well as in magnitude of concentration, is the result ready to leave the laboratory. Even then, an open mind for the followup of the results of a second analysis in an independent laboratory is necessary.

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Applications of Gas Chromatography–Mass Spectrometry in Residue Analysis of Veterinary Hormonal Substances and Endocrine Disruptors

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1. INTRODUCTION

1.1. Anabolic Agents and Endocrine Disruptors

In Europe, the word "hormones" has a very bad reputation because of the possible danger for public health of residues of some of these products in foodstuffs of animal origin. Toxicologists have demonstrated that DES (diethylstilbestrol, a synthetic estrogen) is a potential carcinogen [1–3]. In human medicine, analogous experiences with DES were found (the so-called DES-daughters) [4]. Recently, several cases of poisoning have occurred in Spain and France due to the consumption of liver from animals treated with clenbuterol [5,6]. Moreover, some environmentally persistent alkyl-phenolic compounds (such as nonylphenol) and perhaps other chemicals show estrogenic activity [7]. These "environmental" estrogens are brought up in relation to the decreasing quality of human sperm and regarded as an assault on the male.

The fields of growth promoters and endocrine disruptors are very large and present many challenges. Treatment of cattle with anabolic components may be detected by the residues present in plasma, excreta, meat, or organs of the animals. In regulatory control at the farm, plasma, urine, and/or feces of the animals may be sampled. At the slaughterhouse, tissue as well as excreta are available for sampling. At the retail level (butcher, supermarket) or in case of import/export, sampling is restricted to tissue only. Finally, all kinds of matrices (powders, cocktails, fodder) circulating on the (black) market must be analyzed for the presence of illegal substances.

The issue of the presence of endocrine disruptors in the environment has initiated a large research effort [8]. The variety of sample materials for estrogens is very large and ranges from pure substances to extremely low levels in surface water.

1.2. Analytical Aspects

For studies unraveling the endocrinology of humans, steroids, especially cortisol and its metabolites and precursors, have been extensively analyzed. Many studies were published on the hydrolysis of glucuronic and sulfate conjugates, the extraction of the analytes, and the subsequent derivatization before gas chromatography (GC) [9]. The coupling of GC with flame ionization detection has played a major role in this field. However, with the availability of reasonably priced benchtop mass spectrometers in the 1980s, the analysis of anabolic steroids using GC–mass spectrometry (MS) within the fields of doping analysis and veterinary residue analysis started to gain popularity quite rapidly. Automated GC–MS instruments enabled high-throughput analysis under routine conditions, for example, for doping analysis during the Olympic Games and for veterinary residue control in cattle, when GC–MS was generally regarded as a very expensive technique.

In this chapter, the application of GC–MS or GC–tandem mass spectrometry (MS–MS) for residue analysis for different types of hormonally active substances is discussed. In addition, special attention is focused upon the forensic use of GC–MS.

2. ANABOLIC STEROIDS

2.1. Introduction

Many analogs of the endogenous steroids 17β-estradiol, 17β-testosterone, and progesterone (Fig. 1) have been developed by the pharmaceutical industry for therapeutic purposes. Functional groups have been modified or added, by small or larger chemical modifications, to alter the strength and mode of action, to enable oral administration, and to influence other pharmacokinetic properties.

In general, a straightforward sample pretreatment is carried out (Table 1). The number of steps depends on the sample matrix and the levels of the substances to be analyzed. For the analysis of anabolic steroids in human urine (doping control), the degree of cleanup needed is generally lower than the corresponding analysis of anabolic steroids in the urine of cattle. In the latter case, high-performance liquid chromatography (HPLC) is often routinely used to further purify the extracts obtained.

2.2. Deconjugation

The deconjugation of steroids is an essential step in the analysis. In general, an enzymatic deconjugation is carried out using commercially available Helix pomatia juice (glucuronidase and sulfatase) or bacterial glucuronidase. Earlier studies describe the use of acid hydrolysis instead of enzymatic hydrolysis. A serious drawback of acid hydrolysis is the greater chance of unwanted reactions such as decomposition and degradation of the target analytes. Because of a lack of suitable conjugated standard analytes, the yield of the deconjugation is difficult to verify. Only a few studies have been published, and some data are available from nonpublished studies. In summary, a 16-hr deconjugation with Helix pomatia juice at 37°C has proven to be the best generally applicable approach, although unwanted side effects may occur.

2.3. Sample Cleanup

Although GC–MS provides a specific method of detection, the sample cleanup plays an important role in the final result. Often the procedures are used for multianalyte analysis destined to detect a number of chemically and/or structurally related analytes in one run. Especially with anabolic steroids, this implies the coextraction of many endogenous steroids and steroid metabolites that may be present at levels far higher than the target levels of the analytes (0.5 to 50 μg/L or kg). Furthermore, other sample constituents can interfere with the analysis and influence the final result both in a qualitative way (false positive or false negative result) as well in a quantitative way. Therefore, it is important that a particular method, if possible, is compared with other methods slightly differing in the extraction procedure [10].

Although limited in the range of analytes to be extracted in a single analysis, immunoaffinity chromatography (IAC) has clear advantages in the selectivity and purity of the isolated fraction [11]. For the confirmation of stanozolol with high-resolution GC–MS, IAC was successfully applied [12]. By combining several types of antibodies raised against different steroids, columns can be prepared to selectively extract mixtures of steroids [13–15].

