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Development and optimisation of an improved derivatisation procedure for the determination of avermectins and milbemycins in bovine liver

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A robust procedure has been developed to overcome the instability problems experienced with the fluorescent derivative of eprinomectin. The procedure involves addition of acetic acid, together with the typical reagents methylimidazole and trifluoroacetic anhydride, to produce a fluorescent molecule that can be determined by high performance liquid chromatography (HPLC) with fluorescence detection. Derivatisation is completed in 30 min at 65 °C. This derivatisation procedure was shown to be suitable, also, for the related compounds, moxidectin, abamectin, doramectin and ivermectin. A multi-residue method for these compounds in bovine liver has been developed using the derivatisation procedure. Samples are extracted with acetonitrile; followed by clean-up on deactivated alumina and C18 solid phase extraction (SPE) cartridges. The method was validated using bovine liver fortified at levels of 4 and 20 μg kg^{-1} with the drugs. The mean recovery ranged between 73 and 97%. The intra- and inter-assay variations showed relative standard deviations typically of $<6\%$ and $<14\%$, respectively. The limit of quantitation of the method is 2 μg kg^{-1} (ppb).

Introduction

A number of methods have been reported for the multi-residue analysis of avermectin and milbemycin drug residues in tissue[1–5] and milk.[6,7] Many of these methods are incomplete in that they do not analyse for eprinomectin because of the poor stability of its fluorescent derivative. Some authors have reported on-line pre-column derivatisation of eprinomectin using automated systems.[8,9] As an alternative, a post-column method has been developed, which uses a photochemical reactor for reagentless derivatisation.[6] Ali *et al.*[4] have described the formation of a stable fluorescent derivative of eprinomectin using elevated temperatures and longer incubation periods. The work reported here describes a modification of the latter method which provides a quicker and more robust procedure. Acetic acid has been added to the derivatisation mixture and following heating at 65 °C for 30 min, a stable eprinomectin derivative is formed.

Experimental

Reagents and equipment

Water, methanol (both HiPerSolv grade) and acetonitrile (HiPerSolv and Pesticide grades) were obtained from BDH (Merck, Poole, Dorset, UK). Triethylamine, phosphoric acid and glacial acetic acid (Analytical grade) were from BDH. *N*-Methylimidazole and trifluoroacetic anhydride (Analytical grade) were obtained from Sigma (St. Louis, MO, USA). Dow-Corning Z-1219 (silanising reagent) and sodium sulfate (anhydrous granular) were from BDH. Deactivated alumina was prepared by heating neutral alumina (Merck, Darmstadt, Germany) at 500 °C overnight and adding water (6 g) to the alumina (44 g), followed by mixing for 45 min. Solid phase extraction (SPE) cartridges were prepared in the laboratory using deactivated alumina (2 g), packed into SPE cartridges (6 ml) between polyethylene frits (International Sorbent Technology, IST, Glamorgan, UK). Bond Elut™ cartridges (C18, 100 mg) were from Varian (Harbor City, CA, USA).

The HPLC system consisted of a model 600 HPLC pump with a model 717 autosampler and model 420-ac fluorescence detector, excitation wavelength 365 nm and emission wavelength 470 nm, all from Waters (Milford, MA, USA). The separation was carried out on a stainless-steel analytical column (150 × 3.9 mm id) packed with Novapak C18 material (Waters), and equipped with a guard column containing μBondapak C18 material (Waters). The column temperature was maintained at 30 °C. The mobile phase, consisting of methanol–acetonitrile–1% triethylamine and 1% phosphoric acid in water (61 + 30 + 9.0, v/v/v), was pumped at 1 ml min^{-1}. A Shimadzu (Dusseldorf, Germany) CR-5A integrator (chart speed 5 mm min^{-1}, attenuation 7) was used for recording and processing chromatograms.

