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Measuring faecal steroid metabolites with enzyme immunoassays (EIA) on microtitre plates using biotinylated steroids as labels

Contents:

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Abbreviations: Antibody (AB), biotin label (BL), double distilled water (DDW), microtitre plate (MTP)

I. Extraction (see also Palme et al., 2013)

It is advisable to freeze samples immediately after defecation (in order to prevent possible degradation of metabolites by bacterial enzymes), and store them at -20°C until analysis.

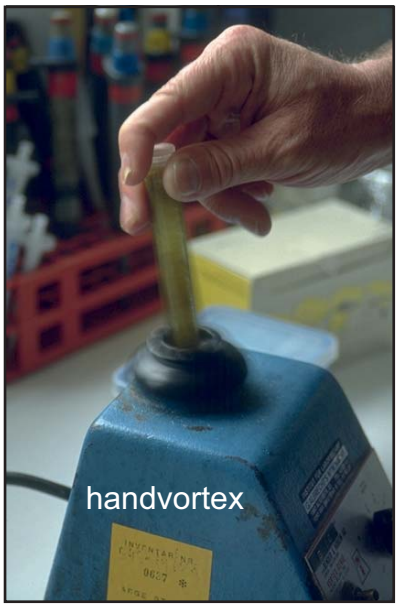
Weigh 0.5 g of wet faeces
Add 5 ml 80% methanol* (premixed; or 4 ml 100% methanol and 1 ml water)

(80% methanol gave the highest yield of naturally occuring metabolites after administration of radiolabelled glucocorticoid in all species examined so far: sheep: Palme et al., 1997; horse: Möstl et al., 1999; carnivores: Schatz & Palme, 2001; for references, see Info-letter)

In some animals (e.g., rodents) it may be necessary to weigh a smaller portion (0.05 or 0.1 g) and add an adequate amount of alcohol (e.g., 1 ml, 80%, Touma et al., 2003, 2004)
In birds, we recommend extracting droppings with 60% methanol, as the metabolites are more polar.

Vortex for 30 min on a multivortex or 1-2 min on a handvortex and centrifuge (2500 g; 15 min).

*(we use methanol for analysis; No. 1.06009.2500, Merck)



a) When high amounts of metabolites are present in the faeces:
Dilute an aliquot of the supernatant with assay buffer (1+9), and store it at -20°C until analysis with EIA.

In most species this simple extraction procedure (=suspension) will suffice.
(e.g., Palme et al., 1997, 1999 etc.)

b) However, if only small amounts of metabolites are present (e.g., horses - Merl et al., 2000) further steps have to be included:

Transfer 1 ml of the supernatant into a new vial
add 5 ml diethylether + 0.25 ml 5% $NaHCO_3$ (Merck 106329)
vortex for 10 sec., centrifuge and freeze
transfer supernatant into new vial and dry it down (under a stream of N_2 and/or in a heat block set at ~60°C), redisolve in 0.5 ml EIA buffer
50 µl are used for the EIA (dilution factor = 110)

II. Measurement with EIA

1. Buffers and solutions

1.1. Coating buffer

1.59 g Na_2CO_3 (Merck 106392 or Sigma S-7795)
2.93 g $NaHCO_3$ (Merck 106329 or Sigma S-6014)
dissolve and fill up to 1 l with DDW, adjust to pH 9.6 with (about 10 ml) HCl (1 mol/l)

1.2. HCl (1 mol/l)

920 ml DDW + 80 ml 37 % HCl (Merck 100317 or Sigma H-1758)

1.3. Assay buffer

2.42 g Trishydroxyaminomethane (Merck 108382 or Sigma T-1503), 20 mmol/l
17.9 g NaCl (Merck 106404 or Sigma S-9625), 0.3 mol/l)
1 g Bovine serum albumin (Sigma A-4503)
1 ml Tween 80 (Merck 822187 or Sigma P-8074)
dissolve and fill up to 1 l with DDW and adjust to pH 7.5 with (about 17 ml) HCl (1 mol/l)
filter through Sep-Pak® C18 (see 1.3.1.)

