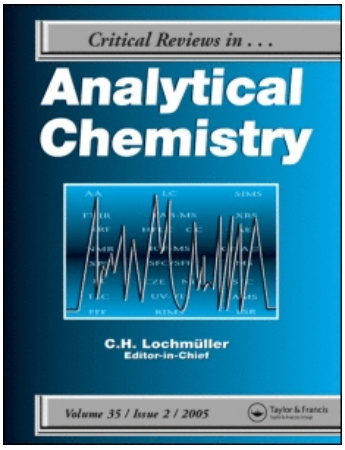

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Analytical Methods for the Detection of Corticosteroids-Residues in Animal-Derived Foodstuffs

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Analytical Methods for the Detection of Corticosteroids-Residues in Animal-Derived Foodstuffs

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Reliable and practicable analysis of veterinary drug residues in food-producing animals represented an important measure to ensure consumer protection. Tha main attention in this text is on the analytical methods for corticosteroids-residue detection. The progress of rapid screening methods and the use of accurate liquid chromatography-tandem mass spectrometry (LC-MS-MS) for the quantitative detection of corticosteroids were discussed. The challenges and achievements in this field, as well as an outline of future perspectives and promising alternatives for well-established procedures, were summarized. Developing highly sensitive analytical multi-residue methods to measure very low levels of corticosteroids is one aspect that should, and will, receive dedicated interest in the near future.

Keywords Animal-derived food, corticosteroids (CORT), residues, analytical methods

INTRODUCTION

Recently, there has been plenty of attention paid to the occurrence of veterinary drug residues in animal-food products. In modern agricultural practice, veterinary drugs are being used on a large scale and administered as feed additives or in the drinking water for therapeutic and prophylactic purposes (1). Many of these substances exhibit adverse properties when used in a wrong or an abusive way (e.g., with a withdrawal period that is too short or using growth-promoting hormones to stimulate the growth by various mechanisms).

Corticosteroids are synthetic analogues of hormones widely used in veterinary medicine. They are frequently used to combat inflammatory diseases and immunologically mediated diseases in animals. Their illicit use as feed additives in animal fattening is well known, despite the fact that they are illegal for such purposes. Corticosteroids include two classes of steroid hormones naturally synthesized in the adrenal cortex from cholesterol: mineralocorticoids and glucocorticoids. Chemical modifications to the cortisol molecule (the main natural glucocorticoid) have generated derivatives with increased therapeutic potency but reduced min-eralocorticoid effects. Table 1 presents the names, the chemical structures and the molecular weight of the corticosteroid drugs used most in the veterinary field.

The reason for such fraudulent practice is that they affect water retention in meat and also lipid metabolism, facilitating the effects of other growth promoters. Corticosteroids increase the number and affinity of mineralocorticoids (e.g. aldosterone) that influence the electrolyte-water balance. Glucocorticoids have important functions upon carbohydrate metabolism, protein and calcium metabolism, and produce the potent anti-inflammatory and immunosuppressive activities. Often corticosteroids have activity in both groups (2). The potential for adverse effects towards animals and humans and the introduction of harmful residues into the human food chain should be avoided. So, the EU has banned their administration for fattening purposes and established Maximum Residue Limits (MRLs) (see Table 2) for some of them.

Corticosteroids-residue control represents an important measure in ensuring consumer protection; reliable data have to be made available to enable adequate risk evaluation and subsequent action. Selection of the most appropriate analytical methods and their thorough validation is an indispensable requirement for putting into practice reliable corticosteroids-residue control. In other words, sophisticated and robust analytical methods must be developed. A variety of methodologies is available for screening and confirmatory analysis. Analytical methods like enzyme-linked immunosorbent assays (ELISAs) and gas chromatography (GC) are based on screening and those suspected non-compliant samples are then confirmed through methods based on the use of gas or liquid chromatography coupled to mass spectrometry, or other sophisticated methodologies and analytical instrumentation, for accurate characterization and confirmation. Several hundred thousand samples, mainly bovine, porcine samples, and various matrices (e.g., muscle tissue, milk,

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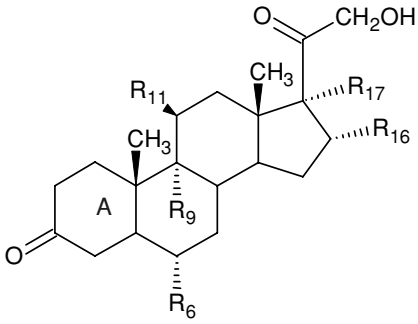
TABLE 1
Name, structures, molecular weight of main corticosteroids in veterinary medicine



Compound	A	R_6	R_9	R_{11}	R_{16}	R_{17}	M_w
1. Beclomethasone	$\Delta^{1.4}$	–	–Cl	–OH	$-CH_3$	–OH	408.9
2. Betamethasone	$\Delta^{1.4}$	–	–F	–OH	$-CH_3$	–OH	392.5
3. Cortisol	Δ^{4}	–	–	–OH	–	–OH	362.5
4. Cortisone	Δ^{4}	–	–	=O	–	–OH	360.5
5. Desoxycocortisone	$\Delta^{1.4}$	–	–	–OH	–	–OH	346.5
6. Dexamethasone	$\Delta^{1.4}$	–	–F	–OH	$-CH_3$	–OH	392.5
7. Fludrocortisone (I.S.)	$\Delta^{1.4}$	–	–F	–OH	–	–OH	380.5
8. Flumethasone	$\Delta^{1.4}$	–F	–F	–OH	$-CH_3$	–OH	410.5
9. Methylprednisolone	$\Delta^{1.4}$	$-CH_3$	–	–OH	–	–OH	374.5
10. Prednisolone	$\Delta^{1.4}$	–	–	–OH	–	–OH	360.5
11. Prednisone	$\Delta^{1.4}$	–	–	=O	–	–OH	358.4
12. Triamcinolone	$\Delta^{1.4}$	–	–F	–OH	–OH	–OH	394.4

kidney and liver), are analyzed each year. The characteristics of corticosteroids (non-volatile, polar, etc.) make them good candidates for liquid chromatography (LC) separations. Meanwhile, liquid chromatography coupled to multidimensional mass spectrometry (LC-MS^2) methods provides a powerful alternative, combining rapidity, specificity and sensitivity, so this methodology allows unequivocal identification of what are usually minute quantities (low ppb levels) in complex matrices.

