KAMIYA BIOMEDICAL COMPANY

Cortisone Chemiluminescent CLIA kit

For the quantitative determination of cortisone in dried fecal extracts, urine, saliva and serum

Cat. No. KT-715

For Research Use Only.

PRODUCT INFORMATION

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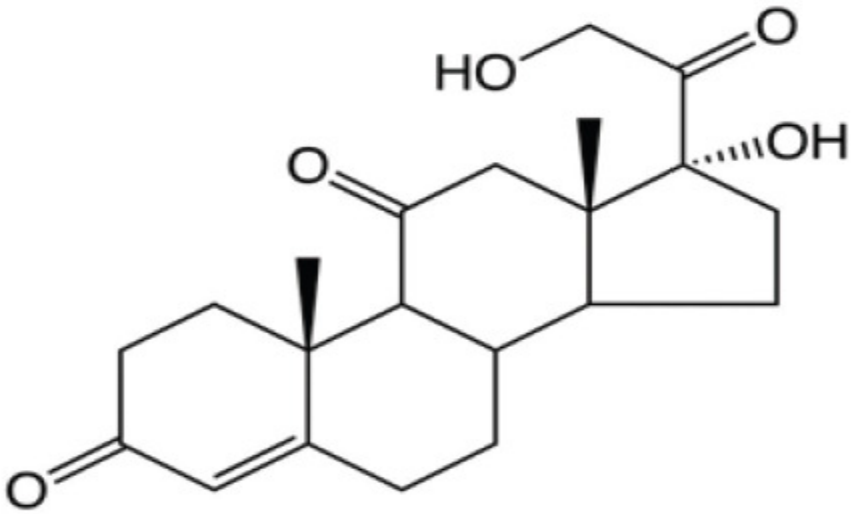
BACKGROUND

Cortisone ($C_{21}H_{28}O_5$, Kendall's Compound 'E') was identified by Mason, Myers and Kendall in 1936 as Compound E extracted from bovine suprarenal gland tissue that had the qualitative but not quantitative activity of cortin. The presence of multiple cortin-like compounds led the authors to speculate that the study of Compound E would reveal the nature of cortin. Compound E is now called cortisone and the more active Compound F, cortisol, and the concentrations of these two glucocorticoids vary due to the activity of two 11ß-hydroxysteroid dehydrogenases (11-HSD). While most tissues have the ability to express either enzyme, 11ß-HSD1 is found primarily in the liver where it converts cortisone to cortisol while 11ß-HSD2 is found in tissues such as the kidney where cortisol receptor binding is required. 11ß-HSD2 deactivates cortisol to cortisone, prohibiting receptor activation. This glucocorticoid "shuttle" helps to initiate and regulate the anti-inflammatory response, making cortisone one of the modern "wonder drugs". Monitoring the ratio of cortisone:cortisol has applications in diabetes, obesity, metabolic syndrome, osteoporosis, and chronic fatigue syndrome in addition to adrenal diseases. Cortisone and cortisol concentrations exhibit a predictable diurnal pattern and can be measured in extracted dried feces, or in serum, plasma, saliva and urine. A recent publication has suggested that salivary cortisone is a good surrogate marker for serum cortisol.



PRINCIPLE

The Cortisone Chemiluminescent Immunoassay kit is designed to quantitatively measure Cortisone present in extracted dried fecal samples, urine, saliva, and serum samples. Please read the complete kit insert before performing this assay. This kit measures total cortisone in serum and plasma and in extracted fecal samples. A cortisone calibrator is provided to generate a calibration curve for the assay and all samples should be read off the calibration curve. Calibrators or diluted samples are pipetted into a white microtiter plate coated with an antibody to capture rabbit antibodies. A cortisone-peroxidase conjugate is added to the calibrators and samples in the wells. The binding reaction is initiated by the addition of a polyclonal antibody to cortisone to each well. After a two hour incubation the plate is washed and the chemiluminescent substrate is added. The substrate reacts with the bound cortisone-peroxidase conjugate to produce light. The generated light is detected in a microtiter plate reader capable of reading luminescence. The concentration of the cortisone in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

COMPONENTS

Coated White 96 Well Plates

A white plastic microtiter plate(s) with break-apart strips coated with goat anti-rabbit IgG.
1 Each

Cortisone Calibrator
Cortisone at 1,000 ng/mL in a special stabilizing solution.
50 µL

Cortisone CLIA Antibody
A rabbit polyclonal antibody specific for cortisone.
3 mL

Cortisone CLIA Conjugate Concentrate
A cortisone-peroxidase conjugate concentrate in a special stabilizing solution.
1 mL

Conjugate Diluent
Contains special stabilizers and additives.
3 mL

Assay Buffer
One plate kit uses a ready-to-use Assay Buffer. Five plate kit uses a 5X concentrate that should be diluted with deionized or distilled water.
50 mL

Dissociation Reagent
1 mL
NOTE: Dissociation Reagent is to be used only with Serum samples.

