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Review

Liquid chromatographic purification and detection of anabolic compounds

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ABSTRACT

The role of liquid chromatography within methods of analysis for steroids, related compounds and β-agonists in biological samples is discussed. Special attention is given to the application of liquid chromatography in sample preparation and extract clean-up. Different forms of liquid chromatography, including immunoaffinity chromatography, are compared and evaluated. Methods for confirmation based on gas chromatography–mass spectrometry and cryotrapping Fourier transform infrared spectrometry are discussed.

CONTENTS

1. INTRODUCTION

Throughout the EEC, the use of xenobiotic anabolic agents is prohibited in food-producing animals. However, for over 30 years a variety of compounds have been used for purposes of growth promotion. The number of compounds used within a particular group, *e.g.*, steroid hormones, is still increasing and new classes of compounds find their way into the "black market' of growth promoters.

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One example of the latter is are the phenylethanolamines, primarily developed as human or veterinary drugs working on the β_2-adrenergic receptor. Well known examples of these so-called β-agonists are clenbuterol and salbutamol.

During the past 30 years, analytical chemists have developed a variety of analytical methods to monitor the presence of residues in biological samples. Liquid chromatography (LC) has been demonstrated to be a key technique. The relative importance is to a large extent based on the wide variety of separation mechanisms and application forms, ranging from binary solid-phase extraction (SPE) to highly efficient, small-particle high-performance liquid chromatographic (HPLC) systems. These different forms allow the use of LC during both sample preparation (clean-up) and final analysis.

The objectives of an analysis can be very different. Within residue analysis for (illegal) growth-promoting agents the compounds used are frequently exogenous. In these cases, as there are no tolerance levels, only unambiguous identification is necessary. However, there are instances where in addition to identification quantification is also necessary, *e.g.*, in forensic analyses for estradiol and testosterone, which are endogenous compounds also used for growth promotion. These different objectives of an analysis impose different demands on the techniques and procedures used. Attention will be focused, however, on methods suitable for combination with spectroscopic procedures for detection and identification.

Analytical methods for residue analysis as a rule contain three different steps: (i) preparation of a primary extract, (ii) extract clean-up and (iii) detection, identification and determination. Frequently, however, the distinction between these steps is not very clear. In a number of methods the detector is placed on-line with the equipment used for extract clean-up, *e.g.*, in HPLC with UV detection. However, also within these applications HPLC can be considered as an extract clean-up procedure prior to detection.

A detailed discussion of methods for the preparation of a primary extract is beyond the scope of this paper, even though it is often neglected. Especially the extraction of analytes from tissues is frequently not evaluated in sufficient detail, the main reason being the absence of good reference materials with incurred residues.

The use of HPLC and SPE techniques is part of most, if not all, modern analytical procedures for steroids and β-agonists at residue levels. Immunoaffinity chromatography (IAC) is one of the more recent additions to the number of techniques available to the analyst. IAC is not a new technique, but within residue analysis applications are relatively recent [1–7], of special interest being multi-residue applications. The use of IAC has been reviewed before [8]. IAC combines the ease of use of SPE with the selectivity of advanced chromatographic (HPLC) systems.

The number of detectors that are sensitive and selective enough to be applied on-line with LC is limited because the solvents used are not compatible, *e.g.*, immunochemical detection after reversed- or normal-phase LC, and the technology of coupling is still under development and not (yet) available in a large number of laboratories not specialized in this technique, *e.g.*, with LC–MS. Therefore, LC separations are frequently followed by off-line detection. Within toxicological and residue analysis, immunochemical and mass spectrometric techniques are widely used, the latter most frequently in combination with gas chromatography (GC–MS).

2. ANALYTICAL PROCEDURES

This section describes analytical procedures for the detection and identification of anabolic steroids and related compounds and of β-agonists in biological materials. The procedures are based on spectroscopic methods for identification.

2.1. Determination of anabolic steroids and related compounds

Methods used for the determination of anabolic compounds are frequently multi-residue methods based on SPE, IAC or HPLC for extract clean-up. Here the procedures suitable for extract clean-up are described and compared. Details can be found in the EEC manual on reference methods and materials [9].

The analytes involved are summarized in Table 1. Fig. 1 shows structures of representatives of the different groups of compounds, the androgens NT, T, MT, Bol and Tb, the estrogens EE2, E2, DES, DE, HEX and Z and the gestagens MPA, CMA and MGA.

TABLE 1

STEROIDS AND RELATED COMPOUNDS USED FOR GROWTH PROMOTION

Analyte	Abbreviation	CAS Registry No.	Formula	Molecular mass
17β-19-Nortestosterone	βNT	434-22-0	$C_{18}H_{26}O_2$	274.3
17α-19-Nortestosterone[a]	αNT	4409-34-1	$C_{18}H_{26}O_2$	274.3
17β-Testosterone	βT	58-22-0	$C_{19}H_{28}O_2$	228.4
17α-Testosterone[a]	αT	99-999-9	$C_{19}H_{28}O_2$	288.4
17α-Methyltestosterone	MT	58-18-4	$C_{20}H_{30}O_2$	302.4
Boldenone	BOL	846-48-0	$C_{19}H_{26}O_2$	286.4
17β-Trenbolone	βTb	10161-33-8	$C_{18}H_{22}O_2$	270.4
17α-Trenbolone[a]	αTb	80657-17-6	$C_{18}H_{22}O_2$	270.4
17α-Ethynylestradiol	αEE2	57-63-6	$C_{20}H_{24}O_2$	296.4
17β-Estradiol	βE2	50-28-2	$C_{18}H_{24}O_2$	272.2
Medroxyprogesterone acetate	MPA	71-58-9	$C_{23}H_{24}O_2$	386.5
Clormadinone acete	CMA	302-22-7	$C_{23}H_{24}ClO_4$	405.0
Megesterol acetate	MGA	595-33-5	$C_{24}H_{32}O_4$	384.5
Zeranol	Z	26538-44-3	$C_{18}H_{20}O_5$	322.4
Taleranol[a]	TAL	42422-68-4	$C_{18}H_{20}O_5$	322.4
Diethylstilbestrol	DES	56-53-1	$C_{18}H_{20}O_2$	268.4
Dienstrol	DE	84-17-3	$C_{18}H_{18}O_2$	266.4
Dexestrol	HEX	84-16-2	$C_{18}H_{22}O_2$	270.4

[a] Not an anabolic itself but a metabolite of the previous compound.

