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Effect-based detection of synthetic glucocorticoids in bovine urine

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ABSTRACT

Challenges to testing for the illicit use of anabolic substances in meat-producing animals stem from the production of new synthetic compounds and the administration of low-dose cocktails to circumvent detection by the surveillance schemes of European Union member states. This work evaluated for the first time GR-CALUX, a highly sensitive reporter gene assay, as a screening tool for the detection of synthetic glucocorticoids in bovine urine. In order to verify the effect of natural corticosteroids on the method, the bioassay was tested first using blank urine samples collected at the farm and the slaughterhouse. Next, the dose–response curves were measured for the most commonly used synthetic glucocorticoids. The bioassay's ability to detect them in spiked and incurred samples of bovine urine was then evaluated. Finally, its performance was compared against a commercially available ELISA kit ordinarily used in screening activities. GR-CALUX performance did not appear to be influenced by physiological levels of endogenous corticosteroids in the farm samples, whereas an increase in these hormones might invalidate the analysis in samples obtained at the slaughterhouse. Using pure compounds, GR-CALUX showed a high sensitivity toward the synthetic glucocorticosteroids tested in order of relative potencies: flumethasone ≫ dexamethasone > betamethasone > methylprednisolone > prednisolone. As expected, the bioassay failed to detect the prohormone prednisone. The results obtained from analysis of the spiked and incurred specimens reproduced those of the blank samples and the pure compounds. 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 appeared to be less susceptible to matrix effects than ELISA.

Keywords

bioassay, screening, CALUX, veterinary drug residues, anabolic steroids, animals

Introduction

Glucocorticoids are a group of steroid hormones including natural and synthetic derivatives. The most important natural molecules are cortisol and cortisone, which are secreted by the adrenal cortex and involved in physiological processes such as stress response, inflammation, immune function and hydroelectrolyte balance (Nozaki 2001; Cantiello et al. 2009). Synthetic derivatives such as dexamethasone and prednisolone are widely used as therapeutic agents in veterinary medicine for the treatment of metabolic diseases, shock and flogistic conditions (Bozzetta et al.

2011; Cannizzo et al. 2011; Ferranti et al. 2011; Nozaki 2001). Natural and synthetic glucocorticoids can be divided into 11-hydroxy active forms (e.g., cortisol and dexamethasone) and 11-keto inert forms such as cortisone and prednisone, which are prohormones (Connolly et al. 2009).

Natural and synthetic derivatives exert their actions by interacting with glucocorticoid receptors (GR), members of the nuclear hormone receptor family that also comprises other steroids, retinoid and thyroid hormone receptors. Once activated, GRs translocate from the cytosol of target cells to the nucleus, where they bind to specific glucocorticoid response elements within the regulatory DNA sequences where they transcriptionally regulate the expression of glucocorticoid-responsive genes (Yudt & Cidlowski 2002; Necela & Cidlowski 2003; Pascussi et al. 2003).

Synthetic glucocorticoid administration in animals is primarily for therapeutic purposes. Though their use is strictly regulated by European Union member states (EC 1996a, 1996b, 2010), their illegal use as growth promoters, either alone or in combination with other molecules, anabolic steroids or β-agonists, is a widespread practice to improve meat quality and quantity in food-producing animals. Indeed, numerous studies on cattle have shown improvements in feed intake, weight gain, feed conversion ratio and carcass characteristics (Stolker & Brinkman 2005; Capolongo et al. 2007; De Brabander et al. 2007; Cannizzo et al. 2011).

One of the recommended matrices for monitoring illegal glucocorticoid use in live animals is urine, which is readily available before slaughter and which contains the highest hormone concentration. Usually, this matrix is first screened by immuno-enzymatic methods, followed by MS-based techniques to confirm the identity of the hormone residues detected.

In the European Union there is no established MRL for glucocorticoids in urine; only for dexamethasone a minimum required performance level (MRPL) of 2 ng ml^{-1} has been set (CRLs 2007). According to previous studies, the same limit is usually applied as a required performance level (RPL) for the other corticoids in this matrix (Andersen et al. 2008; Kolanović et al. 2011).

