Hormonal effects of prohormones

Novel approaches towards effect based screening in veterinary growth promoter control

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Thesis

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Abstract

Within the European Union the use of growth promoting agents in cattle fattening is prohibited according to Council Directive 96/22/EC. Interestingly, there is not a black list of substances, but 96/22/EC states that all substances having thyrostatic, estrogenic, androgenic or gestagenic activity are prohibited. Besides abuse of the "classical" synthetic steroids there is a tendency towards misuse of natural steroids and prohormones. Prohormones are compounds that exhibit limited or no hormonal activity but are direct precursors of bioactive hormones and are intended to be converted to full active hormones via enzymatic processes in the body. However, knowledge about metabolism, the mode of action and excretion profiles in cattle is often unclear, and methods to detect abuse of prohormones in livestock production are lacking. Therefore, the aim of this thesis was to get insight into the hormonal action of prohormones and to develop novel *in vitro* and *in vivo* screening methods allowing effective surveillance on the illegal use of prohormones in livestock production. Hereby the emphasis was on developing effect based approaches to better meet Council Directive 96/22/EC.

The bioactivity of a wide variety of supplements which contained prohormones were tested using a yeast androgen bioassay. For supplements containing solely prohormones the value of this bioactivity based screening appeared to be limited as they require metabolism to become active. Therefore, screening methods for animal feed, supplements and preparations were set-up by using the same yeast androgen bioassay in combination with bovine liver models as well as enzymatic and chemical deconjugation procedures to mimic *in vivo* metabolic bioactivation. The use of either bovine liver S9, liver slices, pure enzymes or alkaline hydrolysis showed that prohormones could be activated, resulting in a significant increase in bioactivity as determined by the androgen yeast bioassay.

For the detection of prohormone abuse at the farm and/or slaughterhouse the usefulness of 'omics' based profiling techniques was investigated. Within this scope a comprehensive metabolomics based screening strategy for steroid urine profiling was developed. Comparison of urinary profiles revealed large differences between the profiles of controls and dehydroepiandrosterone (DHEA) as well as pregnenolone treated animals. Moreover this steroid urine profiling approach allowed identification of biomarkers for treatment by specific prohormones. This resulted in respectively 7 and 12 specific mass peak loadings which could potentially be used as biomarkers for pregnenolone and DHEA treatment.

In addition, the feasibility of a liver gene expression profiling approach was investigated to monitor the effects of DHEA treatment at the transciptome level. It was shown that identification and application of genomic biomarkers for screening of DHEA abuse in cattle is substantially hampered by biological variation. On the other hand, it was demonstrated that comparison of pre-defined gene sets versus the whole genome expression profile of an animal allows to distinguish DHEA treatment effects from variations in gene expression due to inherent biological variation.

Altogether the results of this thesis increase the knowledge about the metabolism and bioactivation of prohormones *in vitro* as well as *in vivo*. Based on this knowledge, a panel of new effect based concepts and screening methods was developed that complement and improve the current testing programs. These new concepts will facilitate better implementation of the European ban on growth promoters in livestock production as described in Council Directive 96/22/EC.

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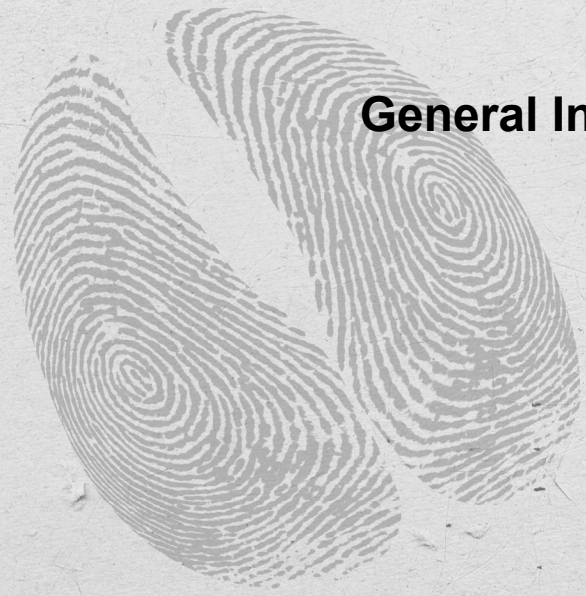
Chapter 1

General Introduction



1.1. History, legislation and monitoring

In modern livestock production special meat producing breeds in combination with sophisticated feeding strategies are employed to assure optimal growth thereby maximizing economical benefits for the farmer. However, to further increase productivity, farmers are tempted to use illegal growth promoters like anabolic steroids, thyrostatics and ß-agonists.

One of the first experiments with growth promoters in ruminants dates back from 1947 showing improved growth and feed conversion in heifers as a consequence of diethylstilbestrol (DES) administration [1]. The recognition of the growth promoting properties of estrogens, either alone or in combination with androgens led to their introduction as a tool to increase meat production. In 1955, the USA allowed DES-containing ear implants in cattle and since then, not only in the USA but also in Europe, a wide range of compounds came available for growth promoting purposes comprising synthetic as well as natural hormones. Because of the awareness that residues of growth promoters in meat may lead to disturbance of homeostatic hormone levels and might adversely affect consumers health [2,3], the use of anabolic agents for growth promoting purposes has been forbidden in The Netherlands since 1961 by a decree of the Commodity Board of Livestock and Meat (PVV) [4]. This consequently raised the need for effective methods to control and monitor abuse of growth promoters and since then various biological, histological, chemical and immunological based screening and detection methods were developed (Figure 1). One of the first screening methods to track abuse of estrogens was based on histological examinations of the prostate and Bartholin's gland in veal calves [5,6]. When animals were found suspect the urine was checked by using more specific chemical and immunochemical methods like thin layer chromatography (TLC) and immunoassays (ELISAs and RIAs). In the beginning of the 1980s the illegal use of estrogens strongly reduced in favor of other anabolic compounds and cocktails. As a result it was harder to identify treated animals due to the fact that histological evaluations showed more variations and alterations were less pronounced [7]. Consequently, control measures shifted to the already existing detection/conformation methods like TLC and immunoassays later followed by more specific hyphenated techniques like gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to mass spectrometry [8]. These techniques allow targeted detection and quantification of a limited number of pre-selected analytes in one single run. Nowadays a typical analytical strategy for residue monitoring is in general a two-step approach. At first, a low cost screening method is applied which is optimized to prevent false negative results. Secondly, a confirmation method is used to confirm any positive screening result thereby

preventing false positive results. For both screening as well as confirmation procedures gas and liquid chromatography in combination with mass spectrometric detection (GC- and LC-MS/MS) are used extensively and are considered state-of-the-art in veterinary control. Apart from screening by GC- and LC-MS, traditional methods such as ELISAs and RIAs are still used as well. Moreover, the last years a lot of effort is invested in development of novel effect based methods, like biosensors and receptor mediated bioassays which have shown to be very useful for screening groups of compounds with a similar mode of action [9]. In case of the illegal use of hormones this is of particular interest for the detection of new, possibly unknown compounds that can escape from detection by chemical analytical methods.

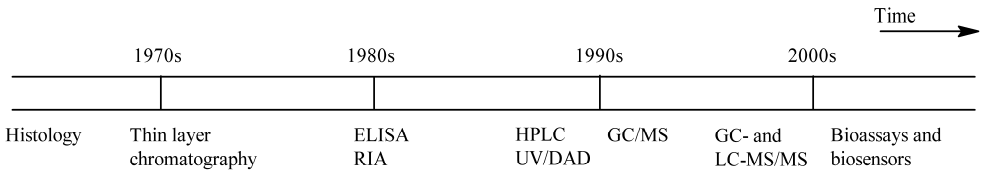


Figure 1: In time evolvement of methods routinely used for monitoring growth promoter abuse.

In The Netherlands, nowadays control is largely governed by European Union legislations. The oldest EU directives date back from 1970 specifying the additives that can be used in animal feeds [10]. Since then, EC legislation was continuously amended and updated until in 1988 EC legislation 88/146/EC became effective which totally prohibited the use of all hormonal growth promoting substances for fattening of farm animals [11]. The latter was replaced in 1996 by Council Directive 96/22/EC which states that; "*Member states shall prohibit: the administering to a farm or aquaculture animal, by any means whatsoever, of substances having a thyrostatic, oestrogenic, androgenic or gestagenic action and of beta-agonists"* [12]. Here it should be noted that the ban described in this directive does not comprise a black list of substances, but explicitly prohibits groups of substances based on their bioactivity. This bioactivity character is even more pronounced in the Dutch national legislation which prohibits the use of substances that exhibit any hormonal activity [13].

To ensure compliance with EU legislations, requirements for residue analysis are described in Council Directive 96/23/EC [14]. This primarily includes sampling and investigation procedures, indications for sanctions in case of non compliance and rules for reporting of monitoring programmes. Also within the framework of Council Directive 96/23/EC, technical guidelines and performance criteria are described in Commission Decision 2002/657/EC [15]. At a national level these EU legislations are implemented in

residue monitoring programs regulating sampling of animal matrices and residue analysis therein. Analysis of the samples taken is performed by routine or field laboratories (RFLs). In each member state the RFLs are coordinated and controlled by a national reference laboratory (NRL) designated by the national government. The results of the implementation of these national residue monitoring plans in the EU Member states are included in European Commission annually reports [16]. Based on the result of these regulatory residue testing programs a realistic overall estimate of the misuse of compounds in the European Union can be made (Figure 2).

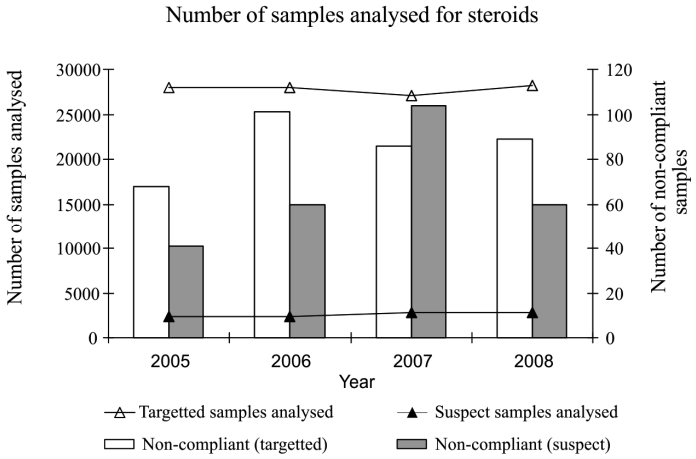


Figure 2: Number of bovine samples analyzed for steroids in the European Union. Shown are the total targeted and suspect samples analyzed as well as the number of samples found non-compliant in the period of 2005 up to 2008 [16].

For example in 2008, a total of 28171 targeted samples from bovine origin were taken for steroid analysis of which 0.31 % was found non-compliant (0.18% excluding cortico-steroids) [16]. In absolute numbers these were 89 non-compliant samples for a total of 12 substances. In addition, 2842 suspect samples were taken as part of the residue control which refers to samples taken as a consequence of either non-compliant results or a suspicion of illegal treatment. Of these 2842 samples 60 were found non-compliant for a total of 6 substances. Besides the classic synthetic steroids a trend is observed towards natural steroid hormones, used alone or in combinations. Moreover, inspections of livestock farms in the Netherlands occasionally turn up feed or herbal additives and preparations containing so-called prohormones. These compounds exhibit limited or no hormonal activity but are direct precursors of bioactive hormones and are intended to be converted to full active hormones via enzymatic processes in the body.

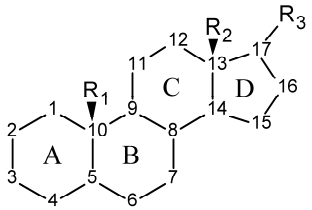
Sport doping control faces similar problems and it appears that for muscle growth the same products are used by athletes as in cattle fattening. Until a few years ago prohormone containing supplements (e.g. dehydroepiandrosterone (DHEA), 4-androstene-diol and 4-androstenedione) were sold as over the counter supplements in the USA and could be easily obtained by Dutch consumers via the internet. Since January 2005, when the Anabolic Steroid Control Act became effective in the USA, prohormones were added to the list of controlled substances [17]. From that time selling and possession of prohormones and prohormone containing supplements was banned and prohormones could only be obtained with a medical description [18]. Drug monitoring in sports is governed by the World Anti-Doping Agency (WADA) which, in contrast to Council directive 96/22/EC, publishes a list of prohibited substances which include all anabolic steroids as well as their precursors [19]. On this list however, there are closed and open classes. A closed class means that control laboratories must screen for only the compounds listed (e.g. endogenous anabolic steroids). An open class means that control laboratories must screen for compounds that have similar chemical structures or biological activity to those named in the list, this to avoid missing new designer steroids.
Despite the observed increase in prohormone abuse in livestock production, knowledge about metabolism, the mode of action and excretion profiles of prohormones in cattle is limited. Moreover adequate screening and detection methods to prove prohormone abuse are lacking because biomarkers are most times not known or are not above highly fluctuating endogenous levels. The main purpose of this thesis research is to get insight in the hormonal action of assumed prohormones and to develop novel *in vitro* and *in vivo* screening methods allowing effective surveillance on the illegal use of prohormones in livestock production. Hereby the focus was on precursors of the natural anabolic steroid 17ß-testosterone and on DHEA in particular.

1.2. Steroid (pro)hormones

Chemical structure and nomenclature of steroids

The chemical structure of steroid hormones consists of a polycyclic C_{17} steran skeleton named cyclopentaneperhydrophenanthrene which has three condensed cyclohexane rings (A, B and C) and a cyclopentane ring (D). Depending on the presence and location of methyl and alkyl side chain groups the parental steroid structures are classified as pregnane (C_{21}), androstane (C_{19}) or estrane (C_{18}) (Figure 3) [20]. For steroids, systematic as well as trivial names are widely used. Systematic names are applied according to the rules for steroid nomenclature formulated by the IUPAC (International Union of Pure and

Applied Chemistry) [21]. Hereby, the parental steroid structure (preg-, androst- and estr-) is the basis for denomination and prefixes and/or suffixes are added to indicate the presence



	R_1	R_2	R_3
Pregnane	CH_3	CH_3	C_2H_5
Androstane	CH_3	CH_3	H
Estrane	H	CH_3	H

Figure 3: The cyclopentanoperhydrophenanthrene structure together with the parental structures of pregnane, androstane and estrane listed [20].

and location of substituents and double bonds. Any number of prefixes is allowed, while only one suffix may be used which is chosen according to the preference list: acid, lactone, ester, aldehyde, ketone, alcohol, amine and ether. Multiple prefixes are written in alphabetical order. For natural steroids the most frequently occurring substituents are the hydroxyl goup (-ol or hydroxyl-) and the oxo group (-one or oxo-). The atoms or side groups which are oriented in the direction below the plane of the paper are denoted with and α and these atom bonds are shown as broken lines in structural formulas. When the orientation is in the direction above the plane of the paper this is denoted with a ß and these atom bonds are depicted as thickened solid lines in structural formulas. If there are two groups attached to the same carbon atom, only the orientation of one of the groups is included in the systematic name. In unsaturated steroid molecules the parental suffix (-ane) is replaced by -ene (-adiene or -en) and the location of the double bond is indicated by the lower number of the two carbon atoms involved in the bond. If similar substituents exist, like two double bonds or two hydroxyl groups, the prefixes di, tri, etc. are added before the suffix or prefix. For example, the systematic name of the prohormone DHEA is 3ß-hydroxy-5-androsten-17-one as the molecule exists of an androstane skeleton with one double bond between C5 and C6, a hydroxyl group at the C3 and an oxo group located at the C17 position respectively (Figure 4). However it should be noted that, similar to many other steroids, this compound is known by many other (trivial) names like prasterone, fidelin, androstenolone, anastar, dehydroisoandrosterone, 3ß-hydroxy-etioallocholan-5-

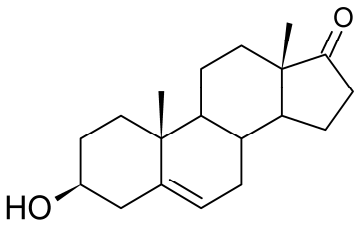


Figure 4: Structure formula of 3ß-hydroxy-5-androsten-17-one (DHEA).

ene-17-one, trans-dehydroandrosterone and 5,6-dehydroisoandrosterone.

Biosynthesis and metabolism of natural steroids

Natural sex steroid hormones are mainly produced in the gonads and adrenal glands and play an important role in growth, behavior, organ functioning and development of secondary sex characteristics. Testicular and ovarian steroid hormone production is controlled by the pituitary gonadotropins luteinizing hormone (LH) and follicle stimulation hormone (FSH). In this process prohormones like DHEA and 4-androstenedione mainly serve as intermediates for the production of more potent sex hormones and only 10-25% of the DHEA and 5% of the DHEA-sulfate produced in the gonads are secreted directly in the blood [22]. Biosynthesis of prohormones like DHEA and 4-androstenedione also takes place in the adrenal cortex and is induced by corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) which stimulate the release of adrenocorticotropin (ACTH) from the pituitary gland [23].

In both gonads and adrenals, steroid synthesis is stimulated by binding of LH, FSH or ACTH to their corresponding receptors located at the cell surface. Upon receptor binding, conformational changes stimulate adenylyl cyclase and subsequently increased production of cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) and phosphorylation of cholesteryl esterase which leads to an increase in intracellular concentrations of cholesterol. This extra-mitochondrial cholesterol is transported by steroidogenic acute regulatory (STAR) protein to the inner membrane of the mitochondria where side chain cleavage by CYP11A1 (or desmolase) turns cholesterol into pregnenolone. Secondly, cAMP affects long-term regulation of CYP11A1 by binding to cAMP regulatory elements on the CYP11A1 gene resulting in increased RNA transcription and increased levels of the CYP11A1 enzyme. Subsequent to CYP11A1 activity, pregnenolone moves to the cytosol were further conversion takes place (Figure 5). In addition ACTH stimulates the production of mineralocorticosteroids and glucocorticosteroids in respectively the zonae glomerulosa and reticularis [23]. This includes cortisol a glucocorticoid which provides the negative feedback on CRH, AVP and ACTH via the hypothalamo-pituitary system.

Transformation of pregnenolone by 3ß-hydroxysteroiddehydrogenase (3ß-HSD) or CYP17A1 are competing reactions, and are respectively known as the Δ4- and Δ5-pathway. Interestingly, in humans and bovines the Δ5-pathway is preferred while rodents mainly utilize the Δ4-pathway [24]. In the Δ5-pathway pregnenolone is hydroxylated by CYP17A1 to 17α-hydroxypregnenolone, and afterwards converted to DHEA via side chain cleavage by 17,20-lyase activity by the same CYP17A1 enzyme. 3ß-HSD completes

the pathway by oxidation of DHEA to 4-androstenedione. In contrast, the Δ4-pathway starts with metabolism of pregnenolone by 3ß-HSD with progesterone as a product. Progesterone also serves as a substrate for CYP17A1 and can be metabolized to 17α-OH-progesterone and further converted to 4-androstenedione by the 17,20 lyase activity of CYP17A1.

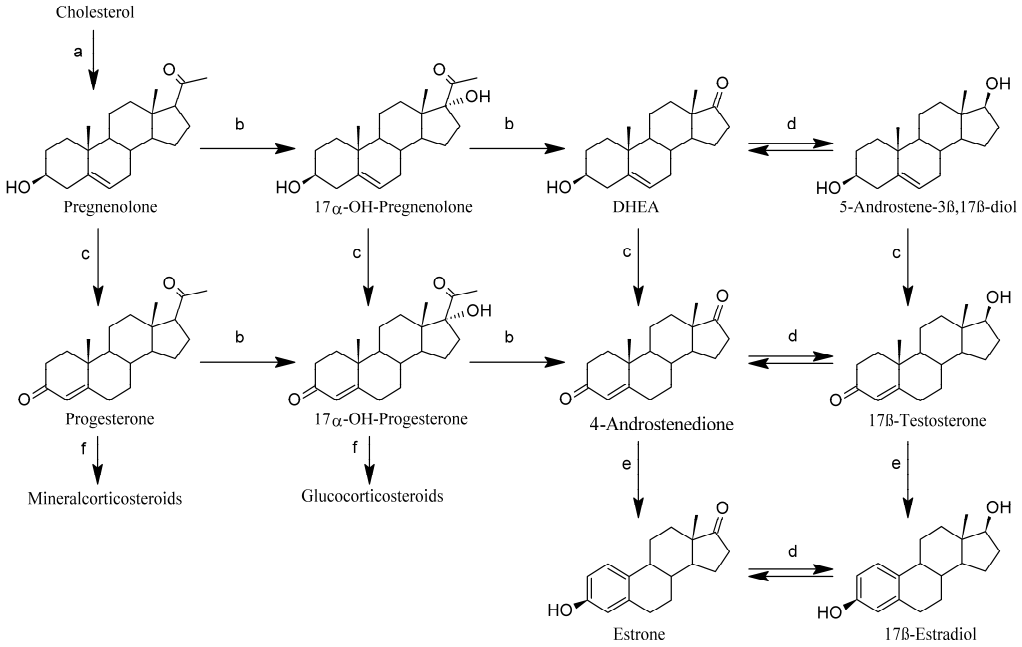


Figure 5: Steroid biosynthesis pathway; a - Cytochrome P450 11A1 (CYP11A1) b - Cytochrome P450 17A1 (CYP17A1) c - 3β-Hydroxysteroid dehydrogenase/isomerase (3β-HSD) d - 17β-Hydroxysteroid dehydrogenase (17β-HSD) e - Cytochrome P450 19A1 (Aromatase) f - Cytochrome P450 21 A1 (CYP21A1).

Phase I metabolism

Steroidal prohormones are extensively metabolized *in vivo* and after exogenous administration only small amounts of the parent substances are excreted via the urine. The most important metabolic phase I reactions include oxidation or reduction at the C3 and C17 position by hydroxysteroid dehydrogenases (HSDs), reduction of double bounds by 5-reductases, aromatization of the A-ring by aromatase and hydroxylation at various positions of the parental steroid structure by cytochrome P450 (CYP) activity (Figure 5).

3-Hydroxysteroid dehydrogenase (3-HSDs)

3ß-HSDs are most abundantly present in steroidogenic tissues but are also found in various other tissues like liver, kidney, brain, skin, lung and adipose tissue. The widespread distribution of 3ß-HSD expression indicates that this enzyme plays an important role in the intracrine formation of sex steroids in large series of peripheral target tissues. Molecular cloning experiments have shown that at least three isoforms of 3ß-HSD are expressed in rat, six isoforms in mouse and two in humans (types I and II) [25]. Human 3ß-HSD type I is mainly found in the placenta and peripheral intracrine tissues, while type II is almost exclusively expressed in the adrenals and gonads [26]. In contrast to humans, up till now only one 3ß-HSD type has been identified in bovine species [26,27]. Under 3ß-HSD activity, 3ß-hydroxy-5-ene steroids are transformed into 3-oxo-4-ene steroids. This two step transformation involves a dehydrogenase and an isomerase reaction. The first step in dehydrogenation of DHEA requires NAD^+ as a cofactor resulting in 5-androstenedione and reduced NADH. Subsequently, this reduced coenzyme NADH, induces a conformational change in the enzyme protein that activates the isomerase reaction converting 5-androstenedione into 4-androstenedione [28]. Also $NADP^+$ can serve as a cofactor in these reactions but was reported to be relatively ineffective [25].

17-Hydroxysteroid dehydrogenases (17-HSDs)

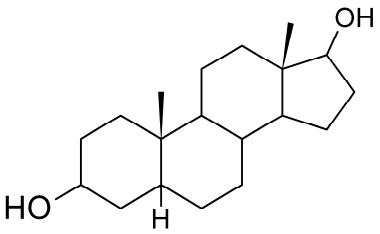
17ß-HSDs catalyze the interconversion of 17-ketosteroids into their corresponding 17ß-hydroxy steroids. At present 14 different mammalian 17ß-HSDs have been identified [29] which are with the exception of 17ß-HSD type 5 all members of the short chain dehydrogenase/reductase (SDR) family. SDRs constitute a large protein family of oxidoreductases, present throughout species. 17ß-HSD enzymes are acting on a large set of substrates and are responsible for reduction or oxidation of hormones, fatty acids and bile acids *in vivo*. Although named as 17ß-HSDs, reflecting the major redox activity at the 17ß-position of the steroid, several 17ß-HSDs are able to convert multiple substrates at multiple sites, such as at the C3 position of the steroid ring. Most of the 17ß-HSDs have bidirectional capabilities, catalyzing either the oxidative or reductive reaction in the presence of the cofactors $NAD(P)^+$ or NAD(P)H respectively. The intracellular location of the enzymes is diverse and different 17ß-HSDs have found to be located in the cytosol, microsomes, mitochondria and peroxisomes. Also differences in enzymatic activity are observed across tissues and organs. These observations along with kinetic studies have demonstrated that although the enzymes have multifunctional capabilities, most have preferential substrate specificity and directionality *in vivo* [30].

Epitestosterone, the 17α-epimer of testosterone, is abundantly present in the urine of men and equals the concentrations of testosterone. Although the exact pathway of formation is still a subject of research, it is believed that epitestosterone is formed by conversion of 17-keto steroids by 17α-HSD activity. This is supported by a study where oral administration of hundreds of milligrams of 4-androstenedione showed an increase in the urinary excretion rate of epitestosterone [31] and a study in human embryonic kidney (HEK-293) cells showing conversion of 4-androstenedione to epitestosterone [32]. Another hypothesis is that epitestosterone is formed by 3ß-HSD oxidation of androst-5-ene-3ß,17α-diol in the testes [33] this precursor in turn, might be formed as a by-product in the synthesis of androsta-5,16-dien-3ß-ol from pregnenolone [34]. In humans it has been shown that interconversion of testosterone and epitestosterone is negligible. As a result, the T/E ratio is utilized as a parameter for detecting abuse of 17ß-testosterone in human sports doping [35]. Compared to humans, other animal species such as rabbits, guinea pigs, mice and calves show great differences in excretion ratio of T and E [36, 37]. This is likely caused by the activity of 17α-hydroxysteroid which is much higher than in men and catalyses a conversion of 17ß-testosterone into epitestosterone. Similar as in humans, the pathway of epitestosterone formation in bovines is far from clear, and in spite of the fact that the testis was identified as a source of endogenous epitestosterone, no interconversion of testosterone and epitestosterone was observed in the testes of bulls, [37]. On the other hand, in castrated bovines it has been demonstrated that 37% of the conversion of testosterone in epitestosterone takes places in peripheral tissues like the blood and liver [38].

5α- and 5ß-reductases

The initial and rate limiting step in metabolism of 3-keto-4-ene steroids, such as testosterone, is the reduction of the C4-C5 double bond by 5α- or 5ß-reductase activity. Both 5α- and 5ß-reductases require NADPH as a cofactor and are located mainly in the liver but also in kidneys, adrenals, skin and testis. In humans, two isoenzymes of 5α-reductase are identified, namely type 1 and type 2; the former exists predominantly in the skin whereas the latter is located mainly in the prostate [39]. Intracellular, 5α-reductase is primarily located in the endoplasmatic reticulum while 5ß-reducatase is mainly located in the cytosol. The extent of 5α- and 5ß-isomers formed depends on the structure of the steroid such as differences in the D-ring structure. For example metabolism of testosterone to its 5α- and 5ß-isomers occurred in a ratio of 1:6 whereas for 17-keto-metabolites (androsterone and etiocholanolone) the ratio was 1:1 [40]. In cattle 5α and 5ß metabolites of testosterone and its corresponding 17-keto metabolites are known,

but no detailed information is available on their 5α/5ß ratio.
In relation to bioactivity the orientation of these hydrogen atoms at the C5 position is very important. This can be illustrated by the relative androgenic potencies (RAP) of eight isomers of androstanediol which vary in α- and ß-configurations of the hydrogen atom at the C5 position and the hydroxyl-groups attached at the C3 and C17 position of the steroidal skeleton (Figure 6).



5x-Androstane-3x,17x-diol

H	C_3	C_{17}	RAP
α	α	α	nr
α	α	β	0.15
α	β	α	nr
α	β	β	0.22
β	α	α	nr
β	α	β	nr
β	β	α	nr
β	β	β	0.0033

Figure 6: Eight isomers of androstanediol and their relative androgenic potencies (RAP) as determined in the RIKILT yeast androgen bioassay (extracted from Bovee et al. [41]) (nr = no response).

It is shown that the more active compounds have a 5α-configuration which results in a more straightened steroidal structure which better fits the ligand binding pocket of the androgen receptor. Compared to 5α-androstane-3ß,17ß-diol, the 5ß-iosmer has a relative androgen potency (RAP) that is about 67 times lower than that of the 5α-isomer. In addition, it is shown that the ß-configuration of the hydroxyl group located at C17 seems to play an important role in ligand binding, as all isomers with the hydroxyl group in the 17α-position are not active [41].

Hydroxylation by cytochrome P450 enzymes
Endoplasmatic reticulum-bound cytochrome P450 enzymes play an important role in the oxidative metabolism of lipophilic compounds such as steroids [42]. In mammals the microsomal cytochromes are predominantly present in hepatic tissue where they catalyze NADPH-dependent mono-oxygenation reactions. In contrast to the extensive knowledge about human and rodent cytochrome P450 enzymes, the characterization of the

cytochrome P450 system in livestock is still far from clear [43]. Although these reactions are highly species as well as compound specific, cytochrome P450 3A is considered to be the most important enzyme family involved in steroid hydroxylation.
Within the scope of steroid urine profiling in humans, formation of hydroxy-metabolites upon DHEA and 4-androstenedione administration is extensively studied and reviewed by Van de Kerkhof [44]. For DHEA the main hydroxylation pathways are 7α-, 7ß- and 16α-hydroxylation [45,46]. 16α-hydroxylation of DHEA to 16α-OH-DHEA is one of the most described hydroxylation reactions of DHEA as this metabolite is present in relatively high levels in urine [46, 47]. In addition to the hydroxy-metabolites also 7-keto-DHEA has been detected in human urine which is probably formed through dehydrogenation of the 7α- or 7ß-OH-metabolites of DHEA. Based on in vitro as well as in vivo experiments the main reactions for 4-androstenedione are 4-, 6ß-, 16α-, 16ß- and 19-hydroxylation. The fact that for 4-androstenedione the same pathways are found as for hydroxylation of testosterone [48] suggests that these reactions are specific for androst-4-ene-3-one steroids. In cattle, data about hydroxylation of steroids is limited to experiments with 17ß-testosterone [49].

Aromatase
Aromatase (CYP19) is also a cytochrome P450 enzyme which converts androgens into estrogens by aromatizing the steroidal A-ring using NADPH as a cofactor [50]. In most vertebrates aromatase enzyme activity is observed in the gonads, brain, placenta, adipose tissue, bone and various fetal tissues [51]. Among species the aromatase protein is highly conserved and compared to humans, bovine aromatase shows 86% homology in amino sequence identity and exhibits equal enzyme activity in the gonads [52].

Phase II metabolism
Phase II reactions involve conjugation reactions of the polar functional groups formed as a result of phase I metabolism. These conjugation reactions occur at polar moieties like hydroxyl and carboxyl groups resulting in increased water solubility and consequently a more effective excretion via the urine. For steroids the main phase II reactions are sulfonation and glucuronidation which occur at the hydroxyl moiety at the C-3 and/or C-17 position of the steroid molecule. In humans, it is observed that 3α-hydroxy steroids are mainly conjugated with glucuronic acid while the 3ß-hydroxy steroids predominantly show sulfate conjugation [40]. Sulfoconjugation involves the transfer of a sulfonate (SO_3^-) from a donor molecule to the hydroxyl acceptor site. These reactions are catalyzed by sulfotransferases, which are located on the membranes of the Golgi complex and in the

cytosol, and require the cofactor 3'-phosphoadenosine 5'-phosphosulfonate (PAPS) which is the sulfonate donor for all sulfotransferase reactions [53]. In humans sulfates of steroid precursors, especially DHEA-sulfate, play an important role as precursors in the peripheral biosynthesis of active steroid hormones. In order to become active the sulfate group is removed by sulfatase activity.

The main site of glucuronidation is the liver, although glucuronidation also is observed in intestines, kidney and prostate tissue. The enzyme family responsible for catalyzing glucuronidation are called uridine diphosphate-glucuronosyltransferases (UGT) which include multiple isoforms and catalyze conversion of a wide variety of exogenous and endogenous compounds. The two families of UGT (UGT1 and UGT2) consist of more than 35 enzymes found in various species [54]. In humans, mainly the isoenzyme UGT1A and UGT2B subfamilies are involved in steroid hormone phase II metabolism [55] where UGT2B17 is one of the main human UGTB isoforms with a high affinity for C19 steroids such as DHEA, testosterone and dihydrotestosterone [56].

Natural occurring levels of steroid prohormones

The biological role and endogenous levels of prohormones, like DHEA, has been extensively studied in humans, primates and rodents and showed significant differences in blood plasma levels between species [57]. In most mammals plasma DHEA and DHEA-sulfate levels are in the low nanomolar range. In humans, DHEA is also present in the nanomolar range while DHEA-sulfate is present at micromolar levels [58]. This is due to the high secretion rate of sex steroid precursors by the adrenal glands in humans which is completely different from laboratory animals like rats, mice and guinea pigs where no significant amounts of steroids are generated outside the gonads [59]. Also in bovines the adrenal contribution to plasma DHEA is believed to be limited [60]. Although the base levels of potent androgens and estrogens in cattle have been investigated intensively in the past, data describing endogenous levels of steroid precursors are limited (Table 1). In bull calves circulating DHEA plasma level were shown to be in the range of 100-1100 pg/ml [61,62] which is interestingly not significantly different from levels observed in heifers and dairy cows [61,63]. In cows levels of DHEA-sulfate were found to be significantly lower than those of DHEA [57,60] suggesting that DHEA (and not DHEA-sulfate) may be considered as the most important circulating precursor of androgens and estrogens in bovines. Steroid precursor levels in beef from bulls and steers are approximately 1000 times lower than levels found in plasma. In beef from bulls median values were reported to be 670 pg/mg for pregnenolone, 10 pg/mg for 17α-hydroxyprogesterone, 240 pg/mg for DHEA and 390 pg/mg for 4-androstendione [64].

Table 1: Plasma levels of steroid precursors (in pg/ml) observed in bulls and heifers as determined by radioimmunoassays (RIA).

	Bulls (n=5)	Bulls (n=10)	Heifers (n=10)	Heifers (n=6)	Cows (n=12)
Age	13-14 months	9-11 months	Unknown	12-14 months	3-5 years
Reference	[62]	[62]	[61]	[63]	[63]
Pregnenolone	nd	250-1200	250-4000	nd	nd
17α-Hydroxyprogesterone	nd	20-100	25-325	nd	nd
DHEA	557 (± 44)	100-1100	50-400	173-259	317-374
4-Androstenedione	nd	25-140	10-100	nd	nd

nd = not determined.

Anabolic actions of prohormones

Anabolic and androgenic actions of prohormones like DHEA appear to occur primarily through the metabolites formed in peripheral tissues. Hereby, the rate of formation of sex steroids strongly depends on the level of expression of specific androgen and estrogen synthesizing enzymes in the tissue. When the site of formation is the site of action this phenomena is described as 'intracrinology' [65]. Through this intracrine activity, locally produced androgens and estrogens exert their action in the same cell in which the synthesis takes place. [58]. Inside the cell these potent steroids exert their activity by binding to hormone specific receptors. After dimerization, the receptor-ligand complex undergoes conformational changes and is translocated to the nucleus. Upon binding to the DNA, hormone specific gene transcription is initiated and followed by translational processes and protein synthesis.

In the illegal circuit and on the internet a wide variety of prohormone containing supplements and creams are marketed which claim to enhance active hormone levels in the body [18]. In principle the justification of these claims are based on the activity of steroid enzymes present *in vivo* which catalyze reactions such as:

- Conversion of an α- or β-hydroxyl group attached to C3 by 3α-HSD or 3ß-HSD enzymes in a C3-oxo group.

- Conversion of the $\Delta^{5(6)}$ double bond to a Δ^4-C3-oxo group by Δ^4,Δ^5-isomerase activity. This conversion probably also takes place under influence of the acidic environment in the stomach.
- Combined conversion of a $\Delta^{5(6)}$ double bond in combination with the 3ß-hydroxyl group by 3ß-HSD/Δ^4,Δ5-isomerase in a Δ^4 steroid with a C3-oxo group.
- Conversion of a C17 oxo group by 17ß-HSD enzymes in a 17ß-hydroxyl group [66].

In earlier days, only prohormones which are part of the testosterone biosynthesis pathway like DHEA, 5-androstenediol, 4-androstenedione and even pregnenolone were marketed. This however expanded to compounds which are not (directly) part of the steroid biosynthesis pathway like 4-androstene-3ß,17ß-diol (which exhibits also direct anabolic and androgenic activity) and precursors of synthetic steroids like boldione which can be easily converted to their active equivalents by 3ß-HSD or 17ß-HSD activity [67] (Figure 5).

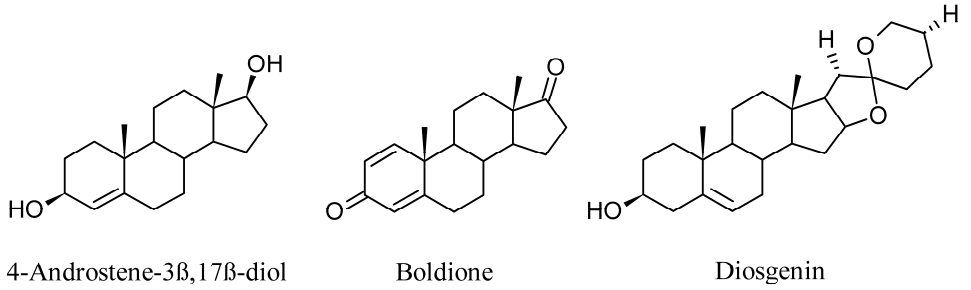


Figure 7: Molecular structures of prohormones.

But also compounds with saturated steroid nuclei like precursors and metabolites of dihydrotestosterone (DHT) are sold as prohormones. This in spite of the fact that DHT itself exhibits mainly androgenic activity and only limited anabolic activity. These compounds cannot serve as precursors of 17ß-testosterone, which is a more anabolic active compound, as the reduction of the double bond between C4 and C5 is irreversible. Also supplements containing herbs, herbal extracts and plant derived compounds are marketed for their prohormonal and subsequent anabolic activity. Compounds like diosgenin [68] (Figure 7) and fytosterols such as ß-sitosterol [69] are suggested to elevate androgen levels via the DHEA-pathway. However, the anabolic properties of these kinds of compounds are even more questionable and not much is known about the *in vivo* mode of action as well as metabolic transitions, especially not in bovines.

1.3. Bioassays for androgen activity screening

Although large differences in androgenic activities are observed between anabolic steroids, no anabolic compound is known without any androgenic property. Therefore bioassays developed for screening for anabolic steroids mostly use androgenic effects (e.g. binding to the androgen receptor) as an endpoint. In this paragraph a short overview is given of the types of bioassays used for screening on androgen activity and their response upon exposure to prohormones.

In vivo bioassays

The golden standard for evaluating androgen receptor (AR) agonistic and antagonistic properties of compounds is still the Hershberger assay [70]. This *in vivo* assay is employed by using either immature or castrated rats receiving the substance under investigation by oral gavage or subcutaneous injection for a period of ten consecutive days. When screening for potential anti-androgenic activity the rats are co-exposed to the test substance and testosterone propionate. The weights of five different androgen dependent tissues are determined: the ventral prostate, seminal vesicles, levator ani-bulbocavernosus muscle, the paired Cowper's glands and the glans penis. When, compared to a control group, the weight of two of these tissues are statistical significantly changed, the compound under investigation is scored positive for androgenic or anti-androgenic activity. Data presented in Table 2 show androgenic activity, anabolic activity and the average anabolic/androgenic quotient (Q) of four steroidal precursors [71]. After subcutaneous injection of the test compound weights of the ventral prostate, seminal vesicles and levator ani muscle were determined using 17ß-testosterone as the biological standard of comparison which was set to 100. DHEA and 5-androstene-3ß,17ß-diol showed limited androgen and anabolic activity while the activity of 4-androstenedione was a factor 2-3 less than that of 17ß-testosterone and 4-androstene-3ß,17ß-diol however showed androgenic and anabolic effects comparable to 17ß-testosterone.

Table 2: Androgenic and anabolic activity and average anabolic/androgenic quotient (Q) of steroidal precursors in rats. The standard of biological evaluation is expressed as 100% activity [71].

	Androgenic activity	Anabolic activity	Q
DHEA	<10	<10	-
4-Androstenedione	30-40	30-50	1
5-Androstene-3ß,17ß-diol	20	10	0.5
4-Androstene-3ß,17ß-diol	125	95	0.8
17ß-Testosterone	100	100	1

Application of such *in vivo* tests to measure androgenic activity has the advantage of including the ADME-parameters; absorption, distribution, metabolism and excretion and can also describe the disposition of a compound within an organism. However, these *in vivo* tests are slow, expensive, difficult, and not very sensitive due to differences in preparation and weighing of the tissue involved. As a result, and for obvious ethical reasons, this animal test is not suitable for routine high through-put screening.

In vitro bioassays

As an alternative for *in vivo* bioassays various *in vitro* bioassays have been developed for use in clinical practice as well as for screening purposes on (anti)androgenic activity of chemicals and environmental samples or when steroid hormone abuse is suspected [9,72]. In general, the principles of these *in vitro* bioassays are based on either receptor binding, cell proliferation (the A-screen) [73], or receptor binding in combination with transcription activation of reporter genes. Receptor binding assays using androgen receptor ligand binding domains are the most simple *in vitro* models to describe the affinity of a ligand for the androgen receptor [74-76]. The binding affinity of DHEA to the androgen receptor was observed to be 2000 times lower than that of 17ß-testosterone, 50 times lower than that of 4-androstene-3ß,17ß-diol and 20 times lower than that of 4-androstenedione and 5-androstene-3ß,17ß-diol (Table 3). In addition DHEA showed also affinity for the estrogen receptor. Hereby the ERß was preferred over the ERα [77].

Table 3: Relative androgen receptor binding affinity (RBA) of prohormones and 17ß-testosterone compared to DHT [74].

	RBA
DHEA	0.037
4-Androstenedione	0.86
5-Androstene-3ß,17ß-diol	0.79
4-Androstene-3ß,17ß-diol	1.7
17ß-Testosterone	69
DHT	100

The main disadvantage of receptor binding assays is that they cannot distinguish agonistic from antagonistic properties as the transcription activation step is not included. For this purpose several receptor based transcription activation reporter gene assays have been developed, based on mammalian as well as yeast cells, each having their own advantages and disadvantages.