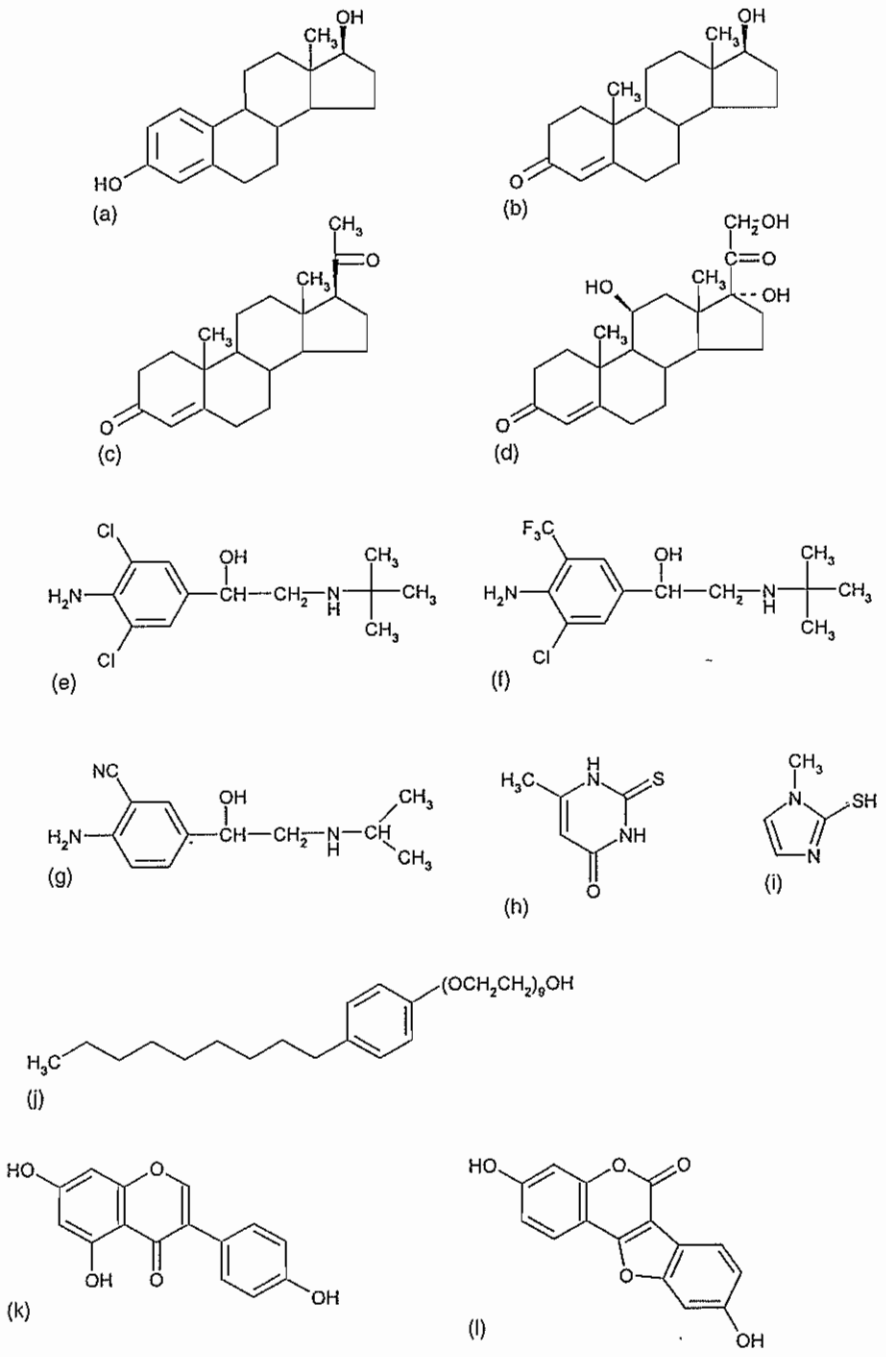

Table 1 Scheme of Analytical Procedures

Urine, bile, liver	Tissue (meat)
Deconjugation	Homogenization
Extraction and purification (L–L, SPE, IAC)	Extraction and purification (L–L, SPE, IAC)
HPLC fractionation	HPLC fractionation
Derivatization	Derivatization
GC–MS analysis (SIM or full scan)	GC–MS analysis (SIM or full scan)

2.4. Derivatization

The need for derivatization procedures applicable for large ranges of analytes to be analyzed within a single method especially necessitates the continuous optimization of this relatively small but very important part of the analytical procedure [16]. The use of internal (deuterated) or external standards is important to check the derivatization yield, which occasionally can show large variations. Because an extensive description of the derivatization itself, despite its importance, is not within the scope of this chapter, the major derivatization procedures are briefly discussed [16,17].

2.4.1. Silyl Derivatives

The majority of analytical procedures employ silylation as the derivatization reaction. Many reagents and combinations have been described. Depending on the catalyst present in a silylation mixture, keto functional groups can be converted into enol-trimethylsilyl (TMS) derivatives. Iodine ions (I^-) in the well-known N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with ammonium iodide and dithioerythritol (1000:2:4 v/w/w) mixture are often used for this purpose [10, 18,19].

2.4.2. Acylated Derivatives

The most commonly used acylated derivative is the heptafluorobutyric acid derivative. An advantage is the high-mass ions generation of high mass ions increasing

Figure 1 Basic chemical structures of growth promoters and endocrine disruptors. (a) 17 β-estradiol, (b) 17 β-testosterone, (c) progesterone, (d) cortisol (hydrocortisone), (e) clenbuterol, (f) mabuterol, (g) cimaterol, (h) methylthiouracil, (i) tapazole (methimazole), (j) nonoxynol-9, (k) genistein (isoflavone), and (l) coumestrol (coumestane).

the specificity. Furthermore, the response of the signals obtained is high, enabling sub-nanogram quantities to be analyzed. A nice example is the determination of 17β-estradiol in bovine plasma [20].

A special use of a combination of acylation and silylation for the GC–MS analysis of stanozolol in calf urine has been described [21,22]. A similar approach has been used for indolalkylamines [23].

The growing interest in GC–combustion–isotope-ratio MS (GC–C–IRMS) has intensified the use of acylation using acetic acid anhydride [24–33]. The advantage is the introduction of a limited number of carbon atoms in order to minimize the effect on the $^{12}C/^{13}C$ ratio.

2.4.3. Methoxylated Derivatives

A specific reagent used for the derivatization of keto groups is methoxylamine or ethoxylamine. The resulting derivative is a stable oxime. This derivatization can be used alone or in combination with silylation. Examples are described for boldenone [34] and chlorotestosterone (clostebol) [35–37].