Though the number of samples resulting in non-compliance at European Union controls is relatively low (EFSA 2012), analysis of illegal preparations finds that sex hormones, glucocorticoids and β-agonists are still being used despite the bans in effect in member states (Courtheyn et al. 2002; Nielen et al. 2003). One possible reason for the few positives found so far could lie with the difficulty of the analytical techniques currently used in control programmes to detect new compounds. Screening and confirmatory GC-MS and multi-residue LC-MS/MS rely on a list based on known molecules. In addition, the multi-analyte screening ability of enzyme immunoassays depends on the limited degree of cross-reactivity of the antibody used (Divari et al. 2010). Compounding the problem is the increasing practice of administering low-level drug cocktails with considerable biological activity. Such illegal mixtures enhance financial gain while escaping detection by surveillance programmes (Connolly et al. 2009).

Recognising this trend to animal doping, novel screening assays have been developed to detect accurately trace residues in samples of animal origin and new compounds or low-dose cocktails. Among these innovative methods are assays employing indirect biomarkers, like receptors (Odore et al. 2006), metabolites (Courant et al. 2009), proteins (Gardini et al. 2006) or gene expression (Toffolatti et al. 2006; Reiter et al. 2007), and in vitro system for effect-based detection of the analytes, like histopathology-based methods (Bozzetta et al. 2011; Cannizzo et al. 2011; Vascellari et al. 2012) and reporter gene assays (RGAs) (Willemsen et al. 2002, 2005; Schumacher et al. 2003; Connolly et al. 2009; Bovee et al. 2011). RGAs based on stably transfected cell lines may provide one of the most specific, responsive and biologically relevant means to screen biological samples for hormone activity, with a high throughput potential (Bovee et al. 2011, 2012). The assay is based on the receptor-mediated mechanism of action of steroids, in which reporter gene expression is a culmination of a molecular cascade of events involved in receptor transactivation. With this strategy, trace levels of new and unknown compounds, as well as drug cocktails consisting of individual compounds, can be detected by directly measuring their biological activity.

A wide variety of RGAs for glucocorticoid detection have been described (Willemsen et al. 2002, 2005; Schumacher et al. 2003; Connolly et al. 2009; Bovee et al. 2011), but only two assays are commercially available to date: the yeast glucocorticoid biosensor and the chemical-activated luciferase gene expression (CALUX) in vitro bioassay based on human bone cells (U2OS). Of the two, only GR-CALUX is suited for sample analysis requiring a low sensitivity (Bovee et al. 2011, 2012).

The aim of this study was to evaluate, for the first time, the GR-CALUX assay as screening tool for the detection of synthetic glucocorticoids in bovine urines. The study was carried out in three phases:

- Evaluation of the effect of natural corticosteroids on the method. In order to assess the effect of these hormones, the assay was tested using blank samples collected at the farm and the slaughterhouse from animals kept in controlled conditions.
- Measurement of the dose–response curve for the synthetic glucocorticoids most commonly used in veterinary practice and illicit treatments (dexamethasone, betamethasone, prednisolone, prednisone, methylprednisolone and flumethasone).
- Assessment of the assay’s ability to detect synthetic glucocorticoids in spiked and incurred samples of bovine urines.

GR-CALUX performance was compared with that of a commercially available ELISA kit usually adopted in screening activities for the detection of synthetic glucocorticoids in bovine urine.

Materials and methods

Reagents and chemicals

Diethylether and acetonitrile were supplied by Sigma-Aldrich (Milan, Italy). Sodium hydroxide and hydrochloric acid were purchased from Carlo Erba Reagenti (Milan, Italy). β-Glucuronidase/aryl-sulfatase was from Roche Diagnostics (Mannheim, Germany). Analytical standards of prednisone, prednisolone, 6α-methylprednisolone, flumethasone, betamethasone, dexamethasone, cortisone, cortisol, 20β-dihydrocortisol and 6β-hydroxycortisol were purchased from Sigma-Aldrich.
For chemical analyses, stock standard solutions of the analytes were prepared in acetonitrile at a concentration of 1 mg l^{-1} and stored at −20°C in the dark.
A pool of blank urine was supplied by Test Veritas (Trieste, Italy). Material BOV11 from the reference set Real Check Bovine Urine was obtained by pooling urines from non-treated animals and characterised in terms of content of synthetic and natural steroid hormones with confirmatory methodologies.
For the spiked samples, stock standard solutions of dexamethasone, betamethasone, prednisolone, methylprednisolone and flumethasone were prepared in methanol at a concentration of 100 mg l^{-1} and stored at −20°C in the dark. Working solutions were prepared by serial dilution in double-distilled water.

