

Determination of selamectin in dog plasma by high performance liquid chromatography with automated solid phase extraction and fluorescence detection

Jean-François SUTRA^a, Marie-Christine CADIERGUES^b,
Jacques DUPUY^a, Michel FRANC^c, Michel ALVINERIE^{a,*}

^aLaboratoire de Pharmacologie-Toxicologie, INRA, 180 Chemin de Tournefeuille,
BP 3, 31931 Toulouse Cedex 9, France

^bÉcole Nationale Vétérinaire, 23 Chemin des Capelles, 31076 Toulouse, France

^cUnité Associée INRA de Physiopathologie et Toxicologie expérimentales,
École Nationale Vétérinaire, 23 Chemin des Capelles, 31076 Toulouse, France

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Abstract – A method is described for the determination of selamectin in dog plasma, using High-Performance Liquid Chromatography (HPLC) with fluorescence detection (excitation and emission wavelengths fixed at 355 and 465 nm, respectively). The fluorescent derivative was obtained by condensation reaction with trifluoroacetic anhydride and N-methylimidazole. The method employs 1 mL plasma samples and gives linear calibration graphs ($r = 0.999$) over the concentration range studied ($0.5 - 50 \text{ ng}\cdot\text{mL}^{-1}$). Automatic solid phase extraction using the benchmate procedure was used for sample preparation. This method permits the determination of selamectin at levels as low as $0.1 \text{ ng}\cdot\text{mL}^{-1}$ and its suitability was demonstrated by a pharmacokinetic study on a dog receiving the therapeutic dose (Spot-on administration).

selamectin / plasma / high-performance liquid chromatography / kinetics / dog

Résumé – Dosage de la sélamectine dans le plasma de chien par chromatographie liquide haute performance avec une extraction en phase solide automatisée et une détection fluorimétrique. Cet article décrit une technique analytique qui permet de doser la sélamectine dans le plasma par chromatographie liquide haute performance, après formation d'un dérivé fluorescent (λ_{max} excitation = 355 nm et λ_{max} émission = 465 nm). Ce dernier composé est obtenu grâce à une réaction de condensation utilisant l'anhydride de l'acide trifluoroacétique et le N-méthylimidazole. La technique requiert 1 mL de plasma et la courbe de calibration obtenue est linéaire dans la gamme de concentrations étudiée ($0.5 - 50 \text{ ng}\cdot\text{mL}^{-1}$). Une extraction en phase solide automatisée a été employée pour traiter les échantillons. Cette méthode présente une limite de détection de $0.1 \text{ ng}\cdot\text{mL}^{-1}$ et

* Correspondence and reprints

Tel.: (33)5 61 28 51 37; fax: (33)5 61 28 53 10; e-mail: malviner@toulouse.inra.fr

apparaît efficace pour conduire des études de pharmacocinétique dans différentes espèces. Un exemple de cinétique plasmatique chez le chien ayant reçu une dose thérapeutique de sélamectine (Spot-on) est décrite.

sélamectine / plasma / chromatographie liquide haute performance / cinétique / chien

1. INTRODUCTION

The therapeutic potential of the avermectin/milbemycin family of endectocides has not been fully achieved for companion animals, either because of idiosyncratic toxicity in Collie dogs or a lack of activity against key parasites, particularly fleas.

Selamectin is a novel avermectin, structurally related to doramectin, which has a unique combination of safety in all breeds and potency against both external and internal parasites of dogs and cats [4]. Selamectin, or 25-cyclohexyl-25-de(1-methylpropyl)-5-deoxy-22,23-dihydro-5(hydroxy-imino)-avermectin B1 monosaccharide, is shown in Figure 1.

Administered topically as a simple "spot-on" application, selamectin is effective against ticks, ear mites, sarcoptic mange and certain intestinal helminths; it prevents heartworm diseases, and is highly potent against fleas [3].

Considering this, to date, no information is available on the pharmacokinetics of selamectin, and the development of a method for the determination of the drug in plasma is required.

In the present experiment, we describe a High Performance Liquid Chromatography (HPLC) determination of selamectin combined with an automatic solid phase extraction procedure and a fluorescent derivatisation. Furthermore, in order to test the applicability of the method to *in vivo* pharmacokinetic studies, the drug was administered to a dog.

