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Recent developments in the use and abuse of growth promoters

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Abstract

During the last few years, control within the European Union (EU) for illegal growth promoters in cattle and pigs revealed only a limited number of positives. Analysis of illegal preparations, however, showed that steroids, often (esters of) natural hormones, and β-agonists are still used. Corticosteroids, controlled to a much lesser extent, seem to have become the most important group, while even thyreostats remain. Alarming information was obtained from specific investigations in which a large variety of products were found, some of which had never been reported to be misused in the field of growth promotion. For β-agonists and quinoxaline compounds, analogues of known compounds are synthesised. Other compounds are readily available as they are registered as growth promoters in some countries outside Europe or are allowed for specific veterinary purposes. Some classes of veterinary drugs are misused for their secondary pharmacological effects, e.g. benzodiazepines as feed intake enhancers and non-steroidal anti-inflammatory drugs (NSAIDs) as pale meat-making agents. Several non-traditional substances are suspected to be used in the field of breeding animals. This is the case for growth hormones (GHs) and all substances acting over this anabolic compound, as for instance, orally GH secretagogue. Moreover, ecdysteroids, which according to old Russian studies, have anabolic activity, are actually very easy to purchase on the Internet. Recent findings in different classes of growth promoters are discussed in detail.

Keywords: Growth promoters; Thyreostats; Anabolics; Ecdysteroids; Somatotropin; β-Agonists; Corticosteroids; Quinoxalines; Benzodiazepines; Non-steroidal anti-inflammatory drugs

1. Introduction

Although the intense control of illegal growth promoters within the European Union (EU) member states, the number of reported positives in cattle and pigs for substances with estrogenic, androgenic or gestagenic effects, β-agonists, corticosteroids and thyreostats in the last few years was very limited.

In a recent comparative study on the legislation on illegal use of growth promoters in the EU [1], results of control in cattle and pigs for these substances in the year 2000 showed only positive findings in six countries. Five countries reported positive findings for β-agonists and substances with estrogenic, androgenic or gestagenic effects with bovines, one country also

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reported the illegal use of corticosteroids. With porcines only three countries reported positive samples for substances with estrogenic, androgenic or gestagenic effects, and two countries reported positive samples for β-agonists. For feeds, only seven countries reported that these samples were taken. All samples from this matrix seemed to be negative for the above-mentioned groups of substances.

Based on this information, it could be concluded that the illegal use of growth promoters has decreased, as some years ago much larger numbers of positives were found in many countries as, e.g. for clenbuterol.

The results obtained on samples of preparations (vials, syringes, needles, etc.), however, are in high contrast with these reassuring figures. Only two countries, however, mentioned that sampling of these objects takes place. The results show that steroids, more and more directed to (esters of) natural hormones, and β-agonists, are still used. Corticosteroids, controlled to a lesser extent, seem to have become the most important illegal growth promoters, while even thyreostatic substances remain. Even more alarming information was obtained from specific investigations in which a large variety of products were found, some of which had never been reported to be misused in the field of growth promotion. For β-agonists and quinoxaline compounds, analogues of known compounds are synthesised, while non-traditional substances are suspected to be used in the field of breeding animals.

2. Discussion

The real extent of the illegal use of growth promoters seems to be much less favourable than the one observed based on the positive findings on animals in the slaughterhouse and farm. The determination of residues of drugs in matrices of biological origin indeed is often very complex, while also precautions are taken by the manufacturers, distributors and users of these compounds.

Difficulties observed in the analysis:

- some drugs are strongly metabolised and the metabolites might still be unknown or their standards not available;
- recoveries for certain drugs are much lower than for others within the same group (e.g. cimaterol and/or salbutamol versus other β-agonists);
- sensitivity for one drug can be much lower than for another within the same group;
- demonstration of exogenous administration of natural hormones remains problematic.

Measures taken by the manufacturer, distributor and/or user:

- use of very low individual doses by combination of different products of the same group (e.g. different androgens or estrogens in the same cocktail);
- use of low doses of compounds belonging to different groups, having additional effects or synergic effects (e.g. corticosteroids and β-agonists in one cocktail);
- use of products known to be hard to analyse because of considerable metabolism (e.g. stanozolol);
- application of synthetic analogues of known growth promoters;
- use of products known not to be examined in the member state.

The last remarks prove the well-organised way of distribution. The fight against the illegal use of growth promoters in stock farming indeed has led to strong indications of the existence of criminal networks. So several specific characteristics of organised crime are observed, like structured associations that last in time and the pursuit of direct or indirect profits. Commercial or other structures are employed to hide or facilitate the crimes, which are concealed or facilitated by the use of intimidation, threat, violence or corruption.