1.3.1. Filtration of buffer through Sep-Pak® C18

Sep-Pak® classic C18 cartridge (360 mg; Waters WAT051910)
rinse with 5 ml methanol (Merck 106009), followed by 10 ml DDW (done by hand with a syringe),
connect column to tubing of peristaltic pump (flow rate of 2 to 10 ml/min),
discard the first 10 ml of the filtrated buffer,
collect buffer in clean bottle.

1.4. "Second" coating buffer

3.146 g Trishydroxyaminomethane (see 1.3.)
23.3 g NaCl (Merck 106404 or Sigma S-9625)
13 g BSA (Sigma A-4503)
1.3 g Sodium azide (Merck 106688)
dissolve and fill up to 1.3 l with DDW and adjust to pH 7.5 with (about 40 ml) HCl (1 mol/l),
filter trough SEP-PAK C18 (see 1.3.1.).

1.5. Washing solution

0.5 ml Tween 20 (Merck 822184); add 2.5 l DDW

1.6. Substrate buffer for peroxidase

1.36 g Sodium acetate (Merck 6267) = 10 mmol/l
dissolve and fill up to 1 l with DDW and adjust to pH 5.0 with (~8 ml) 5 % citric acid (Merck 100244)

1.7. Enzyme solution for Streptavidin-reaction

30 ml assay buffer (see 1.3.)
+ 0.001 ml Streptavidin-POD-conjugate (=0.5 U; Roche 11 089 153 001, 500 U)
mix on a magnetic stirrer a few minutes before use
(the working solution has to be prepared immediately before use!)

1.8. Substrate solution for peroxidase

30 ml of substrate buffer 1.6.
+ 0.5 ml 3,3',5,5'-Tetramethylbenzedine (0.4 %) - Store in a dark bottle!
(0.4 % = 0.4 g [Fluka 87748] in 100 **g*** Dimethylsulfoxide [Fluka 41641])
+ 0.1 ml H_2O_2 (0.6 %; 0.3 ml H_2O_2 [35 %, Merck 108600] + 17.5 ml DDW)
mix gently on a magnetic stirrer a few minutes before use
(the working solution has to be prepared immediately before use!)

*not ml, as it is very viscous

1.9. Stop reagent: 2 mol/l H_2SO_4

900 ml DDW + 100 ml H_2SO_4 (95-97 %; Merck 100731)

2. Coating of microtitre plates (with anti-rabbit IgG or Protein A)

For 30 MPT, prepare a solution of 1 mg IgG (Sigma R2004-5x1MG; add 1 ml DDW to a portion of 1 mg) dissolved in 750 ml coating buffer (see 1.1.)*
Dispense 0.25 ml/well of diluted IgG to the MTP (F96 MaxiSorp, No. 442404, Co. Nunc, Denmark).
Incubate the plate at room temperature overnight.
Discard the solution and refill each well with 0.3 ml "second" coating buffer (see 1.4.)
Cover the filled MTP with parafilm and dust cover (Nunc 264623), and keep it at room temperature until use. You can use the MTP after 3 hours. Store the plates no longer than 4 weeks at room temperature. (Blot plates dry and freeze them at -20°C for longer storage.)

*As an alternative you may use Protein A for coating (but we had recently serious problems with high NSB). For one MTP, prepare a solution of 50 µg Protein A (Sigma P-7837; available in portions of 5 mg - add 0.5 ml DDW - take 5 µl) dissolved in 25 ml coating buffer (see 1.1., 2 µg Protein A/ml).

3. Reagents (stock solutions; relevant only in our lab)

Keep all stock solutions frozen at -20°C until use.
Waterproof colour label marked on small vial indicates different content as follows:

Blue: Antibody (AB)
Red: Biotin-labelled steroid (BL)
Green: Standard

Dilute 0.01 ml of a stock solution (1 mg steroid per ml methanol) with 20 ml of assay buffer.
Mix for 2 minutes in the supersonic bath, wait 3 hours and fill portions of 0.05 ml into new vials.
One vial contains 25,000 pg of the respective steroid.

