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Determination of ivermectin in bovine plasma by column-switching LC using on-line solid-phase extraction and trace enrichment*

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Abstract: Ivermectin is a potent avermectin anthelmintic, derived from the soil organism *Streptomyces avermitilis*. Because of its potency, it is administered in very low doses (200 μg kg^{-1}), giving rise to plasma levels in the ppb region, and hence sensitive methods are required for its determination in biological samples. A method is described for the determination of ivermectin in bovine plasma, based on a commercially available C_{18} column employing a mobile phase composed of acetonitrile–water–isopropanol (40:40:35, v/v/v). Extraction from plasma is carried out on-line using coupled-column technology; switching between the extraction and analytical columns is achieved using a six-port column-switching valve. The method is linear over the range 2–100 ng ml^{-1} (r = 0.9994) and has a limit of detection of 0.8 ng ml^{-1} (signal-to-noise ratio = 3). Reproducibility of the method, expressed as the overall mean relative standard deviation, is 6.7% (intra-assay) and 3.8% (inter-assay). The mean recovery of the drug from plasma is 76.4%. The method is simple and economical in its execution and is sufficiently reproducible to obviate the need for an internal standard.

Keywords: *Ivermectin; plasma levels; LC; on-line solid-phase extraction; column switching.*

Introduction

The avermectin anthelmintics are extremely potent anti-parasitic agents, derived from the fermentation products of the soil organism *Streptomyces avermitilis* [1]. Ivermectin is the most useful and widely used derivative of the avermectins, comprising a mixture of two homologues containing at least 80% of 22,23-dihydroavermectin B_{1a} (the more potent homologue) and less than 20% of 22,23-dihydroavermectin B_{1b} (Fig. 1) [2]. Ivermectin is effective against a wide variety of immature and adult nematode and arthropod parasites in animals such as sheep, cattle, pigs and horses [2]. Its mode of action involves potentiation of the effects of γ-amino butyric acid (GABA), a neurotransmitter which mediates inhibitory signals from interneurons to motor neurons in parasites. By augmenting the effects of GABA, ivermectin causes neuromuscular blockage, thus resulting in flaccid paralysis and eventual death of the parasite. It is ineffective against cestodes and trematodes, as GABA is not involved as a neurotransmitter in these parasites [1], an observation that supports the hypothesis regarding its mode of action [2]. Although the animals to which ivermectin is administered also utilize GABA, they are not adversely affected by the drug as it does not readily cross the blood–brain barrier, so that it has little effect within the central nervous system [3]. Ivermectin is well absorbed when administered orally or parenterally and has a potent and prolonged duration of action. For example, a terminal half-life of 178 h was observed following i.v. administration of 0.2 mg kg^{-1} to sheep [4]. Most of the administered dose is excreted in the faeces and the remainder in the urine [1]. The drug tends to be concentrated in the liver and fat, with minimal residues being detected in muscle and kidneys. Most of the drug is recovered as the unchanged parent compound, there being no significant metabolism.

Measurement of ivermectin in serum or plasma is important for the determination of the drug's therapeutic window, particularly in the control of blood-sucking pests, such as the cattle tick *Boophilus microplus*. In addition, new sustained-release formulations are constantly being investigated for their potential

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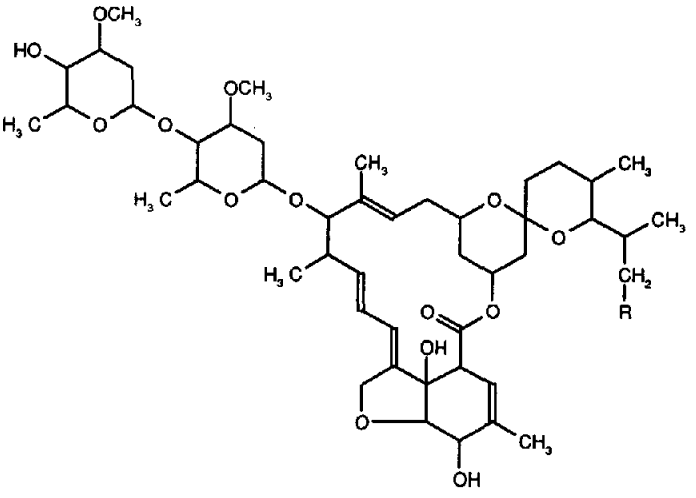

