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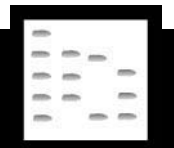
VENOMS: CHROMATOGRAPHY

See **III / NEUROTOXINS: CHROMATOGRAPHY**

VETERINARY DRUGS: LIQUID CHROMATOGRAPHY



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For the analysis of residues of veterinary drugs, liquid chromatography (LC) is of increasing importance: some of these molecules are polar, heat-sensitive and/or difficult to analyse by gas chromatography–mass spectrometry (GC-MS). Moreover, LC is the method of choice for components of high molecular mass. Since the introduction of benchtop LC-MS instruments, there has been an increasing number of publications on the application of this technique in the field of residue analysis.

Equipment

In LC a large variety of packed columns are in use but most residue separations are carried out with some kind of reversed-phase material based on modified silicas (RP-18, RP-8, etc.). Hitherto, in our laboratory, a particle size of 5 μm with column dimensions 150×2.1 mm has been commonly used. For a laboratory involved in residue analysis under accreditation, the daily reproducibility of the chromatogram from column to column is very important (see section on quality criteria, below). In the future, column material of smaller particle sizes (3 μm) may be used routinely, allowing faster separation, higher sample throughput and better limits of detection.

The nature of the mobile phase depends on the column used. In most cases a mixture of water and an organic solvent such as methanol or acetonitrile is used. Special LC grades of solvents are necessary. For analysis of residues, gradient elution is a must. In most cases the column has to be cleaned from interfering components after each run by a gradient. As well as organic solvents, a number of chemicals may be added to the mobile phase (buffers and chelating agents) but the compatibility of these products with the detector should be checked. For LC-MS only volatile components (e.g. trifluoroacetic acid) can be used and this limitation sometimes hinders the transformation of an LC into an LC-MS^n method.

Autoinjection is a must for the routine analysis of residues of veterinary drugs, not only for higher sample throughput but also for reproducibility in the validation of the results. However, particular attention should be drawn to the danger of cross-contamination with such injectors, especially in combination with LC-MS which has low detection limits.

Detectors

For screening purposes universal detectors such as UV and light-scattering detectors are used. However, for the confirmation of suspect samples more is required than just retention time and detector response. Since the results of laboratory analysis may have a serious impact on individuals and companies, false positives must be avoided at any price. For example, a sample of poultry feed, analysed by ion chromatography, was suspected to contain KSCN (a thyreostatic drug). Both the retention time and co-chromatography met the quality criteria. However, the presence of KSCN was so unlikely that the effluent was collected and mixed with Fe^{3+} (to give a red colour with SCN). This test was negative. Later on, it was found that the sample contained acetylsalicylic acid, which is often used in poultry rearing, and that the two molecules are not separated in the chromatographic system used.

More analytical evidence could be gathered by using a diode array detector (DAD). However, at low concentrations of the analyte and/or dirty samples, interferences are very likely. With a fluorescence detector more specific analysis at lower detection limits can be performed but in most cases some kind of derivatization of the analyte is needed.

The mass spectrometric detector is very important in residue analysis: the most common interfaces are electrospray (ES) and atmospheric pressure interface (API).

Quantification

Quantitative analysis is necessary for residues of legal veterinary drugs having a maximum residue limit (MRL). The method used must have limit of quantification of (at least) half the MRL. The validation of quantitative method is very time-consuming and expensive. Therefore, qualitative LC is often used for analysis of residues of illegal substances (with a so-called zero tolerance). However, quantitative methods always have a qualitative aspect (a value for the correct substance) while qualitative methods always contain a quantitative background (e.g. the estimation of peak intensities). This quantitative aspect is reflected in the so-called action limits: levels of residues which an efficient laboratory should be able to reach (e.g. 2 p.p.b. for anabolics). In our laboratory, qualitative data (residue present or not) are transferred into quantitative data as follows: a large number of samples (e.g. 50 urines of different origin) are spiked with several anabolics at a certain level (e.g. the action limit) and analysed. The percentages 'detected spikes' are calculated. A 95% detection levels is statistically accepted. So, it could be stated to the inspection services: 'if a sample contains the spiked level, the residue will be detected with a 95% probability'. Higher or lower levels will be detected with higher or lower probabilities. It should also be mentioned that quantification of one signal (e.g. a UV absorbance) is easier than quatification of a complex signal (e.g. a mass spectrum). However, complex signals give much more information. Internal standards play an important role in quantification in residue analyses. For LC-MS the availability of deuterated standards is often a limiting factor.

