

The Cortisol Steroid Levels as a Determinant of Health Status in Animals

Akefe Isaac^{1*}, Yusuf Ibrahim², Adamu Andrew³, Dung Edward⁴ and Abraham Solomon³

¹Physiology Department, Faculty of Veterinary Medicine, Ahmadu Bello University, Nigeria

²Pharmacology and Toxicology Department, Faculty of Veterinary Medicine, University of Maiduguri, Nigeria

³Veterinary Teaching Hospital, Faculty of Veterinary Medicine, University of Abuja, Nigeria

⁴Physiology, Biochemistry and Pharmacology Department, Faculty of Veterinary Medicine, University of Jos, Nigeria

Abstract

The veterinarian is daily faced with the challenge of diagnosing several disease conditions in different animals. Quantifying cortisol levels has been used in the evaluation of anxiety and distress initiated by infectious and non-infectious disease conditions, mismanagement, transportation, adverse environmental temperature and surgical operations. The hormone-cortisol has been implicated in several immunologic and metabolic processes and can thus serve as a marker to monitor animal welfare. Several factors affect the specific test to be carried out and which sample is to be used. Recently, RIA has been identified as the gold standard for determination of cortisol levels. However it also has its own shortcomings hence the need to utilize other assay techniques such as ELISA, fluorescence techniques and chemical assays which are more common in our environment. There is also prospect for the analysis of cortisol using non-invasive samples such as finger nails as well as ear wax for enhanced test results.

Keywords: Cortisol; Health; Disease; Animals; Elisa; Radioimmunoassay

Introduction

Cortisol (hydrocortisone; 11, 17, 21-trihydroxy-pregnene-3, 20-dione) is a 21-carbon steroid secreted by the adrenal cortices, and is the most physiologically active of the naturally occurring glucocorticoids [1]. In livestock animals, quantification of cortisol levels has been used in the estimation of stress and pain instigated by poor management, castration without local anesthesia, inappropriate environmental temperature, transportation, and ailment [2]. Physiological functions of cortisol include control of carbohydrate metabolism, electrolyte homeostasis, water circulation, anti-inflammatory and immunosuppressant processes. Cortisol is also associated with various physiological and immunologic activities [3]. Assessment of blood cortisol concentration is widespread in large animals; to monitor impacts of prevailing farming practices on animal health and welfare [2].

Synthesis and regulation of cortisol

Cortisol is a glucocorticoid engendered by the adrenal cortex following stimulation by adrenocorticotrophic hormone (ACTH). It unites with two intracellular receptors; the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) [4]. Among the two receptors, the MR has greater binding affinity for cortisol, being occupied by cortisol at concentrations inadequate enough to stimulate the GR. 311 β -Hydroxysteroid dehydrogenase (Type 2; 11 β -HSD2) translates cortisol to the sedentary cortisone [5]. This enzyme is predominantly expressed in mineralocorticoid aimed tissues including colon, salivary gland, and kidney where it functions to protect the MR from excess glucocorticoid. Individuals with low levels of this enzyme exhibit a syndrome called apparent mineralocorticoid excess which features hypokalemia and hypertension. 11 β -HSD1 enzyme is an important rheostat of the concentration of intracellular glucocorticoid, catalyzing the continuous production of cortisol from cortisone [6]. Visceral adipose tissues obtained from obese individuals increases 11 β -HSD1 function in contrast to that obtained from normal individuals. Cortisol formidably boosts adipocyte differentiation; developed visceral adipocytes exhibit high levels of the glucocorticoid receptor. Cortisol circulates majorly in the form of bound protein,

the larger being joined to corticosteroid binding globulin [7]. The half-life of circulating cortisol is about 80 minutes, with roughly 1% excreted without alteration in urine. This expelled fraction is known as urinary "free cortisol" and if the renal activity is functional, it will reflect the level of circulating non-protein bound cortisol. After catabolic breakdown in the liver, cortisol is expelled into the urine as dihydro and tetrahydro-derivatives joined to glucuronic acid [6].

Factors affecting cortisol concentration

The circulating cortisol is usually subject to a circadian timing, with maximum levels reached at 8-9 a.m. and minimum at midnight. Concentrations are usually higher during pregnancy and in subjects receiving increased dose of estrogen therapy [6]. Abnormal cortisol concentrations may result from stimuli such as fear, hypoglycemia, fever, trauma, shock, and despair. In abnormal states of the HPA axis, increased or decreased cortisol values may be obtained [8]. Tumors of the adrenal gland and ectopic or pituitary adrenocorticotrophic hormone-producing cancers are commonly correlated with over-production of cortisol (Cushing's syndrome), while adrenal paucity may result in under-production of cortisol (Addison's disease) [5]. Production of glucocorticoids increases during stress, as such, cortisol may serve as a biomarker of stress. Cortisol levels surge with age, and are habitually elevated in key depressive disorder, hypertension, and AIDS. Cortisol concentrations also vary due to, diet, temperature, relative humidity and physiological states. Therapeutic treatment using glucocorticoids may induce cognitive impairment, low bone density, hypertension, and increased risk of occurrence of type II diabetes (Table 1) [4].