Figure 1
Structure of ivermectin homologues: R = H: 22,23-dihydroavermectin B_{1a}; R = CH_3: 22,23-dihydroavermectin B_{1b}.

advantages in terms of lower dosing frequency. Determination of the drug in serum or plasma as opposed to whole blood is preferred, due to the ease of sample handling and, more importantly, because systemic concentrations are mainly confined to the non-cellular fraction [5]. Liquid chromatographic (LC) methods have previously been described for the determination of ivermectin in plasma [5, 6], serum [7], tissues [8] and whole blood [5]. Although in the latter case it was possible to determine ivermectin at levels as low as 4–5 ng ml^{-1}, the method involved a lengthy sample preparation stage and was based on normal-phase chromatography, using a complicated quaternary mixture as mobile phase.

Because of the demand to determine extremely low levels of ivermectin and since it has a relatively low extinction coefficient, methods have been widely reported in which fluorescent derivatives have been exploited [5, 8–11]. Although these offer low limits of detection, the need for a derivatization step considerably complicates the procedure and losses or lack of reproducibility of the reaction usually necessitate the use of an internal standard. Residues of ivermectin in biological matrices have also been determined using chemical ionization MS–MS [12] and LC in combination with a reversed isotope dilution assay [13]. More recently, a reversed-phase LC method was described which involved detection by UV absorbance at 245 nm [7]. Although sample clean-up was achieved quite easily using solid-phase extraction, two different cartridges were required for each analysis and 5 g of sample was needed to permit a detection limit of 5 ppb.

In the present work a simple reversed-phase LC method is described for the determination of ivermectin in bovine plasma. Samples are introduced onto an extraction column for solid-phase extraction (SPE) and the method employs a column-switching approach in that sample clean-up is carried out on-line with the analytical separation. The method is reproducible, linear over the range 2–100 ng ml^{-1} and sensitive, using ultraviolet absorbance detection at 245 nm. Its principal advantages over earlier methods are that it employs a simple and economical method of sample preparation, has a low limit of detection without derivatization and is sufficiently reproducible as to not require an internal standard.

Experimental

Materials and reagents

Ivermectin injectable solution was purchased from Merck, Sharpe and Dohme (Dublin, Ireland). Deionized water was obtained by passing distilled water through a Milli-Q (Millipore, Milford, MA, USA) water purification system. LC grade acetonitrile, methanol, and isopropanol were purchased from Labscan Analytical Sciences (Dublin, Ireland). All other chemicals were used as received from BDH (Poole, Dorset, UK) and were of AnalaR grade. Drug-free bovine plasma, supplied by the National Food Centre, Dublin, was screened, pooled, divided into 10-

ml aliquots and frozen until required. The drug was separated on a Spherisorb ODS1 (10-μm) column (25 cm × 4.5 mm i.d.) (HPLC Technology, Macclesfield, UK). It was protected by a guard column packed with Corasil (Waters Associates, Milford, MA, USA) C_{18} pellicular packing material (37–50 μm). The mobile phase consisted of acetonitrile–water–isopropanol (40:40:35, v/v/v), delivered at a flow rate of 1 ml min^{-1} by a Waters Model 501 HPLC pump (pump B). For direct injection pump B was connected directly to the analytical column via a Rheodyne (Cotati, CA, USA) Model 7125 two-position, six-port injection valve fitted with a 100-μl loop. For column-switching, the injector was fitted with a 1-ml loop and connected to a Rheodyne Model 7000 two-position, six-port switching valve (Fig. 2) and a second pump (pump A), which was used to deliver the loading/washing solvent acetonitrile–water (40:60, v/v) at a flow rate of 1 ml min^{-1}. The pre-concentration (extraction) column (25 × 2.1 mm i.d.) was dry-packed in-house with Corasil C_{18} pellicular material and was attached to the switching valve as shown in Fig. 2. Eluted components were detected by UV absorbance at 245 nm using a Shimadzu (Kyoto, Japan) SPD-6A UV detector and the resultant peaks were recorded by a Linseis (Selb, Germany) Model 16512 x-t recorder at a chart speed of 200 mm h^{-1}. Ivermectin was then quantified by measurement of peak height as a function of concentration.