2.5. Gas Chromatography–Mass Spectrometry Analyses

2.5.1. Gas Chromatography–Mass Spectrometry

As was also discussed in Chapter 18, the analysis of anabolic steroids, other growth promoters, and estrogens has long been closely linked to GC–MS. The low levels in complicated sample materials containing large amounts of closely related substances that are prone to interfere with the analysis, require the use of capillary GC for separation. Remarkably, in (human) doping analysis, split injection has been the method of choice for many years. In the field of veterinary anabolics, this injection technique is not appropriate for this purpose. The target levels are often below 10 μg/L or μg/kg, necessitating the use of splitless injection. Careful maintenance of the GC injector and the column are essential for successful measurements. In most laboratories, fused-silica nonpolar or low-polarity columns, 25 to 30 m in length are used. In particular cases, columns of up to 60 m in length have been used to optimize the separation of different isomers [38].

An additional cleanup of the extracts can be achieved by using GC–GC with column switching. Rozijn et al. [39] have shown that a HPLC cleanup step can be omitted and replaced by GC column switching under routine conditions.

The use of deuterated standards is important for both qualitative and quantitative analysis. In the last decade, many substances have been synthesized by laboratories or chemical companies.

2.5.2. Gas Chromatography–Mass Spectrometry in Multianalyte Analysis

The GC–MS technique is especially suited for multianalyte analysis. Within our laboratories, a large number of anabolic steroids, stilbenes, and resorcylic acid lactones (RALs) such as zeranol are analyzed within one run. After an initial screening with two ions per analyte, suspected samples are reanalyzed using four ions per analyte in selective ion monitoring (SIM) mode, full-scan mode (ion trap) or MS–MS mode (minimum two daughter ions). Using a quadrupole mass spectrometer, the preparation of the instrument settings, i.e., the acquisition parameters, is time consuming. After maintenance of the gas chromatograph (removal of a small polluted part of the column) or column changes, the acquisition parameters of the mass spectrometer must be checked and readjusted.

In Table 2 the mixture of standard analytes and deuterated internal standards used for the calibration of the GC–MS is shown. The analytes were derivatized with MSTFA++ resulting in the formation of TMS derivatives of both hydroxyl and keto groups. The number of derivatization products formed (often di-TMS, tri-TMS, or isomers) is also included in Table 2. The presence of more than one product has the disadvantage that the minimum detectable level is increased. An advantage is that a combination of products enhances the selectivity and the certainty of the identification.

2.5.3. Gas Chromatography–Tandem Mass Spectrometry

After the development of triple quadrupole mass spectrometers and, moreover, the availability of benchtop ion-trap mass spectrometers, MS–MS has greatly extended the possibilities in the laboratory.

Advantages of MS–MS are the decreased background noise due to the isolation of the parent ion and the subsequent fragmentation. In this way, similar fragments not originating from interferences (sample constituents or column bleed) with a mass comparable to the parent ion cannot be present and will not interfere with the analysis [40]. Recent (unpublished) work in our laboratory demonstrated the power of GC–MS–MS (ion trap) as an important tool for the identification of chloroandrostenedione (CLAD), a metabolite of chlorotestosterone, at a level of 0.2 μg/L in urine samples of cattle.

2.6. Use of Mass Spectrometry as a Confirmation or Identification Tool

The use of MS is often regarded as the ultimate tool for confirmation or identification. Confirmation is generally regarded as identical to identification, with respect to the unambiguous determination of the identity of the analyte. However,

Table 2 Selection of Ions for Multianalyte Steroid Screening Using a Mass Selective Detector

Standard analyte	Remark	RT (min.)	Ions selected for SIM acquisition:	Analyte number
Hexestrol		9.939	207	1
Diethylstilbestrol-d6 (*cis*)	istd	9.354	418; 386	2
PCB-138	GC standard	9.81	360	3
Diethylstilbestrol (*cis*)		9.833	412; 383	4
Hexestrol-d4	istd	9.939	209	5
Dienestrol		10.012	410; 395	6
Dienestrol-d2	istd	10.016	412	7
Diethylstilbestrol-d6 (*trans*)	istd	10.019	418; 386	8
Diethylstilbestrol (*trans*)		10.039	412; 383	9
5α-Estrane-3β, 17α-diol		11.204	332; 242	10
Nortestosterone-17α		12.170	418; 194	11
Trenbolone-17α-1-TMS		12.370	342; 211	12
Boldenone-α		12.401	430; 206	13
Estradiol-17α		12.539	416; 285	14
Nortestosterone-17β-d3	istd	12.653	421; 406	15
Nortestosterone-17β		12.691	418; 194	16
Stanolone (dihydrotestosterone)		12.836	434; 405	17
Boldenone-β		13.018	430; 206	18
Estradiol-17β		13.031	416; 285	19
Estradiol-17β-d3		13.067	419; 285	20
Testosterone-17α		13.232	432; 417	21
Trenbolone-17β-d2-1-TMS	istd	13.258	344	22
Testosterone-17β		13.279	432; 417	23
Trenbolone-17β-1-TMS		13.279	342; 211	24
Testosterone-17β-d2	istd	13.289	434; 419	25
Ethynylestradiol-3-methylether-1-TMS (mestranol)		13.491	382; 367	26
Methandriol		13.742	448; 343	27
Norethynodrel (iso-1)		13.799	442; 427	28
Norethindrone		14.109	442; 427	29
Norethynodrel (iso-2)		14.127	442; 427	30
Delta(9)-11-dehydromethyltestosterone	derivatization standard	14.208	444; 339	31