***Corresponding author:** Akefe Isaac, Physiology Department, Faculty of Veterinary Medicine, Ahmadu Bello University, Nigeria, Tel: +234-8034986335, +234-9024424771; E-mail: akefeisaac@yahoo.com

Received October 24, 2017; **Accepted** November 17, 2017; **Published** November 22, 2017

Citation: Isaac A, Ibrahim Y, Andrew A, Edward D, Solomon A (2017) The Cortisol Steroid Levels as a Determinant of Health Status in Animals. J Proteomics Bioinform 10: 277-283. doi: [10.4172/jpb.1000452](https://doi.org/10.4172/jpb.1000452)

Copyright: © 2017 Isaac A, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Methods of measuring cortisol levels

Cortisol can be assayed in many matrices including blood, serum, feces, sweat, urine, hair and saliva. Serum cortisol levels range from about 25-800 nM (9-300 ng/ml) and averagely 90-95% of the cortisol is protein-bound. Urinary cortisol is unbound, but its concentration depends on tubular and glomerular function. In saliva, roughly 67% of cortisol is unbound. There is usually a good correlation between cortisol concentrations in saliva and serum [6]. Other matrices include cortisol in organs such as bone, liver and pancreas (Figure 1).

Hair cortisol analysis using ELISA technique

This is a unique type of enzyme linked immunosorbent assay. A minimum of 10 mg of hair from the scalp end was used for each

sample. The hair is weighed on a scale, incised into tiny pieces using surgical scissors, and transferred into a disposable glass scintillation vial before addition of 1 ml of methanol. The scintillation bottle is sealed and incubated all night (~16 h) at 52°C while rocking (Gyromax® Amerex Instruments Inc.) [9]. Following incubation, the supernatant is separated into disposable glass culture tubes and evaporated in a dry bath (Thermolyne® Dri-Bath) under nitrogen (Techne® Sample Concentrator) until entirely dry. Following removal of methanol, the sample is resuspended in 150-250 µL of buffered saline at pH 8.0. Samples are mixed for one minute followed by another 30 seconds until they are well vortexed [8]. The cortisol in the hair samples is the assayed using the Salivary ELISA Cortisol kit© (Alpco Diagnostics®, Windham, NH) as per the manufacturer's directions.

Basically, an enzyme catalyses the hydrolysis of substrates that produce a coloured product. The extent of colour change is used to quantitate the amount of labeled hormone-antibody complex that is present [10] (Figure 2). Thus, the reference range for cortisol levels in hair of healthy non-obese individuals is 17.7-153.2 pg/mg of hair with a median of 46.1 pg/mg [9].

Advantages

It does not involve any invasive procedure and can be carried out untrained persons. Samples can be stored at room temperature and be sent by mail, making it potentially useful in population studies. Further, levels reflect average hormone levels over the last two months, as opposed to blood, saliva and urine samples, which reflect acute or daily cortisol levels. Another important characteristic of measurement

Species (n=10)	Baseline (± SEM)	Stressed (± SEM)
Sheep	0.8 ± 0.4	1.2 ± 0.5*
Goat	1.0 ± 0.2	1.5 ± 0.1*
Cattle	1.7 ± 0.1	3.8 ± 0.7*
Horse	5.1 ± 0.2	12.2 ± 0.4**
Camel	2.1 ± 0.5	3.3 ± 0.3*
Monkey	14.8 ± 0.7	30.0 ± 0.2**
Cat	8.2 ± 0.5	10.1 ± 0.3*
Man	18.5 ± 0.1	34.2 ± 0.6**

Values having superscripts *, ** are significantly different (P<0.05) and (P<0.01) respectively, compared to other groups.

Table 1: Cortisol Levels in Different Animal Species (µg/Dl) [15].

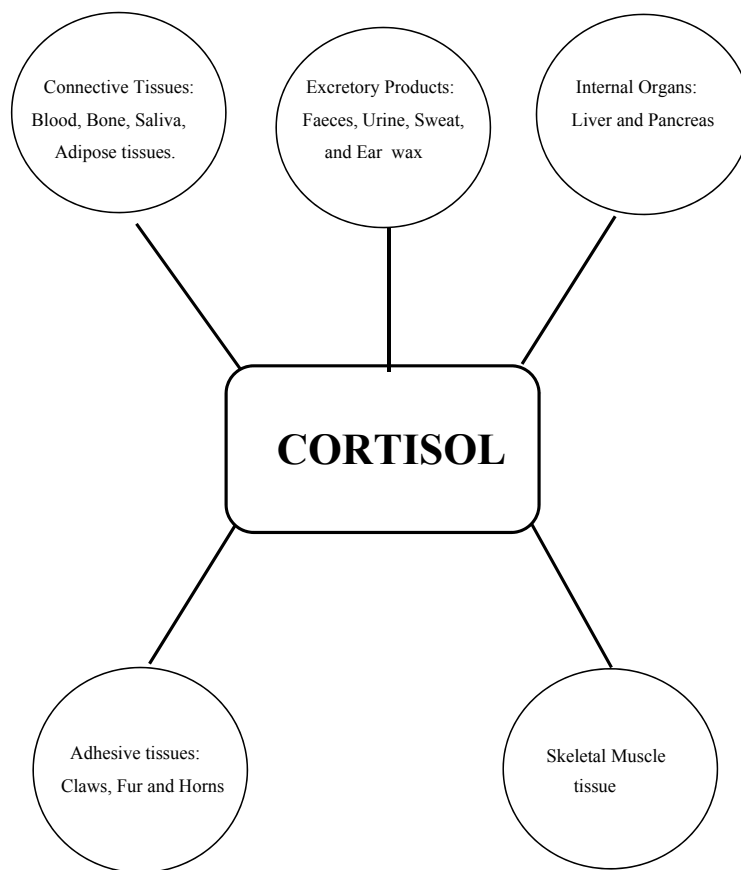


Figure 1: Matrices for measuring cortisol levels in animals.