4. Working dilutions

4.1. Standard

Dispense 0.15 ml (0.2 ml when lyophilized) of assay buffer to one portion of standard vial, shake and wait 20 min.
Dilute this solution 1 : 2.5 seven times. Mix well after each step *(500 pg till 2 pg per 10 µl).*

- In our lab, this is done with a Hamilton Microlab dispenser 1000 (0.09 ml standard + 0.135 ml assay buffer).
- Alternatively, you may take the whole portion of the standard vial (0.05 ml) with the 0.15 ml assay buffer, and add another 2.3 ml assay buffer (you have to transfer everything to a larger vial). This results in a concentration of 500 pg per 50 µl assay buffer, which needs to be further diluted (1:2.5; 1 ml + 1.5 ml buffer).

In case of very sensitive assays, make 9 dilutions and omit the first two *(80 pg till 0.3 pg per 0.01 ml).*
Standard, antibody and biotin label solution have to incubate 20 minutes before you can work with them.

4.2. AB and BL

See special labelling for each individual EIA.

EIAs available for glucocorticoid metabolites:
See page 9.

EIAs available for oestrogens and gestagens/androgens' metabolites: Please ask!

EIA working place in our lab

5. Assay procedure

5.1. Plate washing

Before use, wash coated MTP (see 2.) three times with washing solution (1.5.).
Remove the rest of liquid by blotting the MTP on paper towels.
Do not touch the underside of the plate.

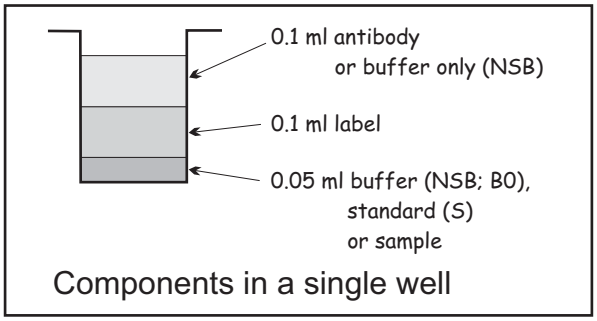
5.2. Pipetting of standards, pool and samples

Dispense assay buffer for nonspecific binding (NSB) and zero binding (0), standards (4.1.), pool X and Y (PX, PY), and sample into the MTP (see Fig. below) prepared earlier (5.1.). We use the Hamilton Microlab dispenser 1000. It takes 0.01 ml from each sample and adds 0.04 ml assay buffer to dispense a total of 0.05 ml (but you can also prepare the standard concentrations per 50 µl as previously described and add that by hand).

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S3	S7	2	6	10	14	18	22	26	30	34
B	NSB	S3	S7	2	6	10	14	18	22	26	30	34
C	B0	S4	PX	3	7	11	15	19	23	27	31	35
D	B0	S4	PX	3	7	11	15	19	23	27	31	35
E	S1	S5	PY	4	8	12	16	20	24	28	32	PX
F	S1	S5	PY	4	8	12	16	20	24	28	32	PX
G	S2	S6	1	5	9	13	17	21	25	29	33	PY
H	S2	S6	1	5	9	13	17	21	25	29	33	PY

NUNC Denmark MaxiSorp

Example of arrangement of standards, pools and samples on the MTP



Components in a single well

5.3. Dispensing of BL

Dispense 0.1 ml biotin-labelled steroid into each well.
For all pipetting steps, use a multi-pipette.

5.4. Dispensing of AB

Dispense 0.1 ml antibody solution into each well.
(Attention: In case of NSB use assay buffer instead of AB.)

Cover the MTP with parafilm and dust cover (Nunc 264623), shake (mild) the MTP overnight at 4°C.

5.5. MTP washing after incubation
Decant incubated MTP, and wash the MTP four times with cold (4°C) washing solution (1.5).

