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Phytosterols and anabolic agents versus designer drugs

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Abstract

Cholesterol is a well-known component in fats of animal origin and it also is the precursor of natural hormones. Phytosterols appear in plants and only differ slightly in structure from cholesterol. An important difference however is the low absorption in the gut of phytosterols and their saturated derivatives, the phytostanols. As a result, there is time for all kind of reactions in faecal material inside and outside of the gut. Determination of the abuse of natural hormones may be based on gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Abuse of natural hormones changes the $^{13}C/^{12}C$ ratio of some metabolites during a relatively long time. The formation of (natural) hormones in the gut may interfere with this method. Designer drugs are mainly known from sports doping. In animal fattening, designer drugs may be used as well. Small changes in the structure of (natural) hormones may lead to a new group of substances asking for new strategies for their detection and the constatation of their abuse.

Keywords: Cholesterol; Phytosterols; Phytostanols; Anabolic agents; Designer drugs

1. Introduction

In this work a link is made between three items which have, at first sight nothing to do with each other, namely phytosterols, anabolic agents and designer drugs. Cholesterol is a well-known component in fats of animal origin and it also is the precursor of natural hormones. Phytosterols appear in plants and have a chemical structure similar to that of cholesterol. An important difference however is the low absorption in the gut of phytosterols and their saturated derivatives, the phytostanols. Consequently, there is time for all kind of reactions in faecal material inside and outside the gut. As an example some reactions, taking place in faeces, with boldenone are given.

Determination of the abuse of natural hormones may be based on gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Abuse of natural hormones changes the $^{13}C/^{12}C$ ratio of some metabolites during a relatively long time. The formation of (natural) hormones in the gut may interfere with this method. Substitution of animal fats with vegetable fats in animal nutrition may also be an altering factor in the pattern of natural hormones. According to literature, in the environment, degradation products from phytosterols may for example, act as endocrine disruptors.

Designer drugs are mainly known from sports doping. Examples are norbolethone, desoxymethyltestosterone and tetrahydrogestrinone. In animal fattening, designer drugs may be used as well: small changes in the structure of (natural) hormones may lead to a new group of substances asking for new strategies for their detection. Laboratories are using very targeted analyses as MS-MS and may therefore miss molecules with small

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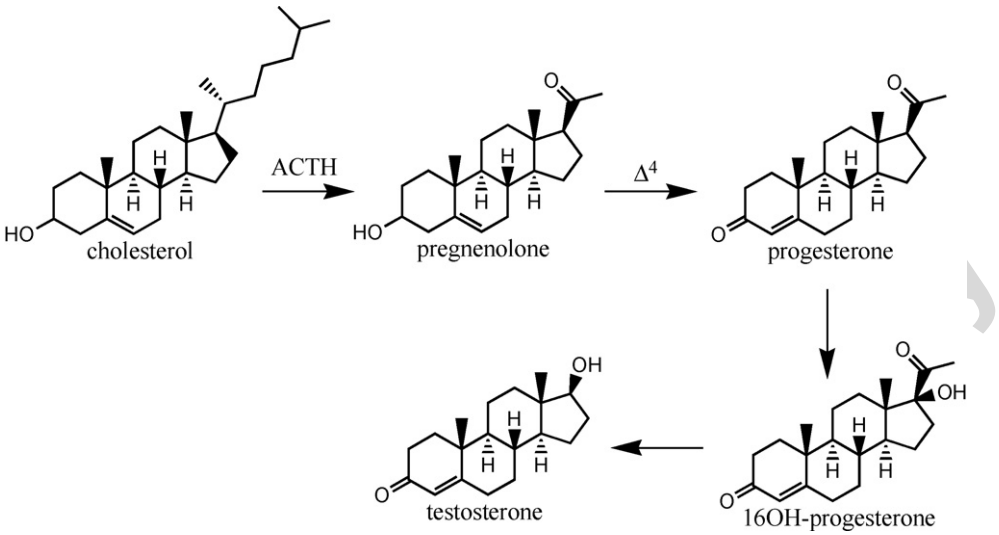

Fig. 1. Pathway: hormone synthesis out of cholesterol.

changes in structure and molecular mass. In this work, some new designer anabolics and their relation to existing substances are given.