Generally, it is important to convince customers (e.g. inspection services) that very reliable quantitative analysis of many samples with low detection limits in a short time for a very low price is not possible.

Special Features of LC

LC-MS^n

The first benchtop LC-MS-MS machine based on a modification of an ion trap was introduced in 1996. In tandem MS an ion (e.g. the molecular ion) may be chosen as parent ion, isolated and concentrated in the trap, while all other ions are ejected. Afterwards the speed of the ion is increased: the ion collides with He present in the trap and fragments. The fragment ions (daughter ions) are measured. The daughter ions are theoretically derived from the parent ion only, but in practice some interference is still present (**Figure 1**).

With quadrupoles, MS-MS is normally the end of the story. In an ion trap one daughter ion may be concentrated and fragmented over and over again. In theory, MS^n opens the way to a significant reduction of the clean-up of the sample. However, fewer and fewer ions of the analyte are present and the signal-to-noise ratio competes with the ability of the apparatus to detect ions. In practice MS^2 is only needed for analysis of most residues with LC-MS.

LC as Clean-up in Residue Analysis

Some hyphenated techniques are claimed to be so specific that they only need minimum sample clean-up. In our experience this is not yet true for the analyses of all residues (e.g. anabolics in complex

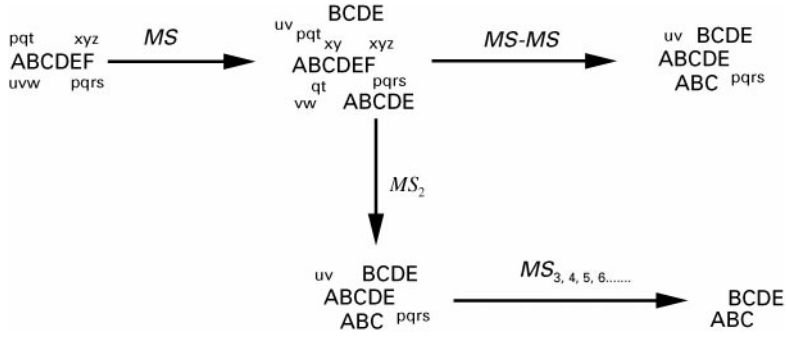

Figure 1 MS (ABCDEF, analyte; pqt, xyz, uvw and pqrs, interferences); MS-MS on ABCDEF; MS^n: formation of granddaughter and grandgranddaughter ions.

matrices at the p.p.b. ($\mu g\,kg^{-1}$) level). The clean-up of the primary extract needs special attention. LC purification adds a considerable value to the specificity of the method and influences the reliability of the results in a positive sense. By fraction collection, very clean extracts are obtained and the limit of detection is substantially decreased.

Immunological methods can also be coupled to LC to eliminate interfering substances.

Quality Criteria for the Use of LC in Residue Analysis

Minimum quality criteria for the identification of residues using different analytical techniques have been published in the European Commission (EC) directive 93/256. For LC the EC has specified the following quality criteria for methods of analysis which may be used for confirmatory purposes:

1. The analyte should elute at the retention time which is typical for the corresponding standard analyte under the same experimental conditions.
2. The nearest peak maximum in the chromatogram should be separated from the designated analyte peak by at least one full peak width at 10% of the maximum height.
3. The absorption maximum in the spectrum of the analyte should be at the same wavelength as those of the standard analyte within a margin determined by the resolution of the detection system. For diode array detection this is typically within ± 2 nm.
4. The spectrum of the analyte above 220 nm should not be visually different from the spectrum of the standard analyte for those parts of the two spectra with a relative absorbance $\geqslant 10\%$. This criterion is met when the same maxima are present and no observed point in the difference between the two spectra is more than 10% of the absorbance of the standard analyte.
5. For confirmatory purpose, if the method is not used in combination with other methods, then co-chromatography in the LC step is mandatory.

