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A review of analytical strategies for the detection of 'endogenous' steroid abuse in food production

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Detection of the abuse of synthetic steroids in food production is nowadays relatively straightforward using modern techniques such as gas or liquid chromatography coupled to mass spectrometry (GC-MS/MS or LC-MS/MS, respectively). However, proving the abuse of 'endogenous' (or naturally occurring) steroids is more difficult. Despite these difficulties, significant progress in this area has recently been made and a number of methods are now available.

The aim of the current review was to systematically review the available analytical approaches, which include threshold concentrations, qualitative 'marker' metabolites, intact steroid esters, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), longitudinal testing and 'omics' biomarker profiling. The advantages/disadvantages of these methods are considered in detail, but the choice of which to adopt is dictated by a number of practical, political, and economic factors, which vary in different parts of the world. These include the steroid/species combination requiring analysis, the matrix tested, whether samples are collected from live or slaughtered animals, available analytical instrumentation, sample throughput/cost, and the relevant legal/regulatory frameworks. Furthermore, these approaches could be combined in a range of different parallel and/or sequential screening/confirmatory testing streams, with the final choice being determined by the aforementioned considerations.

Despite these advances, more work is required to refine the different techniques and to respond to the ever increasing list of compounds classified as 'endogenous'. At this advanced stage, however, it is now more important than ever for scientists and regulators from across the world to communicate and collaborate in order to harmonize and streamline research efforts.

Keywords: steroids; endogenous; natural; analysis; food production

Introduction

The use and abuse of steroids in food production

Because of their potential anabolic effects, and for economic reasons, some steroids have been used to boost the mass and quality of animal carcasses in food production.[1–3] Although there are a number of steroid preparations authorized for this purpose in countries such as the USA, the use of growth promoters (also including non-steroidal products such as the oestrogenic compound zeranol, growth hormone (somatotropin), thyreostats and β2-agonists) are banned within the EU.[4] The reasons for this ban were highlighted in two reports from the European Commission in 1999 and 2002, which concluded that the presence of hormones in meat products may potentially be harmful to human health through endocrine disrupting or carcinogenic mechanisms.[5,6] However, several opinions have disagreed with the conclusions of the European Commission; for example, two subsequent reports published by the UK Veterinary Products Committee.[7,8]

Detection of steroid abuse in food production

In order to enforce the ban on hormone use in food production, EU Council Directive 96/23/EC[9] and EU Commission Decision 2002/657/EC[10] lay down the requirements for residue testing.

Figure 1 and the following paragraphs briefly summarize the evolution of the most common analytical techniques used in the direct detection of steroids in biological matrices. More detailed review articles concerning the analysis of steroids in general have also been published.[11–16]

Analytical detection and diagnosis of steroid abuse in animals intended for food production follows three conceptual stages: screening analysis, confirmatory analysis, and diagnostic interpretation. Screening analysis is designed to rule out samples where there is no evidence of steroid abuse. Confirmatory analysis is designed to unequivocally identify the presence of a steroid/metabolite, by applying the identification criteria of Commission Decision 2002/657/EC[10] (although an update of these criteria in

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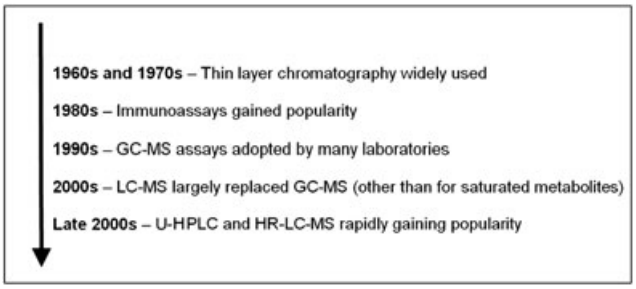

Figure 1. Summary of the evolution of steroid screening techniques used in residue analysis.

response to the recent developments in analytical technologies has recently been proposed.[17] Confirmatory analysis according to 2002/657/EC[10] does not necessarily indicate the source of the identified compound. Diagnostic interpretation looks to identify the source of the identified steroid/metabolite, i.e. the result of abuse rather than of natural occurrence. *However, to simplify the discussions in the remainder of this review, the term 'confirmation' will be used to refer to an analytically confirmed finding that is diagnostically interpreted to be the result of abuse.*

Initially, thin layer chromatography-fluorescence detection (TLC/FL) was widely used.[18,19] Immunoassay techniques such as enzyme-linked immunosorbent assays (ELISA) became popular in the 1980s and 1990s, but were largely replaced in the late 1990s and early 2000s by more definitive mass spectrometric-based techniques such as gas- and liquid-chromatography mass spectrometry (GC- and LC-MS, respectively).[15,20] Also, the recent emergence of higher resolution LC equipment allowing the use of sub-2 μm particle sizes and high flow rates (ultra high pressure liquid chromatography or UHPLC) allow for metabolites with similar molecular masses and retention times to be resolved more easily, which results in shorter analytical run times.[21]