For corticosteroids, the analysis system consists of the following elements:

1. animal species and matrix selection;
2. type of material (incurred matrix material, calibrant and matrix blank);
3. selection and order of processing steps;
4. suitable analytical methodology for characterization measurements; and
5. value-assignment process (certified value and associated uncertainty).

In this review, we present an overview of the methodological advances in the analysis of corticosteroids residues in animal, derived food. We discuss the theoretical and technical aspects of screening and confirmatory methods for the detection of corticosteroids residues, and envisage the future role of well-established procedures and new options that are relevant to their analysis.

TABLE 2
The EU maximum residue limits (MRLs) for dexamethasone, betamethasone, prednisolone and methylprednisolone

Corticosteroids	Muscle	Liver	Kidey	Milk
Dexamethasone	0.75 μg/kg	2 μg/kg	0.75 μg/kg	0.3μg/kg
Betamethasone	0.75 μg/kg	2 μg/kg	0.75 μg/kg	0.3μg/kg
Prednisolone	4 μg/kg	10 μg/kg	10 μg/kg	6 μg/kg
Methylprednisolone	10 μg/kg	10 μg/kg	10 μg/kg	Not for use in animals from which milk is produced for human use

SAMPLE PREPARATION

Suitable Sample Material

The type of sample material is the first selection that has to be made when setting up a monitoring program.

Since the drug concentrations in the edible parts of an animal have to be below the MRL, these matrices are therefore of most interest and, because kidney and liver are the target organs for corticosteroids (dexamethsaone, betamethsone, predinisolone, and methylpredinisolone), the drug concentrations in these organs are higher than in, e.g., heart or lungs. One disadvantage of selecting animal organs or muscle is that they can be analyzed only after slaughtering.

Another group of samples frequently used to monitor substances are animal feed and drinking water. Feed is difficult to analyze because the matrix is abundant in proteins and carbohydrates. However, the drug concentrations in feed are usually much higher than in animal tissues; consequently, drugs can be more easily detected.

It also may be attractive to analyze feces, urine, hair and plasma, in which the drug concentration is elevated. They are mostly used to monitor prohibited substances and can be taken prior to slaughtering. The advantage is that the animals can be destroyed to prevent them from reaching the market after the "non-compliant" results are obtained. Although, for corticosteroids urine represents a good tool (3); hair is currently used in forensic analysis and should be a matrix of choice for long-term corticosteroid misuse determination—that is, when residues cannot be detected any more in urine or manure.

Sampling and Sample Preparation

An effective isolation from the complex biological matrices is necessary prior to the determination of corticosteroids, because of the low residue levels of corticosteroids or their metabolites in the investigated biological matrices. Sample treatment is of major importance when physicochemical techniques are used. The main objectives of sample treatment are (1) removal of macromolecules and other matrix constituents that may affect the detection, and (2) the enrichment of the analytes for the purpose of achieving the required low limits of detection that are often below the μg/kg or ppb range. Most sample preparation procedures include an extraction followed by one or more clean-up steps.

Extraction

Most published methods are based on the analysis of the free corticosteroids. So,Sample extraction might be combined with de-fattening, protein precipitation, and a deconjugation step for compounds excreted in the urine (e.g., glucuronides or sulphates). Hexane is often used to achieve the aim of defatting (45). Corticosteroids have a great affinity to the protein binding. Corticosteroids -protein binding can be destroyed by several methods, which include protein denaturation (trichloroacetic acid, TCA) (5), enzymatic/chemical hydrolysis and protease treatment to quantitatively recover protein-bound residues, or degrading the matrix efficiently (5, 44, 45, 48, 53, 55, 57, 61, 62). *Helix pomatia* juice, which contains β-glucuronidase and arylsulphatase, is widely used to the release of corticosteroids from glucuronide and/or sulphate conjugates when they have to be determined in urine or liver. Sometimes cleaner extracts can be obtained by using the two specific enzymes instead of the juice. The use of enzymatic digestion prior to purification is attracting renewed interest in recent years. Van den hauwe et al. (55) optimized enzymatic hydrolysis conditions using liver samples of a calf treated simultaneously with beta and dexamethasone.

Solvent extraction can be carried out using different solvent or mixtures (e.g., acetonitrile, ethyl acetate, dichloromethane, methanol or water). Methanol and acetonitrile simplify this part of the process because they can simultaneously precipitate the proteins and extract the corticosteroids.

For a number of corticosteroids screening tests are available which require limited sample clean-up. In other cases, combinations of homogenization, purification and enrichment, or filtration and centrifugation steps in the off-line or on-line mode are often performed.

Purification

Purification as a sample preparation method plays a very important role in various analytical fields. Biological materials (e.g., urine, blood, saliva, hair and tissue, and milk) contain many endogenous compounds that can cause interferences during analysis or affect target analytes. A successful isolation of target analytes from biological matrices is needed prior to the instrument analysis.

Purification was often composed of three parts: (1) purification and enrichment—including liquid-liquid extraction, immunoaffinity clean-up, solid-phase extraction, dialysis, size-exclusion chromatography, etc.; (2) filtration and centrifugation—removal of solids; (3) derivatization—pre- or post-separation labelling or analyte conversion. Liquid-liquid extraction (LLE), liquid-solid extraction (usually solid-phase extraction (SPE)), ion-exchange and reversed-phase cartridges, Extrelut columns (diatomaceous earth), gel-permeation chromatography are often used for the purification of samples.

SPE on commercially available disposable columns has become the most common technique for selective analysis of corticosteroids from a variety of biological matrices today (4, 5, 44, 47, 48), while classical methods have employed liquid–liquid extraction (46, 48). LLE and SPE were also used in combination: after analyte isolation by means of LLE, the drugs were subsequently enriched by using a suitable SPE procedure (48). For the rest, the introduction of various types of co-polymeric sorbents (SiOH, Oasis-HLB, NH^2) has helped to make SPE a more robust extraction technique. Taking account of the characteristic C_{17}side-chain of the corticosteroids, the efficient purification of SiOH-SPE was often exploited because of strong interactions between solid-phase silanol groups and the corticosteroids polar groups in C_{17}, C_{20} and C_{21} (4, 44, 49, 63).