Dianabol-d3	istd	14.218	447; 206	32
Dianabol (methylboldenone)		14.263	444; 206	33
Zearalanon-3-TMS (iso-1)		14.439	521; 307	34
Methyltestosterone-17α-d3	istd	14.514	449; 301	35
Methyltestosterone-17α		14.533	446; 301	36
Ethynylestradiol-17α		14.593	440; 425	37
Norethynodrel (iso-3)		14.610	442; 427	38
17α-Ethynyl-testosterone (ethisterone)		14.709	456; 301	39
Zearalanone-3-TMS (iso-2)		14.712	521; 307	40
Zearalanone-3-TMS (iso-3)		14.964	521; 307	41
4-Chloroandrostendione (iso-1)		15.141	464; 449	42
Zearalanol-α-d4-3-TMS		15.174	437; 307	43
Zearalanol-α-3-TMS		15.263	433; 307	44
Zearalanone-3-TMS (iso-4)		15.369	521; 307	45
Zearalanol-β-d4-3-TMS		15.372	437; 307	46
Zearalanol-β-3-TMS		15.449	433; 307	47
Norgestrel		15.449	456; 316	48
Zearalenone-3-TMS		15.490	519; 305	49
4-Chlorotestosterone (clostebol) (sio-1)		15.555	466; 431	50
17α-CH3-androstan-17β-ol-3-one (mestanolone)		15.567	446; 287	51
Norethandrolone		15.643	446; 287	52
Zearalenol-α-3-TMS		15.950	431; 305	53
Chloroandrostenedione (iso-2)		16.029	464; 449	54
Progesterone iso-1		16.098	458; 443	55
Zearalenol-β-3-TMS		16.202	431; 305	56
2-Methoxy-ethynylestradiol		16.244	470; 455	57
Progesterone iso-2		16.451	458; 443	58
Chlorotestosterone (clostebol)		16.510	466; 431	59
Fluoxymesterone (3-TMS)		16.702	552; 462	60
Fluoxymesterone (2-TMS)		16.992	480; 390	61
4-Bromoestradiol		17.001	496; 365	62
Stanozolol-1-TMS		18.712	400; 385	63

Mw = molecular weight of the derivative.
istd = Deuterated internal standard.

there is a difference between the identification and the assurance that no other substance can lead to the same result. Specifically with regard to the presence of isomers, this distinction is of great importance as is shown in the example in section 2.6.3.

2.6.1. Interferences Related to the Presence of Endogenous Metabolites of Steroids

Using a routine GC–MS procedure in SIM mode for the screening and subsequent identification of anabolic steroids in the urine of cattle, relatively high levels (>50 μg/L) of 17 α-hydroxy-19-nortestosterone (epi-nortestosterone) were likely to be present. In animal experiments with calves after intramuscular treatment with a 17β-nortestosterone ester, it was observed that the levels in urine generally vary between 2 and 10 μg/L for 17β-nortestosterone and between 5 and 20 μg/L for the 17α-metabolite [41]. Closer examination revealed that the ions detected (m/z 346, 331, 256, and 215) were generated by the presence of a reduced metabolite of testosterone, i.e., one of the eight possible tetrahydrotestosterone (THT or androstanediol) isomers. The difference in retention time in that particular case was only a few seconds, approximately 2 to 5 scans. By analyzing all THT isomers, the 5β-androstan-3β, 17β-diol (*bbb*THT) was identified as the interfering substance. Remarkably, all four ions selected of 17α-nortestosterone were present in the spectrum of the interference, although the relative intensities were different (Fig. 2). Applying different margins for the relative intensity ratios will lead to the fulfilment of an increasing number of ion ratios (Table 3). Allowing a larger margin than 10%, i.e., 40%, yields three ion ratios and thus a positive identification.

2.6.2. Criteria for Forensic Analysis in Doping, Crime Investigation, and Detection of Anabolics in Cattle

A number of approaches have been used dealing with the difficult question how many ions are sufficient for an unambiguous identification. Sphon [42] used a simple approach using a (per definition) limited mass spectral library. He concluded that three ions were sufficient to distinguish DES from all other library entries. Many laboratories followed this three-ion approach. Within the European Union, a four-ion approach was officially adopted [43,44]. For the development of these criteria, the approach as described by Pesyna et al. [45] was used to estimate the chance for the occurrence of a steroid with that combination of four ions and their relative abundances based on data from mass spectral libraries. For nortestosterone, this chance, regarded as a chance for a false positive result, was estimated as 1 to $2.7*10^8$.

In many publications reviewed, the use of GC–MS as a tool for unambiguous identification is mentioned. When looked at in detail, a more specific description of the data evaluation is missing or only sparsely mentioned. If a quadrupole mass spectrometer was used, the (diagnostic) ion or ions selected for the measurement are mentioned and often shown in chromatograms. However, the precise way in which the complete mass spectrum or the selected ion spectrum was evaluated is infrequently discussed. Infrequently, the relative intensities of the ions measured are compared with the relative intensities obtained for analytical standards.

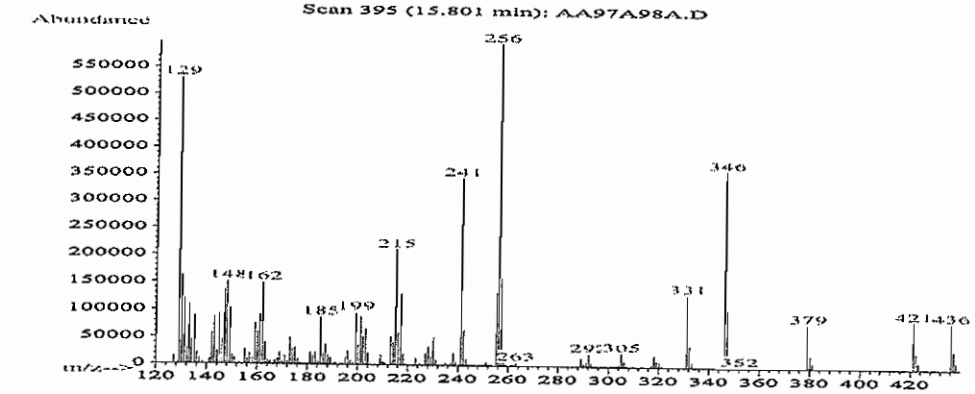

Figure 2 Mass spectra of the TMS derivatives of 5β-androstan-3β, 17β-diol (*bbb*THT) and 17α-nortestosterone (αNT)

Table 3 Effect of the Margin Applied on Fulfilling the Ion Ratio Criteria Applicable for 17α-Nortestosterone in Case an Endogenous THT Isomer Is Present

