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Screening of 4-androstenedione misuse in cattle by LC–MS/MS profiling of glucuronide and sulfate steroids in urine

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ABSTRACT

The use of anabolic agents in food producing animals is prohibited within the European Union since 1988. The illegal use of natural steroid hormones control is however still a current challenge, especially regarding the limitations of existing screening methods. In this context, the present study aimed to develop a new screening approach based on the emerging 'untargeted profiling' concept, but with a special emphasis on steroids phase II conjugated metabolites, in the scope of revealing potential biomarkers signing a fraudulent administration of 4-androstenedione. After extraction and separation of the urinary glucuronide and sulfate steroid fractions, each one was analyzed separately by UPLC–MS/MS using the precursor ion scan acquisition mode. This approach was carried out in order to monitor product ion characteristic of sulfate (*m*/*z* 97) and glucuronide (*m*/*z* 113) functional groups, and then to fish for any potential conjugated steroid leading to these ionic species after fragmentation. After statistical analysis, 86 metabolites (33 from steroid compounds and 53 from other unknown substances) were highlighted as potential biomarkers of 4-androstenedione abuse. After application of several robustness criteria, 26 metabolites (whom 5 were unambiguously structurally identified), were finally selected to build a statistical model which could be used as new diagnostic tool for screening purposes.

1. Introduction

Using natural steroid hormones as growth promoters in cattle is banned since 1988 within the European Union [1,2]. However, steroids can be employed for their anabolic properties in animal breeding farms both fraudulently in UE and legally in other countries (for example USA, New Zealand or South Africa) [3–5]. An efficient control is therefore required with respect to that regulation. Relevant confirmatory methods such as the isotopic deviation measurements (δ^{13}C) in urine by gas-chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) [6,7] or by identification of administered ester forms in hair by GC–MS/MS or LC–MS/MS [8–11] already exist. However the screening of an exogenous administration of natural steroids still remains a difficult and current challenge. Indeed the high variability of the urinary concentration levels of endogenous steroids, which is influenced by many external and inter-individual factors [12,13] does not allow setting relevant reference values for typical untreated animals possibly used for screening purpose. Then, new approaches based on relatively focused metabolite profiling [14–21] or more global metabolomic fingerprinting [22–28] to reveal some direct or indirect biomarkers of growth promoter abuse have emerged in the past few years. Depending on the nature (focused or untargeted) of the considered profiling approach, different acquisition modes in mass spectrometry can be used [29] from the selected reaction monitoring mode (SRM) [14,16,30] for targeted profiling to the full scan mode for untargeted metabolomics [23–27]. Between these two most commonly used profiling approaches, some interesting and promising alternative approaches may be also considered although clearly less employed until now [31–35], which are based on the specific detection of a family of compounds using constant neutral loss or precursor ion scan. These particular acquisition modes were already used to perform profiling of mercapturic acids [31], sulfoconjugates [32] and anabolic steroid metabolites [33–35].

Also in phase 1 metabolism, metabolic disturbances have been noted, the exogenous administration of steroid hormones has already been proven to induce some metabolic disturbances in phase II metabolism [16,30,36,37]. In that context the aim of

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this study was to monitor urinary steroid phase II metabolites using a precursor ion scan method in order to screen for a 4-androstenedione (4-AED) abuse in cattle. Based on a previously described sample preparation procedure and liquid chromatography separation system [14], acquisitions using precursor ion scan mode was developed and implemented to generate sulfate or glucuronide urinary profiles from control and 4-androstenedione treated animal. These profiles were then compared to highlight potential biomarkers of exposure, and to finally build statistical diagnostic model for suspecting an administration of 4-androstenedione in cattle.

2. Experimental

2.1. Chemicals, reagents, materials

Reference steroid compounds were purchased from Steraloids (Newport, RI, USA), Sigma–Aldrich (St. Louis, MO, USA) or National Measurement Institute (NMI, Pymble Australia). Each standard stock solution was prepared at 1 mg/mL in ethanol. Working solutions were prepared by diluting stock solutions in ethanol. All the solutions were stored at −20 °C.

Methanol, ethanol, and ethyl acetate were of analytical grade, and acetonitrile was of HPLC grade. All solvents were purchased from Carlo-Erba Reagents (Rodano, Italy). Formic acid and 32% ammonium hydroxide were respectively obtained from Sigma–Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Deionized water was produced with Nanopure system from Barnstead (Dubuque, IA, USA). SPE SAX 1 g cartridges were purchased from UCT (Bristol, PA, USA). For the synthesis of androstanediol sulfate, the sulfur trioxide pyridine complex was purchased from Sigma–Aldrich and pyridine from Merck.

2.2. Samples

The animal experiments described hereafter were conducted according to an ethically approved protocol. A first experiment was carried out as a proof of concept, in order to highlight urinary metabolites making possible the discrimination between samples collected before and after administration. One heifer (13 months old) was treated (oral route) with a single high dose (250 mg) of 4-androstenedione (Vetranal, Sigma–Aldrich). Samples were collected 4 days, 3 days and just before treatment (−4, −3, 0) and 4 h, 20 h, 30 h, and 2, 3, 4, 7, 8, 9, 10, 11 days after injection (respectively noted 0+, 1m, 1a, 2, 3, 4, 7, 8, 9, 10, 11). All collected urine samples were frozen at −20 °C directly after sampling. A pool of all these samples was also realised and used as quality control (QC) in any subsequent batch of analyses.

In a second time, a complementary ethically approved animal experiment was conducted in order to confirm the previously highlighted metabolites on an independent set of samples. For that purpose, another heifer was treated one time per day during 5 days with a single low dose of 4-androstenedione (100 mg) by oral route. Samples were collected just before the first administration and 1, 2, 3, 4, 5, 6, 7, 8, 10 days after treatment.

In order to have additional data from control animals for biomarkers robustness evaluation purpose, other compliant samples originated from the 2010 French monitoring plan were also used and included in the present study.

2.3. Sample preparation and LC–MS/MS measurement

The used sample preparation procedure and liquid chromatography separation system were previously developed and described elsewhere [14]. Briefly, a strong anion exchanger SPE was used to extract and separate glucuronide from sulfate compounds. The chromatographic separation was achieved in 16.5 min using and Acquity UPLC® system (Waters®, Milford, MA, USA) and a BEH C_{18} reverse phase column (1.7 μm, 2.1 mm × 100 mm, Waters®). For the identification of some androstanediol sulfate, the same elution gradient was used with a BEH Shield RP18 (1.7 μm, 2.1 mm × 50 mm, Waters®) column instead of the BEH C_{18}.

