Effect based detection of illicit use of synthetic glucocorticoids in meat producing calf

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Introduction

European Member States are required to monitor the use of pharmacologically active substances in food producing animals. The availability of new synthetic compounds and the administration of low-dose cocktails circumvent the detection by the surveillance schemes of European Union Member States. The use of synthetic glucocorticoids (GCs) in livestock production has been widely described[1]. As demonstrated for estrogens and androgens, bioassay activity-based screening combined with chemical identification could provide an optimal way to detect the illegal use of these molecules. In Italy, screening methods for GCs have to demonstrate a detection capability (CCβ) of 2 ng mL^{-1}. Recently, prednisolone traces have been found in several experimental and control bovines, posing doubt about the endogenous production of this hormone. Only for this molecule the level of interest has been rise to 5 ng mL^{-1}. We evaluated the applicability of the reporter gene assay GR CALUX (Fig. 1) for the screening of synthetic glucocorticoids in bovine urine[2].

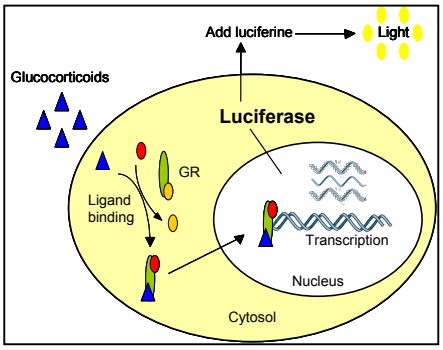

Figure 1: Principle of the GR CALUX - reporter gene assay. The signal dose increases as a result of increasing concentrations of the ligand[3].

Materials and methods

1. Samples: urine samples used in this study were taken from experimental animals reared for 6 months under controlled conditions. Samples were collected after spontaneous urination and stored at -20°C, in darkness, immediately after collection.

2. Evaluation of cross-reactivity of synthetic glucocorticoids in solvent: the glucocorticoid activity of betamethasone, dexamethasone, flumethasone, prednisolone and 6-*α*-methyl-prednisolone was evaluated by preparing serial dilutions of each molecule in DMSO subsequently analyzed by GR CALUX. Ten different concentrations of each molecule were evaluated, in a range between 0 and 100 ng ml^{-1}. Only for prednisolone, concentrations were prepared between 0 and 1000 ng ml^{-1}.

3. Synthetic glucocorticoids cross-reactivity assessment, recovery rate and molecule selection: to assess the presence of a matrix effect able to modify the cross-reactivity calculated on the pure compounds, negative and spiked urine samples were tested. The samples were spiked with 2 ng ml-1 of dexamethasone, betamethasone and flumethasone, with 2 - 3 - 4 ng ml-1 of 6-α-methyl-prednisolone and with 5 ng ml-1 for prednisolone. The recovery rate (RR) of each molecule was also evaluated. It was measured as the ratio between the hormone concentration reviled by GR CALUX (Cr), expressed in dexamethason equivalent, and the real concentration added to the spiked sample (Ca), data was also modified by the REP of the compound assessed (RR = [Cr *

1/REP] / Ca). Furthermore, 13 samples from control animals were analyzed. The molecule with a minimal glucocorticoid activity generating a signal well differentiable from negative samples was chosen for the validation study.
4. Determination of the decision limit (CCα) and the detection capability (CCβ): 24 urine samples from untreated animals were analyzed as such and after fortification by prednisolone. All aliquots were tested in 4 different analytical sessions, spread over the period of a month according to the EC Decision 2006/657.
5. Evaluation of the ruggedness: Youden approach. Variables: pH of buffer K_2HPO_4/KH_2PO_4, pH of buffer $K_2CO_3/KHCO_3$, deconjugation time, extraction time, number of cells, cell exposure time, cell lysis, time.
6. Rating the stability of the analyte in the matrix: a urine sample fortified with 5 ng ml^{-1} of prednisolone was prepared and divided into 5 aliquots. The first aliquot was analyzed immediately, the second after a week, the third after two and so on until the fourth week. The extract of the first aliquot, prepared in duplicate, was also analyzed after 4 weeks from the extraction, in order to assess the stability of the extracts.
7. Rating the specificity: the ability of the GR CALUX to discriminate the analytes of interest from other closely related molecules was assessed. The dose-response curve of each molecule was evaluated in a range between 0 and 1000 ng ml-1 in DMSO by calculating EC_{50} and REP. Then, some urine samples have been fortified with the active molecules (cortisol and aldosterone) and analyzed with the reporter gene assay.
8. Statistical Shapiro-Wilk test was applied to assess the normal distribution of the data obtained from negative and positive urine analysis. The Dixon Q Test was also applied to verify the presence of outliers in the group of negative urines. The F-test was used to assess the ruggedness of the method.

Results and discussion

Cross-reactivity of synthetic glucocorticoids in solvent and matrix; calculation of recovery rates and selection of the molecules for the validation

Table 1 reports the dose-response data. Flumethasone showed the highest glucocorticoid activity. βetamethasone showed effects in line with other studies[4].

Table 1: GR CALUX dose-response data

Compound	EC 50 (ng ml^{-1})	REP
Prednisolone	2.8	0.22
Prednisone	Not active	Not active
Metil-prednisolone	1.08	0.42
Betamethasone	0.68	0.67
Flumethasone	0.25	1.80
Dexamethasone	0.45	1.00

As shown in Table 2, the activity of the molecules have not undergone relevant changes when introducing the urine as matrix, and the results are fully in line with the "expected results" calculated from data obtained from pure compounds. In the frame of this validation activity the molecule with the lowest glucocorticoid activity was used as reference at the level of interest, taking into account to keep a well-differentiable signal from the negative samples. Urines from untreated animals showed hormonal activity comprised between <LOD and 0.66 ng ml^{-1}, thus, it was decided to use prednisolone (Figure 2).

