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Potential of Treatment-Specific Protein Biomarker Profiles for Detection of Hormone Abuse in Cattle

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Supporting Information

ABSTRACT: Targeted protein biomarker profiling is suggested as a fast screening approach for detection of illegal hormone treatment in meat production. The advantage of using biomarkers is that they mark the biological response and, thus, are responsive to a panel of substances with similar effects. In a preliminary feasibility study, a 4-plex protein biomarker flow cytometric immunoassay (FCIA) previously developed for the detection of recombinant bovine somatotropin (rbST) was applied to cattle treated with steroids, such as estradiol, dexamethasone, and prednisolone. Each treatment resulted in a specific plasma biomarker profile for insulin-like growth factor-1 (IGF-1), IGF binding protein 2, osteocalcin, and anti-rbST antibodies, which could be distinguished from the profile of untreated animals. In summary, the 4-plex biomarker FCIA is, apart from rbST, also capable of detecting treatment with other growth-promoting agents and therefore clearly shows the potential of biomarker profiling as a screening method in veterinary control. It is proposed to perform additional validation studies covering high numbers of treated and untreated animals to support inclusion or adaptation of protein biomarker approaches in future monitoring regulations.

KEYWORDS: *biomarker, hormone abuse, veterinary control, multiplex, binding assay, steroids*

INTRODUCTION

Protein biomarker profiling has been suggested as a fast screening approach for detection of illegal treatment practices in human sports doping[1] and in veterinary control[2−6] for detection of banned growth-promoting agents (Directives 2008/97/EC, 96/22/EC, and 96/23/EC[7−9]). Using protein biomarkers has several advantages: First, they are indicative for any substance having the relevant effect and can therefore give a comprehensive first screening result whether any growth-promoting agent was used. Even the use of designer substances of unknown chemical structure could be detected. Second, biomarker profiles remain changed for a longer period of time than the abused substance can be detected in circulation.[10,11] And third, the use of fast screening methods prior to an extensive instrumental confirmatory method is beneficial because of its time savings as it already pinpoints suspicious samples and omits compliant samples from the subsequent analysis.

Nevertheless, up till now, protein biomarker analysis tools were specifically developed for only a specific treatment setting.[12] In this study, we investigated if a previously developed 4-plex biomarker flow cytometric immunoassay (FCIA) for the detection of *protein hormone* recombinant bovine somatotropin (rbST) abuse[4] can also be used to detect treatments with *steroid hormones* such as estradiol (E_2), dexamethasone, and prednisolone. In contrast to rbST, steroids are not species-specific and also exert hormonal effects in humans if residues are consumed. This highlights the importance of a fast screening method for steroid abuse detection in meat production to maintain the highest possible food safety for the customer.

The biomarker panel of the 4-plex FCIA consisted of insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2), osteocalcin, and antibodies produced by cows upon rbST treatment (anti-rbST antibodies). This biomarker panel was specifically developed for the detection of rbST abuse in dairy cattle, and biomarkers were chosen on the basis of information from the literature stating specific changes in biomarker concentrations after somatotropin treatment.[13−19] Nevertheless, three of these four biomarkers, namely IGF-1, IGFBP2, and osteocalcin, are also known to be influenced by steroid hormone treatments such as glucocorticoids and sex steroids.[2,20−27] IGF-1 and IGFBP2 are members of the somatotropin/IGF axis, which is usually involved in growth-related mechanisms and is thereby affected by growth-inducing or -repressing substances, such as growth-promoting steroids.[2,20,22,23,25,26] One possible target of somatotropin, IGF-1, and steroid hormone action is bone, which reacts with an increase or decrease in bone turnover. Osteocalcin is a marker of bone turnover and, therewith, osteocalcin concentrations respond upon administration of bone turnover altering

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drugs.[21,24,27] Therefore, the selected biomarkers might also be influenced not only by rbST but also by other growth-promoting steroids. To prove this, in the here-presented study, plasma samples from an animal study with sex steroid (estradiol) and glucocorticoid (prednisolone and dexamethasone) treatments were analyzed using the previously developed 4-plex FCIA. The obtained biomarker profiles of the treated groups were compared with the ones from a control group. On the basis of the results, the potential of targeted protein biomarker profiling is discussed.

