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An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species

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The monitoring of endogenous hormone plasma levels could be valuable in biomedical, veterinary and pharmaceutical research. A specific high performance liquid chromatography method with diode array detection, for the assay of cortisol, corticosterone and melatonin in animal plasma was developed and validated. The chromatographic separation was achieved on a C_8 reversed phase column with a mobile phase consisting of HPLC-grade water and 35% v/v acetonitrile (pH ± 3.36). The detection was achieved through diode array detection, with two set wavelengths; 245 and 275 nm. The flow rate was at 1 ml/min and the total run time was 50 min. The method was validated according to validation guidelines (Shabir, 2006; US FDA, 2013). The method was found to be linear ($R^2 > 0.99$) over the analytical range (10 to 500 ng/ml) for all three analytes. All the other validation parameters were acceptable and within range. The method was applied to plasma samples from Sprague-Dawley rats and white rhinoceros.

1. Introduction

The measurement of endogenous hormone plasma levels forms a very important part of laboratory and wild animal research. These hormones help to understand and interpret a variety of animal disorders and diseases which are often similar, or related to those seen in other animals, including humans. It also helps in the process of developing new drugs. Hormones are mediator biomolecules secreted by a variety of endocrine glands that are located all over the mammalian body (Tortora and Derrickson 2014). The hormone is secreted from its endocrine gland and enters the bloodstream via the interstitial fluid. The circulating blood system then delivers hormones to their related target cells throughout the body. The hormone system's time of onset of action takes from seconds to hours or days and its duration of effect is generally longer than its release (Tortora and Derrickson 2014). One of the major areas of animal research is stress and stress related disorders. A multitude of hormones (e.g., catecholamines, hormones secreted from the adrenal gland, etc.) are involved in the stress response (Möstl and Palme 2002). Stressful situations trigger the adrenals to respond, which results in an increase in glucocorticoid, mineralocorticoid and/or catecholamine secretion (Möstl and Palme 2002; Koren et al. 2012). These increases form part of the front-line endocrine mechanisms to defend an organism against stressful conditions. In addition, increased hormone levels during stress periods are also part of the hormonal cascade causing parturition in some species. During short-term stress, glucocorticoids improve fitness by energy mobilisation and may change behaviour. However, severe chronic stress (prolonged periods of high cortisol concentrations) may decrease individual fitness by causing immunosuppression and atrophy of tissues (Möstl and Palme 2002). Gong et al. (2015) have shown that serum cortisol and corticosterone in mice are closely correlated with the dynamics that occur during different physiological or pathological (stressful) conditions. They hypothesised that corticosterone is a more adaptation-related biomarker than cortisol during chronic stress, and found that cortisol was a quicker responder than corticosterone during severe acute stress (Gong et al. 2015).

Cortisol and corticosterone are belonging to the corticosteroids, which are produced and secreted by the adrenal cortex gland, both classified as glucocorticoids (Marieb 2006; Sembulingam and Sembulingam 2012). The glucocorticoids are all 21 carbon steroid hormones (Sembulingam and Sembulingam 2012). Cortisol is the glucocorticoid predominantly secreted in larger animals, including humans, whereas corticosterone is the predominant one in rodents (Granner 1985). Plasma samples of two animal species (Sprague-Dawley rat and white rhinoceros) were used to develop and validate the method because of this phenomenon. Figure 1 shows the biosynthesis of cortisol and corticosterone that takes place in the adrenal cortex.

Melatonin is an indolamine secreted by the pineal gland and it plays an important role in the regulation of the circadian sleep–wake rhythm (Sastre Toraño et al. 2000; Çetin et al. 2018). Figure 2 shows that melatonin is synthesised via serotonin from the amino acid tryptophan (Claustrat et al. 2005; Tortora and Derrickson 2014). Research in rats has linked low plasma levels of melatonin to acute stress (physical activity) (Paredes et al. 2005). In another study in rats increased melatonin plasma levels was linked to chronic stress (Dagnino-Subiabre et al. 2006).

