Measurement of Corticosterone Metabolites in Birds' Droppings: An Analytical Approach

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ABSTRACT: Fecal steroid analyses are becoming increasingly popular among both field and laboratory scientists. The benefits associated with sampling procedures that do not require restraint, anesthesia, and blood collection include less risk to subject and investigator, as well as the potential to obtain endocrine profiles that are not influenced by the sampling procedure itself. In the feces, a species-specific pattern of metabolites is present, because glucocorticoids are extensively metabolized. Therefore, selection of adequate extraction procedures and immunoassays for measuring the relevant metabolites is a serious issue. In this review, emphasis is placed on the establishment and analytical validation of methods to measure glucocorticoid metabolites for a noninvasive evaluation of adrenocortical activity in droppings of birds.

KEYWORDS: radioimmunoassay; enzyme immunoassay; cortisol; corticosterone; glucocorticoids; feces; urine; extraction; analytical validation; conservation biology; animal welfare

INTRODUCTION

In vertebrates, the front-line hormones to overcome stressful situations are the glucocorticoids (GCs) and catecholamines. Their increased secretion enhances adaptive physiological responses of an animal.[1–4] The main GCs produced by the adrenal glands are cortisol and corticosterone, the latter dominating in birds.[5,6] Their quantification in blood samples provides valuable information about an animal's endocrine status and can be used as a parameter of adrenocortical activity. Thus, disturbances are assessable,[2,5] although plasma corticosterone concentrations in birds are also subjected to diurnal and annual rhythms,[2,7,8] and corticosterone is also involved in the induction of ovulation in hens.[9]

However, blood sampling itself is critical, as disturbances of the animals will increase the glucocorticoid concentration within minutes,[10] possibly affecting the results. Therefore, in investigations concerning animal welfare, biology, or veterinary medicine, there is increasing interest in measuring glucocorticoid metabolites (GCMs) noninvasively in feces or droppings. This method is feedback free, as samples can be collected without fixation of the animal. Because feces are a complex

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matrix, various other factors may influence the determination of GCM levels. A rigorous validation of the whole analytical procedure is necessary to produce reliable results. Questions regarding collection, storage, extraction, and analysis of fecal samples are reviewed in the following. Emphasis is placed on the establishment and analytical validation of such noninvasive methods for assessing adrenocortical activity in droppings of birds.

CORTICOSTERONE METABOLITES IN DROPPINGS OF BIRDS

GCs are extensively metabolized in various organs, mainly in the liver (for a review, see Ref. 11) and are excreted via the bile into the gut and via the kidney into the urine, mainly conjugated as sulfates or glucuronides. Those products are more water-soluble than the parent steroids.[12] A certain portion of the metabolites is reabsorbed from the gut and again transported to the liver (enterohepatic circulation[6,13]). In the gut, microbial enzymes play an additional role in the conversion of the metabolites. In general, the metabolism includes 5α or 5β reduction, hydroxylations, or reductions of functional groups or side-chain cleavage (C-17,20-lyase) in the case of 17α-hydroxylated metabolites (FIG. 1; see also Refs. 5 and 14).

The best way to investigate the metabolism and excretion pattern of GCs are studies using radiolabeled ($^{14}C/^{3}H$) hormones,[6] although this approach is not possible in every species due to economic or welfare restrictions. If ^{3}H-labeled GCs are used, the radioactive metabolites excreted do not necessarily represent the amounts of formed metabolites because some of the tritium may be lost during metabolism or exchanged for unlabeled hydrogen. These effects do not take place in the case of

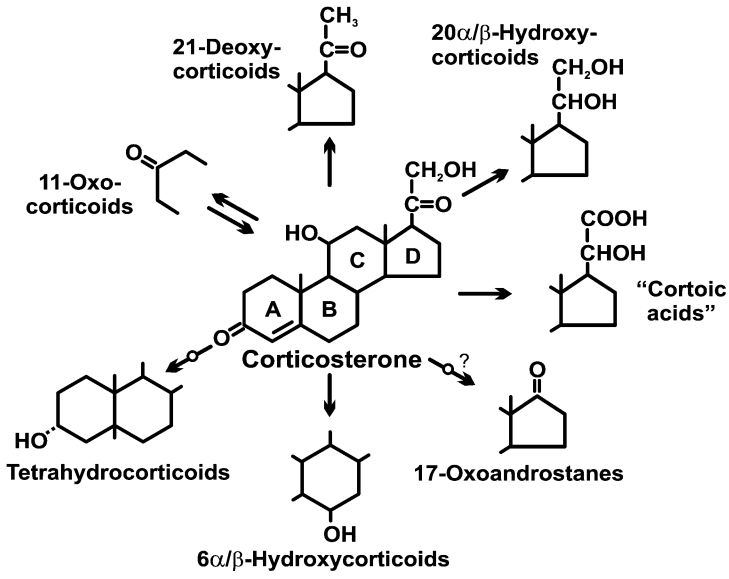

FIGURE 1. Possible pathways of corticosterone metabolism.

^{14}C-labeled GCs, because the ring system of steroids is quite stable. However, ^{14}C-GCs are very expensive, and especially ^{14}C-corticosterone requires a custom synthesis. Because the specific activity of ^{3}H-GC is quite high, the additional mass of these radioactive hormones will not substancially increase the total concentration (radioactive and nonradioactive) in the peripheral blood, and therefore plasma levels of the GCs will remain within the physiological range.

