Artykuł przeglądowy *Review*

Glucocorticoids, their metabolites and their measurement in various animal species

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Summary

Corticoids are a group of vital hormones. Measuring the corticoid concentration in plasma, saliva, hair or feathers, and the amount of glucocorticoids or their metabolites in urine or faeces offers valuable information for clinical diagnosis, livestock management, animal welfare, and conservation biology.

Disturbances of animals influence the endogenous glucocorticoid production, but a careful selection of the sample matrix can overcome some of the sampling difficulties. Some matrices also offer new possibilities, especially post hoc information about the adrenal status. Since most glucocorticoids are excreted as metabolites, the biological activity of these substances is covered in this respect.

Keywords: Cortisol, corticosterone, plasma, saliva, hair, urine, faeces

Definition of the term corticoid and biological activity

Corticoids are chemical substances including steroid hormones or the synthetic analogues. The term "corticoid" therefore covers a group of substances that activate the glucocorticoid receptors in cells independent of the chemical structure. Most of endogenous glucocorticoids are produced by the adrenal glands, but also other organs, such as the lymphoid tissue, the digestive tract or the skin, can produce cortisol or corticosterone (50).

Corticoids are involved in a broad range of regulatory processes, including carbohydrate metabolism, immune response, inflammation, catabolic processes and electrolyte levels (1). Glucocorticoids (such as cortisol or corticosterone) cause metabolic effects, whereas mineralocorticoids (such as aldosterone) are part of the regulatory system controlling the electrolyte and water homeostasis, mainly by increasing sodium retention in the kidney and colon (48).

The action of glucocorticoids covers a broad range of effects at different stages of life. In mammals, these hormones are an important factor in the events leading to parturition (20, 21) and in the maturation of the lungs (49). In general, glucocorticoids help the organism to overcome stressful situations (43). Therefore glucocorticoids are regarded as "stress-hormones", although, from the functional point of view, glucocorticoids are "anti-stress hormones", as they are used to overcome stressful situations. Large amounts of glucocorticoids have anti-inflammatory effects, and the synthetic analogues of these hormones are used in human and veterinary medicine to protect the organism from autoimmunity, inflammation, or overshooting immune responses. They also influence bone metabolism (51), and the long-term action of elevated glucocorticoid concentrations causes osteoporosis. Other effects include increased blood pressure and reduced reproduction. Corticoids also influence brain function (47). It is also well known, that subnormal, as well as too high, corticoid concentrations cause diseases (Morbus Addison, Morbus Cushing; 10).

Endogenous and synthetic glucocorticoids differ in structure. Modifications of the basal corticoid structure cause a higher binding affinity of synthetic glucocorticoid and a prolonged half-life time compared to endogenous hormones. Therefore, the effective strength of these compounds is higher than that of endogenous glucocorticoids. Whereas in high doses glucocorticoids act as catabolic substances, small amounts of synthetic glucocorticoids have anabolic action in cattle. Therefore a monitoring system has to be established to avoid their misuse in the cattle industry (3, 9).

Regulation of glucocorticoid synthesis

In vertebrates, the dominant reactions to environmental or social stimuli are mediated via the hypothalamic-pituitary-adrenal (HPA) axis and the sympa-

thetic nervous system. The classical hormone cascade causing the activation of the HPA-axis starts with an increased production of the corticotropin-releasing factor (CRF), a peptide produced by the hypothalamus stimulating the synthesis of the adrenocorticotropic hormone (ACTH), also a peptide hormone of the pituitary gland. ACTH is secreted into the peripheral circulation and stimulates glucocorticoid production in the adrenal glands. The loop is closed by feedback mechanisms to the hypothalamus and pituitary influencing the production of cortisol and corticosterone, which are the front-line hormones of the HPA- axis. The classical actions of glucocorticoids are mediated via intracellular receptors, but rapid glucocorticoid responses, for example to CRF-induced ACTH secretion, may be caused by effects via non-genomic mechanisms (18).

The above-mentioned hormones do not exclusively regulate glucocorticoid production. Other hormones, such as urocortins (19, 24), arginino-vasopressin (11) and ghrelin (6, 12), also influence the production, and even local regulations (production, activation or inactivation) take place in the peripheral tissues.

