ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 87–96

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Application of 1D ^{1}H NMR for fast non-targeted screening and compositional analysis of steroid cocktails and veterinary drug formulations administered to livestock

A. Lommen [a,*], R. Schilt [a,1], J. Weseman [a], A.H. Roos [a], J.W. van Velde [b], M.W.F. Nielen [a]

[a] *State Institute for Quality Control of Agricultural Products (RIKILT), P.O. Box 230, NL-6700 AE Wageningen, The Netherlands*
[b] *Akzo Nobel Chemicals Research b.v., P.O. Box 9300, 6800 SB Arnhem, The Netherlands*

Received 1 July 2001; received in revised form 17 August 2001; accepted 8 September 2001

Abstract

A fast non-targeted strategy is described for analysis of formulations—meant for administration to live stock—containing growth-promoting agents or veterinary drugs. The use of ^{1}H NMR as a first step universal screening method is applied and used in routine analysis. The implementation of this approach has increased the analysis efficiency considerably. Apart from screening on illegal compounds, ^{1}H NMR information on matrix and thus, indirectly, administration mode, can be present. An ever-growing ^{1}H NMR database is used containing more than 200 reference substances. Based on the ^{1}H NMR screening, decisions for further analysis can be made, such as for instance HPLC fractionation of steroid cocktails and subsequent ^{1}H NMR (and LC–MS) analysis. Examples of unravelling formulations are given in detail including a steroid cocktail containing 15 compounds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: NMR; Screening; Steroid; Anabolic; Veterinary drug

1. Introduction

In livestock production, health-risk related compounds such as growth promoting agents and veterinary drugs may be illegally used for economic reasons. Inspection and control on farms occasionally turn up all kinds of containers, syringes, fluids, suspensions and solids that might be related to the administration of these compounds. The range of compounds that can be encountered varies from estrogens, androgens, gestagens, beta-agonists, beta-blockers, antibiotics, anthelmintics, sedatives, vitamins to non-livestock related compounds. If detailed analysis of each sample were required using traditional analytical means, the number of possible compounds would require a

* Corresponding author. Tel.: +31-317-475438; fax: +31-317-417717.
E-mail address: a.lommen@rikilt.wag-ur.nl (A. Lommen).
[1] Present address: TNO Nutrition and Food Research, P.O. Box 360, NL-3700 AJ Zeist, The Netherlands.

0731-7085/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(01)00613-6

substantial amount of time and money in screening, separation and identification. Detailed analysis is normally in conflict with the limited budget as well as the required short response times for analysis.

Traditionally nearly all research in this field is done by thin layer chromatography (TLC), GC combined with mass spectrometry or LC combined with mass spectrometry [1–5]. Mostly this is a targeted approach towards checking a number of predefined compounds; therefore, this traditional approach is limited. The types of samples described above are normally in concentrations ranging from submilligram to tens of milligrams per millilitre. Therefore an analysis technique like ^{1}H NMR becomes a very feasible alternative. If ^{1}H NMR is to be used, however, the question regarding criteria should be addressed.

Fast screening—often within 1 h—can be done with regard to virtually any compound using straightforward 1D ^{1}H NMR in these types of samples [6]. It is reasonably easy to construct a database of reference ^{1}H NMR spectra for structural comparison and confirmation. For this purpose, a growing database presently containing more than 200 reference substances is used routinely. The obvious advantage of ^{1}H NMR is that it is a universal quantitative detector for any given compound with protons above the normal NMR detection level; furthermore, it can give relative concentrations when more than one compound is present. Most formulations when used on livestock are so concentrated that they need dilution even for a relatively insensitive technique such as ^{1}H NMR. Since ^{1}H NMR is used directly after diluting the sample, information on matrix compounds or solvents may also be available. This may provide additional information on how a compound is administered to livestock.

In a cocktail of compounds in samples of the above type, ^{1}H NMR cannot always give the total composition directly. As with MS-analysis, the identification (exact quantification is not obligatory) of one major, illegally administered compound suffices for screening, since legal action can be taken based on the presence of one compound. Legal action could consist of additional inspection, taking samples from livestock for GC/LC–MS analysis, setting up surveillance, or simply using the NMR result as additional proof in a lawsuit. However, knowing which other compounds are being used illegally, the possible combinations and also the route of administration, can help to fine-tune regulatory control of livestock. For legal proof of usage of banned compounds, GC–MS or LC–MS analysis is required for samples derived from livestock. The choice of matrix, method and compound in MS-analysis could be facilitated by previous ^{1}H NMR results with regard to administration method and types of compound.