THT isomer	Margin (%)	No. ions	m/e	m/e	m/e	m/e
5bA3b17bD	10	1	256			
	15	1	256			
	20	2	256	215		
	25	3	256	215	241	
	30	3	256	215	241	
	35	3	256	215	241	
	40	4	256	215	241	331

2.6.3. Similarity of Spectra

Special attention is drawn to the analysis of isomers with GC–MS. An illustrative example is the analysis of dexamethasone and betamethasone. The analytes are identical with only one difference in the orientation of the 16-methyl group (α resp. β). Usually in reversed-phase HPLC systems, the retention times are almost identical. Underivatized or after derivatization to (tetra-)TMS derivatives, the resulting mass spectra are virtually indistinguishable. Using the oxidation procedure as described by Courtheyn [46,47], the spectra are again almost identical (Fig. 3). However, a difference is seen between the ratio of the two oxidized isomers formed. Dexamethasone has a higher α to β ratio, whereas betamethasone shows a reversed ratio. If a mixture of dexamethasone and betamethasone is present, the difference in ratios cannot distinguish the analytes anymore.

2.7. Use of Mass Spectrometry to Study Metabolism

The use of GC–MS as tool to study the metabolism and excretion of one or more metabolites has been described in many publications. Although this was not the scope of this chapter to present a complete review, a selection of references has been summarized in Table 4.

3. CORTICOSTEROIDS

3.1. Introduction

Similar to the anabolic steroids, a large number of synthetic corticosteroids were developed by chemical modifications of the endogenous corticosteroid cortisol (hydrocortisone) (see Fig. 1). Corticosteroids are widely used e.g., for the treatment of inflammation or dermal diseases. For several years it has become obvious that corticosteroids have gained popularity in the world of the veterinary anabolics [48]. Often (mixtures of) corticosteroids were detected in combinations with anabolic steroids or β-agonists. The mechanism or mechanisms behind the combined use have not been resolved fully. However, a significant effect on the excretion of, for example, clenbuterol has been noticed [49].

Similar to the anabolic steroids, the group of corticosteroids is large. The variations in chemical structures are larger, which has consequences for the analytical procedures.

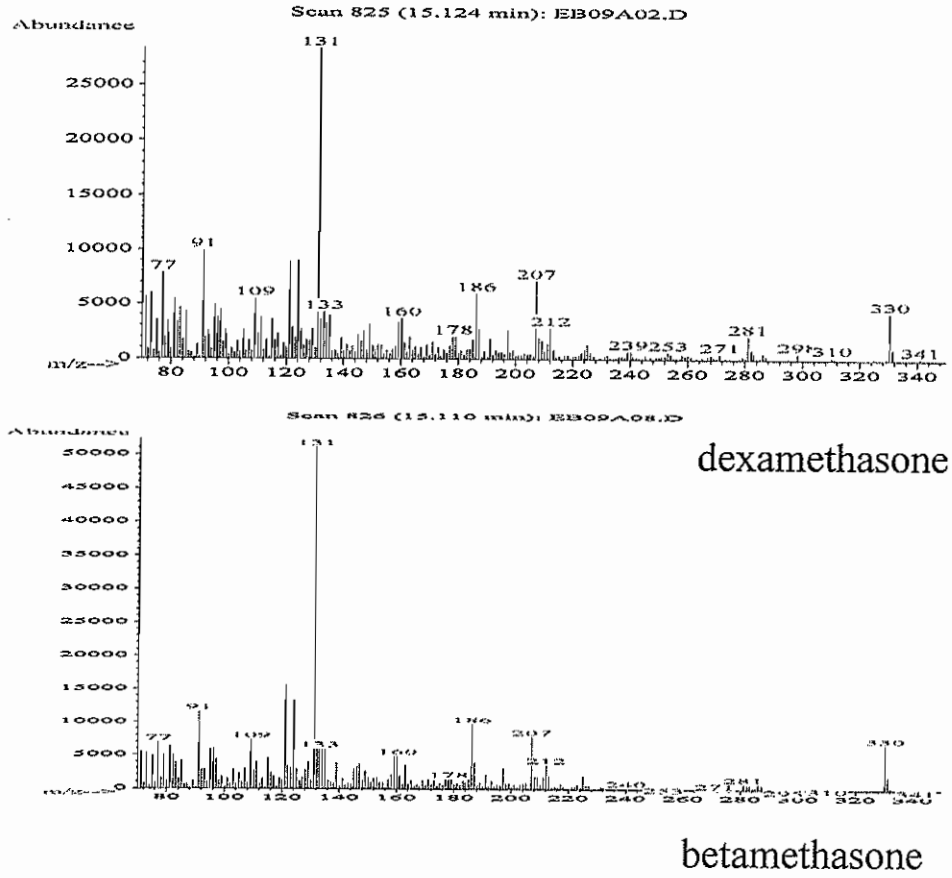

Figure 3 Mass spectra of the oxidized derivatives of dexamethasone and betamethasone.

Table 4 Selection of References Focused on Study of the Metabolism and Excretion of Anabolic Steroids and Their Endogenous Analogs

Analyte(s)	Sample matrix	Extraction and purification	Derivative	Level or range (µg/L or µg/kg)	Reference
Norethisterone and 6 metabolites	Human plasma	Extrelut and DCM	TMS	0.5–8	[99]
Chlormadinone acetate	Veal calf urine	HPLC	HFB	2–50	[100]
Stanozolol and 2 metabolites	Veal calf urine	SPE, IAC	TMS (enol-ether)	1–100	[101]
Stanozolol and several metabolites	Human urine	XAD-2, ether	TMS	≥1	[102]
Stanozolol and 3 metabolites	Cattle urine	SPE	HFB	≥0.001	[103]
Stanozolol and 1 metabolite	Human urine	XAD-2	HFB-TMS	≥2	[104]
Stanozolol	Rat urine	XAD-2	HFB-TMS	≥50	[105]
Norethisterone and metabolites	Human plasma	L–L	MO-TMS	n.d.	[106]
Methandienone and metabolites	Human urine	XAD-2, HPLC	TMS enol-ether	n.d.	[107]
17α-Methylated anabolic steroids	Human urine	XAD-2	TMS enol-ether	n.d.	[108]
Anabolic steroids	Human urine	XAD-2	TMS or TMS enol-ether	n.d.	[17]
Methandienone and related steroids	Human urine	SPE	TMS enol-ether	n.d.	[109]
Methandienone and metabolites	Horse urine	SPE	TMS	n.d.	[110]
Anabolic steroids	Horse urine	SPE	MO-TMS	n.d.	[111]
Anabolic steroids and 6β-hydroxylated metabolites	Human urine	XAD-2	TMS enol-ether	n.d.	[112]
Testosterone	Horse urine and plasma	SPE	TMS enol-ether		[113]

n.d. = Not determined.