The ratio of cortisol to corticosterone in plasma depends on the animal species. In some species, such as ruminants, horses, swine, dogs and cats, cortisol clearly dominates, whereas in others (some rodent species, birds) corticosterone is the dominant glucocorticoid. Whether a species produces cortisol or corticosterone depends on the enzyme equipment of the adrenals, and in most species both glucocorticoids are produced to a species-specific extent (25, 32).

The transport of the hormones from the adrenals to various organs that are the sites of their activity occurs via the blood stream. In blood, the major part of the cortisol or corticosterone is bound to plasma proteins (4), mostly to a glucocorticoid-binding protein (CBG) and to albumin. The non-protein-bound fraction (only 5-10% of the total glucocorticoid concentration in plasma) can diffuse freely, also in tissues, because of the lipophilic nature of these hormones. In many species, the glucocorticoid concentration in blood shows a circadian rhythm (47), but also ultradian patterns of the concentration occur (55) and play a role in the effects of these hormones. Within cells, corticoids are bound to receptors, and the corticoid-receptor complexes cause most of the biological effects.

Metabolism and excretion of glucocorticoids

The half-life time of endogenous glucocorticoids depends on the species as well as on the amount and affinity of CBG in plasma. The glucocorticoid concentration in tissues is not only influenced by the concentration in blood, but is also regulated by the activity of the 11β-hydroxysteroid-dehydrogenase (11β-HSD), which is present in two forms. The type 1 reduces cortisone (an inactive glucocorticoid metabolite) to cortisol (7), whereas 11β-HSD type 2 converts cortisol to cortisone (42) (Fig. 1). This enzyme dominates in mineralocorticoid-sensitive organs. The biological relevance of 11β-HSD 2 is that cortisol can also bind to the mineralocorticoid receptor. The conversion of cortisol to the inactive metabolite cortisone ensures that only aldosterone (this mineralocorticoid is not a substrate for the 11β-HSD 2) is bound to the receptor and there is no or only limited interference by glucocorticoids.

The local "free glucocorticoid" concentration also depends on the temperature of the organ, as CBG releases more protein-bound cortisol at higher temperatures, for example, in inflamed tissues (5).

The predominant organ for glucocorticoid metabolism is the liver. The metabolites produced are mainly ring-A-reduced (5α- or 5β-reduction) and hydroxylated substances. The 5β-reduction of glucocorticoids is in principle the same reaction as the formation of bile acids from cholesterol, since bile acids also have a 5β-configuration (8). The chemical formula of 5α- and 5β-reduced glucocorticoids looks similar, but the shape of the molecules differs, as the 5α-form is plain in structure (similar to the parent hormone), whereas the 5β-form shows a bend between the first two rings. Some of the molecules are also conjugated to form sulfated or glucuronidated metabolites for increasing water solubility. The metabolites excreted differ between species, and also the sex of the animal causes differences in cortisol metabolism (34).