For a complete analysis of a complex cocktail, an HPLC separation followed by ^{1}H NMR (and LC–MS) analysis is required. ^{1}H NMR easily discriminates between steroids, cortico-steroids and other major groups of compounds on the basis of key structural differences identified in the spectra. Here, if an isolated compound does not match any known NMR reference and is still considered a non-matrix compound, MS analysis is optional as a complementary structure elucidation tool. The preferred MS should then be one in which accurate masses can be measured (e.g. ESI-TOF-MS), with which the composition of elements can be calculated. NMR and UV data can be used to limit the possibilities in the composition of elements.

Although not commonly used as a technique in livestock control analysis, a simple 1D ^{1}H NMR-based strategy seems at least very promising with regard to screening of these types of samples and also in channelling these samples into routes for further analysis, if needed. Long-term monitoring of these types of samples with NMR can also give insight as to how illegal compounds are being used and also in how profiles of compounds are changing. This information is of great importance in channelling funds into MS-method development for residue analysis of banned compounds in animal-derived matrices.

Here, we describe new developments in livestock control analysis using ^{1}H NMR to tackle screening and confirmation and to detect and identify possible unknown substances. Examples of screening and also the unravelling of a steroid cocktail are given.

2. Experimental

2.1. Reagents and NMR sample preparation

2.1.1. Standards referred to in this study

From Sigma: acepromazine maleate, beclomethasone, betamethasone, betamethasone 17,21-dipropionate, beta-estradiol, beta-estradiol-3-benzoate, clostebol 17-acetate, fluoxymesterone, furaltadone, methyltestosterone, testosterone 17-cypionate. From Riedel de Haen: chloramphenicol. Gift from Organon N.V. (Oss, The Netherlands): flurogestone 17-acetate, testosterone 17-phenylpropionate, testosterone 17-decanoate, testosterone 17-undecanoate. Gift from D. Courtheijn (ROL, Gentbrugge, Belgium): stanozolol. From Steraloids: dexamethasone, methylboldenone, norethandrolone. From Merck: vitamin B3, vitamin E acetate.

2.1.2. Other chemicals

Methanol-d_4 (Merck 99.8%), deuteriumoxide (Merck 99.95%), trifluoroacetic acid (Sigma), methanol (Fluka; chromatography grade), acetonitrile (Merck, LiChrosolv, gradient grade).

2.1.3. NMR sample preparation

For all reference compounds referred to in this study ca. 1 mg was dissolved in deuterated methanol for the 1D ^{1}H NMR database. For the purpose of screening with 1D ^{1}H NMR, 20 μl of liquid samples or suspensions were diluted to 600 μl in deuterated methanol; NMR samples of solids for screening were obtained by dissolving approximately 1 mg in 600 μl of deuterated methanol. HPLC fractions containing compounds (see Section 2.2) for 1D ^{1}H NMR analysis were evaporated to dryness and redissolved in 600 μl of deuterated methanol.

2.2. Hardware and experiments

2.2.1. Off-line HPLC separation for NMR analysis

The HPLC accessories consisted of an LC-22 pump (Bruker), an HP-1100 autosampler (HP), a Foxy Jr. fraction collector (Isco), a column oven (Bruker) and a diode array (J.M. Aalen). For preparative separation of a steroid cocktail, a Zorbax Eclipse XDB-C8 column (150 × 3 mm; 5 μm; Agilent) was used. The run was performed with a flow of 0.5 ml/min and a gradient from 0.1% TFA in H_2O to 0.1% TFA in acetonitrile. Before loading on the C_8-column, volatile constituents (Fig. 2D: information from ^{1}H NMR screening: propylene glycol) were first evaporated to give an oily solution; 20 μl were injected and every 1 min, fractions were collected for optional ^{1}H NMR analysis.

2.2.2. NMR analysis

^{1}H NMR experiments were performed on a Bruker AMX 400 WB spectrometer. Presaturation was performed on the HDO resonance. A 90° pulse was used; the total relaxation delay was 3.7 s; spectral width was 5000 Hz. The data were acquired in 16 K data points. Before Fourier transformation and phasing, a 1/3 shifted quadrate sine bell filter was applied and a zero-filling to 128 K. Calibration of spectra was achieved by setting the HCD_2-resonance of deuterated methanol to 3.27 ppm. A number of equipment checks were performed on a weekly basis, such as temperature calibration and temperature stability checks as well as line width checks as described elsewhere [7,8].

