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A sensitive method for the measurement of the novel pet endectocide, selamectin (UK-124,114), in dog and cat plasma by chemical derivatisation and high-performance liquid chromatography with fluorescence detection

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Abstract

An analytical method has been developed for the novel pet endectocide, selamectin (USAN, UK-124, 114), in dog and cat plasma to facilitate pharmacokinetic profiling for this compound. The method involves solid phase extraction of the compound and internal standard from plasma followed by chemical derivatisation using triethylamine and trifluoroacetic anhydride. This reaction yields a highly fluorescent product and thus provides a sensitive assay. Using a sample volume of 1.0 ml for dog plasma the assay has been validated over a concentration range of 0.2–40 ng/ml. Due to smaller plasma volumes for cat plasma samples, the assay was validated over a concentration range of 1.0–200 ng/ml using a sample volume of 0.2 ml. The analyte has been shown to be stable for 48 h at room temperature and through three freeze–thaw cycles in dog plasma. The analytical method is highly specific and proved suitable for the analysis of selamectin in dog and cat plasma samples following doses of compound by parenteral and non-parenteral routes.

Keywords: Pet endectocide; Selamectin; High-performance liquid chromatography; Chemical derivatisation

1. Introduction

Selamectin (Fig. 1) is a molecule of the avermectin class which, as a group of compounds, are increasingly used in the treatment of parasitic diseases in animals. Selamectin is a semi-synthetic product produced by chemical modification of doramectin (25-cyclohexyl-5-*O*-demethyl-25-de(1-methylpropyl)avermectin A1a), a fermentation derived avermectin, itself used for the treatment of parasitic infections in livestock [1].

Selamectin has been found to be safe and effective in dogs and cats, against a wide range of ecto- and endoparasites, including *Ctenocephalides* spp., *Sarcoptes scabiei*, *Otodectes cynotis*, *Dermacentor variabilis*, *Toxocara* spp., *Ancylostoma tubaeforme* and immature larvae of the heartworm, *Dirofilaria immitis* [2–4].

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Bioanalysis of avermectins and milbemycins has routinely been enhanced approximately one order of magnitude by chemical derivatisation and HPLC with fluorescence detection compared with HPLC with ultraviolet absorption of the parent molecule [5]. Such methods have been developed for avermectins and milbemycins containing a dihydroxycyclohexene ring including ivermectin [6], doramectin [7] and milbemycin D [8]. This paper describes a solid phase extraction method for the isolation of selamectin and internal standard from dog and cat plasma. The compounds are subsequently derivatised to yield highly fluorescent products which may be analysed by reverse phase HPLC with fluorescence detection. Standard validation criteria [9] were considered during the development of the analytical procedure. The limit of quantification of the method is 0.2 ng/ml for a sample volume of 1 ml.

2. Experimental

2.1. Materials

Selamectin and internal standard, UK-127,053 (structures shown in Fig. 1), were prepared by Pfizer Central Research. Super purity grade methanol and acetonitrile were obtained from Romil (Cambridge, UK). Tetrahydrofuran (SLR grade) was obtained from Fisher Scientific (Loughborough, UK). Triethylamine, trifluoroacetic anhydride and 2.0 M ammonia solution in methanol were obtained from Aldrich (Gillingham, Dorset, UK). The solid phase extraction cartridges (C18 Isolute SPE, 1 ml capacity with 100 mg separating medium) were obtained from Jones Chromatography (Hengoed, Mid Glamorgan, UK).

2.2. Preparation of solutions

A stock solution of selamectin (200 μg/ml) was prepared in methanol in a glass volumetric flask and stored at 4°C. Working solutions (1 and 0.1 μg/ml) were prepared by serial dilution of the stock with methanol, and stored at 4°C. These solutions were freshly prepared for each run, and were used in the preparation of the standard curve. A stock solution of internal standard (UK-127, 053, 200 ng/ml) was prepared in methanol, in a glass volumetric flask, and stored at 4°C. An internal standard working solution was freshly prepared weekly by dilution with methanol, to give a final concentration of 1 μg/ml and stored at 4°C.