2. MATERIALS AND METHODS

2.1. Reagents and equipment

Acetonitrile for HPLC was obtained from Fisher Scientific (Loughborough, UK), and methanol (HPLC grade) obtained from

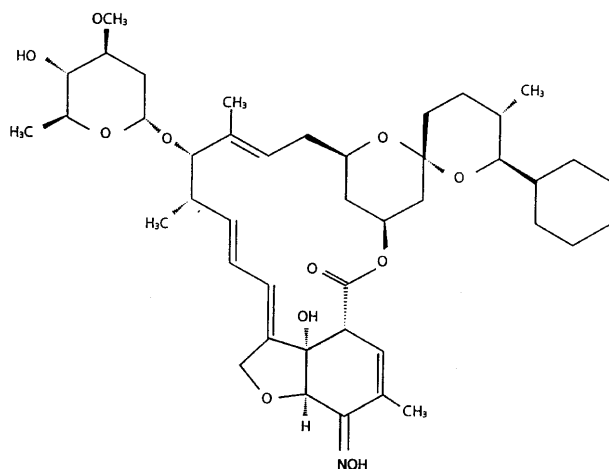


Figure 1. Structure of selamectin.

Carlo Erba (Milan, Italy). Trifluoroacetic anhydride and N-methylimidazole of analytical-reagent grade were purchased from Aldrich (Sigma Aldrich Chimie, St-Quentin-Fallavier, France). Glacial (100%) acetic acid was obtained from Merck (Merck-Clevenot, Chelles, France). Pic B₇ low UV was purchased from Waters (Guyancourt, France). The hydro water purification system (18M Ω cm⁻¹), Millipore (Bedford, MA, USA), was used. Solid phase extraction was performed using Supelclean LC18 cartridges (100 mg, 1 mL) obtained from Supelco (Bellefonte, PA, USA).

The HPLC system consisted of a model 420 pump (Kontron, Paris, France) and a model 360 automatic injector (Kontron, Paris, France), the injection volume was 100 μ L and a model FP-920 fluorescence detector (Jasco, Tokyo, Japan) was connected to a data station Kroma System 2000 (Kontron, Paris, France). The separation was carried out on a stainless-steel analytical column (250 \times 4.6 mm id) packed with Supelcosil LC18 (5 μ m) material (Supelco, Bellefonte, PA, USA). The mobile phase of (0.4 %) acetic acid in water – Pic B₇ – MeOH – acetonitrile (4:0.4:40:55.6; v/v/v/v) was pumped at a flow rate of 1.4 mL·min⁻¹. The detector was fixed at an excitation wavelength of 355 nm and an emission wavelength of 465 nm.

2.2. Standard solutions

The working standard solutions of selamectin used to plot the calibration graphs were prepared by serial dilution of a stock standard solution (1 mg·mL⁻¹) in acetonitrile. The internal standard working solutions of doramectin were similarly prepared from a stock solution (1 mg·mL⁻¹) with acetonitrile to 500 ng·mL⁻¹. Both the stock and working standard solutions were protected from light and stable for at least 3 months at +4 °C.

2.3. Extraction and clean-up procedure

Drug-free plasma samples (1 mL) were fortified with selamectin (0.5, 1, 2.5, 5, 10, 25 and 50 ng·mL⁻¹) using standard solutions in acetonitrile (5-10-20 μ L) and 0.1 mL of a 500 ng·mL⁻¹ internal standard working solution (doramectin) in acetonitrile. The kinetics samples were spiked with the internal standard. The plasma samples were homogenised and a solid phase extraction was performed after 15 min of incubation at room temperature. Acetonitrile (0.75 mL) was added to 1 mL of plasma and 0.25 mL of water. After mixing for 20 min, the tube was centrifuged at 2,000 \times g for 2 min. The supernatant (\leq 2.0 mL) was manually transferred into a tube, which was then placed on the appropriate rack of a benchmate II (Zymark, Hopkinton, MA, USA). Automatic sample preparation was performed as follows (total running time 22.3 min). 1. Conditioning of the cartridge (Supelclean LC.18 Cartridge): positioned on the holder, the column was first conditioned with 5 mL of methanol and 5 mL of water (flow-rate 6 mL·min⁻¹). 2. Loading of the plasma sample: a 2.0 mL volume of plasma sample (supernatant) was applied to the cartridge (flow rate 3.0 mL·min⁻¹). The cartridge was then washed with 2 mL of water, then with 1 mL of water-methanol (75:25; v/v) at a flow rate of 3.0 mL·min⁻¹. Before elution, the cartridge was dried with nitrogen for 10 s, then 2.0 mL of acetonitrile was applied to the column at a flow rate of 3.0 mL·min⁻¹ and the eluate was collected.

