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Analysis of veterinary drugs using supercritical fluid chromatography and supercritical fluid chromatography–mass spectrometry

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ABSTRACT

Packed-column supercritical fluid chromatography has been used for the separation of four veterinary antibiotics (levamisole, furazolidone, chloramphenicol and lincomycin) on an amino-bonded stationary phase utilizing carbon dioxide with methanol modifier as the mobile phase. The effect of change of modifier identity was also studied. Packed-column supercritical fluid chromatography–mass spectrometry employing both moving belt and modified thermospray interfaces was also studied using these compounds.

INTRODUCTION

In a continuation of the sulphonamide studies previously reported [1] the methods employed were used to monitor the possible application of supercritical fluid chromatography (SFC) and SFC–mass spectrometry (MS) to the analysis of four veterinary antibiotics (Fig. 1) which are administered for a variety of reasons.

In veterinary medicine levamisole has been used primarily as an anthelmintic in pigs, sheep and cattle [2]. It has led to improved immunity against coccidiosis [3] and has shown potential in the treatment of bovine diarrhoea [4]. The analysis of levamisole has been demonstrated using both gas chromatography (GC) where the drug could be chromatographed without derivatisation but the column had to be deactivated before use [5–7] and high-performance liquid chromatography (HPLC) which in common with GC requires lengthy clean up procedures [2,8,9].

Furazolidone is a nitrofuran which shows antibacterial and antiprotozoal

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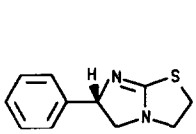

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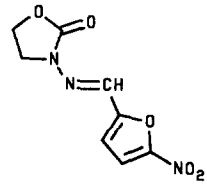

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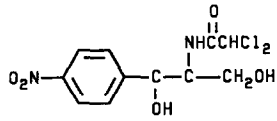

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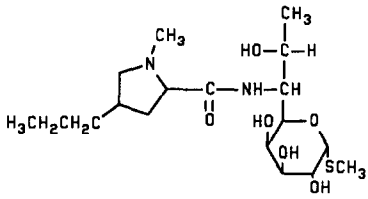


LINCOMYCIN

Fig. 1. Structures of the veterinary antibiotics.

properties. It was withdrawn from use for humans after toxicological studies revealed that the drug was mutagenic and carcinogenic [10]. In veterinary medicine it has been administered in cattle, poultry and swine feeds for therapeutic and prophylactic purposes [11,12] and as a growth promoter [12,13]. Currently furazolidone has a zero tolerance level (no residue) restriction in edible tissue. Furazolidone is extensively metabolised following rapid absorption from the gastrointestinal tract so levels of the drug in meat should be negligible. However once it is transferred to eggs no further metabolism occurs. Combined with a long egg developing time this may lead to unacceptable levels of the drug [14]. Furazolidone has been screened using GC but this is performed on the 5-nitrofuraldehyde intermediate formed on hydrolysis and is non-specific [15]. Numerous HPLC methods have been used but no universal approach has been settled on with clean up procedures and mobile phases employed tending to be complex.

Chloramphenicol has a very broad spectrum of antibiotic activity and has been used in the treatment of disease of pigs, cows and fish [16–18]. However recent concern over toxic side effects in humans has led to restriction or banning of the drug for the treatment of livestock which forms part of the food chain [19]. Numerous analytical methods exist for chloramphenicol and a review of several GC and LC methods has been published [16]. Derivatisation to the trimethylsilyl or heptafluorobutyryl

derivative is required for GC and complex clean up procedures are necessary prior to any chromatographic analysis. Where confirmation of analyte was required it was performed using GC–MS even if HPLC had been used previously [19]. A more recent method used tandem MS to monitor chloramphenicol residues in trout and on-line HPLC–MS–MS to screen chloramphenicol in milk which reduced the extent of clean up required [19].

Lincomycin has been added to feedstuffs, particularly for swine, due to its therapeutic effect against a wide range of clinically important microorganisms [20]. Numerous analytical methods have been applied to the analysis of lincomycin including photometry which suffers from a lack of specificity [21], thin-layer chromatography [22], selected ion monitoring [23], GC [24], HPLC [25–27] and combined chromatography–MS [20]. Selected ion monitoring and GC–MS rely upon formation of the trimethylsilyl derivative prior to analysis and lengthy clean up procedures are vital prior to derivatisation. HPLC has been performed using both refractive index detection which is only useful for concentrated samples and ultraviolet (UV) detection which is also insensitive due to the poor UV absorbing qualities of lincomycin (see Results and Discussion).

