Research on Detection Strategies for Prohibited Veterinary Drugs in Animal-derived Foods

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Abstract Veterinary drugs are a class of pharmaceuticals used in animal husbandry. Residue problems caused by their misuse have become an undeniable concern for food and drug safety. In particular, certain varieties have been widely prohibited both at home and abroad due to their potential risks to human health and the ecological environment. The regulation of such prohibited veterinary drugs has emerged as a critical issue in current food safety research. This paper reviews the specific detection technologies for different classes of prohibited veterinary drugs (tranquilizers, quinolones, amphenicols, sex hormones, nitrofurans, nitroimidazoles, β-agonists, pesticides/insecticides, and arsenic/mercury preparations) according to their chemical structure characteristics, aiming to provide a reference for the development of detection technologies for veterinary drug residues in complex matrices.

Key words Animal-derived food; Prohibited veterinary drugs; Detection technology

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Sample pretreatment is an essential step in the detection of veterinary drug residues. Due to the complex matrix of animal-derived foods and traditional Chinese medicines, as well as the presence of numerous endogenous interfering substances, sample pretreatment steps such as extraction, purification, and concentration are typically required. Common sample pretreatment techniques include liquid-liquid extraction, solid-phase extraction, quick easy cheap effective rugged safe (QuEChERS), and protein precipitation methods.

Commonly used techniques for detecting veterinary drug residues primarily include liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS), and high-performance liquid chromatography-tandem ion trap mass spectrometry (HPLC-IT-MS). Additional methods include GC-MS, capillary electrophoresis-mass spectrometry (CE-MS), and enzyme-linked immunosorbent assay (ELISA). HPLC-MS/MS is currently the most commonly used detection method. It integrates efficient separation with qualitative and quantitative analysis of both single and multiple components[1]. Characterized by high sensitivity, wide applicability, and the ability to perform qualitative and quantitative multi-residue analysis, it is the mainstream technique for detecting veterinary drug residues. HPLC-QTOF-MS offers higher resolution, enabling better differentiation of compounds with very similar mass-to-charge ratios[2] and reducing the rate of false positives. GC-MS offers high sensitivity, accurate quantification, multi-residue detection capability, and suitability for volatile small molecules, serving as a complementary technique to HPLC-MS/MS. CE-MS is cost-effective and versatile, but suffers from poor reproducibility. ELISA is suitable for qualitative screening due to its low cost and high sensitivity. However, it cannot perform multi-residue detection and is prone to false positives.

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Detection Strategies for Different Classes of Prohibited Veterinary Drugs

Tranquilizers

Tranquilizers mainly include benzodiazepines and barbiturates. Certain antipsychotic drugs, such as phenothiazines, also exhibit sedative effects. These substances are associated with adverse effects including drowsiness, withdrawal symptoms, and addiction[3-5]. In fish, their use may also lead to behavioral and developmental changes. Most tranquilizers have relatively low polarity, contain nitrogenous groups, and are weakly alkaline[6-7].

In the pretreatment extraction process, acetonitrile or ethyl acetate can be used as extraction solvents to remove protein interference from animal samples[8]. A certain amount of acid, base, or ammonium acetate may also be added to the extraction solvent to improve extraction efficiency[9]. If necessary, enzymatic hydrolysis with β-glucuronidase/arylsulfatase can be employed to release the compounds[10]. In the purification step, n-hexane or Prime HLB solid-phase extraction cartridges are commonly used for defatting. N-propyl ethylenediamine or C18 is used as adsorbent for dispersive solid phase extraction, and graphitized carbon nanotubes are also used as adsorbent, or MCX is used for specific purification based on weak basicity. Most analyses are carried out using HPLC-MS/MS, with mass spectrometry detectors including ion trap time-of-flight, triple quadrupole, and quadrupole linear ion trap systems.

Quinolones and amphenicols

Quinolones are commonly used to treat infections caused by both Gram-positive and Gram-negative bacteria due to their broad-spectrum bactericidal activity[11]. The overuse of quinolones in

animal husbandry can lead to the development of bacterial resistance and pose potential carcinogenic, teratogenic, and mutagenic risks to consumers.