2.2.3. LC–ESI-TOF-MS analysis

Electrospray ionization mass spectrometry was performed on a Micromass (Manchester, UK) model LCT orthogonal acceleration time of flight instrument coupled with a model HP1050 (Hewlett Packard, Waldbronn, Germany) liquid chromatograph/diode-array UV detection system. The LCT was also equipped with the optional LockSpray dual inlet accurate mass interface, used to supply the phosphoric acid cluster reference compound separately from the LC effluent. The sample (Fig. 2D: information from ^{1}H NMR screening) was first diluted (10 μl in 1 ml of methanol) after which 10 μl was injected. The ESI-TOF-MS system was operated in the positive ion mode at a mass resolution of 4000 (FWHM), and with a specified mass accuracy of 2 mDa. The entire system was controlled by the Masslynx NT version 3.4 data system (Micromass). The exact

mass spectra from the MS analysis were used to calculate the elemental compositions of the peaks in the chromatogram. NMR and UV data were used to restrict the number of possibilities. Full details have been described elsewhere [9].

2.3. NMR database handling

XWINNMR 3.0 (Bruker) running on a 450 MHz PC was used for all ^1H NMR data handling. A special programme was written in Visual C + + 6.0 to convert the ^1H NMR spectra into six files for different categories of compounds. In each file the compounds were written as consecutive experiments in alphabetical order. This enables a fast electronic overlay within XWINNMR for comparisons.

2.4. Validation of identification results

The results of the NMR identification were validated by GC–MS and LC–MS analysis of the same samples in a separate independent laboratory, which is legally qualified in the field of livestock control. The nature of the results is qualitative since quantitative analysis is not needed for legal action.

3. Results and discussion

3.1. Criteria and NMR experiments

^1H NMR contains unique information on conformation and substitution patterns in molecules. However, for confirmation purposes in livestock inspection research and possible legal action taken, criteria for ^1H NMR with regard to experiments and identification should be considered as is customary for mass spectrometric confirmatory residue analysis [9,10].

In deciding on criteria for confirmation with ^1H NMR, the evident strength concerning sensitivity towards local environment is also a potential weakness. In general, ^1H NMR resonance positions are only sensitive in general to changes up to five bonds further away in the same structure, excluding some cases where changes further away influencing total molecular conformation can be reflected in the resonance position. Consequently, it stands to reason that a molecule must be characterised by a spread of proton resonances over the total structure. In the case of, for instance, steroids (a complex group of compounds with different derivatives; Fig. 1), the presence but also the absence of candidate resonances for identification plays a role (Table 2). Candidate resonances are from the aromatic protons, $CH_{(2)}O$ protons (in particular on positions 11 and 21), methyl (in particular singlets on 17, 18 and 19), protons from the ester and possibly relatively unique protons found in certain derivatives. Note that betamethasone and dexamethasone—differing only in orientation of the 16 methyl group (respectively beta vs. alpha)—are easily distinguished (Table 2). Also beclomethasone versus betamethasone (fluor vs. chlorine on C_{10}) show characteristic differences (Table 2). The presence of an ester is established by a downfield shift in resonance position of the $CH_{(2)}O$; this enables the precise localisation of esters (Table 2). Although this works very well, there are some problems with regard to identification and confirmation. It is, in practice, virtually impossible to distinguish between testosterone decanoate and testosterone undecanoate (Table 1). The integral over the broad multiple resonance band at 1.3 ppm often cannot be determined precisely enough due to common impurities at this position; determining the identity as a linear alkyl ester of testosterone is not a problem. In this case, extra information in the form of a molecular mass is required (Table 1).

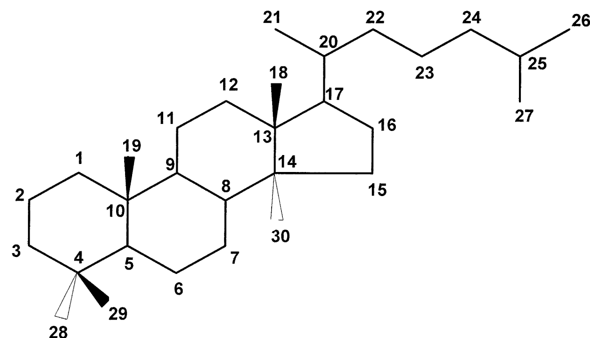

Fig. 1. Basic steroid structure.