5.6. Streptavidin reaction
Dispense 0.25 ml of enzyme solution (see 1.7.) into each well and
incubate the covered plate for 45 minutes at 4°C on a MTP-shaker.

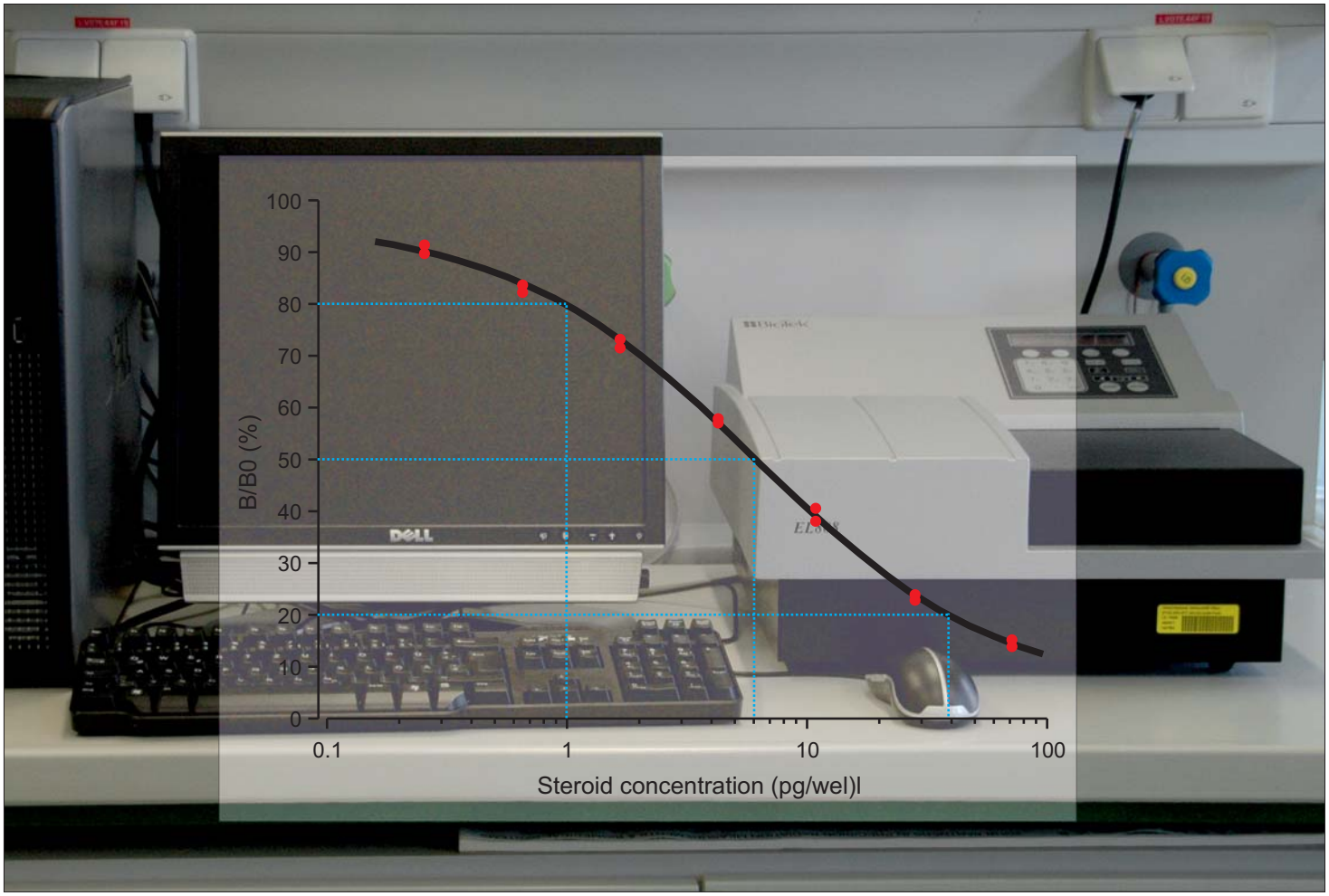
5.7. see 5.5. (second washing)

5.8. Colour reaction
Dispense 0.25 ml of substrate solution (see 1.8.) into each well and
incubate (in the dark!) the covered plate 45 minutes at 4°C (agitating).