2. Sterols

2.1. Cholesterol

Cholesterol ($C_{27}H_{45}OH$) is a soft, waxy, fat-like substance present in all animal fats as well as in some vegetable fats. It is found in the cell membranes of all body tissues and is transported in the blood plasma of every animal. Cholesterol can be present in the free or esterified form and has been assigned many activities, i.e., it has an important role in the structure and function of cell membranes and it has a role in the immune system and in the production of bile. Furthermore, cholesterol is also important as being the precursor of many (natural) hormones, which are synthesized through a complicated multi-step pathway [1,2] (Fig. 1).

The first step in this pathway is the oxidation of the cholesterol side chain, which leads to the formation of pregnenolone and involves the intermediates 20OH- and 20,22diOH-cholesterol. Next, pregnenolone is converted to progesterone through a delta-4 pathway and progesterone is oxidized to 16OH-progesterone. Finally, testosterone is formed. In man there are two main sources of cholesterol: less than 0.5 g a day comes from nutrition and approximately 1 g a day is synthesized endogenously, mostly by the liver, but also by the intestines, adrenal glands and reproductive organs. In cattle, dietary cholesterol is limited since the elimination of animal fat in animal nutrition.

2.2. Phytosterols

Phytosterols or plant sterols are of vegetable origin [3–8]. They have a chemical structure which is similar to that of cholesterol (Fig. 2).

One of the major differences between the two components is located in the side chain at position 17 [9]. An additional double bound can be present at the 22nd position and there can be a substitution of a methyl or ethyl group at position 24. Over 250 sterols and related compounds have been reported to occur in plants. Ergosterol, for instance, is a cholesterol molecule with an additional double bound on the 7th and 22nd position and a methyl group on the 24th position. β-sitosterol has the structure of cholesterol with an additional ethyl group on position 24. Stigmasterol has an ethyl group on the 24th position and a double bound on the 22nd position. Campesterol has a methyl group on the 24th position and brassicasterol has a methyl group on the 24th position and a double bound on the 22nd position. The principal sterol in plant materials is β-sitosterol, usually accompanied by its 22-dehydro analogue stigmasterol. The next most abundant sterol is campesterol. Brassicasterol and avenasterol occur in some plant sources only.

Although cholesterol and phytosterols have a similar chemical structure, their health effects strongly differ (atherosclerosis) [10]. Indeed, based on the chemical structures, phytosterols are as atherogenous as cholesterol (phytosterolemia). However, the low absorption efficiency of phytosterols in the intestine limits this hazardous effect. Phytosterols and stigmasterols are only absorbed in very small amounts in the intestine while cholesterol is absorbed for more than 33–50%. Moreover, the excretion rate

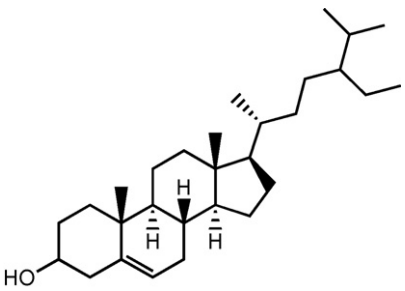

Fig. 2. Chemical structure of β-sitosterol.

of phytosterols is much higher than that of cholesterol. Finally, the presence of phytosterols limits the absorption of cholesterol in the gut, as there exists a competition between both. It appears to be clear that a high baseline dietary intake of plant sterols significantly reduces cholesterol absorption and, despite simultaneous stimulation of cholesterol synthesis, a small but significant reduction of serum cholesterol level can be observed. This explains why the use of vegetable fat, which is rich in phytosterols, decreases the cholesterol content in blood [11–14].

Since the Bovine Spongiform Encephalopathy (BSE) and dioxin crisis all animal fat has been banned out of animal feed and replaced by vegetable material. Since then, the main kind of sterols in animal feed, essentially for cattle, are phytosterols instead of cholesterol.