Data were acquired on a XEVO® TQ-MS instrument (Waters, Milford, MA, USA) operating in negative electrospray ionization (ESI−) mode. Nitrogen was used as desolvation gas at 1000 L/h. Source and desolvation temperatures were set at 150 and 500 °C, respectively. The capillary voltage was set at 2.6 kV. The precursor ion scan (PS) mode was used with argon as collision gas at 0.15 mL/min. As previously described [14], all glucuronide and sulfate compounds – except conjugated estrogens in position 3 –, lead after MS/MS fragmentation to product ions at m/z 113 and m/z 97, which are characteristic of the glucuronide or sulfate forms, respectively. Thus, glucuronide compounds (precursors of m/z 113) were profiled from 440 to 650 m/z with a collision energy set at 28 eV, while sulfate compounds (precursors of m/z 97) were profiled from 340 to 570 m/z with a collision energy set at 35 eV. For both methods, cone voltage was set at 50 V. All MS data were acquired in profile mode. This methodology was used to reveal and select potential biomarkers signing the administration of 4-androstenedione.

Then, a second step was carried out to confirm and semi-quantify these biomarkers, which was based on a targeted SRM acquisition methods both for the sulfate and for the glucuronated compounds. The precursor ions were selected according to the differential signals highlighted during the previous step and products ion were m/z 97 in case of sulfate compounds or m/z 113 in case of glucuronated compounds. The values of capillary voltage, cone voltage and collision energy were the same as those set in the corresponding precursor ion scan mode.

2.4. Data processing

The proposed approach aiming to reveal potential biomarkers of 4-androstenedione administration is based on a rigorous and comprehensive comparison of the sulfate and glucuronide profiles generated from control versus treated animals. At first these complex metabolomic fingerprints have to be deconvoluted to allow conversion of the three-dimensional raw data (m/z, retention time, ion current) to time- and mass-aligned chromatographic peaks with associated peak abundances. For that purpose, several commercial or free software solutions exist like MarkerLynx (Waters), MarkerView (Applied Biosystems/MDS SCIEX), SIEVE® (Thermo Fisher Scientific), MetAlign [38,39], XCMS [40], MetIDEA [41] or MzMine [42]. The principle of these software usually includes 4 major steps namely baseline correction, peak picking (or peak deconvolution), peak alignment and peak integration (collection in each sample of the integrated peak area or height). As a result from the data process, a matrix reporting the abundances of each detected signal (characterised by a couple of m/z ratio and retention time) was generated. This table can be used for statistical analysis. For this study, original data from the used UPLC–MS/MS instrument were first converted to the NetCDF format using the included DataBridge converter (Waters). Since the generated MS data were acquired in low mass resolution and in profile mode, all recorded m/z signals were first converted into their corresponding nominal masses using MetAlign software in order to facilitate the further XCMS processing. The main XCMS parameters were: method = matchfilter, fwhm = 6, sntresh = 3, mzdiff and mzwid = 1 and minfrac = 0.75.

During the biomarker robustness evaluation step, peak heights of potential biomarkers monitored in the targeted SRM acquisition mode were measured with TargetLynx (Waters®, Milford, MA, USA)

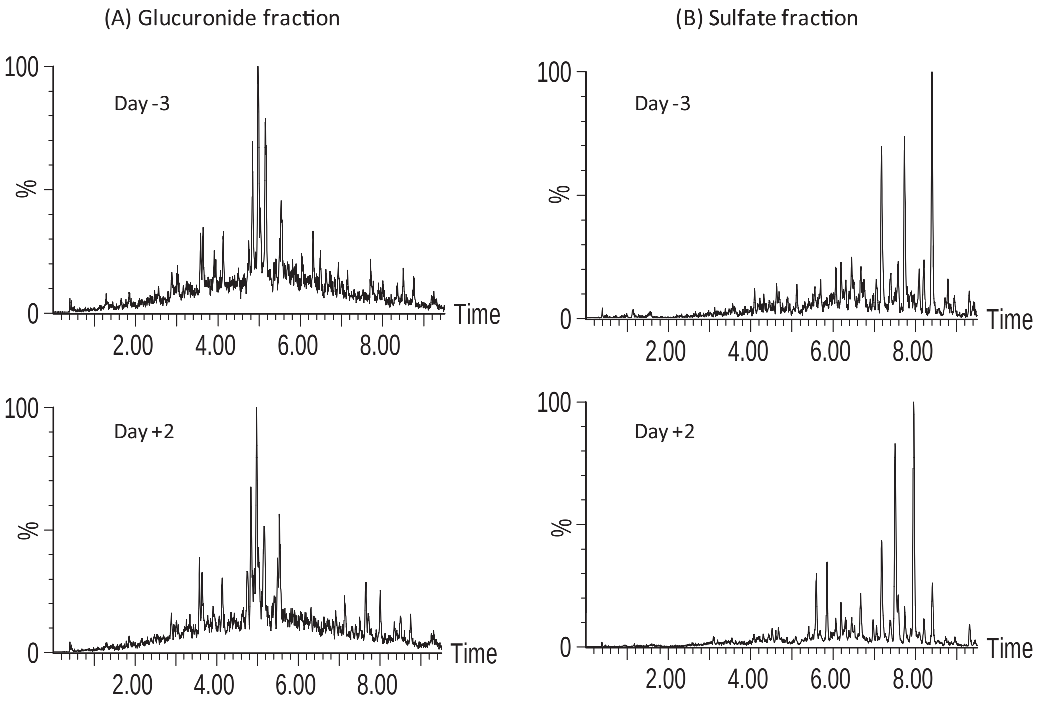


Fig. 1. Precursor ion scan chromatograms from samples collected 3 days before (controls) and 2 day after (treated) the treatment: (A) glucuronide profile and (B) sulfate profile.

software and expressed in relative height (height of analyte/height of internal standard).

2.5. Data analysis

The previous data processing step generates a huge number of variables (*m*/*z*; rt couples). The use of multivariate statistical analyses is then required in order to extract the useful information from the large volume of data generated by such untargeted profiling approach. All these statistical analyses were performed in the present study using SIMCA-P+ software (v.12, Umetrics, Sweden).