Ideally, a sample preparation method should be fast, easy to perform and solvent-free. Modern purification techniques are attractive alternatives to time and solvent-consuming classical extraction methods. Several methods, based on pressurized liquid extraction (PLE) with a commercial solvent extraction (ASE) from Dionex or with home-made devices, have been proposed. The effectiveness of ASE is based on the increases in analyte solubilities that occur at temperatures above the boiling points of commonly used solvents. Draisci et al. (60) developed this extraction protocol for determination of several classes of corticosteroids from bovine liver samples. PLE has been a very effective technique for isolating analytes from fat-containing matrices (20, 21), as it can use water at high pressure and high temperature to extract polar drugs. This innovative extraction technique is based on using high pressure and high temperature to keep the extracting solvent in the liquid state at temperatures above its boiling point; advantages are rapidity, simplicity, small amounts of solvent waste, automated procedures and the possibility of using water as extractant (non-toxic, non-pollutant and cheap). Kurečková et al. (6) used supercritical fluid extraction (SFE) and solid-phase microextraction (SPME) for isolation of four corticosteroids from biological matrices, and demonstrated the eventuality of the use of this fast solvent-free technique in corticosteroids analysis.

Other sample-preparation techniques used for the analysis of corticosteroids residues are ultrafiltration (UF) (50), dialysis and microwave-assisted solvent extraction (MASE) (7). The application of matrix solid-phase dispersion, immunoaffinity extraction (8, 9) and molecular imprinted polymers is reported in a few papers only.

ANALYTICAL METHODS

In practice, a control program is often composed of screening phase and confirmation phase, which use appropriate analytical methods respectively. There are clear differences between screening and confirmatory methods. When samples are suspected to contain an illegal growth-promotor, screening methods are chosen to detect the presence of substances at the level of interest, because avoiding false non-compliant results now is of overriding importance. On the other hand, confirmatory methods are intend to provide full or complementary information enabling the confirmation of the identity of the substance. Both of them have their specific demands, which should be kept in mind when selecting an appropriate analytical technique.

Screening Methods

Coving a large number of samples, screening methods usually aim at providing a "compliant/non-compliant" answer. Thus, there is a need for screening methods that allow the analysis of such a large number of samples in short periods of time. This means that sample throughput will be an important criterion since speed is of the essence. These methods must be able to detect substances at the level of interest. Some false positives (false compliant) are acceptable because they will be further submitted for confirmatory analysis. However, a minimum number of false negative results (non-compliant) must be avoid because they will not be further analyzed.

There are different techniques available for the screening of residues in animal foods. Immunoassay, spectrophotometry (10), high performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and capillary zone electrophoresis (CZE) (11) are the most important techniques used in screening methods.

Immunoassay

For detecting corticosteroids, immunoassays and related immunochemical analytical are widely used in screening methods. Table 3 outlines types of assays. The antibody antigen interaction is highly selective and theoretically enables analytical procedures to be carried out without sample treatment. However, non-specific binding of matrix components that are present in large excess is a distinct problem. In practice, therefore, some form of sample pretreatment is necessary. The assay is performed by bringing the antibodies into contact with the analyte and adding an amount of radio-, enzyme-, or fluorescent-labelled analyte, which competes with the non-labelled analyte for the available binding sites. The amount of labelled analyte bound is then determined directly or after the addition of a suitable substrate that is transformed into a selectively detectable product. Nowadays, most immunochemical residue methods are ELISA, radio-immunoassays (RIA) or fluorescence polarisation immunoassays (FPIA). These methods provide only semi-quantitative analysis and sometimes give rise to false positives, but they continue to be used because of their simplicity in sample preparation, their sensitivity, their speed and their cheapness.

RIA and ELISA are considered to be sensitive techniques for the detection of very small quantities of drug residues in biological matrices. A primary consideration in the immunoassay was to devise a technique which would be adaptable for as many analytes as possible, by ELISA and RIA methods , polyclonal antibodies have been produced, representing the first steps in producing multi-analytes or generic immunoassay screening techniques for corticosteroids residues. In addition, many of them are aimed at the detection of banned synthetic corticosteroids.

Different works have aimed to develop antibodies able to recognize analytes not covered in commercial immunoassays. In corticosteroids immunoassay, it is always required to conjugate carrier protein with a corticosteroids derivative for immunogen preparation to generate antibody. Similarly, enzyme is required to conjugate with corticosteroids derivative to prepare a enzyme conjugate for monitoring antigen–antibody interaction. Thus, there is an increase or decrease of labeled corticosteroids recognition by antibody that affects sensitivity of the assay. Spacer in the enzyme conjugate for hapten can greatly affect the sensitivity of heterologous assay of hapten-like steroids. It may also affect the cross-reactivity for some assays (21). Dexamethasone-21-hemisuccinate, dexamethasone-6- carboxymethyl thioether and