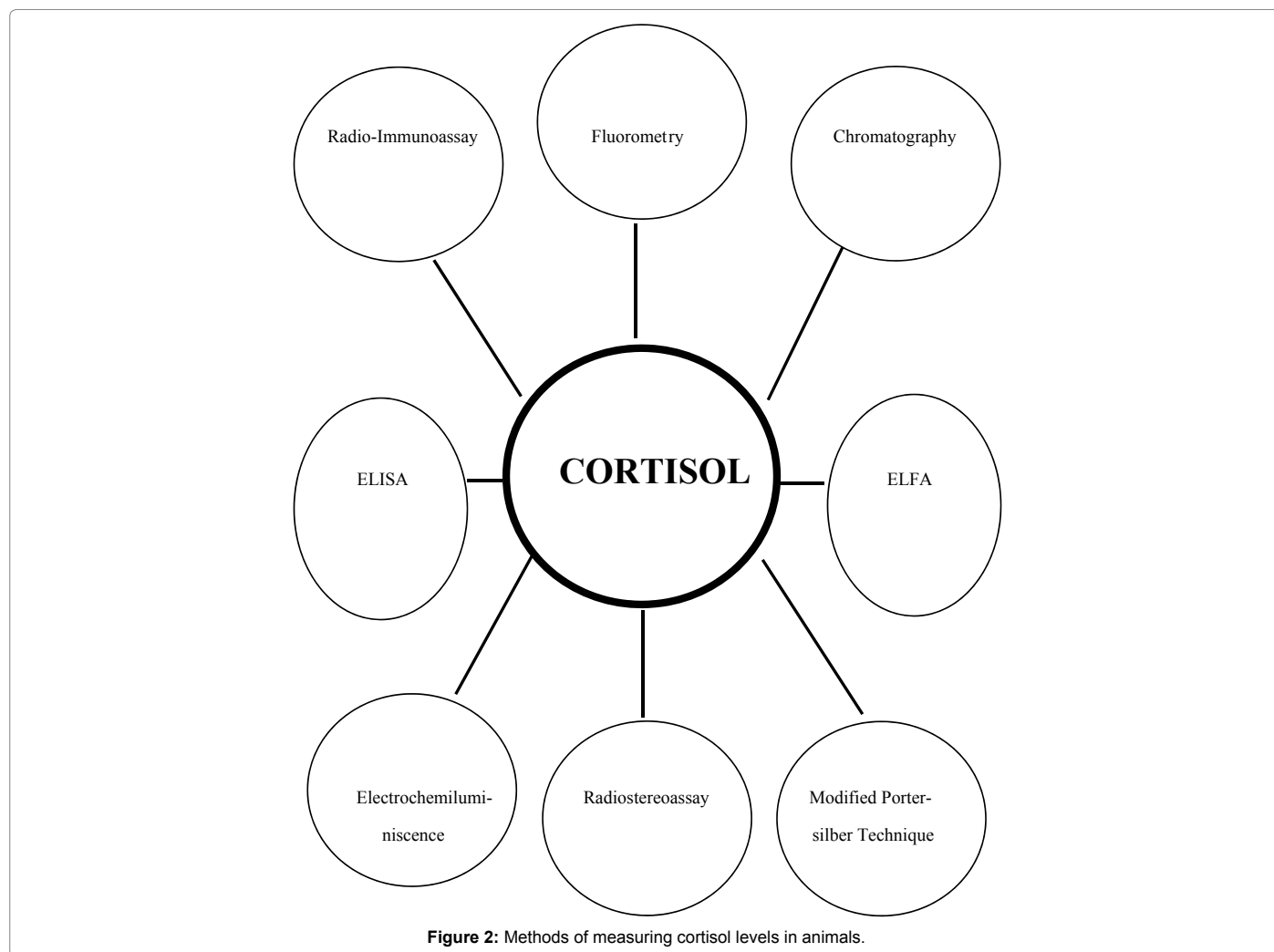


Figure 2: Methods of measuring cortisol levels in animals.

of cortisol in hair is that the levels are not affected by acute stress. Hair cortisol may be important in confirmation of cyclical Cushing's syndrome, and in depression, in which daily cortisol excretion in urine is increased in a higher number of days, but not all days [9].