A principal component analysis (PCA) was first used (unsupervised technique) to check in what extent the analytical – and particularly the instrumental – variability during the different batch of analyses could be neglected or not compared to other sources of variability including our studied factor (i.e. control versus treated animals). Indeed, PCA shows the variance in the data by building new independent variables called principal components (PCs) which are fewer than the original variables. Secondly, using pareto scaling and log transformation, partial least square (PLS) or orthogonal partial least square (OPLS) (supervised techniques) were used to build a model that attempt to explain and predict the status of animals (*Y* variable, i.e. control or treated) from the matrix (*X* variable) but also to select potential biomarkers. The *S*-plot which shows the contribution of each variable on the predictive component was used for that purpose.

A last PCA based on the selected variables was finally used to establish a screening statistical model to suspect 4-androstenedione administration. In this case, pareto scaling was used for relative height of biomarkers.

2.6. Chemical synthesis of sulfate conjugates

In order to identify unambiguously the isomers of 5ξ-androstane-3ξ,17ξ-diol sulfate excreted in urine, the synthesis of all possible isomers was carried out from the 8 free forms according to the method described by Sandhoff et al. [43]. Briefly, a solution of sulfur trioxide–pyridine complex in pyridine (5 mg/mL) was added on each androstanediol isomers (5 μg). The reaction was stopped 15 min later by evaporation to dryness before to dissolve again the dry residue in H_2O/ACN (80:20, v/v) for LC–MS/MS analysis in the precursor ion scan mode specific of the sulfated compounds. The same protocol was used for the synthesis of 6β-hydroxy-androsterone sulfate and 6β-hydroxy-etiocholanolone sulfate.

3. Results and discussion

3.1. Potential biomarkers highlighting

In order to select potential biomarkers of 4-androstenedione administration, only samples from the high dose animal experiment (cf. Section 2.2) were used.

3.1.1. Results preview

Typical examples of precursor ion scan chromatograms obtained from each isolated urinary fraction (sulfate and glucuronide) and each group of animal (control and treated) are shown in Fig. 1. By simple visual examination, the chromatograms obtained from treated and non-treated animals appeared similar for the glucuronide fraction and slightly different for the sulfate fraction. Nevertheless, as it is not “manually” possible to reveal all the information included in the different samples, a systematic and comprehensive data processing procedure using MetAlign and XCMS software was used. Each fraction (i.e. sulfate and glucuronide) was processed independently. The resulting tables obtained were submitted to principal component analysis (Fig. 2). The first two components (explaining in total 38 and 43% of the whole variability respectively for the “glucuronide” and “sulfate” PCA) extracted by this PCA revealed a clear discrimination between sample collected before and after treatment. These first results confirmed our hypothesis that a 4-androstenedione treatment has induced significant metabolic disturbances at the phase II metabolism level.

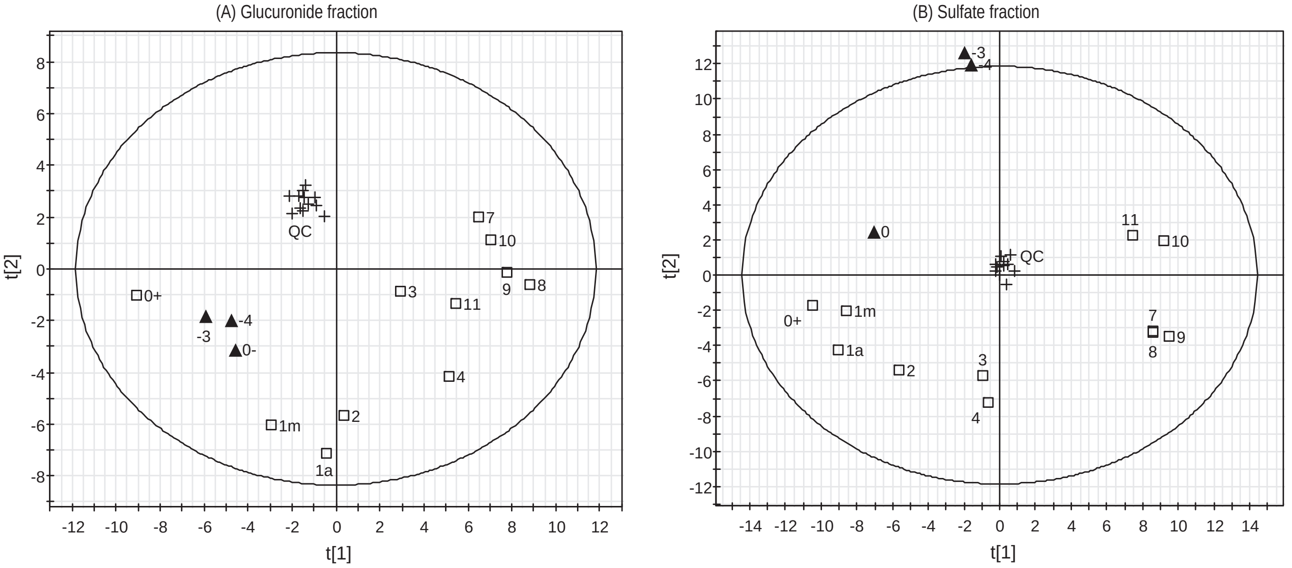


Fig. 2. PCA build from (A) glucuronide profile and (B) sulfate profile (triangle, control urine; square, urine collected after administration; cross, QC samples; numbers indicate the day of urine collection described in Section 2.2).

3.1.2. Quality control

Moreover, QC samples were used to ensure that the differences previously observed were due to metabolic disturbances induced by 4-androstenedione treatment and not to analytical deviations (sample preparation or LC–MS/MS analysis). Indeed, 3 QC samples were extracted and each extract was analyzed 3 times randomly during the sequence. Then, they were processed and finally inputted in the PCA. As shown in Fig. 2, all QC samples were grouped together, demonstrating the reliability of the whole analytical strategy both for sulfate and glucuronide metabolites profiling. Thus, the grouped QC validated that the observed differences between control and treated animals were strictly linked to the 4-androstenedione treatment, on the basis of sulfated or glucuronated metabolites.

