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Metabolomics Approach to Anabolic Steroid Urine Profiling of Bovines Treated with Prohormones

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In livestock production, illegal use of natural steroids is hard to prove because metabolites are either unknown or not significantly above highly fluctuating endogenous levels. In this work we outlined for the first time a metabolomics based strategy for anabolic steroid urine profiling. Urine profiles of controls and bovines treated with the prohormones dehydroepiandrosterone (DHEA) and pregnenolone were analyzed with ultraperformance liquid chromatography in combination with time-of-flight accurate mass spectrometry (UPLC−TOFMS). The obtained full scan urinary profiles were compared using sophisticated preprocessing and alignment software (MetAlign) and multivariate statistics, revealing hundreds of mass signals which were differential between untreated control and prohormone-treated animals. Moreover, statistical testing of the individual accurate mass signals showed that several mass peak loadings could be used as biomarkers for DHEA and pregnenolone abuse. In addition, accurate mass derived elemental composition analysis and verification by standards or Orbitrap mass spectrometry demonstrated that the observed differential masses are most likely steroid phase I and glucuronide metabolites excreted as a direct result from the DHEA and pregnenolone administration, thus underlining the relevance of the findings from this untargeted metabolomics approach. It is envisaged that this approach can be used as a holistic screening tool for anabolic steroid abuse in bovines and possibly in sports doping as well.

In livestock production, growth promoters are used to improve growth rates, feed conversion efficiency, and lean/fat ratios ultimately resulting in economical benefits for cattle fatteners. In contrast to regulations in, e.g., the U.S.A. and Australia, the use of all hormonal growth promoting substances is prohibited within the European Union.[1] To comply with this ban, mandatory monitoring and surveillance programs, based on screening and confirmation concepts, are implemented at a national level.[2] In order to circumvent regulations certain farmers are continuously in search for new growth promoting substances, such as prohormones, of which misuse in cattle fattening is hard to prove. Prohormonal substances do not exhibit hormonal action by themselves, however they are precursors of bioactive steroid hormones. The main precursor of all natural sex steroid hormones, androgens as well as estrogens, is dehydroepiandrosterone (DHEA).[3] In vivo synthesis of DHEA occurs mainly in the adrenal gland where side chain cleavage of cholesterol results in pregnenolone which is metabolized by P450 17α-hydroxylase (P450c17) into DHEA. The compound DHEA itself was not found to exhibit direct androgenic action;[4] however, conversion by peripheral tissues under 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activity is yielding more potent androgens like testosterone (Figure 1).

Routine urine screening is largely performed by using gas chromatography (GC) or liquid chromatography (LC) combined with mass spectrometry (MS).[5] In order to obtain sufficient sensitivity and specificity, GC/MS and LC−MS/MS screening methods are in general based on monitoring of a limited number of ions or MS/MS transitions of known compounds. However, application of these targeted methods do not detect new unknown anabolic steroids or compounds which are absent in the preselected list of target analytes. Moreover, there is a chance of missing abuse of natural compounds, like pregnenolone and DHEA, which might not be significantly above highly fluctuating endogenous levels due to extensive metabolism.

For urine screening including detection of new designer steroids, several more comprehensive screening concepts have been developed. Thevis et al.[6] proposed an LC−MS/MS screening protocol based on the fact that steroids with (partially) common structures show similar product ions, which can be monitored by precursor ion scan acquisition. This idea has been refined by Pozo

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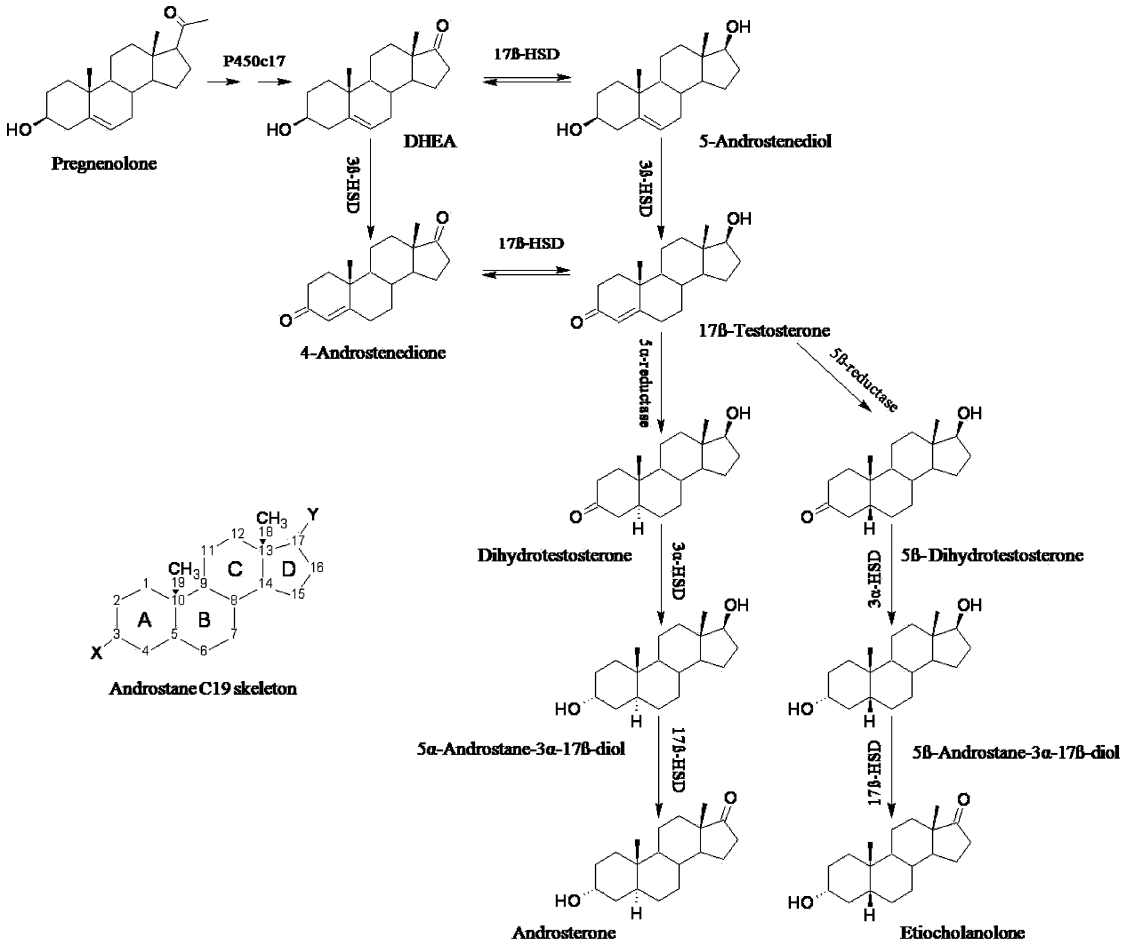