Wash Buffer Concentrate
A 20X concentrate that should be diluted with deionized or distilled water.
30 mL

Substrate Solution A
6 mL

Substrate Solution B
6 mL

Plate Sealer
1 Each

STORAGE
All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED
Distilled or deionized water.

Repeater pipet with disposable tips capable of dispensing 25 µL and 100 µL.

A microplate shaker.

96 well microplate reader capable of reading glow chemiluminescence. All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. **The number of RLUs obtained is dependant on the sensitivity and gain of the reader used. If you are unsure of how to properly configure your reader contact your plate reader manufacturer or carry out the following protocol:**

Dilute 5 µL of the Cortisone CLIA Conjugate Concentrate into 995 µL of deionized water. Pipet 5 µL of diluted conjugate into a white well and add 100 µL of prepared CLIA substrate. This well will give you an intensity close to the maximum binding signal for the assay. Adjust the gain, integration time or sensitivity so that your reader is giving close to its maximum signal.

To properly analyze the data software will be required for converting raw RLU readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer.

SAMPLE TYPES

This assay has been validated for urine, saliva, and serum samples. It has also been validated for dried fecal extract samples. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit.

Cortisone is identical across all species and we expect this kit should measure cortisone from sources other than human. The end user should evaluate recoveries of cortisone in other samples being tested.

SAMPLE PREPARATION

Serum samples need to be treated with the supplied Dissociation Reagent. Addition of this reagent will yield the total cortisone concentration in serum. **Dissociation Reagent is to be used only with Serum samples.** Free cortisone can be measured in saliva and urine samples as directed below.

Dried Fecal Samples

We have a detailed Extraction Protocol available. The ethanol concentration in the final Assay Buffer dilution added to the well should be <5%.

Saliva Samples

Saliva samples should be frozen and thawed, then centrifuged at 14,000 rpm for 15 minutes. The supernatant should be diluted 1:5 to 1:10 with the supplied Assay Buffer prior running in the assay. Ask for our Saliva Sample Handling Instructions.

Urine Samples

Urine samples should be diluted ≥ 1:100 with the supplied Assay Buffer prior running in the assay.

Serum and Plasma Samples

Allow the Dissociation Reagent (DR) to warm completely to **Room Temperature** before use. We suggest pipeting 5 µL of DR into 1 mL Eppendorf tubes. Add 5 µL of serum or plasma to the DR in the tube, vortex gently and incubate at room temperature for 5 minutes or longer. Dilute with 490 µL of supplied Assay Buffer. This 1:100 dilution can be diluted further with Assay Buffer. Final serum and plasma dilutions should be ≥ 1:100.
NOTE: Dissociation Reagent is to be used only with Serum and Plasma samples.

Tissue Culture Media

For measuring cortisone in tissue culture media (TCM), samples should be read off a calibration curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.
Use all Samples within 2 Hours of preparation, or stored at ≤ -20°C until assaying.

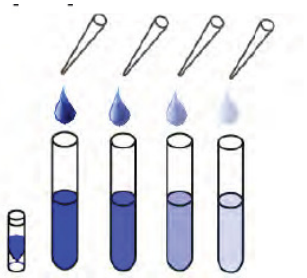
REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all calibrators and samples be run in duplicate to allow the end user to accurately determine corticosterone concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Cortisone Conjugate
The supplied Cortisone Conjugate Concentrate should be diluted 1:4 with the Conjugate Diluent. Once diluted the Cortisone conjugate is stable for one month when stored at 4℃.

Calibrator Preparation
Label nine test tubes as #1 through #9. Pipet 490 µL of Assay Buffer into tube #1 and 250 µL into tubes #2 to #9. Carefully add 10 µL of the cortisone stock solution to tube #1 and vortex completely. Take 250 µL of the cortisone solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #9. The concentration of cortisone in tubes 1 through 9 will be 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312.5 156.3, and 78.1 pg/mL.
Use all Calibrators within 2 hours of preparation.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
Assay Buffer (µL)	**490**	250	250	250	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
Vol of Addition (µL)	**10**	250	250	250	250	250	250	250	250
Final Conc (pg/mL)	20,000	10,000	5,000	2,500	1,250	625	312.5	156.25	78.1

Chemiluminescent Substrate
Mix one part of the Substrate Solution A with one part of Substrate Solution B in a brown bottle. Once mixed the substrate is stable for one month when stored at 4℃.