MATERIALS AND METHODS

Chemicals. 17-β-Estradiol-3-benzoate was obtained from Intervet (Boxmeer, The Netherlands) and dexamethasone-21-sodium phosphate from Fort Dodge Animal Health (Bologna, Italy). Prednisolone was from Novosterol (CEVA VETEM SpA, Agrate Brianza, Italy) and the rbST standard from the National Hormone and Peptide Program of Dr. Parlow (Torrance, CA, USA). IGF-1 standard was purchased from Fitzgerald Industries International (North Acton, MA, USA) and IGFBP2 standard from IBT (Reutlingen, Germany). Osteocalcin standard and mouse anti-osteocalcin antibody were from Haematologic Technologies, Inc. (Essex Junction, VT, USA) and seroMAP microspheres (sets 050, 025, 078, and 084) from Luminex (Austin, TX, USA). Bovine serum albumin (BSA) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were bought from Sigma-Aldrich (St. Louis, MO, USA), and sulfo-*N*-hydroxysuccinimide (S-NHS) was from Fluka (Buchs, Switzerland). Glycine was from Duchefa (Haarlem, The Netherlands), and the polypropylene tubes were from Greiner Bio-One (Alphen aan de Rijn, The Netherlands). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, Germany) and Tween-20 from VWR International (Amsterdam, The Netherlands). Mouse anti-IGF-1 antibody was bought from LifeSpan BioSciences, Inc. (Seattle, WA, USA) and rabbit anti-IGFBP2 antibodies from United States Biological (Swampscott, MA, USA); the filter bottom microtiter plates were from Millipore (Billerica, MA, USA). Goat anti-mouse R-phycoerythrin (PE) antibody and goat anti-rabbit PE antibody were obtained from Prozyme (San Leandro, CA, USA), and goat anti-bovine PE antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The LX-100 flow cytometer was from Applied Cytometry Systems (Dinnington, Sheffield, South Yorkshire, UK) and the software Prism 5 from GraphPad Software, Inc. (San Diego, CA, USA).

Animals and Treatment. Because in the here-presented study only the feasibility of the chosen biomarkers was tested, Directive 2010/63/EU[28] describing the 3-R principle in animal testing was considered, and no new animal experiment was performed. Instead of that, only a limited number of samples, which originated from a previous study conducted at Università di Torino, was available and used to test the developed method. To assess levels of the four biomarkers after treatment of estradiol (E_2), dexamethasone, and prednisolone, plasma samples from an animal study were used. Twenty-four male Charolais bovines (17−22 months) were randomly divided in four groups and received the following treatments: six animals in group E_2 received 0.01 mg (kg body weight)$^{-1}$ per week 17-β-estradiol-3-benzoate by intramuscular injection for 6 weeks. All six animals in group DEX were treated with 0.7 mg day^{-1} dexamethasone-21-sodium phosphate per os for 40 days, and all six animals in group PRED received 15 mg day^{-1} prednisolone per os for 30 days. The control group consisted of 6 untreated animals. These steroid treatments were chosen because they are expected to be illegally used in meat production as growth-promoting agents,[29] and dosages and administration routes were determined on the basis of the literature.[30,31] Blood samples were taken throughout the treatment study; sampling occurred through the external jugular vein with evacuated EDTA tubes to prepare plasma samples by centrifugation at 2000*g* for 20 min, and samples were stored at −80 °C until further use. Plasma samples taken at days 25 (during the treatment) and 43 (after the last treatment) were chosen for analysis. Samples from day 43 reflect the situation of when the animals arrive at the slaughterhouse including a delay after the last treatment (3 days for the E_2- and dexamethasone-treated groups and 6 days for the prednisolone-treated group). The treatment and sampling schedule is depicted in Figure S1 in the Supporting Information. The animal studies were authorized by the Italian Ministry of Health and the Bioethics Committee of the University of Turin.