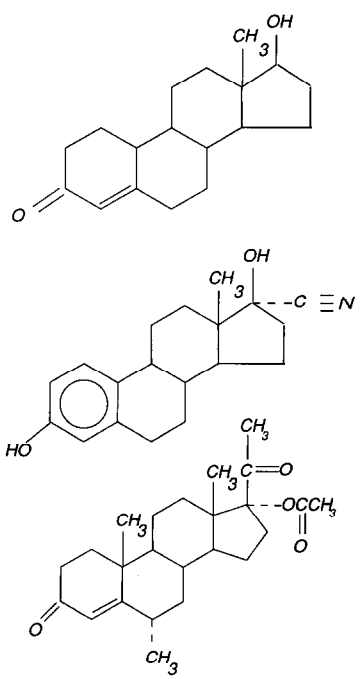

Fig. 1. Molecular structures of nortestosterone (top), ethynylestradiol (middle) and medroxyprogesterone acetate (bottom).

2.1.1. Sample preparation

If available [10], deuterated internal standards are added to the samples prior to analysis for accurate determination and control of false-negative results. Liquids samples (urine or bile) are incubated in the presence of 0.1 ml of β-glucuronidase–sulphatase (*e.g.*, suc'*Helix pomatia* containing 100 000 units of β-glucuronidase and 10 000 units of sulphatase) to deconjugate glucuronide and sulphate conjugates of the analytes (pH 5.2, 2 h at 37°C). After incubation the test portions are extracted with *tert.*-butyl methyl ether (TBME). The combined extracts are evaporated to dryness under a stream of nitrogen in a water-bath at 50°C.

Tissue samples can be extracted either by an enzymatic procedure with subtilisin A [11] or by mechanical (ultrasonic) extraction [9]. As anabolic steroids are frequently injected into the animal in the form of esters, an incubation under alkaline conditions can be applied (KOH in ethanol, 0.5 h at room temperature). After defatting, the extract is ready for further purification.

2.1.2. Extract clean-up with LC

2.1.2.1. Solid-phase extraction. SPE is frequently used for extract clean-up [12,13]. Both normal-

phase, based on silica-type materials, and reversed-phase methods, mainly with C_{18}-modified materials have been described. Typical procedures and elution behaviour are as follows (Sep-Pak columns, Waters–Millipore). With a silica cartridge, the cartridge is washed subsequently with 2 ml of ethanol and 5 ml of isooctane and the residue of the primary extract is dissolved in 5 ml of isooctane and applied to the cartridge. Analytes are eluted with ethanol–isooctane mixtures. With a C_{18} cartridge, the cartridge is washed with 2 ml of methanol and 5 ml of water and the residue is dissolved in water and applied to the cartridge. Analytes are eluted with methanol–water mixtures.

2.1.2.2. Immunoaffinity chromatography. For most of the compounds clean-up by IAC is possible. Several institutes have prepared their own materials. More important, however, several commercial firms supply suitable materials. For multi-residue methods appropriate IAC materials can be combined (MIAC). The procedure for sample preparation by IAC depends on the characteristics of the IAC material used [8]. The following procedure is suitable for the polyclonal rabbit antibodies tested. IAC materials usually have a capacity of 20 ng or more per ml of gel. For multi-residue analysis combinations of gels can be applied. The primary extract is dissolved in 0.05 ml of ethanol. Subsequently, 5–10 ml of water are added and the total mixture is applied to the IAC column. After sample application the column is washed with 5 ml of water and eluted with 5 ml of ethanol–water (1:1, v/v). The eluate is evaporated to dryness under a stream of nitrogen in a water-bath at 50°C or alternatively extracted with TBME. The dry residue is suitable for analysis by GC–MS. Fig. 2 shows the elution of nortestosterone from an IAC column as a function of the ethanol content of the eluent.

2.1.2.3. High-performance liquid chromatography. In addition, clean-up by reversed-phase LC is very effective. The following reversed-phase system has proved to be suitable for extract purification prior to GC–MS: precolumn, Chromguard reversed-phase cartridge; analytical column, Hypersil ODS C_{18} (150 mm × 7.5 mm I.D.); flow-rate, 1.5 ml/min.

The residue is dissolved in 0.10 ml of the HPLC eluent, of which subsequently 0.09 ml is injected into the system. The fractions of interest are collected, usually starting 0.5 min before the retention time of the analyte and ending 1 min later. Sometimes it is possible or advantageous to combine different analytes in a single fraction. In Table 2 an overview is given of suitable solvent compositions and retention times.

The eluent is removed under a stream of nitrogen in a water-bath at 50°C. In a number of instances on-line detection of the analytes at this stage is possible, *e.g.*, in the analysis of highly concentrated extracts of application sites or in samples of urine from veal calves. More details of these methods and the possibilities of, *e.g.*, identification using diode-array UV detection, are reviewed separately.