Figure 1. In vivo androgen biosynthesis: pregnenolone is converted into DHEA under P450c17 activity; subsequently 17β-testosterone is formed via 5-androstenediol or 4-androstenedione due to 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme activity; and 17β-testosterone is converted into unsaturated metabolites under 5α- and β-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD), and 17βHSD activity.

et al.[7] who stated that the combined acquisition of the precursor ion scan of m/z 105, m/z 91, and m/z 77 might be applicable as a screening protocol for most anabolic steroids. Another concept for screening is implemented by using a yeast androgen bioassay for screening calve urine on androgenic bioactivity.[8] In addition, that bioassay was successfully used as an offline LC detector followed by LC−QTOF identification for screening urine on synthetic or unknown designer steroids such as tetrahydrogestrinone (THG).[9]

In human antidoping control, steroid profiling has proven its usefulness by comparing levels and ratios of endogenous produced steroids in urine.[10] Significant variations of endogenous steroid levels and ratios are observed after administration of (pro)hormones[11] including alterations as a consequence of DHEA administration.[12,13] For screening on DHEA abuse in humans, threshold values of 200 ng/mL have been proposed for both DHEA and the DHEA-metabolite 3α,5-cyclo-5α-androstane-6β-ol-7-one.[14] However, because of large differences in metabolism and excretion, a steroid profiling approach using parameters that proved its value in humans, such as testosterone/epitestosterone (T/E) ratios, does not seem to be feasible in cattle.[15]

Recent developments in LC−MS and bioinformatics allow untargeted and unbiased urine profiling approaches which can be adopted from metabolomics research.[16] Ultraperformance liquid chromatography (UPLC)[17] combined with full-scan high-resolution MS such as time-of-flight (TOF) and Fourier transform (FT) ion cyclotron resonance or Orbitrap MS allows more complete chemical profiles of complex biological samples like urine. In addition, the mass accuracy provided by TOFMS and Fourier transform mass spectrometry (FTMS) allows calculation of elemental compositions.[18] Recent performed work by Werner et al.[19] combined UPLC with TOFMS to analyze urine samples from rats treated with phenobarbital. Subsequent comparison of urinary profiles from treated and untreated rats under well-defined laboratory conditions resulted in identification of 14 phenobarbital metabolites not previously reported.

The aim of the present work was to develop a metabolomics based screening strategy for prohormone abuse in real-life urine samples from farm bovines. An untargeted approach is used for

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Table 1. Experimental Setup, Age, and Weights of Bovines Included in the DHEA and Pregnenolone Animal Treatment Experiments

experiment	no. of treated animals (age and weight)	no. of control animals (age and weight)
DHEA no. 1	1 PO and 1 IM (8−9.5 months, 253−290 kg)	3 (6 months, 153−174 kg)
DHEA no. 2	1 PO and 1 IM (8,5−9 months, 253−290 kg)	1 (8.5 months, 275 kg)
DHEA no. 3	1 PO and 1 IM (12.5−13.5 months, 355−410 kg)	2 (13.5−14 months, 350−386 kg)
pregnenolone	4 PO (7.5− 10.5 months, 190−215 kg)	4 (8−9.5 months, 195−240 kg)

detection of differentially accumulating metabolites as a consequence of treatment with the prohormones pregnenolone and DHEA. Urine samples were analyzed by UPLC−TOFMS with the aim to obtain constant and reproducible results, leading to detection of relevant metabolites. Within this context, the a priori focus during development was to ensure method applicability at least for phase I and phase II glucuronide metabolites of steroid hormones but of course also other nonsteroidal metabolites might be picked up. Urine profiles generated by UPLC−TOFMS were processed by in-house developed MetAlign software.[20,21] Through data reduction and alignment, complex chemical profiles were used for various comparisons and searches. Data were analyzed using multivariate statistics followed by identification of signals differential in urine of prohormone-treated versus untreated animals. The mass peak loadings obtained by this untargeted approach were statistically tested for its biomarker potential for DHEA and pregnenolone misuse in bovines. Finally, potential biomarkers were identified based on accurate mass derived elemental composition and retention time comparison with commercially available standards or by LC−LTQ-Orbitrap tandem MS.

EXPERIMENTAL SECTION

Chemicals. DHEA was obtained from Sigma (St. Louis, MO) and was dissolved in Miglyol 812 (Certa SA, Braine-l'Alleud, Belgium) for intramuscular injection. Testosterone-d_3 was purchased from NMI (Pymble, Australia) and testosterone-d_3-glucuronide from NARL reference materials (Pymble, Australia). Pregnenolone and all other steroidal compounds used were obtained from Steraloids (Newport, RI). Acetic acid, formic acid, ammonia, and sodium actetate were of analytical grade and obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Milli-Q-water was purified using a Millipore Milli-Q system (Bedford, MA).

Animals, Treatments, and Urine Sampling. In a time span of $1^1/_2$ years, three independent bovine DHEA treatment experiments were performed using identical treatment and sampling schedules. For obvious ethical reasons it was chosen to perform three small scale treatment experiments, hereby deliberately including inherent biological variations like differences in, e.g., age, origin, nutrition, and disease history. Male Frisian bovines were purchased at the local market and housed for 2−3 weeks before the start of each experiment. Each of the three experiments consisted of two animals of which one was orally (PO) administered capsules containing 1000 mg of DHEA and the other was injected intramuscularly (IM) with 1000 mg of DHEA dissolved in 10 mL of Miglyol 812. Untreated control animals were included in all three experiments, respectively, three animals in the first, one in the second, and two in the third experiment. The pregnenolone experiment consisted of four control animals and four animals which were treated orally with capsules containing 500 mg of pregnenolone. For the DHEA as well as the pregnenolone trial, repeated dose administrations were performed seven times at 24 h intervals. An overview of the experimental setup, including the age and weights of the animals, is shown in Table 1. Before the start of the treatment urine collections were made, and during the animal trials urine was sampled at days 2, 5, and 7 between 08.00 and 17.00 h. The animal study was approved by the Ethical Committee of Ghent University and performed in agreement with local ethical requirements.