In the recent BUFALAW project, co-financed by the European Commission's Falcone programme, the need was repeated for a better knowledge of each other's legislation, in order to facilitate and stimulate international co-operation. It was outlined that the fight against the illegal use of growth promoters in stock farming cannot be organised at a national level alone, because the phenomenon cannot be stopped or slowed down at national borders [1]. In the same way, it need to be emphasised that a better knowledge of each other's methodology, knowledge about the products found and information on metabolism is necessary to improve control. Events like the International Symposium on Hormone and Veterinary Drug Residue Analysis and the EuroResidue Conferences meet those needs.

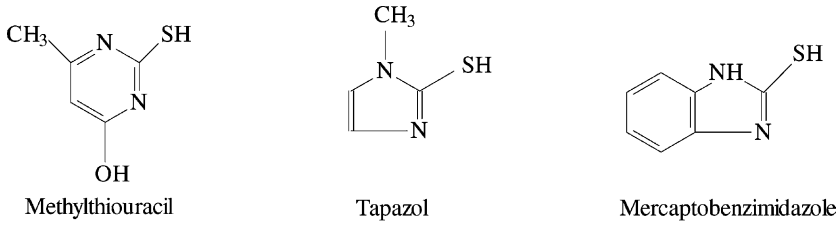

Fig. 1. Chemical structures of some thyreostats.

In the following overview of different groups of growth promoters, findings are discussed in more detail.

2.1. Thyreostats

Thyreostats are a complex group of substances which inhibit the function of the thyroid gland and as a consequence lower the circulating thyroid hormones. The production of thyroid hormones, L-thyroxine (T4) and L-tri-iodothyronine (T3), is decreased.

Administration of thyreostatic drugs to cattle reduces the basal metabolism, lowers gastro-intestinal motility and favours extracellular water retention. Therefore, the abnormal increase in live weight gain obtained with thyreostats mainly consists of an increased filling of the gastro-intestinal tract and an increased water retention by the animal in tissue. In contradiction to some anabolic agents, such as the natural hormones, there is a world-wide agreement on the ban of these drugs: thyreostatic drugs may be harmful to human health, the consumer is misled (water is sold for the price of meat) and the quality of the meat of animals treated with the drugs is inferior (mostly wet and containing less colour). Moreover, there are strong indications that the presence of residues of thyreostats and their metabolites in meat are dangerous for the health of consumers. These products are carcinogenic and teratogenic for the consumer. Consumption of meat contaminated with thyreostats may have caused an increased incidence in Spain of aplasia cutis, a characteristic scalp defect [2].

Thyreostats, important in livestock breeding, can be divided in following two main groups.

- *A group of natural sulphur compounds*: The importance of oxazolidine-2-thiones in cattle fattening stems from its presence in plant material in the form of glucosinolates. The 5-vinyl-oxazolidine-2-thione (goitrin or 5-VTO) derivative is present in large amounts (up to 1%) in the seeds of *Cruciferae* (e.g. rapeseed). Since rapeseed meal is used as a cheap protein source in animal fodder the highly goitrogenic 5-VTO may appear in milk and constitute a health hazard to infants and children who consume milk in large quantities.
- *Xenobiotic thyreostats*: Thiouracils and 1-methyl-2-mercaptoimidazole (tapazol) are synthetic drugs, cheap and readily available on the black market (Fig. 1). They are the most important and most powerful thyreostatic drugs used hitherto. Especially, the use of 6-methyl-2-thiouracil (MTU), in a daily dose of about 5 g per animal over 30 days, has been widespread.

Recently new compounds, such as mercaptobenzimidazole (MBI; Fig. 1) are suspected to be misused in some countries of the EU. The European Reference Laboratory (RIVM, Bilthoven, The Netherlands) warned that a “new” thyreostatic compound was illegally used and not monitored. The detection of MBI was mentioned in a publication of Blanchflower et al. in 1997 with an liquid chromatography–mass spectrometry (LC–MS) method using an atmospheric pressure chemical ionisation (APCI) interface and selected ion monitoring. A multi-residue method for tapazol, thiouracil, methylthiouracil, propylthiouracil and MBI in thyroid tissue using LC–electrospray MS after derivatisation of the compounds with 2-chloro-4-nitrobenzo-2-furazan was described by De Wasch et al. [3].