The choice between SPE, IAC and HPLC depends on a number of parameters: (i) availability; (ii) analyte(s); (iii) number of samples; and (iv) limit of detection needed. To illustrate the efficiency of the different techniques, a comparison was made of the quantitative determination of 17β- and 17α-nortestosterone (NT) in bovine urine. Three test portions of a single sample were pretreated as described. The extracts were purified on an SPE (C_{18} cartridge or an IAC or HPLC column. Fig. 3 shows the GC–MS ion chromatograms (as HFB derivatives) for the molecular ions of 17β- and 17α-NT (m/z = 666) and for the internal standard 17β-NT-d_3 (m/z = 669, added at a level of 2 μg/l).

From these chromatograms it can be concluded

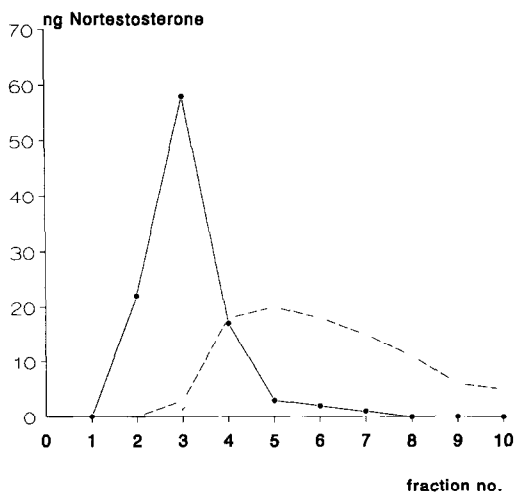

Fig. 2. Elution behaviour of nortestosterone on an IAC column. ● = Ethanol–water (50:50, v/v); dashed lined = ethanol–water (20:80, v/v).

TABLE 2

SOLVENT SYSTEMS AND APPROXIMATE RETENTION TIMES

Compound	Eluent	Retention time (min)
Diethylstilbestrol	CH_3OH–H_2O (65:35)	5.3
Dienestrol	CH_3OH–H_2O (65:35)	6.2
Hexestrol	CH_3OH–H2O (65:35)	5.7
17β-Estradiol	CH_3OH–H_2O (65:35)	4.5
Zeranol	CH_3OH–CH_3CN–H_2O (4:38:58)	4.8
Ethynylestradiol	CH_3OH–H_2O (65:35)	5.0
17β-Trenbolone	CH_3OH–CH_3CN–H_2O (4:38:58)	3.5
17α-Trenbolone	CH_3OH–CH_3CN–H_2O (4:38:58)	4.1
17β-Nortestosterone	CH_3OH–H_2O (65:35)	4.3
17α-Nortestosterone	CH_3OH–H_2O (65:35)	6.7
Methyltestosterone	CH_3OH–H_2O (65:35)	6.7
17β-Testosterone	CH_3OH–H_2O (65:35)	5.3
17α-Testosterone	CH_3OH–H_2O (65:35)	7.6
Boldenone	CH_3OH–H_2O (45:55)	3.2
Medroxyprogesterone	CH_3OH–H_2O (45:55)	9.0
Chlormadinone	CH_3OH–H_2O (45:55)	8.3
Megestrole	CH_3OH–H_2O (45:45)	7.4

that SPE clearly is less effective than HPLC and IAC. The suitability of SPE depends on the limit of detection needed. The sample analyzed contained *ca.* 1 μg/l, approaching the limit of detection after SPE but clearly exceeding it after HPLC or IAC. The main advantage of IAC, however, becomes clear in multi-residue analyses. Similar results to those shown here can also be obtained when 2–6 different IAC materials are combined. The use of HPLC for extract clean-up frequently results in a number of different fractions, increasing the work load for subsequent analytical steps.

2.1.3. Detection and identification

Because of the sensitivity and structure information that can be obtained with GC–MS, this is the method of choice for detection and identification or determination [14,15]. With the system described below both TMS and HFB derivatives can be analysed. Diagnostic ions are summarized in Table 3. The preferred derivative is the derivative used, unless there are strong reasons for using the other derivative, *e.g.*, in multi-residue analyses. After derivatization, the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C and the derivatized residue is dissolved in 0.025 ml of isooctane.

2.1.3.1. GC–MS analysis. The following conditions are used in GC–MS analysis: gas chromatograph, HP 5890 (Hewlett-Packard); GC column, *e.g.* Macherey–Nagel Permabond SE 52; injection, 1–5 μl, splitless, 225°C; column temperature programme, 100–280°C at 20°C/min; temperature of transfer line, 280°C; mass spectrometer, HP 5970 (Hewlett-Packard); aquisition ions, see Table 3.

The evaluation of parameters such as analytical recovery, repeatability and reproducibility are important. To assess the applicability of a method, limits of detection, determination and identification have to be known. For the methods discussed here, the limit of identification is the most important. This limit depends on a number of parameters: sample size, analytical recovery, sensitivity of the detection principle and instrument used and the quality of extract clean-up procedures. The limit of identification additionally depends on the criteria applied. For forensic purposes (reference methods) within the EEC, criteria are laid down [14,15]. For low-resolution on mass spectrometry with EI ionization, at least four diagnostic ions have to be monitored, which have to elute from the GC column simultaneously, and all four must exceed the average noise + 3 S.D. Additionally, the response ratios have to be within ±10% of the corresponding ratio as ob-