In birds, only a few such studies have been conducted so far[15–19] (for a review, see Ref. 6). Following the administration of GCs, a two-peaked excretion curve of radioactivity was found, reflecting urinary (first peak) and fecal (second peak, corresponding to the gut passage time[26]) excretion.[17]

All radiometabolism studies performed so far (for a review, see Ref. 6) demonstrated pronounced species and sometimes even sex differences concerning the formed metabolites.[2,6,16–19] Cortisol or corticosterone is present in fecal samples only in trace amounts, if at all.[6,16–20] In animals with severe diarrhea, however, the situation may be different because there is less time for bacterial metabolism, and albumin or even blood will pass the intestinal mucosa. Until now, definitive characterization of the GC levels in bird droppings is only tentative. In chicken, Rettenbacher *et al.*[17] found that the dominating ^{3}H-labeled metabolites elute in reversed-phase high-performance liquid chromatography (HPLC) after estrone sulfate and before cortisol, which were used as markers.[16–19] They probably resemble conjugated or polar unconjugated (or at least tetrahydroxylated) metabolites. In all species investigated, enzymatic hydrolysis of these ^{3}H-labeled metabolites did not yield large amounts of diethyl ether–extractable radioactivity.[6,18,19] Analyses using mass spectrometry (MS) should be performed to further characterize these metabolites.

COLLECTION AND STORAGE OF FECAL SAMPLES

Sampling and storing is an important issue for steroid analysis. Any problems arising during this process usually cannot be compensated for by analytical skills afterwards. Fecal GCMs are reported to be further metabolized by bacterial enzymes after defecation, depending on environmental conditions.[5,6,21,22] Therefore, feezing samples immediately after defecation is recommended.[2,6,21,23] Fecal GCMs can be treated with heat or by adding acids or alcohol because the steroids are not affected by these procedures (FIGS. 2 and 3). However, storing preserved samples for longer periods may be critical and must be carefully evaluated (for a review, see Touma and Palme,[2] Palme,[22] Millspaugh and Washburn,[21] and Hunt and Wasser[24]).

EXTRACTION

In mammals, fecal steroids have been reported to be not evenly distributed within samples.[21,25] Therefore, homogenizing the feces before analysis is recommended. The sample homogeneity is very important for measuring fecal metabolites, and a certain amount of feces is necessary to represent a random sample. In birds, droppings usually consist of a urinary and a fecal portion, which in some species cannot be separated because the two excreta are already mixed in the cloaca.[26] This fact must be considered because the pattern and amounts of GCMs may differ in the two components of the droppings.

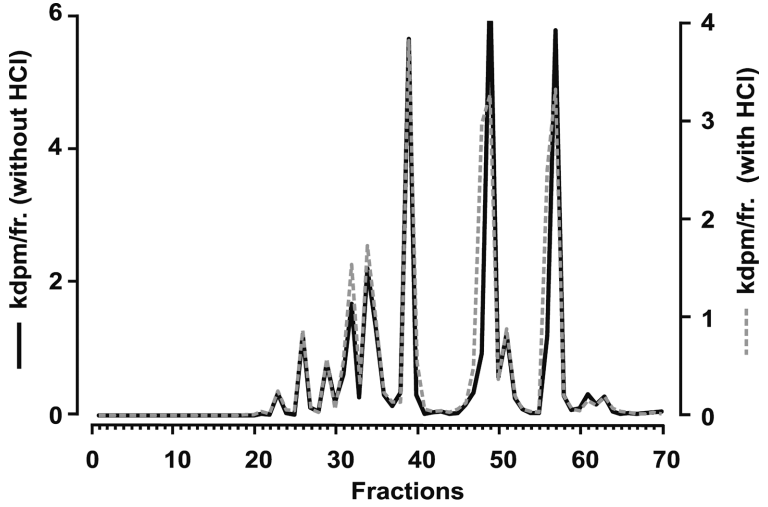

FIGURE 2. Straight phase high-performance liquid chromatographic separation (for conditions, see Palme and Möstl[20]) of ^{14}C-cortisol metabolites derived from an radiometabolism experiment in sheep (Palme *et al.*[25]) immediately after extraction (*straight line*) and after incubation of the extract with concentrated hydrochloric acid for 18 h at 80°C (Möstl *et al.*[27]).

