Biomarkers, May 2008; 13(3): 246–256

informa
healthcare

Plasma biomarker profiling in the detection of growth promoter use in calves

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Abstract
The detection of illicit growth promoter use during meat production within the European Union is reliant on residue testing which is a limiting factor on the number of animals which can be tested and consequently compromises the efficacy of testing procedures. The present study examined a novel detection strategy based on the profiling of plasma component concentrations in response to growth promoter administrations. Calves subjected to nortestosterone decanoate, 17β-oestradiol benzoate and dexamethasone were found to have altered urea, aminoterminal propeptide of type III procollagen and sex hormone binding globulin profiles in response to treatments. These findings demonstrate the potential of using the identification of perturbed profiles within a panel of biomarkers which cover a spectrum of biological activity to reveal growth promoter abuse.

Keywords: *Growth promoters, veal calves, plasma biomarkers, SHBG, PIIINP, urea*

(Received 8 August 2007; accepted 30 November 2007)

Introduction

Whilst the use of anabolic agents in farm animals is known to increase the efficiency of meat production, the use of such compounds within the European Union has been prohibited (Directive 88/146/EEC) since the late 1980s (Stephany 2001). Since that time much work has been devoted to the development of sensitive methods for the detection of drug residues in samples obtained from hormone-treated animals (De Brabander et al. 2007). Developments in this area have resulted in a lowering of the testing detection limit and consequently there has been increased use of illicit endogenous hormones in the form of 'hormone cocktails' (Courtheyn et al. 2002). The low doses of compounds present within these cocktails not only helps evasion of targeted residue testing but also leads to improved treatment efficacy as a consequence of synergistic actions of individual constituents. Such changes in hormone abuse have highlighted the necessity for the development of new techniques which can improve the detection of growth promoter use during meat production. A number of

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500701838593

innovative methods at achieving this have been examined previously and have included the use of receptor concentration determination (Odore et al. 2006) and gene expression profiling (Toffolatti et al. 2006, Reiter et al. 2007).

Another strategy that has emerged recently as a possible tool to aid identification of animals which have been administered anabolic agents focuses on the detection of changes in the proteome of treated animals (Gardini et al. 2006). An extension of such an approach would be to detect perturbations in the circulating concentrations of blood components in response to drug administrations. Plasma constituents that have their levels elevated or depressed in response to treatments offer themselves as potential biomarkers of illicit hormone use. Such a blood-based approach has a number of advantages over current and proposed testing strategies. In contrast to conventional residue analysis which requires test matrices such as urine, or alternative testing procedures such as gene expression profiling which requires biological tissues, blood samples for biomarker profiling can be readily obtained from large groups of animals. In addition, hormone abuse frequently occurs throughout entire herds of cattle as individual animals are rarely hormone treated in isolation, and biomarker profiling offers the potential of cost-effective screening of a greater number of animals compared with alternative techniques.

There is sufficient information in the scientific literature to suggest biomarker profiling might be possible based on evidence that various plasma components have their circulating concentrations altered during growth spurts similar to those that follow administration of androgenic or oestrogenic compounds. Decreased urea levels have previously been suggested to be an early indicator of anabolic activity in cattle treated with various hormone regimes (Hancock & Preston 1990, Hongerholt et al. 1992, Preston et al. 1995). Creatinine and creatine kinase are metabolites connected to muscle functioning whose concentrations may respond to perturbations of normal growth patterns and associated changes in muscle mass (Heymsfield et al. 1983). Sex hormone binding globulin (SHBG) is a glycoprotein that binds and transports testosterone and oestradiol in the circulation (Petra 1991) and the administration of anabolic or oestrogenic compounds has the potential to affect circulating levels of this protein. Collagen is an important matrix protein forming a considerable portion of total body protein and consequently growth exacerbated through growth promoter use is likely to impact on collagen levels. The formation of type III collagen in soft tissues releases into the circulation aminoterminal propeptide of type III procollagen (PIIINP) which has been identified as a potential indicator of anabolic use in humans (Abellan et al. 2005).

