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Validation and robustness testing of a HPLC method for the determination of avermectins and moxidectin in animal liver samples using an alumina column clean-up

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A multi-residue method has been developed for the quantitative determination of moxidectin, abamectin, doramectin and ivermectin in liver samples, with capability for qualitative identification of the presence of eprinomectin. Liver samples are extracted with isooctane, followed by clean-up on alumina-N solid phase extraction (SPE) cartridges. Extracts are derivatised and determined by high-performance liquid chromatography (HPLC) with fluorescence detection. The method was validated using bovine liver fortified at levels of 4 and 20 μg kg^{-1} with the drugs. The mean recovery from bovine liver ranged between 90 and 96%. The intra and inter-assay variations showed RSD typically of $< 5\%$ and $< 10\%$, respectively. The procedure was applied also to ovine and porcine liver, giving similar results. A robustness study, carried out on the alumina clean-up step, indicated that the step is relatively insensitive to method changes. However, significant differences overall were found for the type of alumina and/or commercial SPE cartridge used. The limit of quantitation of the method is 2 μg kg^{-1} (ppb).

Introduction

Avermectins and moxidectin belong to a group of compounds called the macrocyclic lactones, which are used for the treatment of parasitic infections in food-producing animals. Because of the widespread use of these anthelmintic drugs, ivermectin, moxidectin, doramectin, abamectin and eprinomectin, analysis for residues of the drugs in edible tissue has become of major importance. They are lipophilic in nature and therefore are distributed mainly in the liver and fat tissues; as a result these tissues are targeted for residue analysis. In the EU, maximum residue limits (MRLs) for these drugs in liver range from 15 to 600 μg kg^{-1}, depending on the drug and the animal species in question.[1–5]

There are a number of methods available for the determination of the different drug residues in food.[6–14] These methods involve extraction using organic solvents, clean-up by liquid–liquid partitioning and/or solid phase extraction (SPE), and derivatisation of the sample extract, with analysis by HPLC and fluorescence detection. In recent years, with an increase in the number of these drugs available on the market, a number of multi-residue methods have been developed for residues in tissue[15–17] and milk.[18,19] The method described here allows for quantitative analysis for four of the drugs and provides qualitative information on the fifth, eprinomectin. Quantitative analysis of eprinomectin could not be made using this method because of the poor stability of its fluorescent derivative.[12] On-line pre-column derivatisation of eprinomectin has been reported using automated systems.[12,13] As an alternative, a novel post-column method has been developed, which uses a photochemical reactor for reagentless derivatisation.[18] Ali *et al.* have reported recently on the formation of a relatively stable fluorescent derivative of eprinomectin using elevated temperatures and longer incubation periods.[20] For the method described here, a rapid derivatisation procedure was used and the presence of eprinomectin (MRL, 600 ppb) was readily detectable.

The aim of this paper is to report a new SPE method for the rapid determination of avermectin compounds in liver tissue, where traditionally C8 or C18 have been the sorbents of choice for SPE clean-up of avermectins from food products. This method reports the first single step alumina SPE clean-up method for avermectins from tissue; alumina has been used previously but in conjunction with one or more other SPE steps.[20,21] The validation and robustness testing of the developed method are also reported.

Experimental

Reagents and equipment

Water, methanol, acetonitrile, ethyl acetate, 2,2,4-trimethylpentane (isooctane) (HiPerSolv grade) were obtained from BDH (Merck, Poole, Dorset, UK). Triethylamine, phosphoric acid, acetone and hexane (Analytical grade) were from BDH (Poole, Dorset, UK). *N*-Methylimidazole and trifluororacetic anhydride (Analytical grade) were obtained from Sigma (St. Louis, MO, USA). Siliconising solution was from Serva Feinbiochemica (Heidelberg, Germany). Alumina SPE cartridges were prepared in the laboratory using neutral alumina (2 g), (Merck, Darmstadt, Germany), packed into SPE cartridges (6 ml) between polyethylene frits (International Sorbent Technology, IST, Glamorgan, UK). Additional alumina from Sigma and Aldrich (Dorset, UK) were used for robustness testing. Isolute™ cartridges (Al-N, 2 g) from IST, Bond Elut Jr™ cartridges (Al-N, 1 g) from Varian (Santa Clarita, CA, USA), Sep-Pak™ cartridges (Al-N, 2 g) from Waters (Milford, MA, USA), were also used in the robustness study.

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. The separation was carried out on a stainless-steel analytical column (150 × 3.9 mm id) packed with Novapak C18 (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 moxidectin, abamectin, doramectin, ivermectin and eprinomectin (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 moxidectin, abamectin, doramectin and ivermectin) was prepared from the standard stock solution, on the day of use.

