/6/7	An, Et, DHEA, Kan, HAn, Pd, Pt, THS, THE, THF,a-THF
test42	NASA5067(B)	no date	11 mL	nd	38	-
test43	NASA5062 (C)	no date	9 mL	nd	39	-
test44	NASA5185	5/28/2008	6 mL	nd	40	-
test45	NASA5290	10/14/2008	4 mL	nd	41	-
test46	TestosE2 L3	7/29/2009	20 ug each	nd	42	Testosterone, Estradiol

** prepared for identification only

Figures 7 to 11 shows activities in the laboratory of the Zoology department at UFL:



Figure 7: Lizette holding baby alligator



Figure 8: Lizette holding baby alligator



Figure 9: Sample collection



Figure 10: Length & size measurement



Figure 11: Length measurement

Expected Outcomes for Research

The expected outcome of this investigation is the establishment of urine steroid profiling as a diagnostic tool for the evaluation of the endocrine system of crocodilians, e.g. stress and the effects of endocrine disruptive compounds.

Differences in steroid and steroid metabolite quantities and ratios are expected. Differences in levels of excreted cortisol metabolites (tetrahydrocortisone, tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocorticosterone) are also expected.

Ratios of cortisol and cortisone metabolites have also been used for evaluation in humans who suffer from stress-related diseases indicating psychological stress, starvation, obesity, hypo- and hypertension, thyroid abnormalities and tumours of the adrenal. Evaluation of crocodilian urine steroid profiles promises to provide related information.

Plasma investigation of corticosterone levels could give additional information regarding stress-levels in groups of animals from different environments.

Acknowledgements

We thank Dr. Jan Myburgh from the Section Pharmacology & Toxicology, Department of Paraclinical Sciences, Onderstepoort Veterinary Faculty, University of Pretoria, for his support in the arrangements of the study.

We thank the staff of the University of Florida, Department of Zoology for their cooperation, in particular Satomi Khono, who provided lodging to me as student.

We thank Dr. Timothy Garrett for his support and assistance in the GC-MS laboratory of the Howard Hughes Medical Institute, UFL.

We thank IUCN-SSC Crocodile Specialist Group for financial assistance.

Summary: UFL Alligator work – Lizette Bekker: July/Aug 2009

Monday July 27, 2009
Flight from DC to Orlando – arrive 12h11 and drove to Gainsville arrive 17h00.
Met with Professor Lou Guilette and research assistant Satomi Kohno.

Tuesday July 28, 2009
Set-up of chemicals and sample preparation equipment.
Enquired about chemicals not delivered yet.
Sorted urine and plasma samples from freezers.

Wednesday July 29, 2009
Sample preparation: centrifugation, extractions, hydrolysis.
More sorting out of samples in freezers

Thursday July 30, 2009
Sample preparation: centrifugation, 2nd extractions, drying, derivatizing.
Preparation of standard mixtures, drying and derivatizing.
Source derivatization reagents – chemicals not delivered yet!

Friday July 31, 2009
Continue with sample preparation
Injection of test sample to optimize method (time program) on GC-MS
Column I.D. not correct – retention times too long.
Pump down MS to change to new column and condition overnight.

Saturday August 1, 2009
Continue with sample preparation
Start analysis of samples on GC-MS

Sunday August 2, 2009
GC-MS analysis until Monday August 3, 2009

Monday August 3, 2009
Drive back to Orlando in Afternoon

Tuesday August 4, 2009
Fly from Orlando to DC