Although there has been a general shift from GC-MS to LC-MS for drug residue analysis during the past decade, GC-MS has remained an important tool for analyzing saturated steroid metabolites.[22] This is because saturated steroids generally suffer from poor ionization properties under the atmospheric pressure ionisation conditions of LC-MS.[15,23] Although the majority of current urinary screening procedures are based on detection of the 'free' steroid fraction (liberated from its phase conjugates), it may be possible in the future to design assays based on the analysis of intact conjugates.[24,25]

Most recently, robust high-resolution-accurate-mass LC-MS (LC-HRMS) systems displaying excellent mass stability operating at an increased resolution, typically ranging between 7500 and 100 000 full width at half maximum height (FWHM) depending on the type of mass analyzer employed, have become commercially available and have started gaining popularity for sports drug surveillance screening and research[26,27] and food residue analysis.[14,28] Because the data acquired are full-scan analyses of intact $[M+H]^+$ or $[M-H]^-$ moieties at very high resolution, a very large number of analytes can be simultaneously monitored; in real time or retrospectively.

In addition to the classical analytical chemistry techniques that are targeted towards the detection of 'parent' steroids or their metabolites, a number of indirect techniques have recently gained attention. These include immunoassay and receptor based biosensor assays as well as a range of 'omics' biomarker approaches such as metabolomics, proteomics, and transcriptomics.[29–33] Because these methods are targeted towards pharmacological activity rather than individual drug structure, they produce complementary screening data that can be used to indicate whether steroid abuse may have occurred. However, these techniques have yet to find widespread application in the confirmation of steroid abuse, which is typically still achieved by the direct measurement of a steroid or its metabolite.[13]

While the above discussion served to summarize the range of instrumental techniques that may be used to detect steroid abuse, the ability to detect the abuse of each individual steroid is determined by a number of factors. First, the sample needs to be taken from the animal at a time close enough to the point of steroid administration for the concentrations to be above the limits of instrumental sensitivity.

Secondly, the type of matrix used for steroid residue analysis in food and sports drug surveillance differs by a number of variables including the country, the individual authority concerned, whether samples are taken from live animals, at slaughter or from a food-import programme and whether the analyses for a particular analyte are suited to a specific tissue. Other than food import programmes, where analysis of meat (and sometimes organs) are typically required, urine and blood are the most common matrices for testing in both the food and sports residue arenas.[11,34] However, faeces and hair are also important matrices in some countries.[11] When dealing with blood or hair, detection of unchanged 'parent' drug is often considered suitable for determination of drug abuse. However, when dealing with urine or faeces, a large proportion of the excreted dose can take the form of metabolites. This is a particularly important consideration in the case of steroids, which are typically heavily metabolized.[35] It is, therefore, often necessary to conduct metabolism studies in order to determine the appropriate target metabolites for the detection of steroid abuse.

A third consideration is whether the analytical approach is suitable for screening or for confirmation of abuse. Screening approaches are designed to be applicable to the analysis of a large number of samples to rule out those samples lacking evidence of abuse. Screening methods are preferably rapid, cost effective and aim to provide minimal false compliant and a reasonable proportion of false non-compliant results which the laboratory can tolerate on an operating basis. On the other hand, confirmatory approaches (see earlier discussion regarding definition of this term) are designed to provide unequivocal proof of the abuse of a substance and are therefore much more heavily weighted towards avoiding false non-compliant results in order that the data generated is capable of withstanding legal scrutiny and challenge.

Lastly, individual steroids can be broadly classified as either exogenous or endogenous, which impacts on their detection as follows:

Exogenous steroids are known steroids, such as trenbolone. These contain synthetic chemical structures that are thought not to occur naturally. Detection of this class of steroids is relatively straightforward since a purely qualitative demonstration of the presence of these synthetic steroids is all that is required in order to determine abuse (at least in the EU where no maximum residue levels are specified).

Endogenous steroids are also known steroids, marketed or not, such as testosterone, but contain structures that are known to exist naturally.[35] Detection of the abuse of endogenous steroids is more complicated because they are, by definition, natural to some extent and so a simple qualitative demonstration of their presence is insufficient to indicate abuse (at least in relation to

the administered parent drug – see later section on 'marker metabolites'). Furthermore, some of these steroids are also known to be formed as storage or process artefacts following sample collection, which further complicates interpretation of analytical findings.[36,37]

Presence and metabolism of endogenous steroids in food producing animals

Some endogenous steroids such as testosterone, progesterone, and oestradiol are known to be ubiquitous amongst mammals. However, the classification of a steroid as endogenous is a grey area and there are some steroids that may be considered semi-endogenous. This term signifies that the steroid in question has been suspected of being endogenous, but only in certain situations, i.e. in a specific species or at a particular time in the life cycle. Analytical sensitivities for detecting steroids have increased significantly over the years, which have resulted in more and more compounds being suspected as endogenous or semi-endogenous at low concentrations.