Discussion of the Quality Criteria

1. Quality criterion 1 is same for any chromatographic procedure: the retention times of the two peaks, formed by the analyte and the standard, should correspond. Otherwise the analyte clearly differs from the standard. A window of 3% is a reasonable quality criterion. Where a great deviation occurs, co-chromatography may be used (see point 5).
2. Criterion 2 requires a resolution of one between two peaks. However, this quality criterion is not clearly described in the EC document. Here the question might be put whether the criterion should only be required for peaks with the same maximum wavelength. For example, an analyte with maximum absorbance of 430 nm may in practice be readily distinguished from an interfering compound with a maximum of 310 nm, even if they partly co-elute. In LC-MS^n, this criterion will theoretically not be valid if deuterated standards, which nearly co-elute, are used.

3 and 4. These criteria match only LC-DAD. For LC-MS^n criteria have not yet been described.

5. In criterion 5, co-chromatography is required for proper identification of an analyte. The usefulness of co-chromatography may be questioned: co-chromatography may prove that the peak in question is not the analyte but not that the peak is without any doubt the analyte. Moreover, it is important that the concentration of standard analyte added is of the same magnitude as that of the sample.

Examples of LC Methods in Residue Analysis

In this section some examples of LC and LC-MS^n methods for residues of some illegal growth promoters, legal drugs and feed additives are discussed. More extensive information can be found in the Further Reading section.

LC Methods for Illegal Growth Promoters

Thyreostatic drugs The use of these drugs in cattle results in a spectacular weight gain, arising mainly from an increased filling of the gastrointestinal tract and an augmented water retention. In our laboratory a specific thin-layer chromatography (TLC) method for the determination of thiouracil and analogous compounds has been established. For additional confirmation, the final extract of the TLC method could also be analysed by LC-MS^n yielding specific MS^2 and MS^3 spectra (**Figure 2**).

Anabolic steroids The use of anabolic steroids as growth promoters in the fattening of animals is prohibited in all EU member states. GC-MS is the method of choice for a large number of these components. But some compounds such as stanozolol and its most important metabolite in cattle (16β-hydroxystanozolol; **Figure 3**) are difficult.