The present study investigated the variation in the profiles of various plasma components at different time points throughout the period of treatment of calves with a growth-promotant regime typically associated with veal production. Such analysis has enabled the comparison of the concentration profiles of constituents within treated and untreated animal plasma thus addressing their suitability for use as biomarkers of illicit hormone use during beef production.

Materials and methods

Reagents

Dihydrotestosterone and Norit A charcoal were sourced from Sigma (Poole, Dorset, UK). ^{3}H-Dihydrotestosterone was obtained from Amersham Pharmacia Biotech

(Buckinghamshire, UK). Nortestosterone decanoate was sourced from Organon (OSS, the Netherlands) and 17β-oestradiol benzoate and dexamethasone were from Intervet (Boxmeer, the Netherlands). Optiphase Hi-Safe liquid scintillant was from PerkinElmer (Beaconsfield, UK). All water used in these experiments was purified using a Milli-Q Water Purification System (Millipore, Milford, MA, USA).

Veal animal study

Twenty-four crossbred (Holstein Friesian × Fries-Hollands) calves were purchased from conventional commercial farms at 14 days of age. At an average age of 10 weeks and following an acclimatization period of 7 weeks, calves were randomly divided into two control groups (male and female, $n = 6$) and two treatment groups (male and female, $n = 6$). The calves were fed milk replacer with free access to water, roughage and concentrate during the whole experiment. Calves within treatment groups received i.m. doses of 17β-oestradiol benzoate (25 mg) and nortestosterone decanoate (150 mg). This treatment was repeated twice at 14-day intervals after which treated animals received a single s.c. dose of dexamethasone (4 mg). Animals within control groups received matching volumes of peanut oil on indicated treatment days. Blood samples were taken from the anterior jugular vein using lithium heparin Vacutainer tubes (Becton Dickinson, Oxford, UK). After sampling, plasma was acquired by centrifugation at 4°C (1250*g*, 20 min) and stored at −20°C prior to analysis. Treatment and sample collection time points are illustrated in Figure 1 and animals underwent slaughter on day 42 of the study. Animals within the study had an average starting bodyweight weight of 83.5 ± 2.7 kg and changes in bodyweights of all animals were monitored on a weekly basis.

Blood metabolites

Urea concentrations were measured using an enzymatic ultraviolet (UV) method with urease and glutamate dehydrogenase on an Olympus AU600 clinical chemistry analyzer (Olympus Optical, Tokyo, Japan). Creatine kinase levels were determined by a kinetic UV test and creatinine concentrations by a kinetic colour test (Jaffé method) on the Olympus AU600 system.

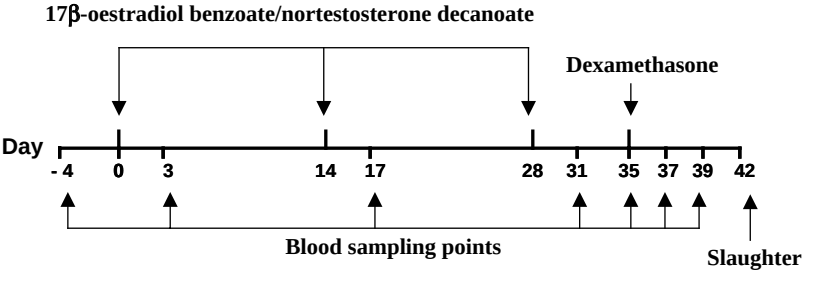

Figure 1. Treatment protocol and sampling collection for veal calf study. Calves within treatment groups received three doses of 17β-oestradiol benzoate (25 mg) and nortestosterone decanoate (150 mg) at 14-day intervals starting at day 0. At day 35 calves received a single s.c. dose of dexamethasone (4 mg). Blood samples were taken prior to treatment at day –4 and at 3-day intervals after each treatment except following dexamethasone treatment where sampling was 2 and 4 days post-treatment.