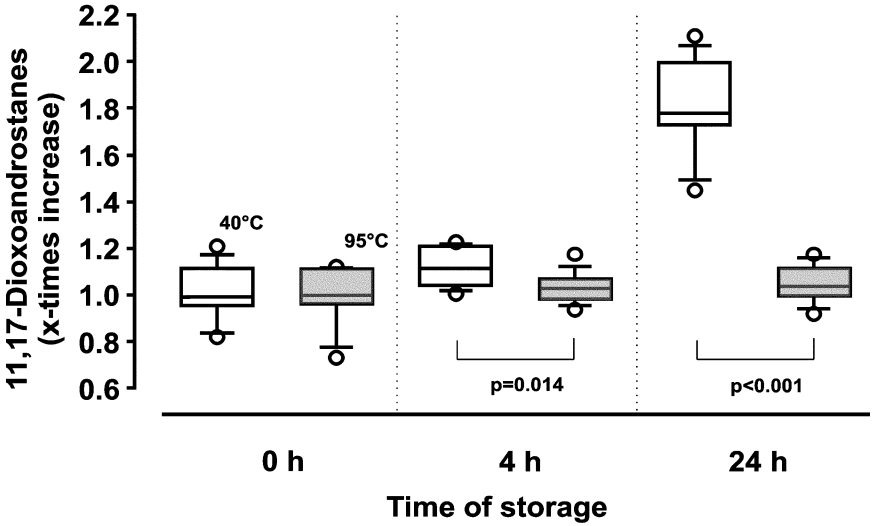

FIGURE 3. Box plot of immunoreactive 11,17-dioxoandrostanes (percent increase) in feces of cows. Samples were defrosted at −40°C and incubated at room temperature (*open boxes*) or at 95°C (*filled boxes*) for 4 or 24 h, respectively (for details, see Möstl *et al.*[27]).

Deep-freezing fecal samples does not destroy bacterial enzymes, which metabolize steroids. If these enzymes are not inactivated before storing the samples (drying, alcohol, or heat), metabolism can continue after thawing. Defrosting the samples by heating (95°C) destroys the bacterial enzymes[27] (FIG. 3). Another possibility is to keep intervals short between defrosting and the addition of organic solvent.

Attention must be paid to avoid carryover effects during weighting of the samples. This is especially important if there are large differences in sample concentrations. In addition, one must address possible health risks caused by parasites, bacteria, or virus particles, because sometimes samples from animals with unknown health status or even sick animals are investigated, and some pathogenic microorganisms, viruses, or prions have zoonotic potential. Some research groups homogenize lyophilized samples before extraction. The powder causes additional risks because particles may be inhaled.

In general, extraction procedures are developed to concentrate the substances of interest and at the same time exclude possible interfering compounds. GCMs of various polarities are present in the feces. Polarity describes the degree to which a compound (steroid) is soluble in water. Additional hydroxyl groups render the steroid more polar (hydrophilic). A separation of hydrophobic steroids from aqueous media using organic solvents (petroleum or diethyl ether) is relatively simple, but dissolves many other lipids also.[28]

Because extraction procedures are time-consuming and costly, assays, which measure an aliquot of the sample directly with no purification, are used to determine steroid hormone levels in plasma samples. Stanczyk *et al.*[29] assessed the reliability of many different commercially available (estradiol and testosterone) kits. Large differences in the obtained results were found between these direct immunoassays. The authors claim that a more thorough analytical validation of the assays is necessary with respect to sensitivity and specificity because matrix influences can play a substantial role. Similar effects must be expected when measuring GC levels in droppings, especially because fecal samples represent a much more complex matrix than plasma samples.

Another way to extract and clean up the substances of interest is minicolumns, filled with material for reversed-phase chromatography (e.g., Sep-Pak C_{18} cartridges from Waters, Milford, MA). Because these columns are too expensive for use in routine analysis, it is recommended that they be used only before HPLC separation of the GCMs.[30,31]

Because radiometabolism studies are not available for most avian species, the extraction procedures must cover a broad polarity range (potentially conjugated and unconjugated steroids); otherwise, substances of interest may be excluded.[17] It should be kept in mind that, for example, diethylether is too apolar to extract tetra- or pentahydroxylated steroids (conjugated steroids are also not extractable using this solvent), and therefore more polar solvents must be used. The more hydrophilic, conjugated steroids (sulfates or glucuronides) can be extracted into organic solvents by forcing the extraction by saturating the water phase with salt (NaCl, ammonium sulfate) and lowering the pH.[28]

Although boiling procedures have been described for extraction of GCMs (e.g., Wasser *et al.*[15]), most authors now use mixtures of methanol or ethanol with water to dissolve the steroids from the feces, which is more a suspension than an extraction.[6,15,22] After shaking (three times for 10 s, using a hand vortex or 30 min with a

multivortex), the vials are centrifuged. We recommend not evaporating the alcoholic extract because redissolving the material in assay buffer can cause problems. Instead, an aliquot of the supernatant is used directly in the immunoassay. Because a higher percentage of alcohol will interfere with the steroid–antibody binding, a further dilution step with assay buffer prior to analysis is performed.

Determination of Recovery Rate

As long as the chemical structures of the immunoreactive metabolites are unknown or the metabolites are not available in radiolabeled forms derived from radiometabolism experiments, a reliable determination of recoveries of GCMs is not possible. Recoveries reported in published studies were based mostly on the extraction of radiolabeled cortisol or corticosterone added to the sample just before processing. Results obtained by Palme *et al.*[32] emphasized that a higher proportion of radioactivity could be extracted from feces after ^{14}C-labeled progesterone was added *in vitro* than with fecal samples containing metabolites of ^{14}C-labeled progesterone injected *in vivo*. This is due to the fact that first, the metabolites are of different polarity, and second, complex interactions between sample matrix and steroids, which can affect extraction efficiency, are less pronounced with steroids added *in vitro*. As a result, the recoveries reported in the literature probably do not reflect the true recovery of the metabolites in the feces, but are over- (or under-) estimates, depending upon the steroid added, the metabolites measured by the immunoassay, and the investigated species. Therefore, recovery testing based on naturally metabolized, radiolabeled steroids infused into the animals should be favored.[6,20,27] The influence of different methanol/water mixtures on the recovery of GCMs in mammals is described in a review by Palme *et al.*,[6] but similar studies are lacking in birds.