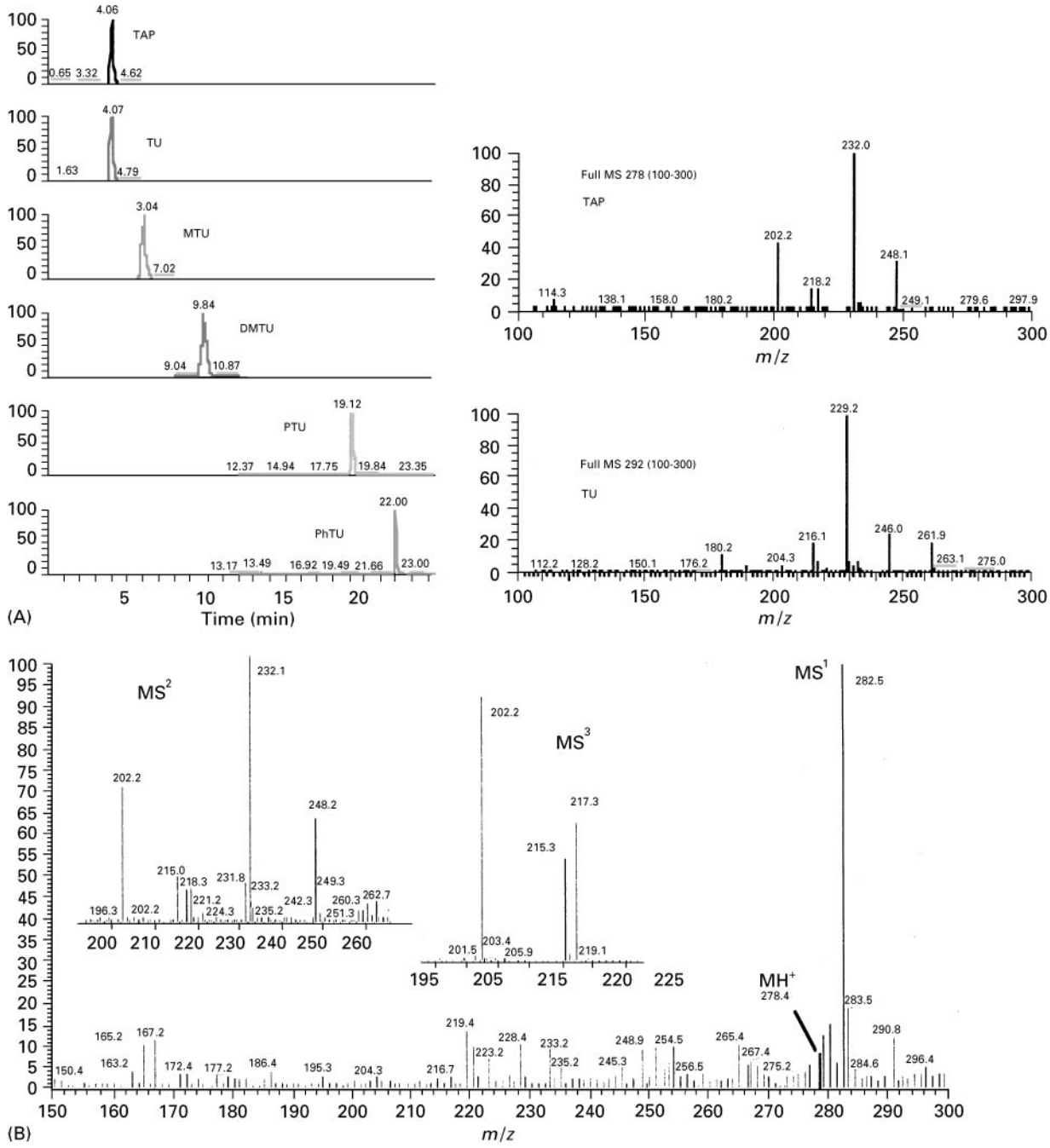

Figure 2 (A) Chromatogram and MS^2 spectra of some thyreostats. Thyreostats: 4(6)-R-thiouracil (R = H (TU); methyl (MTU); n-propyl (PTU); phenyl (PhTU)); TAP, 1-methyl-2-mercaptoimidazole (tapazole); DMTU, (4(5,6)-dimethyl-2-thiouracil). (B) MS^1, MS^2 and MS^3 spectrum of the thyreostat tapazole.

Recently, GC-MS, LC-MS, MS-MS and MS^n methods for this metabolite have been described and compared, in a collaborative study between three Belgian and three Dutch laboratories. It was observed that the spectra obtained on different types of LC-MS systems are clearly different: from one diagnostic ion (in a single quadrupole) to a lot of diagnostic ions with LC-MS^n. This illustrates the difficulty of working out quality criteria for LC-MS^n analysis.

β-Agonists During the 1980s the β-agonists found illegal application in animal breeding (extra weight gain together with a repartition between muscle and fatty tissue). An LC method with post-column

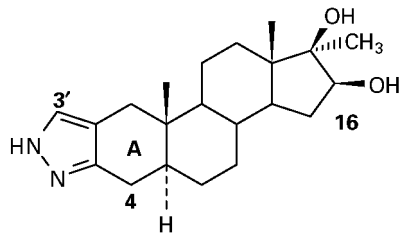

Figure 3 16β-Hydroxystanozolol: the most important metabolite of stanozolol in cattle.

derivatization (with a diazotization mixture) for the determination of clenbuterol and analogues has been described. Later, the very specific detection for anilines was replaced by MS^n detection: it is easier to switch from one analyte to another with an LC-MS system than with a post-column derivatization detector. Moreover, deuterated clenbuterol can be used for quantification. In **Figure 4** a chromatogram of some β-agonists (not all are represented here) and an example of an MS^2 spectrum (tulobuterol) are given.