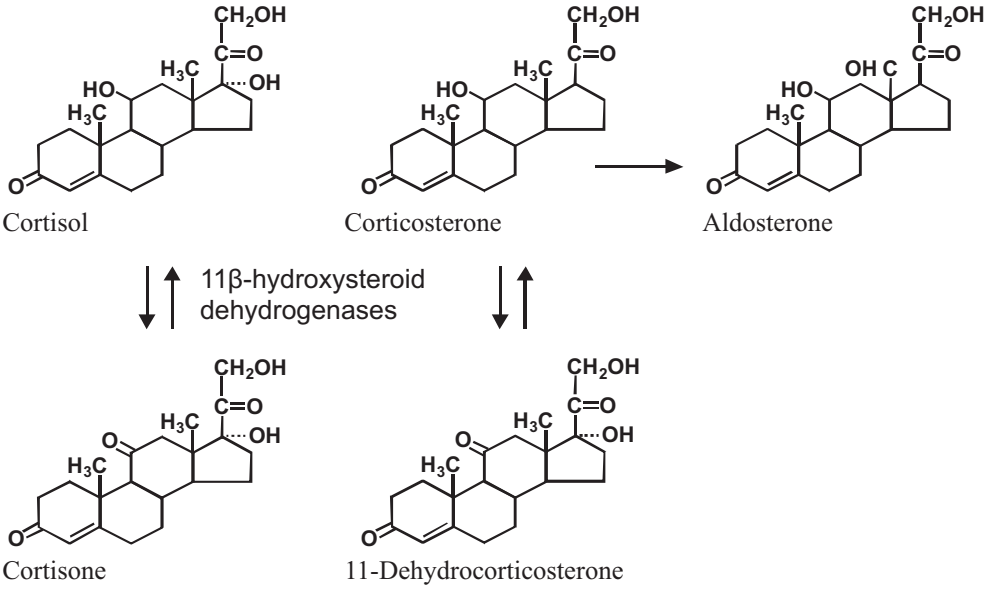

Fig. 1. Chemical formulas of the dominant corticoids and the action of the two 11β-hydroxysteroiddehydrogenases

The percentage of cortisol that is excreted with urine or

faeces varies between species. Among domestic animal species, the extremes are, to my knowledge, the swine, in which only 7% of infused radioactive cortisol is excreted with faeces (34), and the cat, which eliminates most (82%) of the infused radioactive cortisol via this route (44). One has to keep in mind that the percentage of radioactivity in faeces does not represent the percentage of cortisol excreted via bile, because of the re-absorption of substances in the gut (enterohepatic circulation) (26).

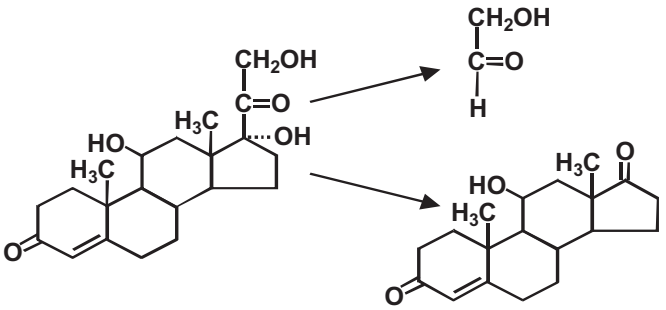

Fig. 2. Side-chain cleaving of cortisol causes the formation of a molecule with a $C_{19}O_3$ structure

Beside reduction, oxidation and hydroxylation, also a side-chain cleavage of glucocorticoids can take place in vertebrates (Fig. 2). The vertebrate enzyme uses substrates with a 17α-hydroxy group, like 17α-hydroxyprogesterone, to form androstenedione or dehydroepiandrosterone (DHEA), and cortisol may also be a substrate, as it has a 17α-hydroxy group, too (corticosterone lacks this group). In some mammalian species, such as ruminants, cats, and some monkeys, substantial amounts of these cortisol metabolites ($C_{19}O_3$ androstanes) are excreted (35). This may be caused by the endogenous production of these androgens, but another source may be the enterohepatic circulation and the reabsorption of these androgens after their production by gut microorganisms. It has been reported that, for example, *Clostridium scindens* can convert glucocorticoids into androgens, and the bacterial enzyme is putatively a transketolase (39) which differs from the enzyme in vertebrates.

Biological activity of glucocorticoid metabolites

Various glucocorticoid metabolites used to be considered of no or low biological activity, but more recent findings demonstrate that at least 5α-reduced cortisol also has glucocorticoid activity (54).

In some species (for example in ruminants and primates), substantial amounts of glucocorticoid metabolites with a $C_{19}O_3$ androstane structure are excreted. Androgenic activity in faeces is an interesting topic even though the activity of the reduced $C_{19}O_3$ androstanes is not known in mammalians. In fish, the 5α-reduced $C_{19}O_3$ androstanes act as endocrine disruptors because of the androgenic activity (15). In cows, androgenic activity in faeces has been known for a long time (41) thanks to the chicken comb test.

The substances causing androgenic effects in bovine faeces have not been identified, but it was shown that substances similar to 11-ketotestosterone (one of the major fish androgens) are present in the faeces of sheep (45). A positive correlation between feed efficiency and the amount of 11,17-dioxoandrostanes in faeces has been reported, which may be an indicator of the biological activity of these cortisol metabolites in ruminants (28). Additionally, the 5β-reduced cortisol metabolite 11-oxoethiocholanolone (an 11,17-dioxoandrostane) is known to be a pheromone in the round goby, a fish species (22).