Sample Preparation. Prior to sample preparation, 5 mL aliquots of each urine sample were lyophilized to determine the dry weights. Next, the volume of nonlyophilized urine aliquots were normalized to 40 mg/mL dry weight by the addition of 0.11−5.83 mL of Milli-Q water. Aliquots of 3 mL were fortified with 20 μL of internal standard (1.5 ng/μL testosterone-d_3 and testosterone-d_3-glucuronide in methanol). Samples were prepared in triplicate on separate days, and if a sample contained less than 40 mg/mL dry residue, a larger sample volume representing 120 mg dry weight was subjected to the following solid phase extraction (SPE) cleanup procedure. To each sample 3 mL of sodium acetate (0.25 M, pH 4.8) was added, and the pH was adjusted to 5.0 ± 0.3 with 4 M acetic acid if necessary. Urine samples were then applied on a reversed phase SPE cartridge (Phenomenex Strata X, 200 mg, 33 μm, 6 mL), previously activated with 12 mL of methanol and 6 mL of Milli-Q water. The cartridge was washed with 6 mL of 0.17 M acetic acid in methanol/water (40:60 v/v) and 6 mL of 0.13 M ammonia in methanol/water (20:80 v/v), dried under vacuum, and eluted with 6 mL of methanol. The SPE eluent was

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evaporated at 45 °C under a gentle stream of nitrogen and reconstituted in 100 μL of methanol followed by adding 400 μL of mobile phase A. Before injection, samples were centrifuged for 10 min at 2000*g*. To include between-day variation, each of the replicates was analyzed in a different measurement series.

Ultraperformance Liquid Chromatography Coupled to Time-of-Flight Mass Spectrometry. Ultraperformance liquid chromatography was performed on a Waters (Milford, MA) Acquity system equipped with a Waters Acquity BEH C_{18} column (150 mm × 2.1 mm i.d., 1.7 μm) which was kept in a column oven at 50 °C. The injection volume was 25 μL, and the mobile phases consisted of (A) 20 mM formic acid in water and (B) 20 mM formic acid in water/acetonitrile (10/90 v/v) at a flow rate of 0.4 mL/min. An isocratic period of 1 min at 100% A was followed by a linear change from 0 to 20% B in 2 min, 20 to 70% B in 20 min, and 70 to 100% B in 2 min. Next, the gradient remained 10 min at 100% B and returned linearly in 1 min to 100% A, remaining at this level for 4 min until the next injection.

The UPLC was directly interfaced with a Waters LCT Premier mass spectrometer equipped with a dual electrospray ionization probe operating in the positive mode (ESI+). The source temperature was set at 120 °C, the desolvation temperature at 400 °C, the capillary voltage at 2500 V, and the cone voltage at 50 V. The cone and desolvation gas flow were 50 and 500 L/h, respectively. A lock mass calibrant of leucine-enkephalin (1 ng/μL) in water/acetonitrile (67:33 v/v) was continuously introduced in the mass spectrometer via the second ESI probe (Lockspray) at a flow rate of 20 μL/min. Data were acquired between *m/z* 80−1000 and processed further in MassLynx 4.1 software (Waters).

Ultraperformance Liquid Chromatography Coupled to LTQ Orbitrap Mass Spectrometry. Identification of a pregnenolone metabolite was carried out on a Thermo Fisher Scientific (San Jose, CA) Accela series U-HPLC system using the same column and identical elution conditions as used in the UPLC−TOFMS experiments, only the injection volume was changed to 20 μL. The LC system was directly coupled to a LTQ Orbitrap XL (Thermo Fisher Scientific) mass spectrometer equipped with an electrospray ionization probe operated in the positive ion mode. The electrospray voltage was 4000 V, capillary temperature 250 °C, sheath and auxiliary gas flow of 40 and 20 arbitrary units, respectively. Precursor ions were isolated in the linear ion trap (LTQ) section at a width of 2.0 *m/z* and collisionally dissociated. Dissociation of *m/z* 317.3 was carried out at a normalized collision energy of 40% and scan ranges were *m/z* 85−400. In the case of *m/z* 285.3, the collision energy was 30% for scan event 1 and 50% for scan event 2, scan ranges were *m/z* 85−350. Data were recorded and processed using Xcalibur software (Thermo Fisher Scientific).

Processing of Data Files. UPLC−TOFMS data generated in MassLynx format were directly imported in an accurate mass version of MetAlign.[20,21] Basically this software performs a baseline correction, accurate mass calculation, data smoothing and noise reduction, followed by alignment between chromatograms, generating data files which are 100−1000 times reduced in size. Next, data were imported in GeneMaths XT (Applied Maths, St. Martens-Latem, Belgium) and ^{2}log transformed. A one-way analysis of variance (ANOVA, $p < 0.01$) with Bonferroni correction was performed to test for differences between groups, days, and routes of administration. To visualize these differences, principal component analysis (PCA) was performed and ANOVA selected mass peak loadings were exported in txt-format to Excel. In search for robust potential biomarkers, additional selection criteria were applied. Fold changes were calculated by comparing the mean of all samples from treated animals versus the mean of all controls. For the DHEA and pregnenolone experiment, mass peaks with respectively a 10- and 5-fold change were selected. Mass peak loadings fulfilling this criteria but with a mean treated signal lower than 200 counts were considered too close to background noise and therefore removed from the selection. Subsequently data were mean centered and hierarchical clustering was done using Cluster and Treeview software.[22] Each of the selected mass peak loadings were evaluated using univariate statistics to determine if they can be used as a biomarker for prohormone detection. A detailed description of univariate modeling can be found in the Supporting Information.

RESULTS AND DISCUSSION

Quality of Analytical Data. Urine samples were analyzed in triplicate, distributing each replicate in random order in a different analysis series. In total, three series containing 109−113 samples each were analyzed by UPLC−TOFMS during a time span of 3 weeks. These series included 15−20 urine samples not belonging to the DHEA and pregnenolone treatment experiments and therefore not considered further in this paper. After every 20 samples, a mixed urine sample was injected to check for consistency during analysis. In addition, each urine aliquot was spiked before SPE cleanup with 30 ng of testosterone-d_3 and testosterone-d_3-glucuronide internal standard. This allowed assessment of retention time stability, consistency of signal intensities, and mass accuracy within and between measurement series. Normalized and ^{2}log transformed mass amplitudes of testosterone-d_3 and testosterone-d_3-glucuronide during measurement series are shown in Figure 2. For testosterone-d_3, differences in mass amplitudes varied between a −2.02 and 1.60-fold change. Mass amplitudes of testosterone-d_3-glucuronide showed higher variability and ranged between a −3.54 and 2.07-fold change. Although some fluctuations are observed, the UPLC−TOFMS system is considered extremely stable: most mass amplitude fluctuations are within a factor 2 without showing up- or down-going trends during the analysis series.