2.3. Phytostanols

The reduced forms of phytosterols, named phytostanols, have an intestinal absorption efficiency which is even lower, nearly zero, than that of their unsaturated derivatives [11–14]. Sterol analysis in intestinal excreta of patients with colectomy showed that 95% of stanol esters consumed had been hydrolyzed, indicating that during the intestinal passage sitostanol and campestanol are mostly in unesterified form [15], which is assumed to be necessary for effective prevention of cholesterol absorption. Phytostanols are also characterized by higher excretion efficiencies than cholesterol molecules and inhibit absorption of cholesterol and plant sterols in the gut. Therefore, phytostanols have the same, or an even higher, cholesterol lowering effect than phytosterols and are added in large amounts (e.g., 2 g/100 g) to some food, e.g., dressing, margarine, cheese and yoghurt. It has been shown that consumption of these preparations as normal dietary ingredients is acceptable and that they can induce a lowering of the serum cholesterol level up to 20%. A long-term reduction of serum cholesterol level to this extent has been calculated to reduce the incidence of heart attacks by about 40% [16]. Examples of phytostanols are stigmastanol and β-sitostanol (Fig. 3).

Phytosterols and phytostanols are normally not produced by the human and animal body, only cholesterol can be formed endogenously. Phytosterols are naturally present in animal feed of vegetable origin while phytostanols are always added exogenously. In Table 1, the sterol concentrations of the most important vegetable fats are given.

The major conclusion that can be drawn from this table is that cholesterol is not only present in animal fat, but also in small amounts in some vegetable fats, like palm oil, soybean oil and rapeseed oil. Although cholesterol is only present in small amounts in vegetable fat in comparison to animal fat, caution has to be paid when using cholesterol as an indicator of animal fat in animal feed.

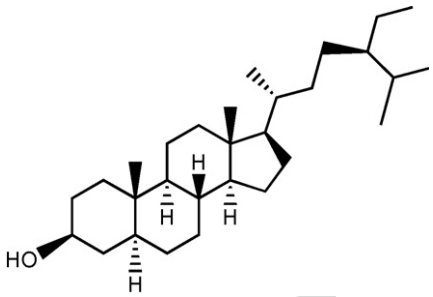

Fig. 3. Chemical structure of a phytostanol.

As mentioned earlier in the article, cholesterol is of importance for the production of human endogenous hormones. Decreasing the cholesterol content of blood plasma by consuming a phytostanol/sterol rich diet could therefore influence hormone balance. Until now, no study has been performed on this matter. Another point of concern is that the phytosterols and stanols we ingest by food, nearly pass unabsorbed through our intestines and thus have the time and the correct environment for all kind of reactions as will be described later.

2.4. Boldenone

Some publications for example, relate boldenone formation to the transformation of phytosterols. 17β-boldenone (17β-Bol), also called 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one, is a steroid with androgenic activity, which only differs from 17β-testosterone (17β-T) by one double bond at the 1-position [17]. Important steroids closely related to 17β-Bol and 17β-T are the 17β-boldenone epimer, i.e., 17α-boldenone, androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED). These two di-keto substances, ADD and AED, are precursors of 17β-Bol and 17β-T, respectively, in humans and different animal species. Their chemical structures are shown in Fig. 4.

Brambilla et al. [18] proved that ADD or boldione, a boldenone precursor, is detected, when corn oil and broth are incubated with calf faeces. According to Sgoifo Rossi et al. [19], the sampling of urine is a very delicate process. In urine, not containing boldenone at "clean" sampling, boldenone could be detected after contamination with faeces during sampling. Pompa et al. and Arioli et al. [20,21] described the neo formation of boldenone in calf faeces and demonstrated that the boldenone concentration increased when rectal faeces were dried and that it

Table 1
Sterol composition of some important vegetable fats

Vegetable fat	%Total sterol	%Total cholesterol	%Total brassicasterol	%Total campesterol	%Total stigmasterol	%Total sitosterol
Palm	0.06	2.6	–	21.4	13.3	60.7
Arachide	0.16	–	0.19	06.2	05.4	68.2
Olive	0.16	–	–	02.8	01.3	82.1
Soy	0.23	0.4	–	18.1	19.9	57.2
Rapeseed	0.74	0.4	11.2	31.6	00.4	52.2