Fortification of samples

For preparation of fortified liver samples, 2.5 g of negative control liver (not containing any detectable analytes) was weighed into 50 ml extraction tubes. A 50 μl portion of 0.2 μg ml^{-1} 1.0 μg ml^{-1} standards solutions were added to give levels of 4 and 20 μg kg^{-1} respectively. Fortified samples were then allowed to sit for 15 min prior to extraction.

Extraction and clean-up

Homogenised liver (2.5 g) was weighed into a 50 ml extraction tube. A 15 ml portion of acetone–water (1 + 1, v/v) was added and the tube contents were mixed using a test-tube mixer (30 s). Isooctane (15 ml) was added and the tube contents were mixed as before, the tube then being centrifuged (10 min, 2000 rpm/1200*g*). After centrifugation, the isooctane layer was transferred to a reservoir connected to an alumina SPE cartridge. The isooctane extract was allowed to pass through the cartridge while the sample was re-extracted twice more with isooctane; 3 × 15 ml volumes of isooctane were used in total and passed through the alumina cartridge. The SPE cartridge was washed with 10 ml hexane–ethyl acetate (70 + 30, v/v) and the analytes were eluted with 8 ml methanol–ethyl acetate (70 + 30, v/v). The eluate was collected in a siliconised test-tube and evaporated to dryness under nitrogen at 60 °C.

Derivatisation

A 200 μl portion of methylimidazole–acetonitrile (1 + 1, v/v) was added to the test-tube, which was stoppered and vortexed for 2 min. A 300 μl portion of trifluoroacetic anhydride–acetonitrile (1 + 2, v/v) 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) and 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 siliconised 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 from fortified sample extracts, as calculated from the standard curve.

Results and discussion

Chromatography and selectivity

The method is based on one previously developed for the determination of ivermectin in bovine liver samples,[22] with the mobile phase being modified to accommodate the additional analytes. The mobile phase of methanol–acetonitrile–1% triethylamine and 1% phosphoric acid in water (61 + 30 + 9.0, v/v/v), was found to be most suitable (Fig. 1). This mobile phase gave baseline separation of the analytes, with the elution order moxidectin (7.8 min), abamectin (11.8 min), doramectin (14.4 min) and ivermectin (19.7 min). All peaks were very symmetrical in nature, with peak asymmetry factors ranging from 1.04 to 1.09. Theoretical plate values were 1589 (moxidectin), 2036 (abamectin), 2363 (doramectin), and 2643 (ivermectin). It was found that eprinomectin, if present, could not be eluted without the addition of 1% triethylamine and 1% phosphoric acid to the mobile phase. Eprinomectin appeared to degrade into three peaks over a short period of time, which prevented its quantitative determination (Fig. 2), but would allow for an indication of its presence to be detected.

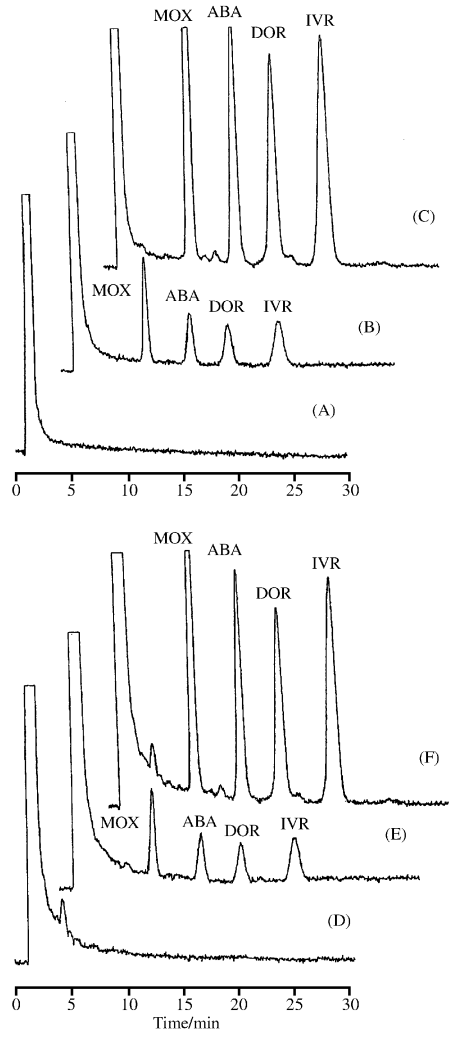

Fig. 1 Chromatograms of 0 ng ml^{-1} (A), 20 ng ml^{-1} (B) and 100 ng ml^{-1} (C) standards and of bovine liver extracts fortified with 0 μ kg^{-1} (D), 4 μg kg^{-1} (E) and 20 μg kg^{-1} (F) moxidectin (MOX), abamectin (ABA), doramectin (DOR) and ivermectin (IVR).