Experimental animals

The breeding plan was designed in adherence to Italian regulations and guidelines for the care and use of experimental animals (D. Lgs no. 193/2006). The study protocol was approved by the Italian Ministry of Health and the local committee for animal welfare. Thirty Friesian male veal calves were divided into two groups and farmed for 6 months under experimentally controlled conditions. Appropriate measures were taken to prevent cross-contamination between the two groups. During the 6 months of breeding, 0.4 mg day^{-1} of dexamethasone 21-disodium phosphate (Desashock) were administered orally, dissolved in milk, to 10 animals for 20 consecutive days; the remaining 20 animals were used as controls. All the experimental animals were slaughtered 12 days after the last dexamethasone administration.

Blank samples

Twenty-three blank urine samples (20 at the farm and three at the slaughterhouse) were collected from the experimental and control animals. At the farm the urine samples were collected, taking care to prevent faecal contamination, after spontaneous micturition, and immediately stored in the

dark at −20°C until analysis. At the slaughterhouse, the urine, when present, was directly collected from the urinary bladder and stored in the dark at −20°C.

Spiked samples

To obtain the lowest background signal deriving from natural corticosteroids, a pool of blank urine obtained by Test Veritas (Trieste, Italy) was used in this phase. Fifteen spiked samples were prepared by adding working solutions to aliquots of blank urine. The samples were fortified with five synthetic glucocorticoids (dexamethasone, betamethasone, prednisolone, methylprednisolone and flumethasone) at three different levels of fortification (1, 2 and 4 ng ml^{-1}). One aliquot was used as a negative control.

Incurred samples

Four urine samples from the experimentally treated animals (approximately 0.5, 1, 2 and 3 ng ml^{-1} of dexamethasone, respectively) were selected after LC-MS/MS analysis. Three urine samples from control animals were used as negative controls. The specimens were collected at the farm, after spontaneous micturition, and were immediately stored in the dark at −20°C until analysis.

GR-CALUX bioassay

The GR-CALUX cell line was developed by BioDetection Systems (Amsterdam, the Netherlands). This bioassay is based on human osteoblastic osteosarcoma U2OS cells transfected with a hGRα expression plasmid and a luciferase reporter construct. The GR-CALUX bioassay was performed as described (Sonneveld et al. 2006; van der Linden et al. 2008). Briefly, cells were cultured in DF medium supplemented with 7.5% foetal calf serum (FCS) and 200 μg ml^{-1} G418. For exposure, GR-CALUX cells were plated in 96-well plates with phenol red-free DF medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS) at a volume of 200 μl per well. The next day the medium was replaced by medium containing a dexamethasone standard curve and the samples to be tested dissolved in DMSO, analysed in triplicate (final DMSO concentration, 1%). For the dexamethasone calibration curve, concentrations in a range of 0–39 ng ml^{-1} were used. After 24 h, the medium was removed, the cells lysed in Triton lysis buffer and luciferase activity was measured using a CentroXS LB90 luminometer (Berthold Technologies, Wildbad, Germany).

GR-CALUX data analyses

Luciferase activity per well was measured as relative light units (RLUs). To determine the EC_{50}, LOD and LOQ of dexamethasone in the GR-CALUX assay, a complete standard curve was included in each plate. The standard curve was fitted using the sigmoidal fit ($y = a_0 + a_1/(1 + \exp(-(x - a_2)/a_3))$ in a spreadsheet developed and provided by BioDetection Systems. The EC_{50} for dexamethasone was calculated by determining the concentration at which 50% of the maximum luciferase activity was reached using the sigmoidal fit equation. LOD was calculated as the luciferase activity generated by the standard zero plus three times the standard deviation; while LOQ was assessed as three times the LOD. To determine dexamethasone equivalents, the luciferase response by samples was interpolated in the linear range of the corresponding dexamethasone standard curve for the same plate.

GR-CALUX urine sample preparation

Extraction and deglucuronidation of urine samples were performed as described previously (Houtman et al. 2009). In short, 2 ml of urine sample were transferred into a glass tube, the pH was adjusted to approximately 7.0, and then 25 µl of β-glucuronidase from Escherichia coli was added (300 units ml^{-1}). Enzymatic deconjugation was carried out in a water bath for 1 h at 50°C. Next, 500 µl of $K_2CO_3/KHCO_3$ buffer were added and the sample was subjected to liquid–liquid extraction by adding 5 ml of MTBE. The tube was centrifuged at 1500 rpm for 5 min and the organic phase was collected and subsequently evaporated under a gentle stream of nitrogen. The final residue was redissolved in 40 µl of DMSO.