3.1.3. Selection of signals of interest

To select the most relevant sulfate and glucuronide metabolites differentially expressed in control versus treated animals, an orthogonal partial least square (OPLS) was performed on the set of 381 (for sulfate) and 333 (for glucuronide) variables extracted by XCMS. The resulting score plot for the sulfate profiles is shown in Fig. 3A. Samples collected before (triangle) and after (square) the administration were discriminated on the predictive component. To visualize which signals (couple m/z_rt) were implicated in this separation, a *S*-plot was built (Fig. 3B). In the *S*-plot, signals were plotted according to their contribution to the predictive component ($p[1]$ axis) associated to their confidence level ($p_{corr}[1]$ axis). Thus, signals located at both ends of the graph correspond to the most influent variables to separate treated from untreated animals, i.e. can be attributed to potential biomarkers of interest. For each signal, an extracted ion chromatogram from the precursors scan was visualized to check the physico-chemical validity of these statistically selected signals as shown in Fig. 3C. After these steps, 59 glucuronated and 90 sulfated metabolites appeared over-expressed while 13 glucuronated and 30 sulfated compounds appeared down-expressed after treatment. In addition, elimination kinetics were drawn over the treatment on the basis of the intensity of each relevant precursor ion. Ions were considered as potential candidate biomarkers if their elimination kinetics had a reliable pattern (Fig. 4). The result was that 86 over the 192 selected signals presented a real excretion kinetic and can reliably be considered as potential biomarkers. Among all the potential biomarkers, 3 different excretion kinetic types can be isolated.

The first and second kinetic types (Fig. 4A and B) were respectively characterised by a later excretion of the metabolite between 3 and 11 days after treatment and by a kinetic showing concentration levels after the administration lower than the basal level (i.e. before administration), certainly due to a feedback mechanism on these metabolites. At this step, no information about the identity of all these compounds was available.

The last one (Fig. 4C) is a kinetic with a rapid excretion between 4 h and 4 days after treatment. One can consider that these metabolites are involved in the primary metabolism of the administered drug (i.e. in the steroids pathway). Thus further studies were carried out in order to identify most of these steroids.

3.2. Identification of steroids as potential biomarkers

Among all our steroid-like potential biomarker candidates, 11 were glucurono-conjugated while 22 were sulfo-conjugated (Table 1). As all conjugated steroids present a MS/MS spectrum with only fragment from glucuronide or sulfate groupment (i.e. m/z 113 and 85 for glucuronide and m/z 97 and 80 for sulfate), studying the fragmentation pattern cannot be informative enough to elucidate the steroid structures. Consequently, our strategy to structurally identify these steroid biomarkers consisted to inject the standard solution (commercially available or in house synthesised) and check the couple retention time and m/z ratio.

3.2.1. Commercially available standard solutions

By injecting all standard solutions available in the laboratory in the same chromatographic conditions than those used for urine sample analysis, and by comparing mass spectra and retention time of standard solutions with those of the selected ions, etiocholanolone glucuronide (m/z 465), epiandrosterone sulfate (m/z 369), epiandrosterone glucuronide (m/z 465), androsterone sulfate (m/z 369) and androsterone glucuronide (m/z 465) were identified unambiguously. These metabolites have already been described as direct metabolites of 4-androstenedione in bovine [14,22], human [44–46] or mice [47].

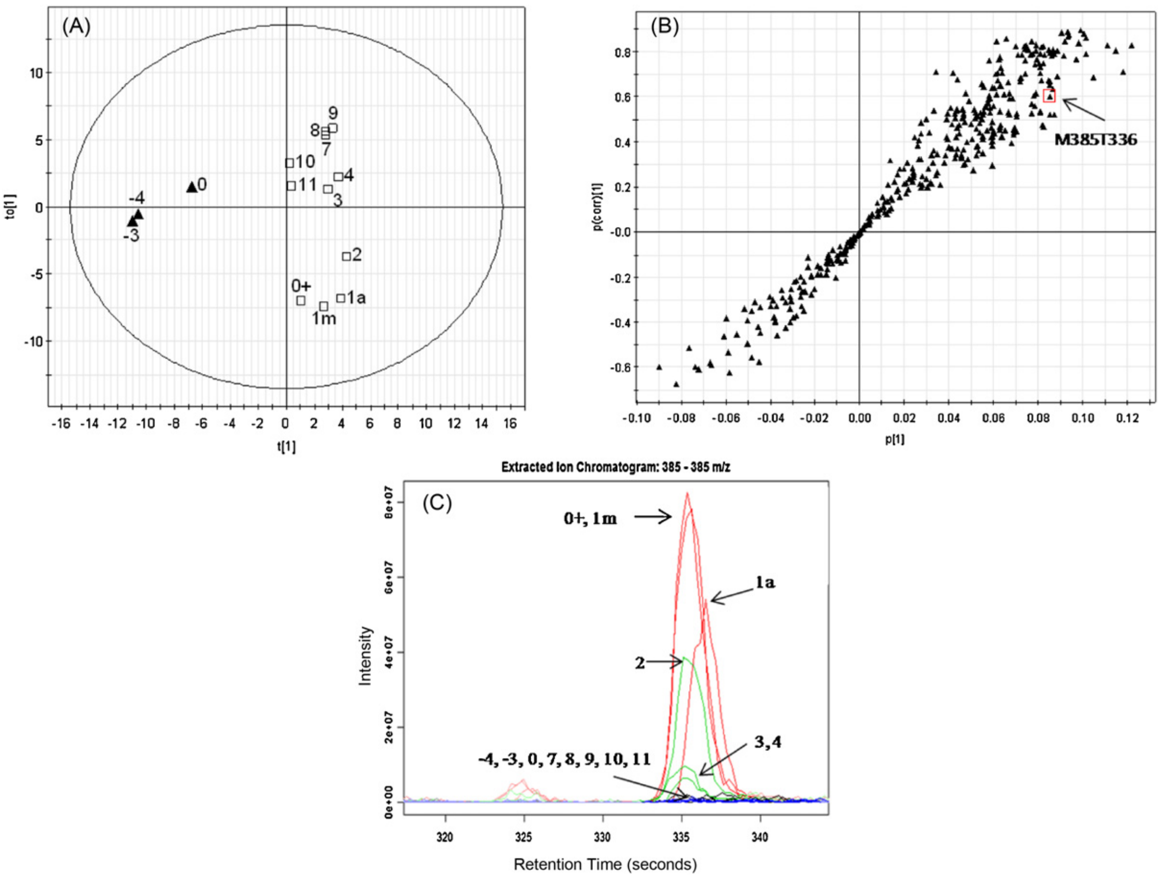
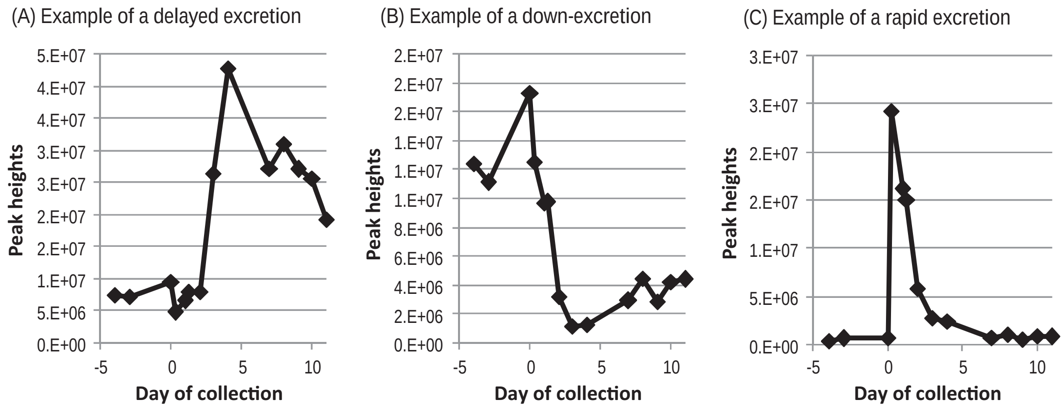