2.2. Substances with androgenic, estrogenic or gestagenic activity

2.2.1. Stanozolol

Stanozolol, first synthesised by Clinton and co-workers in 1959, is clinically used in cases of osteoporosis

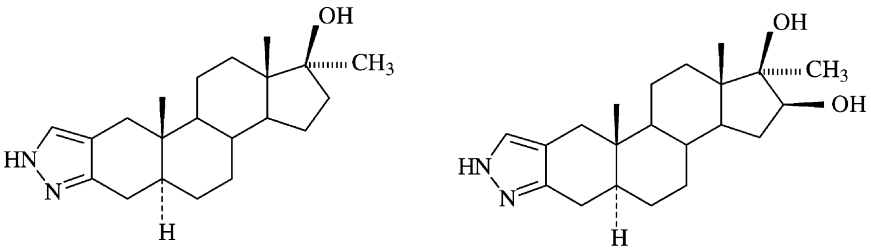

Fig. 2. Chemical structure of stanozolol and its main metabolite.

and deficiency in protein synthesis. Stanozolol or 5α-androstane-17α-methyl-17β-ol (3,2-C) pyrazole most resembles methyltestosterone. Instead of the 3-ketogroup there is a pyrazole ring condensed to the androstane ring system. In the last decade, it was found to be misused on a large scale in animal husbandry. It was often found in injection sites taken at the slaughterhouse: in 1995 it was detected 72 times in the 141 positive injection sites in Belgium. It is metabolised very quickly after administration so that the levels of the precursor molecule in urine are very low, and in this way control via urine is not successful [2]. The metabolite, 16β-hydroxystanozolol, was found to be the marker molecule with bovines (Fig. 2). Detection with gas chromatography (GC)–MS used to be the technique of choice for anabolic steroids but derivatisation and adsorption at the injector was the limiting step for stanozolol and 16β-hydroxystanozolol [4]. LC–tandem MS, nowadays, is the technique of choice for the analysis of both compounds [5]. Elimination profiles for urine and faeces are reported. The same major metabolite, 16β-hydroxystanozolol, was detected in experiments when stanozolol was given orally or intramuscularly to a cow. The results indicate a difference in elimination profile depending on administration: the excretion maximum of 16β-hydroxystanozolol in urine after oral administration was observed sooner than when stanozolol was injected intramuscularly. An extraction procedure was developed for analysis of edible animal matrices (muscle, heart, suet, kidney and liver). Liver is the target matrix followed by kidney. Depending on the time range between administration and slaughter, the equilibrium of stanozolol/16β-hydroxystanozolol shifts in favour of 16β-hydroxystanozolol if the time range is longer. So, it is advisable to look for both analytes in these matrices [6].

During 1999, in Belgium, the inspection services collected 2895 samples at the farm. From these samples, 308 were found to be positive for anabolic steroids of which 302 gave a positive response for 16β-hydroxystanozolol. Most positive samples were found during the summer of 1999, the same period in which residue laboratories had optimised their methods. From September to October, a decline in samples positive for 16β-hydroxystanozolol was observed.

So an important improvement in the residue analysis of stanozolol could be achieved by screening for its marker metabolite, 16β-hydroxystanozolol. In the urine of a dairy cow, treated with 200 mg of stanozolol, 16β-hydroxystanozolol could be detected 17 days after administration, while stanozolol was only detectable for 5 days [2].

2.2.2. 17α-Methylsteroids, norethandrolone and ethylestrenol

It can generally be stated that in vitro and in vivo studies prove that most synthetic anabolics are intensely metabolised. It is possible that large amounts of metabolites are excreted and that they have a prolonged excretion. In addition, the detection of metabolites can add to the reliability of the identification of the administered compounds.

Recently advances have been made in the study of the metabolism of some anabolics.

- The common 3α-hydroxy-5β-tetrahydro metabolite of norethandrolone and ethylestrenol, namely 17α-ethyl-5β-estrane-3α,17β-diol, abbreviated as EED (Fig. 3) was, in Belgium, officially recognised as

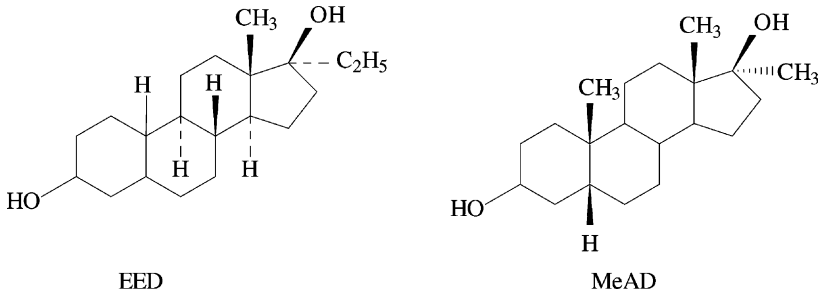

Fig. 3. Chemical structures of EED and MeAD.

a biological marker for the detection of norethandrolone and ethylestrenol. Upon administration of norethandrolone to bovines, EED was the major excretion product in urine and faeces. Compared to norethandrolone and its tetrahydro isomers, it was detected for a longer period of time in excreta. The higher concentration levels and prolonged detection were most pronounced in faeces.