Mass errors of the observed MetAlign calculated accurate mass of testosterone-d_3 and testosterone-d_3-glucuronide were in general below 10 ppm (Figure S-1 in the Supporting Information). Again testosterone-d_3-glucuronide displayed higher variability throughout all measurement series, where testosterone-d_3 showed only in the first analysis series a few outliers above 10 ppm mass error. Overall, it is concluded that the applied full scan analysis of urine samples with high-resolution UPLC−TOF mass spectrometry revealed highly stable and reproducible results.

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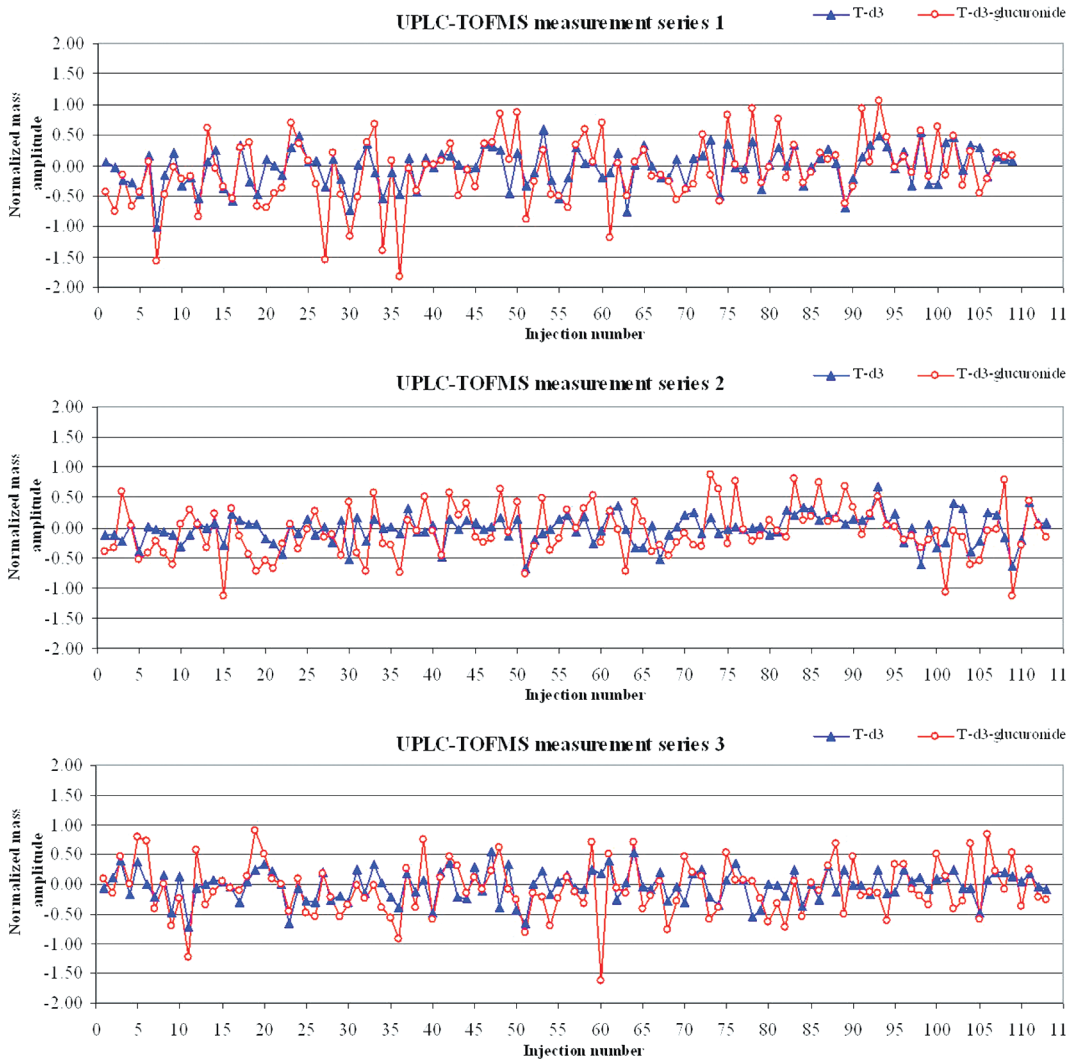

Figure 2. Normalized and ^{2}log transformed accurate mass amplitudes of deuterium labeled internal standards, testosterone-d_3 and testosterone-d_3-glucuronide (30 ng spike added to urine samples before the SPE procedure) observed during UPLC−TOFMS measurements.

Data Processing and Selection of Potential Biomarkers. For the DHEA treatment experiment, aligned UPLC−TOFMS data of all samples were compared by one-way ANOVA ($p < 0.01$) with Bonferroni correction in order to correct for multiple testing. Supervised principal component analysis was applied on the output ($n = 1565$ mass peak loadings) to visualize differences between urine obtained from the control and DHEA treated animals. The projection of the three largest principal components, which represent 44% of the total variance, is shown in Figure 3. Control and treated groups are mainly separated on the *x*-axis. Whereas urine samples from IM and PO treated animals show separation on the *y*-axis. However, potential application in control and enforcement programs is performed without a-priori knowledge about the route of administration. As a result, it is more relevant and desirable to determine potential biomarkers for DHEA treatment independent from the route of administration. The 1565 mass signals obtained after ANOVA were converted back in a Masslynx format. A comparison of urine profiles in total ion chromatogram (TIC) format displaying the average peak amplitude of the controls (A) versus the DHEA treated animals (B) for each of the 1565 selected mass signals is shown in Figure 4. Huge differences are observed between these profiles and because many signals are also observed at lower levels in urine from control animals, they most likely relate to endogenous metabolites, in accordance with expectations following administration of naturally occurring prohormones like DHEA.

In order to identify the most abundant and robust biomarkers, ions with a fold change greater than 10 were selected by comparing the mean of all controls versus the mean of all samples originating from DHEA treatment. In addition, the mean signal of ions following DHEA treatment must exceed 200 counts in order to obtain measurable signals. In total, 180 mass peak loadings (listed in Table S-1 in the Supporting Information) met these additional criteria and together with the top 10 of mass peak loadings found down-regulated, hierarchical clustering (HCA) was performed. First, data were converted in ^{2}log mean centered values followed by HCA on mass peak loadings only. Results of HCA are presented in Figure 4C, where >16 times regulation gets a maximal red or green intensity, representing respectively up- and down-regulation versus the signal average of all samples. The HCA-plot visualizes the presence of discriminating ions which are present in urine originating from both IM and PO DHEA treated animals (area A in Figure 4C). An increase in signal intensity of DHEA IM urine samples at days 2, 5, and 7 is