TABLE 3
Immunoassay methods for corticosteroids residues

Analyte	Type of assay	Basis	Matrix	Sensitivity(LOD)	Cross-reacivity	Ref.
Dexamethasone (DEX)	ELISA	Based on anti-DEX antibody prepared from an immunogen of DEX-21-HS-BSA	Urine	—	Dexamethasone: 100% Flumethasone: 96.0% Betamethasone: 37.0% Triamcinolone: 17.3% Prednisolone: 3.5%	12
Corticosteroids	ELISA	Based on anti-FLM antibody prepared from an immunogen of FLM-human serum albumin	Urine	Flumethasone 2.5μg/L, Dexamethasone 3.1μg/L Betamethasone 12.5μg/L	High-reactivity with several synthetic corticosteroids whilst endogenous corticosteroids such as cortisol gave very low cross-reactivity (< 0.5%)	13
Corticosteroids	ELISA	Based on anti-DEX antibody prepared from an immunogen of DEX-21-HS-BSA with PRE-21-HS-HPR as the coating antigen	Urine Plasma	Urine: 1.45 μg/L Plasma: 0.51 μg/L	Dexamethasone: 100% Flumethasone: 103% Betamethasone: 45% Triamcinolone: 18% Prednisolone: 17 % Endogenous GC: $\leq$ 0.4 %	14
Dexamethasone	ELISA	Based on anti-DEX antibody prepared from an immunogen of 4-CMT-DEX- BSA	—		Betamethasone: 6.7% Prednisolone: 1.6%	15
Cortisol	ELISA	Based on anti-cortisol antibody prepared from an immunogen of cortisol-3-O- CMO-BSA	—	0.5 μg/L	Corticosterone: 1.7% Cortisone: 10% GC: $\leq$ 0.1%	16
Betamethasone	RIA and ELISA	Betamethasone-3-(O-carboxymethyl)oxime-beta-D-galactosidase conjugate as a labelled antigen and 4-methylumbelliferyl-beta-D-galactoside as a fluorescence substrate	Plasma	ELISA: 0.15 μg/L RIA: 2 μg/L	Cortisol: 0.008%	17
11-Deoxycortisol (11-DC)	ELISA	Noncompetitive ELISA. Based on monoclonal anti-DC antibody prepared from an immunogen of 11-DC-BSA	Serum	0.01 μg/L	< 5%	18
6β-hydroxycortisol (6β-OHC)	ELISA	ELISA Based on anti- 6β-OHC monoclonal antibody prepared from an immunogen of 6β- OHC-3-CMO- BSA,OVA,HSA	—	200 μg/L	< 10%	19
Cortisol	ELISA	Non-competitive ELISA based on anti-cortisol antibody prepared from cortisol-3-carboxymethyloxime-BSA	—	0.2 nmol/L	—	20
Cortisol	ELISA	Based on anti- cortisol antibody prepared from an immunogen of cortisol-3-O- CMO and cortisol-21-HS	—	—	< 0.1% with steroids except cortisone, 17 a-OH-progesterone and synthetic corticoids prednisolone.	21
Dexamethasone	RIA	Based on anti-DEX antibody prepared from an immunogen of DEX-21-HS-BSA with TC-ABA-OVA as the coating antigen	Plasma	0.5 μg/L	—	22

ELISA, enzyme-linked immunoassay; LOD, Limit of detection.

dexamethasone-3-(O-carboxymethyl) oxime were synthesized by Shibata et al. (23) for the purpose of obtaining specific antisera used for enzyme immunoassay of dexamethasone. The specificity of each antiserum was assessed by determining cross-reactivity of other corticosteroids by means of ELISA. Anti-dexamethasone-21- hemisuccinate is prove to have lower specificity than others. Nishiguchi et al. (15) obtained an antiserum with high specificity by the immunization of rabbits with 4-(carboxymethylthio) dexamethasone-bovine serum albumin conjugate. Recently new developments tend to prepare multi-residues antibody. So, anti-dexamethasone-21-hemisuccinate is considered to be available since they can recognize several compounds within a group of corticosteroids. However, whether the polyclonal antibody produced by dexamethasone-derivatives is the best choice for the muli-residues detection need further more research. Now, our lab is engaging in the work.

ELISA methods have been developed for the detection of many corticosteroids for many years. A study carried out by Roberts and Jackson (12), developed an ELISA for the screening of dexamethasone in equine urine, which can be used to detect the residues in unextracted diluted urine directly. A successful attempt has been made to raise antibodies against the flumethasone (13), betamethasone, cortisol (16, 20, 21), 11-deoxycortisol (18), and 6β-hydroxycortisol (19). RIA methods for corticosteroids also have been developed by several investigators (17, 22). A routinely used RIA method was compared with an ELISA assay for the determination of betamethasone by Kominami et al. (17), who drew a conclusion that ELISA assay is more sensitive than the RIA, the specificity is also sufficient. However, the precision of the ELISA is inferior to that of the RIA.

Commercial kits have been evaluated in various foods of animal origin following the EU criteria for qualitative screening methods. A large number of ELISA-based methods for corticosteroids residues is commercially available as a kit. For example, there are at least three kits on the market for the detection of the most extensively used dexamethasone, betamethasone, flumethasone, triamcinolone, and prednisolone in urine, milk or tissue and feed samples (Table 4). They are all classical competitive ELISA methods requiring microtiter plates and a spectroscopic reader. In all instances only minimal sample handling is required. Polyclonal antibodies (rabbit anti-dexamethasone-21 derivaties) have been produced for developing ELISA tests for

TABLE 4
Microtitre plate based enzyme immunoassay (ELISA) for the screening of corticosteroids

Analyte	Matrix	Sensitivity (LOD)	Cross-reacivity	Precision	Ref.
Corticosteroids	Bovine urine, liver, milk and feed	0.1 ppb (0.1μg/L)	Dexamethasone: 100% Flumethasone: approx: 100% Betamethasone: approx: 54% Prednisolone: approx: 37% Triamcinolone: approx: 67% Cortisolo: approx: 3% Budesonide: approx: 40% Desonide: approx: 10% Beclomethasone: approx. 16%	Intrassay: CV < 7% Interassay CV < 15%	24
Dexamethasone Betamethasone Flumethasone Triamcinolone Prednisolone	Milk, urine, tissue and feed samples	Dexamethasone Betamethasone Flumethasone: 0.15 ppb. Triamcinolone: 0.45 ppb Prednisolone: 0.75	Flumethasone: 125% Dexamethasone: 100% Betamethasone: 93% Triamcinolone: 33% Prednisolone: 21%	—	25
Corticosteroids like dexamethasone, prednisolone, isoflupredone	Different matrix	—	Dexamethasone: 100% Desoxymetasone: 100% Betamethasone: 87% Prednisolone: 140% Flumethasone: 125% Isoflupredone: 100% Triamcinolone: 81% Cortisol: 16% Prednisone: 10%	—	26

ELISA, enzyme-linked immunoassay; LOD, limit of detection; CV, coefficients of variation.

them, representing the first steps in producing multi-analytes or generic immunoassay screening techniques for these drug residues. Class-specific multi-residue immunochemical tests are employed by preference, since they can recognize several compounds within a group of drugs (see Table 4). However, the possibility of high cross-reactivities should be proven in further research. The limits of detection range from 0.2–1.0 ppb in milk and muscle, to 1–5 ppb in liver. The limits are supposed to be sufficiently low to ensure that animals negative in the test will not contain violative levels of dexamethasone and betamethasone in milk, and meat.