5.9. Stop reaction
Dispense 0.05 ml of stop reagent (1.9)
(blue colour turns into yellow).

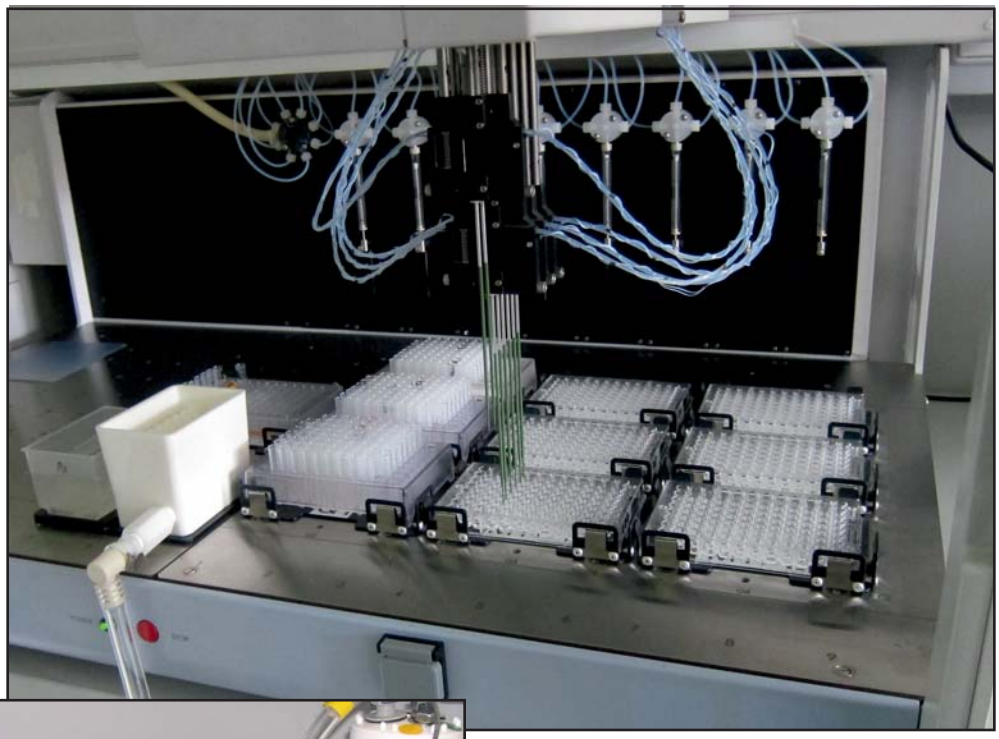
6. Absorbance measuring and calculations

Use an (automatic) MTP reader (in connection with a PC, equipped with a special software for calculation).
We use an ELx808 (Bio-Tek) and Gen 5™)
Reference filter: 620 nm; measuring filter: 450 nm



7. Large scale analysis - special offer!

Large scale sampling is often necessary, especially in studies evaluating adrenocortical activity in animals by measuring faecal glucocorticoid metabolites. We have adapted our lab facilities in order to be able to handle large numbers of samples. So, if it is impossible or unpractical for you to analyse many samples, feel free to contact us (we will analyse them at a reasonable price; especially in case of a collaboration).