2.3. Preparation of calibration curves

Standards at final concentrations of 0.2, 0.5, 1, 2, 5, 10, 20 and 40 ng/ml were prepared by the addition of appropriate volumes of standard solutions of selamectin (0.1 and 1 μg/ml) to 1-ml aliquots of control dog plasma. For analysis in cat plasma the same volumes of standard solutions of selamectin added to 0.2-ml aliquots of control cat plasma provided concentrations of 1, 2.5, 5, 10, 50, 100 and 200 ng/ml. Samples were

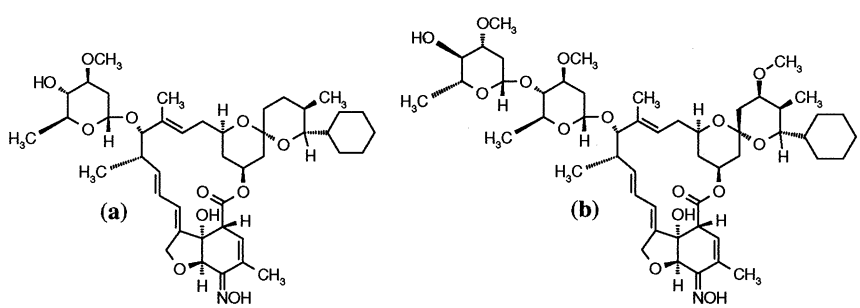

Fig. 1. Structures of (a) selamectin and (b) internal standard, UK-127,053.

thoroughly mixed prior to extraction as subsequently described. For both dog and cat plasma, blank samples were also prepared containing neither selamectin nor internal standard. These were not included as a calibration standard. The calibration curve was calculated by weighted linear regression ($1/y$) of peak height ratio (drug/internal standard) against concentration of calibration samples. The concentration of selamectin in test samples was calculated using the regression line parameters and correcting for any dilution used. Samples in which the concentrations exceeded the highest calibration sample were diluted with control plasma prior to analysis.

2.4. Extraction procedure

Plasma samples were defrosted at room temperature, vortex mixed briefly and then centrifuged ($2500 \times g$ for 5 min) prior to analysis. Accurately measured aliquots of each plasma sample (1.0 ml dog plasma or 0.2 ml cat plasma) were transferred to glass tubes using a dispensing pipette (Gilson, F1000 or F200) and internal standard solution was added (10 μl UK-127,053 at 1 μg/ml in methanol) using a glass syringe (Hamilton, 10 μl volume). To each sample 1 ml of 30% acetonitrile in water was added and thoroughly mixed using a vortex mixer (Jencons Scientific Ltd., UK). The Isolute extraction cartridges were primed by the addition of 1 ml of methanol to each. The methanol was drawn through the sorbent under light vacuum to the manifold. The cartridges were then washed by adding 1 ml water to each and again drawing through under light vacuum. The diluted plasma samples prepared as previously described were added to the cartridges and allowed to drip through under gravity. The cartridges were then washed with 1 ml water and the sorbent dried by application of the vacuum. The cartridges were transferred to tapered glass tubes and eluted with 2×0.5 ml methanol under gravity. The eluate was evaporated to dryness under a stream of nitrogen gas at 37°C using a sample concentrator (Turbovap LV, Zymark, UK). Complete drying of samples was ensured prior to derivatisation.

2.5. Sample derivatisation

Samples were derivatised to give a fluorescent product, using the method of Nowakowski et al. [5]. Triethylamine (100 μl of 50% v/v in acetonitrile) was added to each tube and thoroughly mixed. Trifluoroacetic anhydride (150 μl of 33% v/v in acetonitrile) was then added to each tube and again thoroughly mixed. The samples were concentrated to approximately 75 μl in the sample concentrator at 40°C (approximately 20 min). Ammonia solution (250 μl of 2.0 M in methanol) was then added to each tube and mixed gently and samples again reduced to approximately 75 μl in the sample concentrator at 40°C (approximately 10 min).

Acetonitrile (200 μl) was then added to each tube and mixed thoroughly. An aliquot (200 μl) was transferred to autosampler vials for automated analysis. From each sample 100 μl was injected onto the HPLC analytical column.

2.6. High performance liquid chromatography instrumentation and conditions

Chromatographic separations were performed using a Spherisorb RPB column, 25 cm × 3.2 mm internal diameter (Hichrom Ltd., Reading, UK), and using a mobile phase consisting of 68% acetonitrile, 17% water and 15% tetrahydrofuran, with a flow rate of 0.5 ml/min. The eluent was monitored with a F-1050 fluorescence detector (Merck–Hitachi) and set at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. A Shimadzu LC-6A HPLC pump (Dyson Instruments Ltd., Houghton-Le-Spring, UK) and Merck–Hitachi AS-2000 autosampler, with a 200 μl injection loop, (BDH-Merck, Poole, UK) were used. The chromatograms were integrated using a MULTICHROM chromatography data management system (VG Laboratory Systems, Altrincham, UK).