To date, various stably transfected mammalian cell based reporter gene assays have been developed making use of different cell types and reporter genes [78-84]. The most frequently used reporter genes encode for proteins like ß-galactosidase, luciferase or chloramphenicol acetyl transferase which convert a specific substrate in metabolites with easily measurable luminescence or fluorescence signals. These kind of assays are shown to be very sensitive and considered as biologically relevant for screening on androgenic effects. However, in response to a given test substance not all bioassays show identical results due to differences in metabolic capacities or due to the presence/lack of co-regulators (Table 4). Metabolism by reporter cells results in either activation or inactivation of hormones and subsequent alteration of the bioactivity read out. The latter is not necessarily a disadvantage, in particular when screening for compounds that need metabolic activation, like prohormones. In a recombinant Chinese hamster ovary (CHO) cell line, DHEA showed an EC50 of 1 nM and a relative androgenic potency (RAP) of 0.015 compared to DHT [82] (Table 4). This response is mainly caused by the metabolites of DHEA formed by 3ß- and 17ß-HSD enzyme activity and does not represent the androgenic activity of DHEA. These results indicate that this cell line could be useful for prohormone screening purposes. Other mammalian reporter assays based on MDA human mammary carcinoma cells, human prostate adenocarcinoma PC-3 cells (PALM) and U2-OS bone cells (AR-CALUX) are showing consistent results for DHT, as only a factor 2-3 difference in response is observed between these assays (Table 4). For 4-androstenedione the responses are less consistent and EC_{50} values are observed between 4.5 and 140 nM. The EC_{50} for 4-androstenedione as observed in the MDA and PALM cells is in the same order of magnitude as the response found in the A-screen [73,85]. The EC_{50} observed in the AR-CALUX is thus relatively low and this might be due to the conversion of 4-androstenedione into 17ß-testosterone. DHEA does not show a response in both the AR-CALUX and the MDA cell line which is probably representing its actual androgenic activity. Another androgen reporter assay is based on human embryonic kidney (HEK) 293 cells expressing the human AR and androgen responsive luciferase [80]. Although no exact EC_{50} values are reported for DHEA, 4-androstenedione and 5-androstene-3ß,17ß-diol, all three compounds clearly showed transcription activation in the range of 0.3-100 nM, most likely again as a result of their enzymatic conversion.
Another factor which may reduce specificity in mammalian cell lines is the presence of other endogenous receptors like the progesterone and glucocorticoid receptor (PR and GR). This crosstalk occurs due the fact that the consensus androgen responsive element (ARE) equals those of progesterone and glucocorticoids. As a consequence AREs are also

Table 4: EC_{50} concentrations of prohormones and androgens obtained in various androgen reporter gene bioassays.

			EC_{50} (nM)				
Ref.	Cell system	Endpoint	DHEA	4-AD	5-Adiol	17ß-T	DHT
[84]	AR-CALUX reporter assay derived from U2-OS osteocarcinoma cells	Luciferase activity	>10000	4.5	-	0.66	0.13
[82]	Chinese hamster ovary (CHO) cells.	Luciferase activity (whole cell)	1	0.3	0.2	0.04	0.015
[77]	MDA-MB-453 human mammary tumor cells.	Luciferase activity	nr	-	-	0.5-1	0.5-1
[85]	MCF-7-AR1 human mammary adenocarcinoma cells (A screen)	Cell number	-	59	-	-	0.078
[85]	Human prostate adenocarcinoma PC-3 cells. (PALM)	Luciferase activity	-	35	-	-	0.11
[85]	Chinese hamster ovary (CHO) cells.	Luciferase activity	-	0.27	-	-	0.022
[85]	MDA-MB-453-KB2 human mammary carcinoma cells.	Luciferase activity	-	140	-	-	0.21
[90]	*Saccharomyces cerevisiae*	Luciferase activity	-	500	-	10	-
[91,92]	*Saccharomyces cerevisiae*	EGFP fluorescence	nr	7200	-	76	33

nr = no response; 4-AD = 4-androstenedione; 5-Adiol = 5-androstene-3ß,17ß-diol; 17ß-T = 17ß-testosterone.

activated upon binding by glucocorticoid and progesterone ligand-receptor complexes [86]. Alternatives are found in application of reporter gene assays using host cells which show no steroid metabolism and do not express any endogenous steroid receptors like for instance yeast. Most androgen yeast bioassays developed are based on a *Saccharomyces cerevisae* host strain using either ß-galactosidase, luciferase or enhanced green fluorescent protein (EGFP) as a reporter gene [87-91]. In general, it is shown that these yeast assays are a factor 10-100 less sensitive as compared to mammalian cell based assays (Table 4).

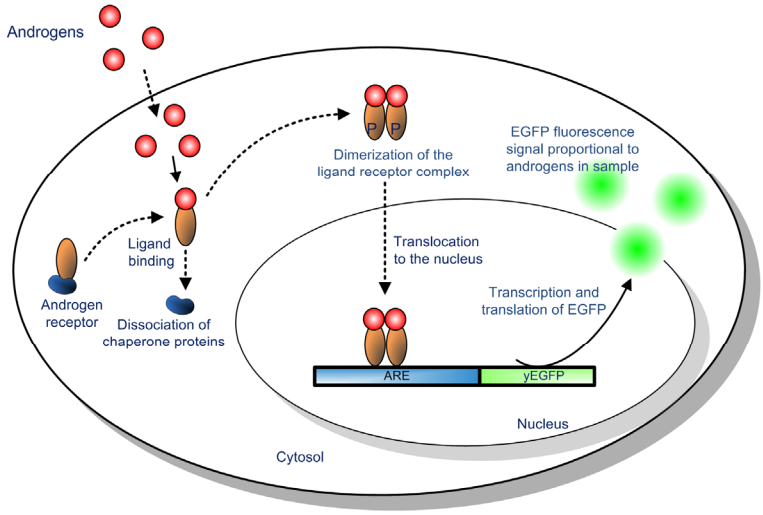


Figure 8: Schematic representation of the mechanism of action of the RIKILT Androgen yeast Assay (RAA).

On the other hand, yeast assays are found to be more robust than mammalian cell assays and can be used for screening of complex biological samples like animal feed, without extensive clean-ups [92]. In this thesis work we used the RIKILT Androgen bioAssay (RAA) which is based on a *Saccharomyces cerevisae* host strain which constitutively expresses the human androgen receptor and possesses an androgen responsive element (ARE) coupled to an enhanced green fluorescent protein (EGFP) reporter system [91] (Figure 8). Upon exposure of this yeast, androgens bind to the human androgen receptor which is present in the cytosol of the cell. After dimerization, the ligand-receptor complex undergoes conformational changes, which enable binding to the androgen responsive element. After translocation to the nucleus, binding to the androgen responsive element results in transcription of the yEGFP reporter gene. The formation of EGFP can be quantified by measuring the fluorescence signal using excitation at 485 nm and emission at 530 nm. This activation of gene transcription takes up to several hours and results in a measurable response already after 6 hours of incubation. However, for practical reasons

dose response curves are normally obtained after 24 hours of incubation, which is a time point that maximum response is reached enabling a more accurate determination of the EC_{50}.

1.4. Advanced mass spectrometry of steroids and metabolites

In the field of residue analysis there is a tendency in moving from targeted methods, mainly based on liquid or gas chromatography (LC or GC) in combination with triple quadropole mass spectrometric (MS-MS) detection, towards full scan MS techniques such as Time-Of-Flight (TOF) and Fourier Transform (FT) Orbital Trap (Orbitrap) or Ion Cyclotron Resonance mass spectrometry [93]. Hyphenation with improved separation techniques like ultra performance liquid chromatography (UPLC) lead to increased chromatographic resolution, speed, peak capacity (number of peaks resolved per unit time in a gradient separation) and sensitivity which allows a more complete analysis of complex biological samples like cell extracts, urine and supplements [94]. Moreover compared to traditional GC- and LC/MS-MS methods which target only preset ions or ion transitions of known molecules, full scan accurate mass spectrometry offers additional possibilities such as retrospective analysis (e.g. determining the presence of newly identified compounds in previous analyses), development of multi-compound/multi-class techniques and the identifications of new (unknown) compounds by accurate mass measurement, elemental composition elucidation assessment and structure elucidation.

Table 5: Number of elemental compositions obtained for the $[M + H]^+$ ions of DHEA (*m/z* 289.2168) and DHEA-glucuronide (*m/z* 465.2488).

		Mass accuracy		No. of
Compound	*m/z* $[M+H]^+$	ppm	mDa	elemental compositions*
DHEA	289.2168	5	1.4	1
		20	5.8	2
		50	14.5	3
DHEA-gluc	465.2488	5	2.3	2
		20	9.3	6
		50	23.3	20

*Elemental composition restrictions: $C_{0–50}$, $H_{0–100}$, $O_{0–10}$, $S_{0–5}$ and double bond equivalent −0.5 to 20, even number of electrons only.

Elemental composition searches limit the number of possible compound identities tremendously when searching in molecule databases such as Scifinder or the Merck index [97 ,98]. The accurate mass of an ion is calculated as the sum of the individual exact masses of all the atoms in the ionized molecule. For example the monoisotopic mass of ^{1}H=1.0078, ^{12}C=12.0000 and ^{16}O=15.9949. In natural steroid hormone analysis, masses of relevant compounds are typically in the range of 200-500 Da and elemental compositions include the atoms C, H, O occasionally S like as for steroid sulfates. When assuming that the mass spectrometer has been calibrated well a mass accuracy of < 5 ppm can point to a single elemental composition option as shown in Table 5 for the DHEA $[M+H]^+$ ion at *m/z* 289.2168 ($C_{19}H_{29}O_2$). A mass accuracy of 20 ppm yields only two elemental composition options ($C_{19}H_{29}O_2$ and $C_{16}H_{33}O_2S$) and 50 ppm already yields three elemental compositions ($C_{19}H_{29}O_2$,$C_{16}H_{33}O_2S$ and $C_{16}H_{33}S_2$). The number of possible formulae increases exponentially as the mass accuracy further decreases and as the molecular mass of a compound increases. As shown for DHEA-glucuronide (*m/z* 465.2488) already six possible elemental compositions within 20 ppm mass accuracy are obtained. The factors affecting mass accuracy typically include the design of the mass analyzer and (the stability of) its mass calibration. The TOFMS instrumentation used in this thesis research allowed the acquisition of accurate masses within 5 ppm, however occasionally only 20 ppm could be achieved.

Subsequent searches of the obtained elemental composition in comprehensive electronic databases such as Scifinder can narrow down the number of tentative identities significantly. In this case $C_{19}H_{28}O_2$ (DHEA) initially gave 2439 possible compound options in Scifinder. After refining this search by only looking for C19 steroid structures and exclusion of isotopes, 136 options were remaining. Since accurate mass measurements as performed by high resolution TOFMS are specific and universal for every compound regardless of the instrumentation used, TOFMS enables the use of accurate mass databases in reducing the number of potential identities [99,100]. In this way accurate mass databases have been used for the identification of androgenic compounds in herbal mixtures and sport supplements [100]. In addition information about isotopic peak ratios, expected retention time and knowledge about the nature of the sample should be used to refine the search leading to the possible identity of the compound. Nevertheless it should be noted that one exact mass yields multiple structural formulas and that within one molecular formula multiple structural isomers are possible (as for example for 5-androstanediol, see Figure 6).

Moreover, controlled MS-MS fragmentation studies in a quadropole-TOF or ion trap-Orbitrap hybrid mass spectrometer can assist in defining substructures and further refine

the number of molecule options. It should be noted however that mass spectrometric structure elucidations have a tentative character and should be preferably complemented by NMR studies. Unfortunately, in practice NMR is often not sensitive enough and the sample matrix too complex. Therefore in EU legislation for residue analysis mass spectrometric comparison with a pure reference standard is being used, thus providing a confirmation of a proposed structure.

1.5. Metabolomics based urine profiling

Historically, steroid urine profiling originates from the field of clinical endocrinology where it is used to detect enzyme deficiencies in newborns by monitoring levels of endogenous steroids in urine [101]. This methodology was adapted and introduced in human doping control in 1983 by Donike et al. [35] and since then has proven its value in detecting illegal use of endogenous steroids by determining levels and ratios of endogenous steroids in urine [35,102,103]. For the detection of these endogenous steroids statistically based threshold values for selected screening parameters have been set. This primarily concerns levels of the parent steroids or ratios such as 17ß-testosterone/17α-testosterone (T/E ratio), androsterone/etiocholonalone, androsterone/17ß-testosterone and 5α/5ß-androstane-3α,17ß-diol [104] but also monitoring of minor (phase I) metabolites are considered as valuable markers for the administration of endogenous steroids [105]. For screening purposes usually a set of endogenous urinary steroids or metabolites is quantified by GC/MS [106,107] offering a suitable basis for individual as well as population-based reference ranges and thus discrimination between undisrupted and altered steroid profiles.

Although limited scientific publications are available regarding alteration of urinary steroid profiles as a consequence of DHEA administration, several urinary profiling parameters for DHEA abuse have been proposed [44,102,103,108,109]. These studies were most likely also the basis for setting the DHEA threshold value at 100 ng/ml for doping control purposes [110]. Recent studies however, demonstrated that urinary DHEA values higher than 100 ng/ml are observed in part of the normal human population [111,112] and therefore alternative threshold values of 200 ng/ml for both DHEA and the DHEA metabolite 3α,5-cyclo-5α-androstane-6ß-ol-7-one have been proposed [112].

When an atypical steroid profile is observed, this usually is followed by confirmation techniques such as gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), a technique to differentiate between endogenous and exogenous steroids based on difference in carbon isotope ratios ($^{13}C/^{12}C$) [113]. Since this is a very complex, time consuming and costly methodology not all samples can be applied to GC/C/IRMS

and hence screening techniques like steroid urine profiling still play a crucial role in detecting misuse of endogenous steroids in doping control.

In livestock production however, misuse of natural occurring (pro)hormones is hard to prove; urinary metabolites are either unknown or profiling parameters which are relevant in humans are failing in cattle due to highly fluctuating endogenous steroid levels or differences in metabolism [114]. On the other hand, recent studies in calves showed that the endogenous steroid profiles were affected upon administration of nandrolone and estradiol [115]. In particular nandrolone administration lead to changes in estranediol profiles which may be used as screening parameters in this context [116]. These findings illustrate that steroid profiling methods have potential to be used as a screening tool in the field of veterinary growth promoter control.

Nevertheless, to facilitate efficient control of anabolic practices there is a need for the development of new more comprehensive screening tools which are able to detect misuse of natural (pro)hormones as well as (new) synthetic steroids in urine. Therefore, as an alternative for targeted steroid profiling, a more comprehensive semi-targeted screening method has been described, which includes also detection of (new) synthetic designer steroids [117]. This LC-MS/MS screening approach is based on the fact that steroids with (partial) common structures show similar product ions in MS/MS analysis which can be monitored by precursor scan acquisition. This approach was refined by Pozo et al. who proposed the combined acquisition of the precursor ion scans of *m/z* 105, *m/z* 91 and *m/z* 77 as a screening protocol for most anabolic steroids [118]. On the other hand, ongoing evolvements in chromatography, mass spectrometry (e.g. TOF and Orbitrap technology) and bioinformatics make it possible to obtain more complete chemical profiles of complex biological samples and enable unbiased profiling approaches which can be summarized in the term “metabolomics”. Application of metabolomics based techniques are providing a snap-shot of the metabolome of a cell, tissue, organ or organism at a certain time point. Unbiased holistic profiling of cellular metabolism products such as sugars, lipids and hormones is focused on measuring as many compounds as possible to generate metabolic fingerprints. Comparison of these fingerprints not only provides information on differences as a consequence of perturbations, e.g. a prohormone administration, but might also lead to identification of metabolites not previously reported to be affected by a treatment [119] (Figure 9).

The last few years metabolomics has also emerged in the field of veterinary growth promoter control as a potential screening tool for untargeted urine steroid profiling [120]. From a historical point of view, nuclear magnetic resonance (NMR) is the most widely used technique for metabolomics purposes. Using NMR combined with multivariate

statistics Dumas et al. [121] was one of the first showing how metabolic perturbations induced by hormonal treatment can be evidenced through characterization of bovine urine samples. However, because of higher performance in terms of sensitivity and specificity, mass spectrometry is becoming more and more used in the field of metabolomics [122]. In particular LC-based methods have shown to be good alternatives and are considered with great interest as a potential global screening tool in steroid metabolome profiling [123]. Recently, several studies in the field of growth promoter control have further demonstrated the efficiency of mass spectrometry based fingerprinting to discriminate between treated and untreated animals [124-126].

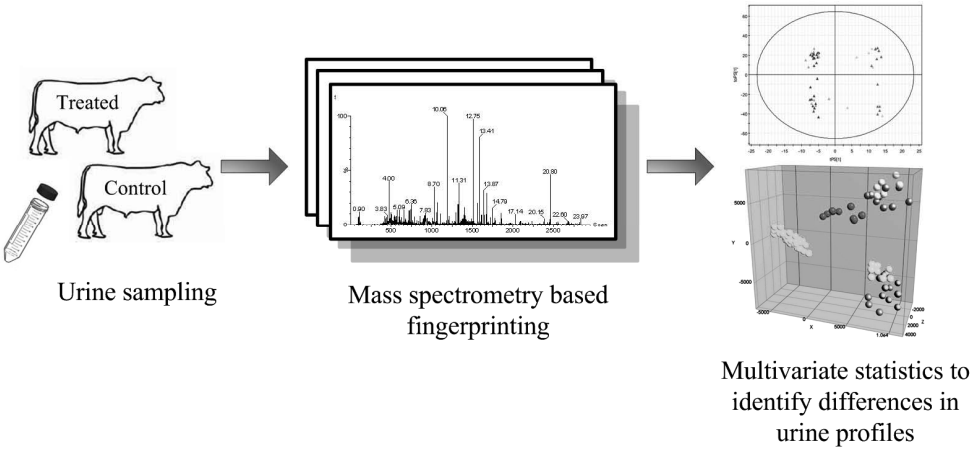


Figure 9: General overview of a metabolomics based strategy for anabolic steroid urine profiling in cattle.

Because full scan mass spectrometric analysis of complex biological samples is generating enormous amounts of data, these approaches demand a highly automated and structured way of data processing. For this purpose several sophisticated bioinformatics tools, such as MetAlign^{TM} have been developed to preprocess and align data allowing an efficient comparison of the obtained profiles [127]. In addition, multivariate statistical tools such as ANOVA, Principal Component Analysis (PCA) and Orthogonal Partial Least Squares analysis (OPLS) are employed to identify differences in urine steroid profiles. Besides highlighting metabolic disruptions upon anabolic treatment, these metabolomics based urine profiling approaches can also reveal new compound specific endpoints which can be used in targeted screening approaches. In this way Anizan et al. [124] demonstrated that PCA not only successfully discriminated between control and 4-androstenedione treated bovines, but also identified 5α-androstane-3ß,17α-diol, 5ß-androstane-3α,17α-diol, etiocholanolone and 5-androstene-3ß,17α-diol as potential

parameters to prove 4-androstenedione abuse. Since these steroids are most likely direct metabolites of 4-androstenedione these findings are underlining the power of such an untargeted approach. Another approach was utilized by Courant et al. [126] using OPLS for both prediction of class membership as well as determination of potential biomarkers for clenbuterol administration. Again it was demonstrated that OPLS-DA statistical analysis permitted discrimination between treated and untreated animals during the treatment course as well as well as several days after the treatment. Among the ions pointed out by OPLS analysis, creatine and 18-hydroxycortisol could be identified as candidate biomarkers for clenbuterol treatment of calves.

1.6. Transcriptome analysis

The transcriptome is the set of total RNA (mRNA, tRNA, rRNA) produced in a cell or tissue at a given time. Analysis of these transcripts provides insight in ongoing processes and can provide detailed mechanistic information on the response to external stimuli. With respect to surveillance on growth promoter abuse, quantification of mRNA can be an attractive complement to the already established (bio)chemical screening and detection methods [128]. Several potential mRNA biomarkers for steroid hormone abuse have been reported for liver [129] or matrices like blood which can also be sampled in the farm phase [130]. Classical methods to determine mRNA levels are Northern blotting and real time RT-PCR. These are targeted techniques for gene expression analysis, using a limited number of preselected genes. Evolvement of the microarray technology however, allows the analysis of thousands of transcripts simultaneously which can describe the whole transcriptome of a cell or tissue in one single experiment. Application of DNA microarrays is therefore becoming more and more the common method to describe the effects of a treatment. After establishing microarrays for humans and classical laboratory test animals like rats and mice, rapid progress in veterinary genomics resulted in the development of commercially available farm animal microarrays, including those for bovines [131]. Although several types of microarrays exist, the most commonly used microarray platforms are those supplied by Affymetrix and Agilent and are based on oligonucleotide probes synthesized/spotted on a glass slide or silicon chip. The use of these bovine microarrays not only can provide detailed information about cellular processes as a consequence of a treatment, but also allow detection of (new) biomarkers which can be used to screen for illegal use of growth promoters [132]. After exposure of cells, tissues or animals to a compound, mRNA is extracted and transferred into fluorescent labeled cRNA (Figure 10). Hereby mRNA is first converted into cDNA using MMLV reverse transcriptase and oligo dT-promoter primer. Subsequently, cDNA is

converted into cRNA by amplification using T7 RNA polymerase and labeled by incorporation of Cy3-CTP or Cy5-CTP. Upon loading of the fluorescent labeled sample onto the microarray, each specific cRNA will anneal to the complementary probe which is unique for a specific gene. By scanning the fluorescence signal intensity the amount of labeled cRNA hybridized to each probe can be determined and a ratio of expression can be calculated.

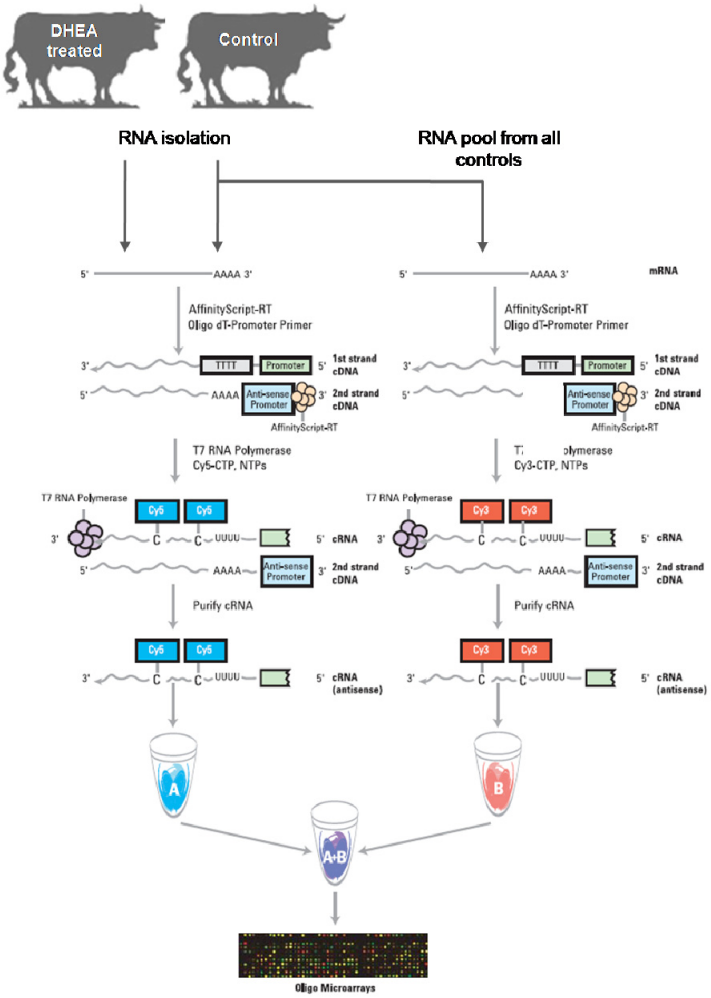


Figure 10: Schematic overview of a microarray experiment using a common reference design. mRNA is isolated from animal tissue, reverse transcribed and converted into fluorescent labeled cRNA using the Agilent microarray fluorescent cRNA synthesis procedure (Agilent Technologies). Cy5 and Cy3 labeled samples are mixed and hybridized on the microarray slide. After scanning, the normalized fluorescence intensities of the Cy5 channel are used to calculate the expression ratios between samples.

The microarray experiments performed in this thesis were using a so called common reference design; each sample (exposed as well as control) is individually labeled with Cy5 and a pool of all control samples is labeled with Cy3. The Cy3 fluorescence signals were only used to normalize the microarray data whereas the Cy5 fluorescence intensities were used to calculate the expression ratios between exposed and controls. Hybridization and scanning is followed by data analysis, starting with normalization [133] and removing of the low intensity spots. As the tissue under investigation does not express all the genes present on the microarray, also a so called 'flooring' procedure is applied. All spots below an arbitrary set threshold are set to that threshold. In this way the spots are not removed but identification of differentially regulated genes within background noise is prevented. In general, the next step is selection of differentially regulated genes by application of a threshold fold change together with a statistical derived *p*-value. To reduce the chance of false positives, most times also other statistical methods are applied to correct *p*-values for multiple testing e.g. by application of a false discovery rate (FDR) [134] or more comprehensive methods like Statistical Analysis of Microarrays (SAM) [135].
Genes above the arbitrary set threshold values are selected for further biological interpretation which is often the most challenging part of microarray analysis. This largely depends on data available in literature, databases such as those at the National Center for Biotechnology Information (NCBI) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [136,137], and a priori knowledge of the biologist who analyses the experimental data. In addition, several bioinformatics tools have been developed to get insight in the function of the genes found regulated and to get an overview of the pathways and processes affected [138,139].
Other approaches like principal component analysis (PCA) and cluster analysis [140] focus on identification of genes showing similar expression patterns. The multivariate statistics utilized by PCA allow a three dimensional visualization of gene expression profiles showing similarities and dissimilarities in the overall gene expression profiles. Alternatively, subsets of genes that contribute to differences between samples can be identified and compared to other gene expression profiles using tools like Gene Set Enrichment Analysis (GSEA) [141].

1.7. Aim and objectives

During inspections of livestock farms in The Netherlands a trend is observed towards abuse of feed and herbal additives and preparations containing prohormones. Up till now this primarily involves natural occurring prohormones like pregnenolone and DHEA,

which are direct precursors of potent androgens and estrogens. However, sometimes also plant derived compounds such as diosgenin or plant extracts are found. Of most of these compounds the *in vivo* effects are unclear and the anabolic action is questionable. Interestingly Council Directive 96/22/EC is based on bioactivity and prohibits the use of compounds with thyrostatic, oestrogenic, androgenic or gestagenic activity. To fully meet this legislation screening should therefore be focused on detection of hormonal activity rather than on detection of the prohormones themselves.

The main purpose of this thesis research is to get insight in the hormonal action of assumed prohormones and to develop novel (effect based) *in vitro* and *in vivo* screening methods allowing effective surveillance on the illegal use of prohormones in livestock production. This could be accomplished by monitoring in the farm phase, like sampling of animal feed, feed additives, urine and blood as well as sampling of tissues in the slaughterhouse phase. Within this scope the bioactivity of a wide variety of supplements, which contained assumed prohormones, were tested on bioactivity by using a yeast androgen bioassay (**Chapter 2**). For supplements containing solely prohormones the value of bioactivity based screening seems to be limited as prohormones show no direct bioactivity and need metabolism to become active. Therefore a screenings method was set -up by using this same yeast androgen assay in combination with bovine liver S9 enzyme fractions to mimic *in vivo* metabolic transitions (**Chapter 3**). Also conjugated hormones like steroid glycosides and esters are not active and do not show any response in bioactivity based screening methods. *In vivo* however, deconjugation of the various groups results in the availability of the bioactive aglycon and subsequent anabolic action. This deconjugation is studied using *in vitro* enzymatic or chemical hydrolysis methods followed by bioactivity screening (**Chapter 4**).

More comprehensive *in vitro* models like precision-cut bovine liver slices allow the combined study of primary hepatic metabolism of prohormones together with evaluation of the hormonal activity of the metabolites formed and their effects on the transcriptome (**Chapter 5**). Ultimately, these *in vitro* test methods could reduce the need for controlled animal studies with banned substances, hereby contributing to the 3 Rs: Reduction, Refinement and Replacement of animal tests.

For detection of prohormone abuse at the farm and/or the slaughter phase we investigated the usefulness of 'omics' based profiling techniques to discriminate treated animals from controls. In human doping control (targeted) profiling of urinary steroid levels is an established method to detect steroid (pro)hormone abuse. However, application of such steroid profiling methods in veterinary control is thought to be of limited value due to highly fluctuating endogenous steroid levels. C**hapter 6** describes a comprehensive

untargeted metabolomics approach to identify the differential regulated metabolites as a consequence of pregnenolone and DHEA treatment. It was aimed to compare the overall urine profiles of treated animals versus controls using multivariate statistics. In the same way gene expression profiling offers the possibility to look at the effects of a prohormone treatment at the transcriptome level. In **Chapter 7** the feasibility of DNA-microarrays as a screening tool for prohormone abuse was investigated at the slaughter phase. Hepatic gene expression profiles of bull calves treated with DHEA were compared with controls to determine differentially expressed genes and to identify biomarkers for DHEA treatment. Finally, the main results and findings are discussed in **Chapter 8**. Implications for further research, for effective surveillance on the illegal use of (pro)hormones and future perspectives are given in that chapter as well.

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Chapter 2

Detection of anabolic steroids in dietary supplements: The added value of an androgen yeast bioassay in parallel with a liquid chromatography-tandem mass spectrometry screening method

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Abstract

Recently we constructed a recombinant yeast cell that expresses the human androgen receptor (hAR) and yeast enhanced green fluorescent protein (yEGFP), the latter in response to androgens. When exposed to testosterone, the concentration where half-maximal activation is reached (EC_{50}) was 50 nM. Eighteen different dietary supplements, already analysed by a liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for the presence of anabolic steroids, were screened for androgenic activity. Eleven samples containing at least one anabolic steroid, with a concentration that was around or above 0.01 mg $unit^{-1}$ according to LC-MS/MS, were also positive in the bioassay. Seven samples did not contain any of the 49 compounds screened for in LC-MS/MS. In contrast two of them were positive in the bioassay. Bioassay-directed identification, using the bioassay as an off-line LC-detector and LC-time of flight-MS with accurate mass measurement was carried out in these two samples and revealed the presence of 4-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol in the first and 1-testosterone in the second supplement, showing the added value of the bioassay in comparison with a LC-MS/MS screening method alone.

1. Introduction

In order to achieve fair play and to fight doping, the World Anti-Doping Agency (WADA) prohibited the use of anabolic steroids in sports. This list of prohibited steroids has grown continuously and several steroids, especially designed to circumvent doping control, have been found. The use and misuse of these substances has been reviewed by Van Eenoo and Delbeke in 2006 and starts with the subcutaneous injections of a liquid obtained from the testis of animals in 1889 and finds its tentative culmination in the discovery of the designer steroid tetrahydrogestrinone (THG) in 2004 [1–3]. The rapid development of mass spectrometry resulted in specific screening methods for anabolic steroids in the 1980s, but the abuse of the endogenous steroid testosterone could not be detected until the testosterone to epitestosterone (T/E) ratio was introduced as a biomarker [4]. Unfortunately, naturally elevated levels of testosterone can occur in some cases and the biomarker ratio (T/E) is not a 100% trustworthy test [5]. In the 1990s several new steroids were commercialised as nutritional supplements. Initially these new steroids were precursors of testosterone, commonly referred to as prohormones, but later on a range of prohormones derived from other steroids than testosterone, including 19-nortestosterone, boldenone and 17α-alkylated steroids, also became available as over-the-counter preparations. The 17α-alkylated steroids were designed to block the metabolism of the 17β-hydroxyl group, which is crucial for androgenic activity, and to improve the oral bioavailability. The 19-norandrogens were designed as anabolic steroids in order to minimise undesirable androgenic side effects. Although the ratio of anabolic versus androgenic activity of 19-norandrogens is improved, most 19-norandrogens have both greater anabolic and androgenic activities [6].

Since 2005 there has been a ban on dietary supplements containing prohormones in the USA, but in order to improve their performance athletes, bodybuilders and even life stylers are tempted to use these kinds of nutritional supplements. Several investigations have shown that the information on the label of these supplements is often misleading or incomplete [7] or that supplements are contaminated with low concentrations of anabolic steroids such as testosterone and stanozolol [8]. In the light of these reports some dietary supplements that were ordered through the internet and intercepted by the Belgian pharmaceutical inspection at the post-office, were analysed by means of a liquid chromatography–tandem mass spectrometry method (LC-MS/MS) for the presence of prohibited anabolic steroids [9]. Results revealed the presence of active anabolic steroids in over 60% of these supplements and it was concluded that most likely the steroids were not deliberately added, but were by-products formed during the synthesis of the prohormones.

Due to the great variety of chemicals with hormone-like activity, both immunochemical and analytical chemical methods have the drawback that they only detect target compounds and are not able to determine biological activity of unknown compounds and their metabolites, this in contrast to biological assays. Receptor-based transcription activation assays can be used to detect all compounds having affinity for a given receptor, both agonists and antagonists [10–13]. Recently we developed a yeast androgen bioassay that expresses yeast enhanced green fluorescent protein (yEGFP) as a measurable reporter protein in response to androgens [12]. This assay is sensitive and highly specific for androgens. Moreover, the assay was proven to be useful to detect the new designer steroid THG in human urine [13] while prohormones such as dehydroepiandrosterone (DHEA), are not active and need metabolic activation before they can be detected [14]. To investigate the performance of this new yeast androgen bioassay 18 dietary supplements, already analysed by an LC-MS/MS method for the presence of anabolic steroids [9], were screened for androgen activity in the bioassay and the outcomes of both methods were critically compared.

2. Materials and methods

2.1. Chemicals

17β-Estradiol, 19-nortestosterone (nandrolone) and progesterone were obtained from Sigma (St. Louis, MO, USA) and 17β-boldenone, 1-testosterone, 4-androstene-3β,17β-diol, 5-androstene-3β,17β-diol, 5α-androstane-3,17-dione, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, 5β-androstane-3β,17β-diol, 17α-testosterone and 17β-testosterone were obtained from Steraloids (Newport, RI, USA). Acetic acid, dimethyl sulfoxide (DMSO), sodium carbonate and sodium acetate trihydrate were obtained from Merck (Darmstadt, Germany). Acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). Chemicals to prepare the growth media for yeast and the preparation of standard solutions in DMSO were as described previously [12]. Water used for LC/TOFMS was purified using a Millipore model Milli-Q system (Bedford, MA, USA).

2.2. Extraction procedure

A stock solution of 17β-testosterone was prepared in DMSO (50 μg mL^{-1}). Supplement samples were grinded and two portions of 100 mg were weighted. One of the 100 mg portions was spiked with 10 μL of the 50 μg mL^{-1} 17β-testosterone stock solution, resulting in a final spiked amount of 5 μg g^{-1}. For extraction, 100 mg of the (un)spiked

supplement samples were mixed with 4 mL methanol and 4 mL sodium acetate pH 4.8. Samples were sonicated for 10 min in an ultrasonic bath and subsequently mixed for 15 min head over head. Samples were centrifuged at 3000 × *g* for 10 min and 4 mL of the upper liquid phase was brought into a glass tube. The pH was adjusted to 4.8 using 4N acetic acid and the extract was subjected to solid-phase extraction (SPE) on a C18 column (0.5 g Varian Bond Elut, Harbor City, CA, USA) previously conditioned with 3 mL methanol and 3 mL sodium acetate pH 4.8. Subsequently, this C18 column was washed with 1.5 mL sodium acetate pH 4.8, 2 mL water, 1.5 mL 10% (w/v) sodium carbonate solution, 2 mL water and finally with 2 mL methanol/water (50/50, v/v). The column was air-dried and eluted with 4 mL acetonitrile. The eluate was applied to a NH_2-column (0.1 g IST, Hengoed, U.K.) that was previously conditioned with 4 mL acetonitrile. The eluate thus obtained was evaporated to dryness under a stream of nitrogen gas and reconstituted in 4 mL acetonitrile. Tenfold dilutions were made in acetonitrile and aliquots of 200 µL were transferred to a V-shaped 96-well plate in triplicate and 50 µL of a 4% DMSO solution was added to each well. To remove the acetonitrile, the plate was dried overnight in a fume cupboard and was then ready to be screened on androgenic activities with the yeast androgen bioassay. Aliquots of undiluted and diluted sample extracts were also investigated with 17β-testosterone spikes afterwards. These extracts spiked after the cleanup were prepared by the addition of 2 µL of a 30-µM 17β-testosterone stock, resulting in a final concentration of 300 nM in the well after adding the 200 µL of the yeast culture. In the same way and in each separate experiment a reagent blank, a negative feed, a negative supplement and corresponding spikes were prepared and used as negative and positive controls.

2.3. Yeast androgen bioassay with fluorescence measurement

The day before running the assay, a single colony from a MM/L agar plate was used to inoculate 10 mL of the selective MM/L medium [12]. This culture was grown overnight at 30 °C with vigorous orbital shaking. At the late log phase, the yeast AR cytosensor was diluted in the selective MM/L medium to an OD value at 630 nm between 0.04 and 0.06. For exposure to standard compounds, aliquots of 200 µL of this diluted yeast culture were pipetted into each well of a 96-well plate and 2 µL of a 17β-testosterone or other stock solution in DMSO was added. DMSO and 17β-testosterone only controls were included in each experiment and each sample concentration was assayed in triplicate. For exposure of yeast to the sample extracts, aliquots of 200 µL of the diluted yeast culture were pipetted into each well already containing the dried extracts as described above. Exposure was performed for 24 h at 30 °C and orbital shaking at 125 rpm. Fluorescence and optical

density (OD) were measured at 0 and 24 h in a Synergy^{TM} HT Multi-Detection Microplate Reader (BioTek Instruments Inc., USA) using excitation at 485 nm and measuring emission at 530 nm. The fluorescence signal was corrected with the signals obtained with the MM/L medium containing DMSO solvent only. Densities of the yeast culture were determined by measuring the OD at 630 nm, but this was only done to check whether a sample was toxic for the yeast cells.

2.4. Bioassay-directed identification of androgenic compounds using LC/TOFMS

The experimental setup for the identification of androgenic compounds consisted of a gradient liquid chromatograph (LC), an autosampler, a dual 96-wells fraction collection system, the yeast androgen bioassay and a high resolution LC/TOFMS system. The gradient LC system consisted of two Knauer model WellChrom K-1001 Pumps (Berlin, Germany), a Knauer high pressure dynamic mixing chamber, a Gastorr model 154 membrane degasser (Japan) and a Spark Holland model Endurance auto sampler (Emmen, The Netherlands). Liquid chromatography was performed using a Waters 150 mm × 3.0 mm i.d. Symmetry column packed with 5 µm C18 material (Milford, MA, USA). The mobile phase consisted of (A) water/acetonitrile (90:10) and (B) water/acetonitrile (10:90) and a gradient elution was used at a flow rate of 0.4 mL min^{-1}, starting at 35% B and linearly programmed to 100% B in 20 min. The column effluent was splitted towards two identical Gilson model FC203B 96-wells fraction collectors (Villiers-le-Bel, France). One 96-wells plate was used for androgenic bioactivity detection using the yeast androgen bioassay. Subsequently, the bioassay suspect well numbers in the duplicate well plate were subjected to LC/TOFMS using a Waters model Acquity LC system equipped with a Waters 150 mm × 3.0 mm i.d. Symmetry column packed with 5-µm C18 material and a mobile phase consisting of (A) water/acetonitrile (90:10) and (B) water/acetonitrile (10:90). Gradient elution was performed at a flow rate of 0.4 mL min^{-1}, starting at 35% B and linearly programmed to 100% B in 20 min. The LC column was directly interfaced to a Waters Micromass (Manchester, U.K.) model LCT Premier MS system equipped with a dual electrospray ionisation (ESI) probe and operated in the positive ion mode at a resolution of 10,000 (fwhm), source temperature 120 °C, desolvation temperature 350 °C, and a cone voltage of 50 V. The second LockSpray ESI probe provided an independent flow of leucine enkephalin lockmass calibrant (10 µL min^{-1}). The lock calibrant data was acquired at a frequency of once per 5 s, using a cone voltage of 50 V. Data was acquired in the centroid mode from 100 to 1200 Da with a scan time of 0.1 s and processed using Masslynx v. 4.1 software. In-source collision-induced dissociation (CID) acquisitions were performed at an aperture voltage of 40, 50 or 60 V, to allow both identification of

small neutral losses and the observation of diagnostic fragment ions. Some of the relative retention time data were obtained using the same LC setup but using a quadrupole MS instead of a TOFMS instrument.

3. Results and discussion

3.1. Bioassay performance

Typical androgen bioassay dose-response curves for several natural and synthetic steroids are shown in Fig. 1. 17β-Testosterone, 19-nortestosterone and 17β-boldenone are potent androgens, but 17α-testosterone hardly gives a response. Fig. 1 shows that 17β-estradiol and progesterone also give some androgenic response in accordance with expectations since the latter two compounds are known to exert also androgenic effects [15,16].

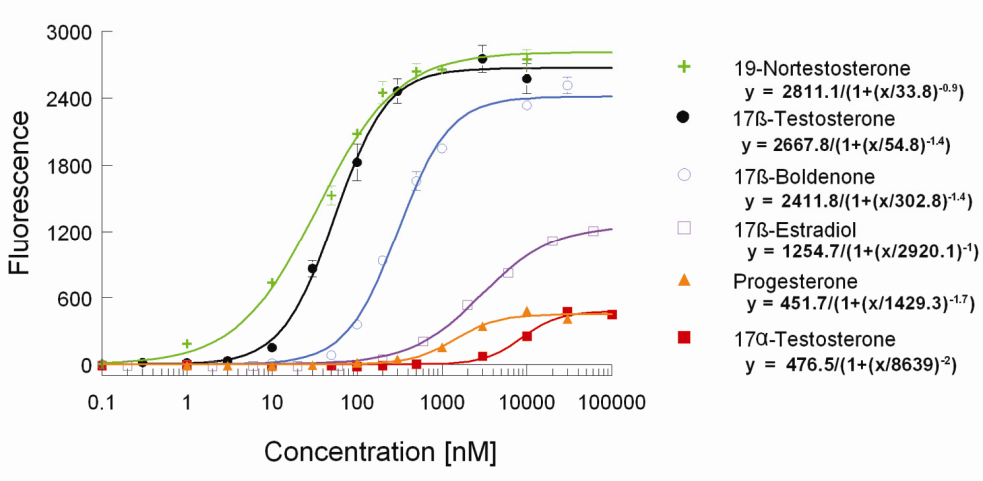

Fig. 1 - Response of different steroids in the yeast androgen bioassay. Exposure to 17β-testosterone (17β-T), 17α-testosterone (17α-T), 19-nortestosterone (19-norT), 17β-boldenone (Bold), 17β-estradiol (17β-E2) and progesterone (P) was started by adding an aliquot of 2 µL of a stock solution of the compound in DMSO to 200 µL yeast culture. Fluorescence was determined after 24 h as described. Fluorescence signals are the mean of a triplicate with S.D. The dose-response curves were fitted using the equation $y = a_0/(1 + (x/a_1))^{a_2}$. *Equal to: response = (max. response−min. response)/(1 + ([agonist]/*EC_{50}*)) ^width of transition.*

Table 1 shows the calculated EC_{50}, i.e. the concentration giving a half-maximum response, and the relative androgenic potency (RAP), defined as the ratio between the EC_{50} of 17β-testosterone and the EC_{50} of the compound, for several compounds. The yeast androgen bioassay showed good sensitivity towards all known androgens tested. The relative inactivity of 17α-testosterone was expected as the 17β-hydroxyl group is crucial

for androgenic activity [17,18]. In a previous study we demonstrated that the RAPs for 5α-dihydrotestosterone, methylboldenone, 17β-boldenone, stanozolol, 17α-methyl-testosterone, tetrahydrogestrinone, 17β-trenbolone and 19-nortestosterone can be explained by their steroidal structure and show a good correlation with (Q)SAR model calculations, while steroid representatives for other hormone receptors, like estrone, 17α-estradiol, 17α-ethynylestradiol and diethylstilbestrol for the estrogen receptor and corticosterone and dexamethasone for the glucocorticoid receptor, show no agonistic response [19].

Table 1. Determined EC_{50} concentrations and relative androgenic potencies (RAP) of compounds in the yeast androgen bioassay.