GR-CALUX dose–response curve

Multiple serial dilutions of six pure compounds (dexamethasone, betamethasone, prednisolone, prednisone, methylprednisolone and flumethasone) in DMSO were prepared in order to assess the sensitivity of the bioassay for these synthetic glucocorticoids. Dose–response curves were fitted using a four-parameter sigmoidal fit on Graphpad Prism (version 5.04 for Windows, Graphpad Software, San Diego, CA, USA). The concentration producing a 50%-fold induction of the maximal response (EC_{50}) and the relative effect potency (REP) as compared with dexamethasone (defined as the ratio between the EC_{50} of dexamethasone and the EC_{50} of the compound) were evaluated for each compound.
Data on the bioactivity of endogenous corticoids were provided by BioDetection System: cortisol (REP = 0.084; EC_{50} = 5.71 ng ml^{-1}) and cortisone (not active).

ELISA analysis

I'screen CORTICO (Tecna, Trieste, Italy) is a competitive enzyme immunoassay for the quantitative analysis of synthetic glucocorticoids in urine, muscle, liver and milk from producing animals. In a previous study, the ELISA kit was validated as a qualitative screening method for the detection of dexamethasone, betamethasone and flumethasone in bovine urine (LOD = 0.25 ng ml^{-1}; detection capability = 2 ng ml^{-1}) (Falasca et al. 2006).
The immunoassay was performed as described in the kit insert; urine samples (1 ml) were prepared by centrifugation at 2000g for 5 min and dilution of the supernatant with dilution buffer (×25).

Synthetic and natural glucocorticoids analysis by LC-MS/MS

An existing LC-MS/MS method (Vincenti et al. 2012) was partially modified to the scope of the present work. Sample preparation and chromatographic separation were conducted without any changes with respect to the above-mentioned paper, while the mass acquisition section was extended to include among the target analytes: 20β-dihydrocortisol, 6β-hydroxycortisol, betamethasone, 6α-methylprednisolone and flumethasone (Table 1).

Results and discussion

Effect of natural corticosteroids

A total of 23 blank urine samples (20 collected at the farm and three at the slaughterhouse) were analysed by GR-CALUX and by a commercially available ELISA kit in this evaluation of the interference effects on the bioassay due to natural corticosteroids, both at physiological concentrations and at the highest stress-induced concentrations.
All the specimens were characterised by LC-MS/MS analyses for the most important natural corticoids and metabolites (Heinig & Bucheli, 2008): cortisone, cortisol, 20β-dihydrocortisol and 6β-hydroxycortisol. The total amount of these physiologic hormones was also quantified.
The results of LC-MS/MS showed different amounts of natural hormones for the samples collected from the farm and the slaughterhouse. Analysis of the farm samples showed values of total natural corticosteroids below 10 ng ml^{-1}; only one sample (no. 08/21) displayed high levels of natural hormones of about 54 ng ml^{-1}. As expected, the three slaughterhouse samples showed the highest level of natural corticosteroids (18.6–140.6 ng ml^{-1}) (Tables 2 and 3).

Applying the GR-CALUX assay to the farm samples, U2OS cells did not exhibit either cytotoxic effects or over-induction of luciferase activity, as described previously for the T47D cells (Willemsen et al. 2002; Connolly et al. 2009).

As measured against the cut-off level used in screening activities (2 ng ml^{-1}), GR-CALUX correctly identified as screening negatives 19 out of the 20 farm samples (Table 2): 18 samples showed a glucocorticoid activity less than 1 ng ml^{-1}; 10 samples showed a hormone activity lower than the LOD of the method (0.078 ng dexamethasone eq. ml^{-1}); and one sample showed activity lower than the LOQ (0.21 ng dexamethasone eq. ml^{-1}). As expected, the sample (no. 08/21) with the highest levels of natural hormones was erroneously identified as being non-compliant (Table 2).

In the three slaughterhouse samples, the stress-related rise in endogenous corticosteroids led to their incorrect identification as 'screening positives' (Table 3).