2.7. Preparation and analysis of QC samples

The intra-day precision and accuracy of the method was investigated by the replicate analysis ($\times 6$) of selamectin in dog plasma at three con-

centrations across the calibration range (0.2, 5 and 40 ng/ml), relative to the multilevel calibration standards. Stability of selamectin in dog plasma and inter-day precision and accuracy were investigated by the analysis of duplicate samples at each of the three concentrations (0.5, 10 and 30 ng/ml). These QC samples were analysed when freshly prepared, following 48 h at room temperature, after three freeze–thaw cycles and following 7 days frozen storage. On each occasion concentrations were calculated from the response relative to freshly prepared calibration standards. In addition, stability in frozen dog and cat plasma samples was assessed by the analysis of replicate QC samples (dog, 29 ng/ml; cat, 142 ng/ml) after storage at less than −20°C for up to 45 days.

3. Results

3.1. Selectivity

Typical chromatograms for control blank and spiked dog plasma are shown in Fig. 2. The retention times of selamectin and UK-127,053 (internal standard) were typically 14 and 11.5 min, respectively. Similar chromatograms were obtained for control and spiked cat plasma. A variable broad peak eluted between 1.5 and 7.5 min, however this did not interfere with the analytes. No interfering endogenous components were observed in blank plasma from six different animals of each species. The use of fluorescence detection following chemical derivatisation combines sensitivity and selectivity for the analysis of selamectin in plasma.

3.2. Recovery

Extraction efficiency was calculated by comparing response for selamectin and internal standard following extraction against spiked solutions in methanol taken through the derivatisation procedure. Extraction recovery varied between 30 and 63% in dog and cat plasma. The variation in recovery must have arisen during the solid phase extraction process, however it was compensated for by similar recovery of internal standard. As no authentic standards were available for the derivatised products, it was not possible to determine the efficiency of the derivatisation procedure.

3.3. Linearity

Calibration curves were shown to be linear from 0.2 to 40 ng/ml in dog plasma (1.0 ml) and 1 to 200 ng/ml in cat plasma (0.2 ml). The use of $1/y$ weighting provided the best overall data fit. A mean coefficient of determination (r^2) of 0.9996 was obtained from five analytical runs in dog plasma and an average coefficient of determination of 0.9997 from two analytical runs in cat plasma using a weighting of $1/y$. Back calculated concentrations for the calibration standards are shown in Table 1. Example chromatograms for blank dog plasma and calibration standards at 0.2 and 40 ng/ml are shown in Fig. 2.

3.4. Precision and accuracy

Precision of the analytical procedure in dog plasma was determined by analysing six identical samples of control plasma containing selamectin at concentrations of 0.2, 5 and 40 ng/ml in a single analytical batch (Table 2). Accuracy and precision were lowest at the low concentration (0.2 ng/ml). However, the mean determined concentration was within 10% of the actual value and the coefficient of variation was less than 15%. At concentrations of 5 and 40 ng/ml the coefficient of determination and the relative bias were less than 5%. Inter-day imprecision and inaccuracy were determined in dog plasma by the analysis of quality control samples on four separate occasions. Duplicate samples at 0.5, 10 and 30 ng/ml were analysed and the results are summarised in Table 3. Precision was again lowest at low concentration with a coefficient of variation of 17% at 0.5 ng/ml.

3.5. Limits of quantitation

The upper limit of quantitation was taken as 40 ng/ml in dog plasma and 200 ng/ml in cat plasma due to acceptable performance of the calibration standards at these levels. The lower limit of quantitation in dog plasma was taken as 0.2 ng/ml