Compound	EC_{50} [nM] in the RAA[a]	RAP[b]
17β-Testosterone (17β-T)	40-75	1.0
17α-Testosterone (17α-T)	8640	0.0063[c]
19-Nortestosterone (nandrolone)	34	1.6
17β-Boldenone	303	0.18
1-Testosterone	38	1.9
4-Androstene-3β,17β-diol	1020	0.049
5-Androstene-3β,17β-diol	n.r.	n.r.
17β-Estradiol (17β-E2)	2920	0.019[c]
Progesterone	1430	0.038[c]

n.r. = no response.
[a] The EC_{50} is the concentration giving half-maximum response. The range in the EC_{50} of testosterone in the different experiments was from 40 to 75 nM.
[b] The relative androgenic potency (RAP) is defined as the ratio between the EC_{50} of 17β-testosterone and the EC_{50} of the compound.
[c] These compounds reach a maximum response that is lower than 70% of the maximum response obtained with 17β-testosterone. The maxima obtained with 17β-estradiol, progesterone and 17α-testosterone are about 50, 20 and 20% respectively.

3.2. Bioassay analysis of supplements

An overview of the ingredients declared on the labels of the supplement samples under investigation can be found in the research paper of Van Poucke et al. [9]. There was not enough material left from sample #11. According to the labels, 14 samples contained one to five prohormones. Nineteen different types of prohormones were mentioned on the various labels of these 14 supplements. Two supplements claimed to contain natural sterols, one to contain β-ecdysterone only and one to contain freeze-dried organs mainly.

Table 2. Bioassay and LC-MS/MS screening results of 18 dietary supplements. LC-MS/MS data adopted from Van Poucke et al. [9].

Dietary supplement	Weight of tablet or content of capsule [g]	Confirmed compounds[a]	Concentration [mg unit^{-1}][b]	Bioassay screening result[c]
1	1.0	17α-testosterone 17β-testosterone	0.02 0.14	+
2	0.55	17β-boldenone 17β-testosterone	<0.01 0.08	+
3	0.30	-	-	-
4	0.50	17α-boldenone 17β-boldenone 17α-nortestosterone 17β-nortestosterone	<0.01 0.13 0.05 0.58	+
5	0.40	17α-boldenone 17β-boldenone 17β-testosterone	<0.01 0.06 0.67	+
6	Powder	-	-	+
7	cream (2 ml)	17α-nortestosterone 17β-nortestosterone 17β-testosterone	0.23 2.54 <0.01	+
8	0.60	17β-boldenone 17β-testosterone	<0.01 <0.01	+
9	0.70	estradiol 17β-nortestosterone 17β-testosterone	1.26 0.32 <0.01	+
10	0.60	-	-	-
11	-	-	-	**Not analysed**
12	0.81	-	-	-
13	0.45	-	-	-
14	0.40	17β-testosterone	0.27	+
15	0.90	17β-testosterone	0.06	+
16	0.40	-	-	-
17	0.50	17α-boldenone 17β-boldenone	<0.01 0.19	+
18	0.55	16-dehydroprogesterone 17α-testosterone Progesterone 17β-testosterone	0.02 0.11 <0.01 0.81	+
19	0.50	-	-	+

[a,b] Confirmed compounds and their concentration obtained by LC-MS/MS [9].
[c] Bioassay screening result. Negative (-) when the response of the undiluted and diluted extract in the yeast androgen bioassay is below 44 (CCα determined with 20 blank animal feed samples, data not shown). Positive (+) when the undiluted or one of the 10 to 10,000 fold diluted extracts gives a response above the CCα.

In addition, seven supplements also claimed to contain plant-derived compounds or plant extracts. Table 2 shows the LC-MS/MS confirmed compounds and their concentration as described by Van Poucke et al. [9].

Recently, we validated the yeast androgen bioassay for the determination of androgen activity in animal feed and here we used a similar method for screening androgenic activity in supplement samples. Therefore, a feed sample that was screened negative was taken as a negative control. Fig. 2 shows the responses obtained in the yeast androgen bioassay with undiluted and diluted extracts of the negative feed control and some of the 18 supplements.

The negative feed control does not give a response, but the negative feed that was spiked with 5 µg 17β-testosterone per gram feed clearly gives a response in the bioassay. This signal decreases in the 10-fold diluted sample and after a 100-fold dilution the signal disappears. Assuming no recovery loss during the sample treatment, the undiluted extract of this spiked feed control would theoretically result in a final concentration of 217 nM 17β-testosterone in the well. According to Fig. 1 this would result in a near maximal response. The 10- and 100-fold dilutions of the spiked feed control would thus theoretically result in well concentrations of 21.7 and 2.2 nM 17β-testosterone, respectively. According to Fig. 1 the 21.7 nM would still give a response while the 2.2 nM would not be able to show a response, thus explaining the observed responses of the spiked feed sample as shown in Fig. 2. The extracts that were spiked afterwards, result in a final concentration of 300 nM 17β-testosterone in the well. These "spike after" controls are performed in order to investigate whether there are disturbing or inhibiting (antagonist) compounds in the sample extract. According to Fig. 2, there are no such compounds present in the extract of the negative feed sample as all three dilutions of the extract give the same response with this "spike after" control. Moreover, the results indicate that the recovery of the cleanup procedure is acceptable as the "spike after" control (300 nM) only results in a little higher response compared to the response of the undiluted spiked feed control (217 nM). Fig. 2 shows that the LC-MS/MS negative supplements #3 and #10 are also negative in the yeast androgen bioassay. The spike controls prove that there are also no inhibiting compounds present in these negative supplements. Similar results were found for the LC-MS/MS negative supplements #12, #13 and #16. Moreover, no matrix effect was observed as the histogram patterns of the negative feed sample and the negative supplements #3, #10, #12, #13 and #16 were similar to that of the chemical blank (data not shown).

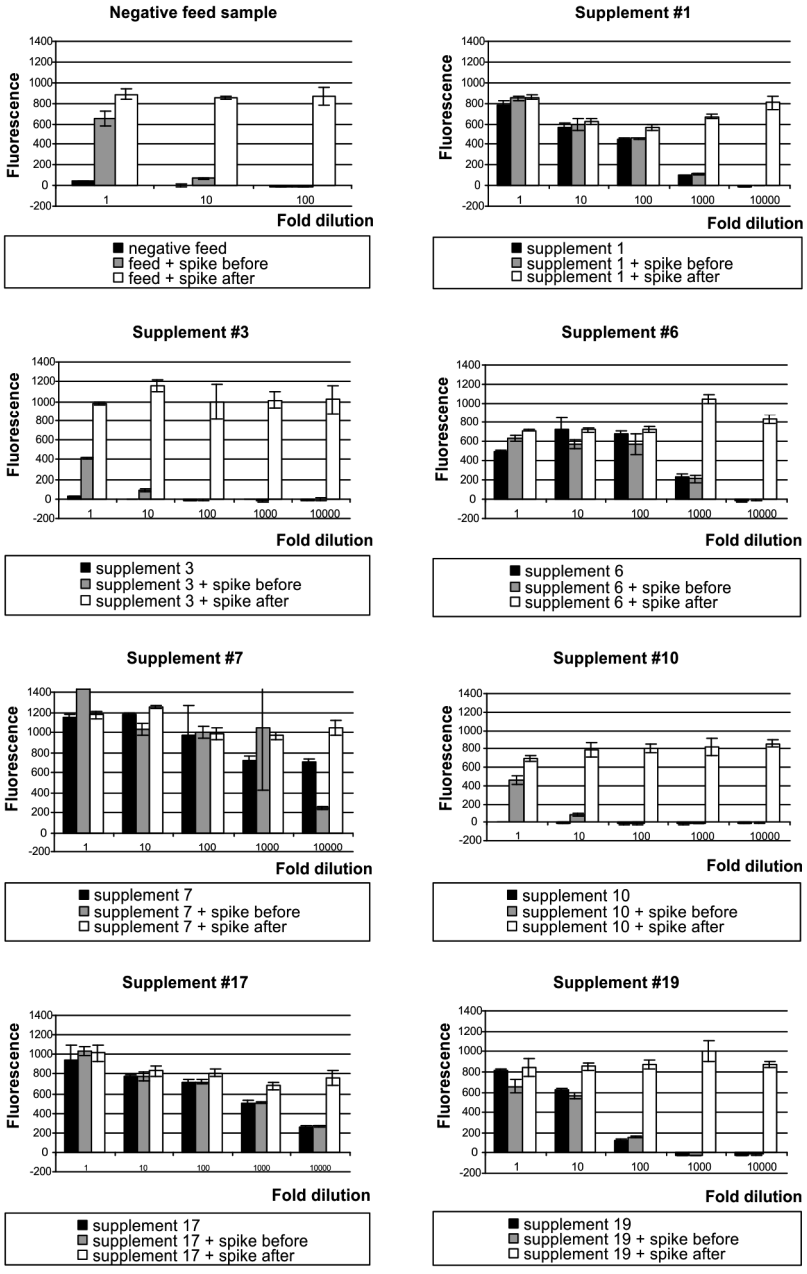

Fig. 2 – Responses obtained with undiluted and diluted extracts of the supplements in the yeast androgen bioassay. Fluorescence was determined after 24 h as described and signals are the mean of a triplicate with S.D.

Fig. 2 also shows the responses of the LC-MS/MS positive supplements #1, #7 and #17. The main active compounds in these supplements are 17β-testosterone, 19-nortestosterone and 17β-boldenone, respectively (see Table 2 column 3). The histogram patterns obtained in the bioassay show that there is at least one potent agonist in the extract of these supplements. The extract of supplement #1 must be diluted more than 100 times before the response declines. After a 10,000-fold dilution the response of supplement #7 is still near maximum while the response of supplement #17 is declining. There is no evidence for the presence of any inhibiting compounds in these supplements. Similar results were obtained with the extracts of the LC-MS/MS positive supplements #2, #4, #5, #8, #9, #14, #15 and #18, showing responses that indicate that these supplements contain potent androgenic agonists.

The bioassay results are included in Table 2 and show that the bioassay screening correlates very well with the LC-MS/MS screening (compare columns 3 and 5). The only differences observed are the LC-MS/MS negative screened supplements #6 and #19: in contrast both of these supplements are screened suspect in the yeast androgen bioassay. The histogram patterns of supplements #6 and #19 in Fig. 2 prove that there is at least one potent agonist in the extract of these supplements. There is no evidence for the presence of any inhibiting compounds.

3.3. Bioassay-directed identification

The bioassay-directed LC-TOFMS system as described in Section 2.4 was used to identify the responsible bioactive compound(s) in supplements #6 and #19. Fig. 3 shows the androgenicity biograms of both supplements obtained by LC-fractionation. The corresponding suspect well numbers in the duplicate 96-well plate were analysed by LC-TOFMS. For supplement #6 we selected fractions 24 + 25, 27, 33 and 35 and for supplement #19 we analysed the combined fraction 31 + 32.

LC-TOFMS analysis of the combined fraction 24 + 25 from supplement #6 resulted in a peak at a retention time of 6.69 min. The reconstructed ion chromatogram and mass spectra of fraction 24 + 25 are given in Fig. 4a and show abundant ions at *m*/*z* 255.2113, *m*/*z* 273.2208, and *m*/*z* 314.2484. The elemental composition of these ions was calculated from the accurate masses. The ion at *m*/*z* 273.2208 appeared to be $[M+H-H_2O]^+$ and originate from a $C_{19}H_{30}O_2$ compound explaining the ions at *m*/*z* 255.2113 $[M+H-2H_2O]^+$ and the acetonitrile adduct ion at *m*/*z* 314.2484 $[M+H-H_2O+C_2H_3N]^+$. Searching elemental composition databases resulted in options like 4-androstene-3β,17β-diol and 5-androstene-3β,17β-diol. However, only the first compound was found to be active in the yeast androgen bioassay (Table 1). After comparing retention times, the androgenic active

compound in fraction 24 + 25 was indeed identified as 4-androstene-3β,17β-diol. Also 400 MHz proton NMR analysis confirmed that supplement #6 contains mainly 4-androstene-3β,17β-diol (data not shown). Since no other steroids were observed by NMR, the remaining active fractions of supplement #6 probably contain quantitatively less abundant compounds. Elemental composition calculations and retention time comparison turned out that fraction 27 contained 17β-testosterone. Supplement #6 was found suspect on 17β-testosterone in the initial LC-MS/MS analysis. However, conformation results did not meet the criteria (data not shown).

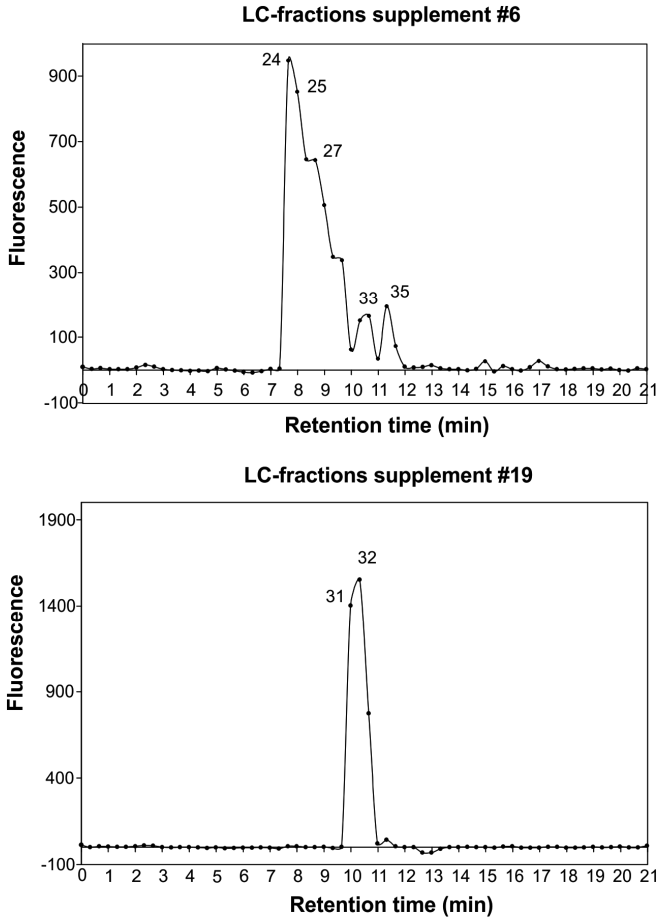

Fig. 3 – Androgenicity biogram of the suspect supplement samples #6 and #19. Extracts were separated on a C18-column and fractions of 20 s were collected and tested in the yeast androgen bioassay. For supplement #6 the 100-fold diluted extract was used and for supplement #19 the 10-fold diluted extract.

Fig. 4b shows the reconstructed ion chromatogram of the less bioactive fraction 33, two peaks are observed at 8.76 and 9.08 min, both showing $[M+H-2H_2O]^+$ ions at *m*/*z* 257, $[M+H-H_2O]^+$ ions at *m*/*z* 275 and $[M+H-H_2O+C_2H_3N]^+$ ions at *m*/*z* 316 (Fig. 4b, inserts 1 and 2), suggesting the presence of, e.g. androstanediol. In total eight different isomers of androstanediol exist, however only 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, and 5β-androstane-3β,17β-diol show androgenic activity in the yeast androgen bioassay, with RAPs of 0.15, 0.22, and 0.0033, respectively [19]. LC/MS relative retention times of these three isomers of androstanediol are listed in Table 3 and are 1.26, 1.22 and 1.08 for 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, and 5β-androstane-3β,17β-diol versus 17β-testosterone, respectively. Comparing this to fraction 33 of supplement #6, the compound responsible for the response in the androgen biosensor is most likely 5α-androstane-3β,17β-diol.

Table 3. Relative retention time of supplement fractions and reference standards by LC/TOFMS or LC/MS.

	Retention times relative against 17β-T [a]	
	LC/TOFMS	LC/MS
Supplement #6		
Fraction 24 + 25	0.89	-
Fraction 27	1.00	-
Fraction 33	1.17 / 1.21	-
Supplement #19		
Fraction 31 + 32	1.17	-
Standards		
17β-Testosterone	1.00	1.00
1-Testosterone	-	1.17
4-Androstane-3,17-dione	-	1.46
4-Androstene-3β,17β-diol	-	0.92
5a-Androstane-3a,17β-diol	-	1.26
5a-Androstane-3β,17β-diol	-	1.22
5β-Androstane-3β,17β-diol	-	1.08

[a] Relative retention time comparison of 17ß-testosterone vs. reference compounds or compounds present in supplement fractions, obtained, respectively by LC/TOFMS and LC/MS.

Because two isomers of androstanediol are observed, the peak eluting at 8.76 min represents an androgenic inactive isomer of androstanediol. Probably 17β-testosterone and the isomers of androstanediol are by-products of 4-androstene-3β,17β-diol present in fraction 24 + 25 and formed during production or storage of the supplement. LC-TOFMS analysis of fraction 35 resulted in a similar mass spectrum as obtained by analysis of fraction 33 (Fig. 4b, insert 1 and 2). This suggests the presence of another androgenic active isomer of androstanediol, however this could not be confirmed by retention time comparison of reference standards using LC/MS.

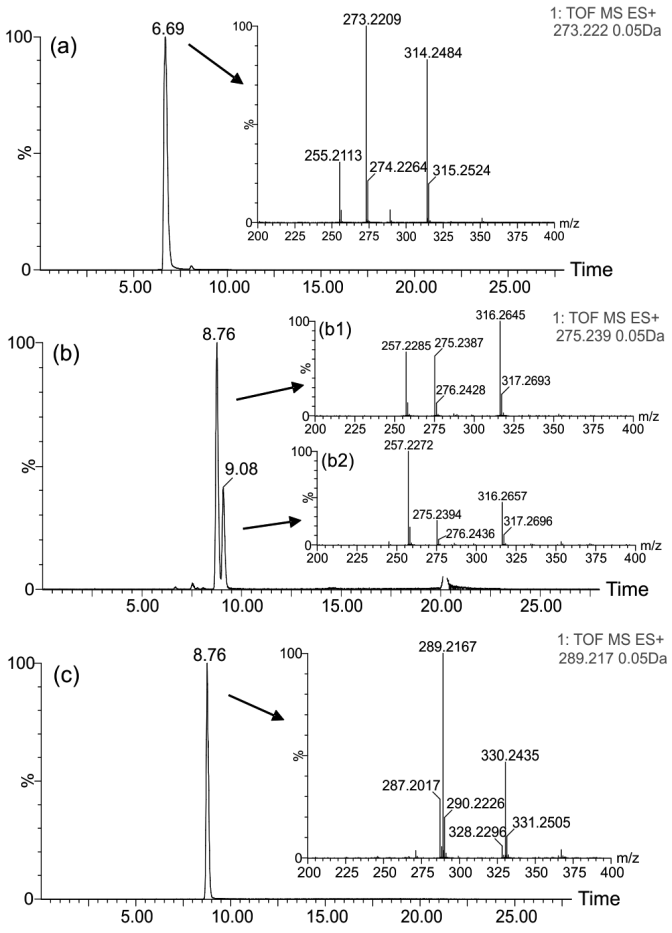

*Fig. 4 – LC-TOFMS reconstructed ion chromatograms (mass window of 0.05 Da) of (**a**) fraction 24 + 25 and (**b**) fraction 33 of supplement #6 and (**c**) fraction 31 + 32 of supplement #19. Chromatogram inserts: the accurate mass spectra.*

Analysing fraction 31 + 32 of supplement #19 with LC-TOFMS, resulted in a peak at a retention time of 8.76 min with an abundant $[M+H]^+$ ion at *m/z* 289.2166 (Fig. 4c). The elemental composition of this ion was calculated as $C_{19}H_{29}O_2$, which is similar to the composition of 17β-testosterone, 1-testosterone and androstanedione. In-source CID spectra of fraction 31 + 32, 17β-testosterone, 1-testosterone and androstanedione using an aperture voltage of 50V are shown in Fig. 5. For 17β-testosterone (Fig. 5b), the $[M+H]^+$ parent ion (at *m/z* 289), $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ ions and two important product ions were observed at *m/z* 97 and 107, which are typical for this compound [20]. In-source CID of androstanedione resulted in abundant product ions at *m/z* 213, 253 $[M+H-2H_2O]^+$ and 271 $[M+H-H_2O]^+$. The product ion spectra of 1-testosterone showed an abundant product ion at *m/z* 187 and less abundant ions at *m/z* 105, 131 and 205 which were also found previously by LC-ESI-MS/MS [21]. The obtained CID spectrum of fraction 31 + 32 of supplement #19 (Fig. 5a) shows high similarities with the CID spectra of 1-testosterone, suggesting the presence of the latter.

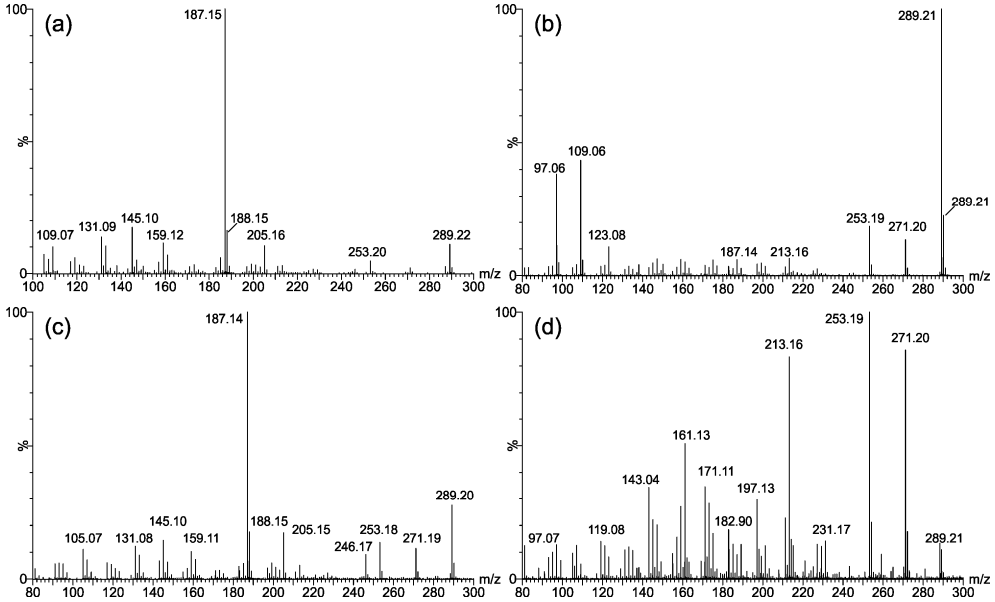

Fig. 5 – In-source CID mass spectra of (a) fraction 31 + 32 of supplement #19, (b) 17β-testosterone, (c) 1-testosterone and (d) androstanedione acquired at an aperture voltage of 50 V.

Comparison of relative retention times of 1-testosterone, androstanedione and the unidentified compound present in fraction 31 + 32 is included in Table 3. Both fraction 31 + 32 and 1-testosterone showed a relative retention time of 1.17 versus 17β-testosterone.

Therefore, it can be concluded that according to the obtained mass spectra, elemental composition, retention times and fragment ion patterns after in-source CID the androgenic compound in fraction 31 + 32 of supplement #19 is most likely 1-testosterone.

3.4. Feasibility of quantification

Although the bioassay has been validated and used as a qualitative screening method for the routine determination of androgenic activity in calf urine and animal feed, the response of sample extracts can be converted to concentrations using a 17β-testosterone standard dose-response curve. The fluorescence response of the undiluted extract of the negative feed sample that was spiked with 5 μg 17β-testosterone per g was 651 which corresponds with a concentration of 143 nM 17β-testosterone in the well. Assuming a final volume in the well of 200 μL, this semi-quantitative approach results in a 17β-testosterone equivalent content of 3.3 μg g^{-1} indicating an overall recovery of 66%. In the same way the 1000 times diluted extract of supplement #1was calculated from the fitted 17β-testosterone standard dose-response curve and its fluorescence response of 110 corresponds with a concentration of 11 nM 17β-testosterone in the well, or a 17β-testosterone equivalent content of 0.3 mg g^{-1}. This estimated content is in the same order of magnitude as the LC-MS/MS determined content of 0.14 mg 17β-testosterone per unit, as a unit of supplement #1 is a tablet of approximately 980 mg and as 17α-testosterone is almost inactive in the bioassay. Overall, this semi-quantitative approach resulted in estimated 17β-testosterone equivalent contents of the supplements that were in at least the same order of magnitude as the LC-MS/MS determined contents.

4. Conclusions

The only way to win the fight against doping is to be one step ahead of the abusers. The fight cannot be won by a strategy of checking urine samples only against a prohibited list (unknown compounds like THG were not found by that strategy). Bioactivity testing of dietary supplements used by athletes is one way to get in front. Here we mapped 18 dietary supplements that might be used by athletes and showed that two supplements, shown negative in LC-MS/MS, were screened suspect in an androgen yeast bioassay. Analysing the bioassay-directed suspect fractions in the duplicate well by LC-TOFMS ultimately identified the responsible compounds showing that this androgen bioassay screening method has a surplus value in comparison with a LC-MS/MS screening method alone.

Acknowledgements

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Chapter 3

Evidence of the indirect hormonal activity of prohormones using liver S9 metabolic bioactivation and an androgen bioassay

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Supplementary material as noted in the text is available via the Internet at:
http://www.springerlink.com/content/7492q80ww4760837/supplementals/

Abstract

Prohormones such as dehydroepiandrosterone (DHEA) are steroid precursors that do not show hormonal activity by themselves. Abuse of these prohormones in cattle fattening is hard to prove because of strong *in vivo* metabolism and the difficulty to detect metabolites which are not significantly above endogenous levels. The aim of the present work was to develop an in vitro assay capable of detecting the indirect hormonal activity of prohormones that might be present in feed supplements and injection preparations. Sample extracts were incubated with a bovine liver S9 fraction in order to mimic the *in vivo* metabolic activation. Subsequently incubated extracts were exposed to a highly androgen-specific yeast bioassay to detect hormonal activity. Metabolic activation of DHEA, 4-androstene-3,17-dione (4-adione) and 5-androstene-3,17-diol (5-adiol) resulted in an increased androgenic activity caused by the formation of the active androgen 17β-testosterone (17β-T), as shown by ultra-performance liquid chromatography and time -of-flight mass spectrometry with accurate mass measurement. The developed in vitro system successfully mimics the hydroxysteroid dehydrogenase (HSD)- and cytochrome P450-mediated *in vivo* metabolic transitions, thus allowing assessment of both bioactivity and chemical identification without the use of animal experiments. Screening of unknown supplement samples claimed to contain DHEA resulted in successful bioactivation and positive screening results according to the androgen yeast biosensor.

Introduction

Within the European Union the use of growth-promoting agents in cattle fattening is banned according to EC directive 96/22 [1]. Interestingly, there is not a black list of substances but EC 96/22 states that all substances having thyrostatic, estrogenic, androgenic or gestagenic activity are prohibited. A trend has been observed from the abuse of synthetic steroids towards natural steroid esters and prohormones. Feed supplements and preparations containing prohormones have the potential to enhance the levels of natural occurring steroids and can be misused in livestock production. After administration and uptake in the blood circulation, peripheral tissues are able to metabolize prohormones into more biologically active androgens and estrogens [2–4]. With respect to androgens this leads to anabolic action and subsequently to increased body weight, muscle strength and improved lean/fat ratios in farm animals [5].

Abuse of prohormones in livestock production is hard to prove: urinary metabolites are unknown or not significantly above highly fluctuating endogenous levels [6]. Chemical methods have the drawback of detecting only targeted compounds of interest. Biological transcription activation assays, however, have the advantage of detecting compounds based on bioactivity. For screening of hormones a wide range of mammalian or yeast cell-based bioassays have been developed [7–10]. These assays focus mainly on ligand-receptor interactions in which activation of a specific receptor is linked to a transcription reporter mechanism. Assays based on mammalian cell lines are in general more sensitive; however, the metabolic capacity of both mammalian and yeast cell based assays is rather limited [11–13]. The latter are relatively easy to use and very robust, making them suitable for screening of samples from practice without complex sample cleanup procedures. In addition, yeast cells lack endogenous receptors and thus lack the potential cross talk from other receptor types [14]. As a result the signals obtained in the yeast androgen bioassay may be associated only with the androgenic properties of the compound or sample extract tested.

In vivo, hepatic first pass metabolism of exogenous and endogenous compounds normally leads to inactivation by phase I and phase II enzymes and the subsequent excretion of the deactivated compounds. However, metabolism can also result in an increased biological activity of a given compound [15]. Prohormonal compounds can be activated by hydroxysteroid dehydrogenases (HSDs). Prohormones such as dehydroepiandrosterone (DHEA), 4-androstene-3,17-dione (4-adione) and 5-androstene-3,17-diol (5-adiol) are direct precursors of potent androgens like 17β-testosterone (17β-T) and dihydro-testosterone (DHT). The 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) enzyme catalyzes the two-step conversion of 3β-hydroxysteroids, like DHEA and 5-adiol, into

4-adione and 17β-T, respectively. 17β-Hydroxysteroid dehydrogenase (17β-HSD) catalyzes the conversion of 17-ketosteroids, like DHEA and 4-adione, into their corresponding 17-hydroxysteroids, 5-adiol and 17β-T, respectively (Fig. 1). These conversions yield an increase of biological activity *in vivo* [2–4]. The action of biologically active androgens is mediated by the androgen receptor (AR). Upon ligand binding, the AR dissociates from its chaperone proteins and is translocated in its active state to the nucleus ready to bind to androgen-responsive elements (AREs) [16]. Binding results in recruitment of coactivators and enhanced transcription of target genes regulating androgenic-anabolic action.

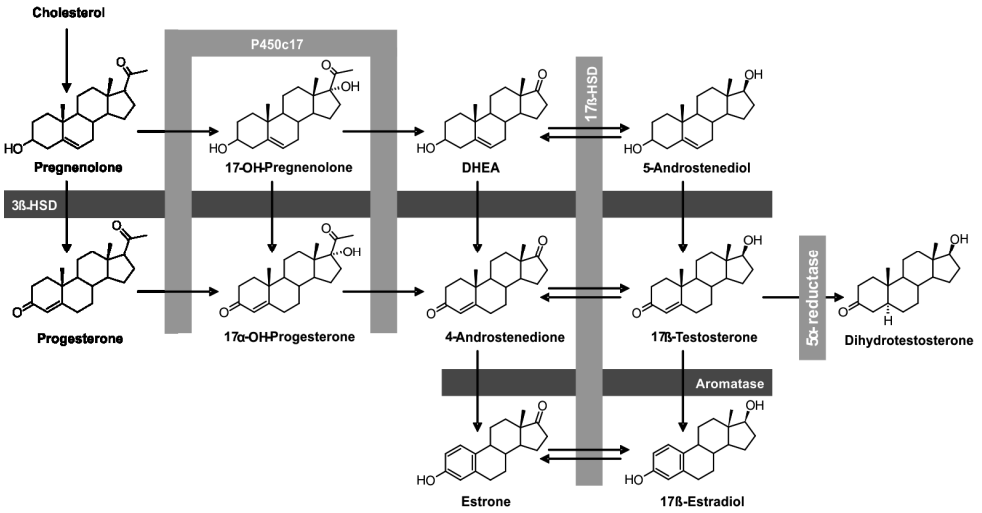

Fig. 1 - In vivo steroid hormone biosynthesis: side chain cleavage of cholesterol results in pregnenolone which is metabolized into DHEA under P450c17 activity. From DHEA, conversions are catalyzed by 3β-HSD and 17β-HSD activity resulting in formation of potent androgens like 17β-T. Reversible reactions (marked by double arrows) depend on cofactor availability (e.g. NADP/NADPH ratios). Estrogens are formed from androgens (4-adione and 17β-T) by aromatase activity.

In this context we explored the combined use of a bovine liver S9-based bioactivation model and a previously developed highly androgen-specific yeast assay based on the constitutive expression of the human androgen receptor (hAR) in combination with an androgen-responsive element coupled to an enhanced green fluorescent protein (EGFP) reporter system. A promising *in vitro* model for evidence of the indirect hormonal activity of prohormones, in accordance with the bioactivity-based legislation [1], has been developed. The results obtained were supported by chemical identification of the metabolites using ultra-performance liquid chromatography (UPLC) and time-of-flight mass spectrometry (TOFMS).

Methods and materials

Chemicals

Dehydroepiandrosterone (DHEA), 5-androstene-3,17-diol (5-adiol), 4-androstene-3,17-dione (4-adione), 17β-testosterone (17β-T), 17α-testosterone (17α-T), 5α-dihydrotestosterone (DHT), 7α-hydroxy-DHEA (7α-OH-DHEA), 7β-hydroxy-DHEA (7β-OH-DHEA), 16α-hydroxy-DHEA (16α-OH-DHEA), 16β-hydroxy-DHEA (16β-OH-DHEA), 11β-hydroxy-DHEA (11β-OH-DHEA) and 19-hydroxy-DHEA (19-OH-DHEA) were obtained from Steraloids (Newport, RI, USA). Sodium acetate, sodium carbonate, sodium chloride, di-sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, ammoniumsulphate, magnesium chloride, potassium chloride, tris (hydroxymethyl)aminomethane (Tris), dimethylsulfoxide (DMSO), hydrochloric acid and acetic acid were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate, NADH disodium salt, NADP disodium salt and NADPH tetrasodium salt were from Roche Diagnostics (Almere, the Netherlands). Acetonitrile, methanol and isooctane were obtained from Biosolve (Valkenswaard, the Netherlands). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were from Difco (Detroit, MI, USA). L-Leucine, bovine serum albumin (BSA), hydroxysteroid dehydrogenase originating from *Pseudomonas testosteroni* and NAD sodium salt were purchased from Sigma (St. Louis, MO, USA). Water used for LC/MS was purified using a Millipore (Bedford, MA, USA) Milli-Q system.

Preparation of liver S9 fractions

Four Frisian bovines (350-430 kg, 13-14 months old) that served as normally fed negative controls in a 6-week animal experiment were sacrificed at the end of the trial. At sacrifice, liver tissue was rinsed with ice cold 0.9% sodium chloride and snap-frozen in liquid nitrogen. Liver tissues were homogenized in twice their volume of Tris-HCl buffer (50 mM, pH 7.4, 1.15% KCl), using a blender. Homogenates were pooled and centrifuged at 10,000 g for 25 min at 4 °C. The supernatant was snap-frozen in liquid nitrogen and stored at -80 °C until use. Protein concentrations in this S9 fraction were determined according to Lowry [17], using the BioRad DC protein assay (BioRad, Veenendaal, the Netherlands) and BSA as a standard. The animal experiment referred to was approved by the Animal Ethics Committee of Gent University, Belgium, in accordance with local ethical requirements.

Prohormone incubations

Incubations of 50 µg (pro)hormones, or of 100 µl final SPE eluent from supplement samples (see Extraction of supplement samples), were carried out in a glass tube containing 0.1 M sodium phosphate buffer (pH 7.4), 10 mM glucose-6-phosphate, 33 mM KCl, 8 mM $MgCl_2$, 2 mg/ml bovine S9 fraction and either 4 mM NAD, NADH, NADP or NADPH. The final volume was 1 ml and the mixtures were incubated at 37 °C in a water bath for 6 h. Single incubations of prohormones and of SPE eluents were performed. Blanks without bovine liver S9 and blanks without cofactor were included to check for nonenzymatic reactions during the incubation period. Reactions were terminated at t=0 and t=6 h with 1 ml acetonitrile, and the reaction products were subsequently subjected to a cleanup, which is similar to a method described by Marwah et al. [18]. In summary, the mixture was centrifuged for 15 min at 3,000 *g*, and the supernatant was transferred to a fresh glass tube. The mixture was extracted again with 2 ml acetonitrile and centrifuged for 10 min at 3,000 *g*. The combined supernatants were evaporated under nitrogen at 45 °C to approximately 0.5 ml. Next, the extract was diluted with 3 ml methanol, centrifuged for 15 min at 3,000 *g* and evaporated at 45 °C under nitrogen until dryness. The residue was dissolved in 200 µl methanol and, following the addition of 1.8 ml water, applied onto a preconditioned reversed-phase solid-phase extraction (SPE) cartridge (Waters Oasis™ HLB, 3 cc, 6 mg). The cartridge was washed twice with 2 ml water and eluted with 2 ml methanol. The SPE eluent was evaporated under nitrogen at 45 °C and reconstituted in 2 ml acetonitrile. Aliquots of 200 µl of this final acetonitrile extract plus 50 µl 4% DMSO were pipetted in a conical 96-well plate (Greiner Bio-One, Germany) and evaporated overnight in a fume cupboard to leave only DMSO the next day.

Extraction of supplement samples

Two authentic supplement samples were extracted. According to the labels, each capsule of supplement A contained 50 mg DHEA and 10 mg vitamin C in a base of rice flour, and each capsule of supplement B contained 500 mg *Tribulus terrestris*, 100 mg 4-androstenedione, 100 mg DHEA, 100 mg lysine and 15 mg zinc amine acid chelate. A 100-mg aliquot of each capsule was extracted according to a method described by Bovee et al. [14]. In summary, sample aliquots were mixed with 4 ml methanol and 4 ml sodium acetate buffer (0.25 M, pH 4.8), sonicated for 10 min and mixed for 15 min in a head over head apparatus. Samples were then centrifuged for 10 min at 3,000 rpm, and 4 ml of the supernatant was collected. The pH of this supernatant was adjusted to pH 4.8 with 4 M acetic acid and applied onto an SPE cartridge (Varian, Bond Elut, C18, 500 mg, 3 ml), previously activated with 3 ml methanol and 3 ml sodium acetate buffer. Next, the SPE

cartridge was washed with respectively 1.5 ml sodium acetate buffer, 2 ml MilliQ water, 1.5 ml sodium carbonate (10% w/v), 2 ml MilliQ water and 2 ml methanol/water (50:50 v/v). The SPE cartridge was dried and eluted with 4 ml acetonitrile. The SPE eluent thus obtained was applied onto an NH2 SPE cartridge (Isolute, 100 mg, 3 ml) previously activated with 4 ml acetonitrile. The run through was collected and evaporated at 45 °C under nitrogen and reconstituted in 4 ml acetonitrile. At this point the SPE eluent is either measured directly in the androgen yeast biosensor or bioactivated according to the procedure as described in Prohormone incubations and then measured by the androgen biosensor. Two sets of spiked supplement samples were prepared to monitor the extraction recovery ("spike before samples") and the potential presence of AR antagonists in the supplements ("spike after samples"). 17β-Testosterone was added to "spike before samples" (5 µg/g) before the sample preparation step and to "spike after samples" (30 µM in DMSO, 2 µl) prior to biosensor exposure.

Recombinant yeast androgen bioassay

Transformants of a *Saccharomyces cerevisiae* strain that express the human androgen receptor (hAR) and an yEGFP reporter system [10] were grown on selective minimal medium plates supplemented with L-leucine. Minimal medium consisted of yeast nitrogen base without ammonium sulphate or amino acids (1.7 g/l), dextrose (20 g/l), ammonium sulphate (5 g/l) and was supplemented with L-leucine (6 mg/l). At day 1, 10 ml minimal medium supplemented with L-leucine (MM/L) was inoculated with a single colony and cultured overnight at 30 °C, in a shaking incubator at 125 rpm. The next day, the overnight culture was diluted in MM/L to an optical density (OD) value between 0.04 and 0.06 at 630 nm. Aliquots of 200 µl yeast suspension were added to each well of a 96-well plate, already containing the DMSO extract of samples and controls as described in Prohormone incubations. A standard dose-response curve of 17β-testosterone was included in each experiment. Plates were incubated at 30 °C for 24 h in a shaking incubator (125 rpm), and fluorescence was measured (485-nm excitation, 530-nm emission) using a Synergy™ HT multi-detection microplate reader (BioTek Instruments Inc., USA). The OD of the yeast was measured at 630 nm after 24 h to monitor for any cytotoxic effects on the yeast cells.

UPLC-TOFMS assignment of substances formed in metabolic bioactivation

Ultra-performance liquid chromatography (UPLC) was performed on a Waters (Milford, MA, USA) Acquity system containing a Waters Acquity BEH C_{18} 1.7 µm, 2.1×50-mm column, with mobile phases (A) acetonitrile/water/formic acid (10:90:0.2, v/v/v) and (B)

acetonitrile/water/formic acid (90:10:0.2, v/v/v), linearly increasing from 20 to 46% B in 5 min at 0.7 ml/min. The column temperature was 45 °C, and the injection volume 20 µl. The column effluent was split 1:1 prior to mass spectrometry.

Mass spectrometry was performed on a Waters QTOF micro instrument equipped with a dual electrospray ionization (ESI) probe and operated in the positive ion mode (ESI) at a source temperature of 120 °C, desolvation temperature 350 °C, desolvation gas flow 700 l/h, ESI capillary voltage of 3,000 V and a cone voltage of 30 V. Phosphoric acid in acetonitrile/water (0.01:50:50, v/v/v) was used as reference solution in the LockSpray™ at a flow rate of 10 µl/min. TOF data were collected between *m/z* 80 and 1,200 and processed using Masslynx v 4.0 software.

Results and discussion

Direct androgen bioassay screening

Androgen bioassay dose–response curves of DHEA and known *in vivo* metabolites 4-androstene-3,17-dione (4-adione), 5-androstene-3,17-diol (5-adiol), 17β-testosterone (17β-T) and 5α-dihydrotestosterone (DHT) are shown in Fig. 2. Nonlinear regression curves were fitted through the data points, and the concentration giving half the maximum (EC_{50}) was calculated.

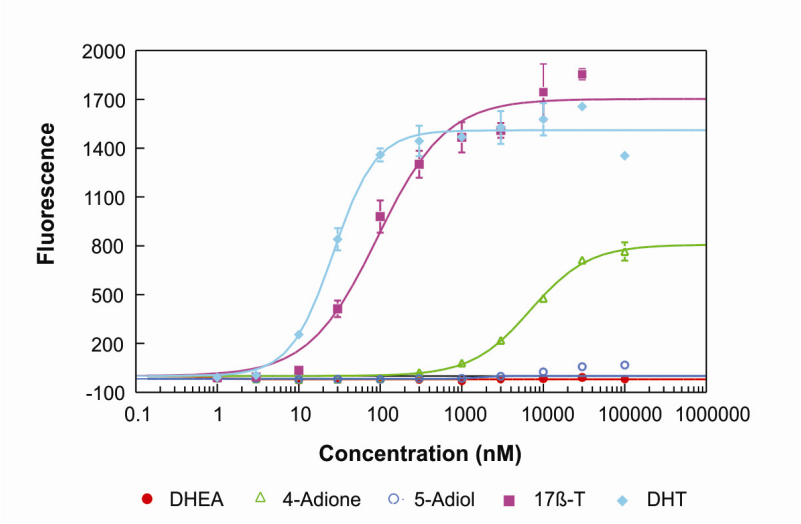

Fig. 2 - Dose-response curves of DHEA, 4-adione, 5-adiol, 17β-T and DHT after 24 h obtained in the androgen yeast biosensor. Fluorescence signals are the mean of an assay triplicate (± SD) and corrected for the signal at t=0 and the blank DMSO.

Exposure of the androgen reporter-gene yeast bioassay to DHEA and 5-adiol did not result in a response of the cells, i.e. no direct hormonal activity was observed *in vitro* in accordance with expectations. In contrast a direct androgenic activity was observed for 4-adione, 17β-T and DHT, with EC_{50} values of 6,900 nM, 92 nM and 26 nM, respectively. It should be noted that the obtained dose-response curve of 4-adione was also caused by a 17β-T impurity (data not shown), apart from the androgenic activity of 4-adione itself.