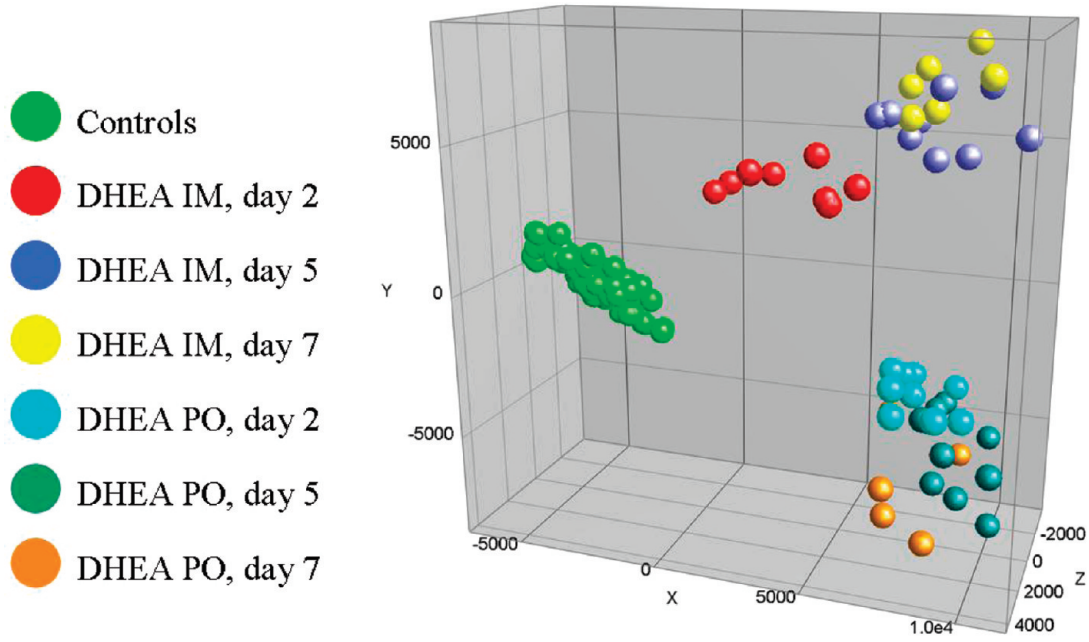

Figure 3. PCA plot of urine samples from control animals (green) and DHEA treated animals (IM and PO at days 2, 5, and 7) after UPLC−TOFMS measurement and ANOVA (p value < 0.01) with Bonferroni correction.

observed. This most likely indicates that repeated treatment of animals with DHEA results in accumulation of metabolites in urine. In Figure 4C, area B, a cluster of ions is shown which are not differentially expressed at day 2, however, showing abundant discriminating signals at days 5 and 7. Mass peak loadings characteristic for DHEA treatment per PO are found in area C, again observing an increasing trend during treatment. No signals were meeting the additional criteria for down regulation (>10-fold change), nevertheless the top 10 (area D) has been included in the HCA of Figure 4C, showing regulation from −1.85 to −7.14.

For selection of potential biomarkers for pregnenolone abuse, a strategy similar to the one followed in the DHEA treatment experiment was applied. Again, data were aligned and ANOVA with Bonferonni correction was performed. Next, mass peak loadings with a p value < 0.01 and a fold change > 5 were selected (n = 163) and applied to HCA (Figure 5). Highly variable signals among samples are observed in area A of the HCA plot, where more robust discriminating markers are found in area B (listed in Table S-2 in the Supporting Information). Moreover, 16 signals were observed to be down regulated >5 times in urine from pregnenolone treated animals (area C in Figure 5 and listed in Table S-3 in the Supporting Information).

Univariate Statistical Analysis. Each of the selected mass peaks, obtained as described in the data processing section (180 for DHEA and 163 for pregnenolone), were evaluated individually in order to determine if they can be used as a biomarker for prohormone detection. For DHEA, all control samples from this experiment are used to estimate the probability density functions of each of the 180 selected mass peaks. In addition, all samples from the DHEA treated bovines are used to determine the number of false negatives. As an example, the probability density function of m/z 255.2078 (RT = 10.98 min) is presented in Figure S-2 in the Supporting Information. The same methodology was applied to pregnenolone data. The number of mass peak loadings yielding no or only a limited number of false negatives for prohormone treatment versus the controls is presented in Table 2. Corresponding mass peak loading for DHEA and pregnenolone are listed in Tables S-4 and S-5 in the Supporting Information, respectively. For screening purposes, a false negative rate of 5% is considered acceptable according to Commission Decision 2002/657/EC.[23] Already seven mass peak loadings from the DHEA treatment comply with this Decision.

From the DHEA and pregnenolone experiment, subsets of mass peaks were selected and tested for false positives using an independent control test set, i.e., respectively, the control samples from the pregnenolone and DHEA experiments. Again the number of false positives was observed to be very low, see the results presented in Table S-6 in the Supporting Information. Thus a good classification is possible for urines from treated and control bovines. It should be noted, however, that although the inherent biological variability was deliberately high, the number of bovines in both groups is still rather small. The reduction of eq 1 to eq 2 as described in the univariate statistical analysis section of the Supporting Information has larger validity for larger sample sizes. Therefore, larger groups are needed to improve the distributions, resulting in more accurate estimations of the number of false positives and false negatives.

Initial Identification of Steroid Related Candidate Biomarkers. Multivariate assigned mass peak loadings responsible for segregation between control and treated animals should be identified. Although complete identification cannot be performed solely on the basis of MS data, the accurate mass signals are very useful for initial identification purposes. Structural characteristics of natural occurring anabolic steroids generally consist of a saturated (androstane) or unsaturated (androstene) skeleton with hydroxyl and/or oxo-groups attached at the 3 and 17-positions (Figure 1). Although large similarities in structures are observed, their electrospray ionization behavior can be completely different.

(23) Council directive 2002/657/EC. *Off. J. Eur. Communities*, **2002**, *L221*, 8.