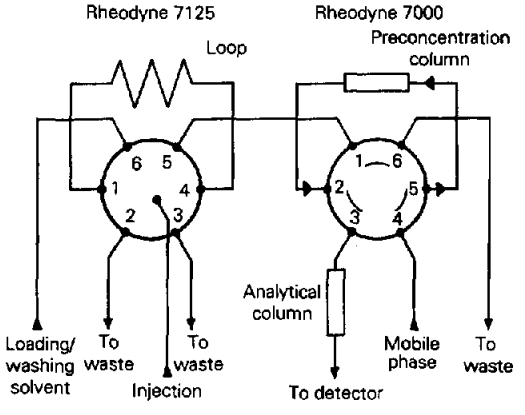

Figure 2
Valve arrangement for on-line solid-phase extraction with column-switching. The sample is injected into a 1.0-ml loop on the Rheodyne 7125 valve, which is connected to the Rheodyne 7000 valve. When this is in position 1, the injected sample is swept by the loading solvent, acetonitrile–water (40:60, v/v), onto the preconcentration (extraction) column. Upon switching the valve to position 2, the mobile phase, acetonitrile–water–isopropanol (40:40:35, v/v/v), is diverted in backflush mode at 1 ml min^{-1} onto the pre-concentration column, where the drug is desorbed and swept onto the analytical column for separation and detection at 245 nm.

Methods

A stock solution containing 100 μg ml^{-1} ivermectin in methanol was prepared. This was diluted (1 + 9) with MeOH–water (1:1, v/v) and used to spike drug-free aliquots of bovine plasma to generate plasma standards of 2, 5, 10, 20, 50 and 100 ng ivermectin per ml of plasma.

For deproteinization of samples, 15 μl of 2 M $ZnSO_4$ and 1.0 ml acetonitrile were added to 2.0 ml plasma. The mixture was homogenized by sonication for 30 s and then centrifuged for 5 min at 3000 rpm. A 1 ml-aliquot of the clear supernatant was injected into the loop on the Rheodyne 7125 valve and was swept onto the concentration column by the loading/washing solvent delivered at 1 ml min^{-1} by pump A. After a 2-min wash period, the Rheodyne 7000 valve was rotated so that the eluent stream from pump B was re-routed in a backflush direction onto the concentration column at 1 ml min^{-1}, thus desorbing the drug and sweeping it onto the analytical column for subsequent separation and detection.

Results and Discussion

Method development

It had already been shown in a number of studies that ivermectin can be separated by reversed-phase LC on octadecyl silica [8, 9, 14–17]. Because ivermectin is highly lipophilic, an eluent containing a high proportion of organic component is required to ensure elution of the compound within a practicable analysis time from a non-polar column such as C_{18} or C_8. Accordingly, on the Spherisorb ODS column employed in this study, the initial mobile phase investigated contained 85% methanol in water, which gave an elution time of 10.5 min for ivermectin. This analytical column was then assembled in a column-switching system incorporating a 10 × 1.5 mm i.d. pre-concentration (extraction) column (Table 1). Many regimens were investigated to minimize retention of plasma interferences on the extraction column, while avoiding elution of ivermectin during the wash cycle. The results of some of the procedures investigated are shown in Table 1. The protocols variously

Table 1
Procedures investigated for optimizing the extraction of ivermectin from bovine plasma