Incubation of prohormones with bovine liver S9 followed by androgen bioassay screening

To study the bioactivation of prohormones, 50 µg of DHEA, 4-adione and 5-adiol were incubated with a bovine liver S9 mix in the presence of either NAD, NADH, NADP or NADPH. Initially, trials were performed with incubations of DHEA with NAD and pure hydroxysteroid dehydrogenase originating from *Pseudomonas testosteroni* containing 3α- and 3β-HSD activity. Incubation of DHEA for 1 h resulted in an increase of response in the androgen yeast assay, caused by formation of 4-adione and 17β-T (results not shown). However, further work was with bovine liver S9 to include the entire species specific liver metabolism.

Results for sample extracts incubated with the S9 mix for 0 and 6 h in the presence of 4 mM cofactor and subsequently screened for androgenic activity in the yeast androgen bioassay are shown in Fig. 3. The androgen assay showed no signal for DHEA at t=0, but after metabolic activation of DHEA with S9 in combination with either NAD, NADP or NADPH, an androgenic activity was measured (Fig. 3a). Application of NADH as a cofactor, however, caused only a slight increase in the bioactivity signal.

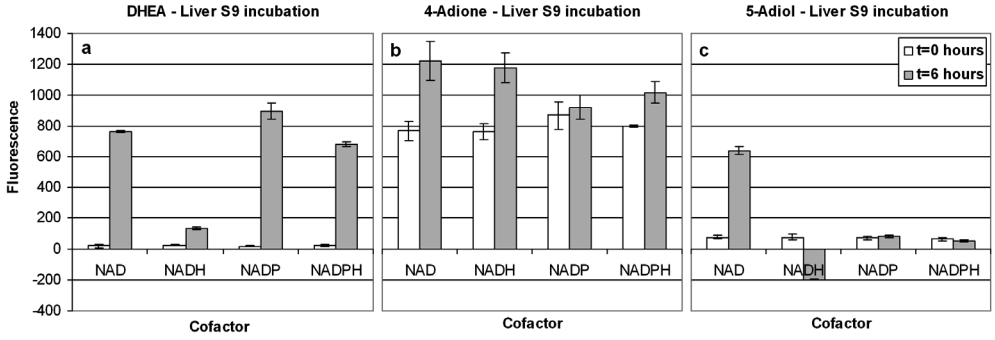

*Fig. 3 - Androgen bioassay responses of (**a**) DHEA, (**b**) 4-adione and (**c**) 5-adiol, before (t=0) and after (t=6) incubation with bovine liver S9 in the presence of different cofactors. Fluorescence signals are the mean of an assay triplicate (± SD) and corrected for the signal at t=0 and the reagent blank.*

The compound 4-adione is already androgenic without metabolic activation. Assuming 100% recovery, the 50 μg 4-adione (containing the 17β-T impurity) without activation would result in a final concentration of 87,000 nM in the well, and in accordance with Fig. 2 this resulted in a maximal fluorescence response of about 800. After S9 treatment the signal increased up to maximal 1,200, suggesting that metabolites are formed that are more active than 4-adione itself (Fig. 3b). Figure 3c shows that 5-adiol can be activated by the S9/NAD combination, but no changes in response were found after using NADH, NADP and NADPH as a cofactor. Whether the differences in response found between NAD and NADP are caused by the high level of specificity of HSDs for either NAD or NADP cannot be stated from the present study. The blanks, without bovine liver S9 or cofactor, showed no increase in response after 6-h incubation (results not shown).

Bioactivation plus androgen bioassay screening of supplement samples

Two supplements, A and B, were extracted and screened directly in the yeast androgen bioassay (Fig. 4a and b). In addition, aliquots of both supplements were spiked before extraction with 5 μg 17β-T per gram sample in order to check the recovery. Assuming no losses, the 17β-T spike would result in a calculated concentration of 217 nM 17β-T in the well. After sample cleanup but prior to the androgen bioassay nonspiked sample aliquots were also spiked with 300 nM 17β-T in the well in order to investigate whether there are any interfering or antagonistic compounds in the extract. According to the dose-response curve shown in Fig. 2, both 17β-T spikes are expected to give maximal bioassay response. Supplement A, stated to contain mainly DHEA, did not give a direct androgen bioassay response, but also the 17β-T spike before and spike after controls failed to give a response. Dilution of the extract, however, resulted in an increase of the spike after control, suggesting that the extract of this preparation contains an antagonist, which could be DHEA. This is in line with earlier performed quantitative structure-activity relationship (QSAR) modeling approaches, where calculations of the free energy after ligand docking and energy minimization of the ligand–receptor complex were plotted against the relative androgenic potency (RAP) [13].

DHEA showed no androgenic activity, but the calculated free energy is low, suggesting a good binding to the androgen receptor. Thus, DHEA shows affinity for the androgen receptor and might compete with bioactive androgens explaining its antagonistic properties. These antagonistic properties of DHEA were confirmed in the androgen yeast bioassay by coexposure of two concentrations of 17β-T, one concentration (70 nM) at half maximum and a concentration (1,000 nM) at full androgen bioassay response (data not shown).

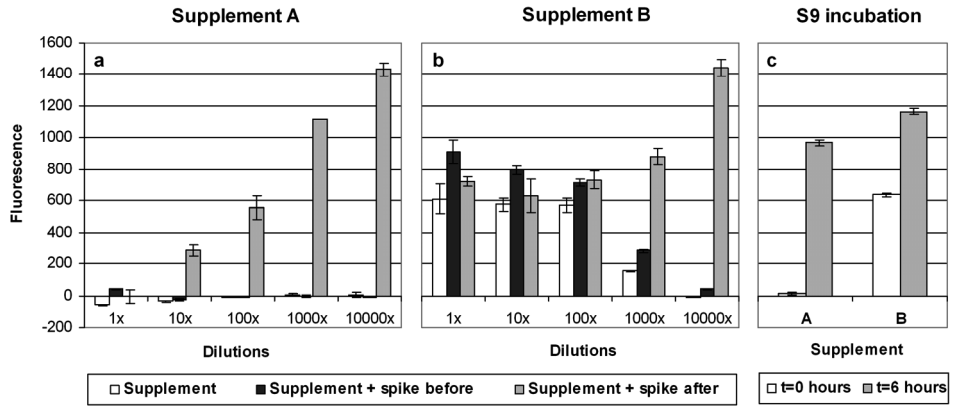

*Fig. 4 - Androgen bioassay responses of (**a**) supplement A and (**b**) supplement B as well as spiked with 17β-T before (217 nM in the well, calculated assuming 100% recovery) and after (300 nM added to the well) extraction and cleanup procedure. Fluorescence signals are the mean of an assay triplicate (± SD) and corrected for the signal at t=0 and the reagent blank.(**c**) Androgen bioassay results after incubation using bovine liver S9 and cofactor NAD.*

Figure 4b shows that supplement B gives a direct androgen bioassay response in accordance with the declared ingredient 4-adione. This response remains at the same level up to a 100-fold dilution of the extract, but at a 1,000-fold dilution the bioassay signal decreases. It should be noted that the "spike after" control does not reach its maximum fluorescence response of about 1,400 and that following dilution of the samples the signal of the "spike after" in the well increases. Again this is due to the antagonistic effects of DHEA, which is also a declared main compound in supplement B. Figure 4c shows the androgen bioassay responses of supplement A and B in the yeast androgen bioassay without and after the metabolic activation with the S9/NAD combination. Supplement A does not give a signal without the activation; however, after 6 h of incubation with bovine liver S9/NAD the signal reaches near maximal response, indicating that bioactive androgenic metabolites have been formed. As mentioned before, supplement B already shows a response; however, after the S9/NAD treatment the signal increases from about 600 to a near maximal response, again indicating that androgenic metabolites have been formed. From these results it can be concluded that supplement A contains pro-androgens showing anti-androgenic properties and supplement B contains both androgens and pro-androgens containing androgenic and anti-androgenic properties.

UPLC-TOFMS analysis

UPLC-TOFMS analysis with accurate mass measurement was used for the identification of the metabolites formed after bovine S9 incubation. The metabolites were identified by

exact mass measurement and elemental composition calculations thereof, followed by comparison of retention times and mass spectra with the data obtained from commercially available standards. Fifty-mDa window reconstructed ion chromatograms were used, being an appropriate window for elemental composition elucidation with a limited chance of false negative results due to mass shifts caused by coeluting isobaric compounds and/or detector saturation [19]. After metabolic activation of DHEA with bovine liver S9 and cofactor NAD (Fig. 5) the main metabolites of DHEA (X) appeared to be 4-adione (IX), 7α-OH-DHEA (II) and metabolite III at a retention time of 0.95 min having an abundant ion at *m/z* 303 and minor ions at *m/z* 285 and *m/z* 267 suggesting a keto-metabolite of DHEA (e.g. 7-oxo-DHEA) or a hydroxy-metabolite of 4-adione. Several minor abundant metabolites eluted after 0.70, 1.07, 1.21, 1.52, 2.09 and 2.29 min of which the last two appeared to be 5-adiol (VII) and 17β-T (VIII), respectively. The identification of 17β-T and 4-adione confirm the bioactivation of DHEA into androgenic substances observed in the androgen bioassay.

Metabolites I and IV are most likely hydroxy-metabolites of DHEA resulting in $[M+H]^+$ ions at *m/z* 305 and $[M-H2O+H]^+$ ions at *m/z* 287. V and VI most likely are keto-metabolites of DHEA or hydroxylated metabolites of 4-adione, showing $[M+H]^+$ ions at *m/z* 303 and $[M-H2O+H]^+$ ions at *m/z* 285. According to ref. [20] metabolism of DHEA employing human liver S9 for 20 min (instead of bovine liver S9 for 6 h) resulted mainly in 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, 16α-OH-DHEA and 5-adiol depending on the cofactor used; no androgenic metabolites like 4-adione and 17β-T were observed. The retention times of DHEA and its metabolites with corresponding exact masses have been summarized in Table 1. UPLC–TOFMS analysis showed that the spectra of 5-adiol, DHEA and hydroxy-metabolites generally were dominated by $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ ions, whereas the base peak in the mass spectra of 4-adione and 17β-T was the $[M+H]^+$ ion, as expected for 3-keto-4-ene steroids [21].

Metabolic activation of 4-adione, containing a 17β-T impurity, with bovine liver S9 resulted in an increase of 17β-T, thereby confirming the increase in androgen bioactivity as observed in the bioassay. Interestingly 17α-T was also formed. For 5-adiol, only metabolic activation using NAD resulted in formation of small amounts 17β-T, in accordance with the bioassay results in Fig. 3b. The fact that DHEA is converted into 4-adione and 5-adiol and the latter two into 17β-T supports that bovine liver S9 contains 3β-HSD/isomerase and 17β-HSD activity. The observed hydroxy-metabolites of DHEA are due to P450 enzyme activity. Indeed in vitro bovine S9 treatment can mimic the in vivo conversions shown in Fig. 1. UPLC-TOFMS analysis showed that supplement A contained only DHEA which is in accordance with what is declared on the label. Apart

from the claimed DHEA and 4-adione, supplement B contained 17β-T and traces of 17α-T, which are possibly by-products formed during production or storage. Metabolic activation of the supplements showed similar metabolite profiles as obtained during activation of DHEA and 4-adione standards, resulting in higher levels of 5-adiol and the more potent androgens 4-adione and 17β-T (see Electronic Supplementary Material).

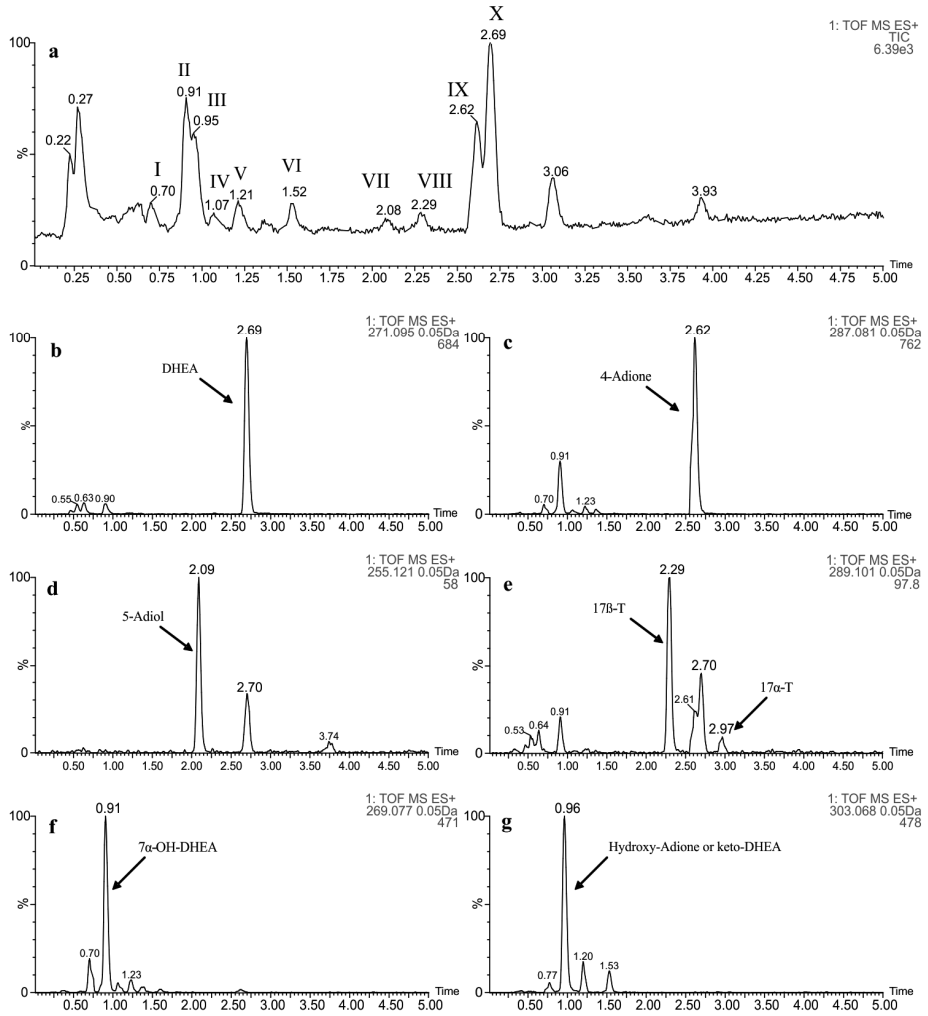

*Fig. 5 - UPLC-TOFMS total ion current (**a**) and reconstructed accurate mass chromatograms of DHEA (**b**), 4-adione (**c**), 5-adiol (**d**), 17α- and 17β-T (**e**), 7α-OH-DHEA (**f**) and a possible hydroxy-adione or keto-DHEA metabolite (**g**) using the accurate mass of the $[M+H]^+$ or $M+H-H2O]^+$ ion and a mass window of 0.05 Da. Conditions: bovine liver S9 bioactivation using cofactor NAD and an incubation time of 6 h.*

Table 1: UPLC-TOFMS analysis data of DHEA incubated for 6 hours with bovine liver S9/NAD

	Retention time (min)[a]	(*m/z*)[b]			Elemental composition	Identified metabolites[c]
		$[M+H]^+$	$[M-H_2O+H]^+$	$[M-2H_2O+H]^+$	$[M+H]^+$	
I	0.70	305.2117 (-0.3)	287.2023 (1.2)	269.1928 (2.3)	$C_{19}H_{29}O_3$	-
II	0.91	-	287.2010 (-0.1)	269.1905 (0.0)	$C_{19}H_{29}O_3$	7α-OH-DHEA
III	0.95	303.1945 (-1.5)	285.1864 (0.9)	267.1781 (0.9)	$C_{19}H_{27}O_3$	-
IV	1.07	305.2077 (-4.0)	287.2038 (2.7)	269.1889 (-1.6)	$C_{19}H_{29}O_3$	-
V	1.21	303.1974 (1.4)	285.1855 (0.0)	-	$C_{19}H_{27}O_3$	-
VI	1.52	303.1966 (0.6)	285.1858 (0.3)	267.1768 (1.9)	$C_{19}H_{27}O_3$	-
VII	2.08	-	273.2220 (0.2)	255.2126 (1.3)	$C_{19}H_{31}O_2$	5-Androstene-3,17-diol
VIII	2.29	289.2197 (2.9)	-	-	$C_{19}H_{29}O_2$	17ß-Testosterone
IX	2.62	287.1981 (-3.0)	-	-	$C_{19}H_{27}O_2$	4-Androstene-3,17-dione
X	2.69	289.2160 (-0.8)	271.2049 (-1.3)	253.1947 (-0.9)	$C_{19}H_{29}O_2$	DHEA

[a]Experimental retention times
[b]Accurate masses of observed ions, with mass error versus the theoretical masses in mDa (in parenthesis)
[c]Compounds confirmed by comparison with a standard

Conclusions

This work has outlined the concept of a bioactivity screening assay for prohormones using a combination of bovine liver S9 bioactivation with androgen bioactivity detection. The prohormone DHEA shows no direct androgenic activity in the androgen yeast biosensor but anti-androgenic properties. On the other hand, DHEA, but also 4-adione and 5-adiol, can be converted by bovine S9 into more potent androgens, resulting in an indirect androgenic hormonal activity. The developed *in vitro* bioactivation system successfully mimics the hydroxysteroid dehydrogenase (HSD)- and cytochrome P450-mediated *in vivo* metabolic transitions, thus allowing assessment of the indirect bioactivity and chemical identification without the use of animal experiments. UPLC-TOFMS analysis confirmed that the tested prohormones are metabolized into the androgenic active steroids 4-adione and 17β-T. In the same manner other prohormones of androgenic bioactive compounds requiring transformation by HSDs at the 3-position and 17-position can be screened for e.g. the prohormones 19-norandrostenedione and 19-norandrostenediol are expected to be converted into the potent anabolic androgenic steroid nandrolone by 3β-HSD/isomerase and 17β-HSD activity. In conclusion we can state that the androgen bioassay can be used for the screening of supplements for the presence of androgens, anti-androgens and prohormones, the last of these following liver S9 bioactivation. The system developed is expected to be equally applicable to prohormone preparations from sports doping.

Acknowledgements

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Chapter 4

Bioassay based screening of steroid derivatives in animal feed and supplements

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Abstract

Receptor binding transcription activation bioassays are valuable tools for the screening of steroid hormones in animal feed and supplements. However, steroid derivatives often lack affinity for their cognate receptor and do not show any direct hormonal activity by themselves. These compounds are thus not detected by these kind of bioassays and need a bioactivation step in order to become active, both *in vivo* and *in vitro*. In this study a comparison was made between different *in vitro* activation methods for hormone esters and hormone glycosides. Testosterone acetate and testosterone decanoate were chosen as model compounds for the hormone esters, representing the broad range of steroid esters of varying polarities, while genistin was used as a substitute model for the steroid-glycosides. Concerning bioactivation of the steroids esters, the efficiency for alkaline hydrolysis was 90-100% and much better as compared to enzymatic deconjugation by esterase. As a result 1 µg testosterone ester per gram of animal feed could easily be detected by a yeast androgen bioassay. When comparing different enzyme fractions for deglycosilation, genistin was shown to be deconjugated most efficiently by ß-glucuronidase/aryl sulfatase from Helix pomatia, resulting in a significant increase of estrogenic activity as determined by a yeast estrogen bioassay. In conclusion, chemical and enzymatic deconjugation procedures for ester and glycoside conjugates respectively, resulted in a significant increase in hormonal activity as shown by the bioassay readouts and allowed effective screening of these derivatives in animal feed and feed supplements.

1. Introduction

The use of anabolic steroids as growth promoters in livestock production is banned within the European Union [1]. To ensure compliance with this ban, requirements for hormone residue analysis are described at the European level and implemented at national levels in residue monitoring programs [2]. Hormone abuse or incidents may be discovered by residue analysis in matrices such as urine, hair and feed. For screening animal feed and supplements reporter gene bioassays have proven their added value in detecting known and unknown steroidal compounds [3-5]. Oral administration of natural steroids via feed or supplements results in low bioavailability due to poor intestinal absorption and extensive hepatic first-pass metabolism. Alternatively, numerous derivatives have been synthesized with the goal to circumvent metabolism and prolong biological activity *in vivo*. Moreover, for efficient uptake, these compounds are often administered via injections, gels or implants. Intramuscular injection of hydrophobic testosterone esters for instance accounts for a slow release in the systemic circulation and by mixing short- and long-chain esters, both short and long term effects are obtained. In addition, steroid esters are also designed to improve the oral availability of steroids since esterification makes steroids sufficiently lipophilic to be incorporated in chylomicrons formed during lipid digestion in the intestine [6]. As a result, testosterone esters are absorbed by the intestinal lymphatic system and enter via this route the systemic circulation, thereby circumventing hepatic first-pass metabolism [7]. The oral bioavailability of testosterone undecanoate was estimated to be 7% and intake of 120-160 mg testosterone undecanoate equals the complete daily production of testosterone in males [8]. Apart from the lipophilicity of the compound, uptake and bioavailability also depend on the lipophilicity of the solvent used and might even be enhanced when taken together with food [6].

Steroidal glycosides constitute a structurally and biologically diverse class of molecules which have been isolated from a wide variety of both plant and animal species [9,10]. Similar to steroid esters, the glycoside group controls the pharmacokinetics and greatly modifies the biological activity of the steroid [11]. *In vivo* experiments with orchiectomised rats that received testosterone glycoside (Figure 1), either orally or intramuscularly, showed significant higher blood levels of testosterone compared to animals receiving oral testosterone [12]. This suggests that androgen glycosides taken orally are less susceptible to hepatic first pass metabolism than their corresponding unglycosilated androgens.

Because the direct androgenic activity of steroid conjugates is often limited and binding to steroid receptors and subsequent biological effects only occur after deconjugation, there is a chance of missing the illegal use of these intact steroid conjugates when screening with

bioassays based on receptor binding and the subsequent transcription activation of a marker gene. In the present work deconjugation steps were developed for steroid esters and glycosides in order to screen for their presence in animal feed and supplements with yeast based bioassays. Testosterone acetate and testosterone decanoate (Figure 1) were selected as model compounds for the development of an enzymatic or chemical hydrolysis method for steroid esters. Due to the lack of an androgen glycoside standard the glycosilated isoflavonoid genistin was used as a steroid-glycoside mimic compound, and combined with an estrogen bioassay screening in order to determine which enzymatic deconjugation was the most suited.

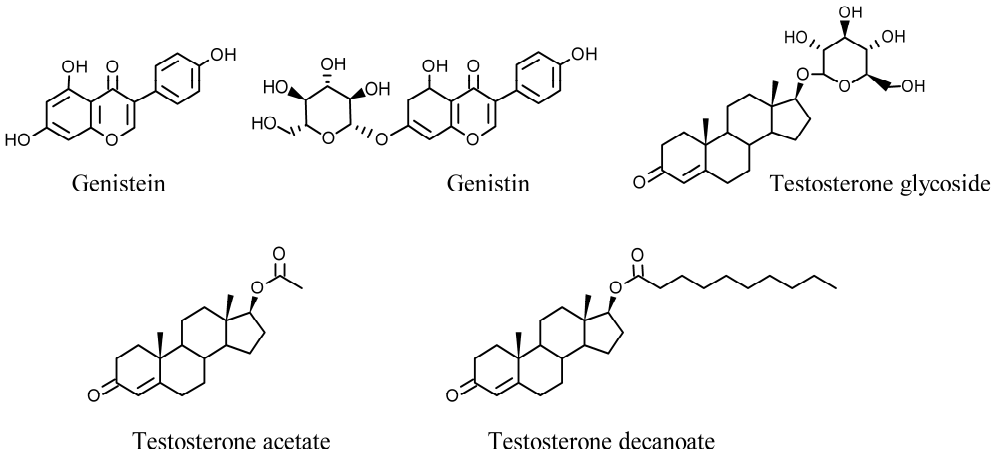

Figure 1: Chemical structures of genistein, genistin, testosterone glycoside, testosterone acetate and testosterone decanoate.

Because the direct androgenic activity of steroid conjugates is often limited and binding to steroid receptors and subsequent biological effects only occur after deconjugation, there is a chance of missing the illegal use of these intact steroid conjugates when screening with bioassays based on receptor binding and the subsequent transcription activation of a marker gene. In the present work deconjugation steps were developed for steroid esters and glycosides in order to screen for their presence in animal feed and supplements with yeast based bioassays. Testosterone acetate and testosterone decanoate (Figure 1) were selected as model compounds for the development of an enzymatic or chemical hydrolysis method for steroid esters. Due to the lack of an androgen glycoside standard the glycosilated isoflavonoid genistin was used as a steroid-glycoside mimic compound, and combined with an estrogen bioassay screening in order to determine which enzymatic deconjugation was the most suited.

2. Materials and Methods

2.1 Chemicals

17ß-Testosterone, 17ß-estradiol, genistin, genistein, esterase (from porcine liver), ß-glucuronidase type H5 from Helix pomatia, ß-glycosidase and L-leucine were purchased from Sigma (St. Louis, MO, USA). Testosterone acetate and testosterone decanoate were obtained from Steraloids (Newport, RI, USA). Sodium acetate, sodium carbonate, acetic acid, formic acid, hydrochloric acid, sodium hydroxide, dimethylsulfoxide (DMSO) and ß-glucuronidase/aryl sulfatase from Helix pomatia were purchased from Merck (Darmstadt, Germany). Dextrose, ammonium sulphate and yeast nitrogen base without amino acids and without ammonium sulphate were purchased from Difco (Detroit, MI, USA). HPLC grade acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). Millipore water was obtained by using a Purelab Ultra system from Elga (Bucks, UK).

2.2 Instrumentation

2.2.1 HPLC analysis

To determine levels of genistein, genistin and testosterone, HPLC analysis was performed on a Waters HPLC system (Waters, Milford, MA) consisting of two model 510 pumps, a model 717 plus auto injector and an automated gradient controller. Chromatographic separation was performed on a Supelcosil LC-18DB 5 µm, 4.6 x 2500 mm column (Supelco, Bellefonte, PA, USA) which was maintained at 40°C in a column oven. The mobile phases consisted of (A) 0.2% formic acid and (B) 0.2% formic acid in acetonitrile. A gradient was run at a flow rate of 1 mL min^{-1} starting at 20% B for 2 minutes followed by a linear increase to 80% B in 8 minutes. Next, the gradient remained 8 minutes at 80% B and returned linearly in 4 minutes to 20% B and remained 3 minutes at this level until the next injection. The injection volume was 50 µL and the column effluent was monitored by a Waters 996 photodiode array detector (Waters) at 200-400 nm and data was retrieved at a single wavelength of 260 nm. The HPLC system was equipped with Empower software (Waters).

2.2.2 LC-MS analysis

Levels of testosterone, testosterone acetate and testosterone decanoate were determined by LC-MS analysis. Liquid chromatography was performed on a Agilent Technologies 1200series system (Agilent Technologies, Santa Clara, CA, USA) containing a Eclipse XDB-C_8 5 µm, 3.0 x 150 mm column (Agilent Technologies) which was kept at 40 °C.

The mobile phases consisted of (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. The injection volume was 50 µL and the flow was 0.4 mL min^{-1}. A step-wise gradient was run starting with 30% B which was linearly increased to 100% B in 10 minutes, kept at 100% B for 10 minutes. Then the gradient returned in 1 minute to 30% B and was kept at this level for 4 minutes until the next injection was started. The column effluent was directly introduced into a Waters model Micromass Quatro micro mass spectrometer operating in ESI positive ion mode. The capillary voltage of the ion source was set at 3000 V and the cone voltage was 30 V. The source temperature was 110 °C, desolvation temperature was 350 °C and the cone and desolvation gas flow were set on 30 L h^{-1} and 600 L h^{-1} respectively. Data was recorded and processed using Masslynx v 4.1 software (Waters).

2.3 Procedures

2.3.1 Alkaline and enzymatic hydrolysis of hormone esters

For alkaline hydrolysis, 1 gram of sample was mixed with 6 mL methanol for 30 minutes in a head-over-head apparatus. The mixture was centrifuged at 3000 x g for 15 minutes and the supernatant was transferred to a fresh tube. The total supernatant volume was adjusted to 6 mL with methanol and deconjugation was performed by adding 750 µL sodium hydroxide (2.5 M) and incubation for 1.5 h at 60 °C in a water bath. After cooling to room temperature, 900 µL hydrochloric acid (2.5 M) was added to stop hydrolysis. Subsequently, 6 mL sodium acetate buffer (0.25 M, pH 4.8) was added and the pH was adjusted to pH 4.8 with acetic acid (4 M). Next, 6 mL of the extract was applied to the solid phase extraction (SPE) clean-up procedure as described below.

For enzymatic hydrolysis, 1 gram of sample was shaken manually with 6 mL sodium phosphate buffer (0.2 M, pH 7.4) and 20 µL of esterase solution (300 U mL^{-1}). Samples were incubated for 3 hours at 37 °C in a shaking water bath and enzymatic hydrolysis was stopped by adding 6 mL methanol. Samples were adjusted to pH 4.8 with hydrochloric acid (2.5 M). After centrifugation for 15 minutes at 3000 x g, 6 ml of supernatant was applied to the solid phase extraction (SPE) clean-up procedure as described below.

SPE was performed on Bond Elute C_{18} SPE columns (Varian, 1000 mg, 6 mL) previously activated with 4 mL methanol and 4 mL methanol/sodium acetate buffer (50/50 v/v). The column was subsequently washed with 3 mL methanol/sodium acetate buffer (50/50 v/v), 4 ml water, 3 ml sodium carbonate (10 % w/v), three times 4 mL water and two times 4 mL methanol/water (50/50 v/v). After drying for 5 minutes under vacuum, the SPE columns were eluted with 2 times 4 mL acetonitrile followed by 2 mL ethylacetate. Next, the acetonitrile and ethylacetate eluate were applied to an Isolute NH_2 column (IST, 500

mg, 3 mL) previously conditioned with 4 mL acetonitrile. The effluent was collected separately, evaporated to dryness under a gentle stream of nitrogen gas and reconstituted in 3 mL of acetonitrile. Next, 200 µL aliquots of these final acetonitrile extracts were pipetted into a 96-well plate in triplicate and 50 µl of 4% DMSO was added. To remove the acetonitrile, plates were dried overnight in a fume hood, remaining only 2 µL of DMSO the next day.

2.3.2 Enzymatic deconjugation of glycoside derivatives

For enzymatic deconjugation of glycoside derivatives, 100 mg of sample was mixed with 4 mL sodium acetate (0.2M, pH 5.2) and 30 µL of either Helix pomatia ß-glucuronidase/ aryl sulfatase, ß-glycosidase or ß-glucuronidase type H5 from Helix pomatia solution was added (each 24 U mL^{-1}). Next, samples were incubated for 3 hours at 52°C in a shaking water bath. After cooling to room temperature, 4 mL methanol was added and samples were mixed head-over-head for 10 minutes. The mixture was centrifuged for 15 minutes at 3000 rpm and 4 mL supernatant was transferred to a fresh glass tube. The pH was adjusted to 4.8 with acetic acid (4 M) and the extracts were subjected to an Oasis HLB SPE column (Waters, 30 mg, 6mL) previously conditioned with 2 mL methanol followed by 2 mL water. Subsequently, the SPE cartridges were washed with 2 mL water and 2 mL methanol/water (50/50 v/v), dried under vacuum for 2 minutes and eluted with 4 mL methanol. A 500 µL portion of the eluate was evaporated to dryness under nitrogen gas at 45°C and resuspended in 500 µL methanol/water (50/50 v/v) for analysis by HPLC. The remaining 3.5 mL eluate was evaporated to dryness under nitrogen gas at 45°C and resuspended in 1.5 mL acetonitrile. Next, 200 µL aliquots of this final acetonitrile extract were pipetted into a 96-well plate in triplicate and 50 µL of 4% DMSO was added. To remove the acetonitrile, plates were dried overnight in a fume hood, remaining only 2 µL of DMSO the next day.

2.3.3 In vitro yeast androgen and estrogen bioassays

Saccharomyces cerevisae transformants expressing either the human androgen receptor (hAR) or the human estrogen receptor alpha (hERα) were grown on selective minimal medium plates supplemented with L-leucine. Supplemented minimal medium (MM/L) consisted of yeast nitrogen base without ammonium sulphate or amino acids (1.7 g L^{-1}), dextrose (20 g L^{-1}), ammonium sulphate (5 g L^{-1}) and was supplemented with L-leucine (6 mg L^{-1}). The yeast androgen and estrogen bioassays were performed as described previously [3,14]. In short, 10 mL MM/L was inoculated with a single colony of the recombinant yeast and grown overnight at 30°C in an orbital shaking incubator at 125

rpm. The next day, this yeast culture was diluted with MM/L until an optical density (OD) value at 630 nm between 0.04 and 0.06 was reached. For exposure to standard compounds, 200 µL aliquots of yeast culture were pipetted into each well of a 96-well plate and 2 µL stock solutions dissolved in DMSO were added. For exposure of the yeast to sample extracts, 200 µL aliquots of yeast culture were pipetted into each well of a 96-well plate already containing the dried extracts as described in sections 2.2 and 2.3. Fluorescence was measured at 0 and 24 hours in a Synergy^{TM} HT microplate reader (BioTek Instruments Inc., USA) using excitation at 485 nm and measuring emission at 530 nm. Standards as well as samples were assayed in triplicate and each fluorescence signal was corrected for the signal at 0 hours and the reagent blank, containing DMSO solvent only. The OD at 630 nm was measured after 24 hours to check whether the yeast was grown well and to determine whether a sample extract was cytotoxic. 17ß-testosterone and 17ß-estradiol standard curves were included in each androgen and estrogen bioassay experiment respectively. Dose-response curves were fitted using the equation $y=a_0/((1+(x/a_1))^{a_2})$. This is equal to: response= (max.response-min.response)/((1+([agonist]/EC_{50}))^width of transition).

3. Results and Discussion

3.1 Androgenic and estrogenic activities of model compounds

The dose-response curves of 17ß-testosterone, testosterone acetate (TA) and testosterone decanoate (TD) as obtained in the yeast androgen bioassay are shown in Figure 2A. Curves were fitted through the data points by non-linear regression and the concentration giving half the maximum response (EC_{50}) was calculated. As expected, TA showed no response in the yeast androgen bioassay. Surprisingly, TD showed a clear response and an EC_{50} value of $1.4*10^3$ nM was calculated, resulting in a relative androgenic potency (RAP) of 0.063 compared to 17ß-testosterone (EC_{50} value of 88 nM). As TD most likely does not bind to the androgen receptor, the obtained response is most probably caused by minor 17ß-testosterone impurities formed during manufacturing and storage of TD or by hydrolysis of TD during sample clean-up or exposure of the yeast. Therefore a TD standard solution was fractionated by a previous described LC system [3] and the fractions were analysed by the yeast androgen bioassay. After fractionation no activity of TD was observed while a clear activity was observed in the yeast androgen bioassay caused by a 17ß-testosterone impurity which was about 1% of the starting amount of TD (data not shown).

The glycosilated isoflavone genistin showed only limited estrogenic activity in the yeast

estrogen bioassay (Figure 2B). Although the maximum response as observed for 17ß-estradiol and genistein was not reached, the EC_{50} of genistin was calculated to be $3.8*10^4$ nM. Genistin was thus at least a factor 10 less potent than its deconjugated equivalent genistein (Figure 1), which showed an EC_{50} value of $2.7*10^3$ nM. As both steroid esters as well as the glycoside conjugate are substantially less potent than their deconjugated equivalents, they can be used as model compounds for the development of deconjugation steps for hormone esters and glycosides prior to the use of yeast based bioassays.

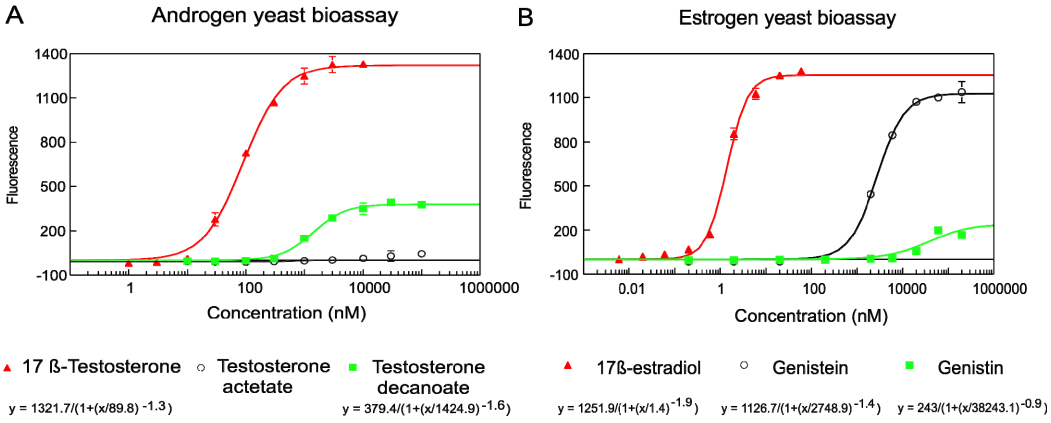

Figure 2: *Dose-response curves of (A) 17ß-testosterone, testosterone acetate and testosterone decanoate obtained in the androgen yeast bioassay and (B) 17ß-estradiol, genistein and genistin obtained in the estrogen yeast bioassay. Fluorescence signals are the mean of an assay triplicate (±SD) and corrected for the signal at t=0 and blank DMSO.*

3.2 Alkaline and enzymatic hydrolysis of hormone esters

A previously established SPE sample clean-up procedure for bioassay based screening of androgens in animal feed was used as a starting point for method development [4]. The new procedure included the addition of an extra ethyl acetate elution step in order to elute also the more lipophilic testosterone esters, as the original method was designed to extract the unconjugated steroids only. Academic standards of 17ß-testosterone, TA and TD were applied to this modified SPE clean-up and the eluate fractions were subsequently analysed in the yeast androgen bioassay. As expected, only testosterone showed a clear response, while no response was obtained for TA and TD (Figure 3, first 8 bars). However, a clear response was obtained in the acetonitrile fraction of TA spiked blanks and the ethyl acetate fraction of the blanks spiked with TD when esterase was added during the exposure of the yeast cells to these fractions (Figure 3, second series of 8 bars). These findings indicate hydrolysis of the testosterone esters by the added esterase and

demonstrates that TA is eluted easily from the column with acetonitrile, just like the unconjugated steroids, while the more lipophilic TD is hardly eluted by acetonitrile and is mainly present in the ethyl acetate fraction.

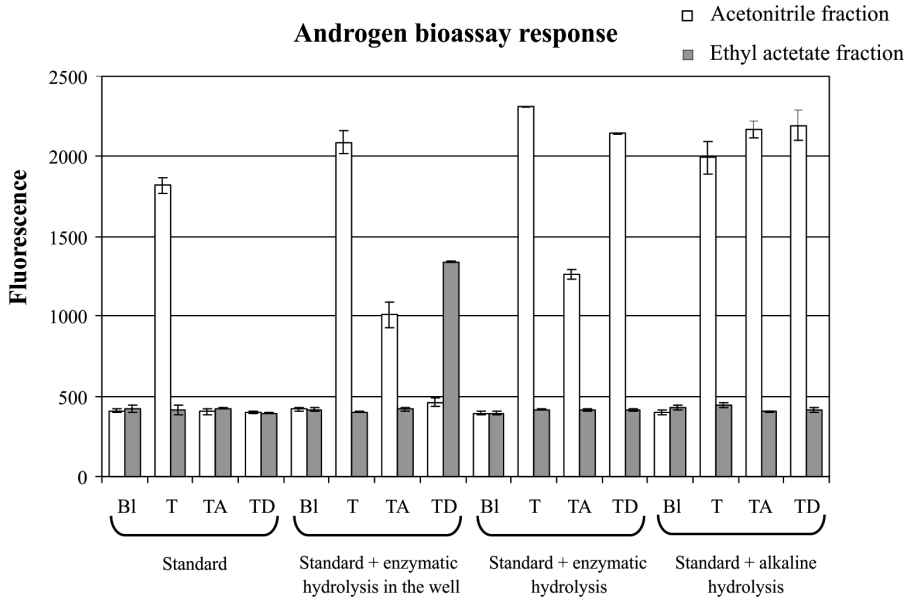

Figure 3: *Androgen bioassay response of acetonitrile and ethyl acetate SPE fractions. Blanks (Bl) and standard solutions containing testosterone (T), testosterone acetate (TA) or testosterone decanoate (TD) were subjected either to enzymatic or alkaline hydrolysis prior to the SPE sample clean-up procedure or afterwards, by enzymatic hydrolysis in the well. Fluorescence signals are the average of an assay triplicate and corrected for the signal obtained at t=0 hours.*

Enzymatic or alkaline hydrolysis of TA and TD before the SPE sample clean-up, revealed strong responses in the acetonitrile fractions only (Figure 3, third and forth series of 8 bars). These findings not only demonstrate both effective enzymatic and alkaline hydrolysis of the T-esters, but once more demonstrates that the unconjugated free testosterone is already completely eluted with acetonitrile. Moreover, on a semi-quantitative level, the results indicate that the recovery for the T-esters is lower than that of the unconjugated testosterone and as a result it is better to perform the activation step before the sample clean-up.

Next, animal feed samples from practice were spiked with 17ß-testosterone, TA or TD. After sample clean-up and exposure of the androgen yeast cells to the sample extracts, the enzymatic hydrolysis performed in the well, by the addition of esterase, showed no significant increase in the obtained responses and only the testosterone spiked feed

samples gave clear positive responses compared to the blank sample (data not shown). However, enzymatic hydrolysis before SPE clean-up resulted in clear responses of all spiked feed samples (Figure 4A). Recoveries for the testosterone spikes were determined by LC-MS analysis and were between 60 to 80%, resulting in maximal responses in the yeast androgen bioassay. After enzymatic hydrolysis also all the TA and TD spiked samples showed a maximal or near maximal response in the yeast androgen bioassay, although only 10 to 20% of the esters were hydrolysed to 17ß-testosterone, as was determined by LC-MS. Moreover, while the recovery of the remaining 80 to 90% of intact ester was 90 to 130% for TA whereas it was only 10 to 30% for TD. This low recovery for TD is probably caused by poor elution of the intact TD ester from the SPE column and/or solubility issues during analysis.

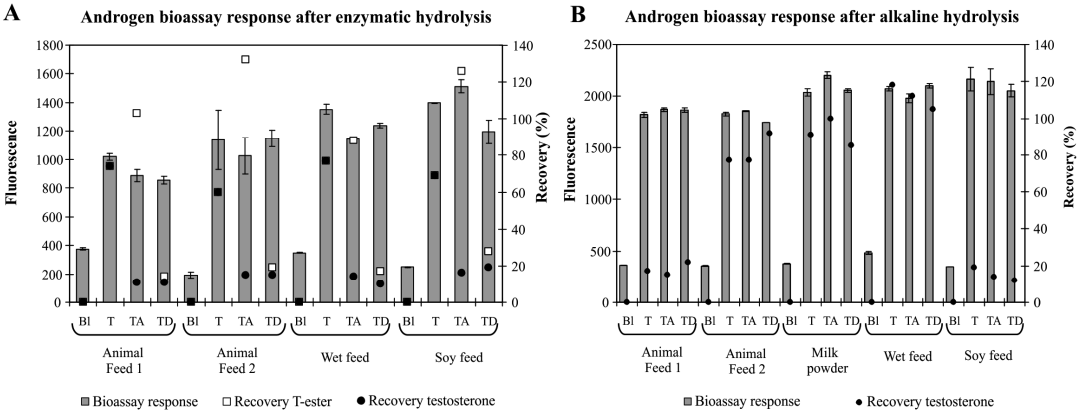

Figure 4: *Androgen bioassay response of blank feed samples and feed samples spiked with 25 µg of testosterone (T), testosterone acetate (TA) or testosterone decanoate (TD) after (A) enzymatic and (B) alkaline hydrolysis. Fluorescence signals are the average of an assay triplicate and corrected for the signal obtained at t=0 hours. The percentage of TA and TD that remained intact (□) and the percentage of free 17β-testosterone (●) formed was determined by LC-MS analysis.*

Alkaline hydrolysis was shown to be more efficient as compared to enzymatic hydrolysis (Figure 4B). After hydrolysis, all spiked samples showed a maximal response in the yeast androgen bioassay. Recoveries of 17ß-testosterone were between 80 and 120% in animal feed 2, the milk replacer and the wet feed sample and surprisingly low for animal feed 1 and the soy feed sample. In case of the TA and TD spiked samples nearly all of the ester was converted to 17ß-testosterone and only small traces of the intact esters were recovered. The applied alkaline hydrolysis procedure is therefore highly efficient, as LC-MS analysis showed that 90 to 100% of the T-esters were hydrolysed. The detection limit of the alkaline hydrolysis procedure was shortly investigated by testing a concentration

range of both the T-esters. This revealed that 1 µg of testosterone ester per gram of feed could easily be detected by the yeast androgen bioassay (data not shown).