The HPLC-DAD method detected melatonin concentrations in units (ng/ml) greater than those reported (pg/ml) by Paredes et al (2005) and Haritou et al (2008). The melatonin molecule is electro-active and can also be easily detected with electrochemical detection (Vieira et al. 1992). This detection technique was thus used to verify the data obtained with DAD detection.

2. Investigations and results

2.1. Methods (standards, buffer, mobile phase and sample preparation)

2.1.1. Preparation of standard solutions

Standard stock solution was prepared with a concentration of 100 µg/ml for each of the following analytes; cortisol, corticosterone

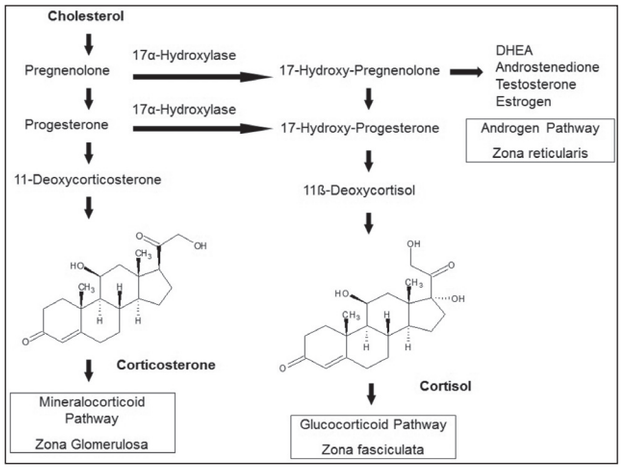

Fig. 1: Adrenocortical hormone biosynthesis, modified from the literature (Sembulingam and Sembulingam 2012).

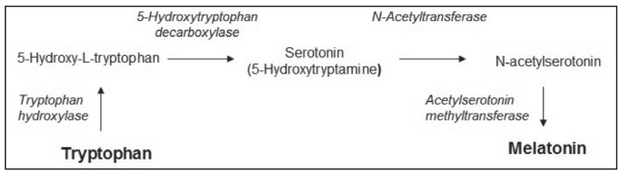

Fig. 2: Melatonin synthesis from tryptophan, modified from the literature (Burtis et al. 2012; Zagajewski et al. 2012).

and melatonin using HPLC-grade water (with 2% methanol) as the solvent. From this stock solution, a range (10 ng/ml to 500 ng/ml) of concentrations were prepared to setup a standard calibration curve, only with HPLC-grade water.

2.1.2. Preparation of the internal standard solution

Standard stock solution of the internal standard, dexamethasone, with a concentration of 100 µg/ml using HPLC-grade water as the solvent was prepared. The working internal standard solution, with a final concentration of 2.5 µg/ml, with an appropriate dilution from the internal standard stock solution using HPLC-grade water as solvent was prepared.

2.1.3. Phosphate buffer saline solution (PBS)

To prepare PBS add 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g disodium phosphate and 0.24 g of monopotassium phosphate to 800 ml of HPLC-grade water and mix. After all the buffer salts were dissolved top up the solution to 1 l. Adjust the pH of the solution to pH 7.4 with hydrochloric acid (Top Tip Bio 2018).

Table 1: Instrumentation settings

HPLC instrument settings:	
Flow rate	1.0 ml/min
Injection volume	100 µl
Run time	55 minutes
Diode array detector settings:	
Wavelength 1	245 nm
Wavelength 2	275 nm
Data collection rate	5 Hz

2.1.4. Mobile phase preparation

Mobile phase for the HPLC coupled to the diode array detector (DAD): A mobile phase consisting of HPLC-grade water and v/v 35% acetonitrile was prepared. The pH of the mobile phase was set at pH 3.36 with glacial acetic acid or formic acid. The mobile phase was filtered through a 0.45 µm nylon filter before use (Agela Technologies).

2.1.5. HPLC-DAD instrument settings

Cortisol and corticosterone were monitored at 245 nm and melatonin at 275 nm (Rizzo et al. 2002; Izquierdo-Hornillos et al. 2005; Viljoen et al. 2012).