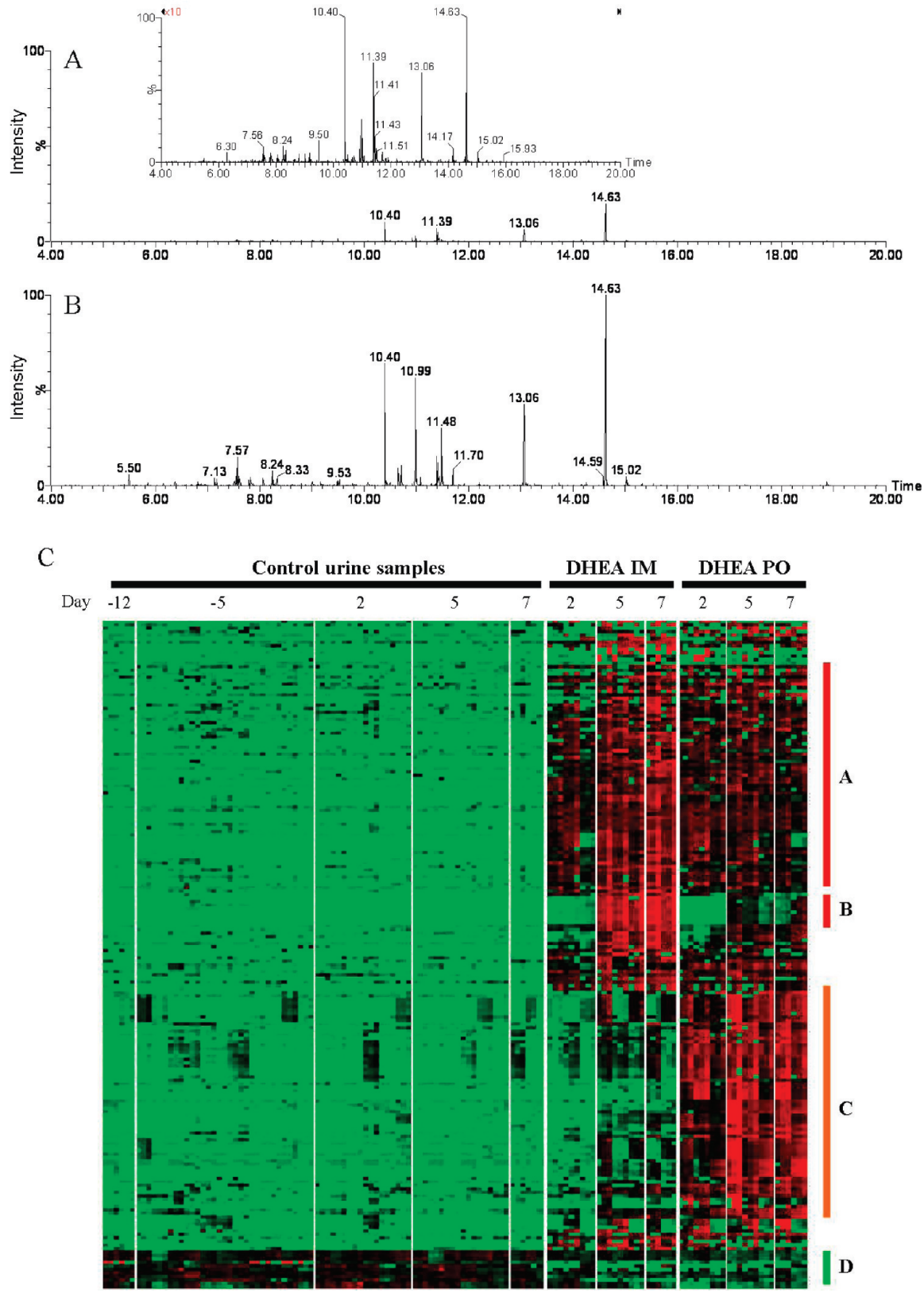

Figure 4. Mass peak loadings with a p value < 0.01 after ANOVA with Bonferroni correction, back converted to total ion chromatogram (TIC) data. Shown is (A) the average of all controls and (B) the average of the DHEA treated group. The insert at chromatogram A shows a 10 times magnification between 4 and 20 min. (C) Hierarchical clustering of ^{2}log transformed and mean centered mass peak loadings showing more than a 10-fold up-regulation. Additionally, the top 10 of mass peak loadings, which are down-regulated is shown (area D). Fold changes were obtained by comparing mean signals of all urines originating from DHEA treated animals versus the mean of all control urines.

In positive ionization mode, it is observed that apart from $[M + H]^+$ ions also abundant $[M + H - H_2O]^+$ or $[M + H - 2H_2O]^+$ ions are formed. This is highly dependent on the groups attached at the C3 and C17 positions and the presence and

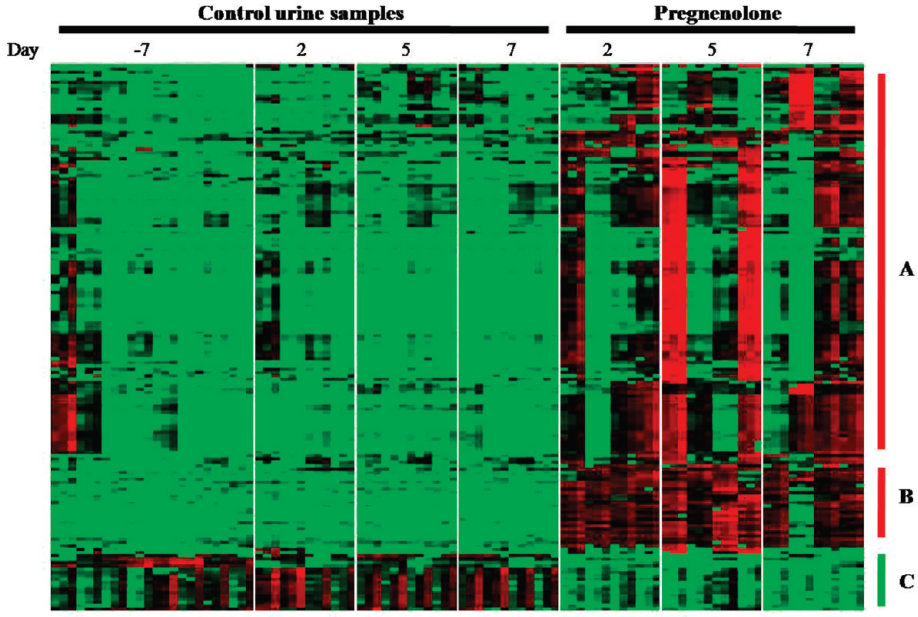

Figure 5. Hierarchical cluster analysis of ^{2}log transformed and mean centered mass peak loadings showing more than a 5-fold increase (areas A and B) or decrease (area C) in signal. Fold changes were obtained by comparing mean signals of all urines originating from pregnenolone treated animals versus the mean of all control urines.