Standard solutions

Ivermectin (Sigma), abamectin (Supelco, Bellefonte, PA, USA), eprinomectin (Merck and Co., Rahway, NJ, USA), moxidectin (American Cyanamid, Princeton, NJ, USA), and doramectin (Pfizer Inc., Groton, CT, USA) were used as standard materials. Standard stock solutions of eprinomectin, moxidectin, abamectin, doramectin and ivermectin (all at 1 mg ml^{-1}) were prepared in methanol. All standard stock solutions were stored at −20 °C. A working standard solution (0.2 μg ml^{-1} of eprinomectin, moxidectin, abamectin, doramectin and ivermectin) was prepared from the standard stock solutions, on the day of use.

Fortification of samples

For preparation of fortified liver samples, 2.5 g portions of negative control liver (not containing any detectable analytes)

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were weighed into 50 ml extraction tubes. A 50 μl portion of a 0.2 μg ml^{-1} or a 1.0 μg ml^{-1} standard solution was added to give fortification levels of 4 or 20 μg kg^{-1}, respectively. After fortification, samples were allowed to sit for 15 min prior to extraction.

Extraction and clean-up

Liver samples were extracted with acetonitrile and cleaned-up by SPE on deactivated alumina and C18 columns as described in the method of Ali *et al.*[4] Two, instead of one, 2 ml volumes of acetonitrile were used to rinse the sample tube and these were added to the C18 SPE cartridge. The eluate was collected in a clean silanised test-tube and evaporated to dryness under nitrogen at 60 °C.

Derivatisation

A 225 μl portion of methylimidazole–acetonitrile (2 + 7, v/v) was added to the test-tube, which was stoppered and vortexed for 2 min. A 225 μl portion of trifluoroacetic anhydride–acetonitrile (2 + 7, v/v) was added and the tube was stoppered and vortexed for 1 min. A 50 μl portion of glacial acetic acid was added and the tube was stoppered and vortexed for 1 min. The derivatised sample extract was filtered through a 0.45 μm filter (13 mm, polyvinylidene difluoride) into a HPLC vial, which was incubated in a fan-assisted oven (30 min, 65 °C). Samples vials were then cooled (4 °C, 3 min) and left at room temperature (12 min) before an aliquot (100 μl) was injected onto the HPLC column.

Calibration

Standards were prepared by adding 0, 25, 50, 100, 250 and 500 μl of the working standard solution (0.2 μg ml^{-1}) to silanised test-tubes, evaporating to dryness under nitrogen at 60 °C and derivatising as described above. Calibration curves were prepared by plotting peak area as a function of analyte concentration (0 to 200 ng ml^{-1}). Recovery was measured from the peak areas obtained for fortified sample extracts, as calculated from the standard curve.

Preparation of eprinomectin derivative for mass spectral analysis

Derivatised eprinomectin free of derivatisation reagents was prepared by injecting standard solutions (1.0 mg ml^{-1}) into the HPLC fluorescence system, with the mobile phase altered to methanol–water (90 + 10, v/v). A 4 ml fraction around the retention time for the derivatised eprinomectin was collected. The fraction was dried over anhydrous sodium sulfate and methanol was evaporated under nitrogen at 60 °C. The eprinomectin derivative was reconstituted in acetonitrile to a concentration of 0.39 mg ml^{-1}, as determined by HPLC.

Mass spectrometry

A dilution of the fractionated eprinomectin derivative in acetonitrile (3.9 μg ml^{-1}) was infused into the mass spectrometer at 10 μl min^{-1} and was analysed in positive ion mode through an atmospheric pressure chemical ionisation interface (LCQ, Thermoquest, Hemel Hempstead, UK). The vaporiser and the capillary temperatures were set to 450 °C and 200 °C, respectively. Sheath gas flow rate was 60 ml min^{-1}. The automatic gain control target was set to 2×10^7 for MS^n and 5×10^7 for full scan MS, the maximum ion time was 50 ms, the number of microscans was set to 3, and the spectra were collected between *m/z* 400 and 1200.