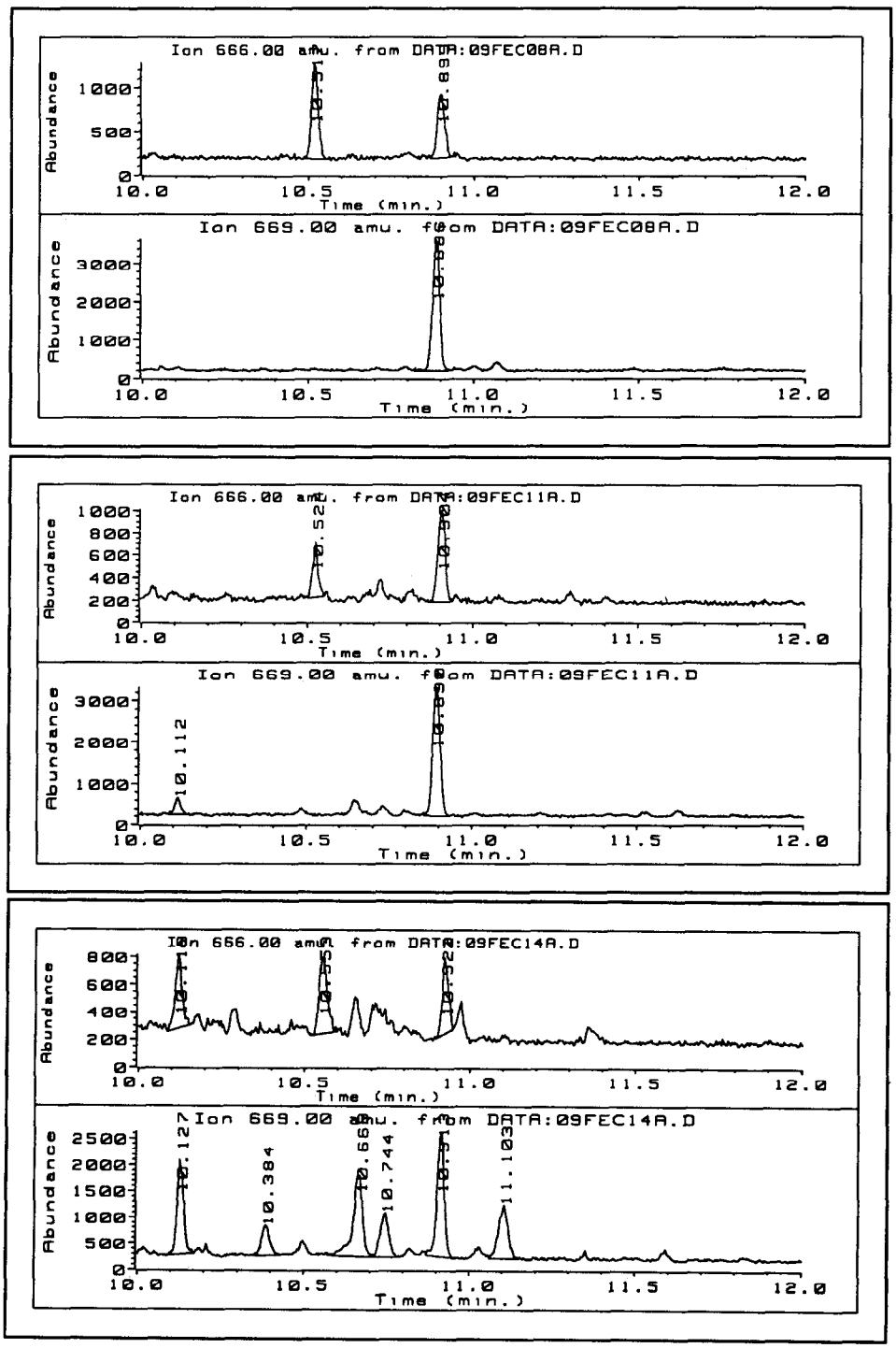

Fig. 3. Comparison of IAC (top), HPLC (middle) and SPE (bottom) for extract clean-up.

TABLE 3

IONS MONITORED DURING GC–MS ANALYSIS

The most suitable derivative is indicated by + +; (+) indicates low yield. The ion used during initial screening is given in italics.

Compound	Derivative		Ions monitored (*m/z*)
	TMS	HFB	
17β-NT	+	+ +	*660*, 453, 306, 133
17α-NT	+	+ +	*660*, 453, 306, 133
17β-T	+	+ +	*680*, 467, 355, 320
17α-T	+	+ +	*60*, 467, 355, 320
MT	(+)	+ +	480, *465*, 369, 355
Bol	+	+ +	*678*, 464, 369, 169
17β-Tb	+ +		*342*, 252, 237, 211
17α-Tb	+ +		*342*, 252, 237, 211
17α-E2	+	+ +	*664*, 451, 409, 356
EE2	+	+ +	*474*, 459, 446, 353
MPA[a]		+ +	*479*, 331, 317, 147
CMA[a]		+ +	540, *497*, 462, 401
MGA[a]		+ +	520, *477*, 421, 381
ZER	+ +	+	538, *433*, 335, 307
TAL	+ +	+	538, *433*, 335, 307
DES	+ +	+	*412*, 397, 383
DE	+ +	+	*410*, 395, 381
HEX	+ +	+	*207*, 191, 179

[a] After alkaline hydrolysis.

tained for the reference compound. For screening purposes, usually only one diagnostic ion (if possible the molecular ion) is monitored. If a sample shows a positive response it has to be reanalysed to fulfil the criteria. Usually this means that the complete procedure is repeated in which the sample is analysed in duplicate, once with and once without the addition of the internal standard because frequently deuterated analogues interfere with the detection of fragment ions owing to losses of deuterium after fragmentation. These criteria result in very different limits of identification.

Fig. 4 shows the EI-mass spectrum of DES-diTMS. The molecular ion of *m/z* 412 contains most of the response, resulting in high sensitivity (50 pg injected), which translates into a limit of detection in real samples of 0.05–0.1 μg/kg or μg/l. This low limit of detection is disadvantageous, however, for the limit of identification. The limit of identification equals the limit of detection for the ion selected in the set of four with the lowest abundance. Therefore, the limit of identification is in the region of 1 ppb, unless other ionization techniques or high-resolution instruments are used.