SHBG-binding capacity assay

The binding capacity of SHBG for ^{3}H-dihydrotestosterone (DHT) was determined using a ligand-binding method that relies on the use of ^{3}H-labelled DHT as tracer and dextran-coated charcoal (DCC) as previously described (Hammond & Lähteenmäki 1983). Briefly, endogenous steroids were removed from samples by 1:100 dilution of plasma with DCC. Following incubation of diluted plasma at room temperature for 30 min, samples were centrifuged (3000*g*, 10 min) and the supernatant used directly for binding assays. Assays were performed in duplicate by incubating 100 µl of 100-fold diluted plasma with either 100 µl of phosphate-buffered saline (PBS) and 100 µl of ^{3}H-DHT (100 000 cpm ml^{-1} PBS), or 100µl of DHT (2000 ng ml^{-1}) and 100 µl of ^{3}H-DHT (100 000 cpm ml^{-1} PBS). Assay tubes were incubated for 1 h at room temperature and then for 15 min at 0°C, after which ice-cold DCC (700 µl) was added to all tubes. Tubes were incubated for a further 15 min at 0°C and then centrifuged for 10 min (2000*g*, 4°C). The resulting supernatants were decanted into scintillation vials, mixed with 4 ml scintillant fluid and radioactivity counted. The SHBG-binding capacity (nmol l^{-1}) was calculated from the amount of ^{3}H-DHT bound to SHBG after correction for sample dilution and subtraction of the amount of radioactivity bound in the presence of excess unlabelled DHT.

Aminoterminal propeptide of type III procollagen assay

The concentrations of plasma PIIINP in experimental study samples were measured using a competitive intact human PIIINP radioimmunoassay kit (Orion Diagnostica, Espoo, Finland). As bovine and human PIIINP show a high degree of sequence homology (Brandt et al. 1984) antibodies produced with these antigens demonstrate high cross-reactivity (Niemela et al. 1984) enabling analysis of bovine samples using human PIIINP assays. Plasma samples were diluted 1:10 in PBS prior to analysis.

Statistical analysis

All data shown are the mean ± SEM. The differences in measured variables between control and treated groups were analyzed using unpaired *t*-test and one-way ANOVA for repeated measurements followed by Tukey's post-hoc test. Due to limited testing capacity not all assays were performed at all time points.

Results

Animal studies

Figure 2 illustrates the percentage bodyweight gain by male (A) and female (B) control and treated calves from the start of the study at day 0 until slaughter on day 42. Both male and female animals undergoing treatment were found to demonstrate an increase in bodyweight over the study period. The detected increase in bodyweight was more pronounced in females, with treated calves exhibiting faster growth and a significantly greater weight gain ($p < 0.05$) compared with matching control animals. The growth rate profiles of animals within treatment group cohorts were not altered following dexamethasone administration at day 35 (data not shown).