3.3 Enzymatic deconjugation of glycoside derivatives

Three different enzyme fractions were assessed for their ability to convert genistin into genistein. Figure 5 shows the rates at which H5 ß-glucuronidase, ß-glycosidase and ß-glucuronidase/aryl sulfatase from Helix pomatia convert genistin into genistein. ß-glucuronidase/aryl sulfatase achieved full conversion within 1 h while the other two enzyme fractions required an overnight (16 h) digestion to achieve a full conversion. The negative control showed no conversion of genistin during the first 7 hours, but after 16 h 17% of the genistin was deconjugated under formation of genistein.

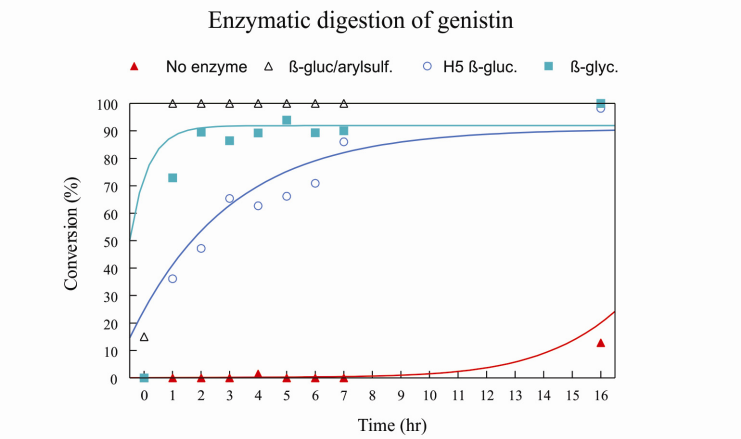

Figure 5: *Genistin standards and feed samples enzymatically digested by H5 ß-glucuronidase (H5 ß-gluc.), ß-glycosidase (ß-glyc.) or ß-glucuronidase/arylsulfatase from Helix pomatia (ß-gluc/arylsulf.) (0.2 U mL^{-1}). Shown is the enzymatic digestion of 25 µM genistin over a period of 16 hours measured by HPLC.*

After development of an efficient extraction procedure, which is described in section 2.3, the three different enzyme fractions were assessed for their ability to deconjugate genistin in real-life animal feed and feed supplement samples. The samples analysed consisted of 4 different soy based feed samples and 2 herbal feed additives that were expected to contain isoflavones as well as glycosilated isoflavones like genistin [13]. Except for feed supplement 1, HPLC analysis confirmed that all sample extracts contained genistein in the range of 2 to 20 µM, which consequently resulted in an initial response in the yeast estrogen bioassay without the addition of an enzyme as shown by the first bars in figure 6. Incubation of the feed samples with ß-glucuronidase/aryl sulfatase for three hours resulted

in a significant increase in estrogenic activity, while incubations with H5 ß-glucuronidase or ß-glycosidase resulted in no or a limited increase in estrogenic activity as compared to the control samples incubated without the addition of an enzyme. These observations were supported by the HPLC data, showing an increase of the genistein levels in the soy feed samples that were treated with the ß-glucuronidase/arylsulfatase enzyme mix.

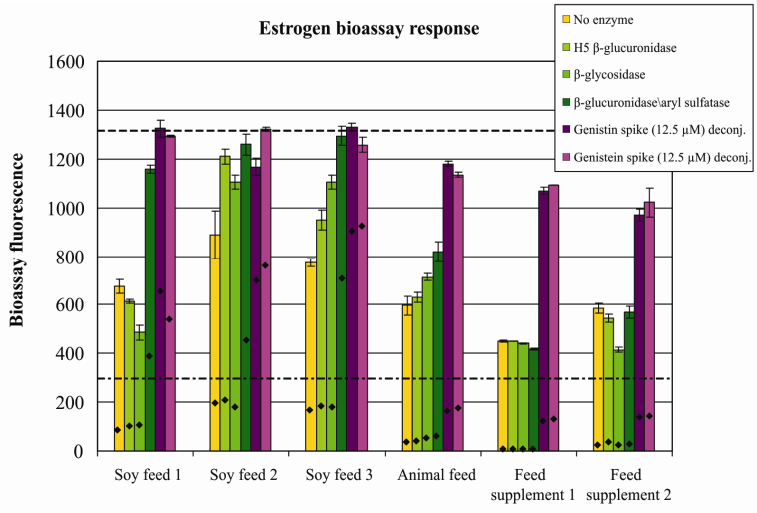

Figure 6: *The estrogen bioassay response of animal feed samples and supplements determined after enzymatic deconjugation. The samples spiked with genistin and genistein were deconjugated with ß-glucuronidase/ arylsulfatase. Bioassay fluorescence signals are the average of an assay triplicate and corrected for the signal obtained at t=0 hours. Mean response of the yeast exposed to the DMSO blank (···), the maximum response of the 17ß-estradiol standard curve (---) and genistein levels prior to deconjugation (●) are included respectively.*

In addition, an attempt was made to get an impression of the conversion efficiency of the ß-glucuronidase/arylsulfatase enzyme mix in these animal feed samples. The samples were therefore spiked with 12.5 µM genistin or 12.5 µM genistein. Upon enzymatic deconjugation a near maximal response was obtained for all these spiked samples, showing almost no difference between the genistin and genistein spiked samples (Figure 6). This indicates that genistin is almost completely converted into genistein and the obtained results are in accordance with the expectations for genistein, as 12.5 µM genistein showed a near maximal response in the yeast estrogen bioassay (Figure 2B).

4. Conclusions

The previously developed yeast based bioassay methods for the screening of estrogens and androgens in feed were not suited to detect inactive steroid conjugates [4, 14]. This

study was intended to develop deconjugation methods in order to activate the inactive steroid conjugates. Testosterone acetate and testosterone decanoate were chosen as model compounds for steroid esters and genistin was chosen as a model compound for a glycoside conjugate. These conjugates were shown to be relatively inactive compared to their free aglycons, testosterone and genistein respectively. Subsequently, it was shown that the hormone esters were most efficiently activated by alkaline hydrolysis while the glycoside conjugate genistin could easily be activated by the ß-glucuronidase/arylsulfatase enzyme mix that is also used for the deconjugation of compounds in calf urine samples [4].

Regarding the sensitivity, for free androgens in wet pulp feed and milk replacers the androgen yeast assay is fully validated according EC Decision 2002/657 [15] at a level of 50 or 100 ng g^{-1} [4]. Although the present study demonstrated that after the alkaline hydrolysis it was still not possible to screen for the presence of testosterone esters as such low levels, 1µg g^{-1} feed could be easily detected by the androgen yeast assay for TA as well as TD. These levels are considered a relevant level for hormone esters to screen for. Together with a previous developed bioactivation protocol for prohormones like DHEA making use of a liver S9 mix [16], a panel of activation steps has been developed. Combined, this results in a comprehensive effect based screening strategy fully meeting EC directive 96/22, which states that all compounds having certain hormonal activity are prohibited [1].

Acknowledgements

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Chapter 5

Bovine liver slices: a multifunctional *in vitro* bioactivation model to study the prohormone dehydroepiandrosterone (DHEA)

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Abstract

Biotransformation of inactive prohormones like dehydroepiandrosterone (DHEA) can lead to the formation of potent androgens and subsequent androgenic responses in target tissues. In the present study a multi-functional in vitro bovine bioactivation model has been developed allowing to study the bioactivation of DHEA and resulting effects on the metabolite, transcript and androgenic activity level. Precision-cut bovine liver slices were exposed for 6 hours to various concentrations of DHEA. Changes in androgenic activity of DHEA containing cell culture media were measured using a yeast androgen bioassay and metabolites were identified using ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFMS). Furthermore, gene expression in the DHEA-treated liver slices was examined using bovine microarrays and gene expression profiles were compared with those obtained with 17ß-testosterone (17ß-T). An increase in androgenic activity was observed in the bioassay upon testing of samples from incubations of DHEA with liver slices and the formation of 4-androstenedione (4-AD), 5-androstene-3ß,17ß-diol, 17ß-T, 7α-hydroxy-DHEA, 7-keto-DHEA and 17α-T could be confirmed by UPLC-TOFMS analysis. Exposure of liver slices to DHEA and the strong androgen 17ß-T resulted in the identification of significantly up- and down-regulated genes and revealed similar gene expression profiles for both compounds. The results obtained indicate that DHEA itself is biologically not a very active compound, but is rapidly activated by liver slices in vitro. Moreover, the data presented successfully highlighted the multifunctional properties of bovine liver slices as an in vitro bioactivation model allowing the assessment of androgen activity or gene expression as effect-based endpoints for prohormone exposure.

Introduction

Steroidal sex hormones are potent compounds and play an important role in development, sexual maturation and behavior of both humans and animals. Human exposure to high levels of steroids results in disturbance of homeostatic hormone levels and is associated with an increased risk in the development of certain cancers (Pike et al., 1993; Hsing, 2001). Especially homeostasis in sensitive populations, like young children, might be easily disrupted as exposure to exogenous hormones, e.g. dietary intake can be relatively high compared to extreme low endogenous hormone levels (Courant et al., 2007, 2008). Residues of hormones and growth promoters in food and feed are thus potential health hazards for consumers. Therefore, within the European Union, the use of growth promoters in animal production is strictly forbidden (EEC Directive 96/22, 1996).

Despite the European ban, hormones are still surreptitiously used by farmers to improve weight gain and feed conversion efficiency. Albeit synthetic steroids are still used, the tendency is moving towards the use of natural steroids and their precursors such as the prohormone dehydroepiandrosterone (DHEA). Although DHEA lacks direct hormonal activity (Rijk et al., 2008), it has the potential to enhance levels of androgens and estrogens *in vivo* (Labrie et al., 1998). In order to prove and prevent fraudulent use of (pro)hormones, implemented monitoring programs focus on analyzing residues in matrices such as urine, feed and hair. For urine, feed and feed supplements, effect-based high throughput screening bioassays for hormones have proven their additional value (Bovee et al., 2009). However, inactive prohormones can not be detected by such screening assays and an additional bioactivation step is needed for these compounds (Rijk et al., 2008).

Because the liver is an important site of metabolism of (pro)hormones, *in vitro* liver models are attractive tools to study biotransformation of (pro)hormones into more or less active metabolites. These models range from simple enzyme preparations like microsomes and liver S9 fractions (Merlanti et al., 2007; Rijk et al, 2008) up to models more close to the *in vivo* situation, like primary hepatocytes (Forsell et al. 1985; Donkin and Armentano, 1993), liver slices (Zalko et al., 1998) and whole liver perfusions (Niles et al., 1961). Each model has its own advantages and disadvantages (Plant, 2004), while liver slices have the advantage that cell-cell interactions, cell heterogeneity and spatial arrangement are maintained. Moreover, liver slices allow investigation of a treatment effect at the gene expression level, as shown for rats (Elferink et al., 2008). Using the biotransformation capacity of liver slices together with gene expression analysis as an endpoint may provide an assay for effect-based screening for the presence of (pro)hormones in preparations and biological matrices.

The aim of the present work was to investigate the potential of precision cut bovine liver slices as a multifunctional *in vitro* model to study bioactivation of DHEA and resulting effects on the metabolite, transcript and androgenic activity level. Following incubation of liver slices with different concentrations of DHEA, changes in androgenic activity of the DHEA containing incubation medium were monitored using a sensitive yeast androgen assay and ultra performance liquid chromatography in combination with time-of-flight mass spectrometry (UPLC-TOFMS) was used to identify the metabolites formed. Subsequently, liver slice gene expression profiles induced by DHEA were obtained using whole genome bovine oligonucleotide microarrays and were compared with profiles induced by the potent anabolic androgen 17ß-testosterone.

Materials and Methods

2.1. Chemicals

17ß-testosterone was purchased from Steraloids (Newport, RI, USA). Dehydroepi-androsterone (DHEA), bovine serum albumin and HEPES were obtained from Sigma (St. Louis, MO, USA). Sodium hydrogen carbonate, D-glucose, EDTA, tris(hydroxymethyl) aminomethane (Tris), calcium chloride dehydrate, potassium chloride, sodium chloride, magnesium sulphate heptahydrate, potassium dihydrogen phosphate and dimethyl-sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Williams' medium E supplemented with Glutamax (WE) and charcoal-stripped fetal bovine serum were obtained from Invitrogen (Breda, The Netherlands). Acetonitrile and diethyl ether were purchased from Biosolve (Valkenswaard, The Netherlands). MilliQ-water used for UPLC-TOFMS was purified using a Millipore MilliQ system (Bedford, MA, USA).

2.2. Preparation and incubation of bovine liver slices

Bovine liver tissue was obtained from the local slaughterhouse and originated from two male animals which were respectively 1.5 years old (380 kg) and 2.5 years old (420 kg). The caudate lobe was removed from the liver and flushed with ice-cold Krebs-Henseleit buffer (pH 7.4) containing 10 mM HEPES and 25 mM glucose. Liver tissue was stored in ice-cold Krebs-Henseleit buffer and transported to the laboratory where cylindrical cores with a diameter of 8 mm were taken out by use of a stainless steel drill press. Next, slices with a thickness of 250-300 mm were prepared using a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL, USA). The most uniform shaped slices were selected and transferred to 6-well culture plates. Each well contained 3.2 ml pre-warmed (T = 39°C) WE medium supplemented with 25 mM D-glucose and 10% charcoal

filtered-stripped fetal calf serum. DHEA and 17ß-testosterone stock solutions in DMSO were added to a final concentration of respectively 0.1, 1, 10, 50 and 100 mM in the well (final concentration of DMSO was 0.5%). Incubations were performed in triplicate, containing 3 slices per well. Control slices were incubated with DMSO-solvent only. Culture plates containing liver slices were incubated in a shaking water bath at 39 °C and continuously gassed with carbogen (95% O_2/5% CO_2). After 6 hours, slices for ATP determination were transferred to a tube containing 1 ml of sonication solution (70% ethanol, 2mM EDTA) and snap frozen in liquid nitrogen and stored at -80°C. For RNA extraction, slices were snap frozen in liquid nitrogen and stored at -80°C. Incubation media were stored at -20°C until analysis.

2.3. ATP determination

For ATP measurements, slices were homogenized by sonication and extracts were centrifuged at 13.000 rpm for 2 minutes. The supernatant was diluted ten times with 0.1 M Tris/HCl, 2 mM EDTA (pH 7.8) and ATP levels were determined by using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) according to the manufacturer's protocol. ATP analysis was performed in triplicate using slices from 3 different wells.

2.4. Extraction of incubation medium

Aliquots of 3.2 ml incubation medium were thawed at room temperature, transferred to a glass tube and mixed with 6 ml diethyl ether. These samples were shaken head over head for 10 minutes, sonicated for 2 minutes in an ultrasonic water bath and centrifuged for 5 minutes at 3000 xg. The organic upper layer was transferred to a fresh glass tube and the medium was extracted again with 3 ml diethyl ether. The combined organic phase was evaporated under nitrogen at 45°C until dryness and reconstituted in 1.5 ml acetonitrile. Next, aliquots of 200 µl acetonitrile extract plus 50 µl 4% DMSO were pipetted into a conical shaped 96-well plate. To remove the acetonitrile, the plate was air dried overnight in a fume hood. A similar procedure was used for samples for UPLC-TOFMS analysis. However, after extraction with diethyl ether and evaporation under nitrogen, samples were reconstituted in acetonitrile/water (10/90 v/v).

2.5. Yeast androgen bioassay

The yeast androgen bioassay procedure used was similar as the method described earlier (Bovee et al. 2007). In short, 10 mL of selective minimal medium supplemented with L-leucine (MM/L) was inoculated with a single androgen yeast colony and grown

overnight at 30°C in a shaking incubator at 125 rpm. The next day, the yeast suspension was diluted with MM/L to an OD at 630 nm between 0.04 and 0.06 was reached. Aliquots of 200 µl yeast suspension were added to the 96-well plate containing samples and controls. Plates were incubated in a shaking incubator for 24 hours at 30°C, 125 rpm. Fluorescence (excitation at 485 nm and emission at 530 nm) was measured at 0 and 24h using a Synergy™ HT multi-detection microplate reader (Biotek Instruments Inc., U.S.A.). Fluorescence signals obtained at 24h were corrected with the signals from 0h and the blank, containing MM/L and DMSO only. After 24h the OD of the yeast culture was measured at 630 nm to check whether the cells had grown well and to assure that the samples were not cytotoxic. In addition, aliquots of sample extracts were spiked with 2 µl of 30 µM 17ß-testosterone in DMSO just before androgen bioassay screening in order to test for androgen receptor antagonistic activity as well.

2.6. UPLC-TOFMS analysis

Ultra-performance liquid chromatography was performed on a Waters (Milford, MA, USA) Acquity system equipped with a Waters Acquity BEH C_{18} column (50 mm x 2.1 mm i.d., 1.7 µm). The column temperature was kept at 45°C and the injection volume was 20 µl. Mobile phases consisted of (A) acetonitrile/water/formic acid (10:90:0.2) and (B) acetonitrile/water/formic acid (90:10:0.2), linearly increasing from 20 to 46% B in 5 minutes at a flow of 0.7 ml/min. The column effluent was split 1:1 prior to mass spectrometry.

The UPLC was directly interfaced with a Waters LCT Premier mass spectrometer equipped with a dual electrospray ionisation probe operating in positive mode (ESI+). The source temperature was 120 °C, the desolvation temperature was set at 350 °C, the capillary voltage at 3000 V and the cone voltage at 50 V. The cone and desolvation gas flow were 50 and 600 l/h respectively. Leucine-enkephalin (1ng/µl) in water/acetonitrile (67:33 v/v) was used as a lock mass calibrant and 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* 100-1000 and processed using MassLynx 4.1 software (Waters).

2.7. RNA isolation and microarray hybridization

Total RNA was extracted from liver slices by homogenization in Trizol (Invitrogen Life Technologies, Breda, The Netherlands). This homogenate was mixed with chloroform and centrifuged at 12000xg for 15 minutes at 4°C. The aqueous phase was transferred to a new tube, mixed with isopropanol, and centrifuged at 12000 xg for 10 minutes at 4°C.

The pellet was washed with 75% ethanol and resuspended in RNase free water. Upon extraction, RNA was purified according to the RNeasy mini kit protocol (Qiagen, Westburg bv, Leusden, The Netherlands) and RNA concentration and quality was determined spectroscopically (Nanodrop technologies) and by automated electrophoresis using the BioRad Experion system (BioRad, Veenendaal, The Netherlands). Only RNA with A260/280 and A260/230 ratios above 1.8 was used for amplification. To generate fluorescently-labelled cRNA, the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA) was used according to the manufacturer's protocol. In short, 1 µg of total RNA was reverse transcribed using T7 tagged oligo-dT primer and labelled with Cy3 or Cy5 (Perkin Elmer/NEN Life Sciences, Boston, MA, USA). RNAs of the control and treated liver slices were individually labelled with Cy5 and RNA of all control slices were pooled and labelled with Cy3. After purification with the RNeasy mini kit (Qiagen), label efficiency and yield were determined using a Nanodrop spectrophotometer (Nanodrop technologies). A mixture of 1 µg of Cy3-labeled cRNA and 1 µg of Cy5-labeled cRNA was hybridized onto a 44k bovine oligo microarray (Agilent Technologies), using Agilent's gene expression hybridization kit. Hybridization was performed at 65°C for 17 hours in a hybridization oven with rotation function (Agilent Technologies). Upon hybridization, microarrays were washed and dried according to Agilent's instructions. Fluorescence measurements were performed using an Agilent Technologies G2565B microarray scanner.

2.8. Microarray data analysis

Fluorescence intensities were quantified using Feature Extraction 8.5 software (Agilent Technologies). Data were imported in GeneMaths XT 1.6 (Applied Maths, St. Martens-Latem, Belgium) and signals below two times background were excluded from further analysis. Subsequently, the data were normalized as described by Pellis et al. (2003). This normalization included correction for the random error, with the median Cy3 signal for each individual spot. Secondly, correction for the systematic error was performed with the median value of the overall Cy5 signal. After normalization, principal component analysis (PCA) was performed to visualize differences between groups. Microarray data were floored by adjusting low intensity spots to a threshold value of 130, hereby reducing the number of less reliable genes. Next, data were ^{2}log transformed and each gene was mean centred. Genes with statistically significant changes in expression were determined by Significance Analysis of Microarrays (SAM) (Tusher et al., 2001). A two class unpaired SAM analysis was performed using a fold change (FC) greater than 1.5 and a false discovery rate (FDR) of less than 0.2%. Hierarchical clustering of microarray data was

performed using Cluster and Treeview (Eisen et al., 1998).

Results

3.1. ATP determination

To assure the quality of the liver slices, ATP levels were determined after incubation of the slices with DHEA and 17ß-testosterone. Incubations with 100 µM DHEA showed a decrease up to 71% of the DMSO blank, while 10 and 1 µM DHEA or 17ß-testosterone showed a slight increase in ATP level compared to the blank (Figure 1). Based on these observations it was decided to use 10, 50 and 100 µM DHEA incubations for monitoring of both biological activity and metabolites formed and the 0.1, 1 and 10 µM DHEA and 17ß-T incubations for gene expression profiling experiments.

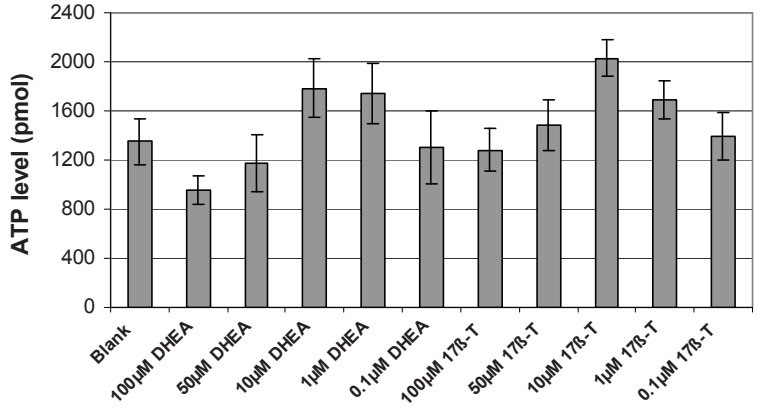

Figure 1 - *ATP levels in bovine liver slices after 6h exposure to various concentrations of DHEA, 17β-testosterone or DMSO solvent only (blank). ATP levels shown are the mean of a triplicate (±Stdev) using 3 different liver slices.*

3.2. Yeast androgen bioassay

After 6 hours exposure of the slices to DHEA, medium samples were extracted and subjected to the yeast androgen bioassay. Medium extracts of the 100 µM DHEA treatment showed a clear response in the androgen bioassay (grey bars in Figure 2). The response decreased in extracts of 50 µM DHEA incubations while incubations with 10 µM DHEA or lower (1 and 0.1 µM DHEA, data not shown) as well as the control incubations of 10, 50, and 100 µM DHEA without slices, showed no response in the androgen yeast bioassay (Figure 2).

To test the samples for androgen receptor antagonistic activity, aliquots of medium extracts were spiked with 17ß-testosterone to a final concentration of 300 nM (per well) and analysed in the androgen yeast bioassay. According to the 17ß-testosterone standard curve this concentration should result in a close to maximal fluorescence signal of approximately 700 (Figure 2). Although all spiked sample extracts gave a clear response, the maximal response was not reached (white bars in Figure 2). However, an increase of the signal was observed in 17ß-testosterone spiked medium extracts from incubations with decreasing concentrations of DHEA. A similar but even more pronounced, antagonistic effect was observed in DHEA incubations without slices.

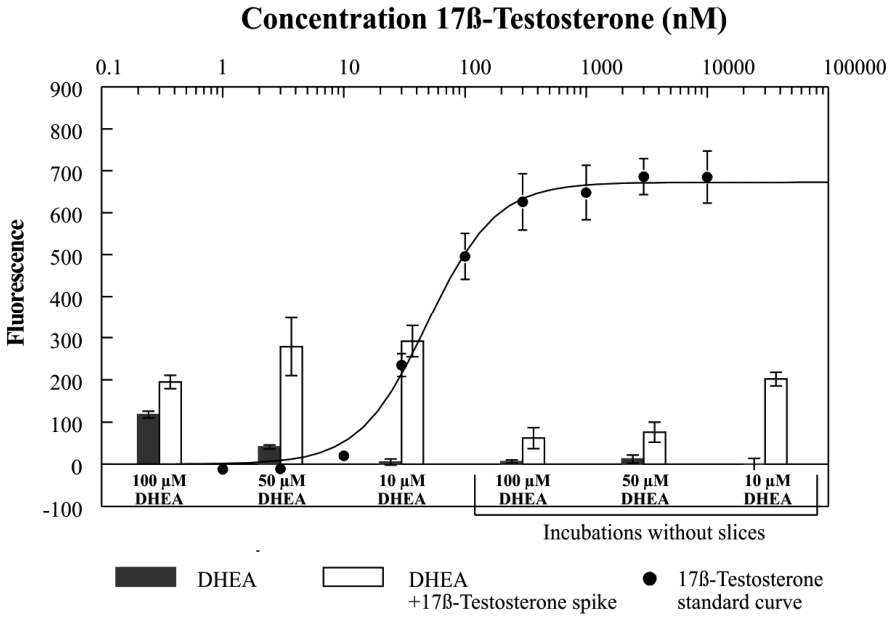

Figure 2 - *Yeast androgen bioassay responses of 10, 50 and 100 µM DHEA incubated with and without bovine liver slices and a 17ß-testosterone standard curve. Fluorescence signals are the mean of an assay triplicate (±Stdev) and corrected for the signal at t=0 hours and the reagent blank. Grey bars represent the direct androgenic activity of the medium extracts and white bars represent the androgenic activity after addition of a 300 nM 17ß-testosterone spike.*

3.3. UPLC-TOFMS analysis

After DHEA incubation with liver slices, medium was extracted and analysed by UPLC-TOFMS analysis with the goal to identify metabolites. Elemental compositions of

metabolites were elucidated using accurate masses and the identity was confirmed by mass spectra and retention time comparison of commercially available standards. Retention times and *m/z* values of the most abundant ion of the analyzed standards are listed in Table S-1.

The base peak intensity (BPI) chromatogram obtained from medium of liver slices incubated with 100 µM DHEA for 6 hours is shown in Figure 3A. The two most abundant peaks are DHEA at retention time 2.88 minutes and a peak at 0.53 minutes which could not be identified, but is most likely a medium component as it is also present in DHEA and 17ß-T incubations without slices (chromatograms not shown).

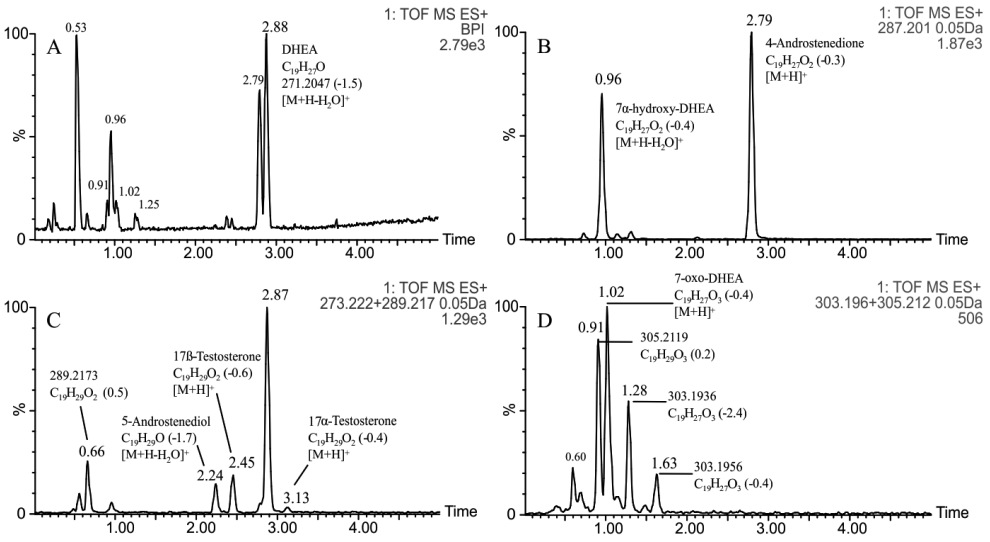

Figure 3 - *UPLC-TOFMS analysis of medium following a 6 hours liver slice incubation with 100 µM DHEA. Shown is (A) the base peak intensity chromatogram and the reconstructed accurate mass chromatograms of (B) m/z 287.2011 (C) m/z 273.2218 + m/z 289.2168 and (D) m/z 303.1960 + m/z 305.2117 using a mass window of 0.05 Da. For other conditions see Materials and Methods section.*

The main metabolite of DHEA is 4-AD which is observed at retention time 2.79 minutes showing a $[M+H]^+$ ion at *m/z* 287.2011 (Figure 3B). Another ion with a *m/z* 287 is observed at retention time 0.96 which is most likely the $[M+H-H_2O]^+$ ion of 7α-hydroxy-DHEA. Figure 3C, shows the reconstructed accurate mass chromatogram of *m/z* 273.2218 + *m/z* 289.2168 showing the $[M+H-H_2O]^+$ ion of 5-androstenediol and $[M+H]^+$ ions of both 17α- and 17ß-T. Several, most likely, hydroxy- and oxo-metabolites of steroids are observed at retention time 0.60, 0.66, 0.91, 1.02, 1.28 and 1.63 minutes (Figure 3D) of which 7-keto-DHEA could be confirmed by retention time comparison. The relative levels

of the identified metabolites were calculated versus the amount of DHEA observed at t=0 incubations (11.1 µg/mL = 100%), by using the peak area of the extracted ion chromatogram and standard curves obtained by plotting the peak area versus the concentration of the standards (0, 0.1, 0.5, and 5 µg/mL). After liver slice incubation 17-24% of the starting amount of DHEA remained unchanged, while summation of the levels of all observed steroidal phase I metabolites plus DHEA resulted in a yield of 33-43% relative to the starting amount of DHEA (Supplemental document S1).
Although small differences in relative intensities were observed, the same metabolite profiles were detected in the 10 µM and 50 µM DHEA incubations in experiment 1 as well as in experiment 2 with slices prepared from a different liver. No metabolites were observed after incubation of 100 µM DHEA without liver slices (data not shown).

3.4. Microarray analysis

RNAs of slices from experiment 1 (0.1, 1 and 10µM DHEA and 17ßT; blank) and experiment 2 (10 µM DHEA; blank) were labeled and hybridized onto bovine arrays. After normalization of the microarray data, unsupervised principal component analysis (PCA) was performed. By reducing the complexity of the dataset, differences in gene expression can be visualized in 3 dimensions. Changes in the same direction are hereby indicative for changes of the same genes. Figure 4A shows the PCA-plot of the first three components covering 78.2% of the total variance. Control and exposed liver slices of experiment 1 as well as experiment 2 are mainly separated on the x-axis. The DHEA and 17ß-T exposed slices of experiment 1 group closely together with the exception of all three slices incubated with 10µM 17ß-T and one 0.1 µM 17ß-T replicate. Based on this observation the 0.1 µM 17ß-T incubation was classified as an outlier and was excluded from further statistical analysis.
Significant regulated genes (FC >1.5 and FDR <0.2%) were determined by Significance Analysis of Microarrays (SAM) and numbers of modulated transcripts are listed in Table 1. The number of significantly regulated genes increased dose dependently, for DHEA as well as 17ß-testosterone, showing in general more down-regulated than up-regulated genes. The Venn diagrams in Figure 4B and C show the number of genes differentially expressed either in one group or in multiple groups of experiment 1. A total of 1048 genes were found to be modulated by at least one DHEA concentration, and a total of 152 genes were found modulated by each of the DHEA concentrations tested (Figure 4B).
Figure 5 shows the outcome of the hierarchical cluster analysis of these 1048 regulated genes for the DHEA treated liver slices as well as for the DMSO blank and 17ß-T treatment groups. It should be noted that replica 3 of the 0.1µM 17ß-T incubation shows

an expression profile which was highly similar to the blank DMSO incubations. This observation justifies again the exclusion of this replicate from the SAM analysis. The genes up-regulated by the DHEA treatment are clustered in area A, showing higher expression levels (increasing red intensity) as compared to the DMSO controls (green). For 17ß-T the same trend is observed, showing a more pronounced regulation at the highest concentration. In area B, genes down-regulated in the DHEA/17ßT treatment groups versus the DMSO controls are clustered together. In general, DHEA and 17ß-T exposed liver slices are showing similar expression profiles, particularly in the upper part of area B. However, a more pronounced down-regulation is observed for the 10 µM 17ß-T exposure.

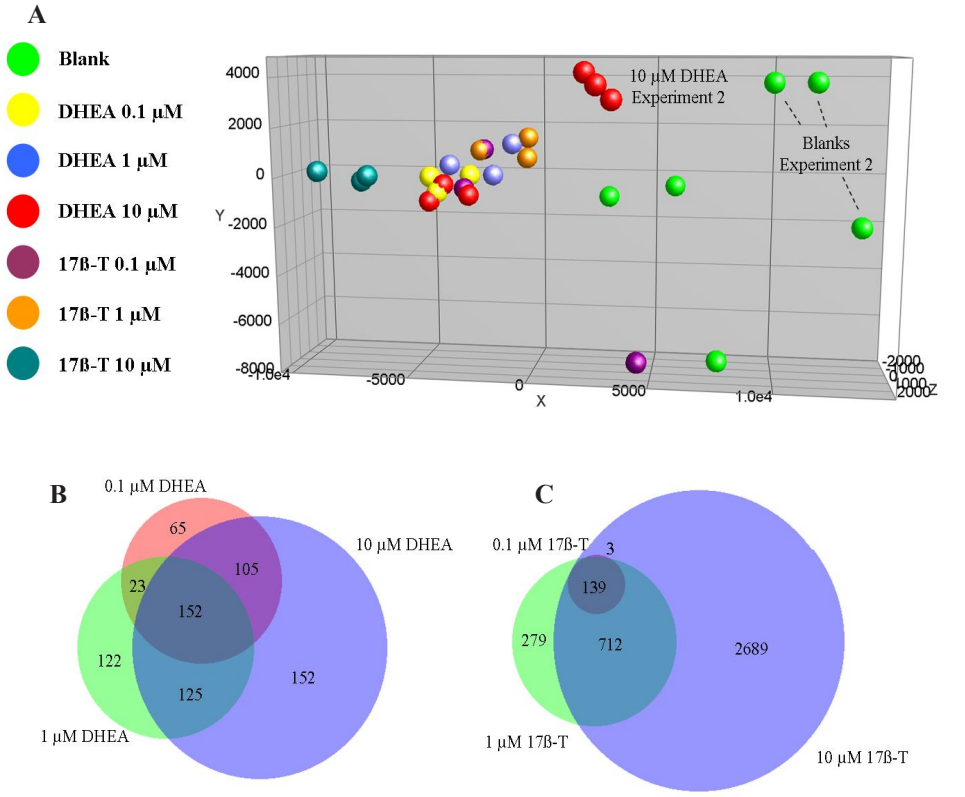

Figure 4. *(**A**) Principal component analysis (PCA) of gene expression profiles in bovine liver slices. The principal components plotted cover 78.2% of the total variance. Spheres in the PCA are representing gene expression profiles observed in control (green) and DHEA (0.1 uM, yellow, 1 uM, blue and 10 uM, red) and 17ß -testosterone (0.1 uM, purple, 1 uM, orange and 10 uM dark green) exposed liver slices of experiment 1. (**B**) Venn diagrams show the overlap between significantly regulated genes observed in bovine liver slices incubated with various concentrations DHEA and (**C**) 17ß-testosterone.*

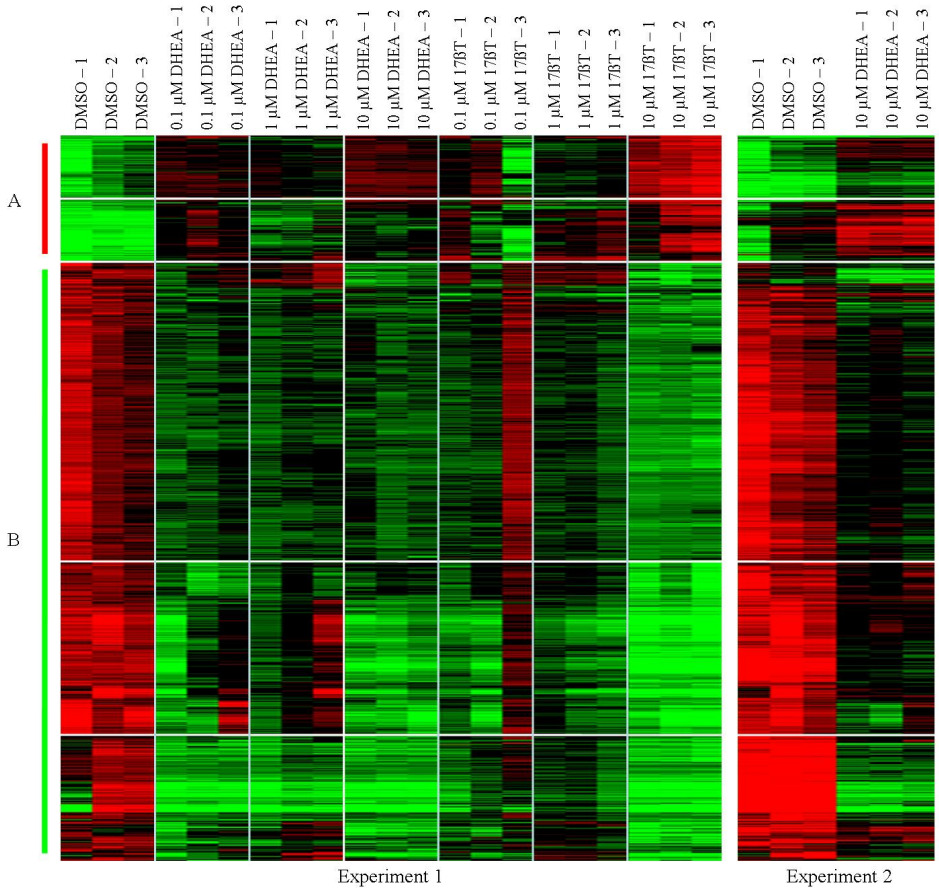

Figure 5. *Hierarchical cluster analysis of microarray probes that were significantly regulated (FC >1.5 and FDR <0.2%) by at least one of the DHEA treatments in experiment 1 (n = 1048). Shown are the responses of these modulated genes in all DHEA (experiment 1 and 2) as well as 17β-testosterone exposed liver slices. Values are ^{2}log transformed and mean centered followed by clustering on genes only using average linkage. Columns and rows represent liver slice incubations and genes respectively. Colors range from bright green (≥ ^{2}log 0.8 down regulated) to bright red (≥ ^{2}log 0.8 up regulated).*

When determining the total number of significantly regulated genes upon treatment of liver slices with 10 µM DHEA in experiment 2, 621 and 789 genes were found to be up- and down-regulated respectively (Table 1), of which 35% overlapped with the genes that were found to be modulated by the 10 µM DHEA exposure in experiment 1 (Supplemental document S2).

Table 1. Number of transcripts significantly changed (Fold change ≥1.5 and FDR< 0.2%) after 6 hours exposure of bovine liver slices to DHEA or 17ß-testosterone.

Dose and compound	Exp. No.	Number	Up	Down
0.1 µM DHEA	1	345	68	277
1 µM DHEA	1	422	10	412
10 µM DHEA	1	869	193	676
0.1 µM 17ß-testosterone	1	142	0	142
1 µM 17ß-testosterone	1	1130	416	714
10 µM 17ß-testosterone	1	3543	1340	2203
10 µM DHEA	2	1410	621	789

Discussion

The main purpose of this study was to investigate the biotransformation of DHEA in bovine liver slices and to examine the feasibility of this model in combination with a yeast androgen bioassay and DNA microarrays for effect analysis. The viability of the liver slices was determined by measuring ATP-levels, as it was previously shown to be a valid quality parameter (Wang et al. 2010). Apart from the 100 µM DHEA incubation, no significant differences in ATP levels between DMSO blanks and treated slices were observed after 6 hours of exposure, thus indicating that with exception of 100 µM DHEA, the other DHEA and 17ß-T concentrations used were not cytotoxic for the liver cells.

While DHEA itself is not active in the yeast androgen bioassay, exposure of bovine liver slices to 100 µM DHEA resulted in a significant increase in androgenic activity, suggesting formation of potent androgenic metabolites. However, medium extracts from incubations of DHEA with or without liver slices that were spiked afterwards with 300 nM 17ß-T, showed a lower activity as expected. The response was lower than that from a 300 nM 17ß-T standard, showing a maximal response in the 17ß-T standard curve. This is likely due to the antagonistic properties of DHEA, as it was observed before that DHEA binds to the androgen receptor but shows no transcription activation in the androgen yeast bioassay (Bovee et al. 2008; Rijk et al. 2008). These antagonistic properties were confirmed by 6 hour control incubations of DHEA without liver slices, showing an increase in bioassay response of the 17ß-T spike with lower concentrations DHEA. Although exposure of liver slices to DHEA concentrations lower than 100 µM did not result in an androgenic response in the bioassay, a decrease in antagonistic activity was observed when comparing medium extracts of the 10 µM and 50 µM DHEA incubations with and without liver slices after spiking with 17ß-T. Here a higher bioassay

response is observed in the DHEA incubations with liver slices as compared to DHEA incubations without liver slices (compare white bars with and without liver slices, Figure 2), suggesting biotransformation of DHEA which results in a decreased antagonistic activity of the medium extracts and/or formation of androgenic metabolites.