Fig. 5 shows the EI-mass spectrum of NT-diHFB. Here the situation is very different. The mo-

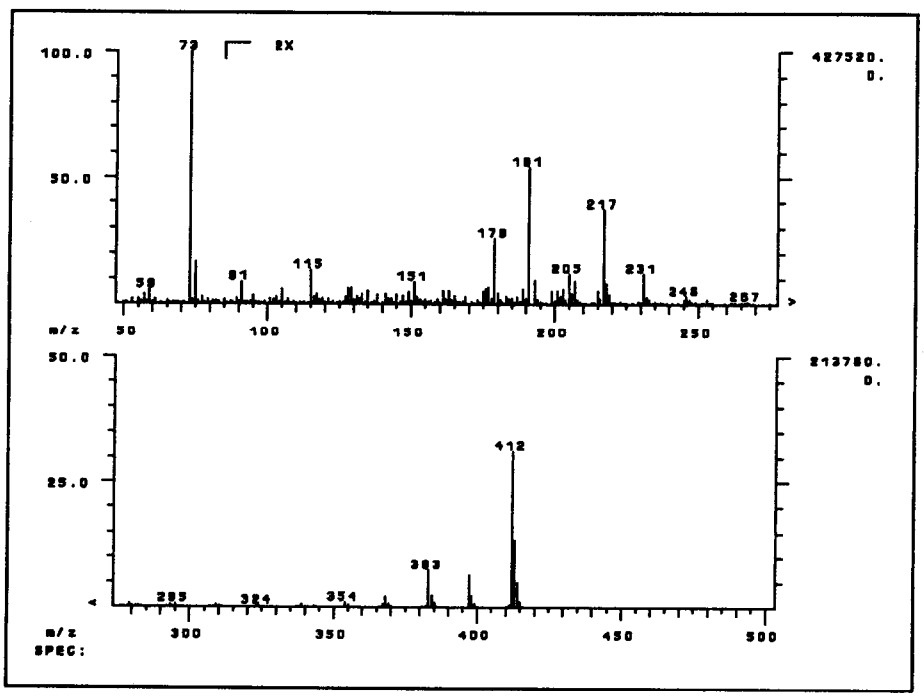

Fig. 4. Mass spectrum of DES-diTMS (EI ionization).

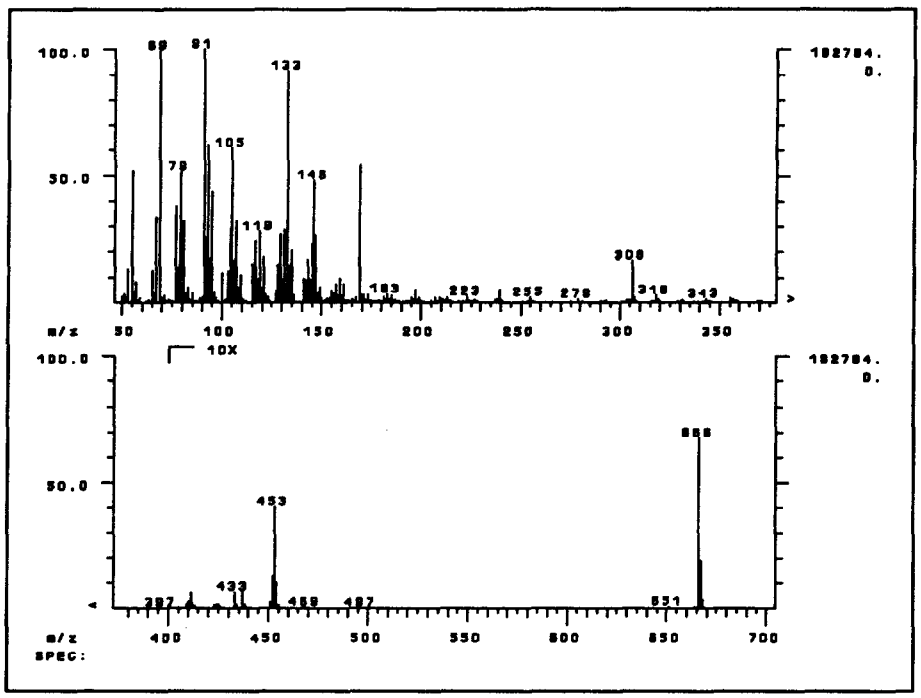

Fig. 5. Mass spectrum of NT-diHFB (EI ionization).

lecular ion of m/z 666 contains a much lower percentage of the total response, which is disadvantageous for the limit of detection (0.3 ppb) but favourable for the limit of identification (0.5 ppb).

The procedures described are currently in use within a number of studies, *e.g.*, national surveillance studies into the occurrence of residues, certification studies for the preparation of certified reference materials (Commission of the European Communities–BCR) and studies to determine the oral availability of (esters of) anabolic steroids. Details of the last type of studies related to nortestosterone and medroxyprogesterone will be published elsewhere in the near future. The combination of a number of different LC techniques resulted in a flexible multi-residue procedure suitable for a variety of purposes, from forensic residue analysis to toxicological studies.

2.2. Determination of β-agonists in biological materials

A shift to a relatively new class of compounds, the phenylethanolamines, primarily developed as human or veterinary drugs working on the β_2-adrenergic receptor, has been observed on the "black market" since 1988. The first of such compounds of which the large-scale misuse as a growth promoter was observed in the EEC was clenbuterol (Fig. 6) [16,17]. Within a few months in the Netherlands and some other EEC countries an effective control strategy was developed and implemented and large-scale monitoring programmes were started. However, in spite of the efficiency of the methods used and the intent of the EEC legislation, the use of β-agonists was not eradicated and a variety of related illegal alternative compounds have appeared, one of the most important being salbutamol, an out-of-patent human and veterinary drug.

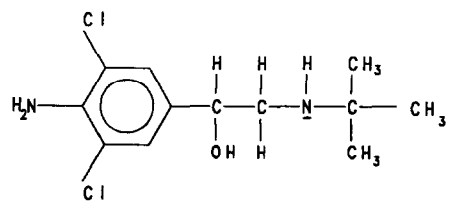

Fig. 6. Molecular structure of clenbuterol.