Limitations

It is restricted to patients with sufficient hair at the posterior vertex and without religious/cultural concerns about taking a hair sample. Hair cortisol levels are not able to determine brief cortisol responses, and cannot be used to determine day-to-day variation. It is not known whether hair cortisol levels vary with hair growth rate, which could be important as the activity of hair follicles is intermittent, consisting of active phase (anagen), transitional (catagen) and resting phase (telogen) [8]. Further, hair growth rate decreases with age, in various diseases (for example, hyper- and hypothyroidism) and varies among ethnic groups. For saliva samples the saliva flow rate has only a minor effect on saliva cortisol levels. By analogy, it is conceivable that for hair cortisol levels the hair growth rate may have limited importance. Each kit also has a different specification. Finally, levels of cortisol in hair may be affected by local synthesis or metabolism in the hair follicles, although the relative contribution of locally produced hormones may be limited. Currently it is not known if hair levels of cortisol vary throughout the various seasons, or if hair growth rate follows a diurnal rhythm [10].

Liquid chromatography

In this chemical assay, purification of cortisol is achieved using open column chromatography, but high performance liquid chromatography is more commonly used as an analytical technique. HPLC uses high-resolution columns containing very uniform particles. These columns can separate very similar molecules with high efficiency, and normally operate under high pressure [3]. Binz et al. [11] used two methods (cortisol/(13)C3-labeled cortisol) were validated in a concentration range up to 500 pg/mg and showed good linearity for both analytes (cortisol: $R(2)=0.9995$; (13)C3-cortisol $R(2)=0.9992$). Slight differences were observed for limit of detection (LOD) (0.2 pg/mg/0.1 pg/mg) and limit of quantification (LOQ) (1 pg/mg/0.5 pg/mg). Precision was good with a maximum deviation of 8.8% and 10% for cortisol and (13) C3-cortisol respectively. Accuracy and matrix effects were good for both analytes except for the quality control (QC) low cortisol. QC low (2.5 pg/mg) showed matrix effects (126.5%, RSD 35.5%) and accuracy showed a deviation of 26% when using cortisol to spike. These effects were likely caused by the unknown amount of endogenous cortisol in the different hair samples used to determine validation parameters like matrix effect, LOQ and accuracy. No matrix effects were observed for the high QC (400 pg/mg) samples. Recovery was good with 92.7%/87.3% (RSD 9.9%/6.2%) for QC low and 102.3%/82.1% (RSD 5.8%/11.4%) for QC high. After successful validation the applicability of the method could be proven [11].

Mass spectrometry

Mass spectrometry can be used to identify the molecular weight, structure and position of functional groups of small molecules. The mass spectrometer produces charged ions from the sample, consisting of parent ion and fragments of original molecule, and then sorts these ions by mass/charge ratio in a magnetic field [12]. The relative numbers and the charge to mass ratio of each ion is typical of a particular compound and can be used to identify the compound structure. It can also be linked to a gas chromatography or liquid chromatography to identify compounds such as cortisol that is separated by chromatography [3]. To better understand depression a LC-MS/MS method for simultaneous determination of F, E THF, 5 α -THF and THE in human urine has been developed and validated. The quantification range was 0.1-160 ng mL⁻¹ for F and E, and 0.2-160 ng mL⁻¹ for the tetrahydro-metabolites, with >86.1% recovery for all analytes. The nocturnal urine concentrations of F, E and tetrahydro-metabolites in 12 apparently healthy male adult volunteers and 12 drug-free male patients (age range, 20-50 years) with a diagnosis of depression were analyzed. A series of significant changes in glucocorticoid metabolism can be detected: F/E ratios and (THF+5 α -THF)/THE ratios as well as F and THF concentrations were significantly higher in depression patients than in healthy subjects ($P < 0.05$); 5 α -THF/F ratios, 5 α -THF/THF ratios as well as 5 α -THF concentrations were significantly lower in depression patients ($P < 0.05$). The results pointed to the decreased 11 β -HSD2 activity and a dysfunction in the 5 α -reductase pathway in depressed patients. This method allowed the assessment of 11 β -HSD1/2 and 5 α / β -reductase activities in a single analytical run providing an innovative tool to explain the potential etiology of depression [13].

Radioimmunoassay technique (RIA KIT)

Radioimmunoassay (RIA) is the "gold standard" technique used to assay serum cortisol [14]. This method is based on competition between unlabelled cortisol and specific quantity of 125I-labelled cortisol for controlled number of binding sites on cortisol specific antibody [15]. Allowing reaction of a fixed quantity of tracer and antibody with varying quantities of unlabelled ligand, the quantity of tracer bound by the antibody is inversely proportional to the concentration of the unlabelled ligand (Figure 2).

Materials & Methods

Cortisol measurement & reagent preparation

Immuno-complex is restrained on the reactive surface of the test tubes. Following incubation, the mixture is discarded, and the radioactivity is estimated in a gamma counter [16]. The quantity of antigen is inversely proportional to the radioactivity estimated in test tubes. By plotting binding values against a succession of calibrators with known amounts of cortisol, a calibration curve is created, from which the anonymous concentration of cortisol in individual samples is determined [13].

Methodology: 1. Equilibrate samples and reagents to room temperature for minimum of an hour before use.

2. Label coated tubes in duplicates of two for each standard (S1-S6), samples (Sx) and control serum (C). Optionally, label two uncoated test tubes for total count (T).