Since all of these drugs could theoretically be administered to swine either in feed premixes or for medical treatment it was felt that all four drugs could be studied in one mixture. We now report the results of experiments which were used to study the separation of these antibiotic standards by packed-column SFC with UV and MS detection.

EXPERIMENTAL

For SFC and SFC–MS a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) modified for SFC operation [28] was used. The chromatograph was coupled to a VG 7070E forward geometry mass spectrometer (VG Analytical, Wythenshawe, U.K.) equipped with a moving belt interface for the acquisition of electron impact and ammonia chemical ionization spectra. Interfacing of the chromatograph with the moving belt interface was effected using a Finnigan MAT thermospray deposition device (Finnigan MAT, San Jose, CA, U.S.A.). The spray depositor was connected in line via a zero dead volume "T" piece between the UV detector exit and the outlet back pressure regulator of the chromatograph, thus effecting a split of the eluent. In order that the full density range of the SFC could be used, the end of the stainless-steel tubing used in the spray deposition device was slightly crimped. The spray deposition device was connected to a 6 V/12 V power supply which was adjusted through a rheostat to prevent freezing of the mobile phase at the tip. This resulted in approximately 50% of the eluent being transferred to the mass spectrometer. For direct introduction SFC–MS a Finnigan MAT 4500 equipped with a thermospray source in the filament-on mode was used. Modification of the source involved replacement of the coiled vapourizer with a straight piece of stainless-steel tubing which was crimped at the end to maintain supercritical conditions. Source heating was performed using a standard thermospray control box. The thermospray source was connected to the SFC system using the stainless-steel transfer line and "T" piece already described. Calibration of the thermospray source was performed using poly(propyleneglycol) of average molecular weight 3000

(Aldrich, Milwaukee, WI, U.S.A.) [29]. Both the Finnigan MAT and VG 7070E mass spectrometers were controlled using a Finnigan MAT INCOS 2300 data system.

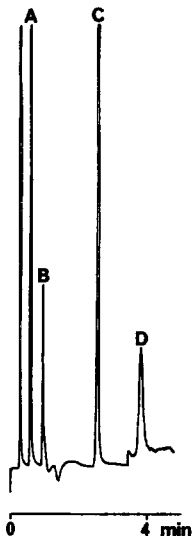
Instrument-grade liquid carbon dioxide supplied in cylinders with a dip tube (BOC, London, U.K.) and glass-redistilled methanol, 2-methoxyethanol, 2-propanol, dimethylformamide and propylenecarbonate were used for mobile phases. The carbon dioxide was introduced directly into the "A" pump of the 1084B and the solvent modifier (usually methanol) was placed in the "B" pump, which was operated in the HPLC mode. The liquid carbon dioxide and the pump heads of the chromatograph were cooled to −25°C using a Neslab RTE-4Z refrigerated bath (Neslab Instruments, NH, U.S.A.).

For SFC, 100 × 4.6 mm I.D. columns packed with 5-μm amino-bonded Spherisorb and 5-μm Spherisorb (Phase Separations, Queensferry, U.K.) were used. Detection was by a UV detector. SFC–MS measurements in the electron impact (EI) mode were performed at 70 eV with a source temperature of 180°C (indicated) and the source housing pressure was in the region $5 \cdot 10^{-6}$ Torr. Chemical ionization (CI) was performed with a source temperature of 100°C (indicated) and a source housing pressure in the region of 0.6 Torr. The belt vapourizer was set at 220°C (indicated). SFC–MS measurements using the thermospray source were all performed in the filament-on mode. The source heater temperature was set at 140°C while the vaporizer temperature was varied.

RESULTS AND DISCUSSION

SFC of veterinary antibiotics

Our objectives in these studies were to evaluate the utility of packed-column SFC for the separation and analysis of antibiotic standards and to investigate the possibility of effecting on-line SFC–MS, using both the moving belt and thermospray interfaces. Initial investigations rapidly revealed that the more acidic stationary phases such as cyano-bonded, ODS2 and silica would be of very little use because both levamisole and lincomycin remained adsorbed to the phase despite the use of high percentages of modifier while furazolidone yielded a badly tailing peak. Chloramphenicol could be screened using silica but due to the fact that all of the drugs eluted from an amino-bonded column all experimental work was performed using that stationary phase. The four antibiotics proved extremely easy to separate and were all baseline resolved even at high percentages of methanol. However detection of the compounds proved more problematical because their absorbance properties were very different due to their contrasting structures. Lincomycin only possesses weak chromophores and even at its wavelength of maximum absorbance (in methanol) at 214 nm it required a high concentration to produce a significant response. When the mixture was chromatographed at 214 nm the peaks due to methanol and levamisole overlapped. In order to minimise the peak overlap and produce a large response for lincomycin a detector sensitivity gradient was used but this resulted in a change in the baseline prior to elution of lincomycin and any changes in the instrumental pumping rate were reflected in the chromatogram despite the low attenuation of the detector (Fig. 2). This was partially countered by chromatographing the mixture at 230 nm where lincomycin showed slight absorbance but baseline changes caused by methanol fluctuation did not occur (Fig. 3). This mixture corresponded to 2 μg of each compound injected