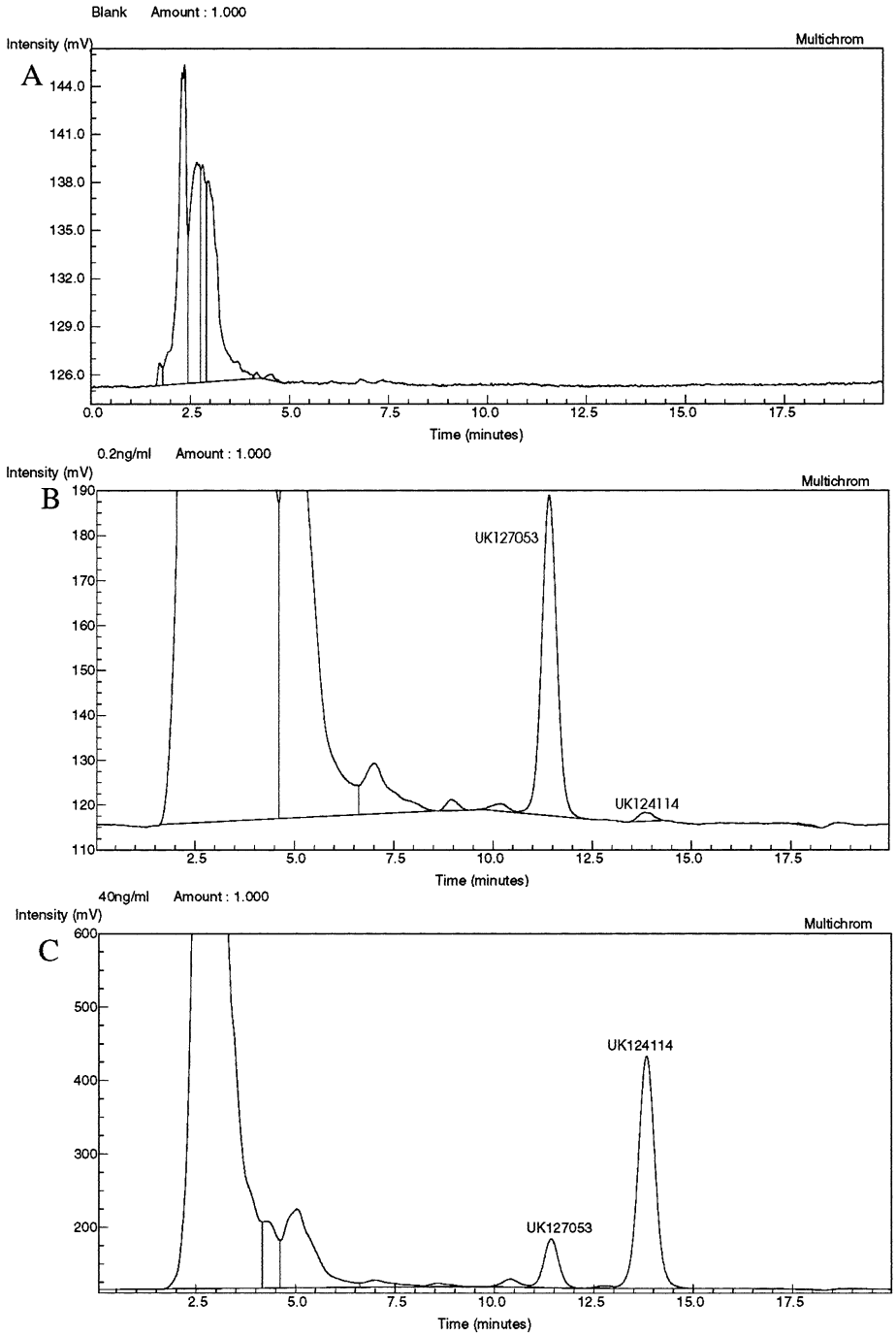

Fig. 2. Representative chromatograms of selamectin and internal standard (UK-127,053) in dog plasma (1 ml). (A) Control plasma blank; (B) control plasma spiked with 0.2 ng/ml selamectin and 10 ng/ml UK-127,053; (C) control plasma spiked with 40 ng/ml selamectin and 10 ng/ml UK-127,053.

Table 1
Calibration curve details for the analysis of selamectin in dog and cat plasma[a]

Selamectin added (ng)		0.2	0.5	1.0	2.0	5.0	10.0	20.0	40.0
Selamectin measured (ng)	Dog plasma	0.19	0.47	1.03	2.25	5.15	9.68	19.7	40.5
	Dog plasma	0.21	0.52	0.87	2.10	5.18	9.98	20.1	40.1
	Dog plasma	0.23	0.47	0.95	1.99	4.82	10.2	19.9	40.3
	Dog plasma	0.22	0.45	0.92	2.03	4.85	9.17	19.9	NR
	Dog plasma	0.17	0.51	1.04	1.94	4.93	9.45	19.6	38.8
	Cat plasma	0.20	0.49	0.97	1.97	4.84	9.32	19.8	39.0
	Cat plasma	0.18	0.50	1.13	1.81	4.86	9.38	19.1	39.6
Mean		0.20	0.49	0.99	2.01	4.95	9.60	19.73	39.72
S.D.		0.022	0.025	0.087	0.14	0.15	0.38	0.32	0.70
Accuracy (relative bias[b], %)		0	−2.0	−1.0	0.5	−1.0	−4.0	−1.4	−0.7
Precision (CV[c], %)		11.0	5.1	8.8	7.0	3.0	4.0	1.6	1.8