TEST SYSTEMS FOR MEASURING GCMS

To analyze fecal GCM levels, two different procedures can be used. Traditionally, complex mixtures of steroid hormone metabolites are analyzed after extraction and derivatization using gas chromatography–mass spectrometry (MS). HPLC–MS is now becoming increasingly popular,[33] as derivatization procedures are not necessary. HPLC using direct UV detection can be applied only if a 4-ene-3-oxo structure is present, as is the case with cortisol, cortisone, or corticosterone. The reduced metabolites cannot be measured with such detectors.

The second approach involves immunoassays. Since 1970, there has been a very rapid increase in publications concerning steroid immunoassays.[34] Immunoassays are much cheaper than methods employing MS, and they allow measurement of many samples within a short time, but they are less specific (see below).

IMMUNOASSAYS

For analysis of steroids, competitive immunoassays are mainly used.[35] This means that the label and the steroid to be measured compete for an antibody binding site. Radioimmunoassays (RIAs) or enzyme immunoassays (EIAs) are mainly used.

RIAs certainly have their merits, such as high precision, robustness, and a long-lasting tradition in performance. Various antibodies and labels for cortisol and corticosterone are commercially available, but because the two hormones are not present in the feces of mammals and birds,[6] these assays rely on cross-reactions with the excreted metabolites. If commercial RIAs are used, cross-reactions with GCMs should be carefully analyzed because the manufacturers mostly give only the data relevant for plasma analysis (see below). Assays especially designed for measuring GCMs are mainly EIAs because radioactive labels for the metabolites are not commercially available, and a custom synthesis or biosynthesis is too expensive.

Antibody Production (Immunogen and Immunization)

To establish an immunoassay, an antibody is required. Therefore, animals must be immunized. Because steroids are too small to act as an immunogen for themselves, they must be linked to a macromolecule—for example, a protein—to be an immunogen. However, most of the steroids do not contain a functional group, which can be linked directly to a macromolecule, and therefore a carboxyl group, for example, must be added. To add these functional groups, one can use a variety of reactive steroid derivatives, including chloroformates, hemicuccinates, carboxymethyloximes, and thioether alkanoic acids.[36] The molecule between the steroid and the protein also acts as a spacer (four to six carbon atoms' distance between steroid and protein seemed to be best suited). The carboxyl group is then used to link the steroid to a protein, for example, by using a mixed anhydride or the carbodiamide reaction. The chemistry of the formation of steroid–protein conjugates was reviewed by Kellie *et al.*[37] If an antibody is raised against this steroid–protein conjugate, the

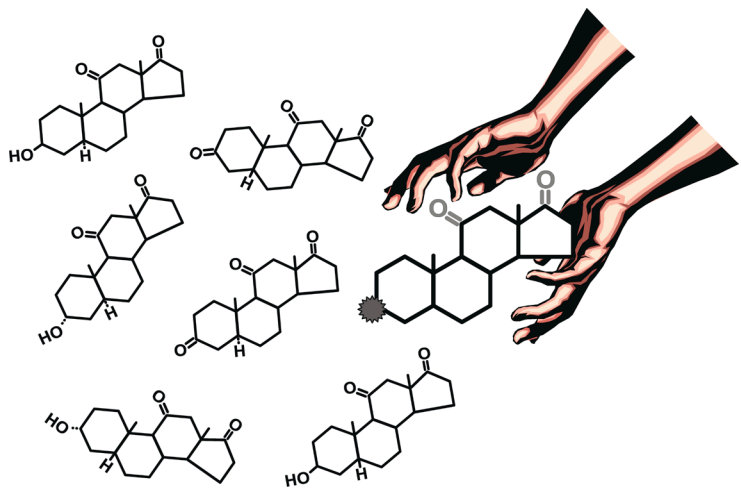

FIGURE 4. Scheme of various 11,17-oxo-androstanes (5α/5β-androstanes, having a 3-oxo or 3α/3β-hydroxyl group), which may show cross-reactions with an antibody raised against 11-oxoetiocholanolone-3-hemisuccinate:bovine serum albumin (e.g., Palme and Möstl[20]). The palpating hands symbolize the antibody, which recognizes only a certain part of the steroid.

functional group in the steroid molecule used for linking to the protein is masked and will not act as an immunological discriminant of the antibody formed (FIG. 4).

For measuring steroid hormones in blood samples, various efforts have been made to increase the specificity of antibodies.[38] For example, many authors described procedures of coupling the steroid at positions remote from functional groups, for example, C-6, C-7, or C-11α to get more specific antisera, using as their basis the fact that potentially cross-reacting steroids are not present in the samples. However, for analysis of fecal GCM levels, the use of highly specific cortisol and corticosterone antibodies must be avoided because those substances are not present in the fecal samples.[6] Therefore, antibodies that are successfully used for measuring fecal GCMs are group specific (e.g., the corticosterone RIA from ICN Biomedicals (Costa Mesa, CA).[15,32]

Because the fecal GCMs vary substantially between species, it may be too time-consuming and costly to raise antibodies against the main metabolites in each species, as this would require establishing a new assay for each species. Unlike in blood samples, in which a highly specific antibody is desired to get a specific assay, most assays for measuring GCMs use antibodies that are more or less group specific (FIG. 4), as due to the masked coupling position, a group of metabolites are recognized by the antibody.