2.1.6. Sample preparation of the plasma samples

Add 4.5 ml of the PBS solution to a 10 x 100 mm screw-capped glass tube followed by 1 ml of the standard solution or test plasma and also 250 µl of the internal standard dexamethasone (2.5 µg/ml). The analytes were extracted with 5 ml of ethyl acetate by mixing the samples for 30 min with a rotating mixing wheel. Hereafter, the samples were centrifuged at 4500 rcf for 15 min. After centrifugation the organic upper layer was transferred to conical tubes and evaporated to dryness under a stream of nitrogen at a temperature of 40 °C. The residue was reconstituted with 125 µl of mobile phase where after the final sample was centrifuged at 1620 rcf for 5 min. The 125 µl final sample was transferred into inserts in vials and placed in the autosampler of the HPLC system for analysis.

2.2. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy, recovery and stability (US FDA, 2013).

2.2.1. Linearity/calibration curve

Linearity was tested by preparing seven standard concentrations as described in section 2.1.2 and analysed in triplicate. The linear regression value (coefficient of determination, r^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir 2006). For calibration, the internal standard method was used with dexamethasone as the chosen internal standard.

2.2.2. Quantification and detection limits (sensitivity)

The limit of quantification (LOQ) and the limit of detection (LOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava and Gupta 2011).

2.2.3. Precision and accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and five determinants for each were done for precision and accuracy. Precision results were expressed as %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA, 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (Shabir, 2006; US FDA, 2013).

2.2.4. Recovery

The percentage recovery of the liquid-liquid extraction procedure was determined by preparing four standard concentrations (10, 75, 250 and 500 ng/ml). These four concentrations were analysed without going through the liquid-liquid extraction procedure and their peak areas determined. Here after the same four concentrations were put through the liquid-liquid extraction process and analysed. The percentage recovery of the liquid-liquid extraction procedure was then calculated with the following equation.

$$\text{Percentage Recovery} = \frac{\text{Peak Area of extracted sample}}{\text{Peak Area of unextracted sample}} \times 100$$

The % recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.2.5. *Stability*

Stability was determined by injecting the three standard solutions (low, medium and high) and two plasma samples directly after sample preparation. The initial injection set was assayed immediately and served as reference values. The samples were re-injected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte will be given as the mean % stability of the samples analysed and ± SD at the given hour.

Table 2: Linear regression line equation and coefficient of determination with the diode array detector

C_8 analytical column	y = mx + c	Coefficient of determination (R^2)
Cortisol	y = 0.0025x + 0.0054	R^2 = 0.9993
Corticosterone	y = 0.0027x + 0.0078	R^2 = 0.9993
Melatonin	y = 0.0018x – 0.0099	R^2 = 0.9991
Linear regression line equation and coefficient of determination with the electrochemical detector		
C_8 analytical column	**y = mx + c**	**Coefficient of determination (R^2)**
Melatonin	y = 1.2003x + 5.8288	R^2 = 0.9999

2.3.3. *Precision and accuracy*

The precision and accuracy results of the four concentrations tested are provided in Table 3. Both the precision and accuracy results were within the acceptable criteria ranges set by the method validation parameters in section 2.2.3. Throughout these four concentrations, the %RSD for both the intra-batch and inter-batch precision was below 4 %. The accuracy of all concentration levels for all of the analytes tested was between 92.64 and 106.78%.

2.3.4. *Recovery*

The percentage recovery for each analyte, measured at the four concentrations tested, were constantly above 90%.

2.3.5. *Stability*

In Table 4, the mean percentage stability results for the all the analytes tested in the three standards and the plasma samples are demonstrated. All the analytes were stable when tested at 24 h and 48 h stored at room temperature in the autosampler tray for the standard samples analysed. The plasma samples analysed for stability had mixed results but in the case of these types of biological samples it is better to analyse them within 24 h after the samples are prepared to ensure sample integrity.

2.4. *Chromatographic results*

Table 5 presents the relative retention times of all the analytes and the internal standard for this method.