[a] Values for the coefficient of determination of the calibration line in dog plasma ranged from 0.9991 to 0.9998 ($n = 5$). The mean equation for the calibration line in dog plasma was $y = 0.0427\ (\pm 0.0049)x + 0.00175\ (\pm 0.00547)$.
[b] Relative bias = [(mean measured value − prepared value)/prepared value]100.
[c] CV, coefficient of variation = (S.D. × 100)/mean; NR, no result.

based on the precision and accuracy following replicate analysis.

3.6. Stability

The stability of selamectin in dog plasma was assessed by the analysis of QC samples under various storage conditions. Mean recoveries ($n = 6$, two at each concentration 0.5, 10 and 30 ng/ml) relative to added amount of selamectin were 102 ± 7, 98 ± 7, 96 ± 8 and $94 \pm 5\%$ for fresh analysis, 48 h room temperature storage, three freeze–thaw cycles and 7 days frozen storage, respectively. Stability in frozen dog and cat plasma samples was further assessed by the analysis of four replicate samples at concentrations of 29 ng/ml in dog plasma and 142 ng/ml in cat plasma. Mean recoveries ($n = 4$) after 0-, 7- and 28-day storage of the dog plasma samples were 104, 101 and 102%, respectively. Mean recoveries ($n = 4$) after 3-, 5- and 45-day storage of the cat plasma samples were 102, 101 and 118%, respectively. The stability of the prepared derivatised samples was assessed by the repetitive analysis of a single bulked sample (nominal concentration of 10 ng/ml), stored in autosampler vials, after 0, 24 and 48 h. Duplicate analysis on each occasion produced average results of 11.4, 11.4 and 11.2 ng/ml, respectively.

Table 2
Intra-day precision and accuracy of the determination of selamectin in dog plasma[a]

Added concentration (ng/ml)	Measured concentration (mean ± S.D., $n = 6$) (ng/ml)	Coefficient of variation (%)
0.2	0.22 ± 0.03	13.6
5	4.93 ± 0.16	3.2
40	40.02 ± 0.54	1.3

[a] Relative bias values were +10, −1.4 and +0.05% at concentrations of 0.2, 5 and 40 ng/ml, respectively.

Table 3
Inter-day precision and accuracy of the determination of selamectin in dog plasma[a]

Added concentration (ng/ml)	Measured concentration (mean ± S.D., $n = 8$) (ng/ml)	Coefficient of variation (%)
0.5	0.47 ± 0.48	17.0
10	11.2 ± 0.28	2.5
30	31.8 ± 1.49	4.7

[a] Relative bias values were −6.0, +12 and +6.0% at concentrations of 0.5, 10 and 30 ng/ml, respectively.

4. Discussion and conclusions

Chemical derivatisation to facilitate sensitive quantitation has been extensively utilised for avermectins containing a dihydroxycyclohexene ring [6,7]. With these compounds the derivatisation procedure results in an intensely fluorescent derivative by dehydration of this function of the molecule. The 5-oximino function of selamectin (and the internal standard, UK-127,053) appears capable of forming a similar derivative which was subsequently confirmed by NMR [10]. The procedure described for the analysis of selamectin subsequently proved suitable for pharmacokinetic monitoring in cat and dog plasma following topical administrations, which resulted in only low levels of circulating drug (data not shown). The procedure is somewhat slow and tedious, however this method should be amenable to automation as previously demonstrated with doramectin [11]. The intra- and inter-day accuracy and precision for this assay were considered satisfactory [9]. Intra-day precision and accuracy values at 0.2 ng/ml were 14 and 10%, respectively, and this was defined as the lower limit of quantitation for the assay in dog plasma. More limited validation has been performed in cat plasma, although essentially the assay performs identically in this matrix. Limited sample volumes available from cat pharmacokinetic studies restricted the limit of quantitation to 1 ng/ml based on a sample volume of 0.2 ml. The analyte has been shown to be sufficiently stable at room temperature, on frozen storage and in derivatised form to allow generation of accurate data.

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