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 found to be 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}. Mean recovery of the analytes was between 84 and 96%. Intra-assay variation was determined by analysing five samples within a single run; RSD were typically at less than 5% with the exception of 8.7% for moxidectin at 20 μg kg^{-1} (Table 1). Inter-assay variation was determined by analysing samples on five different occasions, to evaluate the run to run variation in the method. Mean recovery of analytes was at 90% or higher with RSD at less than 10% (Table 2). The method was evaluated also on ovine and porcine livers fortified with the analytes at 20 μg kg^{-1}. Mean recovery of the analytes from ovine liver was between 80 and 88% and from porcine liver was between 91 and 96%; RSD were typically at less than 5% (Table 3).

Robustness testing

A newly developed alumina clean-up step was included in this method; with extraction and derivatisation procedures adapted from other methods. The robustness of the new step was tested using the following variables: volumes and composition of wash and elution solvents, volume of sample extract applied, and source and amount of alumina. Variation in the volumes and composition of the wash solvent (8, 10 and 12 ml hexane–ethyl acetate at 65 + 35, 70 + 30 and 75 + 25) and of the elution solvent (6, 8 and 10 ml methanol–ethyl acetate at 65 + 35, 70 + 30 and 75 + 25) were tested using the laboratory-prepared alumina cartridge (2 g, Merck). No significant differences were found, with recovery for all drugs under all conditions being at greater than 80%. Similarly, no significant differences were found for variation in quantity of alumina used (1.5 g, 2.0 g and 2.5 g) or for addition of sample extract as 3 × 15 ml or as 1 × 45 ml to the cartridge; recovery for all drugs under the different conditions were at greater than 80%.

Recovery of the analytes appeared to be most influenced by the source of alumina used for laboratory-prepared SPE cartridges and the type of pre-packed commercial alumina cartridges used. Three different sources of alumina were tested, with overall mean recoveries for the analytes (n = 3) being 93% (Merck), 87% (Aldrich), and 90% (Sigma); analysis of variance indicates a significant effect of source of alumina, overall, on analyte recovery (Table 4). A significant effect of analyte, overall, is found, due to the consistently higher recovery of some analytes (*e.g.*, ivermectin) compared with others, but the interaction term (analyte × alumina source) is not significant.

The laboratory-prepared alumina cartridge (2 g, Merck) used in this method was compared with cartridges from three commercial suppliers: BondElut Jr™, Isolute™, and Sep-Pak™. Only the Isolute™ cartridge was identical, in dimensions and alumina weight used, to the laboratory prepared cartridge. The other cartridges differed in dimensions, with the BondElut Jr™ cartridge also differing in weight of alumina (1 g). The Sep-Pak™ cartridge gave lower recovery for moxidectin (68% *vs.* 84–88%) but higher recovery for all of the other analytes (> 93% *vs.* 81–90%); the low recovery of moxidectin using this cartridge may be due to some loss of this relatively polar analyte due to its incomplete elution from the alumina. Overall, mean recovery of the analytes using the laboratory-prepared cartridges were lower compared to the commercial cartridges (84% *vs.* 87–88%). Analysis of variance indicates significant effects of type of alumina cartridge and of

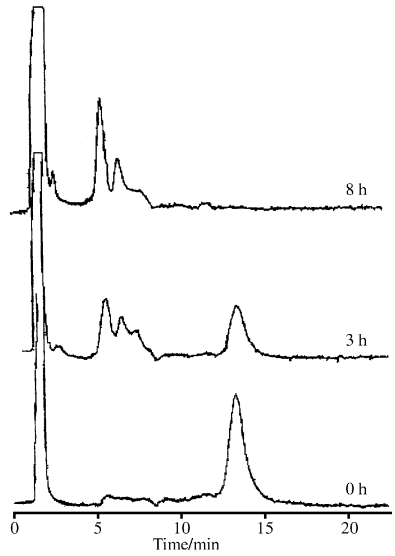

Fig. 2 Chromatograms of eprinomectin standards (100 ng ml^{-1}) after 0, 3 and 8 h storage at room temperature following derivatisation.