Corticosteroids Corticosteroids are also abused in cattle fattening. The weight gain is probably due to secondary effects of the corticosteroids, such as water retention. For the analysis of residues of corticosteroids, GC-MS with negative ion chemical ionization (NCI) detection is still the method of choice. However, for the identification of newly used corticosteroids in injection sites, LC-MS^n offers more identification power than GC-MS (no derivatization; different MS^n spectra).

Anti-infection Agents

This broad range of chemicals is used for both therapeutical and/or growth-promoting reasons. Screening for residues of antibacterials in slaughtered animals is carried out in most states by microbial inhibition tests on kidney tissue. In the case of a positive test, the identity and (in the case of legal drugs) the concentration of the substance should be determined. It is in this aspect that LC and LC-MS methods are mostly used.

Sulfonamides Several LC methods for the determination of sulfonamides have been described. In our laboratory an LC method from the literature was quickly transformed into an LC-MS^n method. In **Figure 5** a chromatogram and MS^2 spectra of some sulfonamides are given. Currently, six sulfonamides are monitored in one run.

Antibiotics For antibiotics such as penicillins, cephalosporins, quinolones, macrolides and tetracyclines a lot of LC and some LC-MS methods have been described. For tetracyclines, for example, ligands (e.g. oxalic acid) have to be added to the mobile phase to prevent extreme tailing. Post-column derivatization (e.g. with $ZrCl_4$) followed by fluorescence detection is

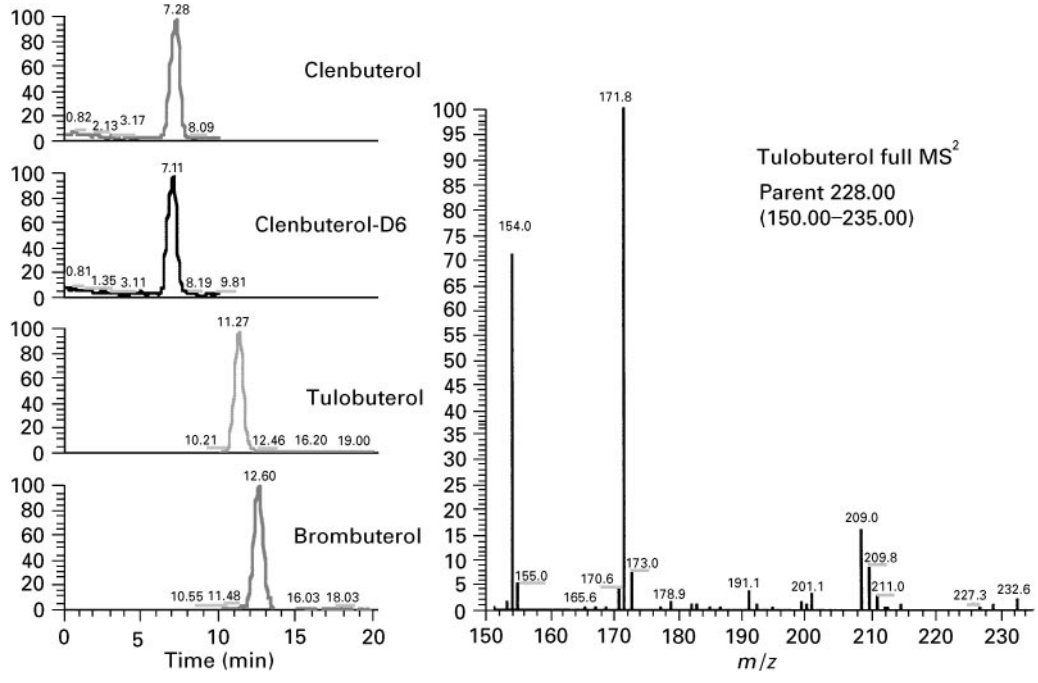

Figure 4 Chromatogram of some β-agonists and MS^2 spectrum of tulobuterol.