Table 3: Accuracy and precision results

Analyte conc. (ng/ml)	Intra-batch (n=5)			Inter-batch (n=5)		
	Measured conc. (ng/ml) (mean±SD)	Precision (%RSD)	Accuracy (%)	Measured conc. (ng/ml) (mean±SD)	Precision (%RSD)	Accuracy (%)
Cortisol						
10 (LOQ)	10.47 ± 1.24	11.82	104.71	10.03 ± 1.17	11.68	100.32
75	76.86 ± 4.71	6.13	102.47	73.19 ± 4.15	5.67	97.59
500	505.72 ± 4.88	0.96	101.14	494.34 ± 17.90	3.62	98.87
Corticosterone						
10 (LOQ)	10.46 ± 1.21	11.61	104.62	9.47 ± 1.21	12.79	94.73
75	74.02 ± 1.82	2.46	98.69	72.33 ± 6.19	8.55	96.44
500	500.79 ± 18.06	3.61	100.16	499.21 ± 14.47	2.90	99.84
Melatonin						
10 (LOQ)	10.17 ± 0.47	4.63	101.74	9.83 ± 1.30	13.27	98.29
75	79.86 ± 5.88	7.36	106.48	69.48 ± 3.83	5.51	92.64
500	529.51 ± 36.81	6.95	105.90	533.88 ± 44.26	8.29	106.78

Note: Intra-batch is where all the standard concentrations were prepared from one standard stock solution and analysed. Inter-batch is where all the standard concentrations were prepared from 5 separate prepared standard stock solutions.

2.3. *Method validation results*

The method was fully validated on a Venusil ASB C_8, 4.6 x 250 mm, 5 µm analytical column.

2.3.1. *Linearity/calibration curve*

The calibration curve constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration ranges (Table 2), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all the analytes.

2.3.2. *Quantification and detection limits (sensitivity)*

The lower limit of detection (LOD) and the lower limit of quantification (LOQ) were determined to be 5 ng/ml and 10 ng/ml for all the analytes respectively.

The following two chromatographic figures are representative of a 75 ng/ml standard (Fig. 3), and a 500 ng/ml standard (Fig. 4), both done with the HPLC-DAD method.

2.5. *Application*

The validated method was applied to plasma samples from white rhinoceros and Sprague-Dawley rats collected in lithium-heparin (green top) and K_3EDTA (purple top) blood collection test tubes respectively. The internal standard method was used to calculate the concentration of each analyte in the samples (Chromacademy 2014).

The following three chromatographic figures represents the two animal species plasma liquid-liquid extraction results.

In Table 6, the concentrations of cortisol, corticosterone and melatonin that were detected and quantified in the plasma samples. The results

Table 4: Stability results for the three standards of the HPLC-DAD method

% Stability (mean±SD) of the analytes at room temperature after 24 h and 48 h.		
Time:	**24 h***	**48 h***
Melatonin (275 nm)	99.14 ± 5.02	87.60 ± 4.70
Cortisol	96.25 ± 5.05	94.12 ± 5.00
Corticosterone	93.97 ± 4.37	92.76 ± 8.12
Stability results of the analytes in plasma samples		
Time:	**24 h***	**48 h***
Melatonin (275 nm)	79,36 %	83,59 %
Cortisol	98,51 %	79,85 %
Corticosterone	85,04 %	89,15 %

*Compared to 100% at t = 0, mean ±SD.

Table 5: Relative retention times of the analytes and the internal standard

Analytes	Relative retention times (min)
1. Melatonin (HPLC-DAD method)	10.0 ± 0.5
2. Cortisol	16.75 ± 0.5
3. Internal standard (dexamethasone)	31.01 ± 0.5
4. Corticosterone	37.45 ± 0.5