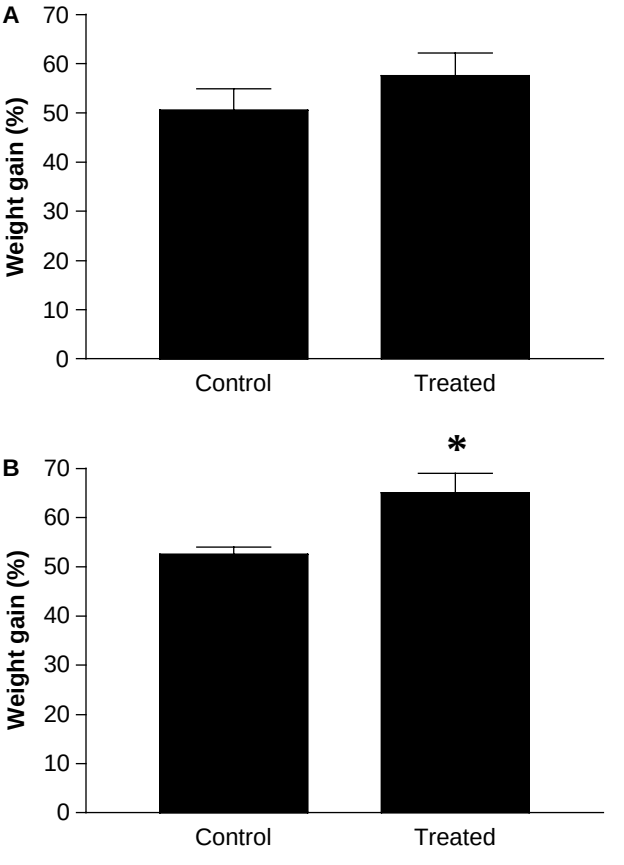

Figure 2. Percentage increase in measured live bodyweight of animals on the veal calf study from the start of the study (day 0) to slaughter (day 42) of (A) male and (B) female control and treated groups. Values shown are mean ± SEM ($n = 6$ per group). *$p < 0.05$ vs control group.

Plasma urea levels

Figure 3 illustrates the urea concentrations detected within plasma of male (A) and female (B) animals at days −4, 3, 17, 31, 35, 37 and 39. By day 3 post-administration of 17β-oestradiol benzoate and nortestosterone decanoate, significantly lower plasma urea levels were found in treated male ($p < 0.01$) and female ($p < 0.001$) calves compared with control animals. Following further anabolic administrations at days 14 and 28, urea levels were found to remain depressed in both male and female treated groups although the difference between control and treated animals was more pronounced within female animals. Dexamethasone treatment at day 35 resulted in an elevation of urea levels in both treated male and female animals with levels on day 37 been significantly increased ($p < 0.01$) relative to levels measured immediately prior to dexamethasone administration. This dexamethasone-stimulated elevation in plasma urea returned levels to those measured in matching control animals at the same time point. However, this increase in plasma urea levels was seen to be transient with urea levels returning to the depressed levels seen prior to dexamethasone administration (day 39).

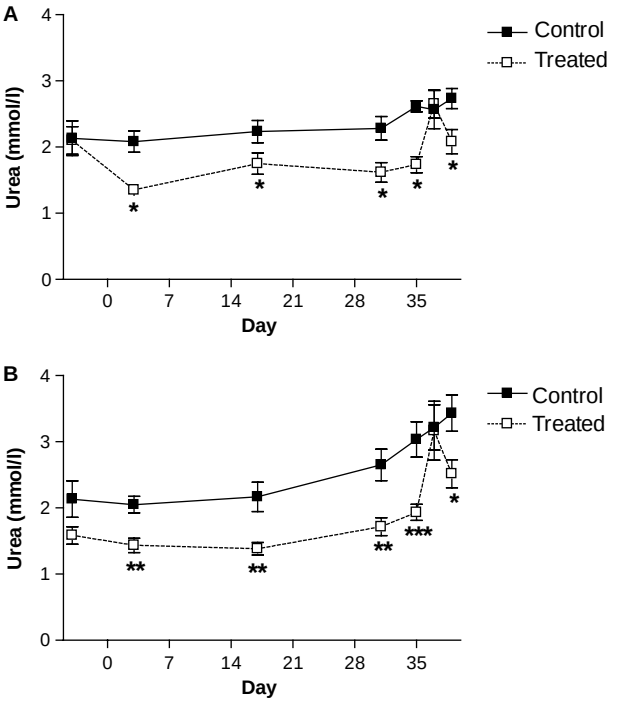

Figure 3. Urea levels of control and treated (A) male and (B) female calves. Values shown are mean ± SEM ($n = 6$ per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ vs control group.