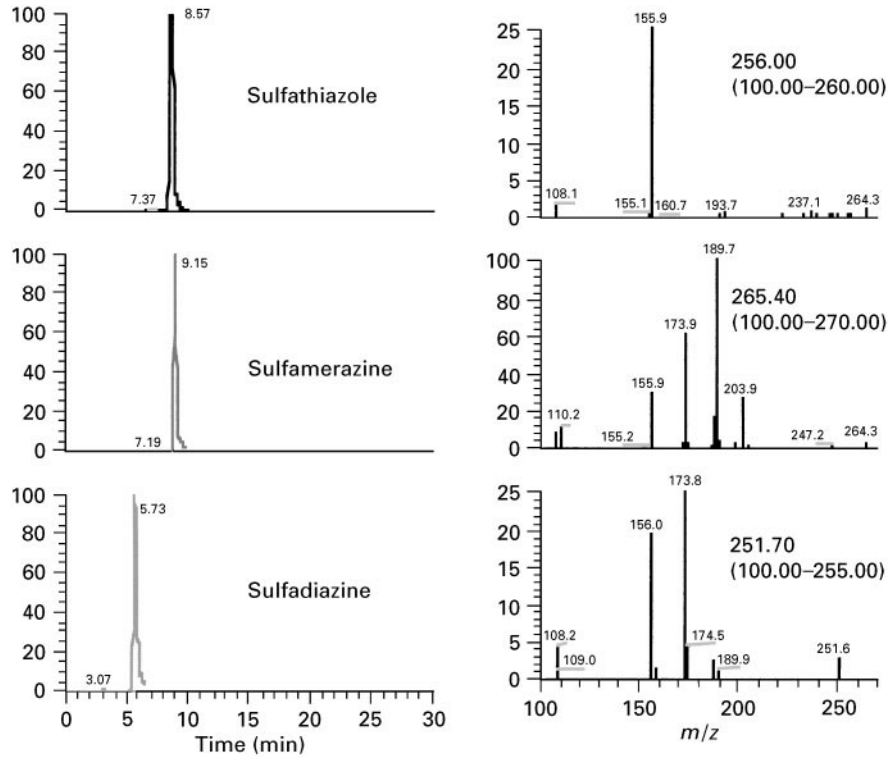

Figure 5 Chromatogram and MS^2 mass spectra of some sulfonamides.

very specific for these molecules yielding very low limits of detection: 0.5–1.5 μg kg^{-1} in comparison with 2–5 μg kg^{-1} with LC-MS.

Antiparasitic Agents

An example of a potent antiparasitic veterinary drug is ivermectine (a macrocyclic lactone disaccharide).