In the recent years, there have been few applications of new immunoassays for the detection of corticosteroids . The demand for rapid, sensitive and accurate methods to screen biological samples has increased in China. In particular, tests that can be completed within minutes or hours would enable processors to take quick corrective actions when corticosteroids are detected. Traditional immunoassay (eg., ELISA and RIA) can't fulfill the mission. Hence, the development of portable, rapid and sensitive technology is crucial for this purpose. In recent years, biosensor technology has been applied to the detection of steroids in foods frequently (27). Molecular imprinting is an inexpensive method for the rapid fabrication of organic polymeric and inorganic network-structured materials that selectively bind a template molecule—in other words, materials that function as artificial antibodies (28). Nanocrystals (quantum dots) and other nanoparticles (gold colloids, magnetic bars, nanobars, dendrimers and nanoshells) also have been receiving a lot of attention recently with their unique properties for potential use in the residues determination (29). Recently, we have developed new immunoassay techniques for the detection of multi-residues of corticosteroids with more sensitivities and timesaving. A new indirect competitive fluorescence-linked immunosorbent assay method for the detection of betamethasone using quantum dots (QDs) as the fluorescence label coupled with secondary antibody was developed. The limit of detection (LOD) of the method could be reached at 0.05 ng/g and it was applied to detect the positive chicken tissues, and was compared with the LC-MS-MS. The relationship between was very well. We also developed the monoclonal antibodies for multi-determination of dexamethasone (DEX), prednisolone (PRE), flumethason (FLM), and triamcinolone (TRA) at the same time. The LOD was 0.2, 0.2, 0.1, and 0.1 ng/g respectively. The commercial kit had been developed and was used in the market in China.

High-Performance Liquid Chromatrography

HPLC is another technique that is often applied to determinate corticosteroids residues for screening aim. The use of HPLC expanded during the 1990s and the availability of automation somehow facilitated its use as a screening technique. The choice of the detection system is very important for selectivity and sensitivity. Usually, the detection of multi-residues is based on a SPE cleanup followed by filtration and injection into a reverse phase HPLC with UV-diode array detection (30). However, sometimes, the UV detector proved to be insufficiently sensitive for the residues detection purpose. So the elaborated detection systems are employed. The method based on immuno- affinity chromatography followed by reverse-phase high-performance liquid chromatrography (IAC-HPLC) with diode array detection (DAD) was developed for dexamethasone detection in feed and drinking water in livestock by Reig (8). Fluorescence detection was shown to be adequately sensitive for the 4 mg formulation of TRA, with the limit of quantification (LOQ) equal to 2.5 μg/L and comparable to the method where six corticosteroids, but not TRA, were determined (31). The other way to improve sensitivity of the HPLC methods for determination of some corticosteroids by HPLC involved pre-column derivatization of the compounds (32). However, when pre-column derivatization is used, the problem is that isomeric forms may appear. The method based on the chemiluminogenic cerium(IV) sulfite reaction involves certain risks during handling. Iglesias et al. (33) used the luminal chemiluminescence (CL) detection system with luminol in the presence of the oxidant hexacyanoferrate (III), in alkaline solution, for the simultaneous determination of nine corticosteroids, thereby avoiding the above problems.

In recent years, micellar liquid chromatography has been developed for the determination of corticosteroids (30, 34–37). M. Capella–Peiró et al. (34) developed a simple and reliable micellar liquid chromatography for detecting seven corticosteroids in creams, ointments and other pharmaceuticals. An isocratic liquid chromatographic method for the determination of betamethasone (BM) and dexamethasone (DM) using micellar mobile phases consisting of cetyl trimethyl ammonium bromide (CTAB) has been developed by R. Gonzalo–Lumbreras and Peña-Garcça-Brioles et al. (35, 30).

Apart from usual screening techniques, many high throughput analytical methods are applied to screening of animal tissues in recent years. Schumacher et al. (38) developed a dual luciferase receptor assay to detect residues or contaminants with corticosteroids activity. The assay was performed by transfection of human cell lines with two reporter constructs followed by the measurement of two distinct luminescence signals, one of which served as internal control to correct for assay variabilities and unspecific matrix effects.

The use of spectrophotometry and TLC for the determination of corticosteroids is very limited nowadays.

Confirmatory Methods

When a screening test indicates the presence of a violative concentration of a drug residue, method selectivity will no doubt be the main criterion because avoiding false non-compliant results now is of overriding importance. In this situation, a confirmatory method is of interest because it provides full or complementary information enabling the confirm the identity of the substance. Several techniques or a combination of techniques are considered suitable for identifying corticosteroids, mainly gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

GC-MS

Many authors have proposed methods based on GC-MS to detect corticosteroids (7, 39–41), including chemical oxidation or derivatization, because this method provides good sensitivity and is sufficiently selective for use as a confirmatory technique. Z. Iqbal et al. reported a procedure for the quantification of endogenous steroids in bovine aqueous humour and vitreous humour by gas chromatography with negative chemical ionization mass spectrometry (GC-CI (−)-MS) (40). The method was also adopted by Olga Huetos Hildago et al. (39) for the detection of dexamethasone in urine of treated cattle after oxidation of the analyte to the 11, 17 diketo derivative using pyridinium chlorochromate. For urine the LOD was 0.2 μg/L. However, GC-MS requires derivatization of the steroids by means of silylation, acylation or oxime/silylation, depending on the properties of the individual steroids. The long separation time, the lack of a universal derivatization agent, the failure of some corticosteroids and problems with chemical rearrangement of others, strongly stimulated the development of LC-MS-based methods.

LC-MS

Today, there is an increasing interest in LC-MS-based procedures,mainly LC-MS^2. It has become the mainstay analytical technique since sample preparation can be simplified without time-consuming derivatization, shorter chromatographic run time and high sensitiveity. When mass fragments are measured using techniques other than full-scan, the system of identification points (IPs) is applied. Commission Decision 2002/657/EC contains detailed information regarding the mass of parent and fragment ions and their relative abundance, a range of techniques and combinations thereof and IPs earned. Antignac et al. (42) presented an interpretation of the concepts introduced in the new 2002/657/EC European decision and proposed a practical guideline dedicated to the validation of analytical methods based on mass spectrometry.