JANUS Automated Workstation
(Perkin Elmer)

Automatic platewasher
(ELx405; Bio-Tek instruments)

Integra VIAFLO 96

8. How to validate an EIA for a given species (see also: Möstl & Palme, 2002; Palme, 2005; Möstl et al., 2005; Palme et al., 2005; Touma & Palme, 2005; Palme, 2019)

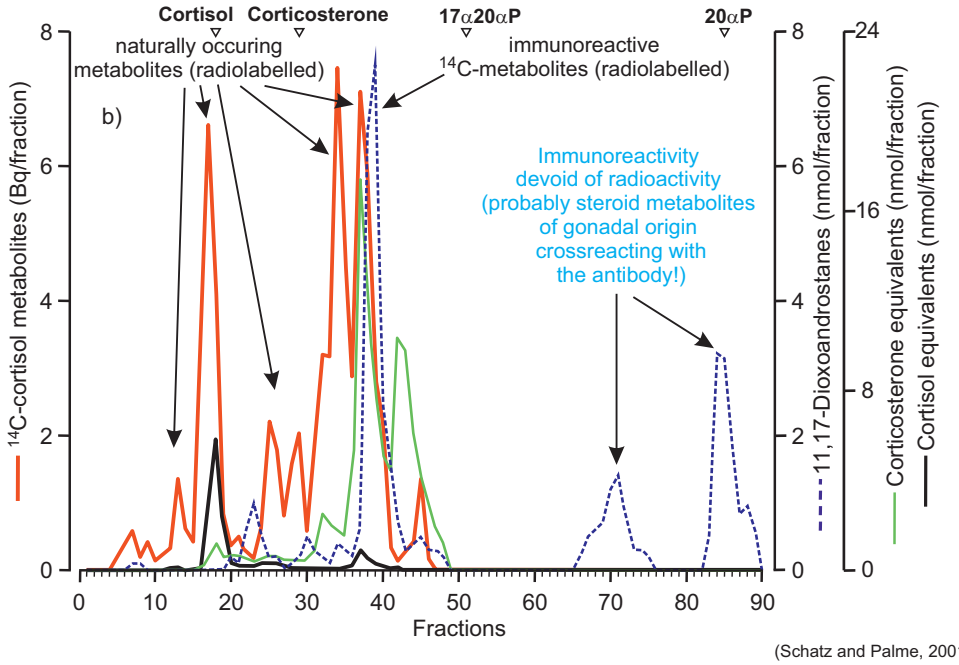
Before planning the experiment, please keep in mind that there is a species-specific delay time between the increase of glucocorticoids in blood and the excretion of the related metabolites in the faeces.

Before application in a new species, a careful analytical and, most important, physiolgical/biological validation of an EIA is mandatory.

8.1. Analytical validation of an EIA

This includes certain criteria like accuracy, specificity (especially cross-reactions with reduced metabolites), sensitivity, precision, and parallelism of the dose response relationship for the standard and the unknown. It is particularly important to demonstrate that the antibody crossreacts with metabolites derived from the GC that are present in the blood. The best method for this is a radiometabolism study (not always possible): After infusion of radiolabelled cortisol (or corticosterone) and the extraction of the faecal sample, a high performance liquid chromatography (HPLC) is performed, and the eluent fractionised and all HPLC-fractions are collected. After measuring the radioactivity and immunoreactivity in the individual fractions, you can see if immunoreactive metabolites coelute with radioactive ones. (They should do so, otherwise the immunoreactive substance does not derive from the radioactive precursor.) One can also partly characterise the metabolites measured by comparing their chromatographic pattern with standards (if available). One can at least compare the immunoreactive substance(s) with the elution position of cortisol or corticosterone.

HPLC-Immunogram of faecal ^{14}C-cortisol metabolites of a male dog



(Schatz and Palme, 2001)

8.2. Physiological/biological validation

Physiological validation means that an increase/decrease in the glucocorticoid production causes an increase/decrease in the substance(s) measured in faecal samples, irrespective of the substance(s) measured. This can be done by injection of ACTH and/or dexamethasone. Additionally, the results of a "stress experiment" (e.g., transportation) may increase the plausibility that an assay measures glucocorticoid metabolites in faecal samples. In most species, lots of different metabolites are formed and excreted. If you have the possibility, you can test which EIA best reflects disturbances.