In Tables 2 and 3, expected data ($\Sigma C_i \times REP_i$) were calculated from the sum of the concentration of each individual endogenous compound (C_i) multiplied by its relative effect potency (REP_i): cortisol = 0.084 and cortisone = 0. Although the GR-CALUX results indicated a clear relationship between the total amount of endogenous hormones and the glucocorticoid activity measured, at the moment approximately one-third of the activity detected in the farm samples and 10–20% in the slaughterhouse samples can be explained by the REP of cortisol and cortisone. Further experiments are needed to assess the interaction between metabolite activity and the method and possible matrix-related effects, other than natural corticoids.

The detection of drug traces by screening for glucocorticoids serves to identify not only their illicit use as growth promoting agents but also their incorrect use as therapeutic agents when administered without a veterinarian's prescription or without recording the treatment in the animal remedies record, and/or not respecting the withdrawal time. Unfortunately, however, lowering the cut-off value has proved almost impossible because of the high background signal related to the natural corticosteroids observed when analysing negative samples and the risk of increasing the number of false non-compliant samples.

The results obtained with GR-CALUX show that by collecting the samples at the farm the screening target concentration can be lowered, thus making this bioassay a useful tool for the detection of treatments with low-dose cocktails and incorrect use of the drugs as therapeutic agents.

In contrast, in the slaughterhouse samples, the stress-related rise in endogenous hormones led to their incorrect identification as 'screening positives'. As reported elsewhere, when fractionation methods are not used to remove the high levels of natural corticosteroids (Connolly et al. 2009), GR-CALUX cannot be applied as the presence of the natural hormones could dominate the glucocorticoid signal in the bioassay. None of these samples exhibited the cell toxic effects described by Willemsen et al. (2002).

ELISA was found to be more susceptible to specific and unspecific binding and matrix effects than GR-CALUX; two out of the 20 farm samples were identified as ‘screening positives’, whereas doubtful results (1.70–1.99 ng ml^{-1}) were obtained for six samples. Unexpectedly, only two out of the three slaughterhouse samples exceeded the screening target concentration (Tables 2 and 3). The ELISA results confirmed the difficulty in reducing the screening target concentration when using this matrix.

GR-CALUX dose–response curve

The sensitivity of the bioassay was also investigated. Figure 1 depicts the dose–response curves for the six synthetic glucocorticoids. A dose-related increase in luciferase activity occurred with dexamethasone, betamethasone, prednisolone, methylprednisolone and flumethasone, but not with the prohormone prednisone.

Table 4 reports the calculated EC_{50} values, the concentration giving the half-maximum response, and the relative effect potency (REP), defined as the ratio between the EC_{50} of dexamethasone and the EC_{50} of the compound, for the six synthetic glucocorticoids tested.
The GR-CALUX bioassay showed sensitivity for five out of the six compounds in the following order of potency:
flumethasone ≫ dexamethasone > betamethasone > methylprednisolone > prednisolone.
Prednisone showed no response in this assay.
GR-CALUX exhibited, using pure compounds in DMSO, a high sensitivity; five compounds had an EC_{50} between 0.13 and 0.99 ng ml^{-1}; only prednisolone presented an EC_{50} of 2.3 ng ml^{-1}. These results are in line with those reported in the literature about this RGA (Schriks et al. 2010; Bovee et al. 2011).
Table 5 shows a comparison between GR-CALUX and other GR RGAs described in the literature: the U2OS cell-based assay was the most sensitive one, with a dexamethasone EC_{50} of about 0.48 ng ml^{-1}, 10 times lower than the less sensitive bioassay. The endogenous hormone cortisol showed a low and comparable activity in U2OS, T47D and HeLa cells. Among synthetic molecules, flumethasone proved to be one of the most active molecules in all the RGAs evaluated, methylprednisolone and prednisolone exhibited a weak glucocorticoid activity in U2OS, T47D and HeLa cells, even if GR-CALUX demonstrated to be the most suitable assay for the detection of these feeble GR agonists (Willemsen et al. 2002; Schumacher et al. 2003; Connolly et al. 2009).

Spiked sample study

In order to evaluate the bioassay's ability to detect synthetic glucocorticoids in bovine urine, 15 spiked urine samples were analysed, and the results were compared with those of the immunoassay. The specimens were fortified with five synthetic glucocorticoids (dexamethasone, betamethasone, prednisolone, methylprednisolone and flumethasone). Prednisone, which showed no activity in the GR-CALUX analysis, was not used in this part of the study.