Table 1
Results of the detailed compositional analysis of a steroid cocktail (Fig. 2D)

Fast screening with NMR	HPLC fractionation and NMR	Theoretical molecular formula	Experimental molecular formula from ESI-TOF-MS	Compound type
	Vitamin B3	$C_6H_6N_2O$	$C_6H_6N_2O$	Vitamin
Stanozolol	Stanozolol	$C_{21}H_{32}N_2O$	$C_{21}H_{32}N_2O$	Androgen
Dexamethasone	Dexamethasone	$C_{22}H_{29}FO_5$	$C_{22}H_{29}FO_5$	Corticosteroid
Fluoxymesterone	Fluoxymesterone	$C_{20}H_{29}FO_3$	$C_{20}H_{29}O_3$	Androgen
Methylboldenone	Methylboldenone	$C_{20}H_{28}O_2$	$C_{20}H_{28}O_2$	Androgen
Flurogestone 17-acetate	Flurogestone 17-acetate	$C_{23}H_{31}FO_5$	$C_{23}H_{31}FO_5$	Gestagen
	Methyltestosterone	$C_{20}H_{30}O_2$	$C_{20}H_{30}O_2$	Androgen
Norethandrolone	Norethandrolone	$C_{20}H_{30}O_2$	$C_{20}H_{30}O_2$	Androgen
	Betamethasone 17,21-dipropionate	$C_{28}H_{37}FO_7$	$C_{28}H_{37}FO_7$	Corticosteroid
Clostebol 17-acetate	Clostebol 17-acetate	$C_{21}H_{29}ClO_3$	$C_{21}H_{29}ClO_3$	Androgen
Beta-estradiol 3-benzoate	Beta-estradiol 3-benzoate	$C_{25}H_{28}O_3$	$C_{25}H_{28}O_3$	Estrogen
	Testosterone 17-phenylpropionate	$C_{28}H_{36}O_3$	$C_{28}H_{36}O_3$	Androgen
Testosterone 17-(cypionate)	Testosterone 17-cypionate	$C_{27}H_{40}O_3$	$C_{27}H_{40}O_3$	Androgen
Testosterone 17-((un) decanoate)	Testosterone 17-((un)decanoate)	$C_{29}H_{46}O_3/C_{30}H_{48}O_3$	$C_{29}H_{46}O_3$ [a]	Androgen
	Vitamin E-acetate	$C_{31}H_{52}O_3$	Not detected	Vitamin

Compounds are listed on the basis of retention on C_8. Italics are used to indicate incomplete evidence for compounds as detected in the fast screening with NMR.
[a] Actually detected as the acetonitrile adduct: $C_{31}H_{49}NO_3$. This confirms the decanoate ester.

^{1}H NMR resonances can also be sensitive to, for instance, temperature, pH and solvent composition. Therefore the use of ^{1}H NMR in confirmation other than through spiking should also rely on very well specified experimental conditions. In some pH-dependent situations, addition of TFA should be considered.

When relying on comparison to a database of reference spectra for confirmation, differences in position of chosen resonances from the same molecule should not deviate more than 0.5 Hz compared to the reference spectrum of the same molecule in a database. Coupling constants should not deviate more than 0.2 Hz. Furthermore, relative amplitudes should also be within a 10% margin in comparison to the reference spectrum. In practise, an expert can assess these criteria most easily by overlaying spectra electronically and scaling these on each other. If spiking is the mode of confirmation action, resonance positions, multiplets of spike and original compound of interest should, of course, be indistinguishable.

3.2. Screening using ^{1}H NMR

In practise, screening of samples can best be done by examining the aromatic and alcoholic region of the ^{1}H NMR spectrum. On the basis of an expert opinion a sub-database can be chosen, which provides reference spectra that can be electronically laid over the spectrum of interest. This either leads to direct identification/confirmation or gives indications to similar structures in the database. If substructures can be identified, search routines in for instance the electronic version of the Merck Index can be used to come up with likely candidate molecules [11]. These candidates in turn can be added to the database and checked.

Furthermore, it could also be concluded that due to complexity, an HPLC separation is necessary followed by sequential ^{1}H NMR and/or MS analysis of fractionated HPLC peaks.

Four examples of ^{1}H NMR spectra of samples are given in Fig. 2A–D.

In Fig. 2A (fluid sample) a spectrum is given in which acepromazine maleate in almost pure form is present. Acepromazine is easily protonated due to the presence of amines. Therefore, in principle, this spectrum does not have to coincide with a reference spectrum and the use of TFA to drive the protonation to the full extent should normally be considered. In this case, however, the reference spectrum in the database was of acepromazine maleate and gave a perfect match indicating the absence of other pH influencing compounds. Indicative are the seven aromatic proton resonances between 6.9 and 7.7 ppm, the N–CH_2 resonances at 4.10 and 3.16 ppm, the C–CH_2–C resonances at 2.15 ppm, the two N–CH_3 resonances at 2.73 ppm, the $COCH_3$ resonance at 2.56 ppm and lastly the aromatic resonance of the equivalent protons of maleate at 6.22 ppm. The original