Those metabolites can be measured using the same assay, because the antibodies show mostly sufficient cross-reaction with steroids, which differ from each other only at positions close or at the position, where the hapten was linked to the carrier molecule. For example, several steroids with two oxo-groups at positions C-11 and C-17 in common [11,17-dioxoandrostanes (11,17-DOAs)] are recognized by an antibody raised against 5β-androstane-3α-ol-11,17-dione-3-HS:BSA (bovine serum albumin;[20] FIG. 4).

Most of the antibodies used for steroid analysis are raised in rabbits because a successful immunization of an animal results in enough material for many assays (working dilutions of antibodies using double-antibody techniques are often greater than 1:10,000). To get higher titers, adjuvants must be applied for immunization, and immunization protocols may take several months to obtain suitable antibodies.[39,40]

Labeled Steroids

In radioimmunoassays, tritiated or iodinated steroids are used, and a highly specific activity of the label increases the sensitivity of the assay system. Since the early 1970s, an increase in sensitivity of steroid immunoassays has been achieved by introducing a so-called heterology. This means that the labels do not use the same steroid, site, or bridge as the hapten used for raising the antibody (steroid heterology, bridge heterology, or site heterology;[41] Fig. 5).

For EIAs, mainly horseradish peroxidase (HRPO) or alkaline phosphatase (AP) is used. In competitive immunoassays, mostly the label and not the antibody (immunosorbent), is enzyme linked. Therefore, these assays should be called EIAs and not enzyme-linked immunosorbent assays because the antibody is not labeled. Another way to label the steroids is to link them to biotin. In both cases, the same biochemical procedures can be used to link the steroid derivates with a carboxyl group (COOH) to a protein (e.g., using the ε-amino group of lysine). Biotin derivates with an amino group plus a spacer [Biotin-PEO-LC-Amine = biotinyl-3,6,9-trioxaundecanedi-

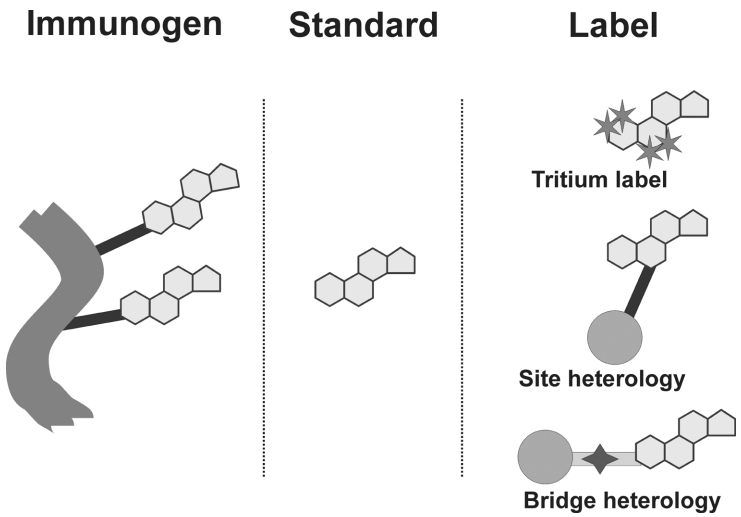

FIGURE 5. Steroid linked to a protein carrier to be used as immunogen for the antibody production, standard, and various labels (tritium label with four tritium atoms at positions C1, C2, C6, and C7 scheme of a potential label with site or bridge heterology).

amine;[46] Biotin-PEO-Amine = biotinyl-3,6-dioxaoctanediamine, 5-(biotinamido)-pentylamine[20]] are commercially available from, for example, Pierce (Rockford, IL).

Biotin is tightly bound by avidin or streptavidin. Streptavidin linked to enzymes like HRPO or AP is commercially available and is used to detect the biotin label. The use of biotin gives a higher specific activity of the system than the direct linking of the steroid to enzymes, as is used in direct labeling. The best ratio of enzyme to steroid achievable by direct linking is 1 to 1, but in many cases more than 1 mole of steroid is bound per mole of enzyme. On the other hand, using streptavidin–enzyme conjugates gives a ratio of two to three enzymes per mole of streptavidin, which results in a higher sensitivity of these assays.

Second-Antibody Technique

In many EIAs, a double-antibody technique is used, which means that the antisteroid antibody is not directly bound to the polystyrol surface of a microtiter plate, but a so-called coating antibody. That antibody was raised in another species as the antisteroid antibody and is directed, for example, against rabbit immunoglobulin G. The coating antibodies used in EIAs are purified mainly by affinity chromatography to give a high coating efficiency for the specific antisera.[42]

ANALYTICAL VALIDATION OF AN IMMUNOASSAY

An established immunoassay must be analytically validated for each given species under investigation. This includes certain criteria, such as accuracy, specificity

(cross-reactions), sensitivity, precision, and parallelism of the dose–response relationship for the standard and the unknown.[a]