Fig. 3. A) OPLS build from sulfate profile (triangle, control urine; square, urine collected after administration; numbers indicate the day of urine collection described in Section 2.2) and (B) its associated *S*-plot. (C) Example of reliable extracted ion chromatogram for the ion M385T336 (m/z 385, rt = 336 s) selected from the *S*-plot.

3.2.2. Standard synthesis

Three selected ions presenting a fast elimination kinetic and m/z ratio (m/z 371) corresponding to androstanediol sulfates were also highlighted. In order to confirm the androstanediol sulfate identification, the synthesis of sulfo-conjugates was carried out from the 8 free forms of androstanediols commercially available using the trioxide sulfur pyridine complex. As results, only the mono sulfated forms (3-sulfate or 17-sulfate) were observed in our analytical conditions, i.e. separation on BEH C_{18} and detection with a precursor ion scan of m/z 97 (Fig. 5A). By comparing the retention time of all the synthesized products with the ones coming from real sample (sample collected 30 h after administration), the 5α-androstane-3α,17α-diol sulfate (rt = 8.73 min) and the 5α-androstane-3β,17β-diol sulfate (rt = 6.72 min) were unambiguously identified (Fig. 5A). For the third androstanediol signals highlighted (rt = 8.06 min), there was a coelution between 2 synthetic compounds: the 5α-androstane-3β,17α-diol sulfate and the 5β-androstane-3α,17β-diol sulfate (Fig. 5A). Thus, an analysis of both the real sample and the two synthesized androstanediol sulfate was carried out with a different chromatographic column to separate the coeluted isomers. As a result, using a BEH Shield RP18 column instead of a BEH C_{18}, all isomers were separated (Fig. 5B). This confirmed that the 5α-androstane-3β,17α-diol sulfate is the third one excreted after 4-androstenedione treatment.

However, by using this approach, the position of the sulfate group (position 3 or 17) cannot be determined and future work will


(A) Example of a delayed excretion

(B) Example of a down-excretion

(C) Example of a rapid excretion

Fig. 4. Example of excretion kinetics for (A) a metabolite with a late excretion, (B) a down excreted metabolite and (C) a metabolite with a rapid excretion.

Table 1
Highlighted metabolites showing a rapid excretion after 4-androstenedione treatment and considered as steroids.

Conjugation	*m*/*z*	Retention times (min)	Identification	Robust
Sulfate metabolites	415 (×2)	7.87; 7.95	–	Yes (×1)
	413 (×2)	7.38; 7.49	–	Yes (×1)
	403 (×4)	3.14; 3.37; 3.43; 3.79	Potential dihydroxy-androstanediol	No
	401	3.7	Potential dihydroxy-androstan-3/17-ol-3/17-one	Yes
	387 (×6)	4.22; 4.62; 5.94; 6.09; 6.39; 6.82	Potential hydroxy-androstanediol	Yes (×4)
	385 (×2)	5.58; 5.69	Potential hydroxy-androstan-3/17-ol-3/17-one	No
	371 (×3)	6.78	5α-Androstane-3β,17β-diol	No
		8.09	5α-Androstane-3β,17α-diol	Yes
		8.37	5α-Androstane-3α,17α-diol	Yes
	369 (×2)	7.68	Epiandrosterone	Yes
		8.02	Androsterone	No
Glucuronide metabolites	483 (×2)	5.08; 5.39	Potential hydroxy-androstanediol	Yes (×1)
	481 (×3)	4.67; 5.21; 5.65	Potential hydroxy-androstan-3/17-ol-3/17-one	Yes (×3)
	467 (×3)	7.5; 7.65; 9.24	Potential androstanediol	Yes (×1)
	465 (×3)	7.4	Epiandrosterone	Yes
		8.38	Etiocholanolone	No
		8.49	Androsterone	Yes