Table 2. Number of Mass Peak Loadings with 0 to 7 False Negatives Observed in Urine Samples Originating from DHEA (n = 6) and Pregnenolone (n = 4) Treated Bovines Compared to Their Corresponding Control Population

DHEA		pregnenolone	
false negatives[a]	no. of mass peak loadings (p = 180)	false negatives[a]	no. of mass peak loadings (p = 163)
0	3	0	0
1 (2%)	1	1 (3%)	0
2 (4%)	3	2 (6%)	0
3 (6%)	4	3 (8%)	3
4 (8%)	2	4 (11%)	3
5 (10%)	2	5 (14%)	0
6 (13%)	3	6 (17%)	1
7 (15%)	2	7 (19%)	2
≫	160	≫	154

[a] The number of false negatives are within the parentheses as the percentage relative to the total number of DHEA (n = 48) and pregnenolone (n = 36) treated urine samples, respectively.

position (C4−C5 or C5−C6 configuration) of the double bond.[24] Moreover phase I and phase II metabolism can cause steroid hydroxylation, oxidation, reduction, and glucuronidation. With administration of DHEA, a minimum of C19 for phase I and C25 for phase II glucuronides is expected for steroid metabolites in urine. Typical elemental compositions of these steroid ions are listed in Table 3. Taking into account losses of water, adduct formation, and combinations thereof, the elemental composition of most of the mass peak loadings listed in Table S-1 in the Supporting Information can be directly linked to steroidal structures. Because of in-source fragmentation and adduct formation, a single compound can produce several m/z signals at the same retention time. Combined analysis of the obtained differential mass peak loadings can therefore provide insight into the identity of the differential regulated urinary metabolites. For example, multivariate analysis yielded several signals at retention time 13.06 min (Figure 6). The most abundant ion is observed at m/z 484.2903 ($C_{25}H_{42}NO_8$). This is probably a NH_4^+ adduct of the compound, explaining the fragment and adduct ions observed at 489.2457 $[M + Na]^+$, m/z 431.2408 $[M + H - 2H_2O]$, m/z 291.2301 $[M + H - Gluc]^+$, m/z 273.2224 $[M + H - Gluc - H_2O]^+$, and m/z 255.2145 $[M + H - Gluc - 2H_2O]^+$. Note that the ion observed at m/z 660.3133 is probably the result of in-source adduct formation yielding a $[M + NH_4 + Gluc]^+$ ion and not a diglucuronide (which would be expected at a shorter retention time). Together, this suggests a molecule with a mass of 466 which could be a glucuronide of androstane-ol-one or androstenediol (Table 3). Relative retention time comparison of commercially available standards versus the deuterium labeled internal standards revealed that this compound is most likely etiocholanolone glucuronide. This is in accordance with literature stating that ionization of etiocholanolone-glucuronide is yielding mainly $[M + NH_4]^+$ and $[M + Na]^+$ ions due to lower proton affinity as compared to some androstene glucuronides.[25] Etiocholanolone is a relevant urinary metabolite known to originate from (exogenous) DHEA[26] and used as a parameter in steroid urine profiling.[11] Using intact steroid glucuronide metabolites in LC−MS/MS routine screening was proven to be an effective targeted analysis method.[27] Similarly, other potential biomarkers can be assigned, although the limited availability of steroidglucuronide standards is an obstacle for full confirmation of the hypothesized identity.

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Table 3. Elemental Compositions and Theoretical Accurate Masses of Some Androstane and Androstene Steroid Related Ions

	$[M + H - 2H_2O]^+$	$[M + H - H_2O]^+$	$[M + H]^+$	$[(M + O) + H]^+$	$[(M + Gluc) + H]^+$
androstene-x-ol-y-one[a]	$C_{19}H_{25}$	$C_{19}H_{27}O$	$C_{19}H_{29}O_2$	$C_{19}H_{29}O_3$	$C_{25}H_{37}O_8$
	253.1956	271.2062	289.2168	305.2117	465.2488
androstenediol	$C_{19}H_{27}$	$C_{19}H_{29}O$	$C_{19}H_{31}O_2$	$C_{19}H_{31}O_3$	$C_{25}H_{39}O_8$
	255.2113	273.2218	291.2324	307.2273	467.2645
androstenedione	$C_{19}H_{23}$	$C_{19}H_{25}O$	$C_{19}H_{27}O_2$	$C_{19}H_{27}O_3$	$C_{25}H_{35}O_8$
	251.1800	269.1905	287.2011	303.1960	463.2332
androstane-x-ol-y-one[a]	$C_{19}H_{27}$	$C_{19}H_{29}O$	$C_{19}H_{31}O_2$	$C_{19}H_{31}O_3$	$C_{25}H_{39}O_8$
	255.2113	273.2218	291.2324	307.2273	467.2645
androstanediol	$C_{19}H_{29}$	$C_{19}H_{31}O$	$C_{19}H_{33}O_2$	$C_{19}H_{33}O_3$	$C_{25}H_{41}O_8$
	257.2269	275.2375	293.2481	309.2430	469.2801
androstanedione	$C_{19}H_{25}$	$C_{19}H_{27}O$	$C_{19}H_{29}O_2$	$C_{19}H_{29}O_3$	$C_{25}H_{37}O_8$
	253.1956	271.2062	289.2168	305.2117	465.2488

[a] x and y are denoting the position (C3 or C17) of the keto- as well as the hydroxygroup, of which the latter could be in the α- or β-configuration.

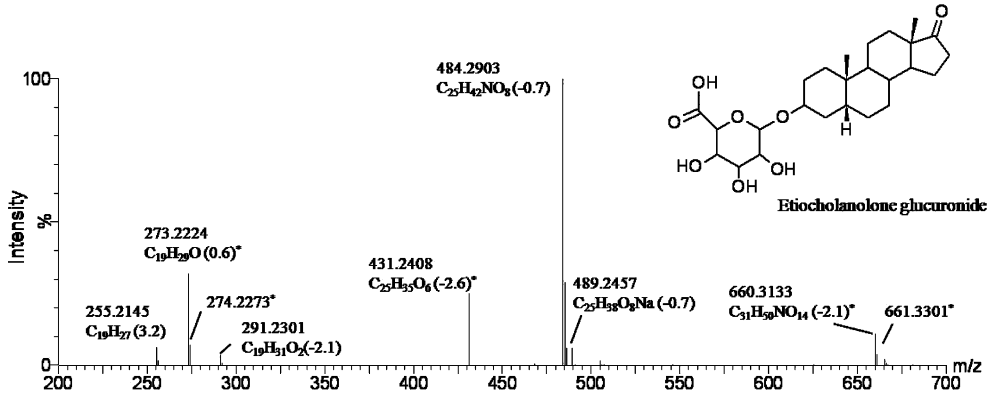

Figure 6. Differential mass peak loadings (p value < 0.01 after ANOVA) obtained at retention time 13.06 min (combining scans 1543−1545). Signals showing >10-fold change compared to the mean of all controls are marked with an asterisk. Accurate mass values are the averages from aligned peaks of all urine samples. The mass error (in millidaltons) versus the theoretical mass of the displayed elemental composition is shown in parentheses. As confirmed by retention time and spectral comparison, this biomarker is most likely etiocholanolone glucuronide.

