Identification of residues by LC-MS. The application of new EU guidelines

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New EU guidelines for identification and quantification of organic residues and contaminants are established to guarantee efficient and reliable residue control of prohibited growth promoting agents, *e.g.* steroids and beta-agonists, and veterinary drugs. Reliability includes the reliability of the identification with spectrometric techniques. The guidelines take into account the implications of a detected violation. The unambiguous identification of a prohibited compound needs more information on the structure of the analyte than the identification of a registered veterinary drug of which the mass fraction detected exceeds the established Maximum Residue Limit. For detection using mass spectrometry this is reflected by the number of fragment ions that must be detected.

Introduction

The use of steroids and beta-agonists for enhancing animal growth is prohibited within the EU whereas the use of veterinary drugs is strongly regulated [1]. Based on EU legislation each Member State is obliged to operate an effective residue control programme, in short the "Annual National Plan" (ANP), to be submitted to the European Commission. This residue control programme must be based on the general principles and guidelines laid down in relevant Commission Decisions [2].

For over a decade now it is realised that the diversity and dynamics of the compounds to be tested for, being either prohibited or legal, ask for a flexible system of developing and validating analytical methods. The solution was found in setting up sets of minimum performance characteristics which have to be fulfilled by methods to be used for residue control of veterinary drugs, including growth promoting compounds, natural and environmental contaminants. Mass spectrometry, either in combination with gas chromatography (GC-MS) [3] or liquid chromatography (LC-MS) [4] plays a key role in residue analyses and subsequently in the related Commission Decisions. For confirmatory (reference) purposes, these criteria discriminate between prohibited (Group A) and legal (Group B) compounds. Confirmatory methods are methods that provide full or complementary information enabling the analyte to be identified unequivocally at the level of interest.

This paper discusses the general principles of verification of the identity of small molecules in (LC)-MS as applicable in residue analyses for veterinary drugs.

Identification points

For confirmatory purposes, the concept of identification points was introduced in the guidelines for data evaluation [5]. For the identification and confirmation of a Group B (legal) compound, it is necessary to collect 3 identification points. For the identification and confirmation of a Group A (illegal) compound it is necessary to collect 4 identification points. The number of identification points "earned" depends on the technique used. However, for Group A compounds a technique based on molecular spectrometry is mandatory. In practise this means that MS techniques are used in most cases.

MS methods are suitable only following either an on-line or off-line chromatographic separation. If full scan spectra are recorded in single MS, a minimum of four ions must be present with a relative intensity of ≥ 10 %.

If mass fragments are measured using other than full-scan techniques, a system of identification points must be used to interpret the data. Table I shows the number of identification points that each of the basic mass spectrometric techniques can earn.

However, in order to qualify for the identification points required for confirmation:

a) A *minimum* of at least one ion ratio must be measured, and

b) *All measured ion ratios* must meet the criteria, and

c) *A maximum* of three separate techniques can be combined to achieve the minimum number of identification points.

The allowable tolerances for the ion ratios are summarised in table II.

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Table I. The relationship between a range of classes of mass fragment and Identification Points earned.

MS technique	*Identification Points earned per ion*
Low resolution mass spectrometry (LR)	1.0
LR-MS^n Precursor ion	1.0
LR-MS^n Transition products	1.5
High resolution mass spectrometry (HR)	2.0
HR- MS^n Precursor ion	2.0
HR-MS^n Transition products	2.5

Footnotes:
1) Each ion may only be counted once.
2) GC-MS using Electron Impact ionisation is regarded as being a different technique to GC-MS using Chemical ionisation.
3) Different chemical derivatives of an analyte can be used to increase the number of identification points only if the derivatives employ different reaction chemistries.
4) Transition products include both daughter and granddaughter products

Table II. Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques.