3.2. Sample Cleanup

The sample cleanup for corticosteroids is rather similar to that used for anabolic steroids. The polarity is somewhat higher, allowing the possibility to separate the two groups. Also IAC has been described [50].

3.3. Silyl Derivatives

The presence of a number of hydroxyl groups enables the formation of silyl derivatives. However, from the analysis of corticosteroids, it was observed that sterically hindered functional groups could not be derivatized or only with a low yield. Applications of special mixtures of derivatization regents using the strongest catalysts (TriSil TBT, for example, from Pierce) in combination with prolonged heating have been successful for cortisol metabolites; however, they are less suitable for fast routine analysis. Bagnati et al. [50] have described the use of a TMS derivative of dexamethasone and betamethasone in bovine urine and McLaughlin and Henion [51] described the analysis of a TMS derivative of dexamethasone in tissue.

3.4. Oxidized Derivatives

Due to the relative instability of silylated corticosteroids, alternatives have been developed by Her and Watron [52] and Courtheyn et al. [47]. For dexamethasone and structurally related analytes, an oxidation reaction with pyridinium chlorochromate has proven to be useful in several laboratories. Later, the procedure was improved by changing the oxidizing reagent to dichromate and reducing the reaction time. Furthermore, the application of a hydrolysis step permitted triamcinolone acetonide to be included in the analytical procedure [46]. As described above, special attention should be given to the identification of dexamethasone and betamethasone because of their similar mass spectra and inadequate peak separation.

4. BETA-AGONISTS

4.1. Introduction

Substances with β(2)-adrenergic action can be used as anabolic agents to increase growth, enhance performance, i.e., carcass quality (less fat and more meat: "repartitioning"), and increase profits [53]. European Union legislation (EU directive 96/22/EC) prohibits the use of hormonally active anabolics (anabolic steroids, thyrostatic agents, and β-agonists) in animal fattening. In the EU, member-state monitoring and meat inspection programs are carried out to detect

illegal use. However, as has been already observed in regulatory control, the number of possibly used β-agonists is large. Small chemical modifications have led to the detection of chemically closely related analogs of clenbuterol, cimaterol, and mabuterol (see Fig. 1). However, the variation of molecular structures that could show a repartitioning effect via β-adrenoceptor activation can be much larger [54–56]. A multitude of GC–MS methods using different derivatives have been described [57,58].

4.2. Sample Cleanup

For current regulatory control for β-agonists in, for example, the Netherlands, a two-stage approach has been developed. This consists of screening methods based

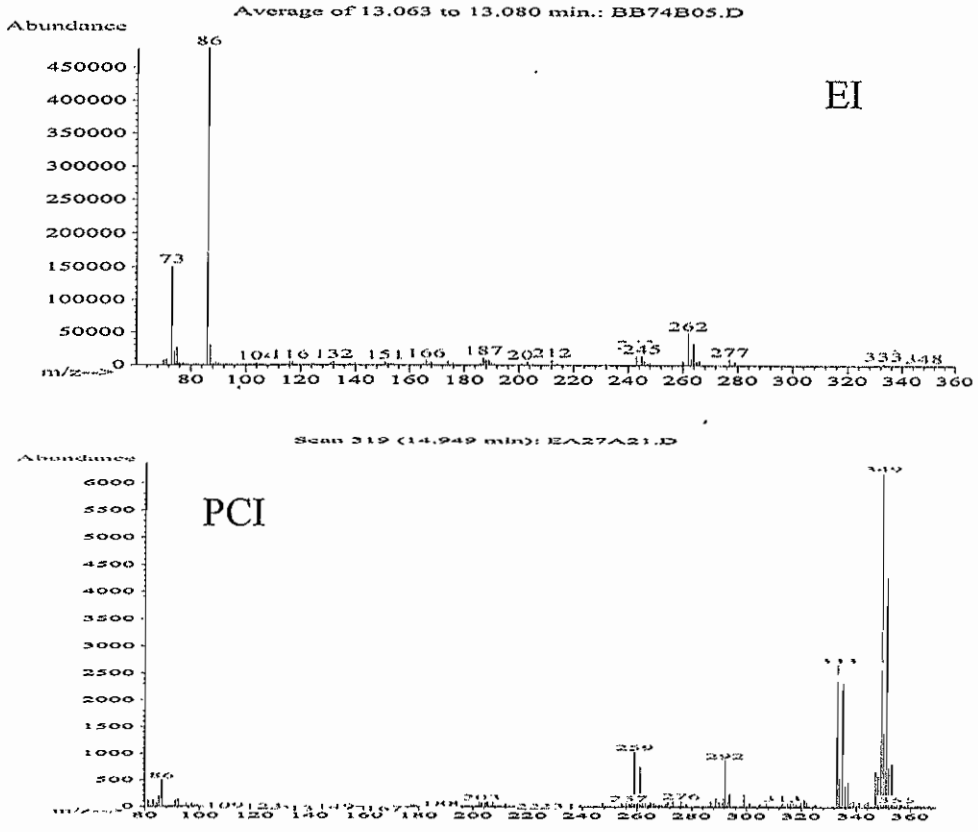

Figure 4 Mass spectra of the TMS derivative of clenbuterol in EI-mode and in PCI-mode (methane).

on enzyme immunoassays (EIA) [59,60], and confirmatory methods using IAC [61] in combination with GC–MS [62].