2.4. Derivatisation

The formation of the fluorophore of selamectin was achieved by using a previously described process for moxidectin [1]. The dry extract was dissolved in 100 μ L of 1 N-methylimidazole solution in acetonitrile (1:1; v/v) and 150 μ L of trifluoroacetic anhydride solution in acetonitrile (1:2; v/v) were added to initiate the derivatisation.

After mixing (< 30 s), the solutions were transferred into autosampler ambered glassware vials, to avoid photoisomerisation of the derivatives under ambient light conditions [6] until the injection of an aliquot (100 μL). HPLC analyses were carried out within 6 hours, to avoid the degradation of the fluorescent derivatives [5].

2.5. Calibration

Calibration graphs for selamectin, in the range of concentrations from 0.5 to 50 $\text{ng}\cdot\text{mL}^{-1}$, were prepared using dog drug-free plasma samples. The fortified plasma samples were taken through the procedure and assayed by HPLC. Standard curves for selamectin were constructed by plotting peak area ratio (selamectin/doramectin) versus drug concentrations. The calibration curve was fitted using a weighted ($1/X^2$) linear least squares regression analysis. The linear regression obtained between peak area ratio and analyte concentration was best described by the equation $Y = a + bx$ where x is the concentration of selamectin ng/mL , b the slope, Y the value of area ratio of the peak and a the intercept.

The extraction recovery of selamectin was measured by comparing the peak areas obtained from spiked plasma samples with the peak areas resulting from the direct injection of standards performed through the derivatisation procedure. The recovery and precision of the procedure was ascertained by processing replicates of plasma samples containing a known amount of selamectin by the proposed method.

2.6. Selectivity and specificity

Control plasmas from three different dogs were prepared and assayed by the described method, except that these control samples were not spiked with the analyte or internal standard. The specificity of the method was demonstrated by the lack of interfering peaks

at retention times of selamectin and doramectin. The selectivity of the assay was evaluated by comparison between the retention times of selamectin and doramectin and those of several other semi-synthetic macrocyclic lactones (moxidectin, abamectin, ivermectin and eprinomectin).

2.7. Drug disposition study

In order to test the application of the method to in vivo pharmacokinetic studies, a selamectin Spot-on commercial formulation (Stronggold 240 mg, Pfizer, Orsay, France) was applied to a beagle dog at a dose rate of 6 $\text{mg}\cdot\text{kg}^{-1}$. Blood was withdrawn via the antebrachial vein, at 0, 1, 2, 3, 4.5, 5, 6, 7, 8, 9, 10, 12, 13, 14, 21, 28, and 35 days after treatment, into heparinised tubes. The plasma was immediately separated and stored at $-20\text{ }^\circ\text{C}$ until analysis. The C_{max} , T_{max} and half-life time were graphically determined.

3. RESULTS

A typical chromatogram, shown in Figure 2A, displays excellent peak symmetry for a 5 ng standard of selamectin and a 10 ng standard of doramectin (internal standard). Under the described conditions, the retention time of selamectin was 9.67 and that of doramectin 15.61 min. The lack of interferences in the separation shows the high specificity of the method. The retention times of moxidectin, abamectin, ivermectin and eprinomectin in these HPLC conditions were: 8.32 min – 12.25 min – 30 min – no peak, respectively. The large difference between retention times of selected drugs demonstrates the high selectivity of the method.