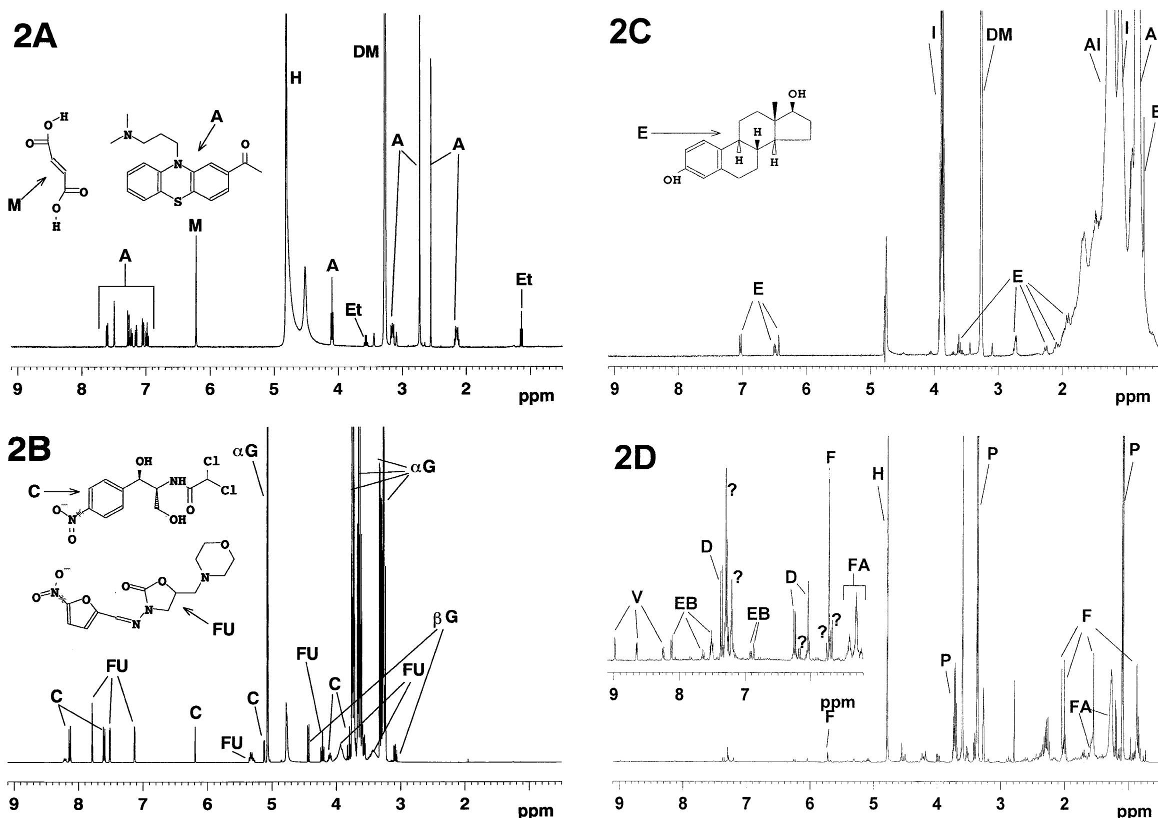

Fig. 2. ^{1}H NMR spectra (see Section 2) for screening of samples (diluted/dissolved in CD_3OD). (A) A water-based solution of acepromazine. (B) Furaltadone and chloramphenicol in a solid glucose containing matrix. (C) 17-Beta-estradiol saturated in an isopropanol/alkane mixture. (D) Oil and polypropylene glycol-based steroid cocktail. Abbreviations: A, acepromazine; Al, alkane; C, chloramphenicol; D, dexamethasone; DM, deuterated methanol; E, estradiol; EB, 17-beta-estradiol benzoate; Et, ethanol; F, flurogestone acetate; FA, fatty acids; FU, furaltadone; alpha-G/beta-G, alpha-glucose/beta-glucose; H, HDO; I, isopropanol; M, maleate; P, propylene glycol; V, vitamin B3.

solvent is water (suppressed by solvent presaturation) since no other solvent signal except for a minor ethanol contamination is found. Acepromazine is a water soluble sedative; it can either be administered orally or intramuscularly.