Each compound was added at three different levels of fortification (1, 2 and 4 ng ml^{-1}), and namely at the screening target concentration (2 ng ml^{-1}), at half and twice that level, respectively. One urine sample was used as a negative control. As reported in Table 6, the results of the GR-CALUX analyses agreed with those obtained in the previous phase, exhibiting a good correlation between concentrations expected and revealed: flumethasone showed the highest glucocorticoid bioactivity, a concentration above 1 ng ml^{-1} easily exceeded the cut-off level between screening positive and negative samples. Dexamethasone and betamethasone showed a lower activity than flumethasone. The concentrations detected by the bioassay were closely related to the fortification levels. According to the low glucocorticoid activity shown by these molecules, none of the fortification levels of prednisolone or methylprednisolone reached the screening target concentration currently used in screening phase.

The result obtained by analysing the negative control sample confirmed the low activity of natural hormones on GR-CALUX (< LOD), and the possibility of lowering the screening target concentration using this assay.

As previously demonstrated, RGAs can easily detect 2 ng ml^{-1} of the most active glucocorticoids in a wide range of matrices, like urine and liver. However, they might fail to differentiate low doses of prednisone and methyl-prednisolone from the mean signal of negative samples (Willemsen et al. 2002; Schumacher et al. 2003; Connolly et al. 2009).

In general, GR-CALUX showed advantages over the other RGAs, exhibiting a higher sensitivity towards the less active synthetic compounds, and allowing an easier and quicker sample preparation; without any step of SPEs. Also in this phase urine samples did not show cytotoxic effects when analysed by U2OS cells.

The results of the ELISA analyses were very close to those obtained by the bioassay. Flumethasone showed the highest cross-reactivity; in fact, the concentration of 1 ng ml^{-1} exceeded the cut-off level. The amount of dexamethasone and betamethasone detected by ELISA was related to the fortification level, whereas prednisolone and methylprednisolone showed the lowest cross-reactivity, and none of the fortification levels reached the cut-off value. The negative control sample displayed a 10-fold higher glucocorticoid concentration than that obtained by the bioassay.

Incurred sample study

The LC-MS/MS analysis revealed dexamethasone residues in almost all urines collected from treated animals, from the second to the 20th day of treatment (average concentrations between 0.9 and 3 ng ml^{-1}); after the end of the treatment, at the first, second and third day of sampling, it was found in 70%, 30% and 20% of the analysed specimens, respectively.
Dexamethasone residues were not detected in samples collected from control animals, and from treated animals before the first administration and at 10 days after the end of the treatment.
Some incurred samples were analysed using both GR-CALUX and the ELISA kit. Four urine samples (about 0.5, 1, 2 and 3 ng ml^{-1} of dexamethasone, respectively) were selected after LC-MS/MS analysis; three urine samples from control animals were used as negative controls.
As reported in Table 7, both GR-CALUX and ELISA exhibited a dose-related detection of the dexamethasone contained in the incurred samples. ELISA showed a higher signal in the analysis of the blank samples than that obtained by GR-CALUX.

Conclusions

Despite the limited number of non-compliances found in European Union controls, examination of illegal preparations and results of specific investigations have exposed the depth and breadth of the continuing illegal use of growth promoters in livestock production (Courtheyn et al. 2002; EFSA, 2012). The analytical methods employed in European Union control programmes for measuring steroid residues fall short of capturing the complexity of illicit practices. One plausible explanation for the limited non-compliant samples found so far is the use of newly developed compounds, though a more likely reason is the increasingly widespread trend of administering low-level drug cocktails (Connolly et al. 2009). As previously demonstrated for oestrogens and androgens (Nielen et al. 2006; Tooriaans et al. 2010), the best way to detect the abuse of new or unknown steroid compounds is by combining bioassay activity-based screening with chemical identification (Houtman et al. 2009).
Here we report the results from a preliminary assessment of a highly sensitive reporter gene assay as screening tool for the detection of glucocorticoids in bovine urine samples. The sensitivity of GR-CALUX indicates that it can easily detect the most widely used synthetic glucocorticoids. The bioassay resulted as being less influenced by the biological matrix than common screening tools. This suggests that because GR-CALUX could lower the target concentration for an ongoing screening activity, it can be a useful tool for the detection of treatments with low-dose cocktails and the incorrect use of these drugs as therapeutic agents.
The possibility of performing the RGA in 96-well plates makes this bioassay almost comparable with a common ELISA kit, and amenable to robotic automation, thereby providing a high-

throughput format necessary to process a large number of samples. The ELISA applied in the study proved to be more rapid than the bioassay, allowing the full analysis of urine samples in a few hours. On the contrary the U2OS-based assay required a longer exposure to the sample, of at least 24 h, but being a 'live assay' it allowed the continuing self-production of new test plates, resulting in it being cheaper.