Measurement of endogenous glucocorticoids

Blood plasma. The concentration profiles of cortisol (or corticosterone) offer interesting insights into physiological and pathophysiological processes. The measurement of the glucocorticoid concentration is mandatory in the diagnosis of hypo- or hyperadrenocorticism. As glucocorticoids increase in response to disturbances and show circadian and episodic changes in concentration, the measurement of a single plasma sample can only be considered as a snapshot of the adrenal activity, and does not necessarily reflect the long-term situation. Therefore dynamic tests, such as ACTH stimulation or dexamethasone suppression tests, have to be performed (10).

The disadvantage of using blood samples as a matrix for measuring glucocorticoid concentration is that sampling itself causes stress and an increase in these hormones within a relatively short time. To follow secretory patterns, in many cases more than one sample may be necessary, which may cause additional stress.

The analysis can be conducted by established immunoassays or physical/chemical methods, such as a combination of chromatography and mass spectroscopy. In plasma samples, the concentration of protein-bound plus unbound cortisol (corticosterone) is usually determined because the plasma samples are extracted with organic solvents. The concentration of the non-bound hormone is measured only in special cases, as this analysis is more costly and time consuming.

Saliva. As the non-protein bound fraction of glucocorticoids can diffuse within tissues, saliva can be used as a matrix (14, 16). Saliva sampling is minimally invasive, and animals can be habituated to sampling – in certain species (primates), even self-sampling is possible after some training (2). The concentration in saliva is a parameter of the free glucocorticoid fraction in plasma, as protein-bound glucocorticoids cannot pass the membranes. Saliva sampling is done with cotton swabs. As the sampling matrix may absorb glucocorticoids, special swabs are available tested to have low absorption for cortisol.

Hair and feathers. The measurement of cortisol in hairs was first conducted in humans, and it is claimed that the results of the analysis in different sections of the hair represent the levels of glucocorticoid produc-

tion at the time when the hair was formed (13, 27). The limitation of this method is that in many animal species, the hair cycle is more complex than in humans (31). Additionally, one has to keep in mind that a local production of glucocorticoids in the skin may take place (23). In humans, the hormones that lead to glucocorticoid production in the adrenals (CRF, ACTH) are also locally produced in the skin, and there is a local regulation of this glucocorticoid production (46). Also external influences, such as UV-light or rain, may alter cortisol concentrations. Considering all these aspects, the analysis may be either a tool for the local production of cortisol, as in skin diseases, or the urgently needed long-term parameter for glucocorticoid production. The extraction procedure is more work intensive than in the case of plasma, as the sample has to be minced and extracted with organic solvents (mainly methanol).

Urine. Until now, most of the work has been done with human urine, and the metabolites are relatively well known. The improvement in the diagnosis of endocrine diseases and doping controls are important drivers of this research. In human medicine, the collection of urine for 24 hours is a standard procedure in monitoring the activity of the adrenals. This eliminates the influence of circadian and ultradian rhythms, and sampling is easy. In veterinary medicine, the collection of urine for 24 hours is possible only under experimental conditions. The analysis can be done with or without extraction and with or without hydrolysis to convert conjugated substances into unconjugated ones (56). The pre-analytical procedure has a large influence on the result measured, and this has to be kept in mind, especially when comparing results obtained in different laboratories.

The problem of the varying concentration in urine, caused by different water intake, can be compensated for by using creatinine as a reference substance, as creatinine is produced by the organism in a relatively constant amount (40).

The specific cortisol (corticosterone) analysis in urine samples is more challenging than the analysis in blood, as a number of closely related glucocorticoid metabolites are present in urine. The results measured by immunoassays represent in many cases the "immunoreactive substances" present in urine (38). Unlike in human medicine, in veterinary medicine the assays used in laboratories are not optimized for a given species with respect to the species-specific pattern of glucocorticoid metabolites excreted via urine. This analytical difficulty may cause the wide range of reference values reported in the literature.