Mobile phase	Extraction column	Wash regimen	Plasma pre-treatment	Result
MeOH–H_2O (85:15, v/v)	C_8*	MeCN–H_2O (2:8, v/v) (5 ml)	None	Impurity peak
MeOH–H_2O (85:15, v/v)	C_8*	Buffer pH 7 (5 ml)	None	High background
MeOH–H_2O (85:15, v/v)	C_8*	25 or 40% MeCN (5 ml)	None	High background
MeOH–H_2O (85:15, v/v)	C_{18}*	25 or 40% MeCN (5 ml)	None	High background
MeOH–H_2O (85:15, v/v)	C_{18}*	MeOH–buffer pH 7 (3:7, v/v) (5 ml)	50% plasma + 50% MeOH or MeCN	Impurity peak; low recovery
MeCN–H_2O (75:15, v/v)	C_{18}*	MeOH–buffer pH 7 (3:7, v/v) (5 ml)	None	Poor peak shape
MeCN–H_2O (75:25, v/v)	C_{18}*	MeCN–buffer pH 7 (3:7, v/v) (5 ml)	PCA + MeCN precipitation	Poor peak shape
MeCN–H_2O–IPA (40:40:35, v/v/v)	C_{18}*	MeCN–buffer pH 7 (3:7, v/v) (5 ml)	PCA + MeCN precipitation	Good blank; low recovery
MeCN–H_2O–IPA (40:40:35, v/v/v)	C_{18}*	MeCN–buffer pH 7 (3:7, v/v) (5 ml)	$ZnSO_4$–MeCN–plasma (0.1:1.0:2.0, v/v/v)	Low recovery
MeCN–H_2O–IPA (40:40:35, v/v/v)	C_{18}†	MeCN–H_2O (3:7, v/v) (5 ml)	$ZnSO_4$–MeCN–plasma (0.1:0.7:1.2, v/v/v)	50% recovery: impurity peak
MeCN–H_2O–IPA (40:40:30, v/v/v)	C_{18}†	MeCN–H_2O (3:7, v/v) (5 ml)	$ZnSO_4$–MeCN–plasma (0.1:0.7:2.0, v/v/v)	Impurity peak
MeCN–H_2O–IPA (40:40:40, v/v/v)	C_{18}†	MeCN–H_2O (3:7, v/v) (5 ml)	$ZnSO_4$–MeCN–plasma (0.1:1.0:1.2, v/v/v)	Impurity peak
MeCN–H_2O–IPA (40:40:35, v/v/v)	C_{18}†	MeCN–H_2O (3:7, v/v) (5 ml)	$ZnSO_4$–MeCN–plasma (0.015:1.0:1.2, v/v/v)	65% recovery
MeCN–H_2O–IPA (40:40:35, v/v/v)	C_{18}†	MeCN–H_2O (3:7, v/v) (2 ml)	$ZnSO_4$–MeCN–plasma (0.015:1.0:2, v/v/v)	75% recovery

*Extraction column 10 × 1.5 mm i.d.; C_8, octylsilane silica packing; C_{18}, octadecylsilane silica packing.
†Extraction column 25 × 2.1 mm i.d.
Key to abbreviations: MeOH, methanol; MeCN, acetonitrile; IPA, isopropylalcohol; PCA, perchloric acid; buffer pH 7, 0.05 M phosphate buffer.

involved changing the analytical column eluent and the washing/loading solutions, changing the packing material in the extraction column and in the analytical column, in addition to exploring different plasma treatments prior to injection.

It became apparent that it was impossible to obtain adequate recovery of ivermectin with minimal endogenous interferents using this system. Thus a larger pre-concentration column was examined. By altering the proportions of acetonitrile and zinc sulphate added for deproteinization at the pre-treatment stage, adequate removal of interferences was achieved with a recovery of approximately 65%. Recovery was further improved (to ~75%) by decreasing the wash-time from 5 to 2 min, although a shorter wash-time led to the appearance of peaks co-eluting with ivermectin.

It became clear that the eluent for the analytical column required adjustment. Isopropanol was found to be a necessary component in the eluent, since a mobile phase composed only of methanol–water required a higher organic phase content for elution of ivermectin from the analytical column (with consequently higher levels of interfering peaks), whereas an acetonitrile–water eluent produced an asymmetric peak for the drug. It was found that the amount of isopropanol in the mobile phase was quite critical: with a smaller proportion of isopropanol, endogenous interferences retained on the extraction column co-eluted with ivermectin on the analytical column, whereas with a greater proportion of isopropanol, a greater number of interferences were removed from the extraction column.