Sample Pretreatment, Microsphere Preparation, and Assay Procedure. For microsphere preparation, 100 μg mL^{-1} rbST standard, 100 μg mL^{-1} IGF-1, 10 μg mL^{-1} IGFBP2, and 75 μg mL^{-1} osteocalcin were covalently coupled to color-encoded seroMAP microspheres (sets 050, 025, 078, and 084, respectively) by using the sulfo-NHS−EDC coupling chemistry as described before.[4,11,32,33] Standard curves were prepared with IGF-1, IGFBP2, and osteocalcin standard proteins in plasma-matched buffer [80 mg mL^{-1} BSA in phosphate-buffered saline (PBS)]. Obviously no standard curve can be produced for the biomarker anti-rbST antibodies. For the generic plasma pretreatment procedure, 25 μL of glycine solution I (27.5 mM glycine, pH 0.5) was mixed with 25 μL of plasma or standard sample in a polypropylene tube under constant vortexing. After 60 min of incubation at room temperature, 50 μL of glycine solution II (400 mM glycine, 0.3% m/v SDS, pH 10) was added, and samples were further diluted by addition of 1.9 mL of 0.1% BSA in PBST (0.05% v/v Tween-20 in PBS), which led to an overall dilution of the plasma by 80 times. Detection assays for IGF-1, IGFBP2, and osteocalcin are of competitive format, where free protein in the blood sample inhibits binding of the primary detection IgG to the respective protein-coupled microsphere. The assay for detection of anti-rbST antibodies is a direct assay, where the antibodies originating from the blood sample directly bind to rbST-coupled microspheres. The 4-plex FCIA procedure was described previously,[4] and the assay principle is depicted in Figure S2 in the Supporting Information. Briefly, 10 μL of primary detection immunoglobulin G (IgG) mixture (1:625 mouse anti-IGF-1, 1:25000 rabbit anti-IGFBP2, 1:100000 mouse anti-osteocalcin) was added to 100 μL of pretreated and diluted sample in a filter-bottom microtiter plate and incubated at 4 °C under orbital shaking for 15 min. Thereafter, 10 μL of microsphere mixture suspension (containing approximately 1250 microspheres per microsphere set) were added to each well and incubated at 4 °C for 1 h under orbital shaking. Microspheres were washed once with PBST, then 125 μL of PE-IgG mixture (1:625 goat anti-mouse PE, 1:1000 goat anti-rabbit PE, and 1:1000 goat anti-bovine PE) was added to each well, and finally the plate was incubated for 30 min at 4 °C under orbital shaking. Then, the plate was centrifuged at 130*g* for 1 min and 125 μL of PBST was added. Thereafter, the plate was put into the LX-100 flow cytometer for measurement. Microspheres from every sample were analyzed in a flow of 1 μL s^{-1} until 50 microspheres per set were counted up to a maximum of 50 μL per sample. Each microsphere set (= each biomarker assay) was identified by its unique color by a red laser, and the fluorescence intensity of the reporter PE attached to each microsphere was quantified by a green laser. Median fluorescence intensities (MFI) were obtained from every analyzed sample, and biomarker concentrations of IGF-1 and osteocalcin were calculated on the basis of standard curves obtained by using the software Prism 5. For IGFBP2, B/B_0 values were used, because the slope of the standard curve was too flat to work with calculated concentrations. When using a standard curve with a flat slope for calculating concentrations, large concentration differences will be obtained even though only a small difference in signal was measured. For anti-rbST antibodies, raw MFI signals were used. The average biomarker levels of the six cows in each group are plotted in Figure 2, and standard deviations are shown as error bars. Biomarker levels of the different treatment groups were compared to their respective time-matched control group by using the unpaired, two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Monitoring biomarker profiles as an indication of possible hormone abuse becomes more and more prevalent in sports doping and veterinary control. Because biomarkers reflect a

biological effect and this effect might also be achieved by other hormonal substances, several treatments can be indirectly detected by altered biomarker profiles. A 4-plex protein biomarker FCIA was previously developed to detect rbST abuse in dairy cattle on the basis of an altered biomarker profile. Biomarkers analyzed were IGF-1, IGFBP2, osteocalcin, and anti-rbST antibodies endogenously produced by the cow as a response upon rbST treatment. The tested biomarkers were shown to be all responsive to rbST in a previous study: IGF-1 and osteocalcin concentrations were increased, IGFBP2 levels were decreased, and anti-rbST antibodies were induced by rbST treatment in dairy cattle.[4] In this work, the 4-plex protein biomarker FCIA was used to test whether beef cattle also show a change in biomarker levels after steroid treatment. Steroids used were a sex steroid (estradiol, E_2) and glucocorticoids (prednisolone or dexamethasone), which are occasionally used in veterinary practice to illegally improve meat production and quality. Plasma biomarker concentrations for IGF-1 and osteocalcin were calculated on the basis of standard curves prepared in plasma-matched buffer (Figure 1). IGFBP2 was