Creatinine and creatine kinase levels

Measurement of plasma creatinine revealed baseline (day –4) levels in male (83.0 ± 1.4 μmol l^{-1}) and female (80.1 ± 1.9 μmol l^{-1}) groups to be similar. Levels of creatinine within control groups increased gradually over the course of the study reaching maximal levels (91.8 ± 3.5 μmol l^{-1}) by day 31 in male animals. Following oestradiol/nortestosterone injections creatinine concentrations within treated groups were found to be unaltered relative to levels observed in control animals. After dexamethasone treatment creatinine levels in both male and female calves were found to elevate slowly, but significant differences ($p < 0.01$) were only observed between control and treated male animals at day 39. Baseline creatine kinase levels in females (182.2 ± 9.2 U l^{-1}) were significantly ($p < 0.001$) higher than males (129.9 ± 8.2 U l^{-1}) but levels in control and treated animals of either sex did not deviate throughout the remainder of the study in response to drug administrations.

SHBG-binding capacity

Figure 4 illustrates the SHBG-binding capacity of plasma from male (A) and female (B) control and treated calves at indicated sampling points. Baseline (day –4) SHBG-binding capacity levels within females were significantly higher ($p < 0.001$) than those observed within males. During the course of the study SHBG-binding capacity levels of plasma from control and treated male animals were found to be comparable at all time points with no detectable change in levels in response to drug administration. In

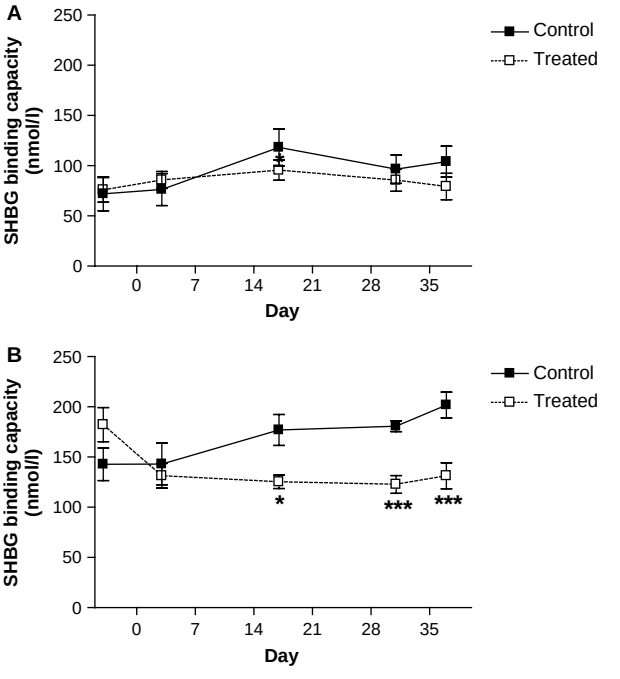

Figure 4. SHBG-binding capacity levels in control and treated (A) male and (B) female calves. Values shown are mean ± SEM ($n = 6$ per group). $^{*}p < 0.05$, $^{***}p < 0.001$ vs control group.

contrast, SHBG-binding capacities of plasma from treated female calves were significantly depressed following treatments. Calves within this group exhibited significantly lower SHBG-binding capacity levels at day 17 ($p < 0.05$), day 31 ($p < 0.001$) and day 42 ($p < 0.001$) relative to levels observed within control animals.

Plasma PIIINP levels

Figure 5 illustrates the PIIINP levels measured in plasma from male (A) and female (B) control and treated calves at indicated sampling points. Female calves were found to possess baseline PIIINP levels which were significantly higher ($p < 0.05$) than those within the male calve cohort. The profile of PIIINP levels in plasma of treated male animals was comparable to that observed within control animals and did not vary over the course of the study in response to oestradiol/nortestosterone injections. Treated male animals exhibited a tendency to have lower PIIINP levels following dexamethasone administration although this did not reach statistical significance. Compared with control animals, PIIINP levels within treated females were found to be significantly elevated in response to oestradiol/nortestosterone injections. This increase appeared to be gradual as increased PIIINP levels were not detected at day 3 following the first of the oestradiol/nortestosterone injections (day 0). Relative to control animals, increased PIIINP levels ($p < 0.05$) were detected at day 17 and day 31 in the treated female group. Dexamethasone administration at day 35 resulted in a rapid and significant ($p < 0.05$) drop in PIIINP levels within females as levels returned to baseline concentrations (day 37).