Results and discussion

Eprinomectin derivatisation

In previous work, stability problems were encountered with the fluorescent derivative of eprinomectin preventing quantitative

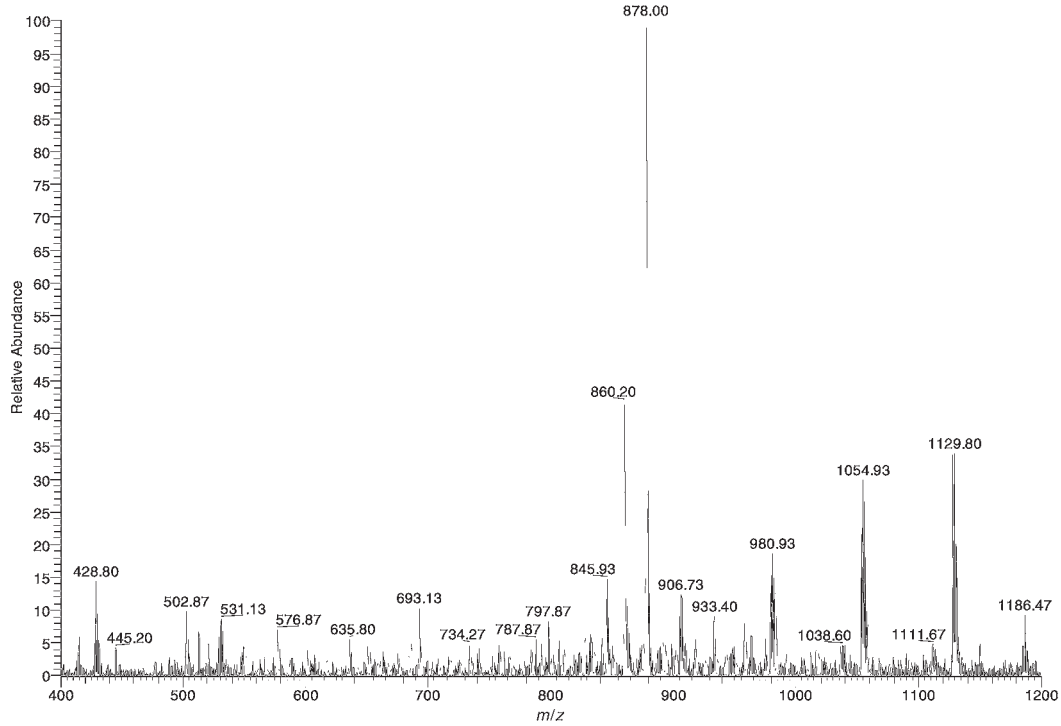

Fig. 1 APCI mass spectrum of derivatised eprinomectin standard.

analysis of this analyte.[5] Rupp *et al.*[10] have reported a method for the production of more stable fluorescent derivatives of ivermectin and doramectin, using an additional derivatisation step, which involves de-esterification of a trifluoroacetyl ester. Since this functional group does not form with the eprinomectin derivative, the method cannot be applied to this analyte. Ali *et al.*[4] demonstrated that by heating the eprinomectin derivative, a more stable earlier eluting molecule was formed.[4] This method was tested in our laboratory, but the expected response and reproducibility could not be achieved.

It was found that by heating at 65 °C for 30 min in the presence of acetic acid a stable eprinomectin derivative was formed. The results of the experiments indicated that acid and to a lesser extent heating, favoured the production of the stable derivative. While it was found that the reaction could proceed by heating alone, the response obtained was only about half that using acid. Addition of acid alone results in the reaction taking approximately 2 h to go to completion, while acid combined with heating reduces the reaction time to 30 min.

This improved derivatisation procedure was used in conjunction with the extraction and clean-up procedure used by Ali *et al.*[4] The C18 elution volume was increased from 2.5 to 4.5 ml to ensure high recovery of eprinomectin. It was noted that the use of certain grades of acetonitrile and polypropylene pipette tips could give rise to interfering peaks in the chromatograms. By using pesticide grade acetonitrile and glass pipettes during sample extraction and clean-up these peaks were eliminated.