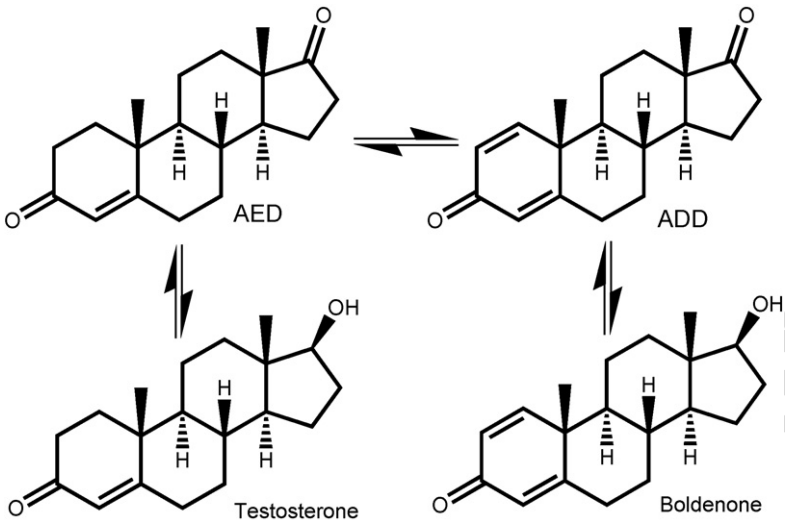

Fig. 4. Conversion of hormones.

increased more than could be explained by the drying procedure alone. Finally, the discrimination between exo- and endogenic boldenone and the incorporation of boldenone esters into hair was discussed by Nielen et al. [22]. Those publications indicate that diverse reactions can occur in and with faeces having a large impact on regulatory control.

3. GC-C-IRMS

GC-C-IRMS is a technique based on the $^{13}C/^{12}C$ ratio in biological material [23,24]. It can be used for the detection of the abuse of natural hormones as it can make an indisputable discrimination between endogenous (produced by the body) and exogenous (administered to the animal) compounds. Two stable carbon isotopes are naturally present in the environment: the ^{12}C isotope (MM 12.00000), which has a relative abundance of 98.89%, and the ^{13}C isotope (MM 13.00335), which has a relative abundance of 1.11%. Depending on the origin of a certain compound, differences in steroid carbon isotopic composition are measured. This forms the base of the GC-C-IRMS technique. At first, the *R* value is calculated. This is the ratio of the heavy isotope to the light isotope. For carbon ($^{13}C/^{12}C$) the reference *R* value is 0.011237199. Usually a standard is used as a reference which in case of carbon is calcium carbonate, also referred to as Pee Dee Belemnite (PDB). The PDB formations are remains of molluscs which are located in a small village of South Carolina. Next, the delta value is calculated according to the following formula. The results are expressed in delta PDB per mille (‰). The delta value is dependent on the source of the compounds analysed.

$$\partial_{\mathrm{PDB}‰}{}^{13}\mathrm{C} = \frac{R_{\mathrm{Sample}} - R_{\mathrm{Ref}}}{R_{\mathrm{Ref}}}$$

Hormones can be present in animal tissues by either an endogenous or exogenous way. Almost all cholesterol in animals is originating from the endogenous way. So, when endogenous cholesterol is metabolized to pregnenolone and further to dehydroepiandrosterone (DHEA), which is then converted to testosterone and its metabolites, an endogenous $^{13}C/^{12}C$ ratio is measured. On the contrary, synthetic hormones, such as testosterone and testosterone esters, are prepared from plant material, for instance from diosgenine or solanidine [25] (Fig. 5).

Diosgenine is the current precursor for synthetic hormones. It is widely available in plant material and its chemical structure is characterized by a steroid nucleus. Other plant derived hormone precursors are known as well, e.g., solanidine and phytosterols. Solanidine is a toxin found in high-starch potatoes. It has a structure similar to that of diosgenine. However, the use of solanidine as a hormone precursor seems to be more difficult compared to diosgenine [25].