Faeces. Samples can be collected easily from all species (including zoo and wildlife animals and birds) (33, 57). Measuring the concentration of glucocorticoids or their metabolites in faeces offers the benefit that the test system provides "post hoc" information and reflects the situation before. As most glucocorticoids are excreted in faeces in a metabolized form (faecal glucocorticoid metabolites, FCMs), assays for the measurement of these metabolites have been developed. It has to be kept in mind that there are differences in the metabolic pattern of glucocorticoids between species and that the amount of authentic cortisol (or corticosterone) can be very low. Therefore, the selection of an assay to measure the dominant metabolites is recommended. As in saliva and urine, the concentration of glucocorticoids (or FCMs) reflects the "free" (non-protein-bound) concentration in plasma.

There is a certain species-specific delay time, compared to plasma levels, caused by the gut passage time. Also the activity of animals is of some importance, as for example in mice the passage time is quicker during active phases of the day compared to inactive periods (52).

The sample has to be homogenous (undigested material has to be removed). The extraction of glucocorticoids and their metabolites from faeces is relatively simple, and the easiest method of extraction is to use methanol (for review see 36).

When faeces is used as a matrix, the analytical situation is even more complex than with urine because glucocorticoid metabolites are further converted by microorganisms of the gut. Microbial metabolism has to be considered, as the microbial enzymes are still active after defecation, and the concentrations of metabolites can change during the storage of samples at room temperature (29).

In radiometabolism studies, it was shown that no detectable radioactive cortisol was present in sheep faeces collected at the time when the peak of radioactivity was excreted. As cortisol, cortisone, and corticosterone may have the same end-metabolites, the exact precursors of FCMs measured in faeces are difficult to identify.

In the case of FCM measurements, the analytical situation is more complex than for urine, as FCMs vary between species, and therefore the assay has to be carefully selected to pick up the main metabolites. A "generalized" FCM assay (53), which could be used in all species, might not fit the dominant metabolites excreted in the species under investigation. The biological sensitivity of such an assay might be poor, and only drastic increases in glucocorticoid production might be detected. For theoretical reasons, the determination of FCMs should reflect small long-term increases in cortisol production over a certain period better than the measurement of total glucocorticoids in plasma samples, because FCM concentration reflects the non-protein-bound glucocorticoid in plasma, and short-term variations, such as episodic fluctuations, are eliminated (37).

Analytical methods

Glucocorticoid concentrations change within a short time (circadian or episodic variations, overruled by stressful events). Therefore, in many cases, quite large

numbers of samples have to be analyzed. The methods used for analysis are immunoassays and mass spectrometry (MS) in combination with chromatographic methods, such as high performance liquid chromatography (HPLC) or gas chromatography.

To obtain a high throughput in the laboratory, "direct" assays (assays without an extraction procedure) would be preferable, but interfering substances can cause serious analytical problems, so in most cases extraction procedures are used before analysis. Immunoassays meet the criteria of high throughput and low cost. The drawback of these methods is that most of these assays react not only with the target molecule, but also with other, structurally related molecules (cross-reactions). As a high amount of metabolites is present in the excreta, this can cause huge differences, which are described in the literature (56). Therefore, immunoassays have to be carefully selected on the basis of a validation of the test systems. In most cases, the results have to be considered as "immunoreactive substances". The problem of the influence of cross-reacting substances is aggravated by the fact that in a veterinary laboratory, samples of different species have to be examined, and there are species differences in glucocorticoid metabolism. The problem of the validity of the tests used and the comparability between laboratories is also present in human medicine.

In the past, gas chromatography/MS was used as an alternative analytical method for measuring steroids. As glucocorticoids are not stable during the process of gas chromatography and decompose at temperatures used for this method, temperature-stable derivatives have to be produced, which is time consuming. The progress in the development of technical resources in laboratories made HPLC/MS available in biological and clinical laboratories (30). The benefit of this method is that the analysis does not require derivatisation and can cover more than one substance per run (as in gas chromatography). Additionally, HPLC/MS offers a high versatility and specificity. The disadvantages of these methods are higher costs of the equipment and lower throughput, but in clinical laboratories the use of MS techniques for measuring hormones will be one of the standard procedures (17).

In conclusion, the monitoring of glucocorticoid activity requires a careful selection of the matrix used and the possibilities of the analysis, especially in the case of glucocorticoid metabolites.

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