The biotransformation of DHEA was monitored by UPLC-TOFMS analysis showing the formation of 4-AD, 5-androstenediol, 17ß-T, 17α-T, 7α-hydroxy-DHEA, 7-keto-DHEA and at least 4 other metabolites that were not identified. According to the elemental composition these are most likely hydroxy- or oxo-metabolites of steroids (Figure 3). Quantification of DHEA and its metabolites resulted in 33-43% yield relative to the starting amount of DHEA. This incomplete yield could be explained by the performed liquid-liquid extraction using diethylether. Hereby the more water soluble phase II metabolites such as steroid glucuronides and sulphates are not extracted while most likely a substantial amount of the steroids is glucuronidated by the liver slices as e.g. shown for 17ß-testosterone (Wang et al. 2010). Moreover, it is not reasonable to expect that all metabolites formed have similar ionization efficiencies in the UPLC-TOFMS analysis. For instance ionization of dihydrotestosterone, an androstane, is 5-10 times less efficient than that of its androstene equivalent 17ß-testosterone, but DHT is a two-fold more potent androgen than 17ß-testosterone (Bovee et al., 2008). Because other androstane steroids are not as potent as DHT, the increase in androgenic activity upon 6 h metabolism of DHEA by liver slices is expected to originate mainly from the formation of 17ß-T and 4-AD. Although 4-AD is approximately a 100 times less potent androgen than 17ß-T, the high levels formed contribute to the observed androgenic activity. Liver slice incubations with 10 µM DHEA showed similar metabolite profiles, but no androgenic activity was perceived in the bioassay probably due to the antagonistic activity of DHEA itself and the low absolute levels of androgenic metabolites formed.

Qualitatively, the bovine liver slice phase I metabolite profiles of DHEA determined in this study are similar to the profiles previously obtained with S9 mixtures prepared from bovine liver using NAD^+ as a cofactor (Rijk et al., 2008). 4-AD and 7α-hydroxy-DHEA were the main metabolites formed, together with minor amounts of 17ß-testosterone, 5-androstenediol and 7-keto-DHEA. Also in human liver S9 experiments the formation of 7α-hydroxy-DHEA as well as of 7ß-hydroxy-DHEA, 16α-hydroxy-DHEA, 7-keto-DHEA and 5-androstenediol was observed (Chalbot and Morfin, 2005). Although 7α-hydroxylation of DHEA has been described as a major pathway in liver (Doostzadeh et al., 1998), metabolite profiles differ between species due to the stereospecificity of hydroxylation by the various P450 enzymes that metabolize DHEA (Miller et al., 2004). Moreover, metabolites observed in experiments using liver S9 fractions, strongly depend on the

cofactor that is used (Chalbot and Morfin, 2005). Liver slices have the general advantage that all phase I and phase II enzymes are present together with their natural amounts of cofactors thus resembling the *in vivo* situation more closely. However, in this case incubation of liver slices with DHEA results in significant lower levels of androgen active compounds as compared to incubations with bovine liver S9 resulting in a lower response in the androgen bioassay (Rijk et al., 2008). This is probably due to the formation of androgen inactive steroid phase II metabolites and therefore liver slices are considered to be a less adequate bioactivation model to monitor the androgenic activity of prohormones.
Regarding DNA microarray analysis, the present study with bovine liver slices shows that DHEA alters hepatic gene expression. This gene expression profile was qualitatively similar to that of the potent androgen 17ß-T. In comparison, in classical androgen-sensitive tissues of gonadectomized mice, DHEA showed gene expression profiles that were highly similar to profiles of the potent androgens dihydrotestosterone (DHT) and tetrahydrogestrinone (THG) (Labrie et al., 2006). These results imply that exogenous DHEA can act as an androgen, but is most probably largely dependent on the expression levels of steroidogenic enzymes in the target cells (Labrie et al., 1991). The gene expression profile of DHEA is most likely caused by its more potent androgenic metabolites like 4-AD and 17ß-T. Similarly, these metabolites are responsible for the observed activity in the yeast androgen bioassay of medium extracts prepared from slices exposed to DHEA. Principal component analysis showed a clear separation in profiles between medium from control slices on the one hand and medium from DHEA or 17ß-T exposed slices on the other hand. For DHEA as well as 17ß-T the number of differentially regulated transcripts increased dose dependently and a significant overlap was observed between both compounds. However, a distinct separation was also shown between experiment 1 and 2, most probably due to obvious experimental and biological variation e.g. differences in origin and background of the bovines of which the livers were obtained.
Rat *in vivo* as well as liver slice gene expression profiles induced by DHEA have been reported before (Depreter et al., 2002; Gu et al., 2003; Werle-Schneider et al., 2006). Although not always regulated in the same direction, in general the genes found regulated by DHEA in the rat liver slices (Werle-Schneider et al., 2006) are also regulated in the bovine liver slices (Figure 6). From these regulated genes, SAM analysis classified PSME1, RPL41, EHHADH, ALDH1A1 and HRG as significant regulated in at least one of the DHEA liver slice exposures. However, genes that were found to be down-regulated in DHEA treated rat liver slices, such as RPL41 and HRG were up-regulated in all DHEA treated bovine liver slices.

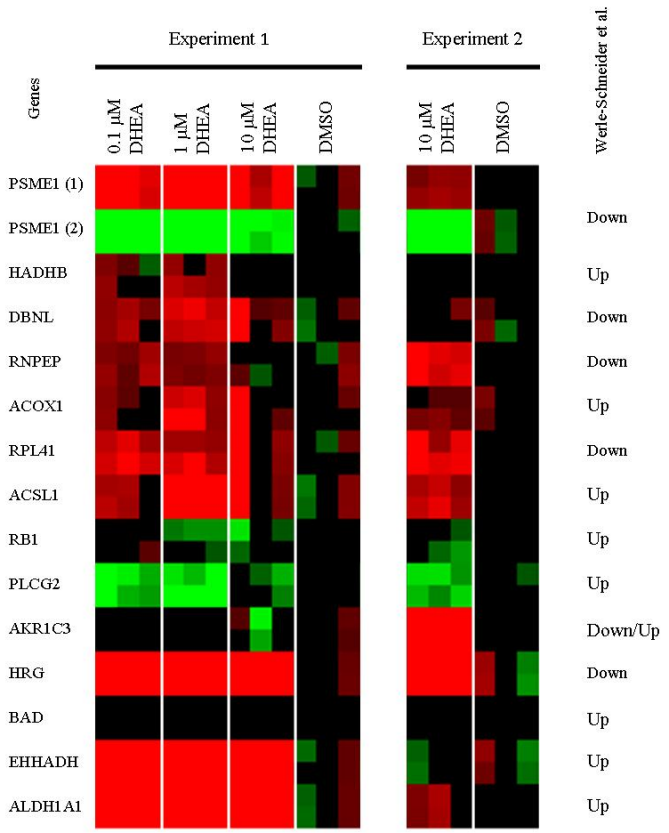

Figure 6. *Heat map of expression in bovine liver slices of genes previously found regulated in rat liver slices treated with various concentrations DHEA [Werle-Schneider et al.]. Red and green colors indicate up regulated or down regulated vs the DMSO control average. Genes classified as significantly regulated by the SAM analysis are marked with an asterisk.*

In conclusion, the present study with bovine liver slices shows that biotransformation of DHEA not only results in an altered bioactivity, but also alters hepatic gene expression. Compared to metabolic conversions induced by bovine liver S9, bovine liver slices are a less efficient model for bioactivation in combination with androgenic reporter gene assays to screen for the presence of prohormones. Probably, this is mainly due to substantial phase II metabolism of DHEA as well as the lower levels of androgenic active metabolites formed. Moreover, preparation of liver slices is very laborious and thus less suitable to use in high through-put screening procedures. On the other hand, due to the fact that they exhibit also phase II metabolism liver slices can serve as an adequate *in vitro* model to study the species-specific metabolism of steroid (pro)hormones. Therefore a more feasible

application of liver slices is foreseen in evaluation of both metabolism of (new) compounds as well as supplements showing up in the illegal circuit of which the *in vivo* mode of action is unclear. Currently, new compounds found in illegal preparations and supplements are tested in small scale animal experiments to obtain knowledge about absorption, distribution, metabolism and excretion kinetics in bovines, and to identify target urinary metabolites. In this process, liver slice models could be included to gather knowledge about metabolism and identification of (new) metabolite biomarkers that could be used for *in vivo* urine screening and hereby reduce the need for animal experiments.

Acknowledgements

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Chapter 6

Metabolomics approach to anabolic steroid urine profiling of bovines treated with prohormones

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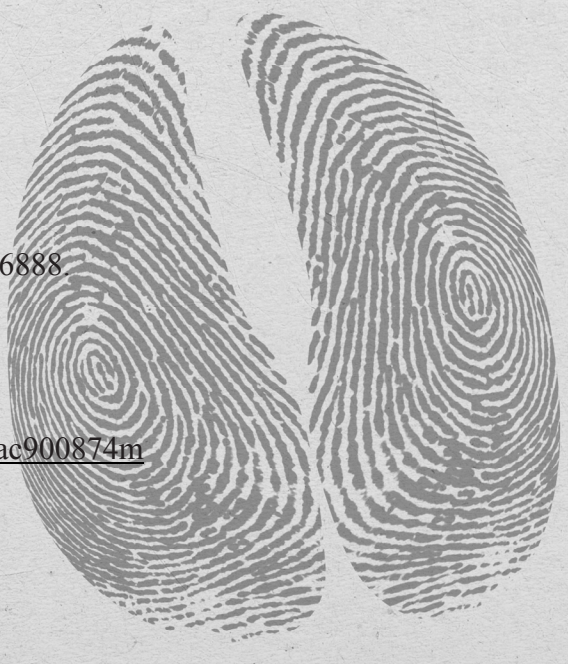

Abstract

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.

Introduction

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 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].

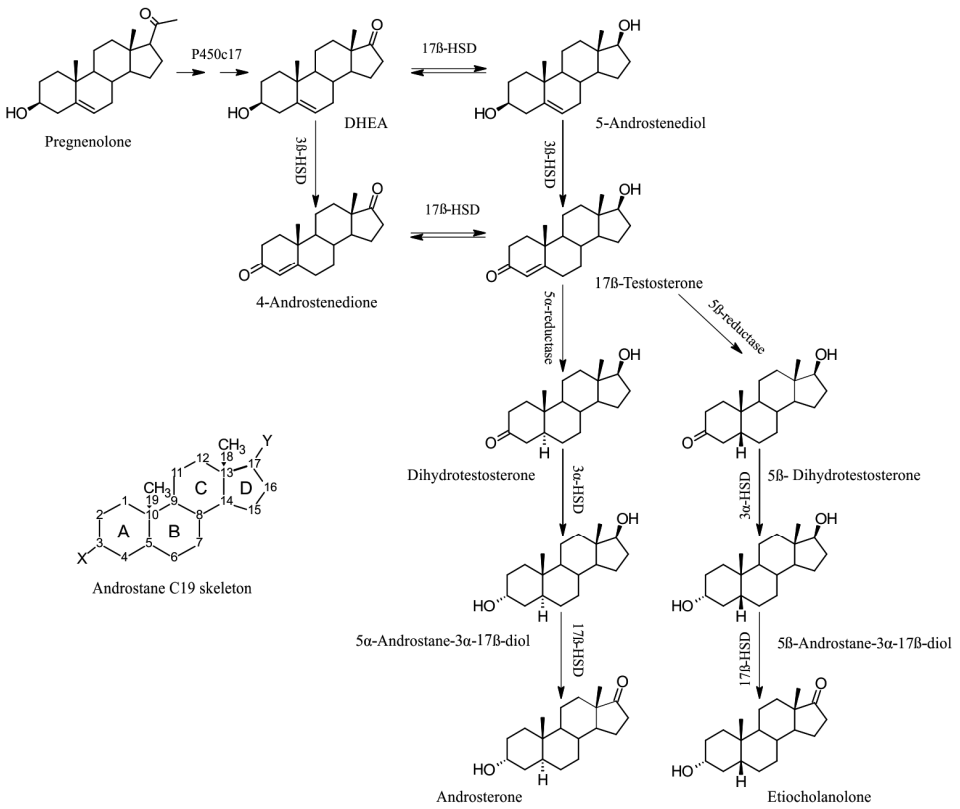

Figure 1 - In vivo androgen biosynthesis: pregnenolone is converted into DHEA under P450c17 activity; subsequently 17β-testosterone is formed via 5-androstenediol or 4-androstenedione due to 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme activity;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

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 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 acetate 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 1½ 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.

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)

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 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.

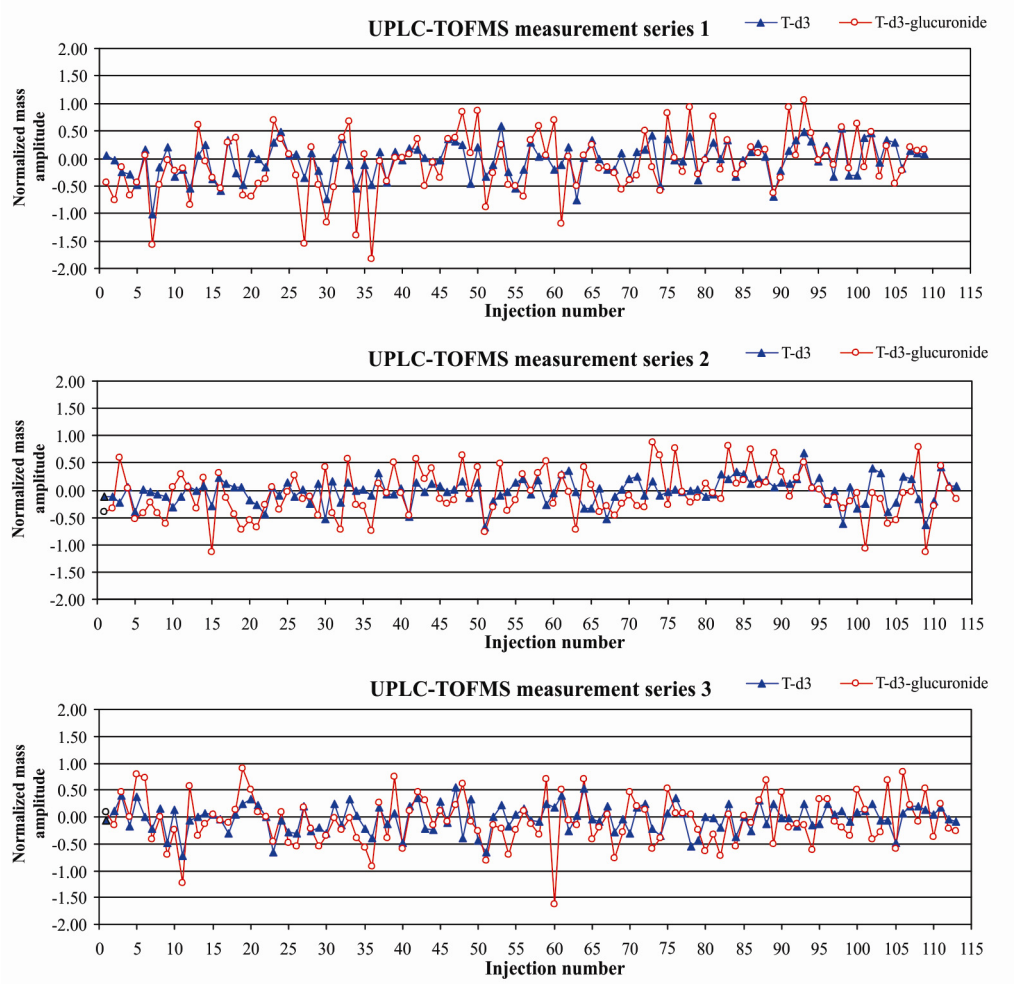

Figure 2 - Normalized and ^{2}log transformed accurate mass amplitudes of deuterium labeled internal standards, testosterone-d_3 and testosterone-d_3-glucuronide (30 ng spike added to urine samples before the SPE procedure) observed during UPLC-TOFMS measurements.

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.

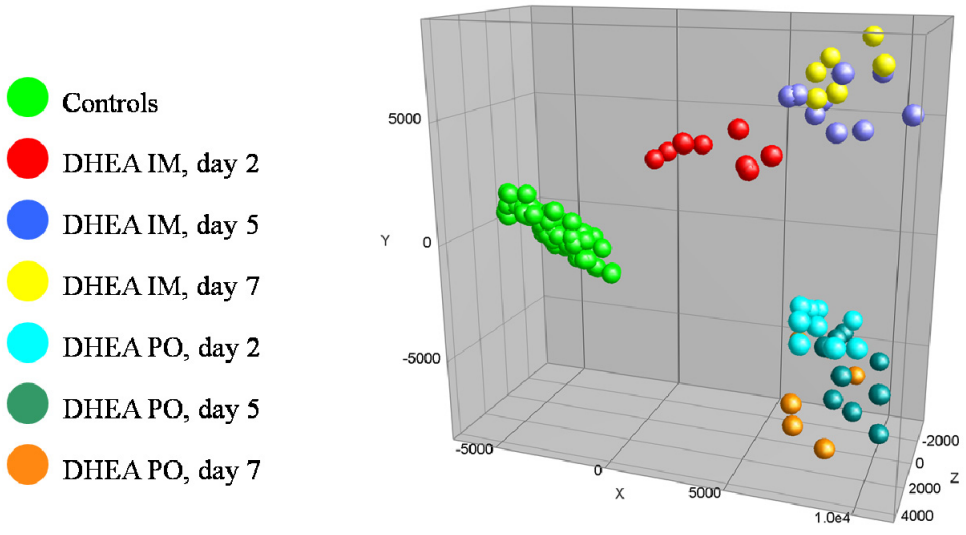

Figure 3 - PCA plot of urine samples from control animals (green) and DHEA treated animals (IM and PO at days 2, 5, and 7) after UPLC-TOFMS measurement and ANOVA (p value < 0.01) with Bonferroni correction.

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 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.

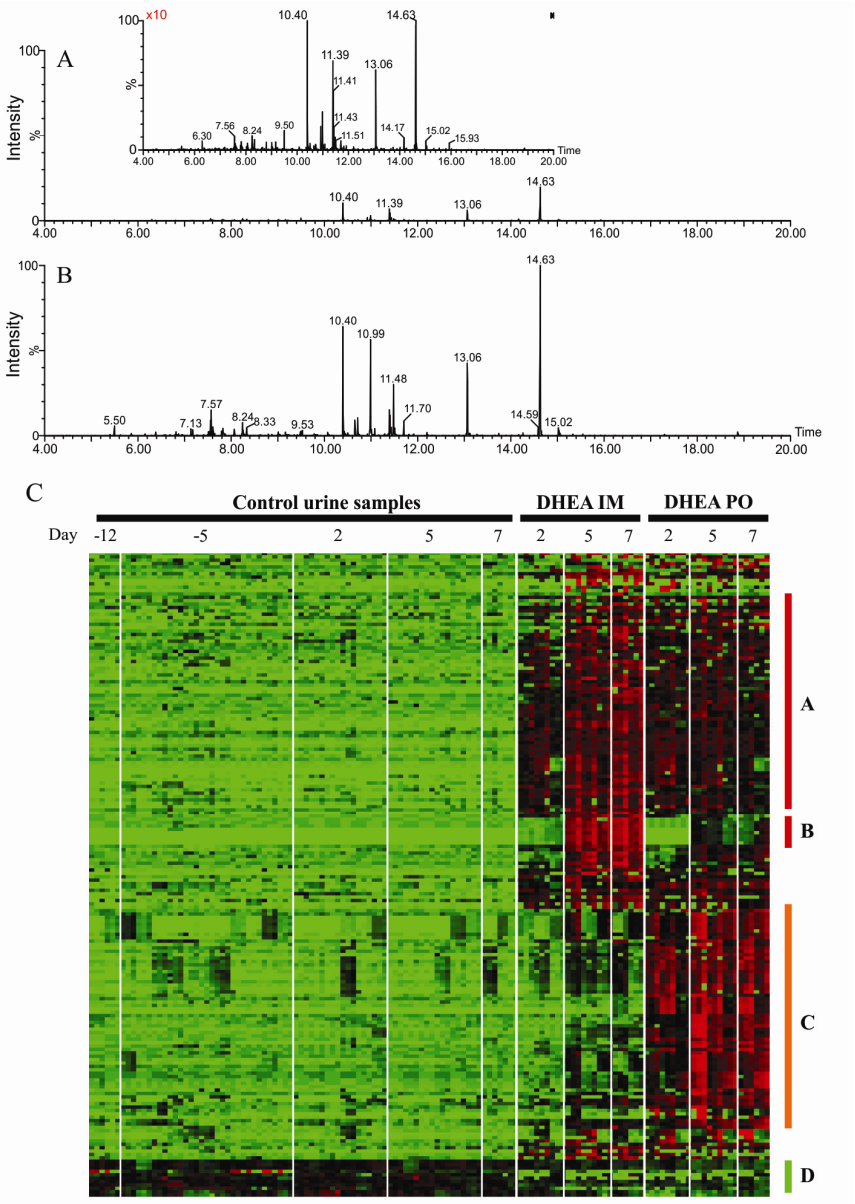

Figure 4 - Mass peak loadings with a p value < 0.01 after ANOVA with Bonferroni correction, back converted to total ion chromatogram (TIC) data. Shown is (A) the average of all controls and (B) the average of the DHEA treated group. The insert at chromatogram A shows a 10 times magnification between 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.

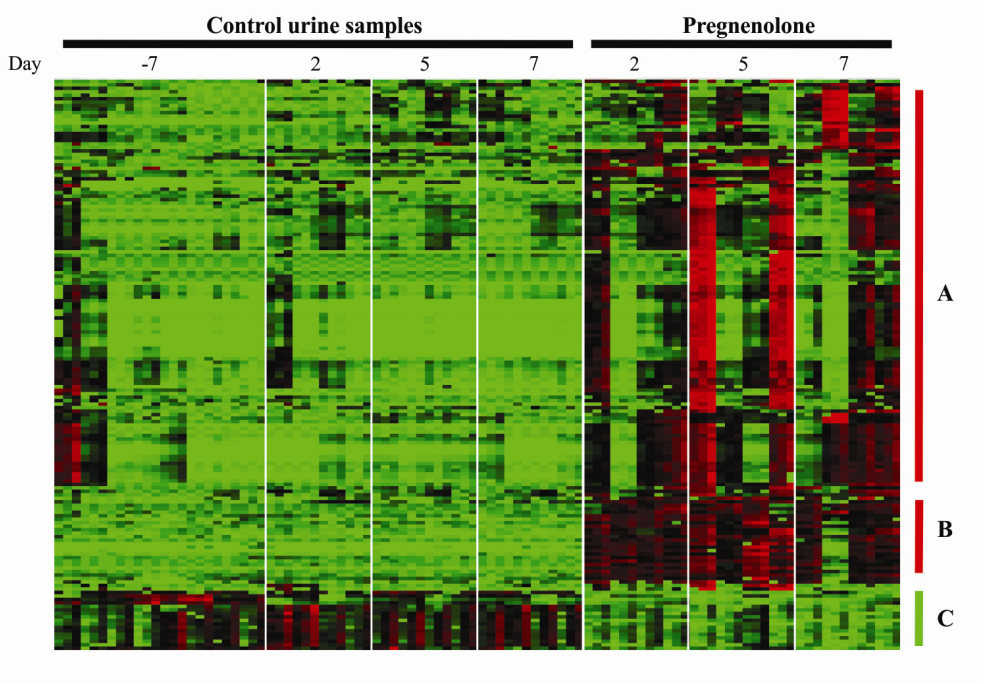

Figure 5 - Hierarchical cluster analysis of ^{2}log transformed and mean centered mass peak loadings showing more than a 5-fold increase (areas A and B) or decrease (area C) in signal. Fold changes were obtained by comparing mean signals of all urines originating from pregnenolone treated animals versus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

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 equation 1 to equation 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.

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.

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. 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 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.

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

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.

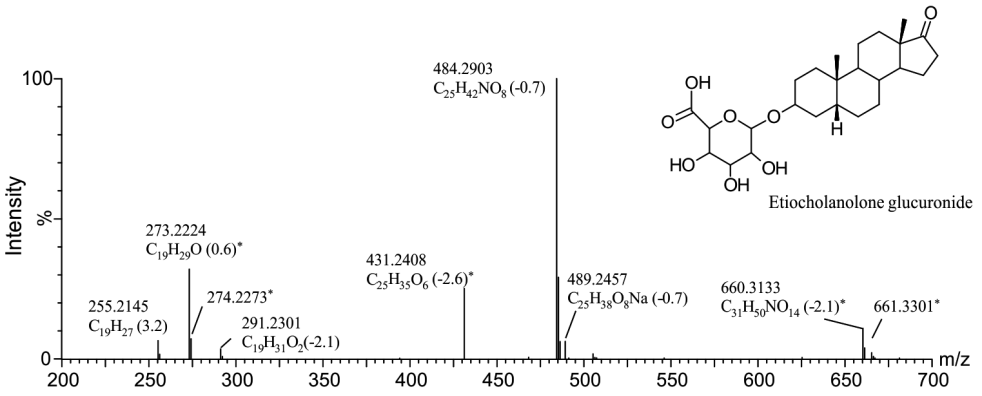

Figure 6 - Differential mass peak loadings (p value <0.01 after ANOVA) obtained at retention time 13.06 min (combining scans 1543-1545). Signals showing >10-fold change compared to the mean of all controls are marked with an asterisk. Accurate mass values are the averages from aligned peaks of all urine 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* 497.31152 ($C_{27}H_{45}O_8$) due to neutral losses of water, a glucuronide moiety (resulting in $C_{21}H_{34}O$ at *m*/*z* 303.26911), 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.

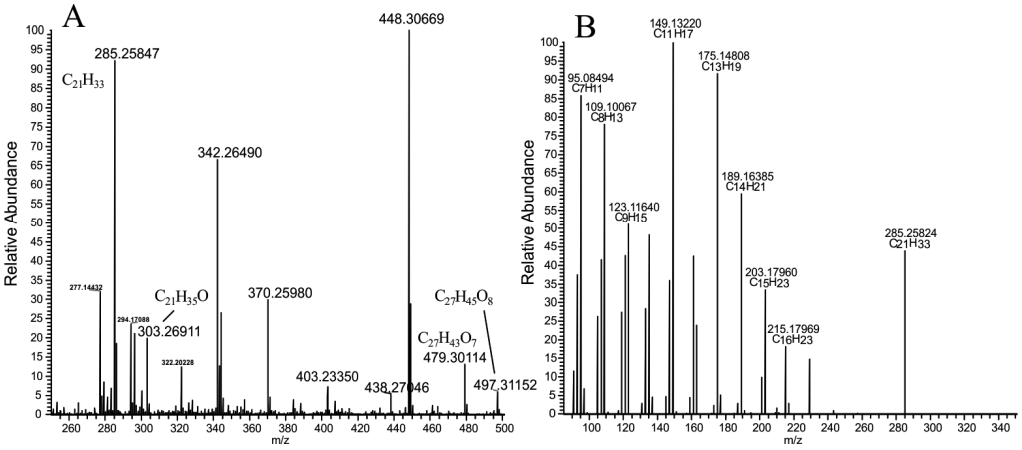

Figure 7 - LTQ Orbitrap mass spectra of an unknown candidate biomarker obtained by untargeted screening of urine from a pregnenolone treated animal: (A) full scan mass spectrum at retention time 16.47 min and (B) MS/MS spectrum from precursor ion m/z 285.3 using a normalized collision energy of 50% and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

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. 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.

Acknowledgement

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Chapter 7

Feasibility of a liver transcriptomics approach to assess bovine treatment with the prohormone dehydroepiandrosterone (DHEA)

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Abstract

Background. Within the European Union the use of growth promoting agents in animal production is prohibited. Illegal use of natural prohormones like dehydroepiandrosterone (DHEA) is hard to prove since prohormones are strongly metabolized *in vivo*. In the present study, we investigated the feasibility of a novel effect-based approach for monitoring abuse of DHEA. Changes in gene expression profiles were studied in livers of bull calves treated orally (PO) or intramuscularly (IM) with 1000 mg DHEA versus two control groups, using bovine 44K DNA microarrays. In contrast to controlled genomics studies, this work involved bovines purchased at the local market on three different occasions with ages ranging from 6 to 14 months, thereby reflecting the real life inter-animal variability due to differences in age, individual physiology, season and diet.

Results. As determined by principal component analysis (PCA), large differences in liver gene expression profiles were observed between treated and control animals as well as between the two control groups. When comparing the gene expression profiles of PO and IM treated animals to that of all control animals, the number of significantly regulated genes (p-value <0.05 and a fold change >1.5) was 23 and 37 respectively. For IM and PO treated calves, gene sets were generated of genes that were significantly regulated compared to one control group and validated versus the other control group using Gene Set Enrichment Analysis (GSEA). This cross validation showed that 6 out of the 8 gene sets were significantly enriched in DHEA treated animals when compared to an 'independent' control group.

Conclusions. This study showed that identification and application of genomic biomarkers for screening of (pro)hormone abuse in livestock production is substantially hampered by biological variation. On the other hand, it is demonstrated that comparison of pre-defined gene sets versus the whole genome expression profile of an animal allows to distinguish DHEA treatment effects from variations in gene expression due to inherent biological variation. Therefore, DNA-microarray expression profiling together with statistical tools like GSEA represent a promising approach to screen for (pro)hormone abuse in livestock production. However, a better insight in the genomic variability of the control population is a prerequisite in order to define growth promoter specific gene sets that can be used as robust biomarkers in daily practice.

1. Background

In the European Union the use of growth promoting substances in livestock production is prohibited according EC directive 96/22 [1]. To ensure compliance with this legislation, requirements for monitoring are described in EC directive 96/23 [2]. At national level, legislations are implemented in residue monitoring programs regulating sampling of animal matrices and residue analysis therein to guarantee fair trade, food safety and public health. Residue analysis in livestock production is in general based on chemical [3], immunochemical or biological [4,5] screening methods followed by mass spectrometry based confirmation methods. Although this strategy seems to work for synthetic anabolic steroids, problems arise when compounds that also occur naturally are used.

Abuse of naturally occurring (pro)hormones is hard to prove since most of these substances are strongly metabolized *in vivo*. Moreover, metabolites are not always known or are present in levels not significantly different from highly fluctuating endogenous levels. This makes it difficult to prove fraudulent use based on quantification of natural occurring compounds. Nowadays, it is observed that misuse of growth promoters in cattle fattening moves towards these natural steroids and steroid esters. Moreover, inspections of livestock farms in The Netherlands occasionally result in the finding of feed or herbal additives and preparations containing so-called prohormones. Prohormones are compounds that exhibit limited or no hormonal action by themselves, however they are direct precursors of active hormones and indirectly affect natural hormone levels. Dehydroepiandrosterone (DHEA) is such a prohormone and is the most abundant occurring precursor of both androgens and estrogens in humans [6,7]. It is claimed that orally taken DHEA improves muscle strength and is therefore illicitly used in sports to enhance performance and appearance [8,9].

Looking for alternatives to support evidence of illegal use of growth promoting substances, gene expression analysis can be an attractive new approach. Several studies demonstrated changes in mRNA expression in bovine tissues upon treatment with growth promoters after performing real-time RT-PCR analysis on a limited number of preselected genes [10-14]. Untargeted transcriptomics approaches using microarrays allow gene expression analysis of thousands of genes simultaneously as well as identification of (new) biomarkers for screening [15,16]. Moreover, microarray data can provide mechanistic insights in cellular processes and pathways and can be used for classification of compounds with the same mode of action (gene expression finger prints) [17,18]. Comparative microarray analysis is therefore in potential a promising screening tool for growth promoter abuse and in particular for prohormones of which the mode of action in cattle is sometimes unclear.

In recent work we used a metabolomics approach to compare urine profiles of control and DHEA exposed bovines [19]. This revealed several urinary steroid phase I and phase II metabolites which are potential biomarkers for DHEA treatment. In the present study we investigated the feasibility of monitoring prohormone abuse at the mRNA level using liver tissue from the same animal experiment. Gene expression profiles of control and DHEA treated animals were compared to determine differentially expressed genes and to identify biomarkers for DHEA treatment.

2. Methods

2.1. Animals and treatment

Male Frisian bull calves were purchased at the local market and housed for 2-3 weeks before the start of the experiment. Treatment with DHEA was repeated in three independent experiments using identical treatment and sampling schedules. Each of the three experiments consisted of two animals of which one was orally (PO) treated with capsules containing 1000 mg DHEA (Sigma, St. Louis, MO, USA) and the other was injected intramuscularly (IM) with 1000 mg DHEA dissolved in 10 ml Miglyol 812 (Certa SA, Braine-l'Alleud, Belgium).

Table 1: Experimental setup, age and weights of bovines included in the DHEA animal treatment experiment.

	Treatment	Age	Weight
Experiment #1	Intramuscular (IM 1)	9 months	290 kg
	Oral (PO 1)	8 months	253 kg
	Control (C 1-1)	6 months	174 kg
	Control (C 1-2)	6 months	172 kg
	Control (C 1-3)	6 months	153 kg
Experiment #2	Intramuscular (IM 2)	9 months	262 kg
	Oral (PO 2)	8 months	210 kg
Experiment #3	Intramuscular (IM 3)	12 months	355 kg
	Oral (PO 3)	13 months	410 kg
	Control (C 3-1)	14 months	368 kg
	Control (C 3-2)	14 months	386 kg
	Control (C 3-3)	13 months	432 kg
	Control (C 3-4)	13 months	350 kg

Administrations were performed seven times, at 24-hour intervals. IM treated animals (n=3, 262-355 kg) were 9-12 months old and PO treated animals (n=3, 210-410 kg) 8-13 months old. Control animals were included in the first (n=3, 6 months old, 153-174 kg) and third (n=4, 13-14 months old, 350-432 kg) experiment. An overview of the experimental setup, age and weights of the bovines is shown in Table 1. Twenty-four hours after the last treatment, the animals were sacrificed and liver tissue was collected, snap-frozen in liquid nitrogen and stored at -80°C until use. The experimental work was approved by the Animal Ethics Committee of Ghent University, Belgium, in accordance with local ethical requirements.

2.2. Microarray analysis

Total RNA was extracted from tissues by homogenization in Trizol (Invitrogen Life Technologies, Breda, The Netherlands) and mixed with chloroform. The lysate was centrifuged at 12000 x g for 15 minutes at 4°C and the aqueous phase was transferred to be mixed with isopropanol which precipitates total RNA. After centrifuging (10 minutes, 12000 x *g* at 4°C) the pellet was washed with 75% ethanol and resuspended in RNase free water. Upon extraction the RNA was purified according to the RNeasy mini kit protocol (Qiagen, Westburg bv, Leusden, The Netherlands). After purification, RNA integrity was determined spectroscopically (Nanodrop technologies) and by gel electrophoresis. Only RNA with A260/280 and A260/230 ratios above 1.8 was used for amplification. To generate fluorescently-labelled cRNA, the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA) was used according to the manufacturer's protocol. In short, 1 µg of total RNA was reverse transcribed using T7 tagged oligo-dT primer and labelled with Cy3 or Cy5 (Perkin Elmer/NEN Life Sciences, Boston, MA, USA). Liver RNAs of the treated and control animals were individually labelled with Cy5 and RNA of all 7 control animals was pooled and labelled with Cy3. After purification with the RNeasy mini kit (Qiagen), label efficiency and yield were determined using a Nanodrop spectrophotometer (Nanodrop technologies). A mixture of 1 µg of Cy3-labeled cRNA and 1 µg of Cy5-labeled cRNA was hybridized onto a 44k bovine oligo microarray (Agilent Technologies), using Agilent's gene expression hybridization kit. Hybridization was performed at 65°C for 17 hours in a hybridization oven with rotation function (Agilent Technologies). Upon hybridization, microarrays were washed and dried according to the Agilent's instructions and fluorescence measurements were performed using an Agilent Technologies G2565B microarray scanner.

2.3. Data processing

Fluorescence intensities were quantified using Feature Extraction 8.5 software (Agilent Technologies). Data were imported in GeneMaths XT 1.6 (Applied Maths, St. Martens-Latem, Belgium) and signals below two times background were excluded from further analysis. Subsequently, the data was normalized as described by Pellis et al. [20]. This normalization included correction for the random error, with the median Cy3 signal for each individual spot. Secondly, correction for the systematic error was performed with the median value of the overall Cy5 signal. After normalization, principal component analysis (PCA) was performed to visualize differences between groups and *t*-test statistics were performed to test for differential expression. Microarray data was floored by adjusting low intensity spots to a threshold value of 130, hereby reducing the number of less reliable genes. Next, spot intensities were ^{2}log transformed and each gene was mean centered versus all samples. Based on these ^{2}log transformed data differentially regulated genes were selected with a *p*-value <0.05 and a fold change >1.5 (> ^{2}log 0.6) in each of the three treatment replicates versus the average from the control animals. Hierarchical clustering of the differentially regulated genes was performed using Cluster and Treeview software [21]. Raw microarray data of the present study have been submitted to ArrayExpress (available at: http://www.ebi.ac.uk) and are stored under experiment accession number A-MEXP-1810.

2.4. Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) is a tool to identify and analyse the differential expression of biologically relevant sets of genes that share common biological functions [22]. Using GSEA, the differentially regulated genes observed in DHEA treated animals versus one control group (e.g. controls of experiment 1) were validated by evaluating this gene set by comparing the same DHEA treated animals versus the other control group (e.g. controls of experiment 3). Therefore, separate gene sets were generated of the differentially expressed genes of respectively IM treated animals (n=3) and OS treated animals (n=3) versus the controls of experiment 1 as well as the controls of experiment 3. For example, the transcripts found significantly up-regulated when comparing DHEA IM versus control group 1 were included in the gene set 'DHEA_IM_vs_CTR1_UP'. In a similar way other gene sets were created for up- as well as down-regulated genes. Next, GSEA ranks all genes on the microarray on differential expression between DHEA exposed and controls using signal to noise statistics, resulting in a list with up-regulated genes at the top and down-regulated genes at the lower end of the list. Each of the pre-defined gene sets was tested against this list and GSEA calculated whether the genes in

the gene set are randomly distributed, enriched at the top or at the lower end of the ranked list. Permutations were performed on gene sets and gene sets were considered significantly affected when the *p*-value was below 0.05 and the false discovery rate (FDR) below 0.25, accordingly to GSEA recommendations [22].

3. Results and Discussion

3.1. Principal component analysis (PCA) and selection of differentially regulated genes

In the present study the potential strengths as well as the pitfalls of microarray experiments using calves from real-life practice were investigated. Three small animal experiments were performed independently using bull calves purchased at the local market. In this way the experimental setup was taking into account the inherent variability needed to investigate the usefulness of bovine-specific microarrays as a screening tool for prohormone abuse in veterinary control. For obvious ethical reasons larger numbers of bovines treated with banned substances could not be justified.

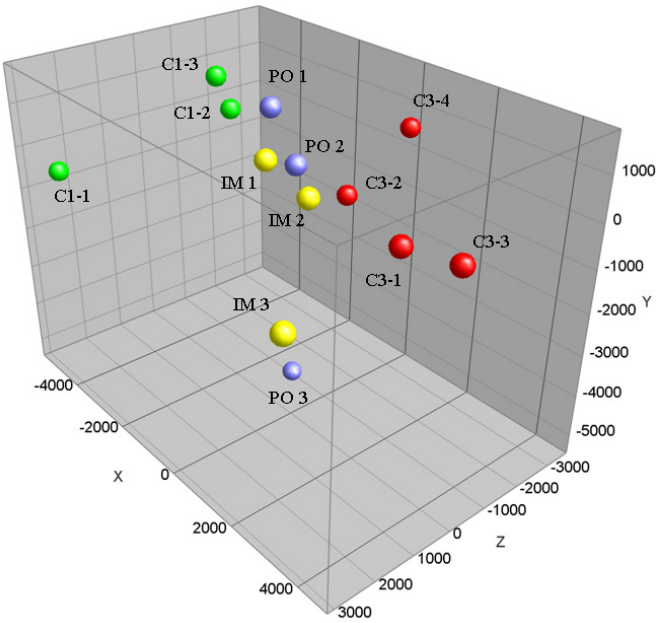

Figure 1 - Principal component analysis (PCA) of bovine liver gene expression profiles. *PCA-plot showing the three principal components of greatest variation which cover 22% (x-axis), 14% (y-axis) and 13% (z-axis) of the total variance respectively. Spheres in the PCA are representing profiles of control animals of experiment 1 (green, C1 1-3), control animals of experiment 3 (red, C3 1-4), orally treated (blue, PO 1-3) and intramuscular treated animals (yellow, IM 1-3) respectively.*

Upon microarray hybridization and data normalization, unsupervised principal component analysis (PCA) was performed to visualize differences between liver profiles of control and treated animals. Figure 1 shows the PCA-plot which is based on the three largest components, representing 49.9% of the total variance. Although there is variation in gene expression profiles of livers of animals treated with DHEA, they are clearly discriminated from those of the controls. However, large differences are observed between the two control groups, whereas the exposed animals (IM 1-3 and PO 1-3) and the control animals of the first experiment (CTR1) are separated along the x-axis while the control bovines of experiment three (CTR3) and the exposed bovines are mainly separated along the z-axis. Based upon the outcome of this PCA, further analysis was focused on comparison of the IM and PO treated animals versus either the total control population as well as the two control groups separately.

	Total number of genes regulated		Up-regulated			Down-regulated		
			DHEA IM		DHEA PO	DHEA IM		DHEA PO
	DHEA IM	DHEA PO	Only	Shared	Only	Only	Shared	Only
Vs all CTR	37	23	27	0	11	9	1	11
Vs CTR 1	306	138	82	16	49	181	27	46
Vs CTR 3	65	65	42	4	27	16	3	31

Figure 2 - Venn diagram comparison of differentially expressed genes. *Differentially expressed genes (p-value <0.05 and fold change >1.5 observed in each of the three treated animals) in liver of intramuscular (IM) and oral (PO) treated animals versus the mean of all controls, the controls of experiment 1 (CTR 1) and experiment 3 (CTR 3). For each comparison the number of unique and shared genes are presented.*

Differentially regulated genes were selected using t-test statistics. A *p*-value <0.05 and a difference of at least 1.5 ($>^2\log 0.6$) fold change, versus the control average, observed in all three biological treatment replicates (either IM or PO) were used as criteria for the selection of differentially expressed genes. An overview of the differentially regulated genes found in the IM and PO treated animals is shown in Figure 2. A total of 37 and 23 genes were found to be regulated in IM and PO treated animals as compared to the total

control group, respectively. Only one of these genes (DMBT) was found differentially expressed (down-regulated) in IM as well as PO treated animals. A hierarchical cluster diagram of all differentially regulated genes is presented in Figure 3. Since many probes were spotted twice or more on the microarray the 37 and 23 genes found regulated are represented by 66 and 39 spots respectively. A detailed description of all regulated genes is listed in Additional file 1.