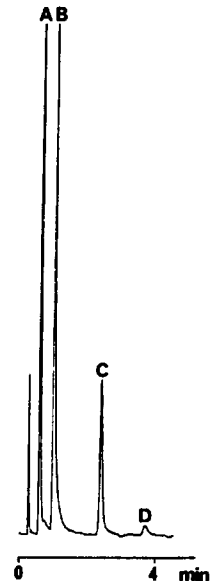


Fig. 2. UV trace (215 nm, range 0.1 a.u.f.s. for 3.5 min then range 0.01 a.u.f.s.) obtained from SFC of a mixture of (A) levamisole, (B) furazolidone, (C) chloramphenicol, and (D) lincomycin on a 100 × 4.6 mm I.D. column packed with 5-μm amino-bonded Spherisorb. The mobile phase was carbon dioxide modified with 15% methanol at a flow-rate of 4 ml/min. Column pressure 351 bar; temperature 75°C.

Fig. 3. UV trace (230 nm, range 0.1 a.u.f.s.) of the veterinary antibiotics. SFC conditions as in Fig. 2.

on-column. The response for chloramphenicol was poor at 230 nm since this was close to its wavelength of minimum absorbance at 240 nm. If chloramphenicol alone were being studied the response would be boosted by monitoring at 280 nm. Sensitivity for levamisole was also poor with detection limits in the low tens of nanogram range while those of furazolidone and chloramphenicol are in the low nanogram range.

One other possible problem, with these compounds was the close proximity in retention of levamisole and to a lesser extent furazolidone which may remain unresolved from endogenous components which would tend to elute close to the solvent peak [1,30]. Simple alterations in the mobile phase facilitated by a change in modifier concentration or column pressure did not greatly affect retention times except at very low methanol concentrations or pressures. Thus in order to maximise the separation of these compounds from the solvent peak the effect of some different modifiers was studied to check the effect of modifier identity on retention. The results are shown in Fig. 4. Due to its weak chromophores lincomycin could not be seen so it is not illustrated.

Ideally a modifier was required which showed a sharp Gaussian peak with a long retention time at high modifier concentrations thus providing scope for further separation at lower percentages of modifier. From these results it would seem that 2-propanol or propylenecarbonate would be most useful for levamisole but the peak obtained using 2-propanol tailed badly. Screening of furazolidone would seem to indicate the use of 2-methoxyethanol especially as the peak shape was comparable to that obtained with methanol.

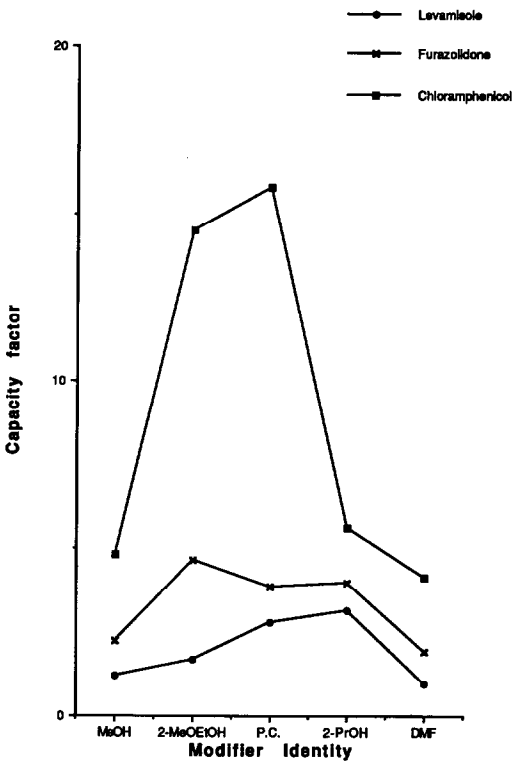


Fig. 4. Effect of modifier identity on the retention times of the veterinary drugs on a 100 × 4.6 mm I.D. packed with 5-μm amino-bonded Spherisorb. The mobile phase was carbon dioxide modified with 20% of either methanol (MeOH), 2-methoxy-ethanol (2-MeOEtOH), propylene carbonate (P.C.), 2-propanol (2-PrOH) or dimethylformamide (DMF) at a flow-rate of 4 ml/min. Column pressure 340 bar; temperature 80°C. Curves: ● = levamisole; × = furazolidone; ■ = chloramphenicol.