Figure 2 summarizes the range of anabolic-androgenic, oestrogenic, progestagenic, and corticosteroids available in proprietary veterinary preparations for which related analytes (whether 'parent' drug or one or more metabolites) have been discovered to be endogenous in one or more mammalian food-producing species. The list of every steroid/species combination that has been reported is beyond the scope of the current review, but the subject has previously been comprehensively reviewed elsewhere.[35]

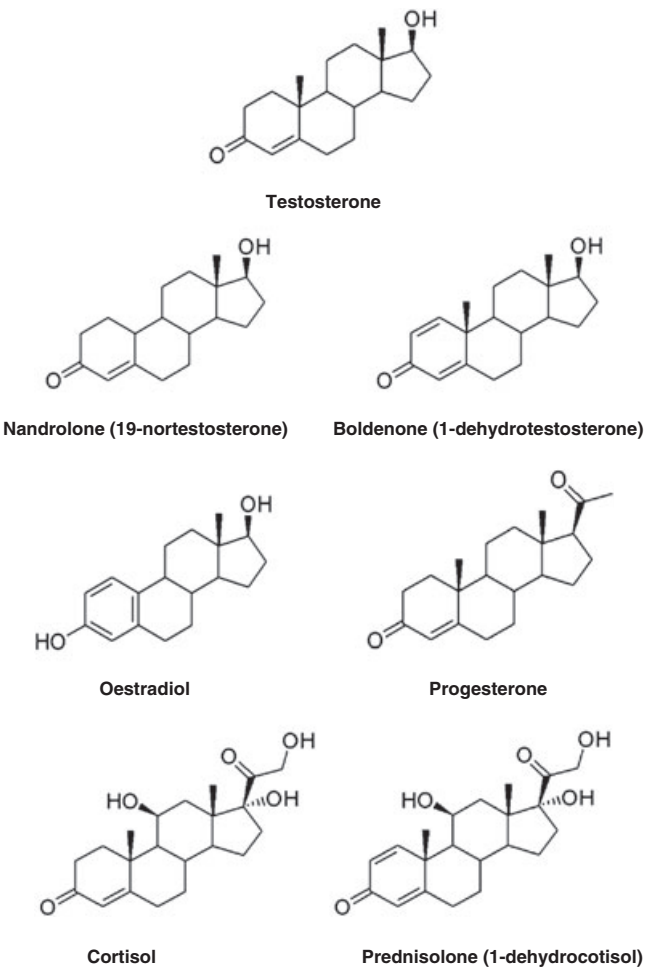

Figure 2. Structures of the steroids for which related endogenous analytes have been discovered in food-producing animals. Reviewed in detail in Scarth *et al.*[35]

Approaches for detecting the abuse of endogenous steroids

Whilst many articles on endogenous steroid metabolism/detection have been published over the years (see individual references given throughout this review), a systematic review of all the available approaches is lacking in the literature. The aim of this paper was therefore to review all of the available analytical approaches and to highlight any important areas of consideration when devising routine testing assays or planning future research.

Although there is still no single technique that is capable of detecting the abuse of all endogenous steroids in all species, significant progress in this area has recently been made and a number of techniques are now available. These approaches, which are reviewed in turn, include: qualitative 'marker' metabolites, threshold concentrations, intact steroid esters, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), longitudinal testing and the developing field of 'omics' biomarker profiling.

The following sections will concentrate on the use of plasma/serum, urine, bile, hair and histological observations for the detection of endogenous steroid abuse. As mentioned earlier, solid tissues such as muscle, liver, fat and kidney are also tested post-mortem in some countries; especially those that operate meat export programmes. However, with the possible exception of the detection of intact steroid esters in an injection site or the potential use of GC-C-IRMS, there is no definitive approach that has been shown to be able to discern endogenous from exogenous in these tissues. Furthermore, the aim of this review was not to list each and every study that has attempted to develop a test for a particular steroid/species combination. Rather, the aim was to highlight the range of available analytical techniques, their potential advantages/disadvantages and some possible future trends in the field.

Marker metabolites

The concept of the marker metabolite method is to screen for a compound that is uniquely detected following the administration of a steroid, but which is not found in untreated animals. For such compounds, a simple qualitative confirmatory analysis is then sufficient to demonstrate abuse; thus making this a very attractive option. For example, the use of boldenone sulphate as a qualitative marker metabolite to demonstrate the abuse of boldenone in cattle has been proposed.[38,39] Furthermore, it has been reported that the isomer 5α-estrane-3β,17α-diol can occur naturally in the urine of pregnant and injured cattle, but that the 17β-isomers 5β-estrane-3α,17β-diol and 5α-estrane-3β,17β-diol are only found following nandrolone administration.[40] The 17β-isomers may therefore be appropriate marker metabolites of nandrolone abuse in cattle.