Mass spectral analysis

Because eprinomectin differs from the other avermectin compounds only by the presence of a secondary amide group, it was considered that this group might be involved either in the derivatisation or in inducing instability of the derivative. To identify the structure of the eprinomectin derivative, a 3.9 μg ml^{-1} derivatised eprinomectin standard was analysed by MS-MS following HPLC fractionation. The two most abundant ions present were at *m/z* 878 and *m/z* 860 (Fig. 1). The ion at *m/z* 878 is the protonated derivative ion of eprinomectin resulting from dehydrative aromatisation, as occurs for the other avermectin derivatives. The ion at *m/z* 860 may be due to loss of water from the *m/z* 878 ion, in the heated interface of the mass spectrometer. Fragmentation of the *m/z* 878 ion produced an ion with *m/z* 789, representing the loss of the neutral fragment, $CH_3CONHOCH_3$. This fragment would correspond with the amide group on the molecule because its particular mass indicates that it contains a nitrogen atom. This fragment loss, together with the most abundant ion at *m/z* 878, indicates that the conversion of the initial derivative does not occur through a loss at the amide group of eprinomectin but through a rearrangement elsewhere in the molecule. The proposed reaction route for the eprinomectin derivative is shown in Fig. 2a and the proposed fragmentation of the derivative ion at *m/z* 878 to produce the ion with *m/z* 789 is shown in Fig. 2b.

Optimisation of the derivatisation procedure

Optimisation of the derivatisation procedure for eprinomectin was carried out using multivariate statistical analysis. A full factorial design was used to examine the effect of each of the factors, temperature, time and volume of acid. This entailed carrying out derivatisation at two levels for each of the factors studied; a total of $2^n + 2n$ treatments (with 5 centre-points) were completed, where n is the number of factors (3) studied. The centre-points were used to provide a measure of experimental error and to detect any curvature in the model. Using the results from these derivatisation treatments, multiple linear regression was used to develop a quadratic equation to fit the data. Response surfaces were then plotted using this quadratic model (Fig. 3). The plot of eprinomectin peak area as a function of reaction time and volume of acid indicated that the optimum reaction time was between 27 and 47 min and that the optimum volume of acid was 33–67 μl. The plot of eprinomectin peak area as a function of acid and temperature indicated similar optimum values for volume of acid. The results indicated that a temperature higher than 65 °C would give a larger eprinomectin peak area, but this temperature was chosen as an upper limit to avoid potential problems of solvent evaporation. From the response surface plots, suitable derivatisation conditions were established to be a derivatisation time of 30 min, 50 μl of acid added and a reaction temperature of 65 °C.

Effect of derivatisation conditions on other analytes

A plot of analyte peak area for the other analytes as a function of each treatment in the central composite design experiment is shown in Fig. 4. This plot indicates that the other analytes are largely unaffected by the variation in the derivatisation