SFC–MS of veterinary antibiotics

In order to combat the problems encountered with UV detection the mixture was chromatographed on-line with the mass spectrometer to evaluate the potential of SFC–MS for the analysis of these drugs.

During initial experiments the antibiotics were chromatographed on-line separately using both electron impact and ammonia chemical ionization. While furazolidone, chloramphenicol and lincomycin yielded sharp peaks which retained chromatographic integrity levamisole showed a large sprawling peak over forty scans which seemed to be due to spreading on the belt surface and would interfere with peaks due to furazolidone and chloramphenicol. When this was repeated at a later date the spray depositor was set at a lower temperature which reduced the risk of precipitation of solutes as the solvation power of the mobile phase increased with lower density. The reconstructed total ion current (TIC) trace for on-line SFC–MS using EI ionization of the four drugs is shown in Fig. 5. This corresponds to an on column injection of 1 μg of each of the drugs. The spectra obtained using EI ionization were of variable quality. Levamisole and furazolidone yielded reasonable molecular ions combined with some

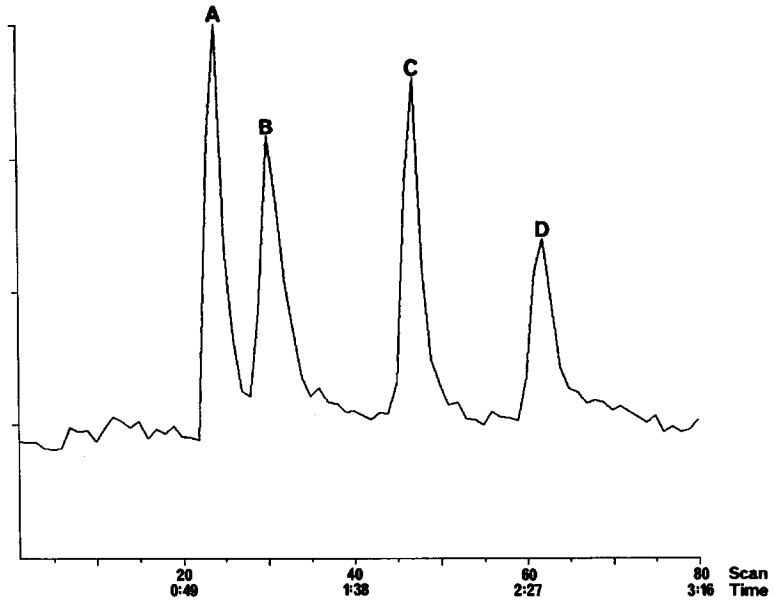


Fig. 5. Computer-reconstructed TIC trace obtained by electron impact SFC–MS of the veterinary antibiotics using a moving belt interface. SFC conditions as in Fig. 2 except the mobile phase was carbon dioxide modified with 20% methanol. Time scale in min:s.

fragmentation information, while chloramphenicol only produced low mass fragmentation data. Lincomycin proved extremely poor as it only showed two ions of intensity > 10% [31]. Ammonia CI spectra were acquired on-line for chloramphenicol and lincomycin (Fig. 6). Furazolidone yielded the $(M + NH_4)^+$ ion as the base peak and also showed a strong molecular ion together with similar fragmentation to that seen using EI ionization. Chloramphenicol showed the protonated molecular ion as the base peak with a strong ammonia adduct ion. It also yielded additional fragment ions between *m/z* 170 and *m/z* 323 which were not seen previously [32]. Lincomycin also yielded molecular weight data though *m/z* 126 remained the base peak [31]. Other diagnostically important ions were obtained though these were all of very low intensity. The spectra are shown in Figs. 7–9 with the relative intensities of important ions shown in Table I.