were expressed in ng/ml. Cortisol, however, was below the LOD in some of the rat samples. The cortisol and corticosterone results were comparable with that found in the literature (Van Heerden et al. 1985; Koren et al. 2012; Viljoen et al. 2012). However, the melatonin concentrations measured in both the species were different to those reported in literature, both the HPLC-DAD and HPLC-ECD method measured the melatonin concentrations in units (ng/ml) greater than those reported (pg/ml) by Paredes et al. (2005) and Haritou et al. (2008) (Paredes et al. 2005; Haritou et al. 2008). However, the melatonin concentrations measured by the two methods, compared well with each other, especially in rhinoceros number 3 after 6 hours of transport, the mixture of rhinoceros plasma samples (Table 6). Furthermore a pharmacokinetic study in human volunteers that were given an oral dosage of a 20 mg capsule of melatonin also measures melatonin concentrations in the ng/ml (mean C_{max} of ±70 ng/ml) analytical unit (Jenjirattithigarn et al. 2014).

3. Discussion

A specific HPLC, coupled to a diode array detector combined with liquid-liquid extraction, was developed and validated for the quantitative analysis of cortisol, corticosterone and melatonin in rat and white rhinoceros plasma samples. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity for all respective analytes calibration ranges was excellent and the coefficient of regression (r^2) was consistently above 0.95. The LOQ and LOD were respectively 10.0 ng/ml and 5 ng/ml for the method. The percentage recovery was acceptable for all the analytes at all the concentrations measured, and the percentage stability was also acceptable for both 24 and 48 h testing for the standards and the plasma samples, however, plasma samples ideally should be analysed within 24 h after sample preparation. The method was found to be selective and specific for cortisol, corticosterone and melatonin, although cortisol was below the limit of detection in some of the rat samples. The higher than expected melatonin levels measured on the HPLC-DAD method were correlated with a HPLC-ECD method. The melatonin levels measured by both methods compared well with each other. That melatonin concentrations can be measured, using these methods, at a higher analytical unit than reported in the literature is novel and requires further investigation once ethical approval is given to get more plasma samples for this analysis.

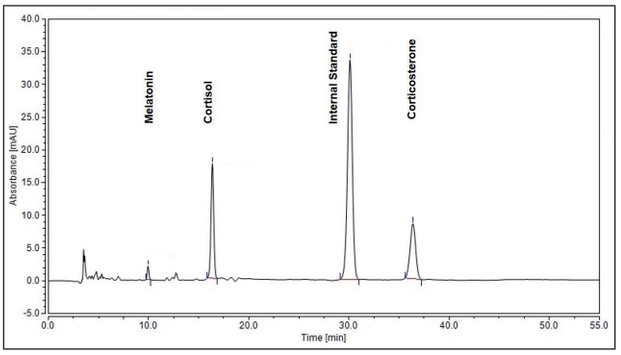

Fig. 3: 75 ng/ml Standard monitored at 245 nm with the HPLC-DAD method.

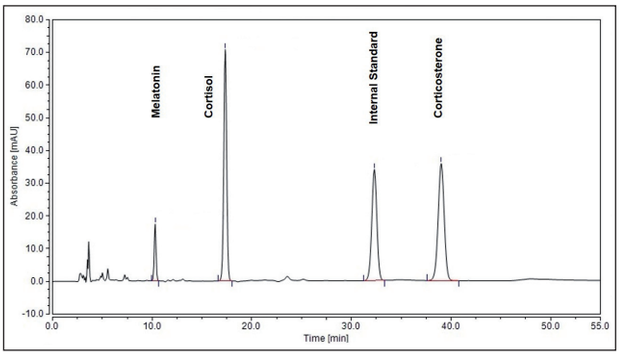

Fig. 4: 500ng/ml Standard monitored at 245 nm with the HPLC-DAD method.

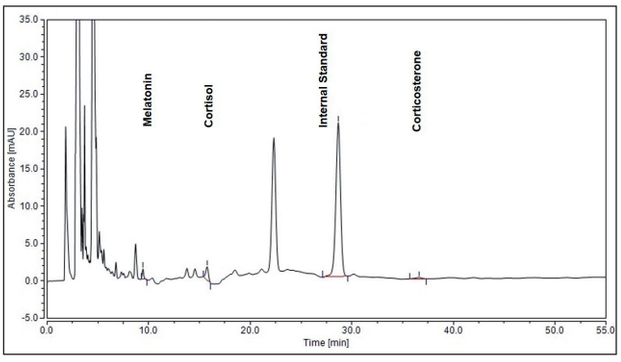

Fig. 5: Rhinoceros plasma sampled at capture and monitored at 245 nm with the HPLC-DAD method.