3. Homogenize all the samples and reagents by gentle blending to avoid foaming.

4. Pipette 10 μ l each of standards, samples and control into the well labelled tubes.

5. Pipette 500 μ l of tracer into each tube.

6. Pipette 500 μ l antiserum into all tubes except T.

7. Firmly fix the test tube rack onto the shaker plate and seal all tubes with a plastic foil. Turn on the shaker and adjust to a moderate speed such that liquid is constantly rotating in each tube

8. Incubate tubes at room temperature for 2 hours.

9. Invert the rack and decant the supernatant from all tubes. In an upside down position place the rack on an absorbent paper for at least two minutes.

10. Count each of the tube in a gamma counter for at least 60 seconds.

11. Calculate the cortisol levels of samples as illustrated in calculation of results.

Sensitivity: 2.9 nmol/l, defined as the concentration, which is 2 standard deviations from the zero standard [13].

Advantage: It is simple, fast, inexpensive and can be automated resulting in a very fast turn-around time [16]. Limitations: The reagents supplied in this kit are optimized to estimate cortisol concentrations in plasma and serum. Frequent freezing and thawing of specimens and reagents should be avoided. Hemolyzed and lipemic specimens should be avoided as they may give false values. Also, kits from different manufacturers should not be mixed or interchanged [16].

Disadvantage: It includes the short shelf-lives of the radioactive reagents, risk of radiation exposure for staff, and the need to dispose of toxic waste (Figure 2) [14].

Acetylcholinesterase competitive ELISA technique

Cortisol ELISA Kit can be used for measuring cortisol in plasma, urine, and other sample matrices. The assay has a range from 6.6-4,000 pg/ml and sensitivity (80% B/B0) of roughly 35 pg/ml [17]. This method is principled on the competition between cortisol and cortisol acetylcholinesterase (AChE) conjugate (cortisol tracer) for a rationed number of cortisol-specific mouse monoclonal antibody binding sites. Since the concentration of the cortisol tracer is fixed while the concentration of cortisol varies, the amount of cortisol tracer that binds to the cortisol mono-clonal antibody is inversely correlated to the cortisol concentration in the well. This antibody-cortisol complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents before addition of Ellman's Reagent to the well. The product of the reaction has a distinct yellow color and absorbs strongly at 412 nm [17]. The color intensity determined spectrophotometrically, is related to the amount of cortisol tracer bound to the well, which is inversely correlated to the amount of free cortisol in the well at incubation; or Absorbance α (Bound Cortisol Tracer) $\alpha 1/(\text{Cortisol})$ [17].

Methodology

Addition of the reagents: 1. ELISA Buffer: Add 100 μ l ELISA Buffer to NSB wells. Add 50 μ l ELISA Buffer to B 0 wells.

If culture medium was used to dilute the standard curve, substitute 50 μ l of culture medium for ELISA Buffer in the NSB and B0 wells (i.e., add 50 μ l culture medium to NSB and B0 wells and 50 μ l ELISA Buffer to NSB wells).

2. Cortisol ELISA Standard: Add 50 μ l from tube #8 to both of the lowest standard wells (S8). Add 50 μ l from tube #7 to each of the

next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. Equilibrate the pipette tip in the standard before pipetting each standard.

3. Samples: Add 50 μ l of sample to each well. Assay each sample at a minimum of two dilutions. Each dilution should be tested in triplicate.

4. Cortisol AChE Tracer: Add 50 μ l per well except the TA and Blk wells.

5. Cortisol ELISA Monoclonal Antibody: Add 50 μ l per well except the TA, NSB, and the Blk wells [18].

Incubation of the plate: Seal each plate with plastic film (Item No. 400012) and incubate at 4°C overnight.

Development of the plate: 1. Reconstitute Ellman's Reagent just before use (20 ml of reagent is sufficient to develop 100 wells): 100 dtn vial Ellman's Reagent (96-well kit; Item No.400050): reconstitute using 20 ml of Ultra-pure water.

2. Empty the wells and rinse severally using Wash Buffer.

3. Add 200 μ l of Ellman's reagent to the well.

4. Add 5 μ l of tracer to the TA wells.

5. Cover the plate with plastic film. Optimum development is achieved using an orbital shaker equipped with a large, flat cover to allow the plate(s) develop in the dark. This assay typically develops in 90-120 minutes.

Reading the plate: 1. Remove fingerprints and dirt by wiping the bottom of the plate with a clean tissue.

2. Remove the plate cover carefully avoiding Ellman's reagent from splashing on the cover. NOTE: Any loss of Ellman's reagent will affect the absorbance readings. If Ellman's reagent is present on the cover, use a pipette to transfer the Ellman's reagent into the well. If excess Ellman's reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with Wash Buffer and repeat the development with fresh Ellman's reagent.

3. Read the plate at a wavelength between 405 and 420 nm. Absorbance may be checked periodically until the B0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when absorbance of the B0 wells are in the range of 0.3-1.5 A.U. (blank subtracted) [19].

The disadvantages of this test includes the high cost of the kit, risk of contamination via staff, time-consuming, and need for staff in every step of the procedure (Figure 2) [17].