Results of GC-C-IRMS are often reproduced in a graph in order to easily compare delta values with the PDB reference

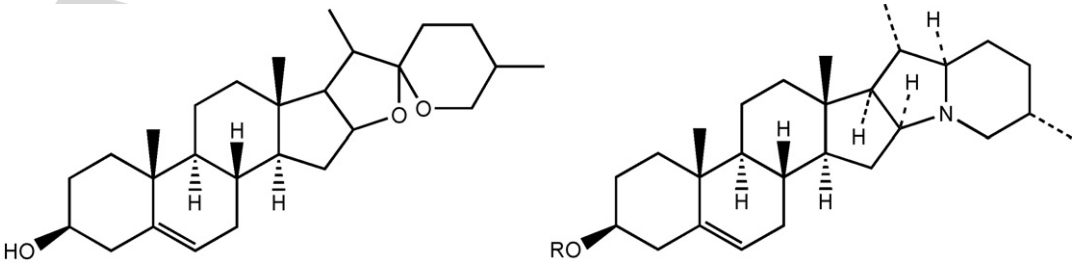

Fig. 5. Chemical structure of diosgenine and solanidine.

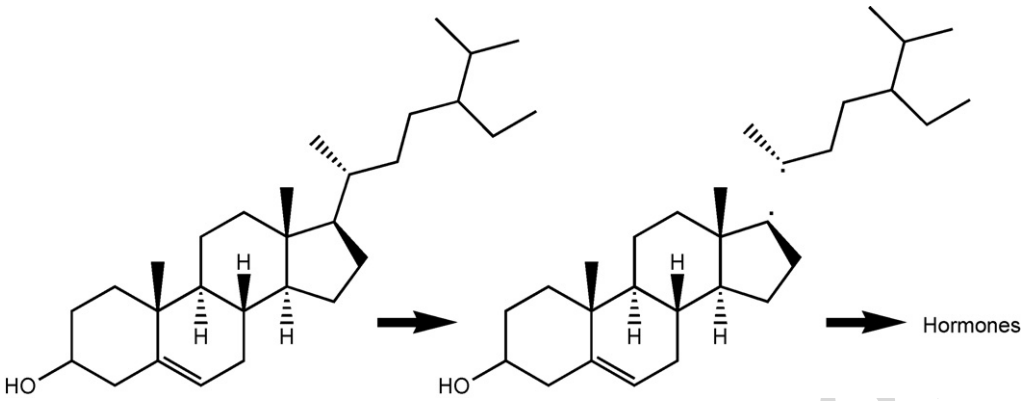

Fig. 6. Cleavage of the side chain.

(1.1237199). Normal delta values for animals fed on C3 and C4 plants are also put in that graph. If animals are treated with synthetic hormones, a different $^{13}C/^{12}C$ will be measured and other, more negative delta values will be calculated.

Conclusion from this chapter is that with the GC-C-IRMS technique laboratories are or will be able to distinguish between non-treated (so-called standard) animals and animals treated with hormonal substances derived from plant material. This is allowed by comparison of the $^{13}C/^{12}C$ ratio of the "suspect" animals with the ratio of "standard" animals. If the *R* value of the samples seems abnormal, it can be concluded that those animals have probably had an exogenous supply of hormones.

As animals nowadays are only fed with plant material, phytosterols instead of cholesterol enter the exogenous way. This may interfere with the results of the $^{13}C/^{12}C$ method.

4. Anabolic agents

4.1. Gynaecomastia

The amount of British men undergoing a breast reducing surgery doubled in 1-year time [26]. The cause would be the involuntary uptake of female hormones in drinking water and hormonally treated meat resulting in gynaecomastia. Those statements, as edited in the press, still are assumptions. Therefore, some other possibilities must be taken into account as well, for example, the use of androgens followed by their aromatisation to estrogens. It is a well-known phenomenon that the use of some androgens in "body building" may result in gynaecomastia. There also are some indications that a shortage in zinc could be involved in this phenomenon. BALCO Labs Inc., a small supplement company in Burlingame, California, has established that somewhere between 70 and 80% of the athletes tested are either "low" or "deficient" in zinc. For instance, among professional football players, the percentage is approximately 73%. Among professional bodybuilders, a depletion or deficiency of zinc also seems prevalent. Om and Chung [27] carried out some experiments indicating that zinc deficiency reduces circulating luteinizing hormone and testosterone concentrations, alters hepatic steroid metabolism, and modifies sex steroid hormone receptor levels, thereby contributing to the pathogenesis of male reproductive dysfunction.