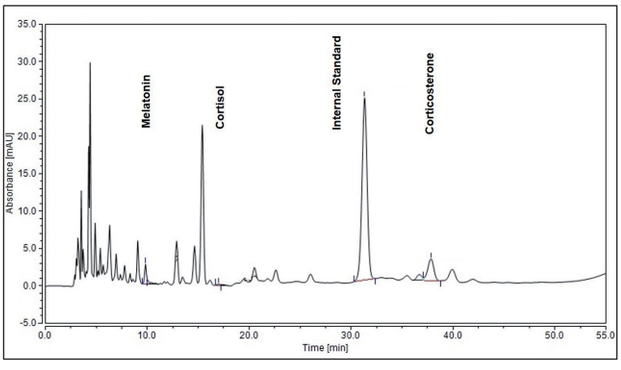

Fig. 6: Rat plasma sample monitored at 245nm with the HPLC-DAD method.

We propose that this method can be applied to drug and behaviour studies in laboratory animals (for example rats and mice) as well as wild animals to determine the stress response to management procedures, and other related disorders in these animals.

Table 6: Rhinoceros and rat plasma extraction results obtained (ng/ml)

	White Rhinoceros samples				2 Male Rats	
Analyte	**Animal 1 captured**	**Animal 1 at released**	**Animal 2 captured**	**Animal 2 after 6 hours of transport**	**Animal 1 sample A**	**Animal 2 sample B**
Cortisol	8,95	20,74	8,93	7,36	BLOD	4,08
Corticosterone	3,50	3,46	2,05	0,69	41.99	31,32
Melatonin with HPLC-DAD method	27,42	22,17	23,25	240,58	105,59	234,22
	Animal 3 captured	**Animal 3 after 6 hours of transport**		**Mixture of rhinoceros plasma samples**		**Mixture of rat plasma samples**
Cortisol	11,51	7,41		17,22		0,91
Corticosterone	2,21	1,37		1,82		30,82
Melatonin with HPLC-DAD method	19.02	42.64		30,33		96,05
Melatonin with HPLC-ECD method	13.89	16.31		19,49		123,18

(BLOD - below limit of detection).

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

Melatonin, cortisol (hydrocortisone), corticosterone and dexamethasone (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water, HPLC grade methanol (MeOH), glacial acetic acid and formic acid. Chemicals used for the sample preparation were sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate, hydrochloric acid and ethyl acetate. All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC columns used was a Venusil ASB C_8, 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g (purchased from Bonna-Agela Technologies, USA).The developed and validated method was applied to prepared white rhinoceros (*Ceratotherium simum*) plasma collected in lithium-heparin blood collection tubes and Sprague-Dawley rat plasma collected in EDTA blood collection tubes. The blood samples were immediately centrifuged after collection to separate the plasma from the blood components. The plasma was transferred to Eppendorf tubes and immediately snap frozen in liquid nitrogen, transferred in dry ice and stored in a -80 °C freezer until day of analysis.

4.1.3. Instrumentation

The chromatographic system for the HPLC-DAD method, consisted of an Ultimate 3000 UHPLC system, equipped with an pump and WPS-3000TSL analytical autosampler, coupled to an diode array detector and Chromeleon® chromatography management system version 7.2 (all obtained from Thermo Fisher Scientific, Waltham, MA USA).

Ethics approval: Ethical approval for this project to use animal (rat and white rhinoceros) plasma was obtained from the animal care, health and safety in research ethics committee (AnimCare) of the Faculty of Health Sciences, from the North-West University (NWU-00252-17-A5), as well as from the Animal Ethics Committee from the University of Pretoria (V067-17).

Conflict of interest: The authors have no conflict of interest to declare.

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