- Another example of an important metabolic route is the hydrogenation of the A-ring structures of the 17α-alkyl anabolic steroids. Results of in vivo experiments and positive routine samples have shown the importance of 17α-methyl-5β-androstane-3α, 17β-diol (MeAD) (Fig. 3) in the detection of three 17α-methylsteroids: methyltestosterone, methylboldenone and methandriol. MeAD is mainly excreted via faeces and is much longer detectable than the three parent compounds [7].

2.2.3. Natural hormones

The use of natural sex steroid hormones and their esters is a still growing problem in the control of residues of growth promoters. In illegal preparations used for growth promotion the natural hormones have become the most popular ingredient. Exogenous administration of these compounds leads to the same hormones and metabolites as the endogenous produced hormones. Therefore, they have to be determined, based on quantitative measurements. However, the concentration of these endogenous hormones can widely vary according to animal species, sex, age and physiological state of the animal. If there is no decision level for the concentration of the natural sex steroid hormones, quantitative measurement of other parameters has to be performed. This can be a metabolite or a biosynthetic precursor of the hormone. For example, the ratio of testosterone/epitestosterone increases after administration of synthetic testosterone. It is difficult, however, to give absolute evidence of illegal use based on the quantification of these natural compounds [8]. A promising technique seems to be GC–combustion–isotope ratio MS. The $^{13}C/^{12}C$ ratio in urine of treated animals differs significantly from the one of untreated animals [9–11]. However, this technique is not (yet) available in most routine labs.

2.2.4. ‘New’ steroids

Next to the steroids longer known to be misused for growth promoting purposes from time to time ‘new’ steroids out of the long list of described substances are encountered. A problem which may arise in this case is the non-availability of standards. Some recent cases of such products found in preparations, injection sites or fat were flugestone acetate, allylestrenol and norclostebolacetate. The structure of the last was elucidated very rapidly by its resemblance to clostebolacetate. At that moment, no standard seemed to be available so that next to MS techniques also nuclear magnetic resonance (NMR) spectroscopy was necessary for the unequivocal identification. An animal experiment has been set up in order to look for the possibilities of its detection in excreta.

2.3. Ecdysteroids

Ecdysteroids are steroids with 27, 28 or 29 carbon atoms with a skeleton characterised by hydroxyl groups at least in positions 2, 3 and 14α, a ketofunction at 6 conjugated with a double bond at 7–8 and multiple alcohol functions on the side chain (Fig. 4).

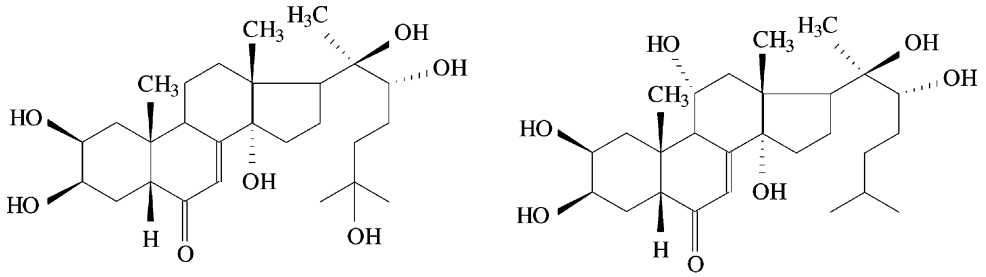

Fig. 4. Structure of some ecdysteroids.