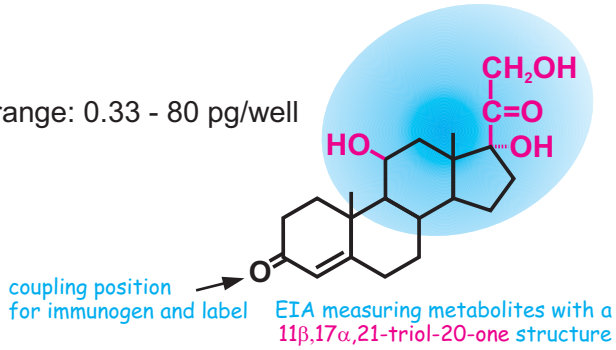
8.3. Other circumstances which may be important

As bacteria metabolise steroids in the faeces, you should check if this metabolism influences your experiment by storing fresh samples for a given time, mimicking the time between defaecation and sample collection in your experiment.
A frequently asked question (by reviewers) is the diurnal rhythm of the excretion. So it may be useful to conduct an experiment to answer this question (see Touma & Palme, 2005).

9. EIAs, produced by our working group, to measure glucocorticoids (or their metabolites) see also references (Info-letter.doc)

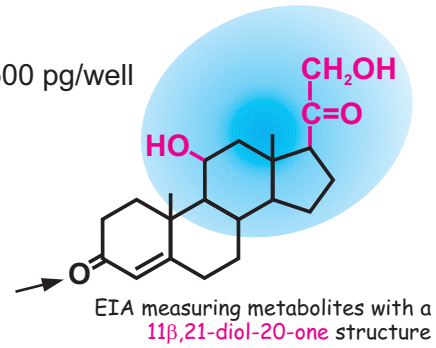
9.1. **Cortisol** (EIA first described: Palme and Möstl, 1997)

Standard: cortisol (4-pregnene-11β,17α,21-triol-3,20-dione); range: 0.33 - 80 pg/well
Antibody: cortisol-3-CMO:BSA (1:20,000)
Label: cortisol-3-CMO-DADOO-biotin (1:100,000)
Application: dogs



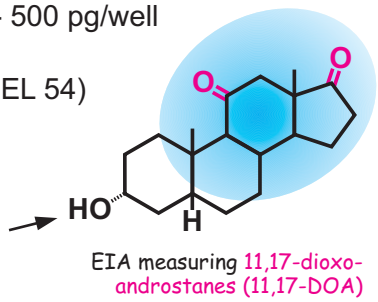
9.2. **Corticosterone** (EIA first described: Palme and Möstl, 1997)

Standard: corticosterone (4-pregnene-11β,21-diol-3,20-dione); range: 2 - 500 pg/well
Antibody: corticosterone-3-CMO:BSA (1:40000)
Label: cortisol-3-CMO-DADOO-biotin (1:200000)



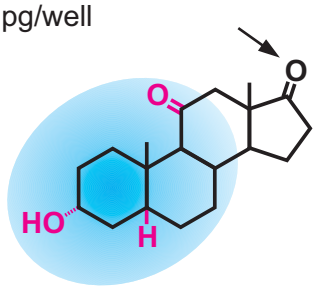
9.3. **11-oxoaetiocholanolone** (Lab-code: **72a**; EIA first described: Palme and Möstl, 1997)

Standard: 11-oxoaetiocholanolone (5β-androstane-3α-ol-11,17-dione); range: 2 - 500 pg/well
Antibody: 11-oxoaetiocholanolone-3-HS:BSA (1:20000; Ak 7/42/95)
Label: 11-oxoaetiocholanolone-3-glucosiduronate-DADOO-biotin (1:250000; EL 54)
Application: various ruminants, primates, horses,...



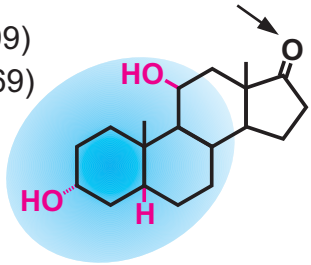
9.4. **11-oxoaetiocholanolone** (Lab-code: **72T**; EIA first described: Möstl et al., 2002)

Standard: 11-oxoaetiocholanolone (5β-androstane-3α-ol-11,17-dione); range: 2 - 500 pg/well
Antibody: 11-oxoaetiocholanolone-17-CMO:BSA (1:60000; Ak 3199/6/96)
Label: 11-oxoaetiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamin (biotinyl-LC; 1:2x10^6; EL 71)
Application: various ruminants, elephants, rodents,...