The ternary eluent finally arrived at thus contained acetonitrile–water–isopropanol (40:40:35, v/v/v) and enabled elution of the ivermectin in a convenient time frame (12.5 min) with minimum interference from endogenous components. The inclusion of isopropanol rather than methanol in the mobile phase made it possible to keep the organic phase content sufficiently low in order to minimize the consumption of organic solvents and avoid precipitation on the pre-concentration column during the backflush step.

Method validation

The complete column-switching method was validated over the concentration range 2–100 ng ml^{-1} ivermectin spiked in plasma. The precision of the assay was evaluated in terms of the variability both between and within batches of replicate analyses over the calibration range. Within-batch or intra-assay variation was determined by analysing four replicates at each of six concentrations. A calibration curve based on the mean peak height values was calculated by regression analysis. Individual peak heights for each point were interpolated on the regression line to yield four new interpolated values of concentration (i.e. 'amount found') at each calibration point (Table 2). The mean, standard deviation (SD) and RSD for each 'amount found' were then calculated and the intra-assay precision was expressed as the mean RSD over the range 2–100 ng ml^{-1}; this was 6.7%. Linearity was assessed on the basis of the correlation coefficient of the regression line used in the intra-assay calculations (Table 2). The correlation coefficient was 0.9994 (n = 6).

Between-batch (inter-assay) variability was calculated by constructing a calibration curve on each of four consecutive days over the same

Table 2
Within-batch analysis: intra-assay precision

Amount added (ng ml^{-1})	Mean amount found (n = 4)* (ng ml^{-1})	±SD	RSD† (%)	Error (%)
100.00	100.76	±4.52	4.49	+7.60
50.00	48.17	±1.70	3.53	−3.66
20.00	20.67	±0.51	2.47	+3.35
10.00	10.00	±0.72	7.2	0.00
5.00	5.09	±0.45	8.84	+1.80
2.00	2.30	±0.31	13.5	+15.00

*Regression data: $y = 0.5147x + 0.0181$; correlation coefficient $r = 0.9994$. The mean amount found represents the average (at each point) of the concentrations obtained by interpolating the individual peak heights found for each of the four replicates.
†Mean RSD, taken as mean intra-assay precision, 6.67% (n = 6).

concentration range. The replicate peak height values were interpolated on the individual regression lines to yield four new values for the 'amount found' at each calibration point. The inter-assay variability was then expressed as the mean RSD over the calibration range (Table 3), giving a value of 3.8% (n = 6).

Recovery of ivermectin from plasma was assessed by comparing the slopes of the regression lines for each of four batches of spiked plasma standards with authentic (aqueous) standards, in all cases following injection of a 1-ml aliquot and column switching. The results of this study presented in Table 4 show that the

Table 3
Between-batch analysis: inter-assay precision

Amount added (ng ml^{-1})	Mean amount found (n = 4) (ng ml^{-1})	±SD	RSD* (%)	Error (%)
100.00	100.32	±0.67	0.67	+3.20
50.00	49.50	±1.28	2.58	−1.00
20.00	20.23	±0.62	3.06	+1.15
10.00	9.86	±0.11	1.12	−1.40
5.00	5.08	±0.26	5.12	+1.60
2.00	2.10	±0.21	10.0	+5.00

*Mean RSD, taken as the mean inter-assay precision, is 3.76% (n = 6).