Relative intensity (% of base peak)	*EI-GC-MS (relative) (relative)*	*CI-GC-MS, GC-MS-MS^n LC-MS, LC-MS-MS^n*
>50 %	± 10 %	± 20 %
> 20 % - 50 %	± 15 %	± 25 %
> 10 % - 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

The main advantage of using identification points is the fact that the verification of the identity can be done in a well-described and internationally accepted way. The arithmetic approach of adding identification points also is helpful in evaluating the total information gathered in those cases where more than one measurement is necessary. Several LC-MS methods use the detection of a (pseudo) molecular ion during the first analyses resulting in 1 identification point (no ratio). This value of 1 can be added to the numerical result of further measurements using other techniques like LC-MSMS and LC-MS^n.

Experimental

The applicability of the criteria for LC-MS methods was evaluated for three analytical methods routinely used in our laboratory.

The confirmation of presence of residues of corticosteroids in samples of bovine urine

After enzymatic deconjugation with a β-glucuronidase/sulfatase mixture (suc d'Helix Pomatia, Brunschwig Chemie, Amsterdam, The Netherlands) in order to deconjugate the analytes the sample is extracted on a SPE (Oasis) column (Waters, Etten-leur, The Netherlands) [6]. The final extract is dissolved in 250 µl LC-solvent of which 200 µl is injected into the LC-MS system (ion-trap LC-MS^n (Finnigan LCQ-system, ThermoQuest, Breda, The Netherlands). Acquisition parameters: vaporiser 500 °C; capillary 150 °C; nitrogen (high purity), 70 p.s.i.; ions are detected in the APCI (+)-MS^2 and MS^3 mode.

HPLC-conditions: mobile phase acetonitrile/water gradient; column: LichroCART® 125-2 mm, Superspher® 100 RP-18, 4 µm particles endcapped (Waters). MS-conditions: Ion trap MS^2 and MS^3 APCI(+)mode; selected ions monitored for Dexamethasone were [MS^2-373; MS^3-355].

For Dexamethasone, Flumethasone and Triamcinolone acetonide, one MS^2 and one MS^3 transition ion are monitored and the relative intensity between of the two-recorded ions is calculated and compared with the ratio as obtained for the corresponding standards. The ions monitored for respectively dexamethasone, flumethasone and friamcinolone acetonide are [MS^2-373; MS^3-355], [MS^2-391; MS^3-371] and [MS^2-415; MS^3-357].

The confirmation of presence of residues of 17β-Trenbolone in samples of bovine muscle

After enzymatic digestion of the tissue (Subtilisin A (Protease, Sigma Aldrich Chemie, Zwijndrecht, The Netherlands)) the aqueous sample is extracted with tertiar butyl methyl ether (TBME) [7]. The primary extract is purified with Immuno Affinity Column (IAC-columns for Trenbolone from Randox® (TB 2186); commercially available from Coring System Diagnostix GmbH (Gernsheim, Germany). The final residue is dissolved in 50 µl of methanol to which 200 µl of water are added. Of this mixture 200 µl is injected into the LC-MS system (LC-MS^n (Finnigan LCQ)). Acquisition parameters: vaporiser 500 °C; capillary 150 °C; nitrogen (high purity), 70 p.s.i.; ions are detected in the APCI (+)-MS^3 mode.

HPLC-conditions: mobile phase acetonitrile/water gradient; column: LichroCART® 125-2 mm, Superspher® 100 RP-18, 4 µm particles endcapped. MS-conditions: Ion trap MS^3 in APCI(+)mode; selected ions monitored *m/z* 197, 211 and 235.

The confirmation of the presence of 17β-Trenbolone is based on measurements by LC-MS^3. Three transition ions are monitored and the ratio between the recorded fragment ions is calculated and compared with the ratio as obtained for the reference standard. The three transition ions monitored for 17β-Trenbolone are *m/z* 197, 211 and 235.

The confirmation of presence of residues of 16β-hydroxy-stanozolol in samples of bovine urine

The analyte 16β-hydroxy-stanozolol is the major metabolite in urine of cattle of the anabolic stanozolol [8]. After enzymatic deconjugation and sample clean-up with SPE the identity of the compound is confirmed with LC-MS ($LC\text{-}MS^n$ (Finnigan LCQ)).