Table 2: GR CALUX cross-reactivity of synthetic glucocorticoids in matrix

Compound	**Fortification (ng ml^{-1})**	**Result (Average) ng ml^{-1} dex EQ**	**Expected result ng ml^{-1} dex EQ**	**Recovery Rate**
Prednisolone	5	0.90	1.1	82 %
Metil-prednisolone	2	0.44	0.84	60 %
	3	0.88	1.26	
	4	1.00	1.68	
Betamethasone	2	1.15	1.34	86 %
Flumethasone	2	4.5	3.60	125 %
Dexamethasone	2	2.00	2.00	100 %

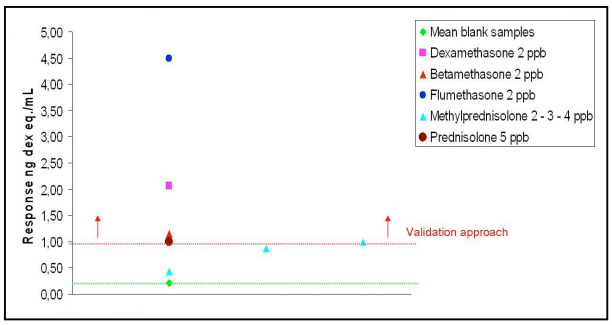

Figure 2: Cross-reactivity of synthetic glucocorticoids in the matrix and the choice of the molecule for validation (prednisolone 5 ng ml^{-1}).

Decision limit (CCα) and detection capability (CCβ)

Figure 3 shows the results of the blank and fortified urine. All fortified samples showed signals well above the CCα; the detection capability was calculated: decision limit (CCα = 0.34 ng ml^{-1}) + 1.64 x the SD standards of positive samples (0.17) = 0.61 ng ml^{-1}.

That's said, no fortified samples were classified as false negative, fully complying the requirements of Decision 657/2002 with a β error <5%. It is to be noted how a false positive rate below the 10% represents a general requirement for a screening test.

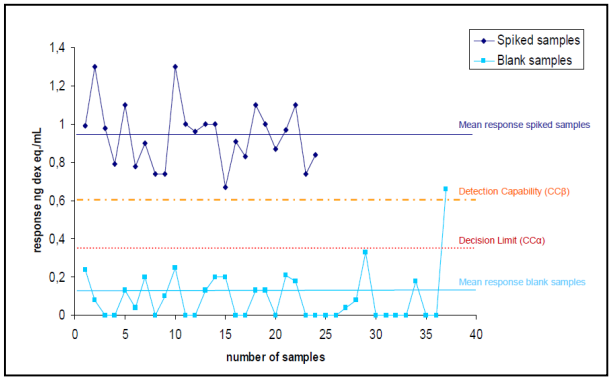

Figure 3: Calculation of the decision limit (CCα) and the detection capability (CCβ).

Ruggedness

A nice robustness emerged, even if particular attention should be paid to the pH of the buffer K_2CO_3/$KHCO_3$, which if too alkaline (pH > 9.5) could reduce the yield of extraction.

Stability of the analyte in matrix

Both the analyte and the extract examined after four weeks have been shown to be stable at least for four weeks.

Specificity

Table 2 reports the GR CALUX specificity and the dose-response data of endogenous molecules. All sex steroids considered showed no activity on the method. Outside of the group of glucocorticoids, only aldosterone presented a certain degree of affinity with GR receptors, enen if 100 fold lower than the dexamethasone, the reference compound (Table 3). Even high doses of aldosterone were not able to generate signals such as to induce "false positive". Interestingly, a reduction of the signal was recorded when examining a sample fortified with high-doses of both prednisolone and aldosterone, compared to samples treated with prednisolone only. This particular aspect should be further investigated assessing the onset of competition phenomena between the analyte and the mineralocorticoid at receptor level (Table 3). Relatively to endogenous glucocorticoids, cortisol

was the only molecule with an important activity both as pure compound than in urine. If stressful situations would generate false positive results remain a concern. Anyway, urine spiked with cortisol set below the CCα (5 ng ml^{-1}), in line with the decision limit of 10 ng ml^{-1}, between the CCα and CCβ (15 ng ml$^{-1)}$ (Table 3).

Table 2: GR CALUX specificity, dose-response data of endogenous molecules

Compound	EC 50 (ng ml^{-1})	REP
Cortisol	3.5	0.12
20β-diidrocortisol	450	0.001
6β-idrossicortisol	650	0.0007
Tetraidrocortisol	-	-
3β- tetraidrocortisol	-	-
Cortisone	-	-
Aldosterone	68	0.007
Progesterone	-	-
17β-estradiol	-	-
Testosteron	-	-
Dexamethasone	0.45	1.00

Table 3: GR CALUX, cross-reactivity of endogenous hormones in the matrix

Compound	Fortification (ng ml^{-1})	Result ng ml^{-1} dex EQ	Expected result ng ml^{-1} dex EQ	Recovery Rate
Cortisol	5	0.21	0.6	33 %
	10	0.36	1.2	
	15	0.60	1.8	
Aldosterone	10	-	-	-
Prednisolone	7	1.00	1.54	65 %
Aldosterone + Prednisolone	10 + 7	0.60	1.54	40 %

GR-CALUX is a promising screening tool for the detection of illicit treatments in meat-producing bovines. Its ability to detect the most commonly used synthetic glucocorticoids was comparable with the ELISA test. Importantly, it showed to be less susceptible to matrix effects than ELISA and potentially offers advantages over existing analytical techniques allowing the detection of new or unknown compounds and low-level cocktails with glucocorticoid bioactivity.

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