The drugs were further investigated by on-line SFC–MS using the thermospray source in the filament on mode. Due to the wide range of structures and functionalities it proved that the ideal conditions for one compound did not necessarily suit another. Thus the mixture was run under conditions that yielded the best response for the mixture rather than for each individual compound. In other experiments most compounds had shown maximum signal when the vapourizer temperature was between 130 and 140°C but it was found that the large response of furazolidone rendered the peaks due to chloramphenicol and lincomycin virtually invisible at these temperatures. Lincomycin produced no response at temperatures below 130°C. The ideal "compromise" temperature for the mixture was found to be 160°C where the response due to furazolidone was slightly quenched while lincomycin had become

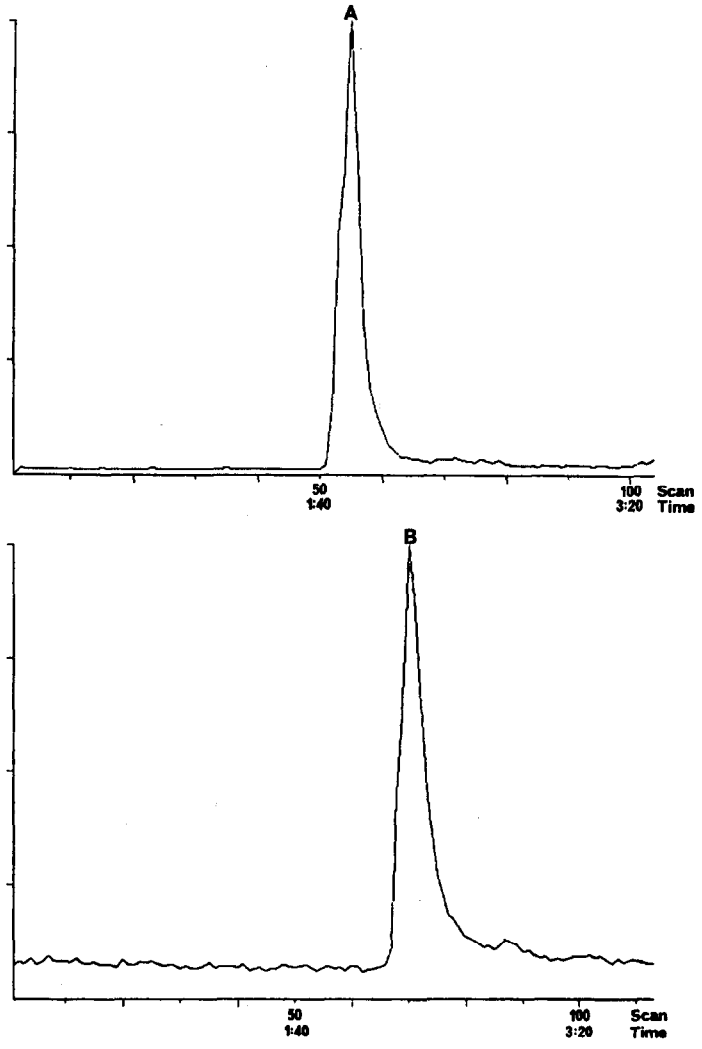


Fig. 6. Computer-reconstructed TIC traces obtained by SFC–ammonia CI-MS of (A) chloramphenicol and (B) lincomycin. SFC conditions as in Fig. 5.

plainly visible. From the reconstructed total ion current trace it can be seen that chromatographic integrity is better retained than with the moving belt interface (Fig. 10) while the run time is reduced because there was no transition time between the spray depositor and the source. If the drugs were being screened singly then each would be chromatographed at its own optimum vapourizer temperature to provide enhanced detection limits.

As previously discussed the inability to separate levamisole from endogenous components within a matrix seemed to be cause for concern. However, when studying change in response with change in vapourizer temperature it was found that

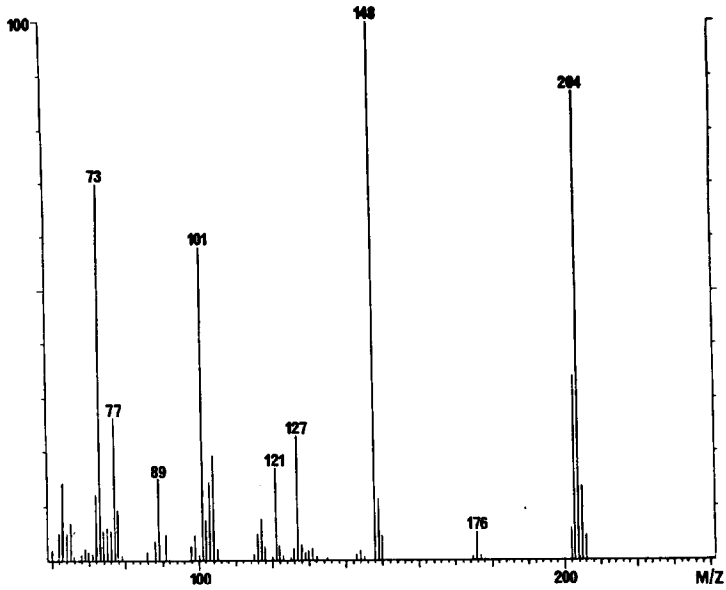


Fig. 7. EI mass spectrum of levamisole obtained by SFC–MS using the moving belt interface.

a significant response was seen at 80°C where no other compounds studied yielded a peak. Thus it may be possible to screen for levamisole at a sufficiently low vapourizer temperature to prevent ionization of other components.