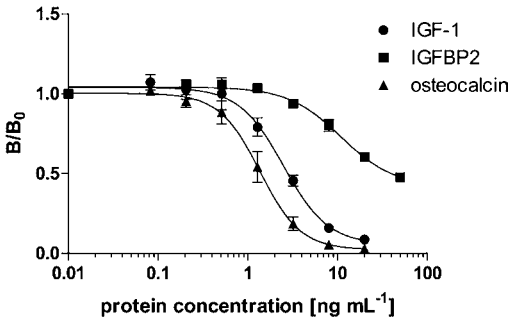

Figure 1. Standard calibration curves for the immunoassays for measuring insulin-like growth factor 1 (IGF-1), IGF binding protein 2 (IGFBP2), and osteocalcin in a competitive format. Standard curves were obtained in plasma-matched buffer.

analyzed using B/B_0 values as already described earlier.[4] For the biomarker anti-rbST antibodies, no standard curve could be obtained; therefore, the presence of these antibodies was expressed as raw median fluorescence intensity (MFI) signals.

Two different time points in all four treatment groups were analyzed. One time point on day 25 was in the middle of the treatment period and reflects on-farm testing, whereas the other one on day 43, which was a few days after discontinuation of the treatments (see Figure S1 in the Supporting Information), reflects when the animals arrive at the slaughterhouse.

Compared to the untreated control group, the three different steroidal treatments had specific effects on the biomarker levels. IGF-1 concentrations were significantly elevated after dexamethasone (+30% IGF-1 on day 25 and +11% IGF-1 on day 43) and prednisolone (+16% IGF-1 on day 43) treatment, whereas no significant effect was observed for E_2 treatment (Figure 2A). The effects of glucocorticoids on IGF-1 levels at different life stages were studied before. Acute glucocorticoid treatment of newborn animals resulted in a stimulation of the somatotropic axis and increased IGF-1 levels.[25,34] In early life stages, glucocorticoid administration had an inhibiting effect on plasma IGF-1 concentrations.[20,22] Nevertheless, glucocorticoid treatment of adults resulted in increased IGF-1 levels again,[23,26] which is also supported by the results of our study, in which 17−22-month-old cattle were treated. As already described by Hammon et al., IGF-1 response upon glucocorticoid treatment depends on age, dose, and duration of the treatment.[22] Furthermore, in untreated cattle, there is a variability in IGF-1 background levels depending on the age and sex of the animal.[35] This variability is, however, not a problem as the age and sex of a tested animal are known to the veterinary inspection services. Therefore, these fluctuations can be easily accounted for following full validation covering different age groups.

For IGFBP2, B/B_0 values were used. Because the IGFBP2 assay is of an inhibition format, increased B/B_0 values represent decreased plasma IGFBP2 concentrations and vice versa. B/B_0 values are shown in Figure 2B. Dexamethasone showed a significant IGFBP2 B/B_0 increasing effect (+11% IGFBP2 B/B_0 on day 25 and +21% IGFBP2 B/B_0 on day 43); thus, plasma IGFBP2 concentration decreased by dexamethasone treatment (Figure 2B). The studies showing an IGF-1 increasing effect also reported an IGFBP2 decreasing effect due to glucocorticoid treatment.[23,25,26] As an exception, Bertozzi et al., who reported decreased IGF-1 levels after glucocorticoid treatment, did not see any effect on IGFBP2 concentrations.[20] In rbST treatment studies, an IGFBP2 decreasing effect was observed together with an IGF-1 level increase as well,[13,36] which might indicate a contrary regulation of these proteins, thus explaining the observed IGFBP2 decrease after dexamethasone treatment in our study. Here, E_2 decreased IGFBP2 B/B_0 values (−12% IGFBP2 B/B_0 on day 25) and, thus, increased plasma IGFBP2 concentrations. In contrast to that, IGFBP2 concentrations were not affected by administration of a cocktail of estradiol and nortestosterone in veal calves.[2] Nevertheless, in rat hippocampus cells[37] as well as in bovine granulosa cells,[38] it was shown that estrogen treatment induced the gene expression of IGFBP2. Furthermore, serum IGFBP2 concentrations were increased in barrows treated with estradiol,[39] which is supported by the findings of our study. Also for IGFBP2, background levels in untreated cattle depend on sex and age and can be accounted for because these parameters will be known in monitoring practice.[40]