ASSAY PROTOCOL

1. Use the plate layout sheet on the back page to aid in proper sample and calibrator identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.

2. Pipet 50 µL of samples or calibrators into wells in the plate.

3. Pipet 75 µL of Assay Buffer into the non-specific binding (NSB) wells.

4. Pipet 50 µL of Assay Buffer into wells to act as maximum binding wells (Bo or 0 pg/mL).

5. Add 25 µL of the Cortisone Conjugate to each well using a repeater pipet.

6. Add 25 µL of the Cortisone Antibody to each well, **except the NSB wells**, using a repeater pipet.

7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 2 hours. If the plate is not shaken signals bound will be approximately 45% lower.

8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.

9. Add 100 µL of the mixed Chemiluminescent Substrate to each well, using a repeater pipet.

10. Incubate the plate at room temperature for 5 minutes without shaking.

11. Read the luminescence generated from each well in a mutimode or chemiluminescent plate reader using a 0.1 second read time per well. The chemiluminescent signal will decrease about 40% over 60 minutes.

12. Use the plate reader's built-in 4PLC software capabilities to calculate Cortisone concentration for each sample.

CALCULATION OF RESULTS

All luminometers read Relative Light Units (RLU). These RLU readings will vary with make or model of plate reader. Average the duplicate RLU readings for each calibrator and sample. Create a calibration curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean RLU's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