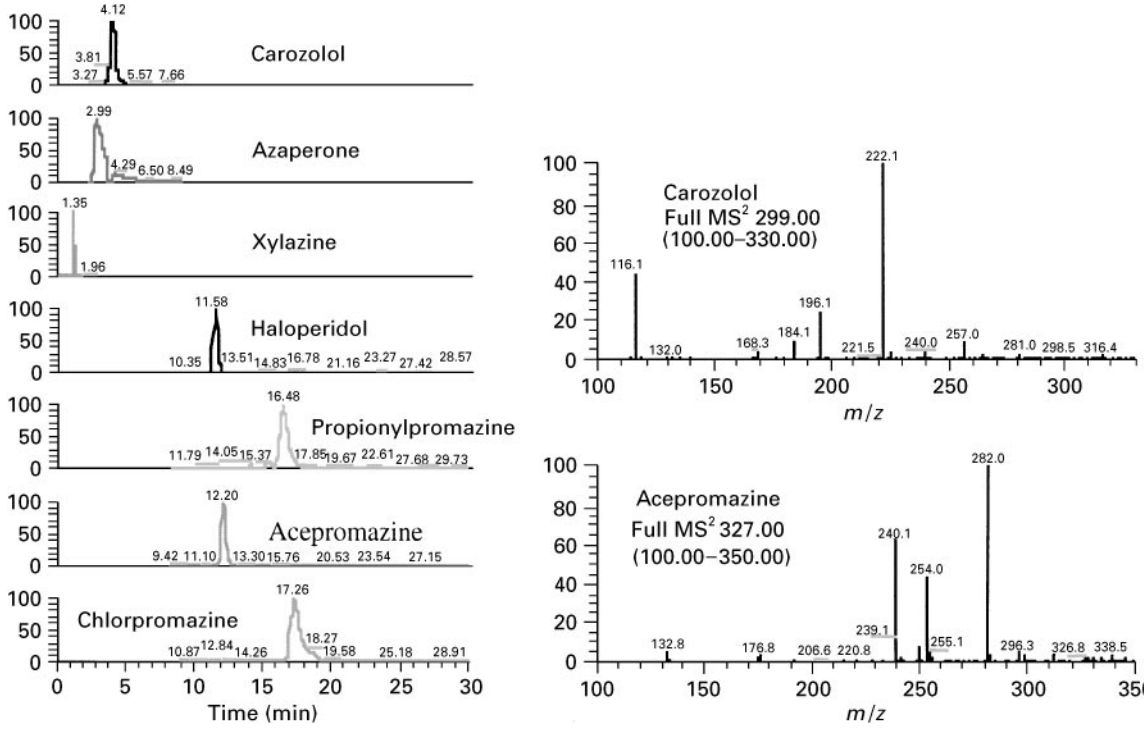

Figure 6 Chromatogram and MS^2 mass spectra of some tranquillizers.

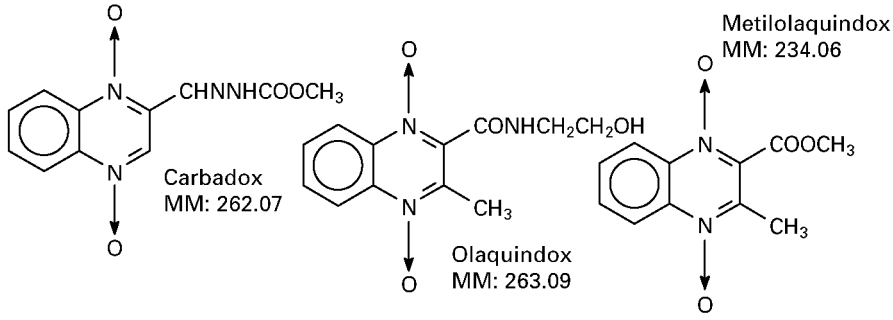

Figure 7 Formulas of carbadox, olaquindox and metilolaquindox.

The drug is effective in low dosages and therefore requires methods with low detection limits (MRL: 15 $\mu g\,kg^{-1}$ in porcine liver). Since the molecule has a high molecular mass, LC is the method of choice. HPLC-UV methods for screening of ivermectine residues in animal tissues and milk have been described. For confirmation the molecule can be derivatized (with methylimidazole–acetic anhydride) and analysed by LC with a fluorescence detector.

Tranquillizers

Tranquillizers may be used illegally to prevent stress during the transport of pigs and bulls to the abattoir. A large number of LC methods have been published for the determination of residues of these components. In our laboratory one method was transferred into an LC-MS^n method with which seven tranquillizers could be determined in a short time. In **Figure 6** mass chromatograms and the MS^2 spectra of these components are given. The data, given in Figures 4–6 were obtained with the same apparatus. This is an illustration of the ease of switching from one analyte to another.

Feed Additives

Some components are not considered as veterinary drugs but as feed additives. Examples are the quinoxalines, carbadox and olaquindox. However, residues of these components may be present in edible tissue as well. Also nonregistered equivalents of these components could be used: as an example, the presence of metilolaquindox was suspected in animal feed: this was possibly a modification of olaquindox with a methyl group. LC analysis gave a chromatogram containing a large peak different from carbadox and olaquindox. However, LC-MS analysis gave a molecular mass less than carbadox and olaquindox. By combining MS with NMR a structure for this molecule was proposed (**Figure 7**). This example illustrates the important of MS in residue analysis.