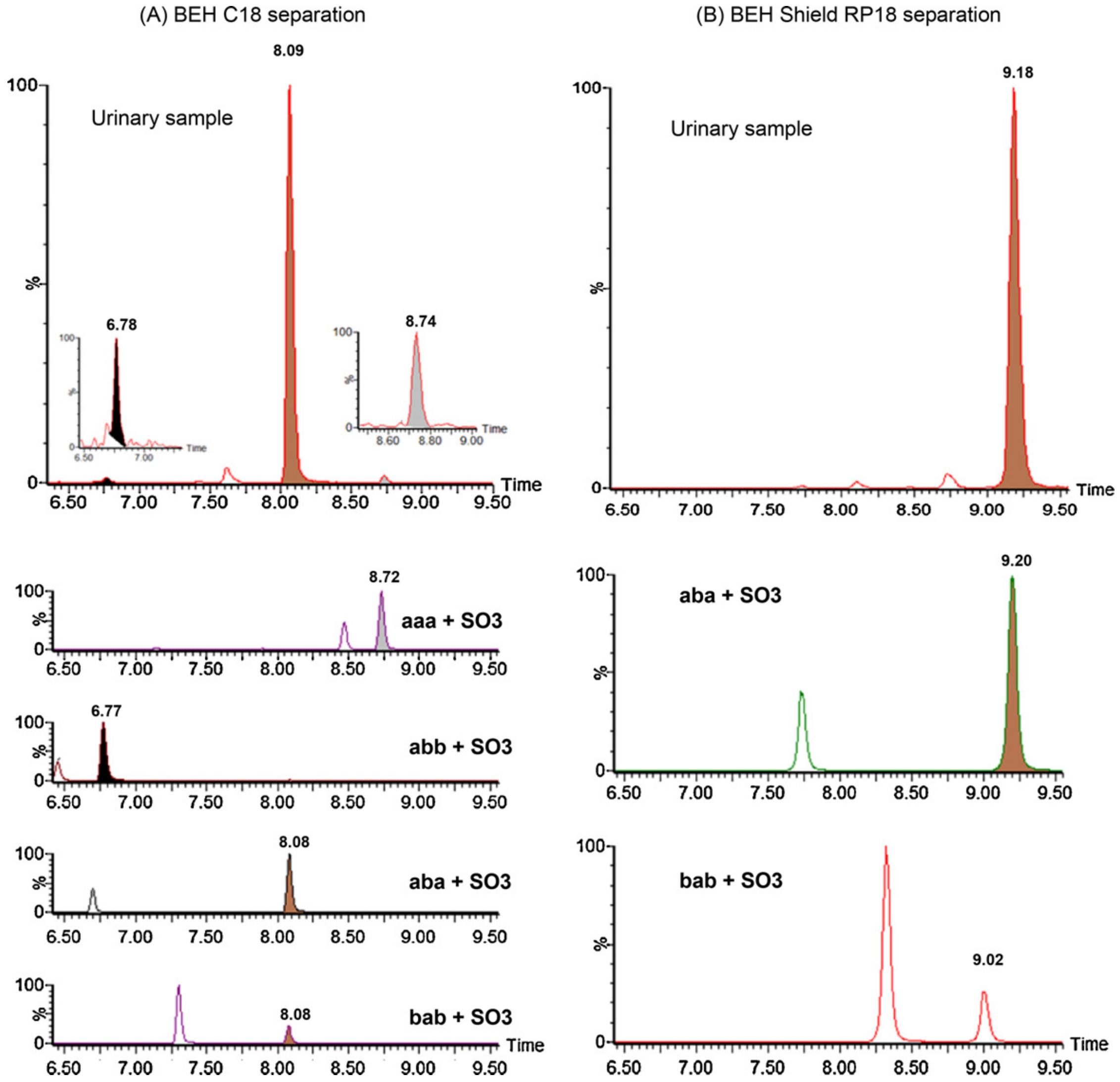


Fig. 5. Extracted ion chromatogram of *m*/*z* corresponding to androstanediol sulfate (*m*/*z* 371) for urine sample and for synthesized androstanediol sulfate obtained with (A) a separation on a BEH C_{18} column and (B) on BEH Shield RP18.

