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

J. F. Sutra[a], C. Chartier[b], P. Galtier[a] and M. Alvinerie*[a]

[a] *Laboratoire de Pharmacologie–Toxicologie, INRA, 180 Chemin de Tournefeuille, BP3, 31931 Toulouse Cedex 9, France*
[b] *Station Régionale de Pathologie Caprine, CNEVA, 60 rue de Pied de Fond, BP 3081, Niort, France*

A method is described for the determination of eprinomectin in plasma using high-performance liquid chromatography with fluorescence detection (excitation and emission wavelengths 355 and 465 nm, respectively). The fluorescent derivative was obtained by a 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 ng ml^{-1}). Solid extraction using the benchmate procedure was used for sample preparation. This method permits the determination of eprinomectin at levels as low as 0.1 ng ml^{-1} and is suitable for the pharmacokinetic study of eprinomectin in animals.

Keywords: Eprinomectin; plasma; high-performance liquid chromatography; kinetics

Eprinomectin or [4″-(epiacetylamino)-4″-deoxyavermectin B$_1$] has recently been approved for use in the European Community as a beef and dairy topical endectocide under the tradename Eprinex pour-on for cattle. Eprinomectin is a new 16-membered macrocyclic lactone that belongs to the avermectin/milbemycin class of potent endectocides.[1,2] It is a new endectocide consisting of a 90 + 10 mixture of two homologues: 4-epiacetyl-amino-4-deoxyavermectins B$_{1a}$ and B$_{1b}$, which differ by one methylene unit at the C-25 portion of the macrocyclic ring[3] (Fig 1). Eprinomectin was selected for development based on its potency and safety profile. One method for the detection of eprinomectin in bovine tissue has been reported,[4] which involves solvent extraction and a solvent partitioning clean-up step. Unfortunately, this method is time consuming and unusable for pharmacokinetic investigations.

Considering that at this moment, there is no information available on the pharmacokinetics of eprinomectin, there is a need for the development of a method for the evaluation of eprinomectin in plasma.

We report here the application of a HPLC determination combined with a solid phase extraction procedure and a fluorescent derivatization. Furthermore, in order to test the applicability of the method to *in vivo* pharmacokinetic studies, eprinomectin was administered to a goat.

Experimental

Reagents and equipment

Acetonitrile and methanol (HPLC grade) were obtained from Carlo Erba (Milan, Italy). Trifluoroacetic anhydride and *N*-methylimidazole of analytical-reagent grade were purchased from Aldrich (Sigma Aldrich Chimie, St. Quentin Fallavier, France). Acetic acid (glacial 100%) was obtained from Merck (Merck–Clevenot, Chelles, France). Pic B$_7$ low UV was purchased from Waters (Guyancourt, France).

The HPLC system consisted of a Model PU980 pump (Jasco, Tokyo, Japan), a Model 360 automatic injector (Kontron, Paris, France) and a Model RF-551 fluorescence detector (Shimadzu, Kyoto, Japan), connected to a data station D450 MT1-EMS (Kontron, Paris, France). The separation was carried out on a stainless steel analytical column (250 × 4.6 mm id) packed with Suplex pkb 100 (5 µm) material (Supelco, Bellefonte, PA, USA). The mobile phase of (0.4%) acetic acid–Pic B$_7$–acetonitrile (42 + 0.4 + 57 v/v/v) was pumped at a flow rate of 1 ml min^{-1}. The detector was fixed at an excitation wavelength of 355 nm and an emission wavelength of 465 nm.

Standard solution

The working standard solutions of eprinomectin used to construct the calibration graphs were prepared by serial dilution of a stock standard solution (0.1 mg ml^{-1}) in acetonitrile. Both the stock and working standard solutions were stable for at least 3 months at +4 °C and protected from light.

Extraction and clean-up procedure

Drug-free plasma samples (1 ml) were fortified with eprinomectin (0.5, 1, 2.5, 5, 10, 25 and 50 ng ml^{-1}) using standard
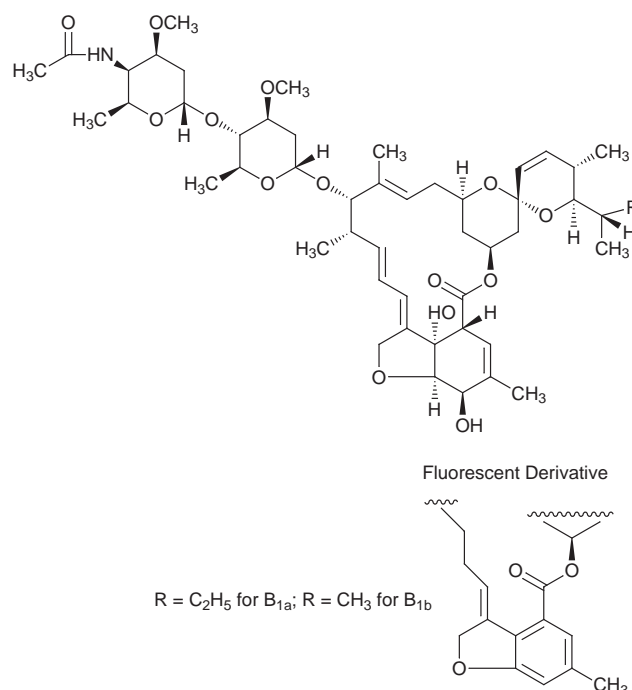