Figure 2C shows a chromatogram obtained from a kinetic plasma sample (4.5 days post-treatment) containing a selamectin concentration of 24.53 $\text{ng}\cdot\text{mL}^{-1}$. Figure 2D shows a chromatogram obtained 35 days post-treatment of the kinetic plasma

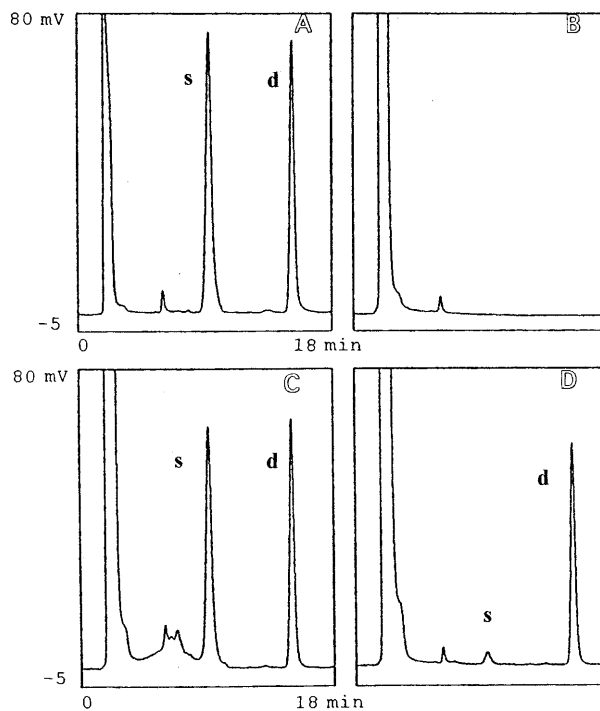


Figure 2. Typical chromatograms (A) standard of (s) selamectin (5 ng) and (d) doramectin (10 ng); (B) blank plasma; (C) kinetic plasma sample containing $24.53 \text{ ng}\cdot\text{mL}^{-1}$ of (s) selamectin and (d) doramectin (10 ng) (D) kinetic plasma containing $1.22 \text{ ng}\cdot\text{mL}^{-1}$ of selamectin and (d) doramectin (10 ng).

containing a selamectin concentration of $1.22 \text{ ng}\cdot\text{mL}^{-1}$.

The extraction recoveries were $92.41\% \pm 2.81$ for selamectin and $89.64\% \pm 3.26$ for doramectin, in the range of concentrations from 0.5 to $50 \text{ ng}\cdot\text{mL}^{-1}$. The linearity of the analytical procedure was tested by using plasma samples spiked in a range of concentrations from 0.5 to $50 \text{ ng}\cdot\text{mL}^{-1}$ (in quintuplicate for each concentration). The average values for slope and intercept ($n = 5$) were 0.05276 ± 0.0029 and $6.8 \times 10^{-4} \pm 3.8 \times 10^{-4}$, respectively. The coefficient of correlation generally exceeded 0.999 ($0.9994 \pm 3.7 \times 10^{-4}$). The recovery rate was $99.86\% \pm 3.56$ in the range of concentrations from 0.5 to $50 \text{ ng}\cdot\text{mL}^{-1}$ for five calibration curves (Tab. I). The inter-assay precision of the method, expressed as the inter-assay preci-

sion was $2.91\% \pm 1.58$ in the range of concentration from 0.5 to $50 \text{ ng}\cdot\text{mL}^{-1}$ ($n = 35$) and the intra-assay precision was $2.85\% \pm 2.40$ for multi-concentration (0.5 to $25 \text{ ng}\cdot\text{mL}^{-1}$) with 12 replicates (Tab. I).

With regard to relative sensitivity, taking a signal-to-noise ratio of two as a criterion, the detection limits of the proposed method using the equipment described were found to be 0.08 ng for selamectin, resulting in a limit of quantification of $0.10 \text{ ng}\cdot\text{mL}^{-1}$.

The selamectin plasma concentrations, evaluated in a dog receiving a Spot-on dose of $6 \text{ mg}\cdot\text{kg}^{-1}$ is depicted in Figure 3. The peak plasma concentration of selamectin observed on the curve ($C_{\text{max}} = 24.53 \text{ ng}\cdot\text{mL}^{-1}$) occurred after 6 days (T_{max}). Thereafter, the plasma concentration decreased progressively, with an apparent terminal half-life of 7 days.