Because the marker metabolite approach is qualitative in nature, care must be taken to ensure that if analytical limits of detection decrease in the future, that the analyte is not discovered to be endogenous at lower concentrations. For example, 19-noretiocholanolone was at one time considered to be a

qualitative marker metabolite for detecting the abuse of nandrolone in the porcine,[41] but was subsequently discovered to be endogenous when more sensitive methods were applied.[42] Therefore, it may be wise to restrict the reporting of marker metabolite results to those that are determined to be at least above the limit of detection (LOD) that was applied in the initial validation study or, depending upon the balance of risk, to apply a safety factor to the LOD below which a compliant result will be reported.

Threshold concentrations

The idea of using a threshold comprises the determination of a concentration above which it is considered statistically unlikely that a result could be produced 'naturally' without producing a high rate of false compliance. Of course, it is important that the concentrations derived from population studies are relevant to the concentrations observed following administration. If they are too high then they may be above those produced following steroid administration and will be useless.

The principle of adopting thresholds to control the abuse of endogenous compounds has long been an accepted approach in both food production[43] and animal sports.[44] In animal sports testing, if used to formally confirm the abuse of an endogenous substance, the standard approach is to set the threshold at a statistical probability of finding a false non-compliance at a rate of 1 in 10 000 in a natural population[44] since this is considered to offer a sufficiently large safety margin to prevent the occurrence of false non-compliances. For food production, an alternative level of probability might be chosen taking into account the risk to the consumer of false non-compliance results. Thresholds for screening may be set at a lower probability, but there then needs to be a secondary mechanism for demonstration of abuse if the confirmatory threshold is not also breeched. In an ideal world, a confirmatory threshold would also be suitable as a screening threshold, but this requires that the threshold is able to produce both low rates of false compliance and non-compliance; an ideal that is seldom achieved.

As an example of this approach, the use of urinary 19-noretiocholanolone concentration data for controlling the abuse of nandrolone in the porcine has recently been reported.[42] Statistical analysis of the population data was carried out in order to suggest screening and confirmatory thresholds for this steroid in the urine of boars and gilts. Because of the non-normal distribution of the data, it was necessary to use a non-parametric method of statistical analysis and the Chebyshev inequality was considered the most suitable as it makes minimal assumptions about the population distribution and produces conservative thresholds relative to methods based on normally distributed data. At a false non-compliance rate of 1 in 10 000 of the normal population, the suggested confirmatory thresholds are 7502 pg/ml for boars and 19 200 pg/ml in gilts. To put these thresholds into context, in a recent study administering 2 mg/kg nandrolone laurate via intra-muscular injection to six boars aged 8–10 weeks, the mean free fraction 19-noretiocholanolone concentration at the last time-point of the study (15 days following administration) was 28,400 pg/ml, with a range of 9600 to 53 600 pg/ml.[41] The suggested thresholds should therefore be able to detect the abuse of nandrolone for several weeks in the majority of treated animals and hence lead to rates of both low false compliance and non-compliance. The validation of these thresholds is significant as there are currently no other published methods available for the detection of nandrolone abuse in the porcine.

In addition to thresholds based on steroid concentration results from a single animal, it is also possible to produce thresholds based on more than one animal within a herd having a result above a certain steroid concentration, i.e. from '2 out of 2' or '3 out of 3' animals tested ('y out of n' animals). The rationale for this approach is that if a steroid is being abused by a farmer, it is likely to be the majority of the herd that is given the drug, not just a single animal. Since threshold concentrations based on a 'y out of n' approach are significantly lower than thresholds based on a single animal, the 'y out of n' approach could lead to a lower false compliance rate for detecting steroid abuse. Whilst this approach may be sufficiently robust for a screening approach in all cases, using it for confirming steroid abuse requires that steroid concentrations are not subject to 'clustering' within herds. Whilst the authors are not aware of any evidence of steroid clustering within steer populations, a biological phenomenon known as the 'Whitten effect'[45] has been reported in female mammals. This phenomenon leads to the synchronization of oestrous in female animals and if it is accepted that some of the high steroid concentration results within a population are caused by oestrous, then this would class as clustering and could lead to false non-compliance results using the 'y out of n' approach.

The first use of the 'y out of n' approach involved the proposal of urinary concentration thresholds for detecting the abuse of nandrolone, boldenone, testosterone and oestradiol abuse in cattle[46,47] For each analyte, the use of the '2 out of 2' and '3 out of 3' animal approaches lead to concentration thresholds approximately 10- and 20-fold lower, respectively, compared with the traditional '1 out of 1' (single animal) approach. However, although the larger 'y out of n' approach allows for a reduction in the concentration threshold, the risk of false compliances increases with n due to potential inter-individual variation in excretion profiles. Therefore, it was suggested that only the '2 out of 2 'approach be used to supplement the traditional '1 out of 1' methods as this is predicted to offer the best balance of a low enough concentration threshold without risking false compliances due to inter-individual variation.