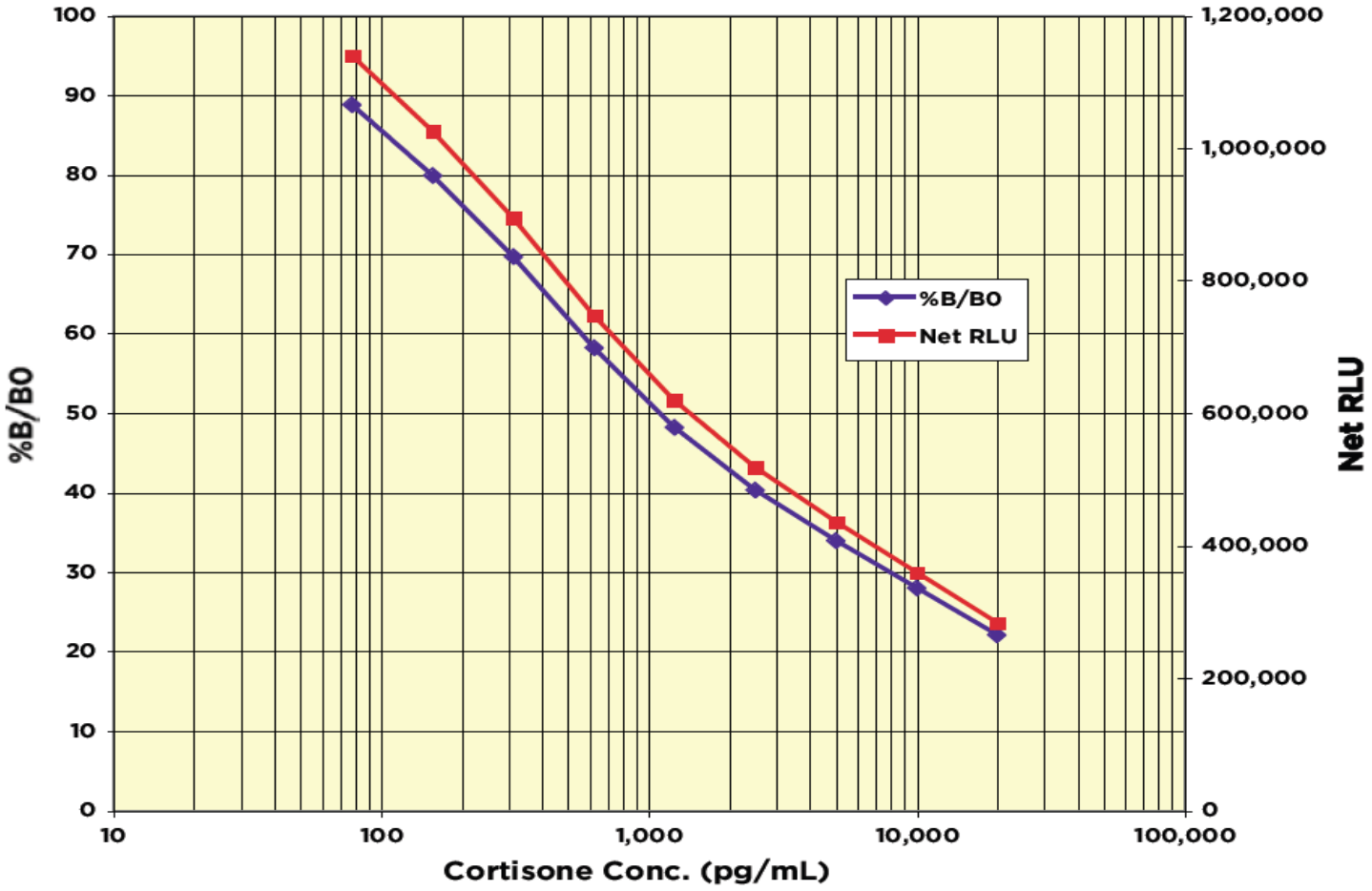
TYPICAL DATA

Sample	Mean RLU	Net RLU	% B/B0	Cortisone Conc. (pg/mL)
NSB	7,660	0		-
Standard 1	290,990	283,330	22.07	20,000
Standard 2	366,485	358,825	27.95	10,000
Standard 3	443,125	435,465	33.92	5,000
Standard 4	525,230	517,570	40.31	2,500
Standard 5	626,385	618,725	48.19	1,250
Standard 6	755,005	747,345	58.21	625
Standard 7	901,865	894,205	69.65	312.5
Standard 8	1,032,540	1,024,880	79.83	156.25
Standard 9	1,147,060	1,139,400	88.75	78.1
B0	1,291,500	1,283,840	100	0
Sample 1	384,985	377,325	29.39	7,879
Sample 2	947,150	939,490	73.18	237.5

Always run your own calibration curves for calculation of results. Do not use this data.

Conversion Factor: 100 pg/mL of cortisone is equivalent to 277.6 pM.

Typical Calibration Curves



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VALIDATION DATA

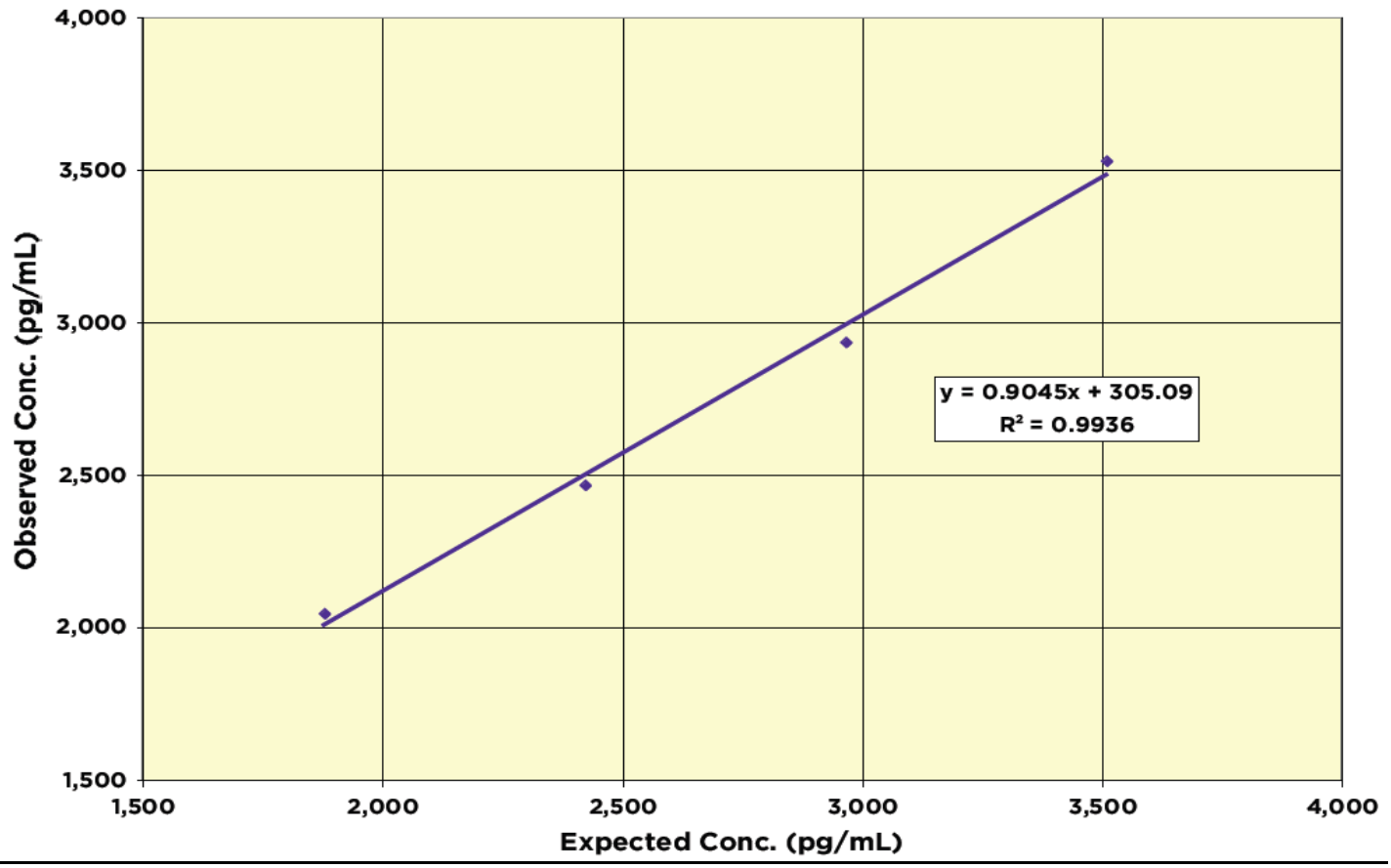
Sensitivity and Limit of Detection
Sensitivity was calculated by comparing the RLU's for twenty wells run for each of the B0 and calibrator #9. The detection limit was determined at two (2) standard deviations from the B0 along the calibration curve.
Sensitivity was determined as 10.6 pg/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the RLU's for twenty runs for each of the zero calibration and a low concentration human sample.
Limit of Detection was determined as 59.6 pg/mL.