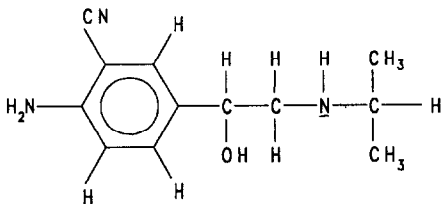

Fig. 7. Molecular structure of cimaterol.

That the use of these compounds is not without risk for the consumer recently became apparent after reports from Spain, where a serious outbreak of poisoning due to the consumption of bovine liver was demonstrated to be caused by the presence of residues of clenbuterol [18]. The majority of the β-agonists known to be used for fattening veal calves and cattle contain either an N-*tert.*-butyl or an N-isopropyl group, *e.g.*, cimaterol (Fig. 7).

A sensitive HPLC method was developed based on post column derivatization suitable for all compounds with an amine function coupled to the aromatic part of the molecule [19]. For compounds such as clenbuterol and cimaterol, very low limits of detection were achieved. An alternative procedure was developed for the simultaneous determination of compounds with either an N-*tert.*-butyl or an N-isopropylgroup. For this purpose antibodies were raised against both clenbuterol and cimaterol by coupling the amine function of the analytes to bovine serum albumin. IAC columns were prepared by mixing the individuel gels obtained by coupling the IgG fractions of the respective antisera. The columns obtained are suitable for the isolation of a large number of β-agonists. The isolated compounds are derivatized (TMS derivatives) and detected by GC–MS. For quantification and quality control, isotopically labelled internal standards are used. The method is applicable to biological matrices such as urine and liver and to animal feeds such as milk replacers and premixes. The method developed has been published in detail elsewere and has been the subject of an EEC-workshop on reference methods for β-agonists [20].

A number of studies have been undertaken to validate the analytical procedure. Most of these experiments were intra-laboratory experiments (repeatability and within-laboratory reproducilibity). In addition, the method was validated in a cooperative inter-laboratory study on animal feeds, organized by the BCR of the EEC and demonstrated during an EEC workshop in Netherlands. The conclusion of these studies was that the methodology used is suitable as a reference method.

Apart from MS, nowadays Fourier transform infrared (FT-IR) spectrometry is also a suitable identification technique in this area. Recently a new type of interfacing of capillary GC and FT-IR spectrometry has been developed. The principle of this so-called cryotraping GC–FT-IR technique (Fig. 8) is condensation of the GC eluates at 77 K on a moving infrared window with subsequent scanning of the trapped compounds by means of FT-IR microscopy [21].

A unique feature of the system is the possibility of carrying out extended post-run scanning of previously condensed compounds. As a consequence, a considerable improvement of the signal-to-noise ratio of the infrared spectra is obtained. The sensitivity is 1–2 orders of magnitude higher than that of conventional GC–FT-IR light-pipe systems and detection limits into the picogram range have been

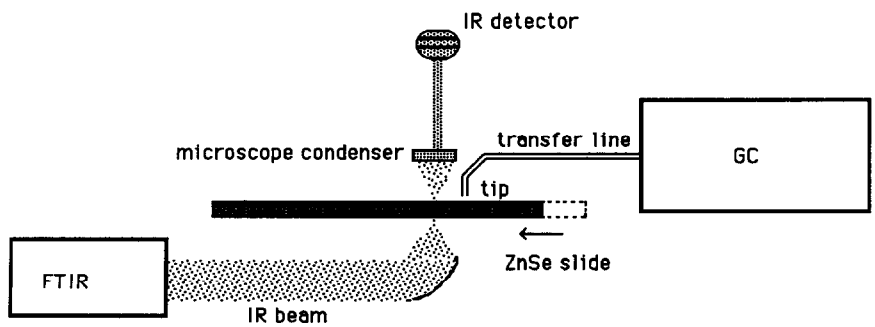

Fig. 8. Principle of cryotrapping GC–FT-IR spectrometry.

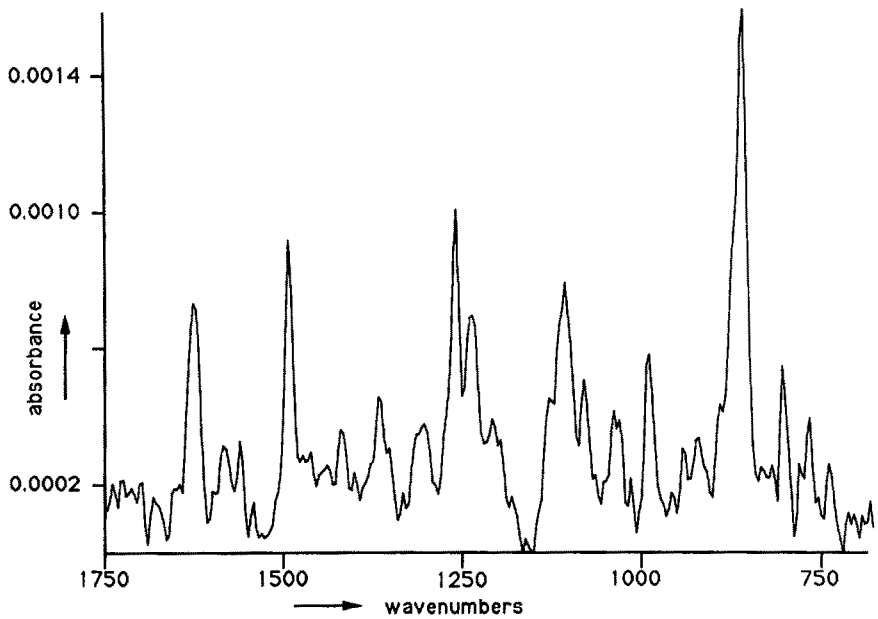

Fig. 9. Post-run spectrum (512 scans co-added) of a sample containing 5 μg/l of clenbuterol-TMS.

reported [22,23]. In view of the high discriminating properties of IR spectrometry, the technique might become a valuable tool in addition to GC–MS.