More than 150 ecdysteroids have yet been identified in plants and in invertebrates, especially arthropods. The main zoo-ecdysteroids are α-ecdysterone (insects) and 20-hydroxyecdysone (also called β-ecdysone, ecdysterone, crustecdysterone, isoinokosterone) (crustaceans). The main phyto-ecdysteroids are ponasterones and makisterone C. In arthropods, ecdysteroids act as moulting hormones, whereas in plants they operate as protective agents against predatory insects. Commercial available ecdysteroids are generally extracted from plants, such as *Leuzea carthamoides* and *Rhaponticum* genus. One of the well-known sources of β-ecdysone is Suma (common name: Brazilian Ginseng, Pfaffia, Para Toda or Corango-acu), a large ground and scrambling vine characterised by deep root system (family: Amaranthaceae; genus: *Pfaffia*; species: *paniculata*). As early as 1963, Burdette found that ecdysterone enhanced the rate of protein synthesis in mammalian tissue; Hikino confirmed the anabolic effects in mice in 1969. By 1976, the Russian scientist Syrov attributed to β-ecdysterone the anabolic effect of Suma extracts, and demonstrated in 1984 its strong anabolic effect in mammals. Suspected in the 1980s to be used by Russian Olympic athletes, Suma was then called the Russian secret [12–14]. Preparations containing ecdysteroids are easily available through Internet: Syntrabol, Ecdysten, Ecdyvone, Ecdybol, Ecdistenum, MethoxyFactor, etc. Five milligrams per kg body weight per day is generally recommended. Ajugasterone C is suspected to have equivalent anabolic properties.

Their numerous alcohol and ketone groups make the determination of ecdysteroids by GC–MS difficult, even after derivatisation. Indeed, LC–MS with mild ionisation, such as ESI in the positive mode, makes a specific (at least four transitions in MS/MS) and sensitive (10–100 pg injected) detection possible. The main trouble for an efficient control is the lack of information regarding metabolism in meat-producing animals, and the difficulty to discriminate between a natural source and anabolic treatment.

2.4. Somatotropin

Growth hormone (GH)—or somatotropin (ST)—is a single polypeptide chain consisting of 191 amino acids (around 22,000 Da), varying considerably between species. The growth promoting effect of GH was shown in rats as early as in the 1920s by Evans and co-workers. They demonstrated that GH increases weight gain [15] and stimulates protein accretion concurrent with a reduction in fat deposition [16]. The effect was not studied in farm animals until the 1950s. Brumby [17] conducted an experiment in cattle, and Turmann and Andrews [18] investigated the effects in pigs. For many years, it was not possible to apply this knowledge to practice due to limited supply of GH. However, with the development of recombinant DNA techniques this has now changed, and porcine GH has been approved since 1995 for commercial use in growing pigs in Australia. Recombinant bovine GH (rbST) is widely used to stimulate milk production in cows, but it is not used commercially for growth promotion. GH cannot be administered orally, but by injection. For cattle, sustainable release formulations for monthly administration are on the market [19]. Four different versions of bST are made in the pituitary gland of the cow, the most abundant being those with a leucine in position 127 (21,819 Da). rbST differs from the endogenous form by the substitution of alanine at

the NH_2-end of the chain by: M- (Posilac, Sometribove from Monsanto), M-D-Q (bSTH, Somagrebove from Cyanamid), M-F-P-L-D-D-D-D-K (Somidobove from Elanco–Eli Lilly), etc. making the differentiation of endogenous ST from the exogenous one possible.

Two approaches are possible for the control of the illegal use of somatotropin in food producing animals: indirect methods, e.g. ELISA, to measure biological tracers, such as IGFI and IGFBP, or direct methods, such as LC–MS/MS (ESI+ ionisation), for the identification of the recombinant form of the administered somatotropin. The first strategy is interesting in plasma or urine to draw conclusions about the negativity or the suspicion of the sample, the second to confirm unambiguously the administration of rbST [20].

2.5. β-Agonists

2.5.1. Analogs of clenbuterol

In several countries clenbuterol, a β2-agonist, is authorised for therapeutic use as a bronchospasmolytic and tocolytic agent in veterinary medicine. In dosages up to 10 times the therapeutic one, clenbuterol has marked anabolic effects [21]. The β-agonists enhance growth efficiency by stimulation of β-adrenergic receptors on cell surfaces. In muscle tissue, β-agonists promote protein synthesis and cell hypertrophy by inhibition of proteolysis. In adipose tissue, β-agonists promote lipolysis. This may result in a reduction of carcass fat up to 40% and an increase of carcass protein up to 40% [22]. This redirecting of cellular energy metabolism in favour of protein synthesis has prompted the referencing of some β-agonists as "repartitioning agents". The remarkable effect on increase of growth rate and the improvement of carcass composition of β-agonists meets a major challenge facing the meat animal industry, namely the increase of lean mass and the reduction of fat content in animal carcasses. For this reason, they have been frequently used in meat-producing animals.