LC can be combined with different mass analyzers, [e.g., quadrupole ion trap (IT), time-of-flight (TOF), quadrupole time-of-flight (QqTOF), quadrupole linear ion-trap (QTRAP) or Orbitrap,] through different atmospheric pressure ionization sources. The polarity and functionalities of corticosteroids allow the use of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) sources, in positive-ion or negative-ion modes, and different modes of instrumental acquisition [multiple reaction monitoring (MRM), product ion scan (ProIS), precursor ion scan (PreIS) and neutral loss scan (Nloss),] for MS detection. Table 5 summarizes information on some selected LC methods used for corticosteroids.

A liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) method was developed by Touber et al. (59), using the latest high-resolution LC column technology, the ultra performance liquid chromatography (UPLC^{TM}), and ESI in the positive ion mode. Residues of 17 corticosteroids from urine samples can be confirmed. The UPLC/TOFMS separation obtained required only 5.5 minutes for all the substances tested. Even the critical pair of DM and BM isomers was almost completely resolved. The LOD and LOQ were determined for most of the analytes in calf urine and found to range from 0.1 to 3.3 μg/L and from 0.4 to 4.4 μg/L, respectively. Because of the full mass range fast scanning TOFMS technology, the method can be easily extended with other banned substances of interest, as demonstrated by the addition of 21 β-agonists to the original analyte mixture in urine, without causing any interferences. However, dedicated UPLC/TOFMS criteria regarding the number of IPs, mass accuracy of parent and fragment ions, ion ratio, and relative retention time haven't be assessed; in order to allow application of this new technology for confirmation of identity as well, that should be considered. Application of the LC-TOFMS system to the elucidation of corticosteroids is still just starting and needs much improvement.

Van den hauwe et al. (53) determined 11 corticosteroids in kidey, muscle and hair samples by means of LC-ESI(-)-MRM. They gave particular attention to the extraction procedure for reducing the number of interfering compounds in the final extract and to improving the LODs; nevertheless, the extraction efficiency cannot be estimated because the authors did not report recovery percentages. The ions were the most abundant ions in the MS spectra of all analytes. (M+formate) /$[M\text{-}H\text{-}CH_2O)^-$. Further dissociations include consecutive losses of H_2O, CH_4, HF and HCL, depending on the analyte. All protocols were successfully validated according to European criteria and real grown sample material was analyzed. Through the analysis of residue concentrations, hair sample residue concentrations could be up to 348 μg/kg; therefore, hair can be an interesting and suitable matrix for detection of synthetic corticosteroids misused in livestock farming.

A comprehensive analytical method based on ultra performance liquid chromato- graphy-electrospray tandem mass spectrometry (UPLC-MS/MS) with negative modes has been developed for the simultaneous determination of 12 corticosteroids residues in milk by Xiaoliang et al. (4). The LOD of the method were from 0.02 to 0.38 μg/kg and the LOQ ranged from 0.07 to 1.27 μg/kg. Average recoveries for the 12 corticosteroids (spiked at the levels of 2 and 0.4 μg/kg) ranged from 69.3 to 94.3%. The shortcoming is that the authors adopted a very tedious, time-consuming extraction procedure that annulled the advantage of this method.

Cherlet et al. (61) developed an LC-APCI (+)-MRM method for determining dexamethasone residues in bovine milk. The deproteinization step was accomplished by adding TCA; SPE on a C_{18} was used to clean-up. The sample preparation is simple and quick but the recovery was low, just 56%. The decision limit (CCα) and detection capability (CCβ) were 0.48 and 0.76 ng/mL, respectively.

The same authors submitted this analytical method to a validation protocol to confirm the occurrence of DM in bovine plasma and tissue (5); LOD were 0.09, 0.13 and 0.33 μg/kg

TABLE 5
Selected confirmatory methods for determining corticosteroids

Analyte	Technique	Sample preparation	Separation	Detection	Matrix	Sensitivity (LOD, LOQ or CC_β) μg/kg or L	Ref.
Dexamethasone-acetate	LC-MS^2	—	—	ESI positive SIM/Full scan	Urine	6	43
Prednisolone-acetate							
9 corticosteroids							
Betamethasone, dexamethasone, corticosterone Tramcinolone methylpredisolone, prednisone	LC-EIMS	C_{18}–SPE/Si-SPE/NH_2-SPE	UltrasphereE UP5ODB C_{18} interchrom (150 × 3 mm, 5μm) ace tonitrile and formiate buffer (pH 3) at 0.25 mL/min	EIMS negative SIM	Hair urines	0.1	44
23 corticosteroids (separation of DEX and BET)	LC-MS, confirmed by LC-MS^2 positive	Hydrolyzed	Reversed phase DB-8 column (75 mm × 4.6 mm, 3 μm) 1.0% acetic acid/methanol at 1.0 mL/min.	APCI positive full scan	Urine	5	45
9 abused synthetic corticosteroids	LC-EIMS	XAD-7 resin L-LPE	Inertsil 3 ODS-3 column (150 × 3 mm, 3 μm), 1 mM ammonium acetate solution(PH 6.8)/acetonitrile(60:40,v/v) at 0.4 mL/min	EIMS negative SIM	Urine	$\leq$ 1	46
12 corticosteroids	UPLC-ETMS	Defatted, Oasis HLB, Si, NH_2-SPE	Waters ACQUITY UPLCTM BEH C_{18} column (100 × 1.0 mm, 1.7 μm). methanol and water with 0.1% formic acid at 0.1 mL/min	ETMS negative SIM	Milk	0.02–0.38	4
Cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide	LC-MS^2	Oasis HLB- SPE	Symmetry C_{18}, ammonium acetate–methanol (pH 3.5)	ETMS negative SIM	Plasma	3.6–7.2	47
Dexamethasone	LC-APCI-MS^2	TCA SPE	PLRP-S polymeric reversed phase column (250 × 4.6 mm, 8 μm), 0.1% acetic acid/ acetonitrile at 1—1.5 mL min	APCI-TMS positive SIM	Plasma tissues	0.09–0.33	5
Flumethasone	LC-MS^2	Enzymatic hydrolysis SPE/LLE	C_{18} Nucleosil (250 × 2.1 mm) MeOH/ammonium formate 1 mM (65/35) at 0.150 mL/min	API-ETMS negative SRM	Urine serum	30 pg/mL (0.03)	48