Recently we applied the technique to the identification of clenbuterol in samples of bovine urine. Fig. 9 shows a spectrum obtained for a sample containing 5 μg/l of clenbuterol. In Table 4 characteristic absorption maxima are compared for a reference spectrum, an extract of a sample of blank urine spiked at 10 μg/l and a sample obtained from a treated animal containing *ca.* 5 μg/l.

3. LC WITH ON-LINE DETECTION

The development of very sensitive UV–VIS detectors and multi-wavelength detection by the diode-array technique has provided an additional tool for the determination of selected anabolic compounds. In addition, column-switching procedures have been developed, allowing LC methods to be more specific and sensitive in combination with automation. We have developed an automated procedure for the determination of trenbolone in urine and muscle. Currently the method is used within an EEC programme on the development of certified reference materials for the determination of trenbolone in urine. For this purpose the previously de-

TABLE 4

INFRARED ABSORPTION MAXIMA (cm^{-1}) OF TMS-DERIVATIZED CLENBUTEROL OBTAINED BY CRYOTRAPPING GC–FT-IR SPECTROMETRY

Reference	Spiked (10 μg/l)	Sample (5 μg/l)
719.5	719.42	721.0
749.9	749.8	749.3
786.2	786.5	786.4
842.0	842.0	842.2
855.1	855.6	–
905.5	906.6	907.9
926.6	926.9	928.2
976.0	975.9	977.0
1027.5	1027.2	1026.0
1067.7	1068.2	1068.6
1095.5	1094.8	1095.6
1195.3	1198.1	1199.1
1229.9	1225.7	1228.1
1250.5	1251.5	1250.5
1296.6	1298.8	1228.1
1360.8	1360.8	1360.0
1415.2	1413.2	1414.4
1488.1	1487.8	1488.6
1561.4	1555.4	1557.9
1581.6	1582.9	1580.4
1621.3	1621.2	1622.9

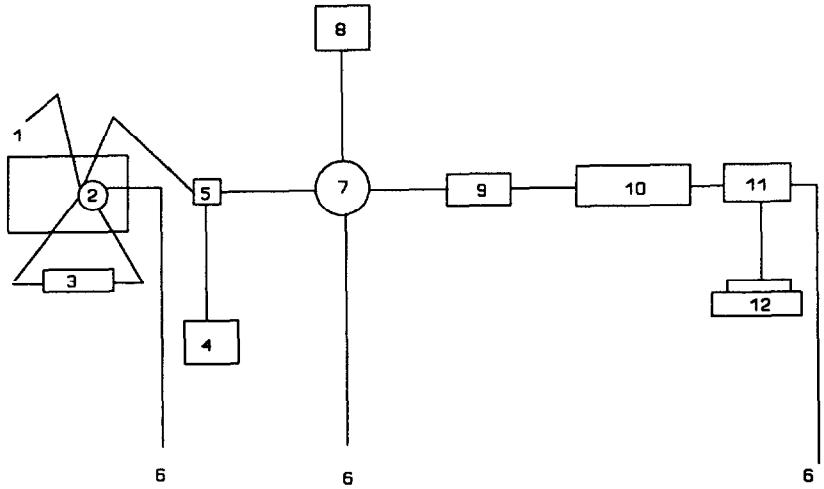

Fig. 10. Column-switching system for IAC–HPLC analyses.

scribed method [1] based on HPLC was automated using a column-switching procedure (Fig. 10).

During step 1, the enzymatically hydrolysed and centrifuged sample of urine (1) is injected directly on to an immunoaffinity column (3). The soft gel (Pharmacia Sepharose 4B Fast Flow) is packed in a glass column (Perstop; 10 × 1.2 cm I.D.) placed within the injection loop of an autoinjector (Gilson 231) (2). An aliquot of 5 ml is flushed through the column, which is subsequently washed with 10 ml of water. During step 2, the bound analytes are eluted with ethanol–water (50:50, v/v). The eluate is diluted on-line (5) with water (4) to a final ethanol content of $<10\%$ (v/v). The diluted eluate is flushed through a reversed-phase concentration column (9) on which the analytes are trapped. During this step the IAC column is in series with the concentration column and the waste (6), thus preventing the build-up of unacceptable pressure over the IAC column. During step 3, valve 7 is switched in such a way that the concentration column is placed in series with the HPLC system [LKB 2150 solvent-delivery systems and a Model 2152 LC controller (10)]. The eluted analytes are detected by measuring their UV absorbance at 350 nm (Kratos Spectroflow 773) (11). If necessary fractions can be collected (LKB Redirac) (12).

For accurate determination, *e.g.*, during homogeneity tests on lyophilized candidate reference materials, the samples are analysed in the presence of a deuterated internal standard, in our laboratory 17β-trenbolone-d_2. In samples of urine the major metabolite is 17α-trenbolone, which is completely separated during HPLC analysis, thus allowing quantification by both HPLC and GC–MS. Strictly this is a pseudo-[M]IAC application, as the antibody binds both 17α-trenbolone and 17β-trenbolone. Fig. 11 shows two chromatograms, one for a sample that does not contain trenbolone and the other from a sample obtained from an animal treated with Trenbolonacetate.

Column switching, combining IAC with reversed-phase LC, has proved to be a highly suitable technique for the determination of trenbolone in samples of urine and tissue.