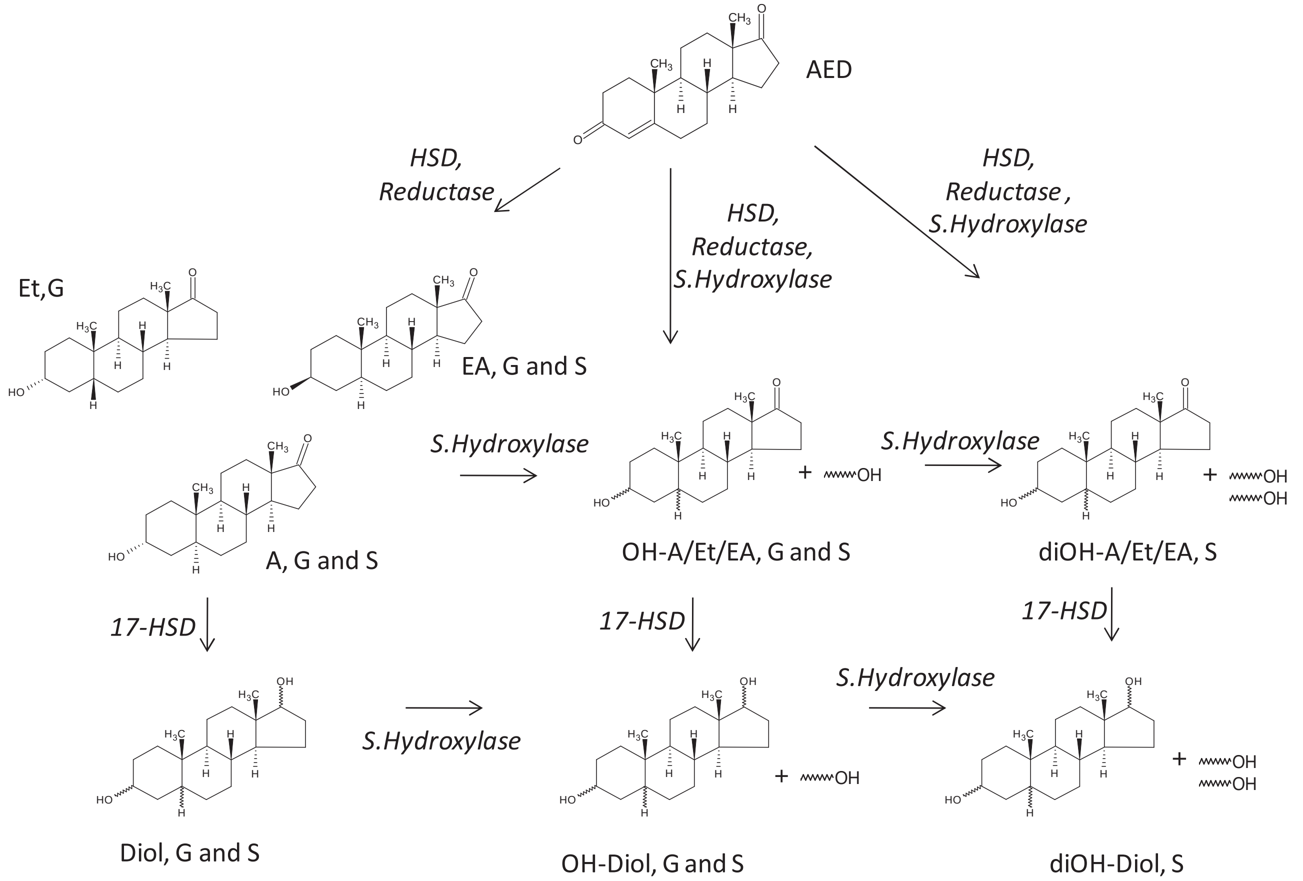


Fig. 6. Proposed 4-androstenedione metabolic pathways highlighted with this work. AED, 4-androstenedione; A, androsterone; Et, etiocholanolone; EA, epiandrosterone; G, glucuronated; S, sulfated; HSD, hydroxysteroid dehydrogenase; S.hydroxylase, steroid hydroxylase.

be devoted to the exact identification of these sulfate compounds with other techniques like NMR or ion mobility mass spectrometry.

In addition, 2 other metabolites with precocious excretion and m/z 385 corresponding to a hydroxyl- or keto-androgen-sulfate were highlighted. Since, the 6β-hydroxy-etiocholanolone sulfate, 6β-hydroxy-androsterone sulfate and 16a-hydroxy-androsterone sulfate have been reported as 4-androstenedione metabolites [44–47], the synthesis of these three compounds was carried out respectively starting from the free related forms. For each synthesis product, 2 peaks were detected corresponding to the mono sulfated forms. However, the retention times of the sulfate products did not match with the ones in the urinary samples. At this

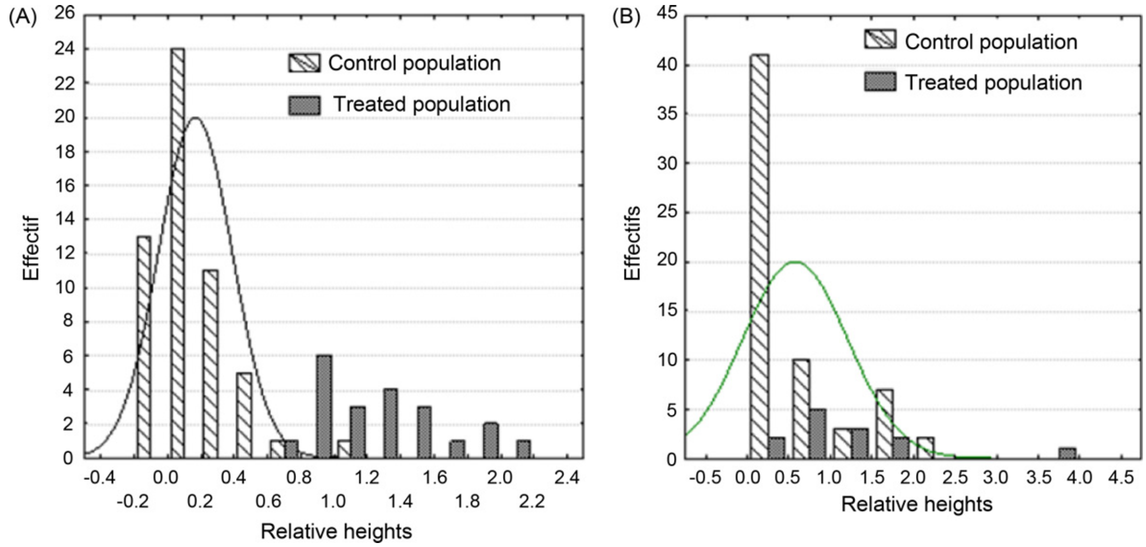


Fig. 7. Example of distribution of relative peak heights (A) for a robust metabolite and (B) for a non-robust metabolite (treated in dark grey and control in hatched).

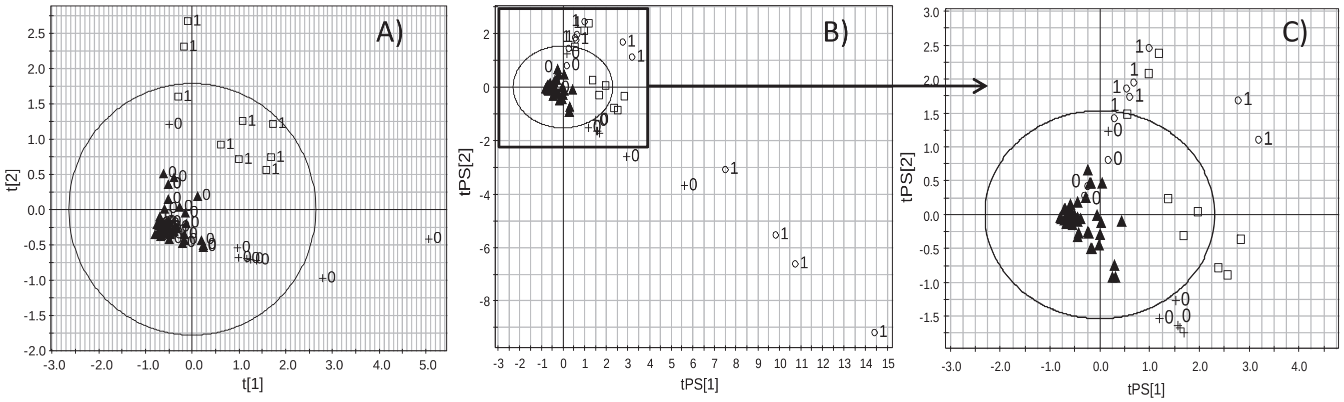


Fig. 8. (A) PCA built from the 26 selected metabolites by the analysis of samples used for metabolites robustness evaluation (cf. Section 2.2). (B) Prediction of the samples from high dose animal experiment (cf. Section 2.2) and of the 8 "different" controls removed from the PCA (A). (C) Zoom of (B) (triangle and square, samples used to build model; cross, 8 "different" controls; circle, predicted samples from high dose animal experiment; numbers indicate the nature of samples: 0 = control samples, 1 = samples from treated animal).

stage, the exact structure of these two metabolites has not been identified. In particular, further investigation would be required to determine the position of the hydroxyl group. It can be however hypothesized that this OH group could be in position 4 since 4-hydroxy-androstendione was detected after 4-androstenedione intake in human [48]. Indeed, we can suppose that 4-hydroxy-androstendione could be also metabolized in 4-hydroxy-etiocholanolone or 4-hydroxy-androsterone by the activities of the 3α/β-hydroxysteroid dehydrogenase and 5α/β-reductase enzymes.