(Continued on next page)

TABLE 5
Selected confirmatory methods for determining corticosteroids *(Continued)*

Analyte	Technique	Sample preparation	Separation	Detection	Matrix	Sensitivity (LOD, LOQ or CC_β) μg/kg or L	Ref.
Cortisone, cortisol, cortexolone, corticosterone, dehydrocorticosterone deoxycorticosterone	LC–ESI-MS	Si-SPE picolinoyl derivatization	Puresil 5C_{18} (150 × 4.6 mm, 5 μm), CH3CN/ 0.25%CH3COOH (45:55, v/v) at 1 mL/min	ESI positive SRM	Saliva	0.05	49
22 corticosteroids	LC-ESI-MS	0.45 um filter	Zorbax Rx-C_8columns (150 × 2.1 mm, 5 μm) water–0.1% acetic acid 0.4 mL/min	ESI positive SRM	Urine	1.0	50
10 corticosteroids	LC-IS-MS	C_{18}-SPE	Novapak C_{18} Waters, 150 × 2.0 mm,4 μm) acetonitrile / 2 mM NH COOH, pH 3.0 at 0.2 mL/min	IS-MS positive and negative SIM	Hair	30–170	51
20 Corticosteroids	LC-MS	C8-SCX-SPE	Reversed phase Supelcosil TM LC-8-DB column (3.3 cm × 2.1 mm, 3 μm; acetic acid/ammonium acetate/methanol at 0.2 mL/min	MRM positive, ESI negative	Urine	5	52
11 corticosteroids (DEX,PRE,BET,FLM,TRA prednisone, methylprednisolone, fludrocortisone, triamcinolone acetonide, beclomethasone, I.S. isoflupredone)	LC-MS^2	Enzymatic digestion SPE	Hypersil Hypercarb column (100 × 2.1 mm, 5 μm) acetonitrile/water (90/10v/v) + 0.3% (v/v) formic acid at 0.22 mL/min	ESI negative MRM	0.3–42.3	Tissue hair	53
Corticosteroids	LC-MS^2	HPLC	Symmetry column C8 (2.1 × 150 mm, 5 μm) water and methanol with 0.02% TFA at 0.3 mL/min	ESI positive MRM	0.07–0.1 nmol/kg	Tissue	54
11 corticosteroids	LC-MS^2	Enzymatic hydrolysis SPE	Hypercarb column (100 × 2.1 mm, 5 μm), acetonitrile /water (90/10 + 0.3% formic acid) at 0.22 mL/min	ESI positive and negative MRM	0.1–0.5	Liver	55
Corticosteroids	LC-MS^2	-	Synergi MAX-RP 80A Phenomenex column (150 × 2.00 mm, 4 μm) 0.1% acetic acid in water at 0.4 mL/min	ESI negative and positive MRM	0.2	Liver, kidney	56

Dexamethasone, flumethasone, fluorometholone, beclomethasone, triamcinolone acetonide	LC-MS^2	Enzymatic hydrolysis, SPE	C18 Alltima Column (2.1 × 150 mm, 5 μm) ,acetonitrile:water (40:60 (v/v)) at 0.3 mL/min	APCI-TMS positive SRM	2	Urine	57
19 corticosteroids	LC-MS^2	SPE	Reversed-phase SupelcosilTM LC-8-DB column(3.3 cm × 2.1 mm , 3 μm); acetic acid, ammonium formate (pH3.8) at 0.2 mL/min	API-ESI negative and positive MRM	–	Urine	58
17 corticosteroids	UPLC-TOFMS	—	Acquity(Waters)BEH- C^{18} column(50 × 2.1 mm., 1.7 μm) 0.1% and 0.2% formic acid in acetonitrile/water; 0.1% and 0.2% acetic acid in acetonitrile/water at 0.35 mL/min	ESI positive	0.1–3.3	Urine	59
Dexamethasone, betamethasone, flumethasone	LC-MS^2	ASE	Reversed-phase Kingsorb C_{18} column (250 × 2 mm, 5 μm), acetonitrile/5 m*M* ammonium acetate /methanol(35:60:5, v/v) at 0.1 mL/min	APCI-ESI negative full scan	1.0	Liver	60
Dexamethasone	LC-MS^2	Protein-removed, SPE	PLRP-S polymeric reversed phase column (250 × 4.6 mm, 8 μm) 0.1% acetic acid in water/acetonitrile at 1–1.5 mL/min	APCI-ESI positive	0.15	Milk	61
6 corticosteroids (triacinolone acetonide, flumethasone, dexamethasone, betamethasone, methylpredinisolone and prednisolone)	LC-MS^2	Enzymatic hydrolysie	Hypersil Hypercarb(4.6 × 100 mm,7 μm), Water/acetonitrile, each with 0.5% acetic acid (10/90,v/v) at 1.5 mL/min	ESI negative	0.2	Bovine liver	62
12 corticosteroids	LC-MS^2	Acid (hair) or enzymatic (urine and meat) hydrolysis, SPE-C_{18},Si-SPE	Octadecyl grafted silica Nucleosil C_{18} AB (50 × 2 mm, 5 μm), methanol/ 0.5% (v/v) acetic acid in water at 0.22 uL/min	ESI negative	2.9–9.3 pg (0.029–0.093,0.04–0.07)	Hair, urine, meat	63

CCβ, detection capabilities; lOD, Limit of detection; LOQ, limit of quantification; LLP, liquid-liquid partitioning; SPE, solid-phase extraction; ASE: accelerated solvent extraction; TCA, trichloroacetic acid; LC-MS^2, liquid chromatography tandem mass spectrometry; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; LC, liquid chromatography, MS, mass spectrometry; SIM, selected ion monitoring; SRM, selected reaction monitoring; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography

and CCβ were 1.8, 1.9 and 2.5 μg/kg for muscle, kidney and liver tissues, respectively. In muscle tissue, low levels of DEX were found which were far below the MRL within 4 days of withdrawal. Much higher levels of DEX were found in kidney and liver tissues. DEX levels fell well below the MRL for liver tissue after 8 days, while for kidney, due to an unknown retention mechanism, DEX still remained above the MRL at that time. The results indicated that liver tissue provides a suitable matrix to monitor the presence of illegal residues of DEX in slaughtered animals and that the conventional therapeutic use of DEX causes drug concentrations in edible tissues that may exceed the MRLs by more than an order of magnitude.