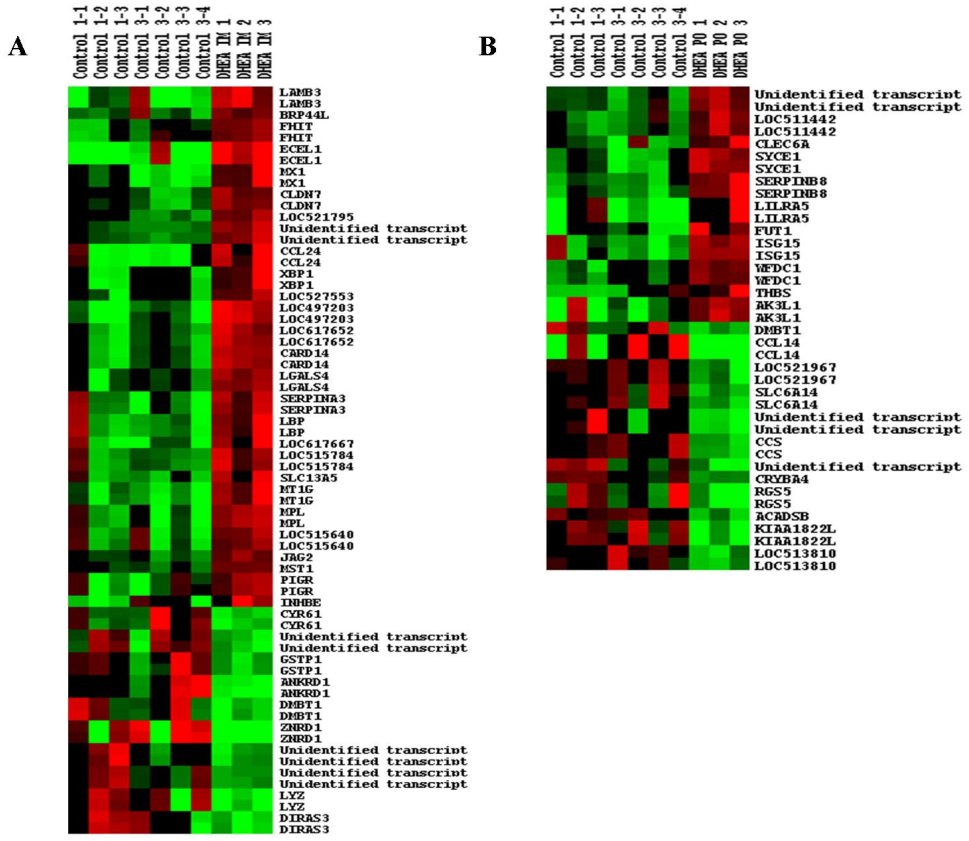

Figure 3 - Hierarchical cluster analysis of significant regulated genes. *Hierarchical cluster analysis (HCA) of genes with a p-value <0.05 and a fold change >1.5 (fold changes calculated for each individual DHEA exposed animal versus the mean of all control animals) for (A) intramuscularly and (B) orally treated animals. Based on ^{2}log mean centered ratios, HCA was performed on genes only using average linkage clustering. Colour scales are ranging from bright red to bright green which correspond with respectively up- or down-regulated genes. Maximum brightness represents a fold change of ≥ 2 (^{2}log mean centered ratios of ≤ -1 or ≥ 1).*

Of the 37 differentially expressed genes in response to the IM DHEA treatment, 4 represented unidentified transcripts and 7 were encoding for proteins with poorly known or unknown function (LOC617652, LOC527553, LOC515784, LOC617667, LOC515640, LOC497203 and LOC521795). Among the 26 transcripts that encode for known proteins, 10 transcripts are involved in immune response and inflammatory processes. Of these latter transcripts XBP1, MX1, LBP, SERPINA3, CCL24, CARD14 and PIGR were found up-regulated and ANKRD1, LYZ and DMBT were down-regulated. The remaining transcripts are involved in various processes like cell growth and proliferation (INHBE), formation of tight-junctions (CLDN7), tumor suppression (DIRAS3), cell proliferation and cell adhesion (CYR61) intra-cellular signalling (JAG2) and cell-cell interactions (LGALS4). Regarding metabolism, the GSTP1 gene was found to be down regulated >1.6 fold in all IM treated animals. GSTP1 mediates glutathione conjugation and plays an important role in detoxification of xenobiotics as well as in uptake and transport of numerous hydrophobic endogenous compounds like steroids [23] Moreover, it has been observed in mouse that the GSTP1 gene contains androgen receptor binding sites which regulate GSTP1 activity in response to androgens [24].

Comparison of PO treated animals versus all control animals revealed a total of 23 differentially expressed genes of which 7 represent unidentified transcripts or encoded for proteins with an unknown function. Again a substantial number of the differentially regulated genes are involved in immune response of which LILRA5, THBS, CLEC6A and FUT1 were found up-regulated and CCL14 and DBMT were down-regulated. Other differentially regulated genes are involved in peptidase inhibition (SERPINB8, WFDC1), G-protein signalling (RGS5) and amino acid transport (SLC6A14). Also regulated is the short/branched chain acyl-CoA dehydrogenase (ACADSB) gene, a member of the acyl-CoA dehydrogenase enzyme family which is involved in fatty acid metabolism. This could point towards regulation of fatty acid metabolism and is supported by a study in which DHEA administration to rats showed significant regulation of genes involved in fatty acid metabolism, including the very long chain acyl-CoA gene which is also a member of the acyl-CoA dehydrogenase enzyme family [25]. Overall it can be stated that the majority of regulated genes are involved in immune response for both PO as well as IM treated animals which is in line with numerous studies reporting the significant immune modulatory properties of DHEA [26].

In principle the above listed genes are potential biomarkers for DHEA treatment. On the one hand, we are aware of the small number of animals used in this study which hampers proper statistics and substantially increases the chance of missing DHEA-responsive genes or detecting false-positive genes. On the other hand, combining and comparing the

data of three independently performed experiments will limit the risk of false-positive genes considerably and results in identification of only the most robustly regulated genes. Therefore, we assessed whether the genes differentially expressed in animals treated with DHEA via one administration route versus animals of one control group would also be affected when compared 1) to other control animals and 2) by the other administration route. To deal with these issues we applied the statistics of gene set enrichment analysis (GSEA). In this way statistical power could be improved and regulated gene sets were tested for their robustness.

3.2. Gene set enrichment analysis (GSEA)

For GSEA we used the genes found to be differentially expressed when the exposed animals are compared with the two control groups separately. For DHEA IM, 306 and 65 genes were differentially regulated versus CTR1 and CTR3, respectively, whereas for DHEA PO, 138 and 65 genes were regulated. As shown in Figure 2, a relatively small number of genes was affected per treatment in both comparisons versus CTR1 and comparisons versus CTR3. Apparently, only a low proportion of genes showed a significant up-regulation or down-regulation of 1.5 or more in both comparisons. GSEA was used as a tool to discriminate DHEA treated animals from non-treated animals on the basis of gene sets generated from genes found to be differentially regulated (Figure 2). Gene sets were compared to the whole experimental dataset and GSEA calculated whether genes within a gene set are randomly distributed, enriched at the top or at the bottom of the ranked list [22]. The advantage of this GSEA approach is that no cut-off is used for determination of differentially regulated genes. Using the whole experimental data set makes that alterations are viewed for as a group of genes instead for individual genes. Gene sets can be significantly affected while changes in expression of individual genes are relatively subtle. For example, the transcripts found to be significantly up-regulated when comparing DHEA IM versus control group 3 (Figure 4A) were included in the gene set 'DHEA_IM_vs_CTR3_UP'. GSEA analysis, using this gene set, showed that the genes where highly enriched in DHEA IM treated animals when comparing versus the CTR1-group (Figure 4B). As shown in Figure 4C, most of the genes are distinctly up-regulated in DHEA IM treated animals, although also variation in gene expression of the individual animals is observed. In a similar way, the other gene sets were compared versus the other 'independent' control group and results are summarized in Table 2. This cross validation showed that 6 out of 8 gene sets were significantly enriched (p-value <0.05 and FDR < 0.25) when DHEA treated animals were compared versus an 'independent' control group. Moreover, gene sets generated on the basis of DHEA IM treated animals showed

significant enrichment in DHEA PO treated animals and vice versa. In total 12 out of 16 gene set comparisons showed significant enrichment, suggesting an overlap in gene expression profiles from IM and PO treated animals which most likely include genes that are differentially expressed irrespective of the manner of DHEA administration.

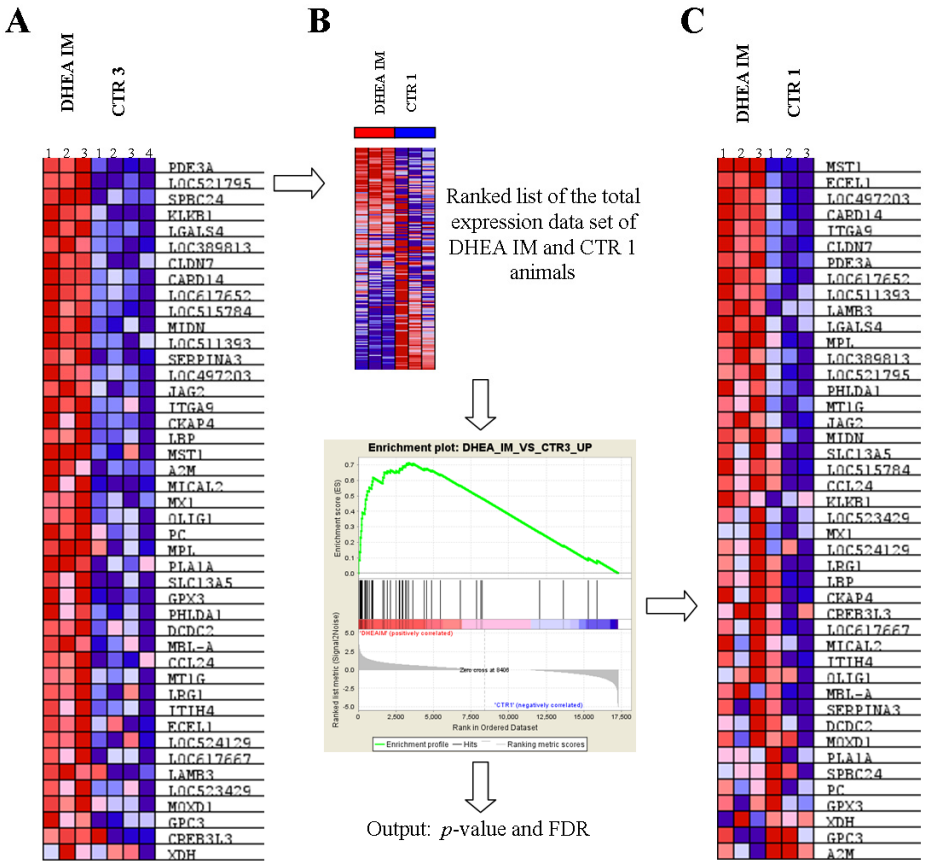

Figure 4 - Overview of the GSEA method applied. *(A) Heat map of the gene set 'DHEA_IM_vs_CTR3' containing all genes found significantly up-regulated (> 1.5 fold and p-value <0.05) when comparing DHEA IM treated animals versus the control population of experiment 3. Colours range from dark red to dark blue representing respectively the highest and lowest expression of a gene. (B) This gene set was compared to the ranked list of the total microarray expression data set of DHEA IM treated and CTR1 animals showing a significant (p-value 0.000 and FDR of 0.000) enrichment of genes in DHEA IM treated animals when compared to the control group of experiment 1. (C) Heat map displaying the genes of gene set 'DHEA_IM_vs_CTR3' in DHEA IM and CTR1 animals.*

Although *in vivo* transcriptomics data of DHEA in liver is limited [25,27], Depreter et al. identified 13 genes which were found to be up-regulated in rat liver [25]. GSEA analysis showed significant enrichment of this gene set in DHEA IM and PO treated animals when these were compared with the controls of experiment 1 (Table 2). These results illustrate that GSEA is a powerful approach for comparative analysis of gene expression data obtained in different settings.

Controlled experiments with bovines have resulted in the identification of genomics based biomarkers which potentially can be used for screening for hormones [10-14,28]. However, when examining bovines from real-life practice, one is dealing with biological variation like age, genetic background, environment, nutrition and disease history. In the current study, this biological variation was deliberately included and was mainly reflected by the large differences in gene expression profiles of the control populations tested. The two control groups in this study showed substantial age differences i.e. the animals in the CTR1, CTR3 and DHEA-treated groups are 6 months, 13-14 months and 8-13 months in age, respectively. Nevertheless, for the DHEA IM and PO treated animals, sets of respectively 37 and 23 genes were found differentially expressed when compared to all controls using standard statistics. These two groups of genes are specific for IM and PO treatment, respectively, and independent from biological factors like age. However, GSEA results showed a correlation between gene expression profiles of IM and PO treated animals, suggesting that there are also effects independent from the route of administration. This is in line with our earlier performed metabolomics study showing large similarities in urine metabolite profiles of IM and PO treated animals as well as metabolites specific for the route of administration [19].

Hence, for application of transcriptomics based screening of bovines for (pro)hormones in practice, the treatment effect should be filtered out from differences in gene expression due to inherent biological variation. Here it was shown that microarray gene expression profiling in combination with statistical methods like GSEA are able to distinguish gene expression profiles of DHEA-treated animals from non-treated control animals. It should be noted that this experiment comprised small numbers of animals and follow up experiments are required to gain statistical power and to obtain a better description of DHEA specific gene sets. Furthermore, the behaviour of such a growth promoter specific gene set should be studied in a broad spectrum of untreated control animals from daily practice, to assure the robustness of the gene set. This underlines the need to obtain more liver gene expression profiles of control animals from slaughterhouses.

Table 2: Significance of gene set regulation after GSEA analysis.

		DHEA IM group compared to CTR1		DHEA IM group compared to CTR3	
Name gene set	**# Genes**	***p*-value**	**FDR**	***p*-value**	**FDR**
DHEA_IM_vs_CTR1_up	98	-	-	0.000*	0.000
DHEA_IM_vs_CTR1_down	208	-	-	0.000*	0.071
DHEA_IM_vs_CTR3_up	46	0.000*	0.000	-	-
DHEA_IM_vs_CTR3_down	19	0.826	0.823	-	-
DHEA_PO_vs_CTR1_up	65	0.000*	0.000	0.751	0.887
DHEA_PO_vs_CTR1_down	73	0.000*	0.000	0.861	0.899
DHEA_PO_vs_CTR3_up	31	0.032*	0.073	0.000*	0.000
DHEA_PO_vs_CTR3_down	34	0.312	0.413	0.000*	0.000
Depreter_et_al_up	11	0.015*	0.031	0.381	0.657
		DHEA PO group compared to CTR1		**DHEA PO group compared to CTR3**	
Name gene set	**# Genes**	***p*-value**	**FDR**	***p*-value**	**FDR**
DHEA_PO_vs_CTR1_up	65	-	-	0.000*	0.000
DHEA_PO_vs_CTR1_down	73	-	-	0.007*	0.002
DHEA_PO_vs_CTR3_up	31	0.173	0.292	-	-
DHEA_PO_vs_CTR3_down	34	0.015*	0.014	-	-
DHEA_IM_vs_CTR1_up	98	0.000*	0.000	0.000	0.479
DHEA_IM_vs_CTR1_down	208	0.000*	0.000	0.000*	0.000
DHEA_IM_vs_CTR3_up	46	0.706	0.738	0.000*	0.000
DHEA_IM_vs_CTR3_down	19	0.009*	0.006	0.002*	0.001
Depreter_et_al_up	11	0.029*	0.030	0.887	1.000

* Significantly regulated gene set.

4. Conclusion

The present study showed that identification of genomic biomarkers for DHEA treatment in cattle is hampered by the large biological variability as compared to genomics experiments with inbred strains of rodents under well-defined laboratory conditions. However, gene expression profiling using whole genome microarrays in combination with predefined gene sets and statistical methods like GSEA showed to be a promising approach to screen for (pro)hormone abuse in livestock production. For application in practice however, a better genomic description of the control population as well as growth promoter specific gene set are needed.

Acknowledgements

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Chapter 8

General discussion and future perspectives:

towards effective screening of prohormones in livestock production

Introduction

Within the European Union the surveillance on veterinary growth promoter abuse is strictly regulated by several legislations. Council Directive 96/22/EC lays down the prohibition of the use of growth promoting agents such as steroid hormones in livestock production [1]. Interestingly there is no list of prohibited substances but 96/22/EC states that all substances having a thyrostatic, oestrogenic, androgenic or gestagenic action and beta-agonists are prohibited in livestock production. In addition Council Directive 96/23/EC encloses specific requirements to monitor anabolic steroids in farm animals and their products [2]. This Directive primarily includes sampling and investigation procedures while technical guidelines and performance criteria for methods in residue analysis are described in Commission Decision 2002/657/EC [3]. In hormone residue analysis, methodologies based on gas and liquid chromatography in combination with mass spectrometric detection (GC- and LC-MS/MS) are presently considered state-of-the-art and are extensively used for screening as well as confirmation purposes. However, most of these methods are limited to screening of a predefined list of hormone residues and can thus not fully meet the legislation 96/22/EC. In this way some kind of discrepancy arises between the bioactivity based ban, as described in 96/22/EC on the one hand and the measures to monitor on targeted (groups) of substances on the other. Therefore, to fully meet the legislation, screening methods should be based on bioactivity rather than on targeted screening of a list of compounds. Within this scope, in recent years significant efforts have been invested in development and implementation of novel effect based methods such as bioassays to detect hormone abuse in the field of livestock production [4].

During inspections at livestock farms in The Netherlands, inspection services are occasionally confronted with animals growing suspiciously fast. Besides these observations also syringes, feed supplements and herbal preparations have been found containing so-called prohormones such as dehydroepiandrosterone (DHEA) and pregnenolone. Prohormones show no or limited direct hormonal activity but might have hormonal effects upon *in vivo* bioactivation. However, knowledge about metabolism, the mode of action and excretion profiles of prohormones is often unclear, in particular in cattle. This thesis research investigated the bioactivity of prohormones upon their metabolism *in vitro* as well as *in vivo* with the goal to develop effective screening and detection methods to detect prohormone abuse in livestock production and to meet Directive 96/22/EC.

Effect based screening of (pro)hormones in supplements and animal feed

Supplements and preparations containing (pro)hormones used in sports also turn up in livestock production and vice versa. As a consequence, the nature of the samples and the methods and requirements for screening are highly similar. In **Chapter 2** we investigated 18 dietary supplements which were previously screened for the presence of 49 prohibited steroids by state-of-the-art LC-MS/MS analysis. After screening by a yeast androgen bioassay two of the supplements which were found negative by LC-MS/MS analysis did show a response. Upon application of the same bioassay as an off-line LC detector in combination with UPLC-TOFMS we were able to identify 4-androstene-3ß,17ß-diol, 5α-androstane-3ß,17ß-diol and 1-testosterone in the bioactive fractions. Because these compounds were not included in the predefined list of 49 compounds they were missed in this targeted LC-MS/MS procedure. This clearly demonstrates the added value of an effect based screening approach where virtually all compounds with androgenic activity are taken into account. This together with bioassay guided identification sets a new benchmark for the analysis of supplements and preparations used in livestock production and sports.

For supplements containing solely prohormones the value of bioactivity based screening seems to be limited. When testing the endogenous prohormones DHEA and 5-androstene-3ß,17ß-diol in the yeast androgen bioassay they showed no direct androgenic activity while 4-androstenedione showed an EC_{50} of 6900 nM (**Chapter 3**). To detect these inactive prohormones in animal feed, supplements and injection preparations, a modified *in vitro* bioassay test system was developed. In order to mimic the *in vivo* metabolic activation, standards as well as sample extracts were incubated with bovine liver S9 fractions and different cofactors before application to the yeast androgen assay. Depending on the cofactor used, this resulted in an increase in androgenic activity for DHEA, 5-androstene-3ß,17ß-diol and 4-androstenedione. After incubation of DHEA with bovine liver S9, UPLC-TOFMS analysis showed that 4-androstene-3,17-dione, 7α-OH-DHEA and an initially unknown oxo-metabolite of DHEA were the most abundant metabolites formed when using $NAD(P)^+$ as a cofactor. The unknown keto-metabolite later turned out to be 7-oxo-DHEA. When using NAD(P)H as a cofactor, metabolism was mainly guided in the direction of 5-androstene-3ß,17ß-diol but again also 7α-OH-DHEA and 7-oxo-DHEA were observed to be major metabolites. In addition also minor amounts of other metabolites are observed which were, based on their accurate mass and retention time, most likely hydroxy- and oxo- metabolites of DHEA, 4-androstenedione or even

17ß-testosterone. An overview of the metabolism of DHEA by bovine liver S9 is shown in Figure 1. Regarding bioactivity, 4-androstenedione and 17ß-testosterone are most likely the main compounds contributing to the bioactivity read-out, as 17α-testosterone, 5-androstene-3ß,17ß-diol and the hydroxy-metabolites of DHEA (data not shown in this thesis) and 17ß-testosterone [5] showed no or limited androgenic activity.

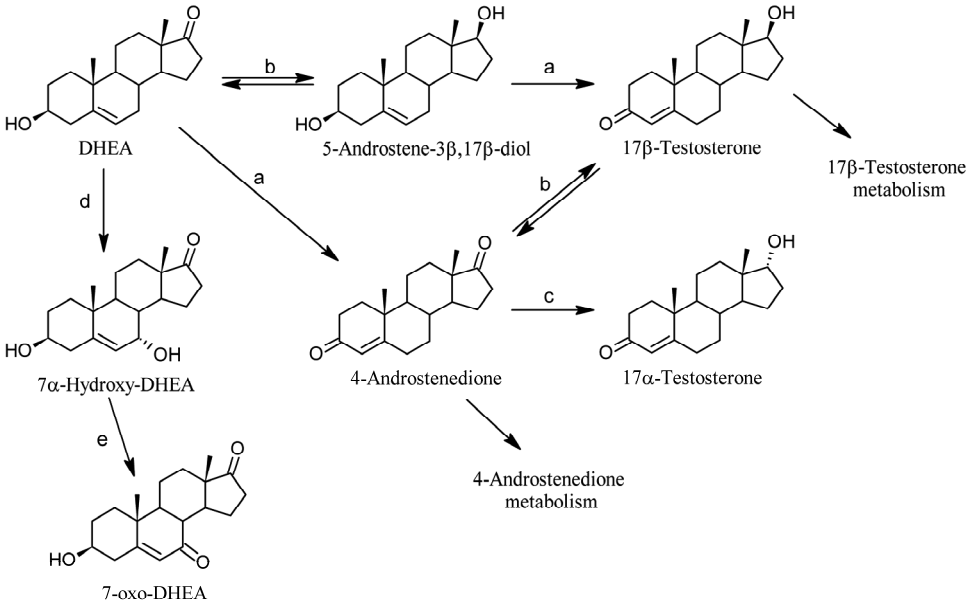

Figure 1: DHEA metabolites observed after incubation with bovine liver S9 and various cofactors. Enzymatic activities involved are: (a) 3β-dehydrogenase/isomerase (b) 17β-hydroxysteroid dehydrogenase (c) 17α-oxido-reductase (d) 7α-hydroxylase (e) 7-hydroxy dehydrogenase.

Metabolic conversion of unknown supplement samples claiming to contain DHEA resulted in successful bioactivation and positive screening results in the androgen yeast bioassay. However, the bioassay read-out was strongly hampered by the androgen antagonistic properties of one of the compounds present in the supplements, most likely DHEA. This antagonistic activity was confirmed by co-exposure of 17ß-testosterone with increasing concentrations of DHEA showing a clear dose dependent inhibition (Figure 2). The activity of 17ß-testosterone was even completely inhibited when the yeast cells were exposed to 70 nM 17ß-testosterone and more than 10 µM of DHEA. This illustrates the necessity to include spike-in controls in bioactivity screening procedures to test for antagonistic action and to rule out that high levels of antagonists, such as DHEA, can mask the presence of direct agonists.

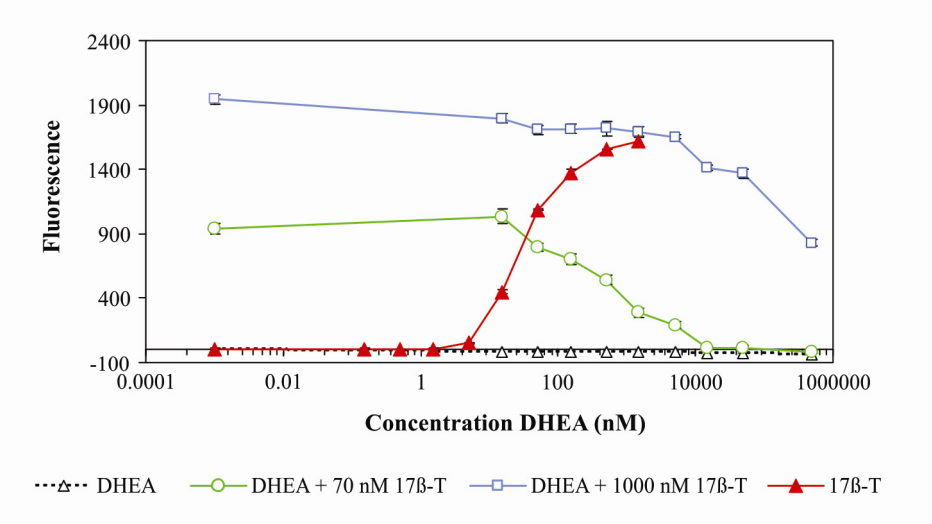

Figure 2: Anti-androgenic action of DHEA. Shown are the inhibition of the response of 17β-testosterone at half maximum (70 nM) and full response (1000 nM) at increasing levels of DHEA. Fluorescence signals are the mean of an assay triplicate (± SD) and corrected for the signal at t=0 hours and the reagent blank.

Interestingly, after incubation of a supplement containing DHEA and 4-androstenedione with bovine liver S9, also high levels of 17α-testosterone were observed in particular when using NAD(P)H as a cofactor. Because this supplement contained apart from DHEA also high levels of 4-androstenedione, these findings are supporting the hypothesis that 17α-testosterone could be formed by 17α-hydroxysteroid dehydrogenase reduction of 4-androstenedione [6,7]. However further research is needed to confirm this observation. In conclusion it can be stated that bovine liver S9 successfully mimicked the *in vivo* hydroxysteroid dehydrogenase and cytochrome P450 mediated metabolic conversions. Using the screening protocol described in Chapter 3, 50 µg of DHEA resulted already in a significant signal in the yeast androgen bioassay. For supplements, application of 100 mg to the proposed screening method is amply sufficient to obtain a positive screening result as they contain in general high levels of prohormones. For example DHEA supplements intended for human use for example contain in general 50-200 mg DHEA per capsule which is equal to the daily intake recommended on the label.

From a bioactivity point of view, steroid derivatives can also be considered as prohormones. Due to the lack of affinity for steroid receptors, intact steroid esters and glycosilated compounds show no or limited direct hormonal activity. Consequently these compounds can be missed in receptor based screening procedures. **Chapter 4** describes

the concept of a yeast bioassay based method for screening of esters and glycosides in animal feed and supplements. To optimize hydrolysis and deconjugation procedures a comparison was made between different *in vitro* activation methods for hormone esters and glycosides. For testosterone esters, the efficiency of alkaline hydrolysis was almost 100% and much better compared to enzymatic hydrolysis by esterase. As a result 1 µg testosterone ester per gram of animal feed could be readily detected by the yeast androgen bioassay. When comparing different enzyme fractions for deglycosilation the glycoside mimic genistin was shown to be deconjugated most efficiently by ß-glucuronidase/ arylsulfatase from Helix pomatia. In conclusion, chemical and enzymatic deconjugation procedures for ester and glycoside conjugates respectively resulted in a significant increase in hormonal activity and allowed effective screening of these derivatives in animal feed and supplements.

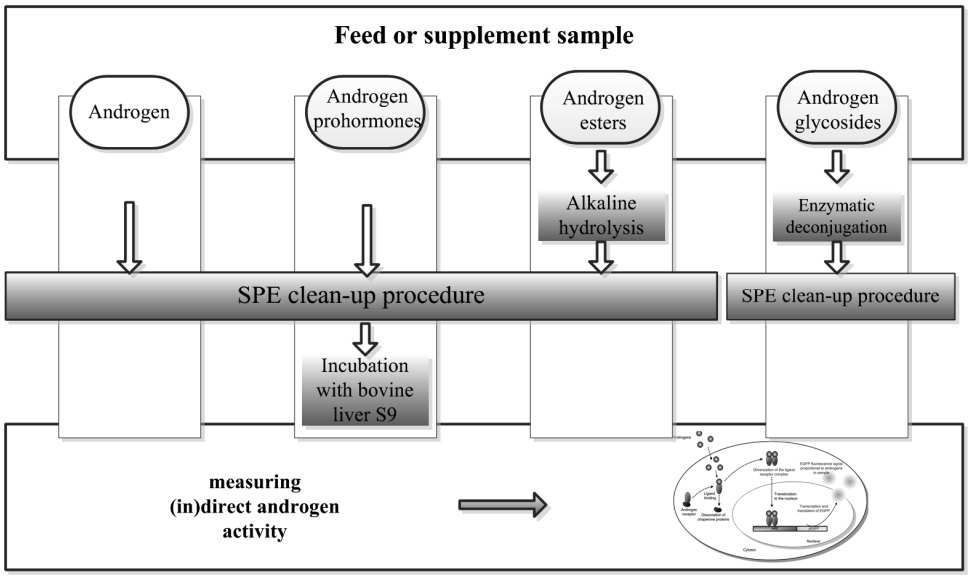

Figure 3: Effect based screening strategies for androgens, androgen prohormones, steroid esters and glycosides. Similar approaches can be foreseen for estrogen and corticosteroid (pro)hormones, esters and glycosides.

To summarize, it can be stated that the previously developed androgen yeast bioassay allowed an effective screening for androgens in animal feed and supplements but is less suited to detect hormonally inactive prohormones. The use of either bovine liver S9, pure enzymes or alkaline hydrolysis showed that prohormones could be activated, resulting in a significant increase in bioactivity as determined by the androgen or estrogen yeast bioassay. Combined, this results in a panel of screening methods for (pro)hormonal

activity that better meets Council Directive 96/22/EC (Figure 3). However, before application in routine use, the proposed qualitative screening methods need to be validated according EC Decision 2002/657.

Compared to enzyme fractions, whole cell systems such as liver slices have the advantage that phase I and phase II enzymes are present together with natural levels of cofactors. Moreover, in liver slices all liver cell types are present maintaining their original interactions and spatial arrangement thus resembling the real *in vivo* situation more closely. In **Chapter 5** the feasibility of bovine liver slices as a model for the bioactivation of prohormones was investigated. After incubation of DHEA with bovine liver slices, UPLC-TOFMS analysis showed that 4-androstenedione, 7α-OH-DHEA and 7-oxo-DHEA were the most abundant metabolites formed, while also minor amounts of 5-androstene-3ß,17ß-diol, 17ß-testosterone and 17α-testosterone were observed. Compared to the earlier performed bovine liver S9 experiments described in Chapter 3, DHEA phase I metabolism observed in liver slices is highly similar to liver S9 incubations using NAD^+ or $NADH^+$ as a cofactor. Application of medium extracts, originating from incubations of liver slices with DHEA, to the androgen yeast assay showed an increase in androgen activity as well as a decrease in androgen antagonistic activity, confirming metabolism of DHEA into more androgen active compounds such as 4-androstenedione and 17ß-testosterone. In this way liver slices can be used as a complementary tool to screen for biologically inactive steroids. On the other hand, because liver slices resemble the *in vivo* situation more closely also a substantial amount of phase II metabolites are formed. Compared to bovine liver S9, bioactivation of DHEA with liver slices is therefore less efficient and can be considered to be a less suitable bioactivation model for screening purposes. Moreover, since preparation of liver slices is very laborious, and the fact that for each screening experiment liver slices have to be prepared freshly, other bioactivation models such as bovine liver S9 are more suitable to use in high through-put screening procedures. Alternatively, the lifespan of liver slices could be extended by cryo-preservation thereby facilitating that one single batch of liver slices could be used for multiple screening procedures. Although this is shown to be a feasible approach for human and rat liver slices, success of these cryopreservation procedures is variable, seems dependent on animal species and often still results in a significant decrease in enzyme activity and cell viability compared to freshly isolated liver slices [8].
At this stage a more feasible application of liver slices is foreseen in evaluation of both metabolism and bioactivation of (new) compounds showing up in the illegal circuit of which the *in vivo* mode of action is unclear. Currently, these compounds are tested in

small scale animal experiments to obtain knowledge about absorption, distribution, metabolism and excretion kinetics in bovines. In this process, exposure of liver slices could be included to gather knowledge about metabolism and identification of (new) metabolite biomarkers that could be used for in vivo urine screening.

Metabolomics based steroid profiling in urine

Urine profiling of endogenous steroid concentrations is an established method to identify abuse of natural hormones in human doping control. In livestock production however, misuse of natural occurring (pro)hormones is hard to prove. Urinary metabolites are either unknown or profiling parameters which are relevant in humans are failing in cattle due to highly fluctuating endogenous steroid levels or differences in metabolism. **Chapter 6** outlined a novel metabolomics based strategy for anabolic steroid urine profiling in the field of livestock production. Urine profiles of controls and bull calves treated with DHEA or pregnenolone were analyzed in triplicate by UPLC-TOFMS. The quality and reproducibility of the analytical procedure was assessed by application of two deuterium labeled internal standards. This demonstrated that the data acquisition was highly stable, both in retention time as well as mass accuracy, within as well as between measurement series. Also the signal intensities were considered as stable since most fluctuations in signal amplitudes were within a factor 2. As a result, changes in metabolite profiles by more than a factor 2 (e.g. the applied thresholds of >5 and >10 times fold change) can be considered to be differential from random variations in the analytical procedure.

Comparison of the urinary profiles using MetAlign™ and multivariate statistics revealed large differences between the urinary profiles of control and DHEA as well as pregnenolone treated animals. For DHEA, cutoff based selection revealed 180 mass signals that were significantly different (fold change >10, *p*-value <0.01) between untreated controls and DHEA treated animals. In addition, PCA and hierarchical cluster analysis revealed also large differences between days and the route of administration. However, because potential application in control and enforcement programs is performed without a priori knowledge about the route of administration it was decided to determine potential biomarkers independent from the route of administration. Consequently biomarkers for DHEA treatment were selected by comparing urine samples of all controls versus all treated animals. In analogy with the DHEA experiment, a similar data selection procedure was performed for the pregnenolone experiment. Here the differences in urine profiles were less distinct since biomarker selection yielded 163 *m/z* values (fold change >5, *p*-value <0.01). Interestingly also mass signals were observed to be significantly down -regulated suggesting negative feedback mechanisms induced by pregnenolone

administration. Yet, within this thesis research no further attempts were made to identify the compounds belonging to those differentially down-regulated masses.

In order to determine if the mass peak loadings selected by multivariate statistics can be used as biomarker for DHEA or pregnenolone administration respectively, all mass signals were evaluated individually by univariate statistics. Hereby the control as well as the treated population was assumed to be normally distributed. Initially the number of false negatives was determined for each mass peak loading individually, by comparing the DHEA and pregnenolone treated bovines to their corresponding control population. For DHEA already seven mass peak loadings showed a false negative rate below 5% hereby complying with Commission Decision 2002/657/EC [3]. Next, the mass peak loadings showing the least false negatives, respectively 12 (for DHEA) and 7 (for pregnenolone), mass peaks were selected and tested for false positives using a small but independent test set of control urines. For DHEA, 10 out of the 12 biomarkers tested showed no false positives, while for pregnenolone 5 out of the 7 potential biomarkers showed no false positives. Although these results are promising, the robustness of these potential biomarkers should be tested against a larger control population of bovine urines to obtain a more accurate estimation of the number of false positives. Apart from identification of biomarkers for treatment by specific prohormones, it is envisaged that application of this holistic methodology is also suitable for general anabolic steroid screening. A possible scenario for implementation of such a steroid urine profiling approach in practice is outlined in Figure 4: upon sampling at the farm, urine samples are measured in triplicate by UPLC-TOFMS according to the method described in Chapter 6. After preprocessing and alignment of the data, the obtained urine profiles are compared to a library of control urines by using multivariate statistics. Based on the statistical deviation from this control population it then can be decided whether a sample could be classified as “compliant” or “suspect”. This inherently raises the need to define thresholds to decide when an urine profile is considered to be differential from a control population. This threshold could e.g. be based on a *p*-value derived by multivariate statistics (e.g. p-value <0.05) or by use of classification tools such as Orthogonal Partial Least Squares analysis (OPLS).

When a sample is classified as “suspect” the observed differential masses can provide extra information about the possible identity of the compound administered and it can be decided to initiate appropriate follow-up actions. When suspecting abuse of synthetic steroids the screening procedure can be followed by confirmatory mass spectrometry analysis to unambiguously confirm the presence of these xenobiotic steroids. When abuse of natural (pro)hormones is suspected follow-up actions are currently limited to investigations at the farm since classical mass spectrometry methodologies are incapable

of discriminating synthetic hormones from the biosynthesized congeners. Nevertheless, based on the screening results, inspection services could focus on farms showing suspicious results by intensifying the sample frequency or perform more detailed investigations in search for suspicious supplements and preparations. These in turn, could be subjected to the yeast androgen bioassay procedures described in Chapter 3 and 4.

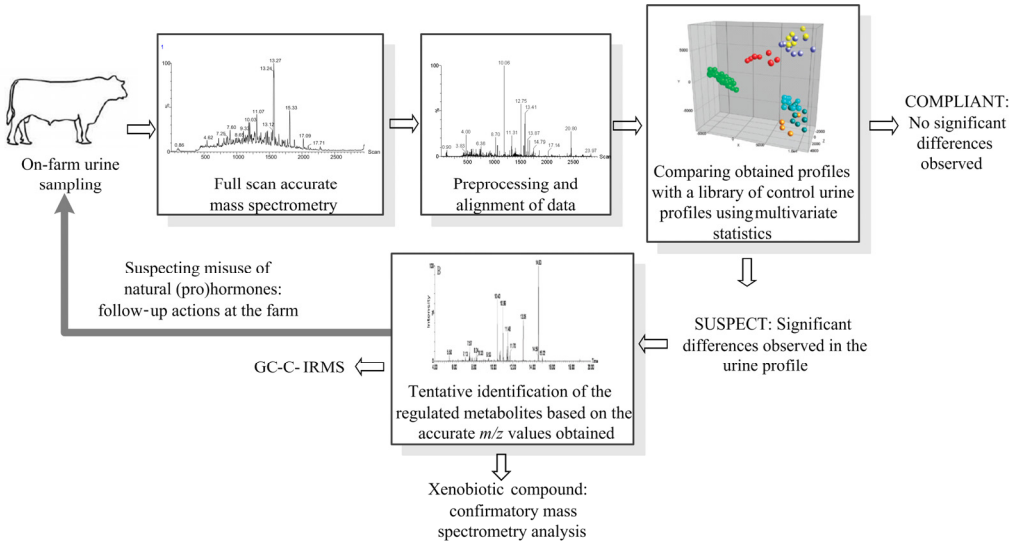

Figure 4: Proposed metabolomics based steroid urine profiling strategy to monitor for prohormone abuse in cattle.

Ideally, suspicion of natural (pro)hormone abuse should be followed by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis of the suspect urine sample to unambiguously confirm the presence of exogenous administered (pro)hormones [9,10]. Although this sophisticated confirmation procedure is successful in discriminating synthetic and natural hormones, this GC/C/IRMS methodology is very complex, laborious and costly. Therefore, unfortunately, this approach is no common practice in veterinary growth promoter control and is currently used in only one National Reference Laboratory in Europe [11]. Another prerequisite for such a holistic screening approach is an ever growing library of control urine profiles reflecting the total control population, thus with inclusion of inherent biological variability such as differences in origin, age, feeding regime and medical history.

Feasibility of a transcriptomics approach for prohormone screening

Surveillance of growth promoter abuse is mainly based on residue analysis in urine, blood, tissue or hair. Alternatively, monitoring differences in transcript levels is in potential a promising screening method to detect abuse of (natural) (pro)hormones, perhaps even when metabolites already have been excreted. In **Chapter 7** we investigated changes in liver gene expression profiles of bovines treated orally (n=3) or intramuscularly (n=3) with 1000 mg DHEA versus two control groups (n=3 and n=4), using bovine 44K oligonucleotide microarrays. In contrast to controlled genomics studies these bull calves were purchased at the local market on three different occasions and aged from 6 to 14 months, thereby reflecting the real-life inter-animal variability.

The significance of the gene expression data obtained from orally (PO) and intramuscularly (IM) treated calves versus all controls were evaluated using t-test statistics. Significant (p-value <0.05) more than 1.5 fold differential regulation of 37 and 23 transcripts for respectively intramuscularly and orally treated animals was shown. In principle these regulated genes could be considered potential biomarkers for DHEA treatment. However we are also aware of the small number of animals used which hampers proper statistics and substantially increases the chance of detecting false-positive genes as well as missing typical DHEA target genes. In addition, for screening purposes this raises the question how specific these genes are for DHEA treatment and consequently the percentage of false positive animals obtained when using these genes for screening purposes. Overall it can be stated that the majority of the regulated genes are involved in immune response for both PO as well as IM treated animals. Although DHEA is reported to have significant immunomodulatory properties it can be expected that these genes are not specific for DHEA treatment nor represent a typical androgenic or prohormonal effect. Moreover, the question is whether the gene expression profiles of the DHEA treated animals in this study will remain differential when they are compared to other groups of control animals. To deal with these issues we applied the statistics of gene set enrichment analysis (GSEA). For IM and PO treated calves, gene sets were generated of genes that were significantly regulated compared to one control group and validated versus the other control group using GSEA. This cross validation showed that 6 out of the 8 gene sets were significantly enriched in DHEA treated animals when compared to an independent control group. This study showed that identification and application of genomic biomarkers for DHEA treatment is strongly hampered by biological variation. On the other hand, it is demonstrated that comparison of defined gene sets versus the whole expression profile allows to distinguish DHEA treatment effects from variations in

gene expression due to inherent biological variation. This observation is a first hint that it is possible to use gene expression patterns to discriminate DHEA treated from control animals and opens the possibility to develop a screening method to control the misuse of (pro)hormones in cattle.

However, as already concluded by Carraro et al. [12], gene expression analysis is not yet the "magic bullet" to detect illicit treatment with growth promoters. Currently, in the field of growth promoter control, gene expression analysis is at the stage of providing proof of principle by performing *in vivo* experiments under controlled settings, ideally resulting in identification of "potential" biomarkers specific for illicit treatment, but application of these genomics based biomarkers for screening in practice is still far away. The next question is then whether the suggested biomarkers are robust and not influenced by biological factors like differences in breed, nutrition, age and medical history and should therefore be tested against a large control population. This can be achieved by obtaining more liver gene expression profiles of "control" animals from the slaughterhouse or, alternatively, even could be retrieved from rapidly growing genome databases such as ArrayExpress or the Gene Expression Omnibus (GEO) [13,14]. Apart from difficulties in identification of robust biomarkers it is foreseen that implementation of such a transcriptomics approach in practice can also be hampered by technical difficulties during e.g. sampling. First of all, for proper comparison of gene expression profiles tissues should be sampled consequently at the same anatomic location of the liver to obtain a homogenous sample without irregularities e.g. the presence of endothelial cells when sampling liver. Moreover, due to sub-optimal sampling conditions at the slaughterhouse, breakdown of mRNA could occur which in turn could cause differences in gene expression analysis. Nonetheless, preliminary evidence from analysis of RNA stability and qRT-PCR repeatability on skeletal muscle samples stored at 4°C suggests that reliable measures of gene expression can be obtained up to 10 days after slaughtering [12]. However, it is not very likely that this long term stability can be obtained in organs which contain high levels of RNases such as liver.

Towards a new control system for prohormones

The fight against growth promoter abuse in livestock production continues to be a major fair trade and food safety related issue. To facilitate efficient control and to try to keep up with the abusers, there is a continuous need for development of new screening and detection methods to cover the broad range of compounds used for treatment. The basis for control on hormone abuse is laid down in Directive 96/23/EC which outlines the measures to monitor certain (groups of) substances and residues while the ban described

in Directive 96/22/EC is based on bioactivity and prohibits the use of all compounds showing androgenic and other hormone-like activity. Within this scope the attention in this thesis has been focused on development of novel effect based screening methods for prohormone abuse. Hereby it is important to realize that according to Commission Decision 2002/657/EC [3], screening methods are used to detect the presence of a substance at relevant levels and with the capability for high sample throughput. Therefore typical screening methods are optimized to examine large numbers of samples in search for non-compliant results and hereby avoiding false compliant results.