Quinolones contain a nitrogen-containing heterocyclic ring and a carboxyl group in their structure, making them amphoteric compounds with relatively good water solubility. Acetonitrile is commonly used as the extraction solvent. Prior to extraction, pH adjustment can be performed using EDTA buffer, or acetonitrile acidified with 1% formic acid. Alternatively, the pH may be adjusted to alkaline conditions using ammonia solution followed by acetonitrile extraction[12]. For purification, methods such as liquid-liquid extraction with n-hexane, freeze-induced liquid-liquid extraction, Prime HLB solid-phase extraction, or enhanced lipid removal dispersive solid-phase extraction can be adopted to eliminate lipids. Adsorbents such as C18 are used to remove other interfering components. Detection methods commonly include HPLC-MS/MS, with mass spectrometry detectors such as Orbitrap, triple quadrupole, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[13]. Other approaches reported in the literature include micellar liquid chromatography, magnetic immunofluorescence assays, and near-infrared spectroscopy.

Amphenicols, represented by chloramphenicol, exhibit broad-spectrum antibacterial activity, but are associated with adverse effects in humans, including hematotoxicity and bone marrow aplasia. Amphenicol compounds are commonly extracted using basified acetonitrile or basified ethyl acetate. Lipid removal is achieved through liquid-liquid extraction with n-hexane, solid-phase extraction with silica, or liquid-liquid extraction with heptane[13]. Purification may also be performed using MCX solid-phase extraction cartridges, taking advantage of the weakly basic nature of amphenicols.

Sex hormones

Sex hormones are classified by origin into endogenous and exogenous hormones. Endogenous hormones mainly include testosterone, progesterone, and estrone. Exogenous hormones can be categorized by chemical structure into synthetic steroid hormones, such as testosterone propionate, trenbolone, and trenbolone acetate, and synthetic non-steroidal hormones, including diethylstilbestrol and hexestrol. Exogenous hormones are commonly used to promote animal growth, induce estrus, and increase body weight. Consumption of animal-derived foods containing exogenous hormones can disrupt the human endocrine system[14] and pose potential risks such as carcinogenicity and damage to the reproductive system.

Acetonitrile is commonly used as the extraction solvent. Due to the generally low polarity of this class of compounds, the use of n-hexane or ethyl acetate for extraction has also been reported. Lipid removal is achieved using Prime HLB cartridges or n-hexane, while purification is carried out by dispersive solid-phase extraction with adsorbents such as C18, PSA, and anhydrous magnesium sulfate[15]. Detection is primarily performed using HPLC-MS/MS, with mass spectrometry detectors such as Orbitrap, triple quadrupole, and ion trap. There are also reports of using ultra-high-performance liquid chromatography alone, with sample pretreatment involving deep eutectic solvent microextraction.

Nitrofurans

Nitrofurans exhibit inhibitory activity against most Gram-positive and Gram-negative bacteria, fungi, and certain protozoa, and have been widely used in livestock and poultry industries for sterilization, disinfection, and treatment of animal diseases. Prolonged or high-dose use of nitrofuran drugs can lead to teratogenic, carcinogenic, and mutagenic effects. Nitrofurans are rapidly metabolized in animals. Their metabolites bind to tissue proteins to form stable compounds, which are released under weakly acidic conditions[16]. Therefore, residues are characterized by measuring these metabolites. However, due to their small molecular weight, poor ionization efficiency, and limited chromatographic separation, derivatization is required prior to detection.

2-Nitrobenzaldehyde is commonly used as the derivatization reagent for samples, typically performed overnight at 37 ℃ for 16 h. Oscillation, heating, ultrasonication, or microwave treatment can significantly shorten the derivatization time. As nitrofuran derivatives exhibit varying degrees of photosensitivity, derivatization should be carried out protected in the dark. After derivatization, lipid removal is achieved using EMR-lipid dispersive solid-phase extraction or liquid-liquid extraction with n-hexane[17], followed by extraction and purification using ethyl acetate or acetonitrile. Detection is primarily performed using HPLC or HPLC-MS/MS, with mass spectrometry detectors such as quadrupole linear ion trap and triple quadrupole mass spectrometers. Surface-enhanced Raman spectroscopy may also be employed for detection.

Nitroimidazoles

Nitroimidazoles exhibit strong antibacterial and antiprotozoal activity, with inhibitory effects against infections and parasites. They are commonly used to treat parasitic diseases such as trichomoniasis and amebiasis. Excessive residues of nitroimidazoles in food can cause adverse effects in humans, including nausea and vomiting, and may lead to teratogenic, mutagenic, carcinogenic, and genotoxic risks, posing a threat to human health.