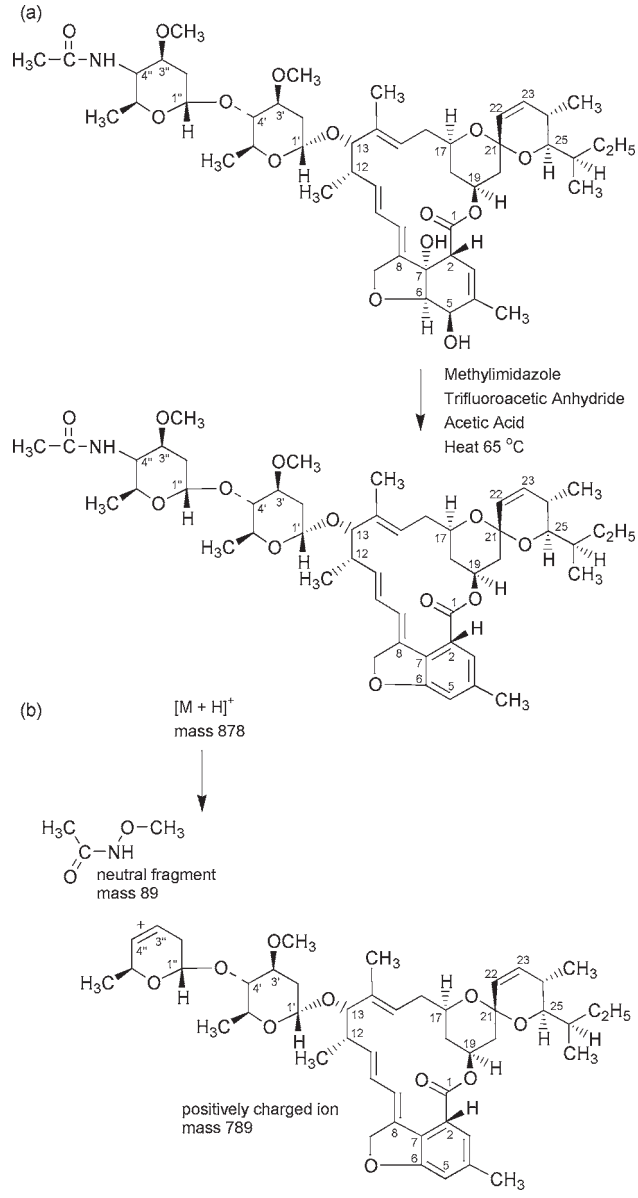

Fig. 2 (a) Proposed reaction route for the preparation of the eprinomectin derivative. (b) Proposed structures of the products of the parent ion (mass 878), protonated derivative ion (mass 789) and neutral fragment (mass 89).

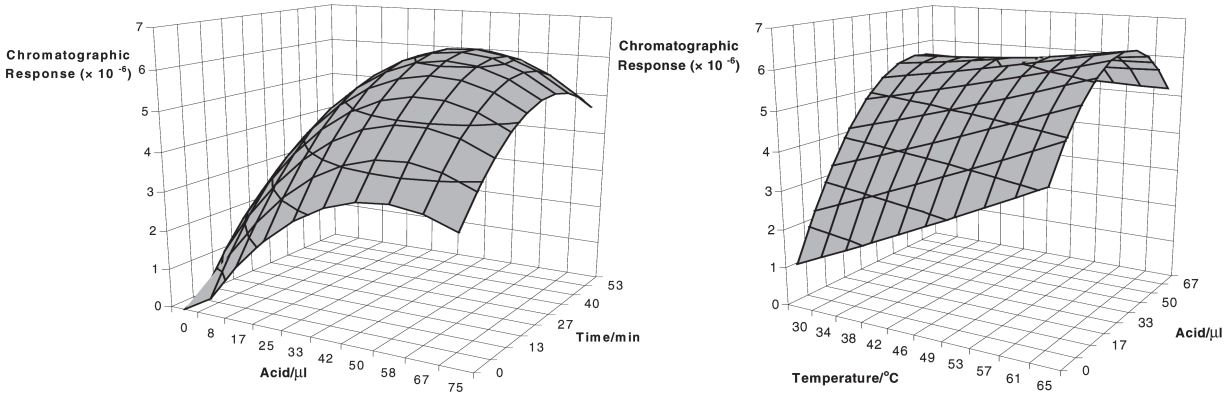

Fig. 3 Response surface plots of eprinomectin peak area as a function of derivatisation conditions.

conditions. Only in the case of eprinomectin, where the derivatisation conditions include no acid (treatment 4) or no reaction time (treatment 16) or a combination of low values for reaction temperature, time and volume of acid (treatment 13) is the response considerably reduced.

Stability study on avermectin and milbemycin derivatives

The stability of the avermectin and milbemycin derivatives was evaluated by analysing a derivatised 100 ng ml^{-1} standard mix at 40 min intervals over a 22 h period. No significant change in the detector response (peak area) for the analytes over this time period was observed; linear equations for detector response against time for each of the five analytes gave regression coefficients (r^2) of between 0.000 and 0.057, indicating that the derivatives produced were stable.