As indicated previously [29,31,32] the spectra obtained using this interface were extremely simple. All of the analytes yielded intense protonated molecular ions which formed the spectral base peaks for levamisole, chloramphenicol and lincomycin while that of furazolidone showed an intensity of 89% of the base peak due to the $[M + CH_3OH + 1]^+$ ion. At 160°C levamisole also showed the protonated dimeric ion at m/z 409 (Intensity of less than 1% of the base peak) which was not seen when the vapourizer temperature was lowered to 80°C. Chloramphenicol displayed some slight fragment ions at m/z 307, m/z 305, m/z 289 and m/z 213 (intensities $< 5\%$) all of which were seen under ammonia CI conditions. The filament- on thermospray spectra obtained for chloramphenicol and lincomycin are shown in Figs. 9 and 11.

CONCLUSIONS

Although the separation of these antibiotics is routine by SFC, UV detection is of limited use because lincomycin can only be seen at microgram levels while the limit of detection for levamisole is also high. Separations were performed using methanol as the modifier but were levamisole or furazolidone being screened separately then propylene carbonate or 2-methoxyethanol may prove more useful as these afford increased separation from the solvent peak (and thus non-polar endogenous components within a matrix).

SFC–MS would seem to be more useful when studying these antibiotics. All can

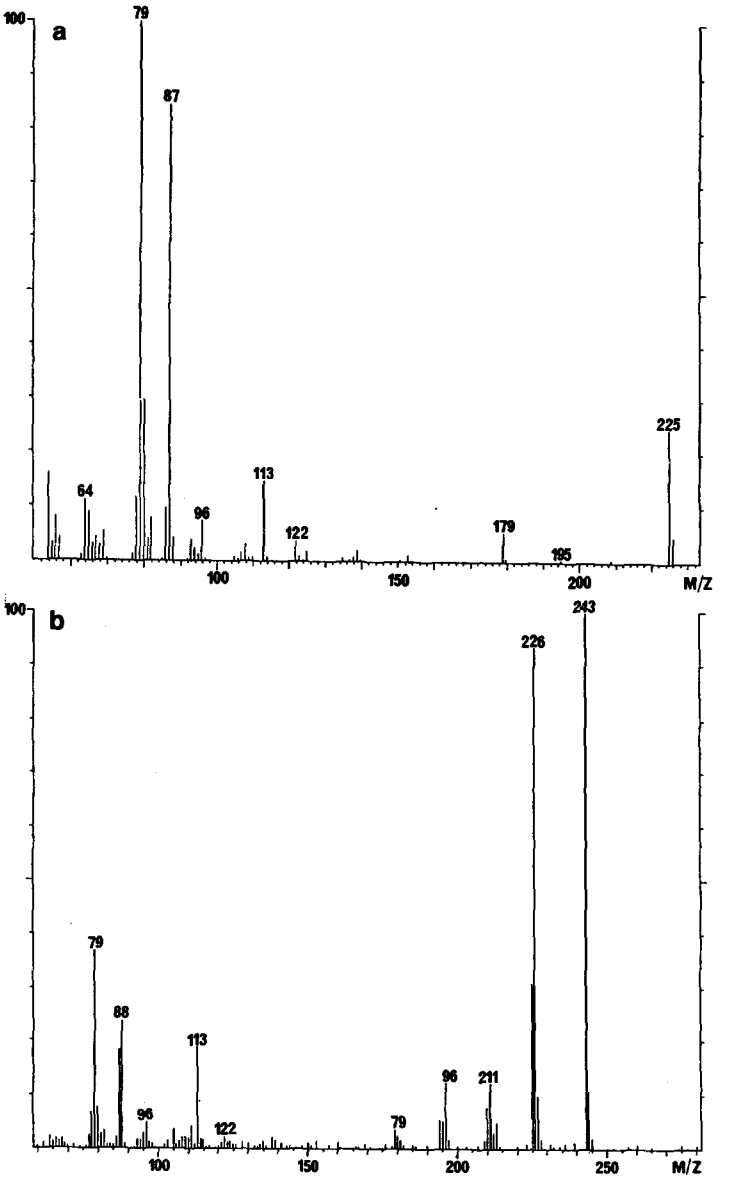


Fig. 8. (a) EI mass spectrum of furazolidone obtained by SFC–MS using the moving belt interface. (b) Ammonia CI mass spectrum of furazolidone obtained by SFC–MS using the moving belt interface.