Linearity
Linearity was determined by taking two serum samples treated with Dissociation Reagent and diluted 1:50 with Assay Buffer, one with a low diluted cortisone level of 1,336 pg/mL and one with a higher diluted level of 4,055 pg/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

Low Serum	High Serum	Observed Conc. (pg/mL)	Expected Conc. (pg/mL)	% Recovery
80%	20%	2,045	1,880	108.8
60%	40%	2,466	2,424	101.7
40%	60%	2,934	2,967	98.9
20%	80%	3,528	3,511	100.5
			Mean Recovery	**102.5%**

Linearity



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Cortisone concentrations were:

Sample	Cortisone Conc. (pg/mL)	%CV
1	7,902	7.9
2	596.1	5.7
3	234.0	10.1

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in thirteen assays run over multiple days by three operators. The mean and precision of the calculated Cortisone concentrations were:

Sample	Cortisone Conc. (pg/mL)	%CV
1	7,904	10.0
2	662.7	11.1
3	262.9	12.9

SAMPLE VALUES

Nineteen random human serum samples were tested in the assay. Neat sample values ranged from 8.8 to 63.3 ng/mL with an average for the human samples of 30.4 ng/mL. (The normal reference range for serum cortisone is 1.3-23 ng/mL.) Six normal human saliva samples were tested in the assay. Values ranged from 5.6 to 16.0 ng/mL with an average of 11.2 ng/mL. Eight normal human urine samples were also tested. The samples read from 49.4 to 268.3 ng/mL.

Dried fecal samples were processed and run in the assay. Samples kindly donated by Dr. J. Williams at the Indianapolis Zoo, which included Amur Tiger, Giraffe, Kudu, Lion, Reeves Muntjac, White Handed Gibbon, White Rhino, and Zebra, were tested and cortisone values obtained ranged from 7.5 to 651 pg/mg dried fecal material.

Palme and Möestl and colleagues have shown that radiolabeled administered glucocorticoids are excreted in differing amounts in urine and feces across species, with fecal excretion ranging from 7% of administered glucocorticoid in the pig to 82% in the cat. Palme has also shown that the peak of fecal concentrations occur at 12 hours for sheep, but takes 48 hours to peak in pigs. It is therefore necessary to evaluate the timing and relative fecal or urine excretion of glucocorticoids for each species.

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)
Cortisone	100%
5α-Dihydrocosterone	31.7%
Prednisone	9.0%
5ß-Dihydrocosterone	4.4%
11-Dehydrocorticosterone	1.0%
Cortisol	<0.1%
Progesterone	<0.1%
Corticosterone	< 0.1%
Estradiol	< 0.1%
Dexamethasone	<0.04%

FOR RESEARCH USE ONLY

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