Validation of the method

Standard curves were prepared using standards at concentrations of 0, 10, 20, 50, 100 and 200 ng ml^{-1}. The curves were linear over this range (r^2 = 0.999). The limit of quantitation for the method, as determined from the lowest standard on the calibration curve (10 ng ml^{-1}), was 2 μg kg^{-1}. The accuracy and precision of the method were determined using bovine liver samples fortified at levels of 4 and 20 μg kg^{-1} (Fig. 5). Mean recovery of the analytes was between 73 and 97%. Intra-assay variation was determined by analysing five samples within a single run; RSD values were at less than 6% (Table 1). Inter-assay variation was determined by analysing samples on five different occasions, to evaluate the run to run variation in the method. RSD values were at less than 8%, with the exception of 14% for eprinomectin at 4 μg kg^{-1} (Table 1).

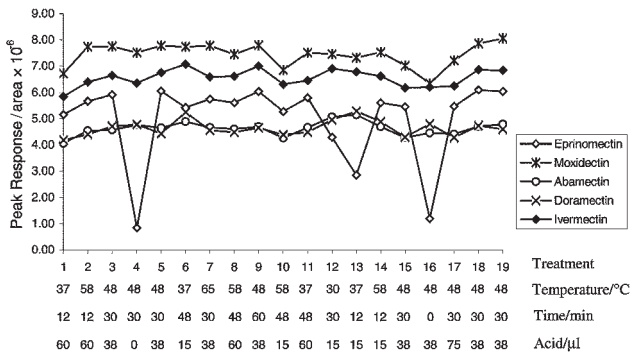

Fig. 4 Effect of derivatisation conditions on analyte peak area response.

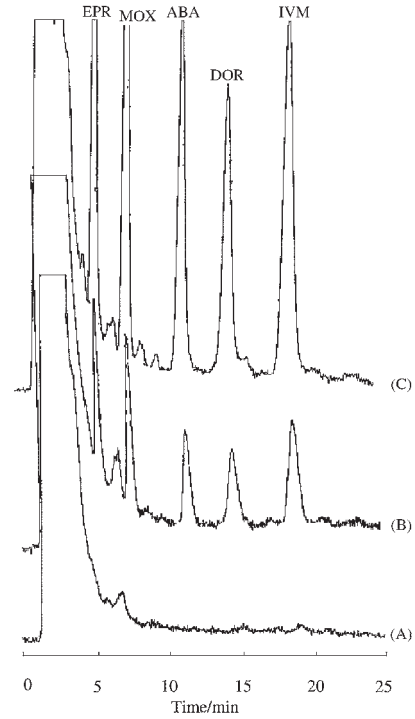

Fig. 5 Chromatograms of bovine liver samples fortified with 0 μg kg^{-1} (A), 4 μg kg^{-1} (B) and 20 μg kg^{-1} (C) of eprinomectin (EPR), moxidectin (MOX), abamectin (ABA), doramectin (DOR) and ivermectin (IVM).

Table 1 Intra- and inter-assay variations for the recovery of the analytes from bovine liver

		Recovery (%)			
		Intra-assay		Inter-assay	
Analyte	Fortification level/μg kg^{-1}	Mean ± s (n = 5)	RSD	Mean ± s (n = 5)	RSD
Eprinomectin	4	74 ± 3.0	4.1	76 ± 10.6	13.9
	20	81 ± 2.9	3.5	84 ± 4.0	4.8
Moxidectin	4	73 ± 1.4	1.9	74 ± 5.3	7.1
	20	85 ± 2.1	2.5	81 ± 5.1	6.3
Abamectin	4	87 ± 5.1	5.8	91 ± 6.6	7.3
	20	89 ± 2.7	3.0	87 ± 5.5	6.3
Doramectin	4	92 ± 2.9	2.9	95 ± 5.2	5.4
	20	90 ± 3.7	4.1	88 ± 7.6	6.7
Ivermectin	4	97 ± 5.7	5.9	91 ± 5.9	6.5
	20	88 + 3.3	3.7	86 ± 5.6	6.5

Conclusion

The paper describes development of an improved derivatisation procedure for eprinomectin. The procedure uses a combination of elevated temperature and acid to produce a stable derivative of eprinomectin. The derivatisation procedure has been shown to be suitable for other avermectins and milbemycins and has been applied in a multi-residue method for bovine liver and has been validated.

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