Most nitroimidazoles are amphoteric compounds with moderate polarity. Extraction with ethyl acetate can remove most water-soluble impurities while retaining the nitroimidazoles. However, since ethyl acetate is not effective at removing proteins, acetonitrile may be used first for protein precipitation. Based on their amphoteric nature, extraction can also be performed using a 2% formic acid solution[18]. Purification can be conducted by PSA dispersive solid-phase extraction or, based on their weakly basic properties, selective purification with MCX cartridges. Magnetic solid-phase extraction may also be adopted. Detection is primarily performed using HPLC-MS/MS or HPLC.

β-Agonists

β-Agonists exist in both free and conjugated forms after metabolism in animals. Drugs such as salbutamol, ractopamine, and terbutaline readily conjugate with glucuronic acid and aryl sulfate in vivo. Therefore, sample pretreatment typically involves enzymatic hydrolysis with β-glucuronidase/arylsulfatase under shaking in a 37 ℃ water bath, or acid hydrolysis using trichloroacetic acid, which also serves to precipitate proteins. The purification

process often involves defatting with n-hexane[19]. Due to their weakly basic nature, cation exchange solid-phase extraction cartridges such as MCX are commonly used. Alternatively, derivatization may be performed followed by detection using GC-MS.

Pesticides

Organochlorine pesticides such as toxaphene and lindane, carbamate pesticides such as carbofuran, and organic nitrogen pesticides such as chlordimeform may be used as insecticides in the rearing of food-producing animals. They can also enter animals and the human body through the food chain. In addition, plant growth regulators such as sodium nitrophenolate may be added to feed to improve feed conversion efficiency and thereby increase production. However, due to their high toxicity[20], they have been prohibited by the Ministry of Agriculture and Rural Affairs for use in food-producing animals.

Based on the characteristics of target compounds and matrices, various detection methods have been reported in the literature. For example, the determination of chlordimeform, amitraz, and their metabolites in honey can be achieved by extraction with 1% ammoniated acetonitrile, followed by purification using a mixture of PSA, C18, and NH_2. For the determination of sodium nitrophenolate in animal-derived foods, extraction with acetonitrile and purification using MAX solid-phase extraction cartridges are peformed. For the determination of sodium pentachlorophenolate in pork, chicken kidney, and fish, extraction can be conducted using 80% acetonitrile in water, followed by purification with QuEChERS. Carbofuran in honey can be detected by extraction and purification using room-temperature ionic liquid-loaded cyclodextrin magnetic nanomaterials.

Prospects

Gradually improving the variety and limit standards for prohibited veterinary drug residues

In China, the management of prohibited veterinary drugs is primarily based on list-based control. However, these lists are scattered across national food safety standards and various announcements issued by the Ministry of Agriculture and Rural Affairs, lacking an integrated class-based management approach and fallback provisions. Additionally, due to historical usage, environmental contamination, and disease treatment, certain prohibited veterinary drugs may present background levels in animals. However, only a few of these substances have established maximum residue limits in GB 31650-2019 and GB 31650.1-2022. It is recommended that the regulatory authorities systematically review and publish a consolidated list of prohibited veterinary drugs, as well as corresponding provisions, to further clarify the residue limit standards for relevant prohibited substances.

Strengthening risk assessment of veterinary drug residues in animal-derived traditional Chinese medicines

Animal-derived traditional Chinese medicines, such as animal-derived foods, also require the use of veterinary drugs during the breeding process. However, the supervision in this field in this area is almost nonexistent at present. Due to China's long-standing tradition of using such medicines and the health philosophy of "preventive treatment of disease", the consumption of animal-derived traditional Chinese medicines in China is substantial, posing potential safety risks. Therefore, it is recommended to strengthen the risk assessment of veterinary drug residues in traditional Chinese medicines based on consumption data of relevant products and safety data of corresponding veterinary drugs, and to establish residue limit standards for veterinary drugs in animal-derived traditional Chinese medicines as soon as possible.

Development of high-throughput detection methods for prohibited veterinary drugs in food

As evident from the detection standards and literature summarized above, current methods for detecting prohibited veterinary drugs are largely categorized by class and fail to cover all prohibited veterinary drug varieties, resulting in low regulatory efficiency and even regulatory gaps. It is recommended to establish a high-throughput screening platform to achieve full coverage of regulated substances. Additionally, a detection strategy combining broad-spectrum high-throughput screening followed by targeted accurate quantification should be promoted, so as to fully leverage the qualitative and quantitative advantages of different mass spectrometry instruments to improve detection efficiency.

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