Enzyme-linked fluorescent assay (VIDAS Assay Technique)

The system uses a fluorescence label such as fluorescein isothiocyanate, which has an excitation maximum at 485 nm and emission maximum at 525 nm. The VIDAS cortisol test is an enzyme-linked fluorescent assay intended for the Mini Vidas system. Immediately after thawing of serum sample, cortisol is measured following the manufacturer's instruction on the Mini Vidas analyzer (BioMerieux S.A., Lyon, France) [20]. As designed by the manufacturer the test has a measurement range of 5.51-2759 nmol/L for serum of human subjects. The analyzers should be cleaned, recalibrated, and operated as instructed by manufacturers.

The Mini-Vidas analyzer is uncomplicated and quick to operate. The Mini Vidas is a compact, swift, automated immunoassay analyzer

requiring recalibration only once every 14 days, optimizing the per result cost. Tests sharing similar protocols may be run together in a single section of the analyzer and each section functions separately from the other. In addition, it is possible to perform a single test with single dose reagents [20]. The promptness of measurement (30 minutes), small sample needed (200L), and wide range of working make the VIDAS method suitable for field studies in farm animals. This method is more relevant when large populations of animals, in particular where the mean value of the group is relevant-such as studies on stress. In contrast, when greater precision and accuracy is required for clinical evaluation of an individual subject, this method is not advisable owing to its comparatively low specificity (76%) [19].

Fluorometric measurement of urinary free hydrocortisone (Cortisol)

Free steroids are extracted from urine with di-chloromethane and washed with aqueous alkali to remove phenolic steroids. Corticosteroids in the washed extracts are then treated with acid-alcohol for fluorogenesis. The 11-hydroxy and C-3 ketone (ring A) are principally accountable for fluorescence, whereas the 17-hydroxy group exerts little influence on fluorescence. Fluorometric measurement of corticosteroid, has brought about the generic term "11-hydroxycorticosteroid" (11-OHCS) to separate such methods from those based on the Porter-Silber reaction (17-OHCS) [21,22].

Overall procedure: 1. Prepare unknown, standard, and blank by adding 2.0 ml of either urine, working cortisol standard, or water, respectively, to labeled 50-ml glass-stoppered centrifuge tubes.

2. Add 15.0 ml of dichloromethane to all tubes, stopper, and shake thoroughly. Complete the extraction by mixing each tube on a vortex-type mixer for 1 min.

3. Allow the phases to separate, then remove and discard the aqueous (upper) layer.

4. Add 2.0 ml of sodium hydroxide (0.1 mol/liter) to all tubes, and mix (vortex) for 1 min. Remove and discard the alkaline (upper) layer.

5. Wash residual alkali from the extracts by adding 2.5 ml water, and mixing the contents of the tubes for 30s (vortex); then remove and discard the aqueous (upper) layer.

6. At zero time (set a timer for 15 min) add 10.0 ml of the washed dichloromethane extracts to 5.0 ml of acid-alcohol reagent (warmed to room temperature) in labeled tubes, stopper, and mix for 30s on the vortex mixer.

7. Immediately remove the dichloromethane (upper) layer from each tube, and discard.

8. Transfer the acid-alcohol phases to square quartz cuvetts (1-cm lightpath), and measure the fluorescent intensity exactly 15 mm from zero time. A small proportion of circulating blood cortisol is excreted in the urine in the unconjugated or free-state and consequently this fraction increases or decreases in accordance with adrenal output. For this reason, fluorometric determination of urinary free cortisol is most informative in detecting hypercortisolism, and has some advantages over colorimetric measurements of 17-hydroxycorticosteroids (17-OHCS) and 17-ketogenic steroids (17-KGS). In addition to its use in assessing hypercortisolism, we have used the fluorometric assay described here to detect hypocortisolism. In Addison's disease and panhypopituitarism, values are found that are clearly lower than those of normal subjects. Drugs such as

spironolactone result in marked fluorescence, thus altering the results. They should not be used prior to the test [21].

Electrochemiluminescence evaluation of salivary cortisol

The electrochemiluminescence (ECL) technique using a regular automated immunoassay analyser was launched to measure serum cortisol levels [2]. It has been reported to provide superior analytical performance even in very low concentration of analyte with shorter turnaround time [16]. This method is faster and convenient. Saliva samples are obtained using oral made of a non-toxic, inert polymer shaped into a 30 x 10 mm cylinder leading to passive droll of saliva [2]. The oral swabs are placed under the front of the tongue for 1 or 2 minutes until they are completely saturated [14]. After which it is kept in a swab storage tube measuring 17 x 100 mm saliva storage tube; consisting of a capped, conical polypropylene centrifuge tube with a different insert allowing saliva to be centrifuged into the bottom of the conical tube [4]. When they are still at room temperature, the tubes were mixed, and immediately centrifuged for 15 minutes at approximately 3,000 RPM. After centrifugation, the swab and small insert are thrown away, and the large outer tube was stored at -80°C until analysis [16]. On the day of measurement, the samples are brought to room temperature. Assays should be performed using only clear saliva, avoiding any sediment present in the bottom of the tube. Salivary cortisol levels is assayed by using an Elecsys Cortisol assay in the same manner as for serum or plasma specimens. Two hundred millilitres of salivary samples are transferred to an Elecsys sample cup and measured using a "cobas e 601" analyser (Roche Diagnostics GmbH; Mannheim, Germany) [2].