Sample 2B (solid sample) is an approximately 1:1 mixture of chloramphenicol and furaltadone in a glucose-containing solid matrix; these antibiotics would probably be administered orally, as concluded from the glucose matrix. Indicative for chloramphenicol are the precise match of aromatic proton resonances of the symmetric nitro-substituted aromatic ring at 8.13 and 7.60 ppm, the $CHCl_2$ resonance at 6.19 ppm, the benzene–CH–O resonance at 5.12 ppm, the CH–N resonance at 4.10 ppm and the two partially obscured inequivalent proton resonances at 3.77 and 3.57 ppm from the CH_2O. The last two resonances could in fact be omitted in the identification since a different chemical group would reflect on neighbouring proton resonance positions (see Section 3.1). Indicative for furaltadone are the CH=N resonance at 7.79 ppm, the nitrofuran ring resonances at 7.51 and 7.13 ppm, the non-morpholino O–CH resonance at 5.32 ppm, the broad morphilino O–CH_2 resonances at 3.94 ppm, the broad morphilino N–CH_2 resonances at 3.43 ppm and one CH–N–morphilino resonance at 4.22 ppm. Three CH–N resonances are obscured by glucose resonances. The morphilino-group is pH-dependent. Since like acepromazine in Fig. 2A, no other pH-influencing compounds are present, furaltadone correlates precisely with the reference although several resonances should be pH-dependent. The broadness of the morphilino resonances is typical for a dynamic structural change in the ring of the morphilino-group. The information present is adequate with respect to identification.

Sample 2C (saturated suspension sample) contains 17-beta-estradiol in the free form in an isopropanol and alkane solvent mixture. It is not likely that isopropanol and alkanes would be administered orally and they are not first choice solvents for intramuscular or subcutaneous administration. However, they may be candidates for a 'pour-on' application in which estradiol is taken up through the skin after evaporation of volatile solvents. In steroids, ring substitutions play a crucial role in the conformation of the different rings. Consequently, the conformation of each ring also influences neighbouring ring conformation. Therefore, a difference in substitution on a steroid is also reflected in proton resonance positions and multiplet structures of protons which are multiple bonds away. This feature is of importance in screening steroid cocktails in which overlap occurs. For a steroid, a limited number of resonances are needed for identification (see for example Table 2). In Fig. 2C the identification of 17-beta-estradiol is given by the aromatic proton resonances of C1 at 7.04 ppm, of C2 at 6.49 ppm and C4 at 6.43 ppm as well as the proton resonances of C17 at 3.62 ppm and the C18-methyl at 0.74 ppm. Several other unassigned protons also match, but are not necessary for identification. ^1H NMR can easily distinguish between 17-alpha and 17-beta conformations using the last two resonance positions.

Fig. 2D (saturated suspension sample) shows signs of a steroid cocktail in an oil (as evidenced by for instance (un)saturated fatty acid signals as indicated) and propylene glycol based matrix. Major steroids found during screening in this sample are flurogestone acetate (gestagen), 17-beta-estradiol benzoate (estrogen) and dexamethasone (cortico-steroid). The oil and propylene glycol (pharmaceutical solvent aids) are probably related to an intramuscular or subcutaneous application. Close examination of Fig. 2D shows that several other steroids could be present in this cocktail (a couple of question marks are indicated; see also Table 1 for educated guesses on the basis of screening alone) which cannot be identified/confirmed due to spectral complexity. The major steroids in sample 2D were identified on the basis of the resonances given in Table 2. For sample 2D there is an evident choice of HPLC for separation and sequential analysis by ^1H NMR and MS so as to facilitate the identification of the other steroids present.

The results obtained by ^1H NMR for the individual compounds were validated by the results obtained from independent LC and GC–MS experiments performed in a separate qualified laboratory. The NMR identifications were confirmed.

Table 2
Assignments of the resonances of the compounds eluting from the C_8 HPLC column

Assignments	C1–H	C2–H	C4–H	O–C11–H	C16–CH3	O–C17–H	C17–CH3	C18–CH3	C19–CH3	C21–CH2–OH	C21–CH3	Special	
Stanozolol-TFA							1.16	0.84	0.77			7.86	Pyrazole-H
Dexamethasone	7.36	6.25	6.04	4.21 F	0.82			0.97	1.55	4.55, 4.21			
Fluoxymesterone			5.70	4.18 F			1.13	0.97	1.55				
Methylboldenone	7.26	6.17	6.02				1.13	0.93	1.26				
Flurogestone 17-acetate			5.72	4.24 F				0.87	1.55		2.04	2.00	Acetate-CH3
Methyltestosterone			5.67				1.15	0.87	1.21				
Norethandrolone			5.76					0.91				0.93	17-Ethyl-CH3
Betamethasone 17,21-dipropionate	7.35	6.26	6.06	4.28 F	1.28			0.94	1.54	4.79, 4.40 E		1.12, 1.09	Dipropionate-CH3
Clostebol 17-acetate						4.56 E		0.85	1.24			3.20, 1.98	C6-H, acetate-CH3
Estradiol 3-benzoate	7.32	6.91	6.87			3.64		0.77				8.12, 7.64, 7.51	Benzoate
Testosterone 17-phenylpropionate			5.67			4.52 E		0.75	1.19			7.22, 7.16, 7.13, 2.88, 2.60	Phenylpropionate
Testosterone 17-cypionate			5.67			4.57 E		0.85	1.21			Several	Cypionate
Testosterone 17-decanoate			5.67			4.58 E		0.85	1.20			1.27, 0.86	Long alkyl, methyl
For comparison													
Betamethasone	7.36	6.24	6.04	4.20 F	1.08			0.97	1.56	4.44, 4.25			
Beclomethasone	7.35	6.24	6.01	4.41	1.09			1.07	1.67	4.44, 4.26			