HPLC-conditions: mobile phase acetonitrile/methanol/water; column: TSK-gel, Super ODS® 50-4.6 mm, 2 µm particles endcapped (TOSO HAAS, Supleco, Zwijndrecht, The Netherlands). MS-conditions: Ion trap MS^3 in APCI(+)mode; selected ions monitored *m/z* 159, 227, 255 and 329.

Acquisition parameters: vaporiser 450 °C; capillary 150 °C; nitrogen (high purity), 70 p.s.i.; ions are detected in the APCI (+)-MS^2 mode. The transition ions with *m/z* values of 159, 227, 255 and 309 are monitored.

Results and discussion

The criteria with respect to the verification of the identity of small molecules with LC-MS, inclusive the extension to multiple MS, as described in the draft revision of Commission Decision 93/256 [5], were developed very recently. The concept of multiple ion monitoring for verification of the identity, however, is already used for more than a decade in GC-MS applications. The applicability of the new criteria was verified in our laboratory for a number of different methods, each using a different type of LC-MS method. All examples are from the field of the detection of the presence of residues of illegal growth promoting compounds. In view of the possible severe trade implications and the public health risks associated with the used of these compounds, the reliability of the analytical methods used is considered of being of the utmost importance.

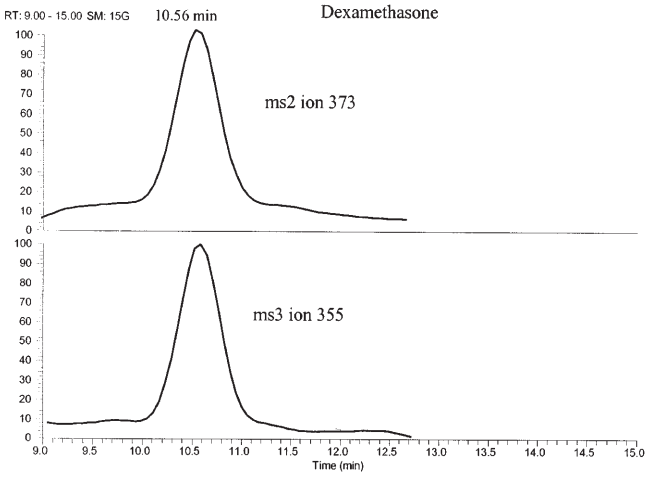

Figure 1. SPE(Oasis)/LC-MS^2MS^3 chromatogram of a fortified sample of hydrolysed urine containing 1 µg/l Dexamethasone (Rt = 10.6 min).

Table III. Intensity ratios for Dexamethasone reference standard and unknown. MS^2 (372.6-373.6), MS^3 (354.6-355.6).

	(MS3/MS2)	
Reference dexamethasone	0,245	
ratio ± 25% (tolerance limits)	0,184	0,306
Average unknown	0,250 ± 0.032	
Result	confirmed	

Figure 1 shows the chromatograms obtained for the $LC\text{-}MS^2/MS^3$ analysis of a sample of bovine urine spiked at a level of 1 µg/l with dexamethasone. The corresponding chromatogram of the blank sample did not show any response.

The evaluation of the ratios is summarized in table III.

According to table I the detection of two transition products yields 3 identification points (1.5 identification points each). However, since the precursor ion also must have been present, an additional identification point can be added. Therefore, the data shown are adequate for the confirmation of the identity of dexamethasone at the level concerned.

Figure 2 shows chromatograms of the monitored transition ions ($LC\text{-}MS^3$) for the confirmation of 17β-Trenbolone in an extract of bovine meat. The sample was found to be "not-negative" during routine screening of imported meat. The initial screening was also performed with LC-MS. The same sample clean-up procedure was used. For Quality Control and quantification, however, a deuterated internal standard, 17β-Trenbolone-d2, was added to the sample prior to extraction. For detection a HP-1100 system with LC-MSD detector (Hewlett Packard, Amstelveen, The Netherlands) was used. Protonated molecular ions with *m/z* 271 and 273 (internal standard) were monitored after APCI. The quantitative result obtained for this sample was 0.8 µg/kg.