If concentration thresholds are based on urine, then an additional consideration that may lead to more effective thresholds is correction of the data for the specific gravity (SG) of the sample. The SG is a measure of the solute concentration of the sample and is therefore an indicator of the hydration status of the animal. For example, when applied to data from UK ovine populations, correction of urinary steroid concentration data for SG led to a reduction in the relative standard deviation of the steroid population data and hence a reduction in the calculated thresholds.[48] An alternative to correcting steroid concentrations for SG would be to use the creatinine concentration. However, results from bovine[47] and ovine[48] population studies in the UK failed to demonstrate a reduction in the relative standard deviation of the data following correction for creatinine concentration.

One disadvantages of the threshold concentration approach is due to the uncertainty surrounding whether population data that are used to set a threshold in one country can be applied to populations in another geographical region due to potential differences in animal breeds, farming practices and environmental conditions. Therefore, it may be sensible to at least conduct a 'partial' population validation study before thresholds are transferred between distinct geographical regions.

Intact steroid esters

Most injectable steroid preparations contain steroids in esterified forms; none of which are known to occur naturally. The direct detection of steroid esters in matrices from an animal may therefore be indicative of steroid abuse. The detection of intact steroid esters in hair has attracted the most attention in literature for this purpose and has already found use in some European labs as a confirmatory technique for proving natural steroid abuse.[49–55] When dealing with hair, however, contamination issues need to be carefully considered in order to eliminate environmental contamination as a possible cause of false non-compliant results.

Until recently, the detection of intact steroid esters in plasma was hampered by their typically low concentrations in this matrix relative to analytical limits of detection.[56,57] However, screening and confirmatory approaches based on the detection of intact testosterone, nandrolone, and boldenone esters below 0.01 ng ml^{-1} in equine plasma have recently been developed.[58] When a testosterone ester is administered as a depot injection, these sensitive assays allow detection of testosterone abuse in female and castrated male equine animals for several weeks longer than the existing (2010) international urinary testosterone concentration thresholds.[59] They also allow the detection of testosterone abuse in intact males for the first time. However, these assays are not suitable for detecting the abuse of steroids that are administered in a non-esterified form and it is also likely that the detection time for the administration of an oral preparation of steroid esters would be reduced compared to that of a depot injection due to the lack of sustained release. Also, the adopting of steroid ester screening assays may require an additional (or at least modified) extraction scheme since steroid esters are typically very non-polar in nature (although the precise log P depends on the nature of the steroid and esterified group). Despite these potential limitations, the detection of steroid esters shows great promise for detecting the abuse of endogenous steroids (at least when they are administered as esters), more studies in other species and matrices are clearly warranted.

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)

Synthetic steroids are most commonly synthesized from organic material derived from C3 plants (often soy), while the diet of food-producing animals is usually a mixture of that from both C3 and C4 plants.[60] The terms C3 and C4 refer to the metabolic pathway used by the plant in synthesizing organic compounds during photosynthesis, utilizing either 3 or 4 carbon-chain metabolites respectively. The significance of this lies in the fact that the two types of pathway display differing degrees of discrimination against ^{13}C and thus result in different ^{13}C to ^{12}C ratios. C4 plants have lower discrimination against ^{13}C than C3 plants, resulting in higher ^{13}C to ^{12}C ratios in C4 plant material.[60] Since steroids produced within the body will derive carbon from both C3 and C4 plant material of dietary sources, the resulting ^{13}C to ^{12}C ratio will be lower after exogenous steroid administration (mainly C3 plant material derived) relative to the endogenous state. GC-C-IRMS can, therefore, be used to detect such differences in the ^{12}C and ^{13}C ratio resulting from the administration of endogenous steroids.

The ^{13}C to ^{12}C ratio determined by GC-C-IRMS is usually expressed as a $\delta^{13}C$ value. The range of $\delta^{13}C$ values for C3 plants (hence also abused steroids) is around −25 to −35‰, whereas for C4 plants is around −11.9 to −15.2‰.[61] For diets usually made up of both C3 and C4 plants, the range of $\delta^{13}C$ values for endogenous steroids is usually somewhere between these two ranges. However, the precise value will depend on the type of diet fed to the animal.[62] The $\delta^{13}C$ values of the steroid or metabolite being measured is usually referenced to an endogenous reference compound (ERC) such as DHEA that is not affected by exogenous steroid administration.[63] This is to ensure that the endogenous make up of the animal has not been affected in some way (i.e. diet) so as to give low $\delta^{13}C$ values for all steroids. A high relative difference between the $\delta^{13}C$ values of the steroid or metabolite and the ERC can therefore be used as an indicator of abuse. As indicated above, diet has a significant effect on the $\delta^{13}C$ value of the endogenous steroid profile.[64] In the UK for example, animals are typically fed a much higher base of C3 plants than in the remainder of Europe, leading to a lower difference in the $\delta^{13}C$ values between administered steroids and the ERC.[61] However, studies by other authors[62,65] have shown that the difference after steroid administration to animals consuming a predominantly C3 diet is still usually sufficient to discern testosterone abuse. It has been shown that after testosterone administration, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA for C4 plant-fed animals (maize) was typically around 10‰, whereas the difference for C3-fed animals (grasses) was typically around 4‰.[65] In the endogenous state, the difference in $\delta^{13}C$ between the testosterone metabolite etiocholanolone and the ERC DHEA was a mean of 0.9‰with a standard deviation of 0.7‰. Applying a confidence interval of three standard deviations to either side of the mean, endogenous $\delta^{13}C$ difference between etiocholanolone and the ERC DHEA allowed a 100% discrimination of samples as either positive or negative.