be run routinely using the moving belt interface providing both electron impact and chemical ionization data though high vapourizer temperature within the spray depositor can lead to precipitation of levamisole and loss of chromatography. Use of the modified thermospray source in the filament- on mode proved ideal for these compounds as no band spreading occurred in the interface so chromatographic

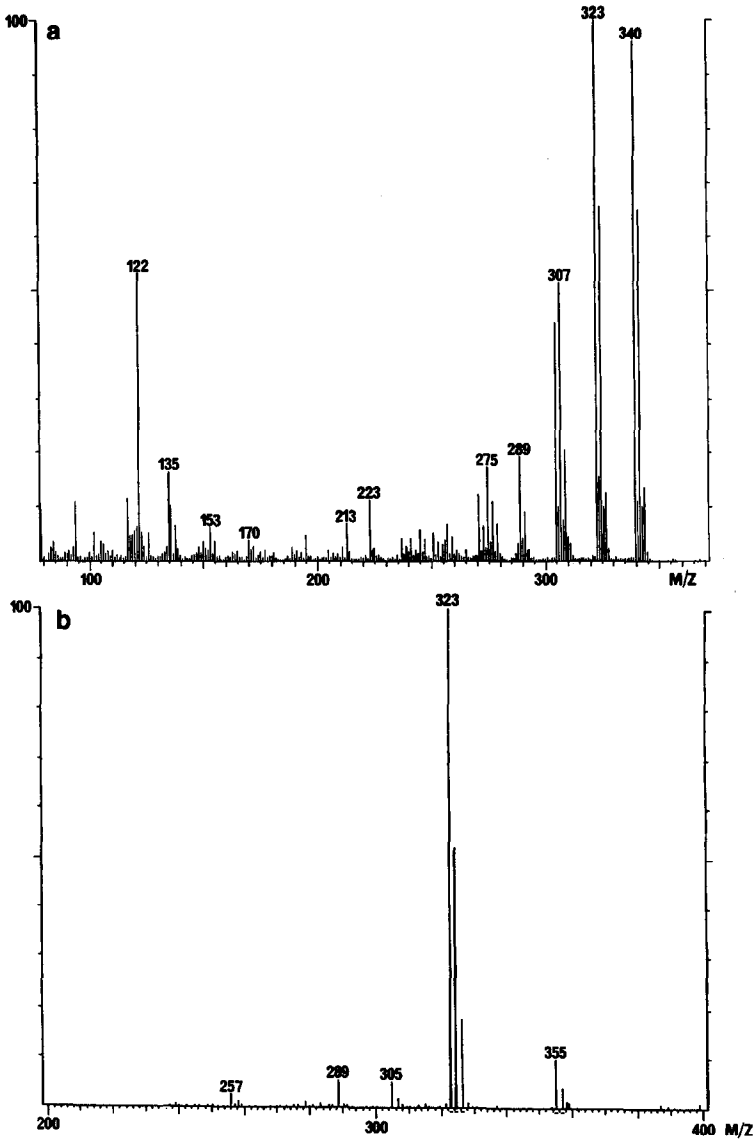


Fig. 9. (a) Ammonia CI mass spectrum of chloramphenicol obtained by SFC–MS using the moving belt interface. (b) Filament-on SFC–MS spectrum of chloramphenicol.

intensity was better preserved than with the moving belt. All of the drugs yielded simple spectra. SFC–MS is a routine analytical technique and could prove to be valuable when screening samples for these compounds.