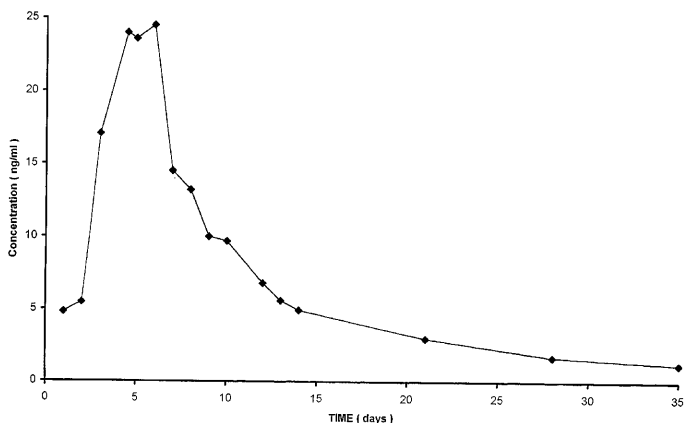


Figure 3. Plasma concentration-time curve for a dog treated with $6 \text{ mg}\cdot\text{kg}^{-1}$ of selamectin (Spot-on administration).

Table I. Recovery rate of selamectin from dog plasma samples fortified with selamectin ($n = 5$) and intra-assay precision ($n = 5$) and inter-assay precision ($n = 2$).

| Concentration added / $\text{ng}\cdot\text{mL}^{-1}$ | Concentration found (mean \pm SD) $\text{ng}\cdot\text{mL}^{-1}$ | Recovery (mean \pm SD)% | Precision (%) | |
|--|--|---------------------------|-----------------|-----------------|
| | | | intra-assay | inter-assay |
| 0.5 | 0.49 ± 0.01 | 97.03 ± 1.99 | 5.33 | 2.05 |
| 1 | 1.05 ± 0.04 | 104.31 ± 3.51 | 6.02 | 3.33 |
| 2.5 | 2.56 ± 0.07 | 102.56 ± 2.70 | 0.57 | 2.63 |
| 5 | 5.01 ± 0.19 | 100.20 ± 3.86 | 3.05 | 3.85 |
| 10 | 9.50 ± 0.55 | 95.04 ± 5.46 | 1.76 | 5.75 |
| 25 | 25.73 ± 0.25 | 102.91 ± 1.00 | 0.36 | 0.97 |
| 50 | 48.47 ± 0.88 | 96.94 ± 1.75 | | 1.81 |
| (mean \pm SD)% | | 99.86 ± 3.56 | 2.85 ± 2.40 | 2.91 ± 1.58 |

4. DISCUSSION

Basic compounds, like selamectin, are partially or completely ionised and often retained by residual silanol groups or silica. Such a dual retention could reduce chromatographic efficiency and produce asymmetric peaks. In this case, the problem was solved by using an ion pair agent (Pic B₇) and a Supelclean LC18 column: a good separation was obtained without any tailing peaks.

The fluorescent derivative of selamectin was obtained by a mechanism similar to those previously reported for ivermectin [2], moxidectin [1], or eprinomectin [7]. This conversion requires less than 30 s at ambient temperature, but the presence of water in the reaction tube can interfere with the derivatisation process. Finally, the fluorescent derivative is stable over a period at least of 6 hours.

A method associating solid phase extraction with a fast derivatisation procedure and

HPLC has been developed for the determination of selamectin in plasma. The automatic sample preparation procedure (using the benchmate II system) gives good reproducibility, owing to the precise control of flow-rates (Cartridge loading, rinsing, elution) and volumes. Furthermore, the Benchmate II performs a gravimetric control of every preparation step, which can be reviewed as an extra control procedure.

A similar method has recently been described [8]. However the latter technique does not use the same fluorescence procedure, and is time consuming (30 min versus 45 s in our procedure). Furthermore, the manual solid phase extraction procedure is slow and tedious. In conclusion, the proposed method, using automatic sample preparation, appears to be efficient for pharmacokinetic studies.

Regarding the extraction procedure and according to other reports [1] about related compounds, the use of solid-phase extraction results in a clean extract with good recovery.

The knowledge of the pharmacokinetic behaviour of antiparasitic drugs is useful to achieve optimal activity. Therefore, the protocol described in the present paper provides a reliable method to detect selamectin in animal plasma at low concentration ($0.1 \text{ ng}\cdot\text{mL}^{-1}$) and could currently be used as a routine technique for pharmacokinetic investigations in dogs.

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