Conclusion

The demands for specificity, reliability, speed and turnover in residue analysis of veterinary drugs are continuously increasing. LC, especially with MS detection, is a reliable analytical technique which should be able to cope with these stringent demands. In comparison with GC and GC-MS, a large range of analytes can be covered and in most cases there is no need for derivatization. It is also easy to switch an LC-MS system from one analyte to another. The lower yield of the LC-MS interfaces and the poorer separation power of LC columns in comparison with GC may be regarded as points to be improved.

The use of illegal alternatives to registered drugs or feed additives poses two important problems for routine inspection: first of all there is no target component. The situation is comparable with the search for a unknown needle in an unknown haystack. Secondly, no analytical standards of the molecule are available. MS (and MS^n) is able to give more information about a suspect peak. The future of LC in residue analysis will depend largely on the possibilities of identification of illegal substances abused and qualitative and quantitative analysis of legal veterinary drugs with LC-MS^n.

See Colour Plate 124.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry; Detectors: Ultraviolet and Visible Detection; Mechanisms: Reversed Phases. **III/Forensic Sciences:** Liquid Chromatography.

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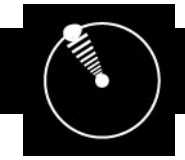
VIRUSES: CENTRIFUGATION



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Viruses have proved to be detrimental as well as beneficial. They are notoriously infectious agents that are at the root of several major diseases in man, domesticated animals, and agricultural crops. However, their attenuated or noninfectious forms have been used as vaccines, enabling the development of immunity against particularly devastating diseases. Recently, replication-deficient viruses have been used as agents for gene delivery and as potential vaccine carriers, as they have evolved efficient mechanisms of infectivity.

Viruses are particulate in nature and are made up essentially of DNA or RNA, wrapped in a predominantly protein coat. They range in size from 20 to 2000 nm (0.02–2 μm) and in molecular weight from 4×10^6 to 2×10^9 Da. Many viruses possess an envelope that is typically derived from the host cellular membrane.

Initial isolation of viruses usually involves centrifugation, particularly density gradient centrifugation (DGC). For almost half a century DGC has been regarded as the most rapid, and reliable preparative procedure for the isolation of highly purified and concentrated virus preparations for subsequent physicochemical and biological characterization. As such, it is used as a benchmark against which alternative methods can be evaluated. To date the technique has permitted the isolation and subsequent characterization of a plethora of viruses belonging to at least 39 major families.

Centrifugal Separations

Although significant improvements in centrifugation hardware have led to increased operational efficiencies, the theory behind centrifugation and the variations of the technique as applied to viruses are well characterized. In a suspension of particles, the rate at which particles sediment when subjected to a centrifugal force depends on the nature of the particles, the nature of the medium, and the magnitude of the centrifugal force. For spherical particles, the sedimentation rate or velocity of the particle depends on a variety of factors as indicated in eqn [1], one of the many forms of the Svedberg equation:

$$dr/dt = [2r_p^2(\rho_p - \rho_m)\omega^2 r]/9\eta \qquad [1]$$

where dr/dt is the velocity of the particle; r_p is the radius of the spherical particle; ρ_p is the density of the particle; ρ_m is the density of the medium; ω is the angular velocity; r is the radial distance of the particle from the axis of rotation; the product $\omega^2 r$ is proportional to the centrifugal force; and η is the viscosity of the medium. It is possible to define a particle in terms of its behaviour in a centrifugal field by manipulation of eqn [1] to yield a simplified version of the Svedberg equation (eqn [2]) that uses the sedimentation coefficient, s, where:

$$s = (dr/dt)/\omega^2 r \qquad [2]$$

For most biological macromolecules, the magnitude of s is about 10^{-13} s, so this value is used as the unit of sedimentation, the Svedberg (S). The sedimentation coefficient for viruses varies between 40 and 4500 S, while for globular proteins it is 2–5 S.

Types of Separations

For a particular viral preparation, the most effective centrifugal separation procedure is one that yields a concentrate with significant recovery of bioactivity