LC−LTQ-Orbitrap Identification. A major candidate biomarker ion responsible for group separation in the pregnenolone experiment is m/z 285.3, eluting at retention time 14.35 min in the UPLC−TOFMS experiments. The possible identity of the molecule yielding this ion is not obvious from Table 3. Therefore this biomarker was characterized with LC−LTQ-Orbitrap tandem MS, showing a retention time shift of 2.1 min compared to the UPLC−TOFMS analysis. Figure 7A shows the LTQ-Orbitrap full scan mass spectrum of a urine sample originating from a pregnenolone treated animal at retention time 16.47 min. The differentially regulated ion at m/z 285.25847 ($C_{21}H_{33}$) is a fragment ion probably originating from m/z 497.311 52 ($C_{27}H_{45}O_8$) due to neutral losses of water, a glucuronide moiety (resulting in $C_{21}H_{34}O$ at m/z 303.269 11), and another water. LTQ-Orbitrap MS/MS analysis of m/z 285 (Figure 7B) shows no fragment ions containing an oxygen atom. The fragmentation pattern is consistent with a precursor ion having hardly any favorable carbon atom for carrying the positive charge. A pregnane or androstane skeleton would be an obvious hypothesis. Although the LTQ-Orbitrap MS/MS findings cannot be confirmed by the lack of available standards, they support the hypothesis of a glucuronide of 3,20-dihydroxy-pregnane.

CONCLUSIONS

The present work has outlined a novel untargeted metabolomics based strategy for anabolic steroid urine profiling in the field of livestock production. Results show that full scan high-resolution UPLC−TOFMS analysis of bovine urine samples generated stable and reproducible profiles. Subsequent accurate mass data alignment combined with multivariate statistical analysis allowed comparison of urinary profiles and highlighted mass peak loadings differentially regulated as a consequence of DHEA or pregnenolone treatment. The mass peak loadings indicated potential biomarkers specific for DHEA or pregnenolone abuse in bovines. Statistical testing of individual mass peak loadings by false negative and false positive classification yielded several robust biomarkers for DHEA and pregnenolone treatment. Validation of those robust biomarkers using an independent test set showed no or limited numbers of misclassifications for the selected mass signals. However, it should be noted that larger control groups are needed to obtain a more complete description of the control group distribution.

Moreover, information about the identity of regulated metabolites as a consequence of prohormone administration was obtained. Observed differences most likely are a direct result of treatment with the prohormones DHEA and pregnenolone, given the fact that most of the differentially mass signals could be ascribed to steroid related structures within 5 mDa mass measurement accuracy. Following an initial identification, some compounds were verified by the analysis of commercially available steroids and steroid glucuronides.

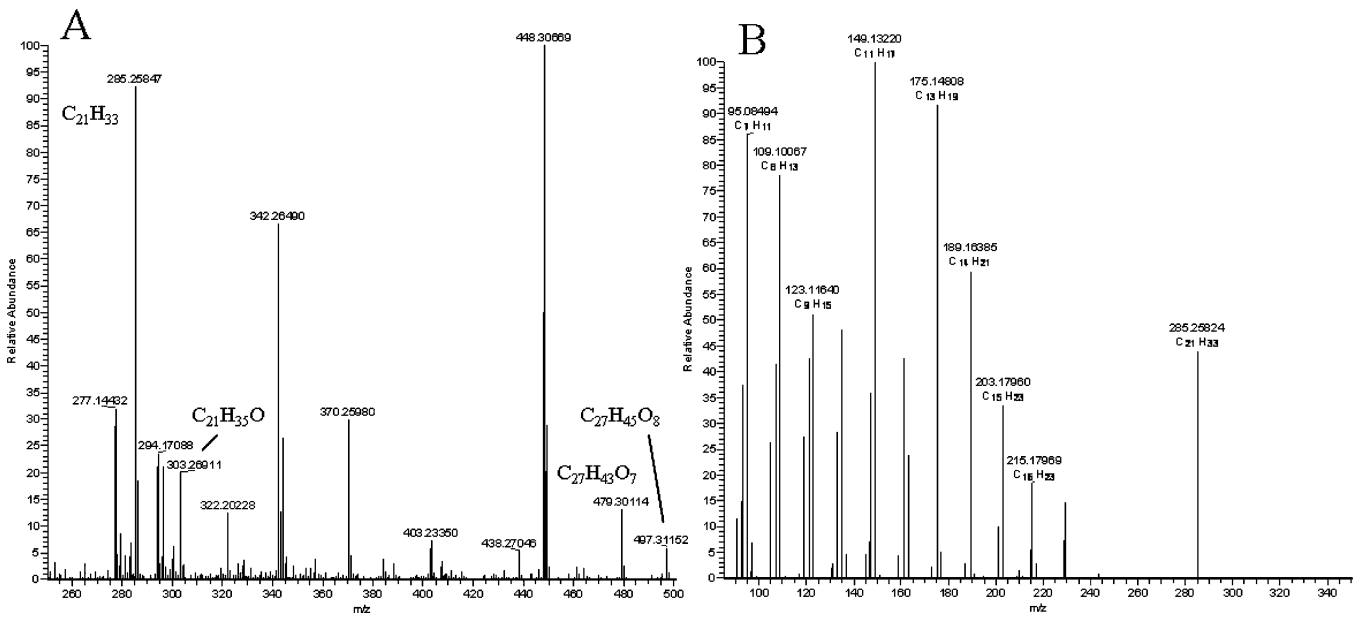

Figure 7. LTQ Orbitrap mass spectra of an unknown candidate biomarker obtained by untargeted screening of urine from a pregnenolone treated animal: (A) full scan mass spectrum at retention time 16.47 min and (B) MS/MS spectrum from precursor ion *m*/*z* 285.3 using a normalized collision energy of 50% and an isolation width of 2.0 *m*/*z*.

In addition, the structure of an unknown steroid glucuronide was elucidated by Orbitrap tandem MS. Nevertheless, the identity of most prohormone derived metabolites remains unclear due to the lack of standards, hereby emphasizing the need for more commercially available standards of steroid metabolites to achieve proper identification.

It is envisaged that application of this holistic methodology is suitable for general anabolic steroid screening purposes in livestock production and eventually in sports doping. Urine profiles of unknown individual animals can be compared with a library of control urine profiles. On the basis of the statistical deviation from this control population and identity of the observed differential mass signals, it can be decided to initiate appropriate follow-up actions. Within this context, future work will be directed at acquisition of additional urine profiles from control animals, in order to obtain a good description of the normal distribution of the control population.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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