Accuracy

The absolute concentrations (true values) of steroid hormone metabolites are difficult to evaluate because the matrix, cross-reacting substances, and the purity of the standards used may influence the results. Whitehead[43] stated that the method used for analysis influences the results, and that a particular result obtained by a particular method may not be true, but may give a correct value. For measuring GC metabolites in feces of mammals or droppings of birds, various laboratories are using different assays and extraction procedures. Usually, the assays consider only some of the metabolites and ignore others. Therefore, concentrations published may vary between laboratories, hindering a direct comparison of the results between different laboratories. In cattle and geese, the influence of using different antibodies tomeasure GC metabolites is shown by Morrow *et al.*[44] and Frigerio *et al.*[45] Because there are different metabolites in feces, the question of which metabolite should be measured is important. For example, in fecal samples of sheep, 27 different metabolites were detected using HPLC–MS.[46] Radiometabolism investigations showed similar results also in various other species (for a review, see Palme *et al.*[6]). So there is a broad range of substances that can be measured as parameters of GC production, and the results differ depending on the metabolite(s) measured. Standardization with respect to the results measured by different laboratories should be one goal.

Taken together, independent of a "true" value, a "good" assay should fulfill some criteria,[47] like to provide appropriate specificity and sensitivity, to be precise, to be robust, and to have a "working range" adequate for the study and to be compatible with the environment.

SPECIFICITY AND CROSS-REACTIONS

If we define specificity according to the fact that the substance measured is substantially unique and identical to the standard used, this definition is quite rigorous.[48] As in most assays for GCMs, there is more than one immunoreactive substance measured, and their chemical structures are not characterized. Thus, a specificity test in such a strict sense as described by Cekan[48] cannot be given in most cases.

Parallelism of diluted samples with a standard curve is sometimes given as a parameter of specificity of an immunoassay. However, as has been already described by Ekins,[49] some cross-reacting compounds will yield dilution curves that give acceptable parallelism results with standards, and parallelism is therefore not a marker of specificity, but a proof of a dose–response relationship.

[a]To convert steroid concentrations given in nanograms per gram to nanomoles per kilogram (SI unit), one multiplies the original value by a factor of 1000 divided by the molecular weight of the standard of the immunoassay used. For example, the molecular weights of cortisol, corticosterone, and 11-oxoetiocholanolone are 362, 346, and 304, respectively.

Because the GCMs in the feces are a complex mixture of different, structurally closely related steroids, testing cross-reactions is important for the analytical validation of an immunoassay.[34] This provides information, which substances, other than the standard, react with the antibody. Tests should include steroids, which are expected to occur in the samples (GCMs) of the species investigated and not only considering the steroid hormones present in the blood. For comparisons between laboratories, we must consider that incubation times have an influence on the concentrations measured because cross-reactions of an assay system are different, depending on whether an assay is in the equilibrium or not. The cross-reactions indicate to which degree a certain steroid reacts in the immunoassay. However, they do not reveal which substances are really measured because other metabolites not evaluated for their reactivity may be present in the sample (especially in the feces). Therefore, a recommended procedure for assessing the presence of cross-reacting substances is the analysis of samples derived from radiometabolism studies. After extraction, an HPLC separation of the metabolites is performed, and the individual fractions of the eluent are collected. An aliquot of each fraction is used for measuring radioactivity, and another aliquot for the immunoreactivity in the respective assay(s). If the immunoreactive substances coelute (at least partly) with radioactive peaks, this is an indicator that an assay can detect metabolites of the parent steroid. Radioactive peaks, occurring without accompanying immunoreactivity, demonstrate that those metabolites are not measured by the assay system. On the other hand, immunoreactive peaks without coeluting radioactivity are an indicator for cross-reacting substances not originating from the substance injected.[5,6,22,31,50] These so-called HPLC immunograms also characterize the immunoreactive substances present in the droppings, but care must be given to possible exclusion of steroid metabolites during extraction, cleanup procedure, or redissolving of the extract in the mobile phase for chromatography. Therefore, it is also advisable to calculate the total amount of immunoreactive metabolites measured (area under the curve) with and without chromatography to check whether significant amounts of immunoreactivity were lost during the whole chromatographic procedure.

Cortisol and corticosterone are both present in plasma of some vertebrates, and Teskey-Gerstl *et al.*[30] showed that different fecal metabolites are formed originating from these two hormones. The authors injected ^{14}C-labeled cortisol and ^{3}H-labeled corticosterone separately in European hares (three animals each). Without such radiometabolism investigations, it is an open question whether the immunoreactive GCMs measured in feces originate from cortisol or corticosterone. For example, a commercially available corticosterone RIA (ICN Biomedicals) is widely used,[15,23,24] but its cross-reactions with the fecal steroid metabolites (5α/5β-reduced steroids) are still unknown. This should be tested, especially to elucidate if the ICN antibody measures cortisol or corticosterone metabolites (both GCs differ only at position C-17; cortisol having a hydroxy group, which is lacking in the case of corticosterone).[6] That may be especially important in animals with cortisol as the main GC in the blood, because in those species the adrenal gland is also capable of secreting corticosterone and both hormones may have different biological functions (the latter acting mainly within the brain). Because this corticosterone antibody shows cross-reactions of less than 1% with cortisol, it probably reacts to an even lesser extent with the reduced cortisol metabolites. Although bacteria can produce many different steroid-transforming enzymes, the presence of a 17α-dehydroxylase was not

reported.[14] Thus, the corticosterone RIA may measure metabolites reflecting blood corticosterone and not cortisol levels. If there is no proof that the substance measured is identical to the standard, it is advisable to use the term "immunoreactive." For example, an antibody raised against 11-oxoetiocholanolone (linked at position C-3 of the molecule) will show cross-reactions with 11,17-dioxoandrostanes (FIG. 4). This description will be more correct than labeling the measured substances as 11-oxoetiocholanolone. In the case of cortisol or corticosterone immunoassays, the standard is not present in the feces,[2,6,18,19] and the substances measured should be labeled as cortisol/corticosterone metabolites. However, some reduced GCMs may have a biological activity[51–53] that is neither known nor proven for the fecal GCMs. Therefore, the term "fecal glucocorticoids" should also be avoided.