The GC-C-IRMS method itself requires a significant amount of sample preparation prior to analysis including hydrolysis, solid-phase extraction (SPE), liquid-liquid extraction (LLE) and HPLC fractionation steps, as the influence of matrix interferences need to be minimized.[61] Derivatization of the extracts followed by separation using GC further purifies the extract before introduction into a furnace. The furnace then combusts the introduced sample, which is then analyzed alongside a reference gas by MS in order to determine the relative levels of ^{13}C and ^{12}C.[66] The absolute sensitivity of the method depends to some extent on the analyte and matrix in question, but 2–10 ng ml^{-1} is typically required, meaning that only some analyte matrix combinations are currently suitable.[67]

The EU ISOSTER GRD1-2001-40085 project[68] successfully validated the use of this technique for detecting testosterone abuse in urine via monitoring of etiocholanolone values in relation to DHEA as the ERC. The method was also applied to steroids in other tissues, but in most cases the steroid or ERC contents were too low to be of use in the technique. In addition to testosterone, it has been shown that detection of oestradiol abuse in the bovine is possible via monitoring the $\delta^{13}C$ value of the urinary oestradiol metabolite epioestradiol (17α-oestradiol) relative to DHEA.[62] GC-C-IRMS has also been used to analyze porcine nandrolone and 19-norandrostenedione concentrations in testicles, liver, and kidney, although post-administration samples were not included.[66]

The laborious nature of the sample-preparation technique currently makes the technique unsuitable for use as a screening tool, but it has already found use as a confirmatory approach for proving testosterone abuse in human sports.[69] The executive

summary of the ISOSTER project GRD1-2001-40085 in 2006[68] showed that the GC-C-IRMS method for detecting testosterone abuse in bovine urine was successfully validated in several European laboratories.

Targeted and untargeted biomarker approaches

The rapidly advancing applications of omics-related technologies, which allow the simultaneous analysis of a large number of compounds within a biological system, have huge potential to transform the way drug residue surveillance detection is performed. The concept behind applying the omics approaches are not to detect the presence of a drug directly, but instead to be able to detect its cumulative biological effect (biomarker) within the animal through either targeted (pre-defined profiling) or untargeted (global-profiling) approaches.[70,71] One of the key advantages of the biomarker approach is that no matter what method of doping is used, then a change in the mRNA (transcriptomics), protein (proteomics), or metabolite (metabolomics) profile should be detectable for drugs with common pharmacology. The definition of a 'normal' versus 'suspect' biomarker profile depends on whether 'latitudinal' or longitudinal' comparisons are applied – both of which are currently being investigated (longitudinal testing is considered separately in the following section).

The following discussion introduces a representative sample of the different 'omics' and other biomarker approaches that have been reported in the veterinary drug residue surveillance area. A full discussion of this topic is beyond the scope of this thesis. For more detailed account the reader is directed to recent reviews on the subject.[30,31]

As an example of a recent transcriptomics approach, the effect of trenbolone acetate plus oestradiol administration on the profile of 38 pre-defined candidate genes in bovine calf blood has been reported.[72] The authors determined gene expression in blood cells by quantifying concentrations of mRNA using quantitative real time reverse transcriptase polymerases chain reaction technology (qRT-PCR) and found that 11 of the 38 candidate genes were influenced by treatment. These included the oestrogen receptor-α, the glucocorticoid receptor-α, the apoptosis regulator Fas, the proinflammatory interleukins IL-1α, IL-1β and IL-6 as well as the major histocompatibility complex II, creatine kinase, myotropin, RNA binding protein 5 and actin-β. Principal component analysis was then applied and was able to discern untreated animals from those treated at days 2 and 16 post-administration.

As an example of a recent proteomic approach, the effect of concomitant oestradiol and nandrolone administration on the plasma concentrations of a range of pre-defined candidate proteins using a number of different detection techniques has been reported.[73] Concentrations of immunoreactive inhibin were found to be decreased following administration of the steroids to male calves for up to 38 days, but not in females. Concentrations of the remainder of the targeted proteins including insulin-like growth factor-1, insulin-like growth factor-binding proteins-2 and 3, luteinising hormone, follicle-stimulating hormone and prolactin were not affected by steroid treatment.