Table 1 Intra-assay variation for the recovery of the analytes from bovine liver

		Recovery (%)	
Analyte	Fortification level/μg kg^{-1}	Mean ± s (n = 5)	RSD
Moxidectin	4	84 ± 2.7	3.3
	20	92 ± 8.0	8.7
Abamectin	4	93 ± 3.3	3.6
	20	90 ± 2.5	2.8
Doramectin	4	88 ± 3.6	4.1
	20	91 ± 2.5	2.8
Ivermectin	4	93 ± 1.4	1.5
	20	95 ± 1.6	1.7

Table 2 Inter-assay variation for the recovery of the analytes from bovine liver

		Recovery (%)	
Analyte	Fortification level/μg kg^{-1}	Mean ± s (n = 5)	RSD
Moxidectin	4	93 ± 5.5	6.6
	20	93 ± 8.6	9.3
Abamectin	4	92 ± 5.0	5.4
	20	90 ± 6.1	6.8
Doramectin	4	90 ± 7.9	8.7
	20	92 ± 6.7	7.3
Ivermectin	4	96 ± 8.7	9.0
	20	96 ± 6.6	6.9

Table 3 Recovery of the analytes from fortified ovine and porcine liver samples

		Recovery (%)	
Analyte	Fortification level/μg kg^{-1}	Mean ± s (n = 5)	RSD
Ovine Liver—			
Moxidectin	20	83 ± 1.6	2.0
Abamectin	20	88 ± 7.8	8.8
Doramectin	20	80 ± 3.1	3.9
Ivermectin	20	85 ± 2.7	3.2
Porcine Liver—			
Moxidectin	20	94 ± 4.3	4.5
Abamectin	20	93 ± 0.4	0.4
Doramectin	20	91 ± 3.0	3.3
Ivermectin	20	96 ± 3.7	3.9

Table 4 Effect of different sources of alumina used in the SPE clean-up on recovery from samples fortified at 20 μg kg $^{-1}$ (n = 3)

	Mean recovery ± s (%)		
Analyte	Merck	Aldrich	Sigma
Moxidectin	90 ± 1.2	85 ± 1.6	85 ± 2.1
Abamectin	90 ± 4.9	85 ± 2.1	89 ± 2.9
Doramectin	93 ± 1.3	87 ± 3.9	92 ± 2.7
Ivermectin	99 ± 1.6	90 ± 3.2	95± 2.7
Overall mean	93.2	86.6	90.1

Analysis of variance—

Source of variation	F test significance
Alumina	$P < 0.001$
Analyte	$P < 0.001$
Alumina × Analyte	$P > 0.05$[a]

[a] Not significant.

Table 5 Effect of different SPE alumina cartridges on recovery from samples fortified at 20 μg kg $^{-1}$ (n = 3)

	Mean recovery ± s (%)			
Analyte	Laboratory[a]	Isolute™	Bond Elut Jr™	Sep-Pak™
Moxidectin	84 ± 2.6	87 ± 0.9	88 ± 3.4	68 ± 6.7
Abamectin	81 ± 3.0	83 ± 1.3	84 ± 2.9	93 ± 3.5
Doramectin	86 ± 3.1	90 ± 1.4	87 ± 2.3	96 ± 3.1
Ivermectin	83 ± 3.1	89 ± 1.8	87 ± 2.1	96 ± 4.4
Overall mean	83.5	87.4	86.6	88.2

Analysis of variance—

Source of variation	F test significance
Cartridge	$P < 0.01$
Analyte	$P < 0.001$
Cartridge × analyte	$P < 0.001$

[a] SPE cartridge prepared in laboratory.

analyte, overall, on recovery (Table 5), with a significant interaction term, also. In summary, three of the alumina cartridges, the laboratory-prepared and two of the commercial cartridges, gave similar recoveries (maximum difference of 5%); markedly different recoveries, both lower and higher, were obtained using the Sep-Pak™ cartridge. These results (Tables 4 and 5) indicate that significant differences may be found in analyte recovery, related to the source of alumina or type of commercial cartridge used, but that in practically all cases recoveries in excess of 80% are obtained for the analytes; the performance of moxidectin on the Sep-Pak™ cartridge illustrates the need to optimise conditions for the particular SPE cartridge being used.

Conclusion

The paper describes development and validation of a multi-residue method for avermectins and moxidectin in bovine, ovine and porcine livers. Robustness testing has shown the method to be relatively insensitive to a wide variety of method changes. However, recovery can be affected by the source of alumina and/or of commercial SPE cartridge used. The method gives quantitative determination of moxidectin, abamectin, doramectin, and ivermectin, and may be used for qualitative identification of the presence of eprinomectin. The method is sufficiently sensitive to determine residues of these compounds well below their MRL values. An analyst may process up to 10 test samples, in addition to quality control samples, in a working day with HPLC analysis in an unattended overnight run.

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