Determination of cortisol by radiostereoisotope assay or competitive protein binding

Corticosteroid binding globulin (CBG) is prepared from plasma sample. The plasma is stripped of steroids with charcoal Norit A in a concentration of 50 mg/ml of plasma, filtered twice using Whatman NQ 50 filter paper (Fisher Scientific Co.) and stored in 1 ml aliquots at 0-10 C. The scintillation fluid is composed of 42 ml of liquofluor (New England Nuclear Co.), 21 ml of absolute ethanol and 6.5 ml of Biosolv-solubilizer (Beckman NQ 184983) in 1 liter of toluene (spectroanalysed grade) [23].

Extraction: A tracer solution of 3H-cortisol 500-600 cpm in 0.1 ml of ethanol is placed in 15 ml stoppered centrifuge tubes and allowed to dry. An anonymous amount of plasma in 0.2-0.5 ml was added. Extraction is performed with two 5 ml aliquots of methylene chloride and each tube shaken in a vortex mixer for 1 minute. The methylene chloride is evaporated under nitrogen, and the sample re-concentrated by dissolving in 2 ml of acetone and re-drying [24].

Thin Layer Chromatography (TLC): The splitting of the steroids is performed by TLC using fluorescent silica gel. The system used to develop the TLC is methylene chloride ethanol 92:8. Cortisone and corticosterone impede cortisol in the binding sites of corticosteroid binding globulin. Solutions of these steroids run rapidly than cortisol in this system and so are effortlessly separated. The rapidity of the spots can be regulated with the concentration of the alcohol. Two and a half cm of the 2 cm wide silica gel stripes are scraped off the plates and diluted with 7 ml of acetone via disposable pipettes containing acetone washed glass wool. The diluent is collected in 5 ml centrifuge tubes in which the complete binding process is performed. The sample is diluted in 2 ml of acetone and a 0.5 ml aliquot is placed in a counting vial to ascertain the percentage of recovery [16].

Binding Procedure: On a standard curve, each set of determinations carried no fewer than four standard replicates and three 0, 10, 20, 40 and 80 ngs replicates. The corticosteroid binding globulin solutions are made by diluting the stripped plasma to a 1.5%. Sufficient 3H-cortisol is added to furnish a final concentration of 10 ng % of the solution. One ml of the CBG-isotope solution is added to each tube of the standard curve and that of unknown samples, and are shaken on a vortex mixer. The samples are incubated at 45 e for 5 min and cooled at 08°C for 10 min. Fifteen mg of fuller's earth are added and each shaken briefly on a vortex mixer, with a further 2 min on a rotating agitator. The tubes are then cooled at 8 C for another 10 min. After centrifugation for 2 min at 2500 rpm, 0.5 ml of the supernatant is placed in a counting vial. Ten ml of scintillation fluid is added to the vial and counted in a Liquid Scintillation Counter (Nuclear Chicago Mod. 720) with an efficiency of 15% [25].

Modified assay of porter-silber chromogens in urine

Acidify an aliquot of filtered urine (from a 24-h. collection) to pH 1 in a 100-ml beaker with 50% sulfuric acid using a PH meter. Saturate 5 ml. of the acidified sample with ammonium sulfate in a 50-ml screwcap centrifuge tube. Add crystals with shaking until only traces remain at the bottom. Add 30 ml. of cold ethyl ether, cap the tubes, and invert twice, releasing the pressure each time by loosening the cap slightly. Extract by vigorous shaking for 60 sec [4]. Permit the layers to differentiate. Centrifuge, if necessary, at 1500 rpm for 1 mm. Aspirate off the aqueous (bottom) layer by means of a blunt-tip needle. Filter into a marked tube through Whatman No. 1 filter paper. Evaporate the ether extract in a hood or under nitrogen. Dissolve the ether residue in 5 ml of methanol. Should any precipitate appear, centrifuge and obtain clear supernatant for color development. The prepared reagent is stored in an amber vial and refrigerated at 40 and Used for 17-OHCS determination after ACTH stimulation [26].

Colorimetry: Prepare and mark tubes or cuvetts divided into 2 groups. Add sulfuric acid reagent to each Group. Thoroughly mix by vigorous shaking (parafilm capping) or stirring by mechanical mixer for 15 sec. Incubate in a 56 (176) serological bath for 20 mins. At the end of the period, place the tubes in an ice bath or cold-water trough for 3 mins. Read the absorbance (0.1) at 410 nm against reagent blank (RB) [27].

Conclusion

Base-line cortisol levels and stress-induced cortisol levels vary in different animal species. Determination of cortisol levels is essential in determining health and disease states. Several factors affect the specific test to be carried out and which sample is to be used. Recently, RIA has been identified as the gold standard for determination of cortisol levels. However it also has its own shortcomings hence the need to utilize other assay techniques such as ELISA, fluorescence techniques and chemical assays which are more common in our environment. There is also prospect for the use of proteomics to evaluate cortisol levels in non-invasive samples such as finger nails as well as ear wax for enhanced test results.

References

1. Ali N, Pruessner JC (2012) The salivary alpha amylase over cortisol ratio as a marker to assess dysregulations of the stress systems. *Physiol Behav* 106: 65-72.
2. Raff H, Singh RJ (2012) Measurement of late night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. *Clinical Chem* 58: 947-948.