4.2. Aquatic environment

The above-mentioned phenomena, caused by hormones in human beings are related to those observed in aquatic animals. The Fenholloway River (Taylor County, FL, USA), for example, contains chemicals that masculinize the females of eastern mosquitofish (*Gambusia holbrooki*). This is evidenced by some female species having elongated anal fins, which normally is a male specific trait. This phenomenon was linked to the presence of phytosterols as the main source of hormone contamination in surface water. These phytosterols originate from wood used in paper mills. When wood, and the phytosterols they contain, are incubated with *Mycobacterium smegmatis*, progesterone, 17α-hydroxyprogesterone, AED and ADD are formed [28]. Indeed, as is the case for cholesterol, the side chain of phytosterols can be oxidized, yielding a steroid nucleus which gives rise to different kind of hormones (Fig. 6).

Cleavage performed by *Mycobacterium* can, for instance, give rise to an AED yield of approximately 68% [29]. Subsequently, AED can be converted to ADD, boldenone and testosterone. As fermented bamboo shoots (*Bambusa balcooa* and *Dendrocalamus strictus*), fast growing plants, are an enriched source of phytosterols, it would be interesting to exploit them for hormone production. Some microorganisms from the 'soibum exudate', involved in microbial bioconversion of phytosterols during fermentation of succulent bambooshoots, have already been isolated and identified as *Bacillus subtilis, B. licheniformis, B. coagulans* and *Micrococcus luteus* [30]. Crude phytosterol was purified to isolate β-sitosterol, which was then subjected to microbial bioconversion using *B. subtilis* yielding a considerable amount of ADD in the presence of a metal chelate inhibitor [30].

4.3. Invertebrates

In our laboratory, the transformation of hormones has been studied with invertebrate animals as an alternative to and partial

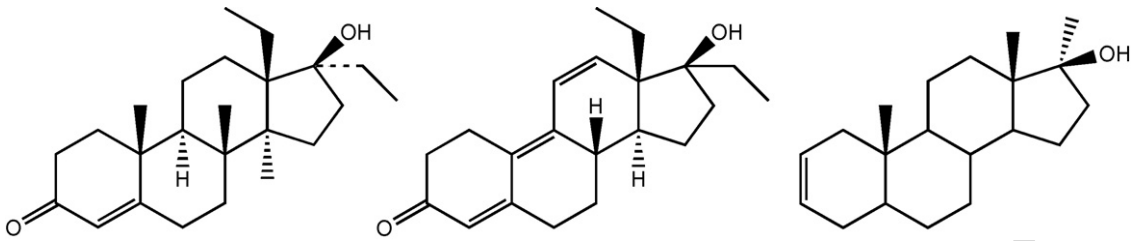

Fig. 7. Chemical structures of NB, THG and DMT.

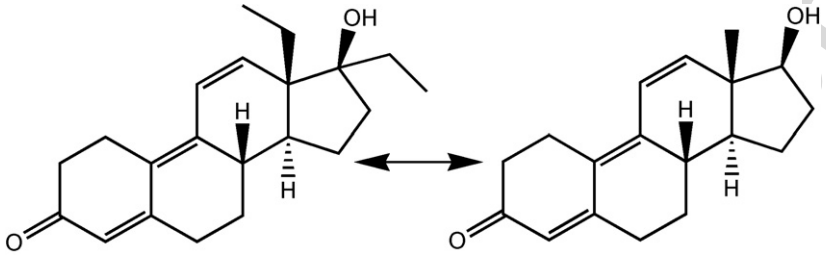

Fig. 8. Comparison of the chemical structures of THG and trenbolone.

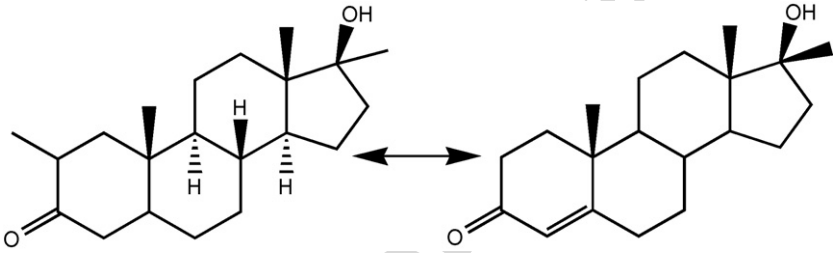

Fig. 9. Comparison of the chemical structures of Superdrol and methyltestosterone.