Both, E_2 (−32% osteocalcin on day 43) and dexamethasone (−41% osteocalcin on day 25 and −49% osteocalcin on day 43) decreased osteocalcin levels (Figure 2C). Known effects of glucocorticoids are their growth-retarding actions on bone. Thus, decreased osteocalcin concentrations were seen before,[2] and it was described that glucocorticoids repress the osteocalcin promoter.[24] It is known that estrogens as well as androgens induce bone formation and repress bone resorption.[27] Nevertheless, previous studies described that they did not see any effect in osteocalcin levels of calves throughout a 28 day sex steroid treatment period with combined treatment of estrogens and androgens.[2] Because osteocalcin is released into the circulation during bone formation[41] and bone resorption,[42] it is a matter of the ratio of bone formation and resorption before and after estrogen treatment whether increased or decreased levels are observed. Furthermore, osteocalcin turnover is also influenced by the somatotropin/IGF system in an endocrine and paracrine manner,[21] which might explain the decreased osteocalcin concentrations observed in our study. As well as for IGF-1 and IGFBP2, background osteocalcin levels in untreated cattle are age-dependent and can be accounted for because these parameters will be known to the veterinary inspection services.[43]

As expected, none of the steroid treatments affected the endogenous antibody response of antibodies directed against rbST (Figure 2D), which are produced by the animal after

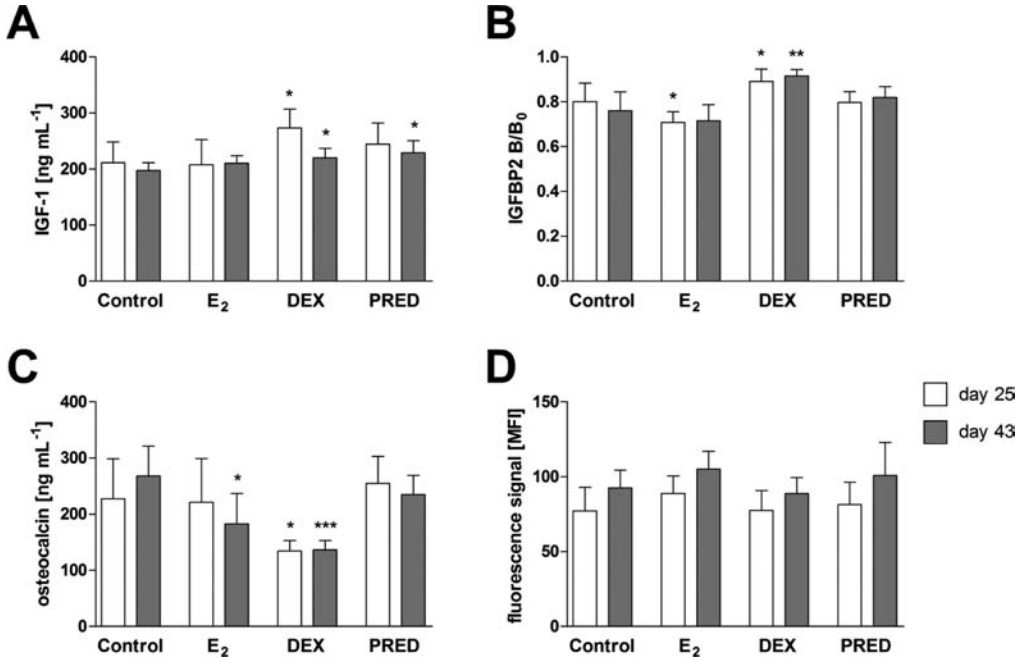

Figure 2. IGF-1 concentration (A), IGFBP2 B/B_0 values (B), osteocalcin concentrations (C), and fluorescence signals of anti-rbST antibody detection (D) obtained from the 4-plex protein biomarker FCIA after treatment with estradiol (E_2), dexamethasone (DEX), or prednisolone (PRED). Each bar represents the average of six cows in one treatment group at one time point; error bars indicate standard deviations. Significance levels were ∗ for $P \leq 0.05$, ∗∗ for $P \leq 0.01$, and ∗∗∗ for $P \leq 0.001$ and were obtained by comparing a treated group at one time point to its time point-matched control group using the two-tailed Student's t test.

treatment with this protein hormone. Thus, this is a biomarker that selectively discriminates the rbST treatment from steroidal treatment practices in cattle.