The study shows that a reporter gene assay based on stably transfected human bone cell (U2OS) is suitable for the qualitative analysis of bovine urine samples. Further investigations will need to focus on the analytical requirements under 2002/657/EC before the assay can be validated as an effective screening method for routine residues analysis (EC 2002; CRLs 2010).

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Image Gallery

Table 1. Essential figures of merit of the LC-MS/MS method.

Analyte[a]	CCα (ng ml^{-1})	LOD (ng ml^{-1})
Prednisone	0.66	
Prednisolone	0.67	
6α-Methylprednisolone	1.20	
Flumethasone	0.63	
Betamethasone	0.61	
Dexamethasone	0.59	
Cortisone		0.1
Cortisol		0.1
20β-Dihydrocortisol		0.5
6β-Hydroxycortisol		5.0

Note: [a]A decision limit (CCα) was assessed for synthetic and forbidden molecules, while in detecting endogenous compounds a limit of detection (LOD) was set.

Table 2. Results of the 20 farm urine samples; the specimens were characterised for endogenous corticosteroids by the LC-MS/MS method and analysed using GR-CALUX and the ELISA kit.

Sample code	LC-MS/MS (ng ml^{-1})					GR-CALUX (ng dex eq. ml^{-1})		ELISA (ng ml^{-1})
	Cortisol	Cortisone	20β-Dihydrocortisol	6β-Hydroxycortisol	Total	Measured	Expected	
08/06	3.1	5.3	1.3	< LOD[c]	9.7	0.73	0.26	0.31
08/10	1.3	2.3	0.9	< LOD[c]	4.5	< LOD[a]	0.11	1.23
08/21	15.7	15.4	9.5	14.1	54.7	3.7	1.32	2.96
08/26	< LOD[c]	0.8	< LOD[c]	< LOD[c]	0.8	< LOD[a]	0	< LOD[b]
08/45	0.5	1.0	0.6	< LOD[c]	2.1	< LOD[a]	0.04	0.95
08/68	< LOD[c]	0.7	< LOD[c]	< LOD[c]	0.7	< LOD[a]	0	0.50
08/71	< LOD[c]	< LOD[c]	< LOD[c]	6.7	6.7	0.68	0	0.61
08/72	1.1	1.5	0.6	< LOD[c]	3.2	0.31	0.09	0.50
08/79	< LOD[c]	0.6	< LOD[c]	< LOD[c]	0.6	0.34	0	0.49
08/88	0.6	1.0	0.9	< LOD[c]	2.5	< LOD[a]	0.05	0.84
09/06	0.6	< LOD[c]	1.3	< LOD[c]	1.9	< LOD[a]	0.05	1.98
09/31	< LOD[c]	< LOD[c]	1.4	7.9	9.3	< LOD[a]	0	1.70
09/32	1.4	0.7	1.4	< LOD[c]	3.5	< LOQ[a]	0.12	1.48
09/35	1.4	1.1	0.8	< LOD[c]	3.3	< LOD[a]	0.12	2.62
09/36	0.8	1.7	1.0	< LOD[c]	3.5	0.95	0.07	1.89
09/38	< LOD[c]	0.5	1.4	< LOD[c]	1.9	< LOD[a]	0	1.78
09/41	1.4	1.2	1.2	< LOD[c]	3.8	< LOD[a]	0.12	1.32
09/42	2.0	1.5	2.5	< LOD[c]	6.0	1.1	0.16	1.93
09/43	1.3	1.5	1.6	< LOD[c]	4.4	0.44	0.11	1.99
09/44	0.7	1.0	1.2	< LOD[c]	2.9	0.33	0.06	1.37

Notes: [a]GR-CALUX: LOD = 0.078 ng ml^{-1}, LOQ = 0.21 ng ml^{-1}.
[b]I'screen CORTICO: LOD = 0.25 ng ml^{-1}.
[c]LC-MS/MS LOD = 0.1 ng ml^{-1} (cortisol, cortisone), 0.5 ng ml^{-1} (20β-dihydrocortisol) and 5 ng ml^{-1} (6β-hydroxycortisol).