Based on the outcomes of this thesis research we propose to implement two methods to screen for prohormones: (1) screening for prohormones in feed and (feed) supplements and (2) a holistic screening method for screening on prohormones in urine. Regarding screening of prohormones in animal feed and supplements we used bioactivation models in combination with an androgen yeast transcription activation bioassay. This allowed effective screening as well as detection of the (indirect) androgenic action of prohormones simultaneously hereby better meeting legislation 92/22/EC. The number of false compliant results in those methods is largely depending on the efficiency of the bioactivation procedures and the sensitivity of the yeast androgen bioassay readout system. For androgens this yeast androgen bioassay was validated according to Commission Decision 2002/657/EC [3] at a level of 50 or 100 ng per gram of animal feed [15]. Hereby the number of false negatives was shown to be less than 5%. Also the percentage of false positives was only 5% but this percentage could be lowered when conformation analysis would include more androgenic metabolites [15]. For DHEA the current level of detection is around 12.5 µg per gram of feed/supplement, and for steroid esters a level of 1 µg per gram of animal feed could be easily detected.

As stated above the second outcome of this thesis research focused on the development of a holistic screening method for screening on (pro)hormones in urine. For introduction of new screening strategies, such as profiling methods, in official control, specific criteria should be discussed as well as an estimation for false positive and negative rates should be made. For the developed steroid urine metabolomics approach samples can be sifted based on the statistical deviation from the control population classifying a sample to be either “compliant” or “suspect”. This inherently raises the need to define thresholds to decide when a urine profile is considered to be differential from a control population. This threshold could e.g. be based on a number of mass peak loadings observed to be significantly (p-value <0.01) above an arbitrary set fold change or by use of more sophisticated classification tools such as Orthogonal Partial Least Squares analysis (OPLS). It should be noted that apart from (pro)hormone administration other compounds/

medicines might disrupt the endogenous steroid profile as well. So occasionally a targeted chemical confirmation (focused on *a priori* known substances) following a suspect result from a more holistic screening approach might be negative. In such a case it might be questioned whether the initial screening should be classified as "false suspect" since a less obvious compound/medicine causing a biological effect can still be considered to violate the ban on hormonal activity as laid down in Directive 96/22/EC indirectly. The number of false negative screening results will depend on the time of sampling following administration, the initial dose administered, the intrinsic bioactivity of the compound and the sensitivity of the current UPLC-TOFMS profiling method. Under de current experimental settings even seven individual mass peak loadings showed a false negative screening rate lower than 5%, which is considered adequate according Commission Decision 2002/657/EC. However, these biomarkers should be tested for their robustness against a larger control population (see also the Future perspectives section). Moreover, combining individual biomarkers could increase statistical power.

Although transcriptomics based screening for (pro)hormone abuse is only in the early phase of providing the proof of principle, gene expression analysis has the potential to discriminate between DHEA treated and control animals based on gene sets. Based on a statistical derived *p*-value retrieved by statistical analysis tools like GSEA it can be determined whether a gene expression profile deviates from gene expression profiles obtained from a library with control animals. Although the number of animals we used as a test set was limited, 6 out of the 8 genesets were found significantly different from the controls using a p-value < 0.05. In such a genomics approach the number of false positives obtained plays an important role and the question is how specific the identified gene sets are for a treatment with the prohormone DHEA. Based on the current results this question is hard to answer and further research is needed.

All together, the current developed methods should be viewed as a screening tool to complement and further improve the testing programs rather than replacing current (confirmatory) tools. This, since current EU legislation still requires unambiguous evidence of drug administration which can only be achieved by classical targeted mass spectrometric based confirmation approaches. On the other hand, it should be noted that legislations in horse racing and human doping control are not ruling out indirect biomarkers. For instance legislations in human doping control state that sufficient proof of an anti-doping rule violation is established by either of the following: "presence of a prohibited substance or its metabolites or markers in the athlete's sample" [16]. While legislations in horse racing state that: "The finding of any scientific indicator of administration or other exposure to a prohibited substance is also equivalent to the finding

of a substance" [17]. In this way also biology-based approaches such as bioassays and measuring levels of genes or proteins could be used as direct indicators of hormone treatment.

Future perspectives

Bioactivation and effect based screening of prohormones

Within this thesis research we investigated the usefulness of bioactivation models making use of pure enzymes, bovine liver S9 and liver slices in combination with a yeast based androgen reporter system. This has resulted in effect based methods to screen for prohormones and steroid derivatives in animal feed, supplements and preparations, which complement the already existing bioassays for screening androgens, estrogens and corticosteroids. However, the most ideal model for bioactivation of prohormones is still the *in vivo* situation where they pass through the complex dynamic processes of absorption, distribution, metabolism and excretion (also known as ADME) directly within the bovine's body. Consequently the prohormone can be extensively metabolized leading to a target site concentration of the parent compound and/or its metabolites which can differ largely from what was expected from the administered dosage. The usage of *in vitro* liver models only highlights one aspect of bio(in)activation, and hence can only partly mimic the *in vivo* situation. Therefore, future research should be focused on investigating other organs to better describe metabolism and bio(in)activation of (pro)hormones. For instance, after oral administration, the acidic environment in the stomach can hydrolyze steroid esters or other steroid derivatives. Moreover, compared to liver, phase I and phase II metabolism in other tissues is often underestimated, this is for example shown in human intestine slices where metabolism of 17ß-testosterone by CYP2C9, CYP2C19 and 17ß-hydroxysteroiddehydrogenase is observed to be at metabolic rates comparable to those in the liver [18]. For other compounds it has been shown that even 60% of the total phase I and phase II metabolism already takes place in the intestine [19]. For this purpose, a wide variety of *in vitro* model systems based on cells or tissue slices have been developed to mimic these biotransformation pathways as well as models for the gastrointestinal tract including the stomach. The latter range from relatively simple *in vitro* digestion models simulating gastrointestinal incubations [20,21] up to complex dynamic gastrointestinal tract models [22]. Ideally, such *in vitro* models for stomach, intestine and liver should be combined to get a better overall impression of metabolism and bioactivation after (oral) administration of (pro)hormones.

Recent advances in microfluidic systems makes it possible to mimic multi-organ interactions which has been termed as a "body-on-a-chip" approach [23,24]. These approaches make it possible to perform inter-organ studies simply by integration of multiple organ models into one single microfluidic device. Together with e.g enzyme fractions or cell cultures this contributes to more realistic *in vitro* systems that can mimic the whole-body response. Using this concept, several microfluidic approaches for testing metabolism-dependent drug toxicity are developed [25] even making use of enzyme fractions such as microsomes which are integrated in a sol-gel bioreactor [26]. Drugs introduced into this microfluidics system diffuse into the layer containing the liver microsomes where subsequent metabolism takes place. In a similar way other *in vitro* bioactivation models such as liver slices [27] as well as slices of other organs such as intestine [17,28] are shown to be easily integrated in those microfluidics systems. It is envisaged that such approaches could be of great value for (pro)hormone screening as they could be used; (1) to mimic the *in vivo* bovine situation allowing to investigate metabolism of (new) (pro)hormones, which eventually may result in new metabolite biomarkers that could be used for urine screening; (2) for screening purposes especially when androgen reporter cells could integrated on those chips allowing to screen for androgenic activity, with and without bioactivation, using a "bovine-on-a-chip" approach (Figure 5).

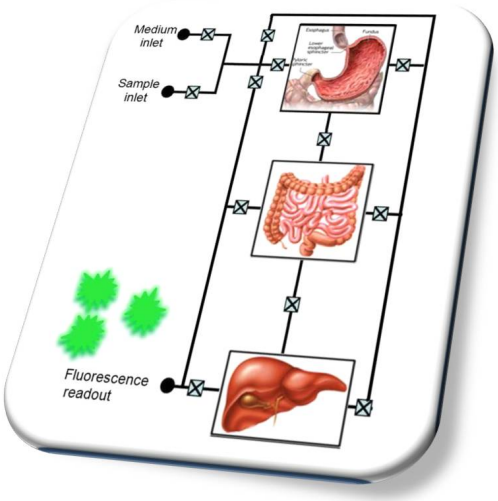

Figure 5: Bovine-on-a-chip.

Screening based on steroid urine profiles

Steroid profiling metabolomics approaches, similar to those discussed in Chapter 6, are considered nowadays with great interest by several European control laboratories as a potential holistic screening tool in hormone control programs [29]. As discussed in one of the above mentioned paragraphs, such an untargeted approach is not only a suitable tool for general anabolic screening purposes but also allows identification of potential candidate biomarkers for specific treatments. For application in practice, additional experiments are essential to assess the robustness of the candidate biomarkers. Therefore, in a follow up study, the 12 biomarkers for DHEA as determined in Chapter 6 were tested against a large control population of bovine urines (>140) sampled at 50 farms throughout The Netherlands [30]. Comparison of the TOFMS signal intensity of each of the 12 selected masses in the samples of the treated animals and the control population resulted in the selection of 5 mass signals as the most robust biomarkers for DHEA treatment (Table 1). All of these 5 biomarkers could be attributed to glucuronidated steroid metabolites. For the most robust biomarker (*m/z* 255.2108 at retention time 10.98 min.) a confidence interval of 4.2 σ is demonstrated between treated and control animals. Combination of the 5 selected biomarkers results in a separation between treated and controls with a confidence interval of 3.47 σ, corresponding to a false positive/negative rate of 0.05%. Consequently, future screening for DHEA abuse could be limited to a targeted LC-MS/MS analysis of those 5 biomarkers, instead of measuring a complete urine profile.

Table 1: Robust biomarkers for DHEA treatment of bovines (extracted and modified from ref [30]).

RT (min)	m/z value (Da)	Deviation (ppm)	Elemental composition biomarker	Fragment assigned	Tentative identity of metabolite
5.47	481.2423	-3.0	$C_{25}H_{37}0_9$	$[M+H]^+$	OH-androstene-ol-one-gluc
7.50	305.2138	-15.0	$C_{19}H_{29}0_3$	$[M\text{-gluc}+H]^+$	OH-androstene-ol-one-gluc
10.62	271.2056	-2.2	$C_{19}H_{27}0$	$[M\text{-gluc-}2H_20+H]^+$	OH-androstene-diol-gluc or OH-androstane-ol-one-gluc
10.70	255.2095	6.5	$C_{19}H_{27}$	$[M\text{-gluc-}3H_20+H]^+$	OH-androstane-diol-gluc
10.98	255.2108	11.8	$C_{19}H_{27}$	$[M\text{-gluc-}3H_20+H]^+$	OH-androstane-diol-gluc

Another prerequisite in this process is the identification of these robust biomarkers. Although TOFMS analysis can provide already a tentative identity (Table 1, column 6), the exact identity of the most robust biomarkers remains unclear due to the lack of

standards. Hereby emphasizing the need for more commercially available standards to achieve proper identification of the biomarkers. Another potential drawback which remains to be investigated may lie in the limited window of detection i.e. the time after prohormone administration during which biomarkers are still significantly different from levels in control urines.

In analogy with steroid profiling for human doping control purposes, investigations are ongoing regarding steroid profiling in cattle urine [31]. This profiling approach is based on the quantitative determination of almost all natural hormones (aglycons as well as glucuronide and sulphate conjugates) present in the steroid hormone biosynthesis and metabolism pathway. While single parameters may not be significantly different from undisrupted control urines, analyzing the whole pathway could increase statistical power allowing to discriminate urine samples from animals treated with (natural) (pro)hormones. By using statistical models such as SIMCA or Discriminant Analysis an unknown urine sample could be tested against a library of control urine profiles, and depending on the outcome classified as "compliant" or "suspect".

Furthermore, to follow-up these urine profiling screening techniques, there is a need for techniques to confirm the abuse of natural prohormones. Therefore, control laboratories should focus on implementation of GC/C/IRMS analysis methods for natural prohormones allowing to unambiguously confirm the presence of exogenous administered natural prohormones.

Transcriptomics based screening

Although application of transcriptomics based screening for hormone abuse is only in the early phase of providing the proof of principle [12,32], gene expression analysis offers the possibility for the implementation of (long lasting) effect-based toxicological endpoints. Because surveillance mainly occurs at the holdings, future gene expression analysis should be focused at samples originating from holdings rather than sampling of organs at slaughter. Within this scope, easy to collect sample matrices such as blood [33] or hair follicles [34] are attractive target tissues to sample for gene expression analysis. Although, for anabolic steroids the proof of principle is already demonstrated for these sample matrices, it remains to be investigated whether gene expression analysis in these matrices is suitable to detect effects of prohormones, as well as whether the methods are applicable in practice. In this process, it is foreseen that whole genome microarrays are used for holistic screening as well as identification of biomarkers for a certain treatment. Application of transcriptomics based biomarkers for screening however, most likely will move to low cost methods such as targeted RT-PCR or small scale multiplex

transcriptome analysis. An example of such an approach is the transcriptomics-based detection of type A trichothecenes in food [35]. For that purpose, human MCF7 breast carcinoma cells are exposed to food sample extracts followed by quantification of 28 selectively amplified target sequences by hybridization on a miniaturized microchip platform which could be scanned with a portable miniaturized USB camera. A similar array based on estrogen-dependent genes is currently employed for the detection of compounds with estrogenic activity [36]. Another low cost/high-throughput procedure is developed by Eppendorf allowing detection of multiple DNA targets (~30-50) in one tube [36]. By integrating multiplex target amplification, amplicon hybridization and real-time detection in one cartridge, this technology combines the multiplex capabilities of a microarray with the wide dynamic range of real-time RT-PCR resulting in a sample-to-result time of less than 6 hours. Although these proposed methods are still more laborious than most reporter gene assays, this is a first step towards application of transcriptomics based methods to screen food and feed for residues and contaminants at low costs and allowing high-throughput.

All together, the results of the current thesis increased our knowledge on the metabolism and bioactivation of prohormones *in vitro* as well as *in vivo*. Moreover, based on this knowledge, we revealed new effect based concepts and prohormone screening methods that complement and improve the current testing programs. Meanwhile the bioactivation concepts described have been validated and fully implemented for the screening of illicit preparations and feed supplements at RIKILT - Institute of Food Safety. These new concepts better meet the European ban on growth promoters in livestock production as described in Directive 96/22/EC. Moreover the *in vitro* concepts developed allow a reduction in animal testing since new unknown (pro)hormone compounds can be tested *in vitro* for bioactivity at the transcript, the androgenic bioactivity and the metabolite level, thereby limiting the need for *in vivo* bovine trials to verification of the *in vitro* experiments only.

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Summary

Summary

Within the European Union the surveillance on veterinary growth promoter abuse is strictly regulated by several legislations. Council Directive 96/22/EC lays down the prohibition of the use of growth promoting agents such as steroid hormones in livestock production [1]. However, there is no list of prohibited substances but 96/22/EC states that all substances having a thyrostatic, oestrogenic, androgenic or gestagenic action and beta-agonists are prohibited in livestock production. In addition Council Directive 96/23/EC encloses specific requirements to monitor anabolic steroids in farm animals and their products [2]. This Directive primarily includes sampling and investigation procedures while technical guidelines and performance criteria for methods in residue analysis are described in Commission Decision 2002/657/EC [3]. In hormone residue analysis, methodologies based on gas and liquid chromatography in combination with mass spectrometric detection (GC- and LC-MS/MS) are presently considered state-of-the-art and are extensively used for screening as well as confirmation purposes. However, most of these methods are limited to screening of a predefined list of hormone residues and can thus not fully meet the legislation 96/22/EC. In this way some kind of discrepancy arises between the bioactivity based ban, as described in 96/22/EC on the one hand and the measures to monitor on targeted (groups) of substances on the other. Therefore, to fully meet the legislation, screening methods should be based on bioactivity rather than on targeted screening of a list of compounds. Within this scope, in recent years significant efforts have been invested in development and implementation of novel effect based methods such as bioassays to detect hormone abuse in the field of livestock production [4]. During inspections at livestock farms in The Netherlands, inspection services are occasionally confronted with animals growing suspiciously fast. Besides these observations also syringes, feed supplements and herbal preparations have been found containing so-called prohormones such as dehydroepiandrosterone (DHEA) and pregnenolone. Prohormones show no or limited direct hormonal activity but might have hormonal effects upon *in vivo* bioactivation. However, knowledge about metabolism, the mode of action and excretion profiles of prohormones is often unclear, in particular in cattle. This thesis research investigated the bioactivity of prohormones upon their metabolism *in vitro* as well as *in vivo* with the goal to develop effective screening and detection methods to detect prohormone abuse in livestock production and to meet Directive 96/22/EC.

In **Chapter 2** we investigated 18 dietary supplements which were previously screened for the presence of 49 prohibited steroids by state-of-the-art LC-MS/MS analysis. After

screening by a yeast androgen bioassay two of the supplements which were found negative by LC-MS/MS analysis did show a response. Upon application of the same bioassay as an off-line LC detector in combination with UPLC-TOFMS we were able to identify 4-androstene-3ß,17ß-diol, 5α-androstane-3ß,17ß-diol and 1-testosterone in the bioactive fractions. Because these compounds were not included in the predefined list of 49 compounds they were missed in this targeted LC-MS/MS procedure. This clearly demonstrates the added value of an effect based screening approach where virtually all compounds with androgenic activity are taken into account. This together with bioassay guided identification sets a new benchmark for the analysis of supplements and preparations used in livestock production and sports.

For supplements containing solely prohormones the value of bioactivity based screening seems to be limited. When testing the endogenous prohormones DHEA and 5-androstene-3ß,17ß-diol in the yeast androgen bioassay they showed no direct androgenic activity while 4-androstenedione showed an EC_{50} of 6900 nM. To detect these inactive prohormones in animal feed, supplements and injection preparations, a modified *in vitro* bioassay test system was developed (**Chapter 3**). In order to mimic the *in vivo* metabolic activation, standards as well as sample extracts were incubated with bovine liver S9 fractions and different cofactors before application to the yeast androgen assay. Depending on the cofactor used, this resulted in an increase in androgenic activity for DHEA, 5-androstene-3ß,17ß-diol and 4-androstenedione. After incubation of DHEA with bovine liver S9, UPLC-TOFMS analysis showed that 4-androstene-3,17-dione, 7α-OH-DHEA and an initially unknown oxo-metabolite of DHEA were the most abundant metabolites formed when using $NAD(P)^+$ as a cofactor. The unknown keto-metabolite later turned out to be 7-oxo-DHEA. When using NAD(P)H as a cofactor, metabolism was mainly guided in the direction of 5-androstene-3ß,17ß-diol but again also 7α-OH-DHEA and 7-oxo-DHEA were observed to be major metabolites. In addition also minor amounts of other metabolites are observed which were, based on their accurate mass and retention time, most likely hydroxy- and oxo- metabolites of DHEA, 4-androstenedione or even 17ß-testosterone. Regarding bioactivity, 4-androstenedione and 17ß-testosterone are most likely the main compounds contributing to the bioactivity read-out, as 17α-testosterone, 5-androstene-3ß,17ß-diol and the hydroxy-metabolites of DHEA (data not shown in this thesis) and 17ß-testosterone [5] showed no or limited androgenic activity. Also metabolic activation of unknown supplement samples claiming to contain prohormones resulted in successful bioactivation and positive screening results in the androgen yeast bioassay.

From a bioactivity point of view, steroid derivatives can also be considered as prohormones that need to be deconjugated to become biologically active. **Chapter 4** describes the concepts for screening of esters and glycosides in animal feed and supplements. These methods are based on alkaline hydrolysis or enzymatic deconjugation followed by screening on bioactivity using yeast bioassays. For testosterone esters, the efficiency of alkaline hydrolysis was much better compared to enzymatic hydrolysis by esterase. As a result 1 µg testosterone ester per gram of animal feed could be readily detected by the yeast androgen bioassay. When comparing different enzyme fractions for deglycosilation the glycoside mimic genistin was shown to be deconjugated most efficiently by ß-glucuronidase/arylsulfatase from Helix pomatia.

To summarize, it can be stated that the bioactivation procedures described in Chapter 3 and 4 of this thesis complement and improve the current effect based screening procedures, resulting in a panel of methods for screening on compounds with androgenic activity as well as prohormones and steroid derivatives. In this way the bioactivity based ban described in Council Directive 96/22/EC can be better met in future.

Compared to enzyme fractions, whole cell systems such as liver slices have the advantage that phase I and phase II enzymes are present together with natural levels of cofactors. Moreover, in liver slices all liver cell types are present maintaining their original interactions and spatial arrangement thus resembling the real *in vivo* situation more closely. In **Chapter 5** the feasibility of bovine liver slices as a model for the bioactivation of prohormones was investigated. After incubation of DHEA with bovine liver slices showed that 4-androstenedione, 7α-OH-DHEA and 7-oxo-DHEA were the most abundant metabolites formed, while also minor amounts of 5-androstene-3ß,17ß-diol, 17ß-testosterone and 17α-testosterone were observed. This resulted in an increase in androgen activity as well as a decrease in androgen antagonistic activity. This confirms metabolism of DHEA into more androgen active compounds such as 4-androstenedione and 17ß-testosterone. On the other hand, because liver slices resemble the *in vivo* situation more closely also a substantial amount of phase II metabolites are formed. Compared to bovine liver S9, bioactivation of DHEA with liver slices is therefore less efficient and can be considered to be a less suitable bioactivation model for screening purposes. Moreover, since preparation of liver slices is very laborious, and the fact that for each screening experiment liver slices have to be prepared freshly this method is less suitable for use as a high through-put screening procedure.

Chapter 6 outlined a novel metabolomics based strategy for anabolic steroid urine profiling. Urine profiles of controls and DHEA or pregnenolone treated bull calves were analyzed by UPLC-TOFMS and compared by using MetAlign™ and multivariate statistics. These comparisons revealed large differences between the urinary profiles of control and DHEA or pregnenolone treated animals as well as between days of sampling and the route of administration. Data analysis showed that dozens of mass peak loadings were responsible for the significant differences (fold change >5x or >10x and a *p*-value <0.01) observed between urine profiles of controls and DHEA or pregnenolone treated animals. In order to determine if the mass peak loadings selected by multivariate statistics can be used as biomarker for DHEA or pregnenolone administration respectively, all mass signals were evaluated individually by univariate statistics. For DHEA already seven mass peak loadings showed a false negative rate below 5% hereby complying with Commission Decision 2002/657/EC [3]. Next, the mass peak loadings showing the least false negatives, respectively 12 (for DHEA) and 7 (for pregnenolone), mass peaks were selected and tested for false positives using a small but independent test set of control urines. For DHEA, 10 out of the 12 biomarkers tested showed no false positives, while for pregnenolone 5 out of the 7 potential biomarkers showed no false positives. Although these results are promising, the robustness of these potential biomarkers should be tested against a larger control population of bovine urines to obtain a more accurate estimation of the number of false positives.
For future application of this holistic methodology in practice, urine profiles could be statistically compared to a "control population" i.e. an ever growing library of urine profiles of untreated control animals. Based on the statistical deviation from this control population it then can be decided whether a sample could be classified as "compliant" or "suspect". Based on these screening results, inspection services could focus on farms showing suspicious results by intensifying the sample frequency or perform more detailed investigations in search for suspicious supplements and preparations. Another option is analyzing the urine sample with gas chromatography/ combustion/isotope ratio mass spectrometry (GC/C/IRMS) to unambiguously confirm the presence of exogenous administered (pro)hormones.

In **Chapter 7** the feasibility of a transcriptomics approach for screening on DHEA was investigated. Liver gene expression profiles of bovines treated orally or intramuscularly treated with DHEA were compared versus two control groups. Large differences in gene expression profiles were observed between treated and control animals as well as between the two separate groups of control animals. Nevertheless, significant ($p<0.05$) more than

1.5 fold differential regulation of 37 and 23 transcripts for respectively intramuscularly and orally treated animals was shown. The majority of the regulated genes are involved in immune response for both PO as well as IM treated animals. It should be noted that the small number of animals used can hamper proper statistics and substantially increases the chance of detecting false-positive genes as well as missing typical DHEA target genes. In addition, for screening purposes this raises the question how specific these genes are for DHEA treatment and consequently the percentage of false positive animals obtained when using these genes for screening purposes. Moreover, the question is whether the gene expression profiles of the DHEA treated animals in this study will remain differential when they are compared to other groups of control animals. To deal with these issues we applied the statistics of gene set enrichment analysis (GSEA). For IM and PO treated calves, gene sets were generated of genes that were significantly regulated compared to one control group and validated versus the other control group using GSEA. This cross validation showed that 6 out of the 8 gene sets were significantly enriched in DHEA treated animals when compared to an independent control group.
On the one hand this study showed that identification and application of genomic biomarkers for DHEA treatment is strongly hampered by biological variation. On the other hand, it is demonstrated that comparison of defined gene sets versus the whole expression profile allows to distinguish DHEA treated animals from controls.

In the last chapter (**Chapter 8**) the results obtained in this research are summarized and discussed and also the future perspectives of the developed concepts set forth. To summarize it can be concluded that this thesis contributes to the knowledge about metabolism and bioactivation of prohormones *in vitro* as well as *in vivo*. The new effect based concepts for prohormone screening described in this thesis complement and improve the current testing programs as well as meeting better the European bioactivity based ban on growth promoters as described in Council Directive 96/22/EC. Moreover the *in vitro* concepts developed allow a reduction in animal testing since new unknown (pro) hormone compounds can be tested *in vitro* for bioactivity at the transcript, the androgenic bioactivity and the metabolite level, thereby limiting the need for *in vivo* bovine trials to verification of the *in vitro* experiments only.

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Samenvatting

Samenvatting

Zoals vastgelegd in Richtlijn 96/22/EG [1] is in de Europese Unie het gebruik van groeibevorderaars in de veeteelt bij wet verboden. Er is echter geen lijst met verboden stoffen, maar 96/22/EG stelt dat alle stoffen met thyrostatische, estrogene, androgene of gestagene activiteit en bèta-agonisten verboden zijn. Daarnaast beschrijft Richtlijn 96/23/EG de specifieke eisen om vee en dierlijke producten te monitoren op anabole steroïden [2]. Deze wet behelst voornamelijk monstername- en onderzoeksprocedures terwijl technische richtlijnen en criteria voor residu-analyses zijn beschreven in Beschikking 2002/657/EG [3]. In hormoon residu-analyse zijn methodes over het algemeen gebaseerd op gas- of vloeistofchromatografie in combinatie met massaspectrometrische detectie (GC - en LC-MS/MS) welke worden gebruikt voor zowel het screenen als het bevestigen van hormoonmisbruik. De meeste van deze screeningsmethodes zijn echter beperkt tot een korte lijst van vooraf bekende groeibevorderaars en kunnen dus niet volledig voldoen aan de essentie in Richtlijn 96/22/EG. Op deze manier is er dus een discrepantie tussen het op bioactiviteit gebaseerde verbod beschreven in 96/22/EG aan de ene kant en de technische middelen om te monitoren op gerichte (groepen) stoffen aan de andere kant. Om volledig aan de wetgeving te voldoen zouden screeningsmethodes op bioactiviteit gebaseerd moeten zijn. De laatste jaren is daarom veel tijd geïnvesteerd in de ontwikkeling en implementatie van nieuwe effectgebaseerde methodes, zoals bioassays, om misbruik van groeibevorderaars in de veehouderij op te sporen [4].
Tijdens controles van veeteeltbedrijven in Nederland worden inspectiediensten echter soms geconfronteerd met dieren die op een onverklaarbare manier verdacht snel groeien. Naast deze observaties worden ook spuiten, supplementen en kruidenpreparaten aangetroffen die zogenaamde prohormonen, zoals dehydroepiandrosterone (DHEA) en pregnenolone, bevatten. Prohormonen hebben geen of geringe directe hormonale activiteit, maar hebben mogelijk wel hormonale effecten na *in vivo*-bioactivering. Kennis over metabolisme, werkingsmechanisme en excretieprofielen zijn echter vaak onduidelijk, met name in vee. Dit proefschrift beschrijft het onderzoek naar de bioactiviteit van prohormonen, zowel voor als na *in vitro*- en *in vivo*-metabolisme, met als doel het ontwikkelen van effectieve screenings- en detectiemethodes om prohormoon misbruik in de veeteelt aan te kunnen tonen en om te kunnen voldoen aan het op bioactiviteit gebaseerde verbod beschreven in Richtlijn 96/22/EG.

In **Hoofdstuk 2** zijn 18 voedingssupplementen, die vooraf met LC-MS/MS waren gescreend op de aanwezigheid van 49 verboden steroïdhormonen, getest op hormonale activiteit met behulp van een androgenen gist-assay. Na het screenen van deze

supplementen, gaven twee eerder negatief verklaarde supplementen een respons in de androgenen gist-assay. Gebruik makend van dezelfde gist-assay in combinatie met ultra performance vloeistofchromatografie time-of-flight massaspectrometrie (UPLC-TOFMS) was het mogelijk om 4-androstene-3ß,17ß-diol, 5α-androstane-3ß,17ß-diol en 1-testosteron in de bioactieve fracties aan te tonen. Omdat deze verbindingen niet in de lijst met 49 vooraf bekende steroïden stonden werden ze gemist door de eerder gebruikte LC-MS/MS procedure. Dit illustreert duidelijk de toegevoegde waarde van een effect-gebaseerde aanpak, waar in principe alle verbindingen met androgene activiteit kunnen worden opgepikt. Samen met bioassay-geleide identificatie is dit een nieuwe maatstaf voor de analyse van supplementen en preparaten die worden gebruikt in zowel de veeteelt als in de sport.

Voor supplementen die alleen prohormonen bevatten, lijkt het gebruik van een op bioactiviteit gebaseerde screeningsmethode van beperkte waarde. De endogene prohormonen DHEA en 5-androstene-3ß,17ß-diol vertonen geen directe activiteit in de androgenen gist-assay, terwijl 4-androstenedion een EC_{50} van 6900 nM geeft. Om deze inactieve prohormonen in diervoeder, supplementen en injectiepreparaten op te sporen, is een alternatief *in vitro*-bioassay testsysteem ontwikkeld (**Hoofdstuk 3**). Om de *in vivo*-metabole activering na te bootsen, zijn standaarden en monsterextracten geïncubeerd met runderlever S9-fracties, gevolgd door een screening in de androgenen gist-assay. Afhankelijk van de gebruikte cofactor resulteerde dit in een toename van de androgene activiteit voor DHEA, 5-androstene-3ß,17ß-diol en 4-androstenedion. Na incubatie van DHEA met runderlever S9, laat UPLC-TOFMS analyse zien dat 4-androstene-3,17-dion, 7α-OH-DHEA en een in eerste instantie nog onbekende oxo-metaboliet van DHEA de meest voorkomende metabolieten van DHEA zijn wanneer $NAD(P)^+$ als een cofactor word gebruikt. De onbekende oxo-metaboliet bleek later 7-oxo-DHEA te zijn. Wanneer NAD(P)H als een cofactor wordt gebruikt, word DHEA voornamelijk in 5-androstene-3ß,17ß-diol omgezet, maar opnieuw zijn 7α-OH-DHEA en 7-oxo-DHEA de meest voorkomende metabolieten. Daarnaast worden ook kleine hoeveelheden onbekende metabolieten waargenomen die, gebaseerd op hun accurate massa, waarschijnlijk hydroxy - en oxo-metabolieten van DHEA, 4-androstenedion of zelfs 17ß-testosteron zijn. Met betrekking tot bioactiviteit, zijn 4-androstenedion en 17ß-testosteron de verbindingen die het meeste bijdragen aan de androgene respons, terwijl 17α-testosteron, 5-androstene-3ß,17ß-diol en de hydroxy-metabolieten van DHEA en 17ß-testosteron geen of zeer geringe androgene activiteit vertonen [5]. Ook metabole activering van prohormoon bevattende supplementen uit de praktijk resulteerde met succes in bioactivering en een

positief screenings resultaat in de androgenen gist-assay.

Vanuit het oogpunt van bioactiviteit zijn steroïd-conjugaten in principe ook prohormonen. Deze verbindingen moeten eerst gedeconjugeerd worden alvorens ze biologisch actief kunnen worden. In **Hoofdstuk 4** worden methodes beschreven voor het screenen op esters en glycosiden in diervoeder en diervoedersupplementen. Deze methodes zijn gebaseerd op alkalische hydrolyse en enzymatische deconjugatie, gevolgd door screening op bioactiviteit met behulp van gist-bioassays. Voor testosteron-esters blijkt alkalische hydrolyse efficiënter te werken dan enzymatische hydrolyse met behulp van esterase. Na alkalische hydrolyse resulteerde een spike van 1 µg testosteron-ester per gram diervoeder al in een positief signaal in de androgenen gist-bioassay. Voor deglycosylering blijkt β-glucuronidase/arylsulfatase van Helix pomatia het meest efficiënt te werken, resulterend in een significante toename van de hormonale activiteit.

Samenvattend kan worden vastgesteld dat de bioactiveringsprocedures beschreven in Hoofdstuk 3 en 4 van dit proefschrift de huidige op effect gebaseerde screenings-procedures aanvullen en verbeteren, wat resulteert in een panel van methodes dat geschikt is voor screening op zowel verbindingen met directe androgene activiteit als van prohormonen en steroïd-conjugaten. Hierdoor kan in de toekomst beter worden voldaan aan het op bioactiviteit gebaseerde verbod zoals beschreven in Richtlijn 96/22/EG.

Vergeleken met lever enzymfracties hebben modellen gebaseerd op cellen, zoals leverslices, het voordeel dat alle fase I- en fase II-enzymen aanwezig zijn samen met de natuurlijke hoeveelheden aan cofactoren. Bovendien zijn in slices alle leverceltypen aanwezig met behoud van natuurlijke interacties en ruimtelijke ordening, wat in theorie een betere benadering van de *in vivo*-situatie geeft. In **Hoofdstuk 5** is de bruikbaarheid van runderleverslices voor de bioactivering van het prohormoon DHEA beschreven. Na incubatie van DHEA met runderleverslices, worden voornamelijk de metabolieten 4-androstenedion, 7α-OH-DHEA en 7-oxo-DHEA gevormd, maar ook kleine hoeveelheden van 5-androstene-3ß,17ß-diol, 17ß-testosteron en 17α-testosteron. Dit resulteerde zowel in een toename van androgene activiteit als in een afname van anti-androgene activiteit en bevestigt het metabolisme van DHEA in meer androgene verbindingen zoals 4-androstenedion en 17ß-testosteron. Daar leverslices meer op de *in vivo*-situatie lijken, wordt er waarschijnlijk ook een substantieel deel aan fase II-metabolieten gevormd. Mede door dit fase II-metabolisme is incubatie van DHEA met runderleverslices vergeleken met runderlever-S9 een minder efficiënt bioactiverings model voor screeningsdoeleinden.

Bovendien is het maken van deze leverslices zeer arbeidsintensief en ook daarom minder geschikt voor routinematige screeningsprocedures.

In **Hoofdstuk 6** is een nieuwe, op metabolomics gebaseerde, strategie voor urine-steroïdprofilering beschreven. Urineprofielen van controle en DHEA of pregenenolone behandelde runderen zijn gemeten met UPLC-TOFMS en vervolgens vergeleken met behulp van MetAlign^{TM}-sofware en multivariate statistiek. Deze vergelijkingen laten grote verschillen zien tussen urineprofielen van controle en DHEA of pregnenolone behandelde dieren evenals tussen de dagen van monstername en de manier van toediening. Na statistische analyse blijkt dat tientallen massa's verantwoordelijk zijn voor deze significante verschillen (>5 of >10x gereguleerd, *p*-waarde <0.01) tussen urineprofielen van controle en DHEA dan wel pregnenolone behandelde dieren. Om te bepalen of deze massa's geschikt zijn als biomarkers voor DHEA of pregnenolone misbruik zijn al deze massa signalen ook individueel geëvalueerd met univariate statistiek. Voor DHEA vertoonde al zeven massa's een percentage vals-negatieven lager dan 5% hetgeen voldoet aan de screeningsmethode eisen van Beschikking 2002/657/EG [3]. Vervolgens zijn op basis van het aantal vals negatieven, respectievelijk 12 (voor DHEA) en 7 (voor pregnenolone) massa's geselecteerd die zijn getest op het aantal vals-positieven versus een kleine maar onafhankelijke set van controle-urines. Voor DHEA vertoonden 10 van de 12 biomarkers geen vals-positieven, terwijl voor pregnenolone 5 van de 7 potentiële biomarkers geen vals-positieve resultaten opleverden. Hoewel deze resultaten veel-belovend zijn, moet de robuustheid van deze potentiële biomarkers versus een grotere controlepopulatie urines worden getest om een betere schatting van het aantal vals-positieven te kunnen maken.
Bij een toekomstige toepassing van deze holistische methode in de praktijk zouden urineprofielen statistisch vergeleken kunnen worden met een "normaal-populatie", dat wil zeggen een groeiend bestand van urineprofielen van onbehandelde controledieren. Het analyseresultaat is dan een statistische waarschijnlijkheid dat het urinemonster afwijkt van een normaal profiel plus een indicatie van de biomarkers die daarvoor verantwoordelijk zijn. Op basis hiervan kan een opsporingsdienst gericht verder zoeken naar preparaten, voer, en andere monsters, dan wel besluiten tot een intensievere gerichte controle (opsporingsfocus). Een andere optie is analyse van urine met behulp van gas chromatography/combustie/isotoop ratio massaspectrometrie (GC/C/IRMS) om het gebruik van exogene toediening van natuurlijke (pro)hormonen onomstotelijk vast te kunnen stellen.

In **Hoofdstuk** 7 is de bruikbaarheid van genexpressieprofilering als een screeningsmethode voor DHEA-misbruik onderzocht. Lever genexpressieprofielen van runderen die intramusculair of oraal waren behandeld met DHEA zijn vergeleken met genexpressieprofielen van (twee groepen) controledieren. Dit resulteerde in grote verschillen in genexpressieprofielen, zowel tussen de controle en de behandelde dieren als tussen de twee controlegroepen onderling. Desalniettemin resulteerde dit in 37 en 23 significant gereguleerde genen (>1.5x gereguleerd, *p*-waarde <0.05) specifiek voor respectievelijk de intramusculair en oraal behandelde dieren. De meeste van deze gereguleerde genen hebben betrekking op immuunrespons en de vraag is dan ook hoe typerend deze genen voor DHEA-behandeling zijn. Ook moet worden opgemerkt dat om ethische redenen slechts een klein aantal dieren in de proefopzet is meegenomen. Dit beïnvloedt de statistiek en hierdoor is de kans op identificatie van vals-positieve genen groter evenals de kans op het missen van DHEA-specifieke genen.
Daarnaast is het de vraag of de gevonden genexpressieprofielen significant verschillend blijven wanneer ze worden vergeleken met andere groepen van controledieren. Daarom is gebruik gemaakt van de statistiek van gene set enrichment analysis (GSEA) waar naar verschillen in genexpressie wordt gekeken op basis van zogenaamde genensets. Voor zowel de intramusculair als oraal behandelde dieren zijn genensets gecreëerd versus de ene controlegroep die vervolgens zijn vergeleken met genexpressieprofielen van de andere controlegroep. Deze validatie toonde aan dat 6 van de 8 genensets statistisch significant waren verrijkt in DHEA-behandelde dieren wanneer deze worden vergeleken met een "onafhankelijke" controlegroep. Aan de ene kant laat dit onderzoek zien dat identificatie en toepassing van genbiomarkers sterk gehinderd wordt door biologische variatie, aan de andere kant is het wel degelijk mogelijk om DHEA behandelde dieren te onderscheiden van controledieren op basis van hun genexpressieprofielen.

In het laatste hoofdstuk (**Hoofdstuk 8)** worden de resultaten van het in dit proefschrift beschreven onderzoek samengevat en bediscussieerd en worden de toekomstperspectieven van de ontwikkelde concepten uiteengezet. Samenvattend kan worden geconcludeerd dat dit proefschrift bijdraagt aan de kennis van metabolisme en bioactivering van prohormonen zowel *in vitro* als *in vivo*. Bovendien kunnen de nieuwe op effect gebaseerde concepten en prohormoon screeningsmethodes beschreven in dit proefschrift de huidige testmethodes aanvullen en verbeteren en kunnen zij tevens beter voldoen aan het op bioactiviteit gebaseerde verbod beschreven in Richtlijn 96/22/EG. Daarnaast kunnen de ontwikkelde concepten bijdragen aan het terugdringen van het proefdiergebruik, daar nieuwe (pro)hormonen eerst *in vitro* kunnen worden geëvalueerd

op bioactiviteit, metabolisme en genexpressie. De noodzaak voor *in vivo*-proeven blijft hierdoor beperkt tot verificatie van de resultaten van de *in vitro*-experimenten.

Referenties

[1] EEC Directive 96/22, 1996. Official J. Eur. Commun. No. L125, 3-9.

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Dankwoord

Dankwoord

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Jeroen

About the author

Curriculum Vitae

Jeroen (Cornelis Willem) Rijk was born on July 15th 1978 in Goes, The Netherlands. In 1997 he finished his higher secondary education at the St. Willibrord College in Goes. In the same year he started the bachelor study Food Technology at the HAS in Den Bosch, with the specialization Product Development. After receiving his BSc degree in 2001 he continued with a master study Food Technology at Wageningen University. Here he followed the specialization Integrated Food Science and conducted his thesis research at the Toxicology department of Wageningen University where he studied the anticarcinogenic properties of isothiocyanates in *Brassica* vegetables. He conducted his second thesis research at Campina Innovations in Wageningen where he implemented vitamin B12 producing bacteria in yoghurt. Afterwards, he continued working for half a year at the packaging development department of Campina.
From February 2005 till February 2009 he was appointed at the Toxicology and Effect Analysis department of RIKILT - Institute of Food Safety where he conducted the PhD project described in this thesis. In May 2009 he was appointed as a postdoctoral researcher at RIKILT where he, until present, works on modulation of steroidogenesis, a project carried out within the framework of the Netherlands Toxicogenomics Centre (NTC).

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Rijk, J.C.W., Bovee, T.F.H., Groot, M.J., Peijnenburg, A.A.C.M., Nielen, M.W.F., 2008. Evidence of the indirect hormonal activity of prohormones using liver S9 metabolic bioactivation and an androgen bioassay. Anal. Bioanal. Chem. 392, 417-425.

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Overview of completed training activities

Conferences and meetings

NTC work in progress, Wageningen, 2005.
5th International symposium on hormone and veterinary drugs residue analysis, Antwerpen, Belgium, 2006.
Annual meeting of NOW CW Studiegroep Analytische Scheikunde, Lunteren, 2006.
NTC work in progress, Wageningen, 2007.
Annual meeting of NOW CW Studiegroep Analytische Scheikunde, Lunteren, 2007.
3rd International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, 2007.
CRL Workshop, Bilthoven, 2007.
NVMS annual meeting, Haarlem, 2007.
Euroresidue Conference on residues of veterinary drugs in food. Egmond aan Zee, 2008.

Courses

Toxicogenomics, Postgraduate education in toxicology, Maastricht, 2006.
Introduction in LC-MS, Hyphen MassSpec, Wageningen, 2006.
Molecular Toxicology, Postgraduate education in toxicology, Amsterdam, 2007.
Biomolecular Mass Spectrometry, Universiteit Utrecht, Utrecht, 2007.
Cell Toxicology, Postgraduate education in toxicology, Leiden, 2008.

Scientific writing, CENTA, Wageningen, 2007.
Project and Time management, Wageningen Graduate Schools, Wageningen, 2010.

Additional activities

RIKILT Seminars, Wageningen, 2005-2009.
Cluster and Prohormone meetings, Wageningen, 2005-2009.
Colloqium Technische Universiteit Dresden, Dresden, Germany, 2008.
Organic Chemistry Colloquia, Wageningen, 2008-2009.
MLW excursions, Wageningen, 2009 and 2010.

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