4.3. Silyl Derivatives

Clenbuterol can be analyzed as a silyl derivative. Unfortunately, the mass spectrum obtained in electron ionization (EI) mode limits the possibilities for an unequivocal identification at lower levels due to the fact that the higher mass ions are only present with very low abundances (Fig. 4). As an alternative, the combination of two analyses, one in EI mode and one in positive chemical ionization (PCI) mode has been successfully used for confirmatory purposes. Furthermore, the combination of two silyl derivatives has been applied [63,64]. The use of alkylboronic acid derivatives has been described, although the gas chromatographic behavior is less favorable [65,66].

5. THYROSTATICS

5.1. Introduction

In contrast to most anabolic agents, there is a general agreement on the ban of thyrostatic drugs: these drugs may be harmful for human health and the meat derived from animals treated with the drugs may be of inferior quality. The weight gain obtained with thyrostatics consists mainly of an increased filling of the gastrointestinal tract and a higher water retention of the animal. Due to the inhibiting effect on the thyroid function by selectively blocking iodine uptake by the thyroid and thus preventing the biosynthesis and excretion of thyroxine (T_4) and triiodothyronine (T_3), the thyrostatics are called antihormonal agents.

The most important and powerful thyrostatic drugs hitherto used are thiouracil and analogous compounds, especially methylthiouracil (MTU) and tapazole (TAP) (see Fig. 1).

5.2. Sample Cleanup

Due to their polar properties, extraction of the thyrostatics is only possible with polar solvents or silica columns. The complexation properties of the thiol compounds offer an elegant alternative method for metal-mediated affinity chromatography [67,68].

Specific procedures for the detection of this group of drugs have been described [67,69,70]. These methods are based on thin-layer chromatography (TLC) in combination with fluorescence induction of the NBD-derivatives (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) of the drugs with cystein, combined with a rapid and selective extraction procedure, based on a specific complex formation of the

was also used by Schilt et al. [68] in a GC–nitrogen-phosphorus detection (NPD) and GC–MS method.

5.3. Derivatization

The thyrostatics can be alkylated to obtain better GC behavior. After extraction, an alkylation with methyl iodide can be made [71]. Alternatively, an extractive alkylation can be performed [68]. Recently, an acylation with pentafluorobenzyl bromide has been successfully applied [72]. After a TLC separation, the thyrostatics can be isolated from the two-dimensional TLC plate and silylated with MSTFA, as was described by Batjoens et al. [73], yielding additional qualitative information.

6. ENDOCRINE DISRUPTORS, SUBSTANCES WITH ESTROGENIC PROPERTIES

Humans are exposed to endocrine disruptors in the workplace, home, community, or during medical care; wildlife are exposed through food and water consumption. Endocrine disruptors are industrial chemicals and environmental pollutants able to disrupt reproductive development in wildlife and humans by mimicking or inhibiting the action of the gonadal steroid hormones estradiol and testosterone, e.g., by binding to estrogen or androgen receptors. The toxicity of these so-called environmental endocrine disruptors is especially insidious during sex differentiation and development due to the crucial role of gonadal steroid hormones in regulating these processes. Published data indicate that chemical exposures may cause alterations in reproductive behavior and contribute to infertility, pregnancy loss, intrauterine fetal demise, birth defects, and ovarian failure in laboratory animals and wildlife.

Data on the association of chemical exposures and adverse reproductive outcomes in humans, however, are equivocal and often controversial. Some studies indicate that chemical exposures are associated with infertility, spontaneous abortion, or reproductive cancer in women. In contrast, other studies indicate that there is no association between chemical exposure and adverse reproductive outcomes. The reasons for such ambiguous findings in human studies are unknown, but likely include the fact that many studies are limited by multiple confounders, inadequate methodology, inappropriate end points, and small sample size. The mechanisms by which chemicals alter reproductive functions in all species are complex and may involve hormonal and/or immune disruption, deoxyribonucleic acid (DNA) adduct formation, altered cellular proliferation, or inappropriate cellular death. There is very little information on the effects of metabolism and intracellular binding proteins on target cell uptake of endocrine disruptors.

Furthermore, individual endocrine disruptors may be agonists/antagonists for more than one endocrine response pathway [74].

A variety of chemicals, ubiquitous in our environment, are suspected reproductive toxicants. Chemicals with known estrogenic or androgenic properties include some herbicides, fungicides, pesticides, e.g., dichlorodiphenyltrichloroethane (DDT), organochlorone pesticides such as methoxychlor and chlordecone (kepone), organophosphate pesticides such as parathion, malathion, and diazanon, phthalates, phytoestrogens, some industrial waste products, solvents such as perchloroethylene, toluene, and styrene, ethylene oxide, 4-vinylcyclohexene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and polychlorinated biphenyls [75,76]. Analytical chemical aspects of two main groups of endocrine disruptors are discussed below: nonionic surfactants, such as alkylphenol ethoxylates, and phytoestrogens.

7. ALKYLPHENOL ETHOXYLATES

7.1. Introduction

Nonionic surfactants, applied for detergency and (de)foaming processes in industry, consist of alcohol ethoxylates (AE) and alkylphenol ethoxylates (APE). Alkylphenol ethoxylates derived from nonylphenol are called nonylphenol ethoxylates (NPE) and comprise about 80% of the total market volume. Over 60% of these detergents (about 300,000 t/a of alkylphenol ethoxylates in Europe and the United States) end up in an aquatic environment via sewage treatment. Here, environmental biodegradation through anaerobic digestion occurs. The metabolites are nonylphenols, short-chain nonylphenol ethoxylates (typically 1 to 3 ethoxylate units), and acids (such as nonylphenoxy acetic acid and [(nonylphenoxy)ethoxy] acetic acid). These metabolites are even more persistent than their parent compounds and may accumulate in food chains [77–79]. These environmentally stable nonylphenols behave like steroid hormones. The metabolite nonylphenol is about 10 times more toxic than the parent compound [80]. Although the small quantity of nonylphenols may not be directly toxic to aquatic wildlife, an alarming long-term effect of estrogen and progesterone xenobiotics is now evident because of accumulation [81]. The observation that almost every river in the United States has been found to contain biologically active amounts of nonylphenol metabolites has recently led to concerns about their impact on the environment and has prompted an increasing effort to survey Western European environments for the presence of NPE.