PRECISION

As parameters of precision control, pool samples must be analyzed in each assay and the coefficient of variation (CV) of their measured concentrations calculated within and between assays (intra- and interassay CV). To detect a potential bias within an assay, these samples should be distributed among samples from the experiment. To monitor the precision more thoroughly, two pool samples with different concentrations (high- and low-level pool) can be used.

SENSITIVITY AND BLANK VALUES

The sensitivity of an assay is defined as the smallest value that can be reliably discriminated from zero values with a 95% probability (two standard deviations from the signal given by the zero blanks).[34,54] Therefore, the precision influences the sensitivity of a test system, and a higher precision will also lower the assay sensitivity. Immunoassays usually have higher variations at both ends of the standard curve and lowest in the middle. In addition, blank effects have more consequences in the lower concentrations, whereas interferences concerning nonspecific binding are more pronounced in the higher concentrations (low optical densities). If possible, the dilution of samples should be performed in such a way that most of the values measured are in the range of the optimal precision of the assay, which is evaluated by a so-called precision profile, which shows the variation of pool samples at various dilutions.

As mentioned earlier, blank values (nonspecific interferences) may be a problem in immunoassays, especially when the concentrations of the metabolites to be measured are relatively low. In this case, interferences from the sample matrix or from the organic solvents used for extraction may cause problems. Because samples are normally not available without GCMs, it may be advisable to perform a suppression test using synthetic GC to check if the assay can detect the lower amount of GC produced by the adrenal glands.[2] The dexamethasone or flumethasone metabolites do not cross-react in immunoassays for corticosterone metabolites, but the adrenal production of GCs is reduced in response to the injection of the synthetic substances. Testing blank extracts (extraction procedures done without feces) will give some information concerning blank values.

The use of highly sensitive assays can reduce a possible blank problem because such assays permit a dilution of the sample extract that is much higher than those of less sensitive assays, and the interfering substances may be decreased to a level where their effect is negligible.

ASSAY SELECTION

Selection of an appropriate assay plays an important part in fecal analysis. There are various immunoassays described, measuring GCMs in fecal samples. Some authors (e.g., Kotrschal *et al.*,[55] Goymann *et al.*,[56] and Wasser *et al.*[15]) used corticosterone assays, which show some cross-reactions with the GCMs in feces. Another approach is the use of assays especially designed to measure groups of fecal metabolites, as for example described by Palme and Möstl[20] and Möstl *et al.*[46] for ruminants. In birds, a tetrahydrocorticosterone EIA (5β-pregnane-3α,11β,21-triol-20-one, a metabolite of corticosterone) was established to measure the corticosterone production in Wilson's storm petrels.[57] The same assay was also used successfully in Adélie penguins.[58]

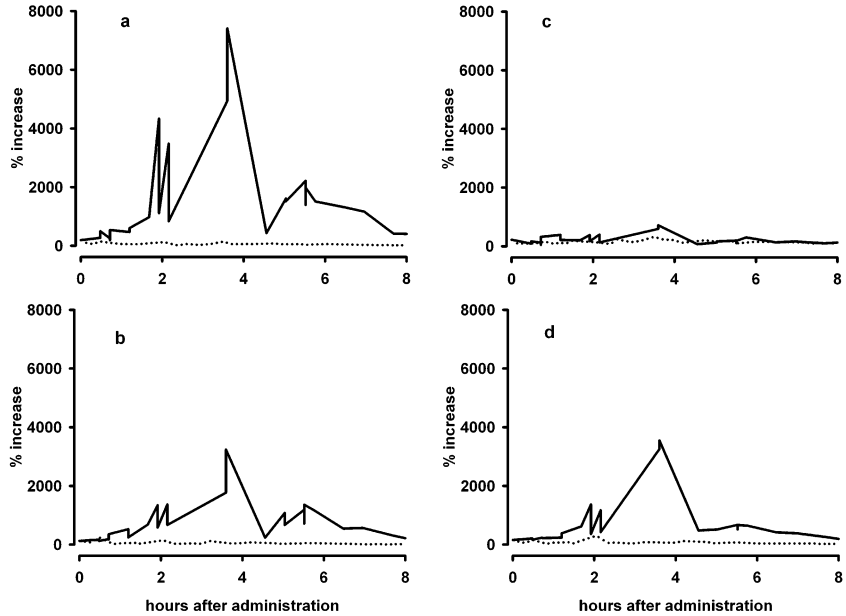

FIGURE 6. Immunoreactive corticosterone metabolites in feces of a gander after ACTH injection. All samples were stored at –20°C after collection and analyzed after methanolic extraction using four different assays: (**a**) 11β-hydroxyetiocholanolone EIA;[45] (**b**) tetrahydrocorticosterone EIA;[57] (**c**) corticosterone EIA;[20] (**d**) 11-oxoetiocholanolone EIA.[46] *Dotted line* represents the basal values measured during the same time on the day before ACTH administration.