Compounds are listed on the basis of retention. ‘F’ after a given resonance position indicates a coupling to a C10-F. ‘E’ after a given resonance position indicates the presence of an ester.

3.3. Detailed analysis using HPLC, ^{1}H NMR and MS

The steroid cocktail in Fig. 2D was fractionated using an analytical C_8 column and subjected to further ^{1}H NMR analysis. LC–ESI-TOF-MS analysis was performed separately [9]. In Table 1 the results from ^{1}H NMR and MS analysis are given. Restrictions from NMR and UV data were used to calculate the most likely elemental compositions from the accurate masses obtained in the LC–ESI-TOF-MS experiments. The analyses of some HPLC fractionated compounds are not given since they were found by ^{1}H NMR to be matrix related. The ^{1}H NMR results, apart from the vitamins, are on the basis of comparison to the database and are further described in Table 2. Assignments of the resonances were done on the basis of common ^{1}H NMR expertise and the comparison of known structures and spectra. Vitamin B3 is identified by its four aromatic resonances at 8.98, 8.65, 8.25 and 7.50 ppm and the typical heterocyclic aromatic coupling constant of 4.8 Hz; TFA was needed. Vitamin E–acetate is identified by the singlet methyls at 2.27 (acetate), 2.04, 1.95, 1.92 (on the aromate), 1.21 (O–C–CH_3) and four doublet methyls between 0.86 and 0.80 ppm (aliphatic part of the molecule). Fifteen compounds could be detected and conclusively identified in this cocktail. On the basis of the ^{1}H NMR database, all compounds could be confirmed, except one testosterone ester for which there is no clarity regarding the length of the ester. The LC–ESI-TOF-MS analysis gives conclusive evidence for a decanoate ester.

4. Conclusion

The initial screening of samples with 1D ^{1}H NMR has increased the efficiency of analysis considerably; often results can be obtained within an hour. Compounds in the type of samples used for livestock administration are in the submilligram to tens of milligrams per millilitre range; they are therefore easily detected by ^{1}H NMR. The lack of sample preparation or separation is a major advantage with regard to analysis time and also in giving a complete picture of the sample (i.e. no compounds are lost and the matrix holding information on the mode of administration is present). The simplicity of the approach, i.e. 30-fold dilution or dissolving in deuterated methanol, has the great advantage of being a robust and easily reproduced experiment with little variation in solvent and thus negligible resonance shifts and good possibilities for a database. This also holds major advantages in defining and adhering to criteria needed for legal consequences.

In contrast to LC–MS or GC–MS analysis the NMR approach has the advantage that no prior knowledge concerning the type of compounds or matrix is necessary. Also advantageous is the lack of sample preparation and fast analysis time in this screening procedure. The high sensitivity of mass spectrometry, obligatory for residue analysis, is not necessary for these types of samples. An additional advantage of NMR is that the information on matrix and thus administration mode can be deduced.

With the advent of on-line LC–NMR a new possibility for analysis of steroid cocktails and drug formulations seems to come into view [12–14]. However, the increased sensitivity of LC–NMR versus conventional NMR is not necessary, since concentrations are adequate for conventional ^{1}H NMR. Also, supposing the compounds of interest bind to the column, the on-line separation can actually become more of a disadvantage than an advantage, since different separation methods will give different solvent compositions for eluting compounds. Differences in solvent composition will give resonance shifts for compounds of interest and thus will complicate a strict adherence to identification criteria necessary for legal action. Furthermore, for technical and financial reasons the preferred organic solvent for LC–NMR is non-deuterated acetonitrile. This solvent has a very prominent signal at approximately 2 ppm, which can obscure important information on ester and methyl resonances. Therefore, in the near future the traditional off-line approach allowing re-dissolving in deuterated methanol seems to be most practical.