7.2. Sample Cleanup

The analytical chemistry of NPE is outlined as follows. The technique most commonly applied to extract nonionic surfactants from aqueous samples has been a

procedure called sublation. In this technique, fine bubbles of a inert gas (helium or nitrogen) are dispersed through the aqueous sample, thereby transferring the surface-active materials into an overlaying organic layer. The coextracted ionic surfactants are subsequently removed by ion-exchange chromatography. Centrifugal partition chromatography has been used as an alternative because of its ability to concentrate sample volumes of several liters into organic solvent amounts of several tens of milliliters. Solid-phase extraction (SPE) has been shown to be a very powerful and robust alternative to the traditional methods of extraction. Using this approach, determination of AE and APE in aqueous samples is possible at the ppb level [82].

The determination of alkylphenols and nonylphenol ethoxylates is challenging due to the complexity of the mixtures, comprised of various isomers with different branching of the alkyl moiety and oligomers (with different numbers of ethoxylate units) [82–85].

7.3. Gas Chromatography–Mass Spectrometry

High-performance LC has become the favored method of analysis for these compounds because of its ability to separate and quantitate the various homologues and oligomers by length of the alkyl and ethoxylate chains [86]. The confirmation of the compounds may be done by GC–MS, after the collection of HPLC fractions.

7.4. Derivatization

Derivatization (using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) or hexamethyldisilazane (HMDS)) is only needed for the analysis of oligomers containing more than 5 or 6 ethoxylate units. Alternatively, high-temperature GC may be used, although analyte degradation may occur [87].

8. PHYTOESTROGENS

8.1. Introduction

Phytoestrogens are chemicals naturally occurring in plants, soy products, legumes, and grains, and thought to affect adversely the female reproductive system of laboratory rats, sheep, and humans ([75] and references cited therein). Phytoestrogens appear to have both estrogenic and antiestrogenic activity. The exact mechanisms and effects of phytoestrogens are still unknown. There is evidence that isoflavones exert a protective effect to cancer risk [88,89]. For example, antitumor activity has been demonstrated by inhibition of tumor cell growth. Also, they appear to act in other ways that may inhibit tumor formation and growth, e.g., elevation of sex-hormone-binding globulin and possibly lowering

effects of isoflavones exist also, especially with children. These are related to knowledge of the role of estrogens at critical stages of development and in mediating reproductive or neuroendocrine disruption in various animal species [91].

The three major chemical types of phytoestrogens that have been identified are flavones, isoflavones, and coumestans. The estrogenic potency of these compounds is variable. The flavones are weak estrogens. The methoxyflavone, tricin, is a constituent of alfalfa and weakly estrogenic in the mouse.

Isoflavones, such as genistein (see Fig. 1) and daidzein, are found in just a few botanical families, e.g., soy. Up to 3 mg/g of genistein and daidzein and their β-glycosides are present in soybeans. Information on isoflavone content in soy products, such as soy milk, tofu, and fermented products such as miso, soy sauce, and tempeh, is given by Coward [92] and Wang [93].

Among the isoflavones, genistein is the most active estrogen with the highest binding affinity for the estrogen receptor. Coumestrol (see Fig. 1), the most potent of the coumestans, has higher binding affinity for the estrogen receptor than genistein. [94].

The metabolic fate of isoflavones, "dietary estrogens," is rather complex. It is of interest that the general pattern of isoflavone conjugates in urine is similar to that of endogenous steroids [94].

8.2. Sample Cleanup

The analysis of isoflavones has been discussed by several authors. The extraction of isoflavones from soy products preferably occurs by acid hydrolysis of the glucoside conjugates followed by a (hot) extraction with acidic methanol, ethanol, or acetonitrile. Extraction of isoflavones from biological fluids, such as serum, plasma, urine, and milk usually occurs using solid-phase extraction methodology [95].

8.3. Gas Chromatography–Mass Spectrometry

Traditionally, GC–MS was applied to determine soy isoflavones and their metabolites in human biological fluids [90,96,97]. Silylation is the most often used method for the derivatization. For quantification, many deuterated standards have been synthesized [97]. Similar to the developments for steroid analysis, LC–MS is more and more frequently used [95,98].

9. GENERAL REMARKS

The widespread application of GC–MS in residue analysis has improved the analytical possibilities enormously and has largely contributed to the quality of the

in the past decade, LC–MS is following this pattern. In general, GC–MS enables measurement at low residue levels with a high degree of specificity. Unfortunately, no estimations of the error probabilities, especially for false positive identifications, are available yet. Currently, a limited number of studies are being carried out to develop models to predict these possibilities. The application of quality criteria destined to objectively evaluate the mass spectrometric results [44] greatly improves the results obtained. However, their general use is still limited as is observed in many publications reviewed. Usually one or more diagnostic ions are mentioned and depicted, whereas no reference is made to the actual procedure followed to compare spectra of pure standards with spectra obtained from analytes present in the sample.

As has happened with GC–MS, there is a clear trend that the use of LC–MS, because of the increasing availability of robust and sensitive instruments, will be very common in the field of the analysis of growth promoters and endocrine disruptors. In many laboratories, LC–MS is used already to detect and identify anabolic steroids, corticosteroids, and β-agonists at low µg/L or µg/kg levels. Looking at multiresidue analysis, LC–MS will earn its position as a very useful technique. The development of quality criteria for LC–MS, similar to the well-known criteria for GC–MS, for confirmatory analysis will certainly boost the possibilities for more general use.

It is expected, however, that it will not fully replace GC–MS because of the (complicated) behavior of several growth promoters (especially steroids) that possess unfavorable LC–MS properties (either too low or too high degree of fragmentation). Furthermore, the resolution obtained with LC is normally lower than that obtained with capillary GC. The modern laboratory will use both GC–MS and LC–MS to solve their analytical challenges.

ACKNOWLEDGMENTS

The authors thank Mr. H. Hooijerink, Mr. E. O. van Bennekom, and Mr. B. Brouwer for their valuable assistance and contributions.

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