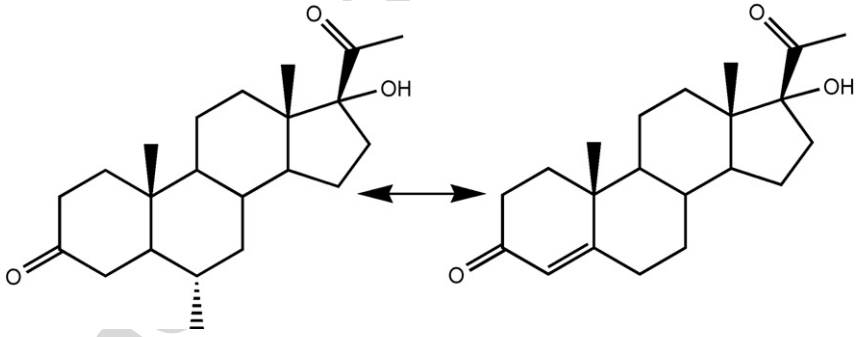

Fig. 10. Comparison of the chemical structures of Methyl-1-P and OH-progesterone.

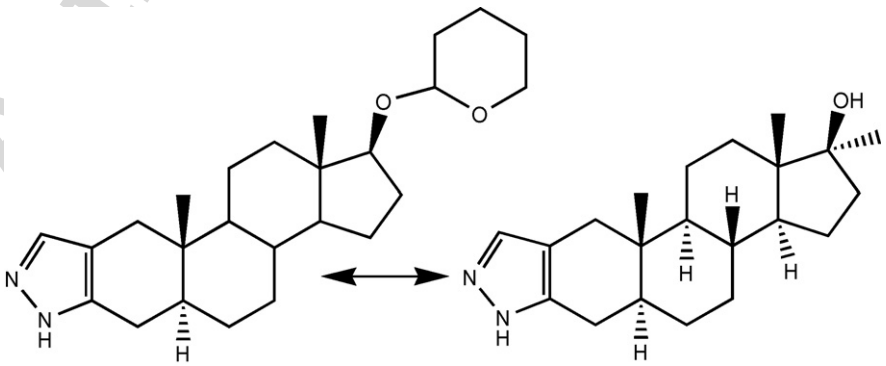

Fig. 11. Comparison of the chemical structures of prostanozol and stanozolol.

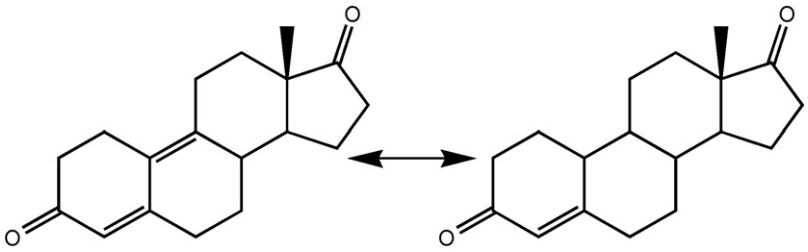

Fig. 12. Comparison of the chemical structures of FINIGenX Magnum Liquid and NAED.

replacement of farm animals in animal experiments. At first, the mysid shrimp *Neomysis integer* was used. *N. integer* is an aquatic invertebrate with a well developed endocrine system. In early experiments, the similarity of metabolisation of stanozolol and clostebolacetate by *N. integer* and the bovine was demonstrated [31]. Later, other transformations as well, a.o. the transformation of phytosterols to hormones, were studied [32]. Recently, the brine shrimp *Artemia franciscana*, and *Lucilia sericata*, a blowfly maggot, was employed. It was demonstrated that maggots of *L. sericata* were able to convert phytosterols into ADD, a boldenone precursor, and testosterone into boldenone [33].