4. LC–MS METHODS

Combined LC–MS is one of the most rapidly advancing coupled techniques. However, its use within the area of steroid and β-agonist analyses has so far been limited, the main reason being the availability of numerous analytical alternatives. The widespread use of GC–MS and the limited resolving power of LC were no stimulus for the development of LC–MS procedures. There are, however, a number of research applications of LC–MS which clearly demonstrate the power of this technique. LC–MS has proved to be particular interesting in metabolism and conjugation studies and in analyses of highly polar corticosteroids.

Shackleton *et al.* [24] developed a method for the

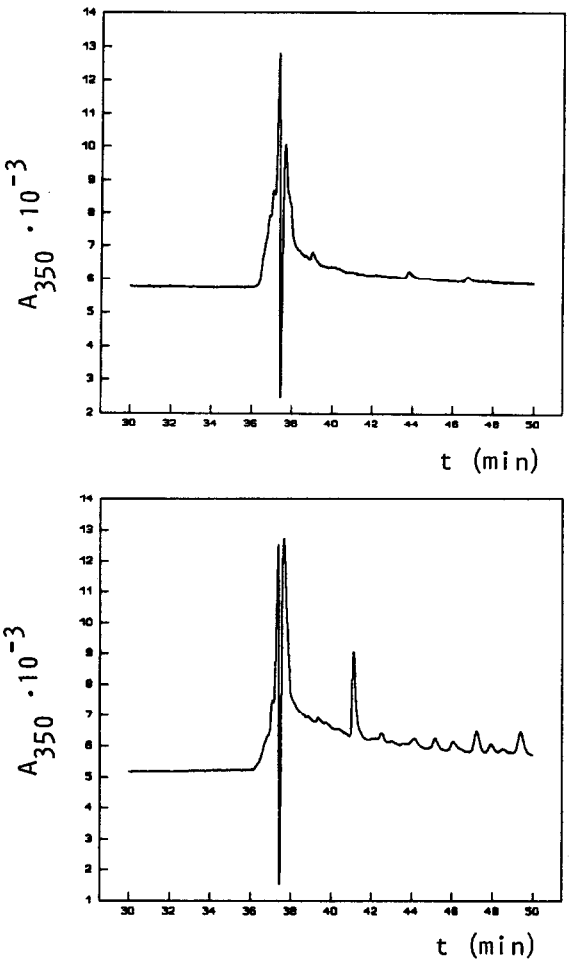

Fig. 11. HPLC separations of samples of urine obtained after on-line IAC–HPLC analysis. Upper panel: blank sample; lower panel: obtained from treated animal.

simultaneous determination of dehydroepiandrosterone sulphate, androsterone sulphate, epiandrosterone sulphate and androst-5-ene-3β,17β-diol sulphate in human serum, based on LC–isotope dilution MS. Poon *et al.* [25] demonstrated the suitability of LC–MS in a study of the metabolism of 4-hydroxyandrost-4-ene-3,17 dione in breast cancer patients. Seven metabolites were identified in samples of urine after hydrolysis, liquid–liquid extraction and reversed-phase LC–MS using a thermospray interface.

Sturm *et al.* [26] identified a number of metabolites of medroxyprogesterone acetate (MPA) in human plasma, a knowledge of which is important as these metabolites could well be responsible for the beneficial effect of MPA in breast cancer patients.

Several applications in which corticosteroids were determined using LC–thermospray MS [27–29] further illustrate the importance of LC–MS, especially in areas where GC–MS is not suitable because of the high polarity of the analytes or when derivatization should be avoided.

5. CONCLUSIONS

The current tendency within residue analysis is the development of either fast screening procedures, frequently based on an immunochemical detection principle, or methods based on GC combined with MS for confirmation. During recent years, however, the distinction between these two types of methods has become less clear because of the improved methods of sample clean-up which allow the selective isolation of groups of compounds. Mixed-mechanism SPE procedures and multi-immunoaffinity techniques are clear examples of LC developments that have greatly contributed to the current state of the art within residue analysis.

Screening methods are methods optimized for maximum sample throughput and a minimum chance of obtaining false-negative results. LC is not a preferred technique within such applications because it still is a relatively laborious procedure. For confirmation analysis, LC is frequently used. The LC–immunogram and LC–diode-array procedures are typical examples of methods used for confirmation. For this purpose the EEC has laid down criteria for positive identification [14,15] with such procedures. Apart from general criteria for assay performance, the amount and quality of information are specified.

A reference method should be able to yield direct structure information on the compound detected. For this purpose, LC has to be combined with one of the advanced spectroscopic techniques such as MS, IR spectrometry of UV–VIS spectrometry. So far MS is the only technique suitable in cases of international dispute. The role of LC, other than for puposes of sample clean-up, within such methods has been limited. LC–MS has found only a limited number of applications within residue analysis. In the near future this situation is likely to change.

LC is a key technique within residue analyses for

steroids, β-agonists and related compounds. The use of immunoaffinity chromatography, column-switching procedures and the further advancement of LC–MS will be areas of future developments.

6. ABBREVIATIONS

BCR	Community Bureau of Reference
BOL	Boldenone
CMA	Chlormadinone acetate
DE	Dienestrol
DES	Diethylstilbestrol
E2	Estradiol
EE2	Ethynylestradiol
EEC	European Economic Community
EI	Electron impact
GC	Gas chromatography
HEX	Hexestrol
HFB(A)	Heptafluorbutyric acid (anhydride)
HPLC	High-performance liquid chromatography
IAC	Immunoaffinity chromatography
ID	Isotope dilution
LC	Liquid chromatography
MGA	Megestrol acetate
MIAC	Multi-immunoaffinity chromatography
MT	Methyltestosterone
MPA	Medroxyprogesterone acetate
MS	Mass spectrometry
NT	Nortestosterone
SPE	Solid-phase extraction
T	Testosterone
TAL	Taleranol
TBME	*tert.*-Butyl methyl ether
TMS	Trimethylsilyl
Tb	Trenbolone
ZER	Zeranol

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