Now, we have reviewed the experience with a variety of LC-MS techniques for the determination of corticosteroid since 2000. We can draw a conclusion that liquid chromatography–electrospray tandem mass spectrometry (LC-ETMS) and liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (LC-APCI-TMS) are currently the most ideal tools for monitoring corticosteroids. By applying the selection reaction monitoring (SRM) mode, these systems achieve the best sensitivity and selectivity. Another recent development is TOF-MS, allowing considerably higher selectivity due to substantially increased mass resolution.

Moreover, coupling chromatographic procedures to novel detection techniques may provide a very sensitive, specific analytical system for residues analysis (64, 65). Zhang et al. (65) evaluated strategies for coupling CL detection with HPLC for simultaneous determination of corticosteroids residues such as triamcinolone, prednisolone, hydrocortisone, cortisone, methylprednisolone, dexamethasone and TCA. The procedure was based on the enhancement effect of corticosteroids on the CL reaction between luminol and the complex of trivalent copper and periodate ($[Cu(HIO_6)_2)^5$), which was on-line electrogenerated by constant current electrolysis. Under optimum conditions, the LOD at a signal-to-noise ratio of 3 ranged from 0.08 to 1.0 μg/kg and the LOQ at a signal-to-noise ratio of 10 ranged from 0.27 to 3.33 μg/kg for seven corticosteroids. The average recoveries for corticosteroids (spiked at the levels of 5–50 μg/kg) in pig liver ranged from 88 to 106%. The result indicated that the proposed HPLC-CL method could be potentially used for the routine monitoring of corticosteroids residues in animal tissues.

CONCLUSION AND TRENDS

The present review shows that many techniques are available and various strategies can be envisaged to determine residues of corticosteroids in biological products. This leaves one with the problem of selecting the most appropriate control system for a specific situation. Some general conclusions regarding residue analysis of corticosteroids in biological samples are presented below. In addition, relevant trends are indicated.

There are clearly differences between screening and confirmatory methods. Screening methods can detect the presence of a substance or a class of substances at the level of interest and mainly include bioassays, while confirmatory methods provide full or complementary information enabling unequivocal identification and, if necessary, quantification of the analyte at levels of interest. The most commonly used analytical techniques (ELISA, GC-MS, and LC-MS) were compared by Pujos et al. (66); they also assessed the possibilities and the sensitivity of each techniques for application to doping tests. The results were presented in Table 6.

TABLE 5
Comparison of methods for analyzing corticosteroids [66]

	ELISA	GC–MS	LC–MS
Advantages			
Preparation	Low	—	—
Analysis	Short	Specific short	Specific
Disadvantages			
Preparation	—	Long obligatory	Long obligatory
Analysis	Non-specific	—	Long (2 injections Low sensitivity for endogenous

Current approaches in corticosteroids-residue analysis offer many advantages, which explain why their acceptance has been so fast and general. However, they also have many obvious disadvantages.

There are several arising problems in this field such as:

1. All these materials have a highly complex composition consisting in the mixture of low amounts of several substances, like a "cocktail" that exerts a synergistic effect giving similar efficiency to the use of a single substance at higher and, thus, detectable amounts. The great variety in samples and residues wait to be analyzed. The complexity of the matrix (animal species, tissue type, and the nutritional regime of the animal) also should be taken into account. Regulatory control of residues of corticosteroids is generally performed by analyzing the particular product, e.g., tissue or milk. All these samples are protein rich (from 3% in milk to 20% in meat) that easily bind with corticosteroids, and also contain significant amounts of divalent and trivalent cations which may form complexes with some corticosteroids, all of which can affect the detection.
2. There are the extremely low part-per-billion levels, at which corticosteroids residue should be analyzed. MRLs for some corticosteroids-residues in different kinds of matrices of different kinds of food-producing animals are given. However, unfortunately, such MRLs do not exist for many of corticosteroids. What's more, legislation may differ considerably in different countries, and, for many food commodity residue combinations, there are no set MRLs or clear guidance of the levels of residues permitted. This makes interpretation

of certain results difficult. So, it's essential to establish a comprehensive and authoritative criterion with international agreements.
3. The techniques for sample preparation are considered from a very classical point of view, which is obsolete in many cases, and looking at them superficially, few differences can be observed.

In addition to these problems, we face more strict requirements for the performance of analytical methods:

1. Efforts should be directed to attain high-throughput methods able to extract a large number of samples in a short time. That has been achieved using less sample, less extracted solution, and shorter clean-up cartridges instead of long, home-made, clean-up columns
2. The availability of screening methodologies facilitates the control of corticosteroids in animal-derived foods, reducing the number of samples to be confirmed through tedious and costly confirmatory analysis. Many ELISA-based screening kits, already available in the market, will probably be routinely implemented in the next few years, increasing the number of screened samples with high sensitivity. The improvements in screening methodologies and its implementation will contribute to a better safety assurance of foods of animal origin.
3. Improving strategies for the confirmation of analyte identity is necessary. Using robust atmospheric pressure ionization interfaces—notably ESI, which is preferred for more polar analytes as are often encountered in the present field of interest, and, next, the introduction of triple-quadrupole and ion-trap multi-stage tandem-MS instruments, almost all major classes of corticosteroids are detected, identified and quantified satisfactorily. The gradual introduction of Q-ToF-MS machines, with their distinctly enhanced selectivity and the possibility to calculate element composition, is expected to improve performance even more in the near future. In addition, the use of Q-ToF-MS should help to improve the performance of methods using IP-based criteria for the confirmation of analyte identity: after all, a "non-compliance" conclusion can have dramatic effects.
4. Coupling chromatographic procedures to immunochemical techniques can provide a very sensitive, specific analytical system for either determination or confirmatory analysis.

To increase applicability of these methods, making the methods more reliable and robust will be interesting challenges to advancing the analysis of corticosteroids in animal-derived food.

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