4.4. Boldenone

In relation to the observations described above, the following remarks on boldenone can be made. If boldenone is detected in one animal of a particular farm, it is mostly found in all animals of the same farm. Boldenone is almost exclusively found in male animals, in which testosterone is present in substantial amounts. The detection of boldenone in excreta of animals is in most cases accompanied with the presence of large amounts of testosterone, but also AED and/or progesterone. This suggests that transformations between hormones and, possibly phytosterols, may play an important role in the boldenone problematic [33].

5. Designer drugs

A designer drug can be defined as an anabolic drug designed to be undetectable. In 2003, a new designer anabol, DesoxyMethylTestosterone (DMT) was detected by the doping laboratories [34]. DMT could be considered as derived from methyltestosterone and was not the first designer drug ever mentioned. Norbolethone (NB) and TetraHydroGestrinone (THG) were already known by that time [35–37]. The chemical structures of the last two hormones are very much alike, just differing from one another by two double bounds. THG is probably the most famous designer drug as many athletes made use of it. Its structure could be compared to that of trenbolone, as it has just an extra ethyl group on the 17th position and an ethyl group instead of a methyl group (Fig. 7).

On the 22nd of April 2006, the website of "Health Canada" advised consumers not to use unauthorized products containing anabolic steroids as they can potentially induce severe health trouble, such as liver disorders and heart problems. They mentioned the following five products: Superdrol, Methyl-1-Progesterone, Ergomax LMG, Prostanozol and FINIGenX Magnum Liquid. In this work, the structures of these "new" anabolics are compared with known substances (Figs. 8–12).

Ergomax LMG turned out to be DMT. Superdrol can be compared to 17α-methyltestosterone as it also has the substitution of a methyl group on the 17th position, the lack of a double bound on the 4th position and a methyl group on the second place. The same changes are observed when comparing Methyl-1-P, the replacer of Methyl-1-testosterone, with hydroxyprogesterone. It also has the substitution of a hydroxyl group on the 17th position, the lack of a double bound on the 4th position and an extra methyl group, this time positioned on the 6th place. Prostanozol is identical to stanozolol, the Ben Johnson hormone, except that it has a totally different group on the 17th position. FINIGenX Magnum Liquid, also known as extra-4,9-diene-3,17-dione, can be compared to norandrostenedione (NAED), the nortestosterone precursor. The only difference is the absence of a double bound on position 9. So, all hormones mentioned only possess minor changes in comparison to hormones known in the normal circuit. It is very likely that methods designed for the detection of the regular hormones (e.g., methylteststerone) can also be applied for the detection of designer anabols (e.g., Superdrol), provided of course that some adaptations are made.

6. Conclusion

Due to the BSE and dioxin crises, animal fat was banned from the feed of farm animals. Therefore, phytosterols became the main exogenous sterol source for farm animals instead of cholesterol. The main difference between phytosterols and cholesterol is the absorption efficiency in the intestine. Cholesterol is absorbed in a large amount (30–50%) while the absorption of phytosterols and their saturated analogues, the phytostanols, is almost zero. Therefore, there is time and also the circumstances for all kind of reactions inside and outside the intestine. It has a.o. been demonstrated that phytosterols can be transformed into all kind of hormones (androstenedione, androstadienedione) by elimination of the side chain at position 17. These reactions may possibly disturb natural hormone balances.

On one hand, conventional methods for the detection of the abuse of natural hormones, as the $^{13}C/^{12}C$ method will have to be adapted, for example by the creation of some kind of so called standard animals. In these standard animals the "normal" hormone patterns (e.g., $^{13}C/^{12}C$ method) should be well known. If, in an animal under investigation, a different pattern is observed, the burden of demonstrating the reason of this abnormal pattern

is on the producer instead of on the competent authority. An example is the regular appearance of boldenone in excreta of farm animals.

On the other hand, all kind of new drugs, often called designer drugs, are regularly being introduced in the (black) market and on the Internet. In most cases, these substances are just variations of old structures. There certainly is a strict need for certified standards of these substances, and analytical methods for their detection, a task for the EU community reference laboratories. However, it is very likely that the existing conventional methods could be adapted for the detection of these "new" designer drugs as well.

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