Fig. 1 Structure of eprinomectin and its fluorescent derivative.

solutions in acetonitrile (5–10 µl). The plasma samples were homogenized and 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 2000*g* for 2 min. The supernatant (≤2.2 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 run time 22.3 min). Conditioning of the cartridge (Supelclean LC18 Cartridge, 100 mg in 1 ml, Supelco): the column, positioned on the holder, was first conditioned with 5 ml of methanol and 5 ml of water (flow rate 6 ml min⁻¹). Loading of the plasma sample: a 2.2 ml volume of plasma sample (supernatant) was applied to the cartridge (flow rate 3.0 ml min⁻¹). The cartridge was washed with 2 ml of water, followed by 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 cartridge at a flow rate of 3.0 ml min⁻¹ and the eluate was collected.

Derivatization

The formation of the fluorophore of eprinomectin was achieved by using a previously described process for moxidectin.[5] The dry extract was dissolved in 100 µl of 1-*N*-methylimidazole solution in acetonitrile (1 + 1 v/v) and to initiate the derivatization 150 µl of trifluoroacetic anhydride solution in acetonitrile (1 + 2 v/v) were added. After mixing (<30 s) an aliquot (100 µl) was injected directly into the chromatographic system.

Calibration

Calibration graphs for eprinomectin in the range 0.5–50 ng ml⁻¹ were prepared using drug-free plasma. The fortified plasma samples were taken through the procedure and assayed by HPLC. Calibration graphs were constructed using the peak area as a function of analyte concentration and least-squares linear regression analysis was used to determine the slope.

The extraction efficiency of eprinomectin was measured by comparing the peak areas obtained from spiked plasma samples with the peak areas resulting from direct injection of standards carried through the extraction procedure. The inter-assay and intra-assay precision of the extraction procedure and chromatography was evaluated by processing replicates of plasma samples containing a known amount of eprinomectin.

Drug disposition study

In order to test the application of the method to *in vivo* pharmacokinetic studies, eprinomectin pour-on formulation was applied to a goat at a dose rate of 0.5 mg kg⁻¹ (Eprinex, Merial, Iselin, NJ, USA). Blood was withdrawn *via* the jugular vein at 0, 8, 12 and 24 h and 1.5, 2, 4, 6, 8, 10, 14, 20, 26 and 30 d after treatment into heparinized tubes. The plasma was separated immediately and stored at –20 °C until analysis.

Results and discussion

Because basic compounds like eprinomectin are partially or completely ionized and often retained by residual silanol groups or the silica; this dual retention could reduce the chromatographic efficiency and produce asymmetric peaks. This problem was solved by using an ion pair agent (Pic B₇) and a highly deactivated column for basic compounds (Suplex pkb100), and a good separation was obtained without any tailing peaks.

Eprinomectin contains hydroxyl groups at C-7 and C-5 (Fig. 1), as does ivermectin or moxidectin. Reaction of eprinomectin with acetic anhydride in the presence of a base catalyst (*N*-

methylimidazole) results in acetylation of the two hydroxyl groups.[6] Consequently, this acetylated derivative undergoes condensation at the C_2—C_7 and C_5—C_6 positions to form a fluorescent derivative having a six-membered aromatic ring conjugated to a butadiene unit. This conversion requires less than 30 s at 25 °C; the presence of water in the reaction tube can interfere with the derivatization reaction. The excitation and emission maxima were 355 and 465 nm, respectively.

A typical chromatogram is shown in Fig. 2A and displays excellent peak symmetry for a 5 ng standard of eprinomectin. Under the described conditions, the retention time of eprinomectin was around 10.8 min. Fig. 2B shows a chromatogram of a corresponding drug-free plasma sample. The lack of interferences in the separation suggests a high specificity for the method. Fig. 2C shows a chromatogram obtained from a kinetic plasma sample containing 7.0 ng ml⁻¹.