The evaluation of the ratio is summarized in table IV.

From table IV it can be concluded that the intensity ratios observed for the signals detected correspond to the reference values determined under identical experimental conditions.

The detection of three transition products yields 4.5 identification points (1.5 identification point each). However, since the precursor ions also must have been present, two additional identification points can be added. Therefore, the data shown are adequate for the confirmation of the identity of 17β-trenbolone. For the same reasons, the initial analysis with LC-MSD did not fulfil the criteria for confirmation. Only 1 identification point was obtained and no ratios were

calculated. The analyses of known blank samples was part of each experiment. None of the signals used for the detection and identification of the analyte was present in any of these samples.

The third procedure evaluated was a recently developed method for the confirmation of 16β-hydroxy-stanozolol. Figure 3 shows the confirmatory data, four ions monitored in MS^2 after APCI, for a sample spiked at a level of 2 μg/l.

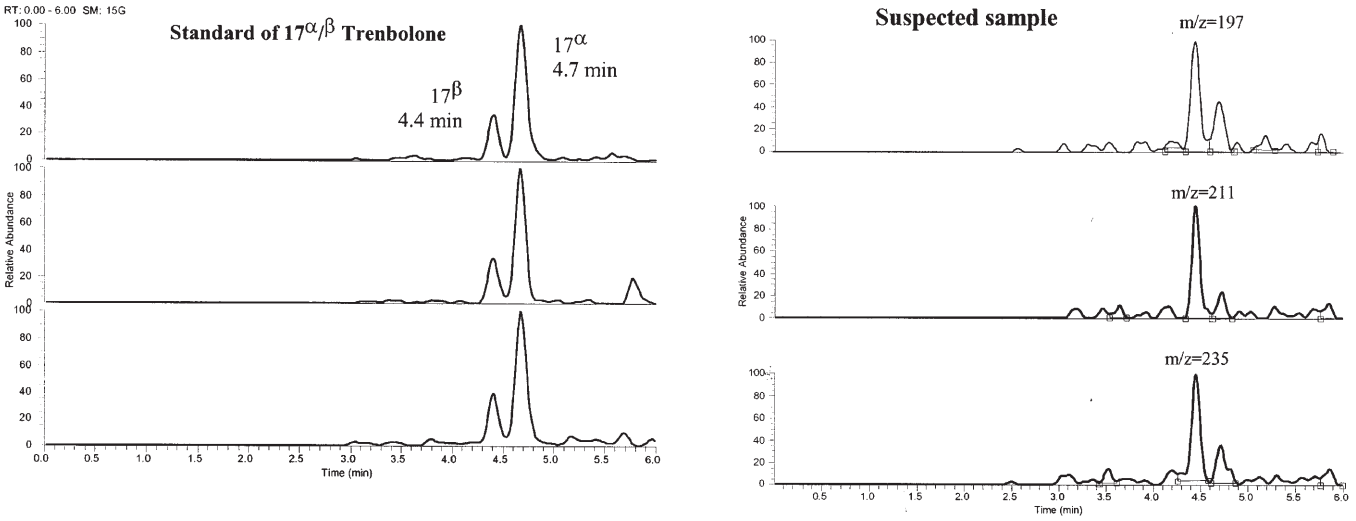

Figure 2. IAC/LC-MS^3 chromatogram of a) Standard of 1 ng of 17α/β-Trenbolone b) bovine meat sample containing 17β-Trenbolone (Rt = 4.4 min).