As an example of a recent metabolomics approach, the effect of androstenedione administration on the profile of phase II steroid conjugate metabolites in bovine urine has been reported.[74] The authors used a combination of targeted (selected reaction monitoring) and semi-targeted (precursor ion scanning) LC-MS/MS approaches to identify altered metabolite profiles. Using the targeted approach, concentrations of epiandrosterone-3-sulphate were found to be upregulated in treated animals, while some unidentified metabolites were found to be upregulated when applying the semi-targeted approach.

As an example of an alternative type of biomarker approach, an interesting study based on 'Histological Screening for illegal administration of growth promoting agents in veal calves' has been reported.[75] This technique showed some potential for using the effects of androgens and oestrogens on male prostate or female clitoris/Bartholin gland as biomarkers of abuse and has recently been followed up by looking at the range of 'normal' histological findings in large numbers of untreated calves.[76] However, the technique can only be applied post-mortem and, to date, has only been adopted for routine screening in a small number of countries.

Whether biomarker approaches are suitable for the formal confirmation of abuse or just as screening tools remains to be determined. However, further studies are clearly warranted in order to investigate their great potential.

Longitudinal sampling

For the threshold and biomarker approaches described in the previous sections, results from individual animals are typically judged to be 'atypical' if they fall outside of a normal reference range within a population. One factor that reduces the success of these approaches is the typically large inter-individual variation within these populations. In human sports, the concept of longitudinal testing has recently been introduced in order to make use of the observation that intra-individual variation is observed to be lower than inter-individual variation. Longitudinal testing, also known as subject-based profiling, relies on the application of a Bayesian statistical approach whereby each individual has their own threshold for a particular variable, which are dependent on their previously determined results. For example, if individual A repeatedly displays lower concentrations of a particular steroid compared to individual B, then the future concentration threshold of this steroid for individual A would be lower than for individual B. Longitudinal testing forms the fundamental basis for the recently introduced Athlete's Biological Passport in human sports testing and a number of publications demonstrating its usefulness in detecting the abuse of natural steroids have been published.[77]

While longitudinal data for a number of steroids in biological fluids from a range of animals has been published, these studies were mainly concerned with assessing the effect of age on physiological parameters and were not designed explicitly with subject based profiling in mind.[78] While fluid matrices such as plasma and urine may be repeatedly sampled over an animal's life, longitudinal studies of solid tissues are generally not possible because this usually means slaughter of the animal (hence only one sample can be taken). Also, animals are often only tested once in life (if at all), which makes them much less amenable to multiple tests over a prolonged period of time compared to athletes that are routinely tested as part of their careers. Furthermore, the financial interest in a particular individual to be tested is also generally much higher in human sports compared to food production where large herds of animals are bred with relatively low profit margins. The overall 'cost per head' for a particular individual is therefore another factor that reduces the likelihood of longitudinal testing becoming a routine paradigm in veterinary drug residue testing programmes. However, it is not beyond the realms of possibility that a cleverly designed sampling model

could be developed that makes the use of longitudinal testing more attractive. Therefore, it is worthy of consideration in any theoretical debate on the possible future of testing for the abuse of endogenous steroids in food production.

Discussion and future perspectives

As is apparent from the current review, while there is still no single technique that is capable of detecting the abuse of all endogenous steroids in all species, significant progress in this area has recently been made and a number of approaches are now available. Some potential advantages and disadvantages of these techniques are listed in Table 1.

The choice of which approach/es to adopt in each situation is dictated by a number of practical, political and economic factors,[79] which are likely to vary in different parts of the world. These include the particular steroid and species combination requiring analysis, the type of matrix to be tested, whether samples are collected from live animals or at slaughter, available analytical instrumentation, sample throughput/cost considerations and the relevant legal and regulatory frameworks in operation. In designing a routine testing stream within a particular laboratory, then a range of parallel or sequential screening/confirmatory combinations of the available approaches are feasible (depending, of course, on each of the aforementioned limitations). For example, if the laboratory wishes to employ just a single technique, then they may choose to implement either the threshold, marker metabolite

Table 1. Summary of some of the potential advantages and disadvantages of the available techniques used for the detection of endogenous steroid abuse