The use of β-agonists as growth promoters is not permitted in the European Community. The illegal use of these active substances resulted in several cases of food poisoning with symptoms, such as tremor, tachycardia and nervousness [23]. The development of a suitable control strategy based on screening methods, such as ELISA, followed by confirmatory analysis with GC–MS and LC–MS on a variety of matrices were very successful in reducing the occurrence of clenbuterol.

Recently a new series of clenbuterol-like compounds occurred in black market preparations in Italy, trivially named as compound A and clenmeterol. Such compounds revealed a stronger lipophilicity (terbutylic group substituted with an aromatic ring). When assayed on in vitro organs, they show a predominant β1-activity (increased heart rhythm). The use of adrenergic drugs with a selective β1-activity could be more effective for repartitioning purposes but increase the toxicological risk on consumer health [24]. Certain modifications on clenbuterol structure can deceive the tests without varying substantially the biological activity. Six new clenbuterol congeners (G4, G5, G6, G8, D5 and D9) were prepared in this way, which were not identified by the common tests. For this reason illicit traffic of new β-agonists for use in animal production is very lucrative [21,25]. Very recently another analogue of clenbuterol was found in several feed samples. The molecular weight is even larger than that of the above-mentioned compounds. The structure has not yet been completely elucidated.

2.5.2. Ractopamine and zilpaterol

Ractopamine is a dietary β-agonist that is approved for use in swine with a 50 μg/kg tolerance in the US but is illegal in Europe. Therefore, control of export of pork to the EU requires the ability to determine the presence of the drug at the μg/kg level. Due to its deviating structure most existing screening and confirmatory tests fail to detect ractopamine. Several European laboratories also included the substance in their confirmatory methods, while recently a screening assay based on optical biosensor detection has been worked out [26].

Zilpaterol, present as an active β2-agonist in Zilmax®, is one of the newer β-agonists officially registered for fattening purposes in cattle in Mexico and South Africa. Zilpaterol–HCl is a powerful β-agonist, which is more effective than ractopamine, but only about one-tenth effective as clenbuterol. Mexican reports conclude that zilpaterol supplementation can have marked beneficial effect on growth performance and carcass yield of feedlot steers. Enhanced growth performance accounts for 55% of the net economic value of zilpaterol supplementation (benefit to the

feeder), while increased carcass cut ability accounts for 45% of the net value (benefit to the meat packer and retailer) [27]. The structure clearly differs from other β-agonists, such as clenbuterol or ractopamine, so that routine screening methods based on immunoassays are not able to detect the new molecule. Dutch and Belgian laboratories recently joined their efforts to develop a confirmatory method for detection and set up an animal experiment.

2.5.3. Natural products

Natural β-agonists like hordenine are given partly to impede detection and also because of their action as such [23]. This biogenic amine is generated during the breakdown of tyramine beginning on the first day of barley germination. It is contained in cacti as well as in different kinds of reeds. The stimulatory action of hordenine on the respiratory and cardiovascular systems is short-lived and appears only when a high dose of the compound is used. The compound is also considered to be a stimulant in horse racing. It is one of the active amines in Zhi Shi, a Chinese herb, which is reported to stimulate the β3-receptors, resulting in breakdown of fat cells [28].

Recently, also, ephedrine was detected several times in feed for pigs. Ephedrine is an alkaloid obtained from species of herbs of the genus *Ephedra*. Ephedrine is a sympathomimetic agent with direct and indirect effects on adrenergic receptors. It has α- and β-adrenergic activity and has pronounced stimulating effects on the central nervous system. It has been used for its bronchodilator properties in the treatment of asthma and respiratory disorders [29,30]. Ephedrine is very popular product for weight control. By stimulating the sympathetic nervous system, the amount of heat produced from food increases (thermogenesis) and, therefore, the amount of fat stored decreases. However, many side effects result from excess of ephedrine. Insomnia, irritability, headaches, nausea, vomiting, difficulties with urination, accelerated hearth rate and muscle twitches are some of the lesser side effects. Prolonged use can lead to disturbances in the heart's rhythm, high blood pressure and amphetamine-like dependency, and eventually heart failure [31].

It is obvious that the obscure situations with natural β-agonists are causing highly unpredictable and possibly dangerous situations.

2.6. Corticosteroids

Corticosteroids are frequently used drugs in human and veterinary medicine, often in combination with other drugs, such as antimicrobial drugs or β-agonists. They can be divided in two groups: mineralocorticoids (e.g. aldosterone) with principal action upon electrolyte and water metabolism, and glucocorticoids with important functions upon gluconeogenesis, glycogen deposition, protein and calcium metabolism, together with anti-inflammatory and immunosuppressive activities. Often corticosteroids have activity in both groups. Examples of natural glucocorticoids are cortisol and cortisone. Dexamethasone and prednisolone are well-known synthetic glucocorticosteroids [30].