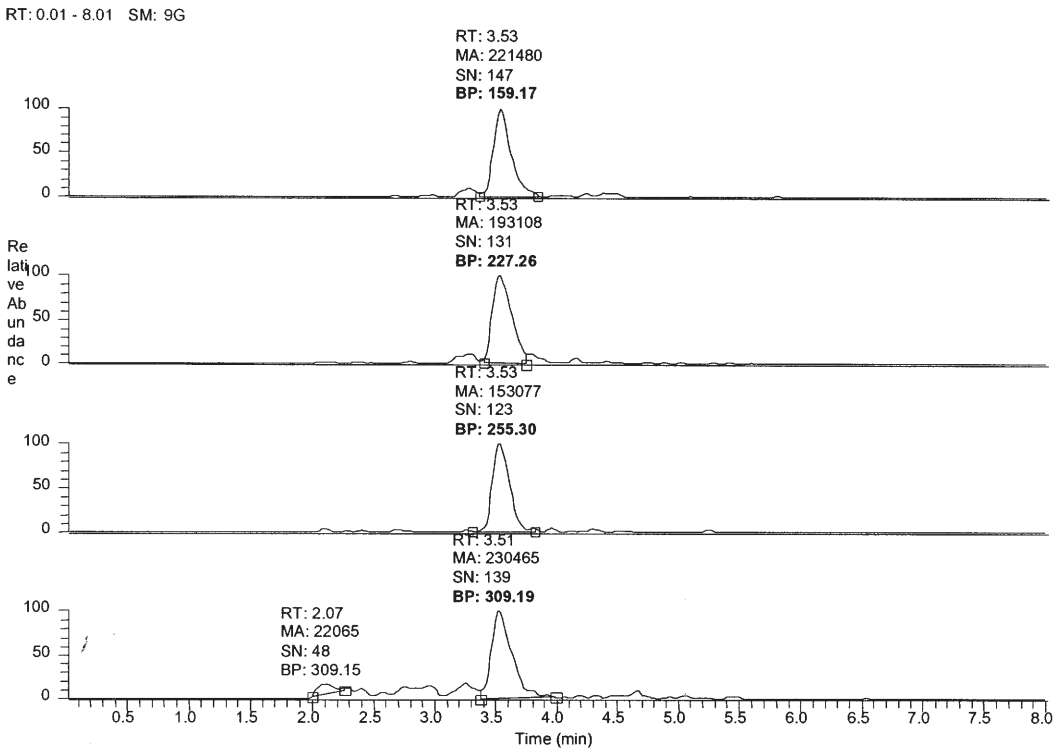

Figure 3. Confirmation 16β-hydroxy-stanozolol in bovine urine (control sample containing 2 μg/l), MS^2 of mass 345 (MH^+) after APCI.

Table IV. Relative intensities of the transition ions applied to EU-criteria.

	Relative Intensities m/z *211/235*	*Relative Intensities* m/z *197/235*	*17β-Trenbolone Confirmed?*
Standard	0.93	0.57	
EU-criteria	0.93 ± 20 % (0.74-1.12)	0.57 ± 20 % (0.46-0.68)	
Suspected sample	0.78	0.57	YES

Together these signals result in 6 identification points, more than adequate for positive identification.

Again, all ratios calculated were in agreement with the reference values and none of the signals was detected in blank control samples.

Conclusions

Confirmation of the identity, especially of illegal compounds, must be performed in a reliable and transparent way. Fixed criteria therefore are essential. These criteria must be established in such a way that the are applicable at relevant levels; typically 1 µg/l for samples of urine and 0.5 µg/kg for tissue samples. On the other hand, they must result in a truly reliable identification, meaning that they must yield adequate structure information. From the three examples shown above, it is concluded that the use of the concept of identification point gives good results. The concept is purely experimentally derived and based on experiences with GC-lrMS. For the past two decades it has been mandatory to detect at least 4 ions when applied in official residue control within the EU. Very few published methods deviate from this general rule. The best known example is the use of only 3 ions for the detection of diethylstilbestrol (DES) in bovine urine as described by Sphon *et al.* (9). Until now, no hard evidence was obtained that, in combination with proper evaluation of the results, false positive results can be obtained in this way.

Even though a fundamental chemometric basis is lacking, these preliminary experiences already indicate the usefulness of this approach. The concept of identification points currently can be considered the most reliable and practical approach for the verification of the identity of residues detected in biological matrices.

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