3.2.3. *Other potential steroids metabolites*

Several other compounds present in the selection with *m/z* values corresponding to oxidised-androgens glucuronide or sulfate (i.e. *m/z* 481 and 483 for glucuronide or *m/z* 387, 401 and 403 for sulfate) were pointed out as biomarker candidates. At that time their structure has not been elucidated, but several hypotheses (Table 1) can be suggested. Indeed, the glucuronide metabolites at *m/z* 481 could be associated with a hydroxy-androstan-3/17-ol-3/17-one (hydroxy-etiocholanolone, -androsterone, -epiandrosterone, -dihydrotestosterone) as described in the literature [44–47]. Concerning the sulfo-conjugate at *m/z* 401, an isomer of a dihydroxy-androstan-3/17-ol-3/17-one could be a possibility. Moreover, it can be assumed that the signals at *m/z* 483, 387, and 403 were directly associated to the metabolites identified at *m/z* 481, 385 and 401. Thus they could be hydroxy-androstanediol glucuronide (*m/z* 483) or sulfate (387) and dihydroxy-androstanediol (*m/z* 403). However all these hypotheses need to be confirmed by a structural elucidation of all these metabolites.

Considering all these suggestions, and even if the aim of this study was not related to a metabolism issue, some metabolic pathways (already known and unknown) appeared to be disturbed after 4-androstenedione administration and were highlighted in this work (Fig. 6). Indeed, if the "classical" androgen degradation pathway (including the etiocholanolone and the androsterone) is well known both in human [46], mice [47] or bovine [14] species, the two other metabolic pathways (including mono-hydroxylated and bi-hydroxylated androgen) were never described in bovine species.

3.3. *Robustness of highlighted metabolites*

In order to evaluate the robustness of the metabolites pointed out as associated with the AED treatment, additional samples originated from a second animal experiment ($n = 9$, treated) as well as from the 2010 French monitoring plan ($n = 64$, controls) were analyzed in the SRM mode. Indeed, as the precursor ion (*m/z* corresponding to the potential biomarkers) and the product ion (*m/z* 97 or 113 respectively for a sulfate or glucuronated metabolites) are easily known, two SRM methods were built: one for the sulfates (41 metabolites monitored) and another for the glucuronides (45 metabolites followed). Then the robustness of biomarkers was evaluated in two steps. The first one consisted in the control of the excretion kinetics of all selected metabolites for the second treated animal (5 AED administrations of 100 mg). Indeed, the metabolites became relevant if its kinetics appeared linked to the administration, i.e. if there was a modification of the excretion after treatment. Forty-five metabolites over the 86 initially selected answered to these criteria and passed this first level of evaluation. The second step consisted in checking that the distribution of the relative height of potential biomarkers in each class (all control versus treated from the two animal experiments) was different (Fig. 7A) or overlaid (Fig. 7B). As a result, among the 45 metabolites previously selected during the first step, 26 (17 steroid like and 9 non-steroid like) presented different distribution for control and for treated animal and were considered definitively robust.

3.4. *Toward a screening model*

The final purpose of this study was to use all robust metabolites in order to build statistical models which can be used for predicting unknown samples. Thus, a PCA was built (Fig. 8A) with all samples except those of the first animal experiment, which was used for prediction. This PCA shows clearly a good discrimination between samples coming from treated (square and number 1) and control animal (triangle and number 0). However, 8 controls (cross and number 0) seemed to be different from the others. They were discarded to build a second model (not shown) with control very well regrouped together and separated from the positive samples along the two first components. Using this last model, samples from our robustness evaluation set, were correctly predicted (Fig. 8B and C). Indeed, samples collected before the administration (circle and number 0) of 250 mg of 4-androstenedione were plotted within the control group and sample collected after the administration (circle and number 1) were predicted outside the control group as suspicious sample. These results indicate a very satisfying false compliant rate in the state and on the basis of these data which is very important for a screening method. Moreover the 8 different control samples (cross and number 0) removed previously were also predicted but far from the control group. As it is normally compliant samples, they can be declared as false suspect. Then, only 12%

(8/67) of control samples are classified false suspect which is a very reasonable value for screening purpose. Indeed, this value is widely better than the highest quantity of false suspect obtained with the current screening method based only on a concentration threshold of 17α-testosterone or etiocholanolone in bovine urine.

Finally, this model, based on many steroids involved in the general metabolic pathway of natural steroids, could be also useful for a better detection of others natural steroids misuse like testosterone, even if it was initially built to monitor 4-androstenedione abuse.

4. Conclusion

The aim of this study was to develop a reliable and robust methodology to perform phase II glucuronide and sulfate metabolites profiling by UPLC–MS/MS for antidoping screening purpose. In this context, a LC–MS/MS profiling method using the precursor ion scan acquisition mode specific of glucuronated and sulfated metabolites was carried out and used to reveal candidate biomarkers of a 4-androstenedione abuse. As a result, some steroidal metabolites involved in the metabolic pathway of 4-androstenedione were highlighted and identified. Several other non steroidal metabolites were also found relevant and need now to be identified. Using these biomarkers (both steroids and non steroids), a screening method based on statistical analysis was proposed allowing the detection of an 4-androstenedione abuse up to 11 days with a 12% false suspect rate and a 0% false compliant. This detection window is largely longer than the one described before [14] based only on few steroids metabolites. However, a sample detected outside the statistical model is not a full proof of misuse and a confirmatory method (GC–C–IRMS analysis or detection of administrated ester) needs to be performed in order to declare it compliant or non-compliant.

Thus the interest of such a semi-focused profiling approach has proved efficiency. Besides, this general approach to select and prove the robustness of biomarkers of phase II metabolism after precursor ion scan acquisition appeared promising and could be extended to other anabolic treatment such as testosterone. Nevertheless, before to use the statistical model developed in this study for routine analysis, this one needs to be confirmed by additional samples and especially by non-compliant samples.

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