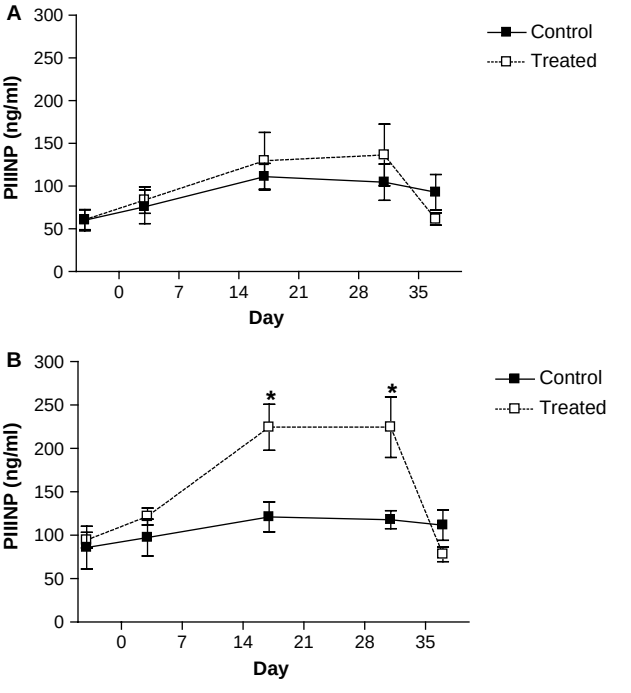

Figure 5. PIIINP levels in control and treated (A) male and (B) female calves. Values shown are mean ± SEM ($n = 4$–6 per group). $^{\star}p < 0.05$ vs control group.

Discussion

This study has demonstrated the potential of using plasma biomarkers to signal exposure of young cattle to illegal growth promoting agents. Plasma urea, in male and female calves, and SHBG and PIIINP profiles in female calves only, were significantly altered following exposure to oestradiol and nortestosterone whilst the administration of dexamethasone also resulted in perturbations to the profiles of plasma urea and PIIINP. Decreased urea levels are indicative of a sustained response to oestradiol/nortestosterone administrations and reflect an increase in the utilization of amino acids for protein deposition and a decrease in protein turnover rates. As reported previously (Istasse et al. 1989), dexamethasone administration resulted in an increase in plasma urea levels reflecting elevated catabolic activity. The effect of this agent on plasma urea proved to be transient with levels returning to previously depressed levels 2 days post-administration which is in line with the duration of activity of this drug (Liapi & Chrousos 1991).

Despite the biochemical evidence of elevated anabolic activity, there was a lack of an associated significant increase in the weight gain of treated males compared with control animals. This may be a consequence of the fact that the endocrine status of young male calves is already geared towards accelerated growth, making additional improvements to the growth rate through administration of exogenous hormones difficult to achieve. The more pronounced increase in bodyweight gain and concomitant reduction in plasma urea levels in treated females suggest that these animals were more responsive to the growth-promotant strategy employed in this study. The increase in weight of treated animals was not of as great a magnitude as

that observed in previous veal studies (Toffolatti et al. 2006), but this may be explained by the fact that these earlier studies utilized older animals which may be more responsive to treatments. There was no observed difference in plasma creatinine and creatine kinase levels between control and treated animals. The gradual increase in creatinine over time in males potentially reflects the increase in muscle mass accompanying normal growth in these immature animals.