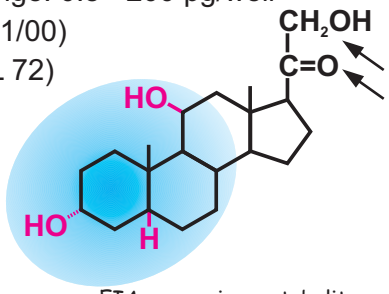
9.5. **11β-hydroxyaetiocholanolone** (Lab-code: **69a**; EIA first described: Frigerio et al., 2004)

Standard: 5β-androstane-3α,11β-diol-17-one; range: 2 - 500 pg/well
Antibody: 5β-androstane-3α,11β-diol-17-one-CMO:BSA (1:20000; Ak 3138/6/99)
Label: 5β-androstane-3α,11β-diol-17-one-CMO-biotinyl-LC (1:100000; EL 69)
Application: various primates, birds, rodents,...



9.6. **Tetrahydrocorticosterone** (Lab-code: **50c;** EIA first described: Quillfeldt et al., 2003)

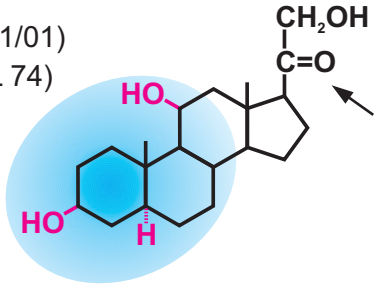
Standard: Tetrahydrocorticosterone (5ß-pregnane-3α,11ß,21-triol-20-one); range: 0.8 - 200 pg/well
Antibody: 5ß-pregnane-3α,11ß,21-triol-20-one-CMO:BSA (1:40000; Ak 50c/51/00)
Label: 5ß-pregnane-3α,11ß,21-triol-20-one-21-HS-biotinyl-LC (1:8000; EL 72)
Application: bird species



EIA measuring metabolites with a 5β-3α,11β-diol structure

9.7. **5α-pregnane-3ß,11ß,21-triol-20-one (**Lab-code: **37e**; EIA first described: Touma et al., 2003)

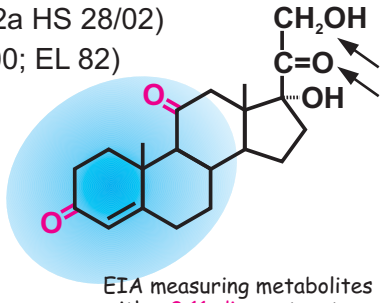
Standard: 5α-pregnane-3ß,11ß,21-triol-20-one; range: 0.8 - 200 pg/well
Antibody: 5α-pregnane-3ß,11ß,21-triol-20-one-CMO:BSA (1:30000; Ak 37e/31/01)
Label: 5α-pregnane-3ß,11ß,21-triol-20-one-CMO-biotinyl-LC (1:30000; EL 74)
Application: various rodents (e.g.: mice; rats and rabbits)



EIA measuring metabolites with a 5α-3β,11β-diol structure

9.8. **Cortisone** (Lab-code: **32a**; EIA first described: Rettenbacher et al., 2004)

Standard: 4-pregnene-17α,21-diol-3,11,20-trione; range: 2 - 500 pg/well
Antibody: 4-pregnene-17α,21-diol-3,11,20-trione-21-HS:BSA (1:20000; Ak 32a HS 28/02)
Label: 4-pregnene-17α,21-diol-3,11,20-trione-CMO-biotinyl-LC (1:5000000; EL 82)
Application: various Galliformes



EIA measuring metabolites with a 3,11-dioxo structure

10. How to calculate the dilution factor (see also Palme et al., 2013)