The difference in structure between steroids and corticosteroids lies in the side chain on C17, with hydroxygroups on C17β and C21 and a ketofunction on C20. Synthetic corticoids are developed to enhance their potency and their glucocorticoid effects. This is mainly achieved by dehydrogenation on $\Delta^{1,2}$, hydroxylation or methylation on C16 and halogenation on C6 and/or C9 [32].

The main therapeutic use is related to their anti-inflammatory and immunosuppressant properties which suppress the clinical manifestations of disease in a wide range of disorders, such as rheumatoid disease, gastro-intestinal disorders (Crohn's disease), renal disorders, skin disorders, asthma, cardiac disorders, organ and tissue transplantation, ocular disorders and neurological disorders. A major drawback of the therapeutic use is the wide range of side effects. Treatment can be by oral administration, intramuscular, subcutane or intravenous injection, or as a topical application [30].

Although it has long been recognised that large doses of synthetic glucocorticoids reduce growth rates and lead to muscle atrophy, dexamethasone and other corticosteroids are frequently used as illegal growth promoters in livestock production. Originally they were often combined with β-agonists and/or anabolic steroids [32]. Cocktails with β-agonists appear to be in use to prevent receptor down-regulation and tolerance in the animal or to affect meat quality by increasing water content [23]. More recently corticosteroids became applied alone. Low doses of glucocorticoids result in improved feed intake, increased live

weight gain, reduced feed conversion ratio, reduced nitrogen retention and increased water retention and fat content [33].

A long list of glucocorticosteroids has been found in illegal preparations or as residue in controlled farm animals, including dexamethasone, betamethasone, prednisolone, methylprednisolone, prednisone, flumethasone, isoflupredone and triamcinolone acetonide. Among the more recent compounds found in preparations clobetasolpropionate and beclomethasonedipropionate can be mentioned. Also esters of the endogenous compound cortisol have been detected, from which the control via residues in biological matrices is practically excluded.

2.7. Quinoxalines

Carbadox and olaquindox are two widely available antibacterial synthetic quinoxaline compounds with growth promoting activity. Both substances were allowed as growth promoter with pigs by Directive 70/524/EEC. The growth promoting effect of both compounds is supposed to be primarily caused by a stabilisation of the intestinal microflora improving the feed conversion and reducing the formation of toxins. In contrary to hormones or hormone-like substances, the growth promoting efficacy does not require systemic receptor-mediated effects interfering with the hormonal balance [34].

The compounds have not been without controversy because carbadox is both mutagenic and carcinogenic in animals, while olaquindox is strongly mutagenic [34–36]. Both substances have been vigorously tested for toxicity due to their extensive absorption and formation of persisting residues. The product licence for these feed additives was withdrawn following publication of Commission Regulation No. 2788/98.

With the ban for carbadox and olaquindox [37], there is a potential risk for the use of unknown quinoxalines. A feed concentrate for poultry was obtained, which was declared to contain a compound named “metil-olaquindox”. LC analysis with diode array detection indeed seemed to confirm the presence of an analogue of olaquindox and carbadox. The original hypothesis, based on the name given to the compound, that the substance was olaquindox with an additional methyl group, however, was left after LC–MS analysis. The probable molecular mass of 234 indeed was lower than those for olaquindox and carbadox, being 263 and 262, respectively. The search for analogues in the literature and on the Internet yielded some compounds like mequidox and mequindox, however, no compound with the correct molecular mass was found. Further research by NMR resulted in methyl 3-methyl-2-quinoxalinecarboxylate-1,4-dioxide (Fig. 5) as the most probable structure [38]. The same compound was found in feed for rabbits in Italy and was called isolaquindox [39]. This compound with a probable comparable or even higher toxicity than the well studied compounds carbadox and olaquindox seems to be present on the black market in many countries, while no methods are available for the control in feed or food. The same can be said for the other analogues. Furthermore, no standards are available. It might be interesting to note for control purposes that the metabolite of the compound discussed, theoretically is the same as for olaquindox and mequidox [40].