The elevated baseline SHBG-binding capacities found in females relative to males may be reflective of the androgen-induced decline in SHBG levels which occurs in males at this stage of development (Elmlinger et al. 2005). Over the course of the study significantly lower SHBG-binding capacities were observed in treated females whilst levels in treated males were indistinguishable from those in matching control animals. Although SHBG binds both oestrogen and testosterone in humans, it appears to behave as an androgen-binding protein in other species (Kouretas et al. 1999). The fact that females are not pre-exposed to high levels of endogenous androgens and therefore possess an increased sensitivity to the androgen component of administered treatments may explain the decrease in SHBG-binding levels in these animals. The physiological control of circulating SHBG levels is thought to involve the opposing effects of androgens which lower, and oestrogens which elevate levels (Petra 1991), whilst other important SHBG regulators include other factors which play important roles in the anabolic growth process. Consequently, parenterally administered oestradiol and nortestosterone are likely to impact on SHBG levels both directly through effects on hepatic output and indirectly through effects on key regulatory factors such as insulin and insulin-like growth factor (IGF)-I (von Schoultz & Carlström 1989). Both short- and long-term use of anabolic steroids in humans has been shown to reduce SHBG levels which remain suppressed for a sustained period post-treatment (Ruokonen et al. 1985, Sinnecker & Köhler 1989). SHBG has a 7-day half-life and demonstrates little diurnal or biological variation over periods of time (Lewis et al. 2006) – attributes which contribute to SHBG's suitability as a candidate biomarker of growth-promotant use.

In this study there was an approximate doubling of serum PIIINP levels within female animals during the period of oestradiol/nortestosterone treatment whereas levels dropped immediately post-dexamethasone administration. PIIINP is released during the formation of type III collagen in soft tissue such as muscles, tendons, fibrous tissues and blood vessels. The increase in PIIINP concentrations through the effects of both growth hormone (Parssinen et al. 2000) and nandrolone decanoate (Hassager et al. 1990) on soft tissue formation has been described in humans leading to suggestions that measurements of PIIINP could be used to detect hormone abuse in sport (Abellan et al. 2005). Oestrogenic compounds have also been reported to increase serum PIIINP levels in humans but not to the same extent as androgenic agents (Hassager et al. 1990). The sharp and rapid decrease in PIIINP levels following dexamethasone administration has been reported in humans (Saarela et al. 2003) and is attributed to a glucocorticoid-induced suppression of type III collagen synthesis.

Although additional work will be required not only to identify further biomarkers but also to determine the normal concentration range of biomarkers in animals of various breeds and age so as to enable accurate identification of suspect profiles, this small controlled study has provided evidence of the potential of such an approach. The major advantage of a biomarker-based detection method which focuses on altered plasma component profiles is that blood for such analysis can be readily obtained at

any point throughout the animal rearing process whilst such a system may be automated enabling high-throughput screening. This is in contrast to the current situation where matrices for residue analysis can be acquired with ease only at the point of slaughter with only a limited number of samples proceeding through laborious analysis. Biomarkers suitable for profiling would ideally have minimal day-to-day or diurnal variations under normal conditions and exhibit a low basal concentration spread within the herd population as a whole. Although such parameters may be hard to achieve using a single biomarker, the use of a panel of biomarkers such as urea, SHBG and PIIINP which represent a wide spectrum of *in vivo* metabolic activities, offers greater sensitivity to detect growth promoter abuse. The use of multiple biomarkers also potentially offers the opportunity for the identification of the type of growth promoting agent administered. Contrasting biomarker responses observed following oestradiol/nortestosterone and glucocorticoid administrations suggest distinct biomarker profiles could be used to distinguish between different compound classes and drug combinations.

In summary, this study has demonstrated the potential of plasma biomarker profiling to signal growth-promotant use during beef production. Testing based on such methodology raises the possibility of improving detection efficiencies through increased sample throughput thus aiding identification of suspect herds.

Acknowledgements

This work was financially supported by grants from the European Commission project 'New technologies to screen multiple chemical contaminants in foods' (project acronym BioCop, contract FOOD-CT-2004-06988) and *Safe*food Ireland (project 04CR/05).

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