Table 4
Drug recovery studies on ivermectin in bovine plasma

Sample type	Regression data*	r	%Recovery†
Aqueous standards	$y = 0.6667x + 0.4040$	0.9992	—
Plasma standards			
Batch 1	$y = 0.5051x + 0.1032$	0.9992	75.6
Batch 2	$y = 0.5217x + 0.0294$	0.9994	78.1
Batch 3	$y = 0.5085x + 0.1636$	0.9998	76.2
Batch 4	$y = 0.5047x + 0.3149$	0.9996	75.6

*Range 2–100 ng ml^{-1} with four replicates at each of six calibration points.
†Mean recovery = 76.4 ± 1.03% (RSD = 1.34%). Recovery data were calculated from the ratio of the regression data for each batch with respect to authentic calibration data for aqueous samples of ivermectin.

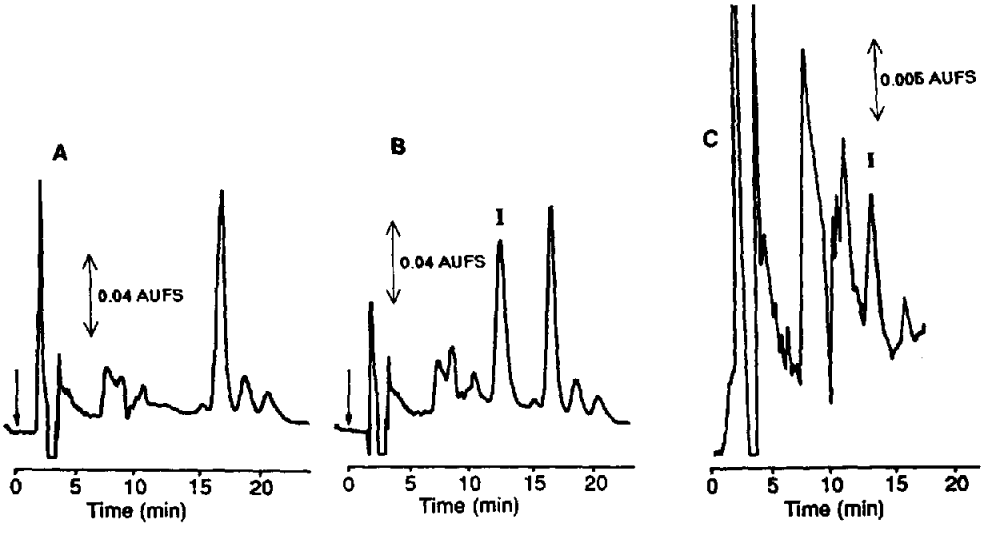

Figure 3
Chromatograms of plasma samples after SPE with column switching. (A) Drug-free bovine plasma, (B) plasma enriched with 50 ng ml^{-1} ivermectin, and (C) plasma enriched with 5 ng ml^{-1} ivermectin. Extraction was carried out on-line using a C_{18} extraction column (cf. Fig. 2). Separation on the C_{18} analytical column was under the conditions described in Fig. 2 and in the text.

mean recovery was 76.4% (RSD = 1.34%; $n = 4$). The minimum detectable concentration was found to be 0.8 ng ml^{-1} of ivermectin in plasma, (signal-to-noise ratio, 3:1). The limit of quantitation was assessed as 2 ng ml^{-1}, this being considered to be the level at which quantitation was consistently possible (RSD between 10 and 15%). Chromatograms of drug-free and ivermectin-enriched plasma are presented in Fig. 3.

Conclusions

A simple and rapid procedure for the determination of trace amounts of ivermectin in bovine plasma has been developed. The method is based on a readily available reversed-phase LC packing material and utilizes the rapid and efficient approach of on-line solid-phase extraction with column switching. Due to the inherent reproducibility of on-line SPE and the fact that the method features the minimum of sample handling, reproducible quantitation at trace levels is possible without the need for an internal standard. The method is cost-effective in that it uses a commercially available column and a concentration column which may be dry-packed in-house with pellicular packing material. The extraction column may be used for up to 10 1-ml injections of bovine plasma, since the high organic content of the analytical mobile phase permits good regeneration of the column surface during the desorption step.

It is anticipated that with appropriate modifications to avoid interference from endogenous components, the method could be applied to the determination of ivermectin in other biological matrices. Work is continuing in this laboratory on the development of a suitable column-switching SPE methodology for the determination of ivermectin residues in salmon tissue.

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