On the basis of analytical validations (e.g., HPLC immunograms) alone, it is not possible to select the best-suited EIA for measuring adrenocortical activity in a species. To achieve that, it is important to perform a physiological and biological validation as well (for details, see Touma and Palme[2] and Goymann[50]), which should prove that changes in activity of the hypothalamic–pituitary–adrenal (HPA) axes are reflected in fecal GCM concentrations measured by the respective assay. For example, a higher increase of GCM concentrations following an ACTH challenge is thought to indicate a better-suited immunoassay.

To show this, data from a recently performed ACTH test in domestic geese are given. Because these birds have a long gut passage time, we also expected we would be able to discriminate the immunoreactive metabolites excreted via the urine (first peak) and the feces (second peak). ACTH (0.25 mg; Synacthen, Ciba Geigy, Switzerland) was administered (i.v.) to four geese (two males and two females). and all droppings were collected for the next 10 h. For comparison, all samples were collected from the same animals during the same time on the day before the stimulation test. We measured various immunoreactive metabolites using assays with antibodies produced against corticosterone,[20] 11-oxoetiocholanolone,[46] tetrahydrocorticosterone,[57] and 11β-hydroxyetiocholanolone.[45] All assays showed an increase in immunoreactive substances measured in the animals after ACTH injection (FIG. 6). As expected, two immunoreactive peaks occurred, representing urinary and fecal metabolites. The baseline-to-peak ratio was highest using the 11β-hydroxyetiocholanolone EIA. Therefore, this assay showed the highest reactivity, and thus may detect smaller changes in GC production in this species than, for example, the corticosterone assay used. These results back up the findings of Frigerio *et al.*[45] There it was shown that adverse weather conditions increased the concentration of the immunoreactive substances measured with the 11β-hydroxyetiocholanolone assay, but not

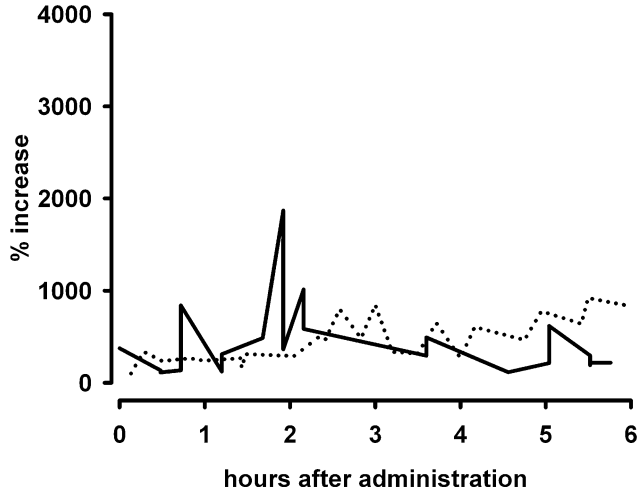

FIGURE 7. Immunoreactive 20-oxopregnanes in droppings of the same gander after ACTH injection. *Dotted line* represents the values measured during the same time on the day before ACTH administration.

those measured with the corticosterone EIA, revealing different biological sensitivities of the assay systems. There is no general rule regarding which assay should be used in birds because the pattern of formed metabolites is species specific. For example, Goymann *et al.*[16] tested the 11-oxoetiocholanolone EIA[20] in stonechats, but did not get satisfactory results. In general, if small changes in GC production must be measured, a careful selection of the assay systems available is advisable. However, a measured increase of immunoreactive metabolites after a stressful event or ACTH injection is not conclusive proof that an assay measures fecal GCMs. The goose samples mentioned earlier were also analyzed using an assay for 20-oxopregnanes,[59] and the results show an increase in these metabolites also. FIGURE 7 shows the data of the same male individual as shown before (Fig. 6). The increase was even more pronounced in the females. This may be explained by the fact that progesterone is the precursor of GCs and also increases after ACTH injection.[60] In addition, these immunoreactive metabolites were excreted predominantly via the urine. In this case, the only way to definitively prove that the metabolites measured with a certain EIA are derived from plasma GC would be an HPLC immunogram of samples from a respective radiometabolism study.

CONCLUSION

It is mandatory to evaluate the immunoassays used for measuring fecal GCMs for each species under investigation. As highlighted earlier in this article, an immunoassay must be carefully validated analytically. Particularly, it should be demonstrated that the antibody used cross-reacts with metabolites derived from the GC present in the blood. In addition, a physiological and biological validation is necessary. A clear alteration of fecal GCMs in relation to basal levels is thought to be indicative of a better-suited immunoassay for measuring adrenocortical activity. However, depending on the actual experiment performed, stability of the fecal GCMs measured with an immunoassay also plays a crucial role. Because this was reported to vary substantially between assays, these may also be criteria for the selection of an immunoassay. Only appropriate assays fulfilling these criteria will allow investigators to also monitor low degrees of disturbances, and thus provide a high biological sensitivity.

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