The biomarker profile results of every type of steroid treatment are summarized in Table 1 and are also compared to the biomarker profile of rbST treatment obtained from a previous study.[4]

Table 1. Treatment-Specific Biomarker Profiles Consisting of Significantly Increased (+), Decreased (−), or Stable (Ø) Biomarker Levels after Treatment

	biomarkers			
treatment	IGF-1	IGFBP2	osteocalcin	anti-rbST antibodies
rbST[a]	+	−	+	+
prednisolone	+	Ø	Ø	Ø
dexamethasone	+	−	−	Ø
E_2	Ø	+	−	Ø

[a]Adapted from Ludwig et al.[4]

In general, some of the changes in biomarker concentrations are rather low, but it always depends on the biological variation in the control group whether an increase of <20% will be detectable or not. If the biological variation in the control group is very low, the <20% change might be sufficient for discrimination. Furthermore, it is not likely that only one biomarker will be indicative for drug abuse. Therefore, always a biomarker profile will be used to identify drug abuse. Combining biomarkers into a profile will be mainly done using statistical models, such as discriminant functions as previously shown for detection of human growth hormone doping in athletes,[44] support vector machines as done for the detection of growth-promoting hormones in cattle,[45] or the statistical model k-nearest neighbors as previously described for rbST detection in dairy cows.[4] If several biomarkers are combined into a statistical model, even a slight change in one biomarker might contribute significantly to the decision making that a sample considered as being positive for drug abuse.

Obviously, biomarkers of growth-promoting drugs are growth-related and therefore also affected by other factors such as growth in early life stages. Therefore, before implementation of the demonstrated method, the measurement of samples from an extensive control population reflecting all age groups would be necessary. Then, for the ultimate method, age correction of the measured biomarker levels would be necessary to determine whether the animals were treated or not. One possibility to do this is to compare the results of an individual animal to the biomarker profile of its respective age-matched control group. In this case, each age group would have a specific age-dependent threshold level for each biomarker. Another approach would be to establish discriminant functions, in which all relevant biomarker levels and the age of the animal are included as factors for the calculation of a final test score. If the score then exceeds a set limit, the sample is considered to be positive. This approach was already followed for the detection of GH abuse in athletes.[44]

As can be seen in Table 1, each treatment influenced the biomarkers in a unique pattern, which potentially allows the reverse conclusion that a certain biomarker profile is indicative for a specific treatment. Thus, when an unknown sample is analyzed by using the 4-plex biomarker FCIA, not only the suspicion that an animal was treated would become clear but also the possible abused class of substances might be indicated, which is crucial information for the subsequent instrumental confirmatory analysis according to Commission Decision 2002/657/EC.[46]

To adapt this 4-plex biomarker FCIA for steroidal compounds in routine veterinary control, a thorough validation

needs to be done, as demonstrated for the detection of rbST.[4] First, a large untreated bovine population, representing all age groups, must be analyzed to get a more detailed impression of the range of naturally occurring biomarker levels. Then, a comparison of animal treatment study results with control results would allow the calculation of true-positive and true-negative rates, thereby considering different steroidal treatment regimens. Furthermore, time course studies need to be done to evaluate whether changes in biomarker levels remain stable after prolonged treatment or whether they decline after a certain period of time in treatment. In Figure 2A, IGF-1 levels are still increased on day 43 after dexamethasone treatment, but they are less pronounced than on day 25. We can only speculate whether this is an effect of a prolonged treatment or whether the observed decrease in concentration versus day 25 is because the treatment was discontinued three days before sampling. Furthermore, results have to be evaluated on the basis of the animal's sex and age. If the selected candidate biomarkers would not be sufficient yet for pinpointing the abuse of a certain substance according to European Union guidelines (Commission Decision 2002/657/EC[46]), then more biomarkers can be simply added to the developed multiplex screening protocol. The addition of other biomarkers might also increase the potential of the method to detect a treatment with a cocktail of steroids. Additional biomarker candidates for detection of anabolic hormone abuse, such as Ir-inhibin or sex hormone binding globulin (SHBG),[2] were suggested already by Mooney et al. and Pinel et al.[47,48]

In summary, the feasibility of a 4-plex biomarker FCIA, initially developed for rbST abuse detection, was preliminary demonstrated for detecting treatment with other growth-promoting agents and therefore clearly highlights the potential of biomarker profiling as future screening methods in veterinary control.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

BSA, bovine serum albumin; E_2, estradiol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; FCIA, flow cytometric immunoassay; IGF-1, insulin-like growth factor 1; IGFBP2, IGF binding protein 2; IgG, immunoglobulin G; MFI, median fluorescence intensity; PE, phycoerythrin; PBS, phosphate-buffered saline; PBST, 0.05% (v/v) Tween-20 in PBS; rbST, recombinant bovine somatotropin; SDS, sodium dodecyl sulfate; sulfo-NHS, sulfo-*N*-hydroxysuccinimide

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