Arguably, ^{1}H NMR is an expensive technique requiring expert interpretation. The price of ^{1}H

NMR is related to the field strength of the magnet. However, presently the price of a 300 MHz NMR machine does not exceed that of a sophisticated LC–MS machine. Decisions made by ^{1}H NMR pre-examination of the type of samples described here could also shortcut analysis costs otherwise made with regard to manpower and other types of sophisticated identification means. ^{1}H NMR can be used as an analysis tool for livestock inspection in legal matters since analysis procedures including checks as well as criteria for identification of compounds can be defined. Together with MS-techniques such as ESI-TOF-MS, its potential in structure elucidation of unknown compounds in steroid cocktails and veterinary drug formulations is extremely strong. This increases the likelihood of detection of unknown compounds in obtained formulations that are used in meat production. In time a better picture can be drawn of the use of illegal compounds enabling better fine-tuning of livestock inspection with regard to which matrix and which compounds GC/LC–MS analysis should be performed on. Information on steroid cocktails is already creating more understanding of the illegal administration of growth promoting agents. Cocktails for intramuscular or subcutaneous administration often contain a combination of estrogens, gestagens, androgens and cortico-steroids. A number of these compounds are present to yield a boosting effect because they are liberated and metabolised nearly completely within a few days; others are slow-release compounds to obtain a long steady state effect. Corticosteroids are thought—apart from increasing appetite and thirst—to be used as masking agents as they decrease the concentration of growth-promoting compounds in urine [15].

Acknowledgements

Akzo Nobel is acknowledged for the ESI-TOF-MS measurements. The authors thank the General Inspection Service (AID) and the Foundation for Quality Guarantee of the Veal Meat Sector (SKV) for providing the sample materials. L.A. Lindsey is thanked for critical reading.

References

[1] K.K. De-Wasch, H.F. De-Brabander, D. Courtheyn, C. Van-Peteghem, Analyst 123 (12) (1998) 2415–2422.
[2] K. Vanoosthuyze, E. Daeseleire, A. Van-Overbeke, C. Van-Peteghem, A. Ermens, Analyst 119 (12) (1994) 2655–2658.
[3] E. Daeseleire, K. Vanoosthuyze, C. Van-Peteghem, J. Chromatogr. A 674 (1-2) (1994) 247–253.
[4] P. Batjoens, H.F. De-Brabander, F. Smets, G. Pottie, Analyst 119 (12) (1994) 2607–2610.
[5] F. Smets, H.F. De-Brabander, P.J. Bloom, G. Pottie, J. Planar Chromatogr. Mod. TLC 4 (3) (1991) 207–212.
[6] R. Schilt, A. Lommen, E.O. van-Bennekom, H. Hooijerink, L. Brouwer, J. de Jong, M.S. Leloux, in: L.A. van Ginkel, A. Ruiter (Eds.), Proceedings EuroResidue IV (2000): Conference on Residues of Veterinary Drugs in Food, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands, 2000, p. 982.
[7] A. Lommen, J.M. Weseman, G.O. Smith, H.P.J.M. Noteborn, Special issue NMR in environmental sciences, Biodegradation 9 (1998) 513–525.
[8] H.P.J.M. Noteborn, J.M. Weseman, R. van de Jagt, A. Lommen, Special issue NMR in biotechnology, J. Biotechnol. 77 (2000) 103–114.
[9] M.W.F. Nielen, J.P.C. Vissers, R.E.M. Fuchs, J.W. van Velde, A. Lommen, Rapid Commun. Mass Spectrom. 15 (17) (2001) 1577–1585.
[10] EEC (1993) document 93/256/EEC, Official J. Eur. Commun. (1993), No L118/64.
[11] The Merck Index: CD-ROM Windows, Version 12.3, Chapman & Hall/CRC, ISBN/ISSN: 1584881291, Publication date: 1/26/2000.
[12] K. Levsen, A. Preiss, M. Godejohann, Trace Trends Anal. Chem. 19 (1) (2000) 27–48.
[13] J.L. Wolfender, K. Ndjoko, K. Hostettmann, Phytochem. Anal. 12 (1) (2001) 2–22.
[14] A. Lommen, M. Godejohann, D.P. Venema, P.C.H. Hollman, M. Spraul, Anal. Chem. 72 (2000) 1793–1797.
[15] M.J. Groot, R. Schilt, J.S. Ossenkoppele, P.L.M. Berende, W. Haasnoot, J. Vet. Med. Ser. A 45 (6-7) (1998) 425–440.