Approach (range of published matrices)	Example references	Potential advantages	Potential disadvantages
Marker metabolites (urine)	[38–40]	- Can be used for both screening and confirmation. - Can be combined with some other screening approaches such as threshold concentrations if the same analytical technique is used.	- Dependent on the analyte in question not being detected endogenously in the future if lower limits of detection are applied.
Threshold concentrations (urine, plasma/serum)	[42–44,46–48]	- Can be used for both screening and confirmation by using different thresholds. - Can be combined with some other screening approaches such as marker metabolites if the same analytical technique is used.	- Questionable whether population data produced in one country can be applied abroad. - Requires at least a 'semi'-quantitative evaluation of all screening data.
Steroid esters (hair, plasma/serum, muscle)	[49–58]	- Can be used for both screening and confirmation. - A definitive measure of abuse since the relevant steroid esters are not know to exist endogenously	- Will only detect abuse if the steroid has been given in the ester form. - May require an additional or at least modified sample preparation scheme as steroid esters are typically very non-polar.
GC-C-IRMS (urine, tissue)	[60–66]	- A definitive measure of abuse if an endogenous reference compounds is used.	- Only suitable for confirmatory analysis as technique is lengthly and costly. - Success depends on the diet of the animal.
Longitudinal testing (urine, plasma/serum – in human sports)	[77,78]	- Potentially produces lower false negative rates than latitudinal threshold or biomarker approaches. - Could (in theory) be used for both screening and confirmation.	- Requires multiple tests over a prolonged period of time, which may lead to the technique being considered too expensive. - Not suitable for purely post-mortem based testing where only one sample is available. - Only applied in the human sports area to date.
Targeted biomarkers (urine, plasma/serum, histological observations)	[31,72–76,83]	- Potential ability to detect endogenous steroid 'cocktails' through measuring the cumulative biological effect.	- More basic research is needed before the efficacy of this approach is validated. - Uncertain whether biomarkers could be used in a confirmatory environment.
Untargeted biomarkers (urine, plasma/serum)	[31,84–87]	- Ability to detect endogenous steroid 'cocktails' through measuring the cumulative biological effect. - Potentially more powerful than targeted biomarkers because the approach doesn't require prior knowledge of what the target analyte would be.	- More basic research is needed before the efficacy of this approach is validated. - Uncertain whether biomarkers could be used in a confirmatory environment. - If highly complex algorithms based on concentrations of multiple analytes are used, there is the potential for cross-validation issues between different laboratories.

or intact ester approaches since each of these are potentially suitable for both screening and confirmation of abuse (depending on the matrix in question). Furthermore, it might be possible combine the marker metabolite and threshold screening approaches if the same extraction and method of instrumental analysis are suitable for each of the relevant steroids; the only difference being that some analytes would be treated qualitatively while others quantitatively. Cost permitting, the laboratory may also wish to employ parallel screening approaches in order to broaden their analytical coverage; for example by using combinations of one or more of the marker metabolite, threshold, intact steroid ester, and biomarker approaches (again, depending on the matrices in question). Furthermore, the laboratory may also wish to consider employing additional and/or alternative confirmatory methods in order to increase their confidence in the interpretation of the results; for example by employing an orthogonal technique such as GC-C-IRMS. Table 1 lists those approaches that are considered suitable for screening, confirmation or potentially both.

As further research is carried out, it is likely that these techniques will be shown to be capable of detecting the abuse of a wider range of steroid/species combinations than is currently possible. However, as analytical limits of detection decrease, it is also likely that further steroids will be discovered as endogenous in food-producing animals. For example, since the time that the review article of Scarth *et al*.[35] was published, the following steroids have been discovered to be endogenous: 19-noretiocholanolone in boars and gilts,[42] a range of different nandrolone metabolites in steers and heifers,[47,80] wethers, rams and ewes[48] as well as prednisolone in cattle.[81] Furthermore, it has recently been demonstrated that some types of wooden crate in which veal calves are housed may contain precursors to Boldenone.[82] Specifically, the related compound androsta-1, 4-diene-3, 17-dione was detected in the wood from calves housing. The wood was also found to contain progesterone, androstenedione and epitestosterone. This raises the possibility that 1-dehydro steroids may be consumed inadvertently by calves due to environmental contamination. In light of these results, a threshold concentration approach might therefore be more appropriate for detecting boldenone abuse compared to a qualitative marker metabolite based method.

Of course, the above considerations are all dependent on the different hormone bans remaining in place. It is possible, in theory at least, that one or more of the bans could be overturned in the future for political or scientific reasons. This would no doubt mean that the requirements for residue testing would change significantly. However, the bans are likely to remain in place for the foreseeable future (and so, therefore, do the efforts of the residue analysts).

Conclusion

Detection of the abuse of endogenous steroids represents a major challenge to veterinary drug residue testing laboratories and the list of analytes falling into this class has increased simultaneously with improved analytical sensitivity. However, significant progress in this area has recently been made and a number of approaches are now available. These include: threshold concentrations, qualitative 'marker' metabolites, intact steroid esters, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), longitudinal testing and the developing field of omics biomarker profiling. The choice of which to adopt is dictated by a number of practical, political and economic factors, which vary in different parts of the world. These include; the steroid/species combination requiring analysis, the matrix tested, whether samples are collected from live or slaughtered animals, available analytical instrumentation, sample throughput/cost and the relevant legal/regulatory frameworks. Furthermore, these approaches could be combined in a range of different parallel and/or sequential screening/confirmatory testing streams, with the final choice being determined by the aforementioned considerations.

Despite these recent advances, more work is still required in order to further refine the different techniques and to respond to the ever increasing list of compounds classified as 'endogenous'. However, at this advanced stage it is now more important than ever for scientists and regulators from across the world to communicate and collaborate in order to harmonize and streamline research efforts.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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