In our opinion this method could be used for surveillance of the drugs in tissues. These drugs showed similar responses to other compounds using the "thermospray" interface so it should give detection limits in the low nanogram range [29] or lower if

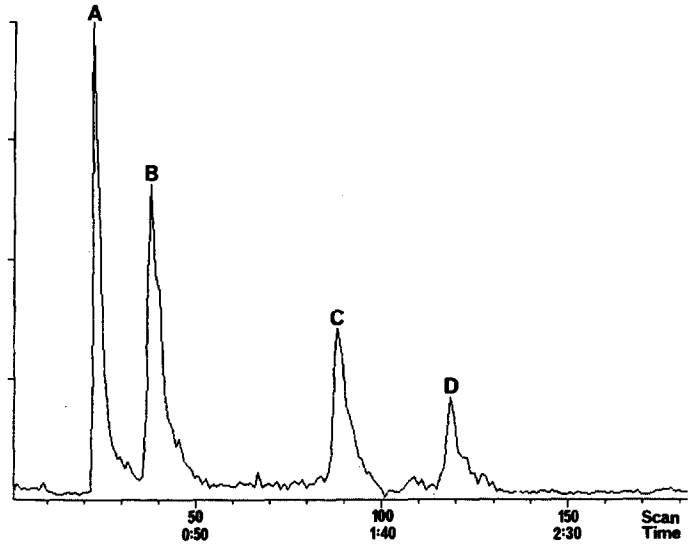


Fig. 10. Computer-reconstructed TIC trace obtained from SFC–MS of the veterinary drugs of the veterinary antibiotics using a modified thermospray interface. SFC conditions as in Fig. 5. The source temperature was 140°C and the vapourizer temperature was 160°C.

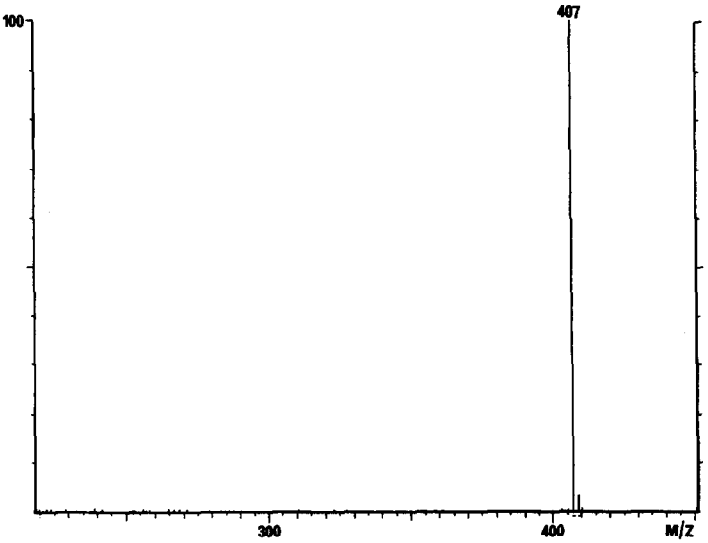


Fig. 11. Filament-on SFC–MS spectrum of lincomycin.

TABLE I

IMPORTANT IONS OF THE VETERINARY ANTIBIOTICS AND THEIR RELATIVE INTENSITIES OBTAINED BY SFC–MS UNDER EI, AMMONIA CI AND FILAMENT-ON THERMOSPRAY (TSP) CONDITIONS

Antibiotic	Mol.wt.	Ionization	Important ions (m/z) and intensities (%, in parentheses)	
Levamisole	204	EI	73(69)	76(26)
			101(58)	104(19)
			127(23)	148(100)
			203(34)	204(100)
		TSP (160°C)	205(100)	206(19)
			409(1)	
		TSP (80°C)	205(100)	206(16)
Furazolidone	225	EI	79(100)	87(85)
			96(8)	113(15)
			179(5)	225(25)
		NH_3 CI	79(38)	88(24)
			113(19)	196(13)
			211(11)	225(30)
			226(94)	243(100)
		TSP (160°C)	226(89)	258(100)
			259(14)	
Chloramphenicol	322	EI	70(86)	77(52)
			106(34)	118(28)
			136(34)	153(100)
			155(40)	170(39)
		NH_3 CI	122(53)	305(44)
			307(51)	309(20)
			323(100)	325(66)
			340(96)	342(65)
		TSP (160°C)	213(4)	289(3)
			305(2)	307(3)
			323(100)	325(58)
Lincomycin	406	EI	82(50)	126(100)
		NH_3 CI	82(5)	126(100)
			127(10)	359(3)
			389(4)	407(52)
		TSP (160°C)	407(100)	

single ion monitoring were employed. These drugs could be present in similar matrices to the sulphonamides [1] and on the basis of their retention times chloramphenicol and lincomycin should have minimal interference from endogenous material, following a similar clean up procedure. Furazolidone and levamisole would be more problematical but hopefully the use of alternative modifers as discussed could help surmount this problem. A further aid to levamisole analysis would be the ability to acquire "thermospray" spectra at low temperatures.

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