3. Keevil BG (2016) LC-MS/MS analysis of steroids in the clinical laboratory. *Clin Biochem* 49: 989-997.
4. Yoon JB, Stephanie S, Annette MK, Sonia J, Andreas H, et al. (2015) The hyporeactivity of salivary cortisol at stress test (TSST-C) in children with internalizing or externalizing disorders is contrastively associated with amylase. *J Psychiatr Res* 71: 78-78.
5. Gold PW, Drevets WC, Charney DS (2002) New insights into the role of cortisol and the glucocorticoid receptor in severe depression. *Biol Psychiatr* 52: 381-385.
6. Friedman TC, Yanovski JA (1995) Morning plasma free cortisol: inability to distinguish patients with mild Cushing syndrome from patients with pseudo-Cushing states. *J Endocrinol Invest* 18: 696-701.
7. Cirimele V, Kintz P, Dumestre V, Gouille JP, Ludes B (2000) Identification of ten corticosteroids in human hair by liquid chromatography-ion spray mass spectrometry. *Forensic Sci Int* 107: 381-388.
8. Loussouarn G (2001) African hair growth parameters. *British J Dermatol* 145: 294-297.
9. Ito N, Ito T, Kromminga A, Takigawa M, Bettermann A, et al. (2005) Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J* 19: 1332-1334.
10. Lavker RM, Sun TT, Oshima H, Barrandon Y, Akiyama M, et al. (2003) Hair follicle stem cells. *J Invest Dermatol Symp Proc* 8: 28-38.
11. Binz TM, Braun U, Baumgartner MR, Kraemer T (2016) Development of an LC-MS/MS method for the determination of endogenous cortisol in hair using (13) C3-labeled cortisol as surrogate analyte. *J Chromatogr B Analyt Technol Biomed Life Sci* 1034: 65-72.
12. Jones RL, Owen LJ, Adaway JE, Keevil BG (2012) Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. *J Chromatogr* 882: 42-48.
13. Debono M, Harrison RF, Whitaker MJ, Eckland D, Artl W, et al. (2016) Salivary cortisone reflects cortisol exposure under physiological conditions. *Science* 3: 34-45.
14. Zhai X, Chen F, Zhu C, Lu Y (2015) A simple LC-MS/MS method for the determination of cortisol, cortisone and tetrahydro-metabolites in human urine: assay development, validation and application in depression patients. *J Pharm Biomed Anal* 107: 450-455.
15. Akefe IO, Elisha K, Esievo KAN, Sani A (2016) Methods of measuring cortisol. *Clinical Chemistry Seminar, Pathology Department, Ahmadu Bello University, Zaria, Nigeria*. 18: 24.
16. Dimitris A, Papanicolaou JA, Yanovski GB, Cutler JR, George P, et al. (2012) A single midnight serum cortisol measurement distinguishes cushing's syndrome from pseudo-cushing states. *J Clin Endocrinol Metab* 32: 443-444.
17. Perogamvros I, Owen LJ, Keevil BG, Brabant G, Trainer PJ (2010) Measurement of salivary cortisol with liquid chromatography-tandem mass spectrometry in patients undergoing dynamic endocrine testing. *Clin Endocrinol (Oxford)* 72: 17-21.
18. Gallagher P, Leitch MM, Massey AE, McAllister Williams RH, Young AH (2006) Assessing cortisol and dehydro-epiandrosterone (DHEA) in saliva: effects of collection method. *J Psychopharmacol* 20: 643-649.
19. Glass AR, Zavadil III AP, Halberg F, Cornelissen G, Schaaf M (1984) Circadian rhythm of serum cortisol in Cushing's disease. *J Clin Endocrinol Metab* 59: 161-165.
20. Tanada N, Kashimura S, Kageura M, Hara K (1998) Utility of caffeine analysis for forensic hair discrimination. *Nihon Hoigaku Zasshi* 52: 233-237.
21. Tunn S, Mollmann H, Barth J, Derendorf H, Krieg M (1992) Simultaneous measurement of cortisol in serum and saliva after different forms of cortisol administration. *Clin Chem* 38: 1491-1494.
22. Adam EK, Till HL, Granger DA (2011) Diurnal alpha amylase patterns in adolescents: associations with puberty and momentary mood states. *Bio Psychol* 88: 170-173.
23. Umeda T, Hiramatsu R, Iwaoka T, Shimada T, Miura F, et al. (2015) Use of saliva for monitoring unbound free cortisol levels in serum. *Clin Chem* 110: 245-53.
24. Pichini S, Altieri I, Pellegrini M, Pacifici R, Zuccaro P (1997) Hair analysis for nicotine and cotinine: evaluation of extraction procedures, hair treatments, and development of reference material. *Forensic Sci Int* 84: 243-252.
25. Spiehler V (2000) Hair analysis by immunological methods from the beginning to 2000. *Forensic Sci Int* 107: 249-59.
26. Wennig R (2000) Potential problems with the interpretation of hair analysis results. *Forensic Sci Int* 107: 5-12.
27. Yamada J, Stevens B, de Silva N, Beyene J, Taddio A, et al. (2007) Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates. *Neonatology* 92: 42-49.