Table 3. Results of the three blank urine samples collected at the slaughterhouse; the specimens were characterised for endogenous corticosteroids by LC-MS/MS and analysed using GR-CALUX and the ELISA kit.

Sample code	LC-MS/MS (ng ml^{-1})					GR-CALUX (ng dex eq. ml^{-1})		ELISA (ng ml^{-1})
	Cortisol	Cortisone	20β-Dihydrocortisol	6β-Hydroxycortisol	Total	Measured	Expected	
6196	30.0	33.6	29.0	48.0	140.6	25	2.5	4.64
6210	4.7	5.7	2.9	5.2	18.6	3.3	0.4	0.58
9446	25.5	17.4	24.7	14.8	82.4	10	2.1	2.28

Table 4. Bioactivity profiles of a range of synthetic glucocorticoid compounds analysed by GR-CALUX.

Compound	EC_{50} (ng ml^{-1})	REP
Flumethasone	0.13	3.60
Dexamethasone	0.48	1.00
Betamethasone	0.57	0.84
Methyl-prednisolone	0.99	0.48
Prednisolone	2.3	0.21
Prednisone	n.a.	n.a.

Note: n.a., Not active

Table 5. Comparison between four mammalian cell-based reporter gene assays (RGAs) in detecting synthetic and endogenous glucocorticoids.

Cells	U2OS (GR-CALUX)	T47D (Connolly et al. 2009)	T47D (Willemsen et al. 2002)	HeLa (Shumacher et al. 2003)
Dexamethasone EC50 (ng ml^{-1})	0.48	0.79	2.44	5.00
REP synthetic molecules				
Dexamethasone	1.00	1.00	1.00	1.00
Betamethasone	0.84	1.95	0.50	1.30
Flumethasone	3.60	1.17	2.53	6.50
Methyl-prednisolone	0.48	0.01	0.35	0.61
Prednisolone	0.21	0.08	n.t.	n.t.
REP endogenous hormones				
Cortisol	0.08	0.15	n.t.	0.08
Cortisone	0.00	0.00	n.t.	n.t.
Corticosterone	n.t.	0.08	n.t.	n.t.

Note: n.t., Not tested.

Table 6. Reporter gene assay (RGA) and ELISA analysis of the spiked urine samples; the specimens were fortified with five synthetic glucocorticoids at three different levels of fortification.

Spiked samples			GR-CALUX (ng dex eq. ml^{-1})		ELISA (ng ml^{-1})
Code	**Compound**	**ng ml^{-1}**	**Measured**	**Expected**	
TV 1	Dexamethasone	4	4.40	4	6.09
TV 2		2	2.20	2	3.27
TV 3		1	0.76	1	2.13
TV 4	Betamethasone	4	5.1	3.36	4.69
TV 5		2	2.00	1.68	2.75
TV 6		1	1.20	0.84	1.72
TV 7	Flumethasone	4	15	14.4	8.31
TV 8		2	9.50	7.2	3.83
TV 9		1	5.90	3.6	2.31
TV 10	Prednisolone	4	0.60	0.84	1.48
TV 11		2	0.54	0.42	1.23
TV 12		1	0.35	0.21	0.97
TV 13	Methylprednisolone	4	1.40	1.92	0.68
TV 14		2	0.48	0.96	0.72
TV 15		1	0.65	0.48	0.79
TV 16	Negative control	0	< LOD (0.078)	< LOD (0.078)	0.61

Table 7. Analyses of the incurred bovine urines containing dexamethasone administered at a growth-promoting dose (0.4 mg day^{-1}).

	LC-MS/MS	GR-CALUX (ng dex eq. ml^{-1})	ELISA (ng ml^{-1})
Blank 1	0.60		0.62
Blank 2		< LOQ (0.21)	0.90
Blank 3	0.68		1.25
Dexamethasone 0.5 ng ml^{-1}	0.56		0.97
Dexamethasone 1 ng ml^{-1}	1.90		2
Dexamethasone 2 ng ml^{-1}	2.27		3.20
Dexamethasone 3 ng ml^{-1}	5.02		4.80

Figure 1. Dose–response curves of several synthetic glucocorticoids analysed with GR-CALUX.