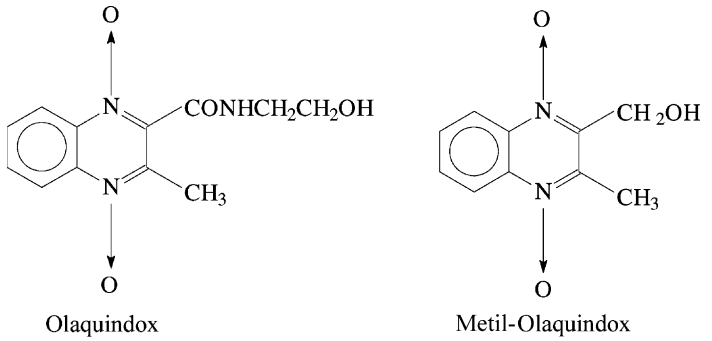

Fig. 5. Structures of some quinoxalines.

2.8. Non-steroidal anti-inflammatory drugs

Probably one of the most commonly used therapeutic drug classes world-wide are the group of non-steroidal anti-inflammatory drugs (NSAIDs). Their anti-inflammatory, analgesic and antipyretic activities are well known and they are widely used to treat inflammatory conditions, fever and rheumatic diseases as well as acute and chronic pain, including osteoarthritis and rheumatoid arthritis, with rather important differences between the different subclasses. Enolic acids and pyrazolon derivatives, salicylic acid derivatives, *p*-aminophenol derivatives, indole indene derivatives, heteroaryl acetic acids, arylpropionic acids, anthranilic acids and alkanones are the different classes that can be distinguished [30,41].

NSAIDs act by blocking the action of cyclooxygenase (COX), the key enzyme in the synthesis of eicosanoids (prostaglandins and related substances) from arachidonic acids. Differences are based on the selectivity of those drugs to block cyclooxygenase 1 (COX1), enzyme responsible for the formation of tromboxane A and prostaglandins and cyclooxygenase 2 (COX2), enzyme responsible for the formation of leukotrienes [42].

In livestock breeding, salicylates are routinely used to condition animals just after transport to reduce the effects of stress. They can be easily administered by oral route, with no relevant side effects. In the market, they are available at convenient price. As well as for therapeutic purposes, the use could be hypothesised as an anti-clotting agent, administered close to slaughtering, to speed up the bleeding just before carcass dissection. A more efficient bleeding could also be responsible for a whiter colour of meat from pigs and calves, relevant in some cases as a quality parameter. At high doses, reduced lipogenesis has been reported, by partially blocking incorporation of acetate into fatty acids [43].

2.9. Benzodiazepines

Benzodiazepines are anxiolitic and sedative drugs, widely used in human medicine. Their activity is mainly based on the receptor competition with gamma aminobutyric acid, a chemical mediator of the central nervous system. They can be classified in long, medium and short acting compounds, according to the structure activity relationship. Such modulation in the pharmacological activity allow a wide range of therapeutic uses, from treatment of insomnia to the pre-anaesthetic medication. In the veterinary practice of pet animals, benzodiazepines can be prescribed for the same scope. In veterinary medicine, benzodiazepam injection is commonly used for anaesthesia induction [44]. The use of medium acting benzodiazepines, such as diazepam has been proposed as an anxiolitic and sedative in sheep transport to prevent stress, limiting leg injury occurrence. A short acting benzodiazepine, brotizolam, is available to stimulate feed intake in weak animals.

The low cost, the practicability of their oral administration, the possibility of stimulating the appetite and tranquillising animals [45,46], thus counteracting the side effects of β-agonists as repartitioning agents, explains their administration in feed on spots close to the consignment. Indeed cocktails of β-agonists with benzodiazepines are in use, the benzodiazepine being intended to “antagonise” the reduction in feed intake caused by β-agonists and/or tremors of the animals [23].

3. Conclusions

Thorough examination of preparations and results of specific investigations prove the continued illegal use of a large variety of growth promoters. Their detection in matrices of biological origin is far from evident by, e.g. extensive metabolism of the compounds and/or the low dosages used. Furthermore, structural changes of known representatives can elude screening and/or confirmatory tests, while new substances and even new classes of compounds are introduced unknown to the laboratories. The increased use for screening purposes of very specific methodology, such as MS with single ion monitoring and even tandem MS, is a handicap in detecting new molecules.

Solutions to improve the efficiency of residue testing programmes might be found in the encouragement of the study of the kinetics and metabolism of the substances in the target animal and of the production of reference standards of compounds and metabolites, isotope-labelled compounds and samples of incurred matrices. The application of screening methods based on different approaches, including group specific

methods and methods based on the measurement of indirect parameters also should be encouraged. The use of histological and physiological indicators should be worked out. Especially, in order to reveal the use of new compounds, combined efforts are necessary, and not only between laboratories, but also between laboratories and inspection services.

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