According to other reports[5] about related compounds the use of solid phase extraction results in a clean extract with good recovery. The extraction recovery was 76.46 ± 3.95% in the range of concentration from 0.5 to 50 ng ml⁻¹. The linearity of the analytical procedure was tested by using plasma samples spiked in a range of concentration from 0.5 to 50 ng ml⁻¹ in quintuplicate for each concentration. The linear regression obtained between peak area and analyte concentration was best described by the equation $Y = a + bx$, where x is the concentration of eprinomectin in ng ml⁻¹, b the slope, Y the area of the peak and a the intercept. The coefficient of correlation generally exceeded 0.999. The inter-assay precision of the
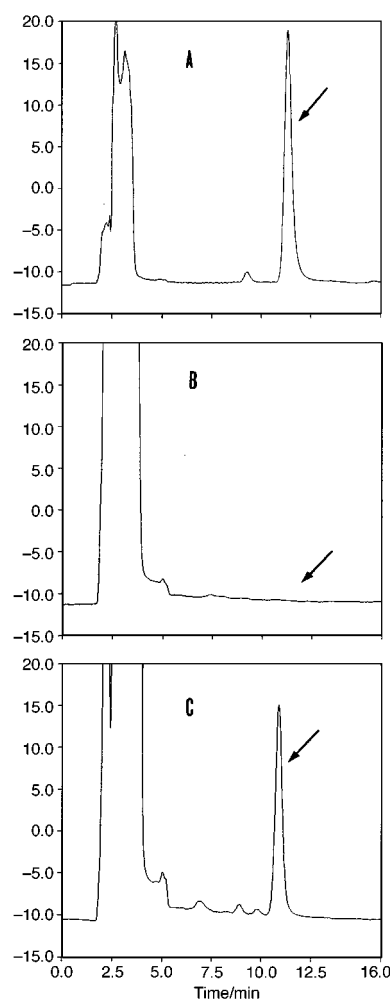

Fig. 2 Typical chromatograms: (A) standard of eprinomectin (5 ng); (B) blank plasma; (C) kinetic plasma sample containing 7 ng ml⁻¹.

method expressed as the RSD was 3.51% in the range of concentration from 0.5 to 50 ng ml^{-1} (n = 5) and the intra-assay precision was 2.87% for an intermediate concentration (5 ng ml^{-1}) with six replicates (see Table 1).

The eprinomectin plasma concentrations evaluated in a goat receiving a pour-on dose of 0.5 mg kg^{-1} is depicted in Fig. 3.

Table 1 Recovery of eprinomectin from goat plasma sample fortified with eprinomectin (n = 5) and inter-assay precision

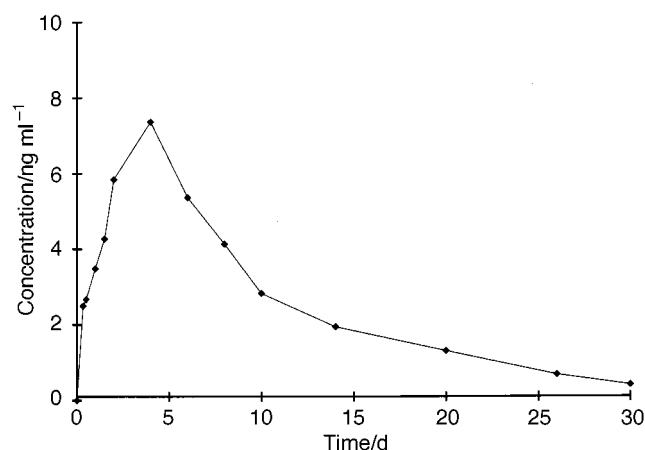| Concentration added/ng ml^{-1}	Concentration found (mean ± s)/ng ml^{-1}	Recovery (%)	RSD (%)
0.5	0.48 ± 0.01	94.4	3.2
1	1.07 ± 0.05	107.5	5.1
2.5	2.56 ± 0.02	102.5	0.8
5	4.76 ± 0.23	95.1	4.9
10	9.64 ± 0.53	96.4	5.5
25	25.16 ± 0.56	100.6	2.2
50	50.65 ± 1.39	101.3	2.7
Average		99.7% ± 4.6	3.5 ± 1.7

Fig. 3 Plasma concentration–time curve for goat treated at 0.5 mg kg^{-1} of eprinomectin (pour-on administration).

The peak plasma concentration of eprinomectin occured after 4 d with a level of 7.36 ng ml^{-1}. Thereafter the plasma concentration decreased progressively with a terminal half-life of 6.62 d.

In conclusion, a procedure, derivatization with solid phase extraction and HPLC, has been developed for the determination of eprinomectin in plasma. The automatic sample preparation procedure using the Benchmate II system gives better reproducibility, owing to the precise control of flow rates (cartridge loading, rinsing, elution) and volumes. Further, the Benchmate II performs a gravimetric control of every preparation step, which can be regarded as an extra control procedure.

The protocol described here provides a reliable method for the detection of eprinomectin in animal plasma at low concentration (0.1 ng ml^{-1}) and is currently being used a routine technique for pharmacokinetic investigations in goats and cattle.

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