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

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Abstract

Moxidectin is a newly developed potent anthelmintic agent with a high potency although present at very low concentration in cattle plasma. A method is described for the determination of moxidectin in plasma using high-performance liquid chromatography with fluorescence detection (excitation and emission wavelengths 383 and 447 nm, respectively). The fluorescent derivative was obtained by a dehydrative reaction with trifluoroacetic anhydride and N-methylimidazole. The method employs 1-ml plasma samples and has linear calibration graphs ($r = 0.997$) over the concentration range studied, i.e., 0.1–10 ng/ml. Solid-phase extraction using the Benchmate procedure was used for sample preparation. Recoveries at low concentrations (0.1–10 ng/ml) were higher than 75%. The limit of quantification was 0.1 ng/ml (C.V. 6.95%). The method is suitable for the pharmacokinetic study of moxidectin after subcutaneous administration to cows.

1. Introduction

Moxidectin is a semisynthetic derivative of nemadectin [1,2], a macrocyclic lactone produced by cultures of *Streptomyces cyanogriseus*. Moxidectin is active at very low doses (0.2 mg/kg) against a wide variety of nematode and arthropod parasites in cow [3] and sheep [4]. Moxidectin is chemically related to the milbemycins [5] and avermectins [6] (Fig. 1), which have activity against a broad spectrum of nematode and arthropod parasites of animals [7]. The molecular structure includes a fused cyclohexene–tetrahydrofuran ring, a bicyclic 6,6-membered spiroketal and a cyclohexene ring fused to the 16-membered macrocyclic ring. Moxidectin is the 23-(O-methyloxime) derivative of nemadectin and differs structurally from ivermectin in having no sugar moiety at the C-13 position and having an unsaturated side-chain at the C-25 position.

The detection of moxidectin in animal tissues using high-performance liquid chromatography with fluorescence detection has been reported [8]. The fluorescent derivative was obtained by a dehydrative reaction with acetic anhydride and pyridine as solvent and catalyst, according to the procedure previously described for ivermectin [9]. More recently, considering the complexity of the procedure, the derivatization conditions were modified to include the use of trifluoroacetic anhydride and N-methylimidazole as the catalyst in acetonitrile [10]. The formation of the fluoro-

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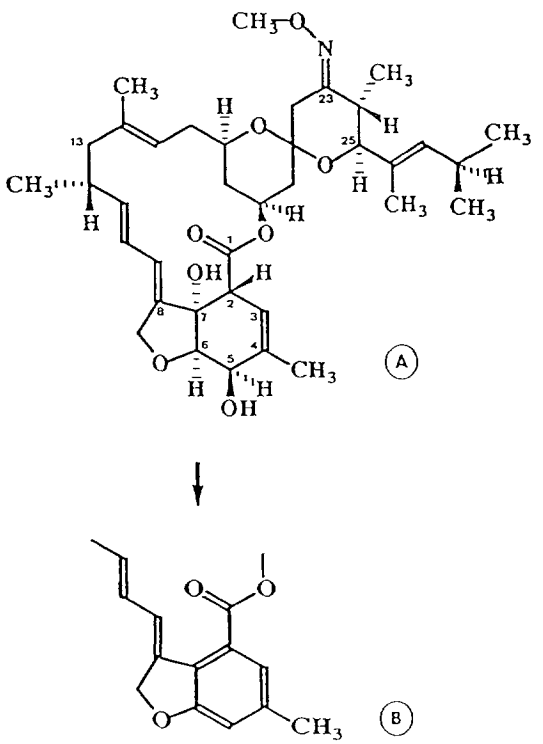

Fig. 1. Structures of (A) moxidectin and (B) its fluorescent derivative.

phore was achieved in 30 s at 25°C and resulted in a very stable derivative of ivermectin. The aim of our study was to ascertain the applicability of the method for moxidectin using the new derivatization procedure.

Considering that there is a little information available at present on the pharmacokinetics of moxidectin in cattle and sheep [11,12] and the very long half-life of the drug [8], there is a need for the evaluation of moxidectin at plasma concentrations significantly below 1 ng/ml.

2. Experimental

2.1. Reagents

Trifluoroacetic anhydride and N-methylimidazole of analytical-reagent grade were purchased from Aldrich (Sigma-Aldrich Chimie, St. Quentin Fallavier, France). Acetonitrile and methanol (HPLC grade) were obtained from Carlo Erba (Milan, Italy). Water was deionized and distilled. Moxidectin was kindly provided by Dr. Asato (American Cyanamid, Princeton, NJ, USA).

2.2. Apparatus

A constant-flow high-performance liquid chromatograph, consisting of a Model PU80 pump (Jasco, Tokyo, Japan), Model 360 automatic injector (Kontron, Paris, France) and a Model RF551 fluorescence detector (Shimadzu, Kyoto, Japan), was connected to a D2500 laboratory computing integrator (Merck, Paris, France).

2.3. Standards

The solution of moxidectin used to construct the plasma calibration graphs was prepared by serial dilutions of a stock solution (1.0 mg/ml in methanol). Both the stock solution and the serial dilution standards were stable for at least 3 months at 4°C.

2.4. Extraction

Drug-free plasma samples (1 ml) were fortified with moxidectin (0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml) using standard solutions (2–10 μl). The plasma samples were homogenized and solid-phase extraction was performed after 15 min of incubation at room temperature.

Acetonitrile (1 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 ($\leq$2.2 ml) was manually transferred into a tube which was then placed on the appropriate rack of a Benchmate II (Hopkinton, MA, USA). Automatic sample preparation was performed as follows (total run time 24.9 min).

Conditioning of the cartridge (Supelclean LC18 cartridge, 100 mg, 1 ml): the column, positioned on the holder, was first conditioned with 5.0 ml of methanol and 5.0 ml of water (flow-rate 6 ml/min). 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). 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. Before elution, the cartridge was dried with nitrogen for 10 s (flow-rate 6.0 ml/min), then, 1.2 ml of methanol was applied to the cartridge at a flow-rate of 3.0 ml/min and the eluate was collected.

2.5. Derivatization procedure

The eluate was evaporated to dryness under a gentle stream of dry nitrogen at 50°C in a water-bath. The dry residue was dissolved in 100 μl of the N-methylimidazole solution in acetronitrole. To initiate the derivatization, 150 μl trifluoroacetic anhydride solution in acetonitrile were added. After completion (<30 s), an aliquot (100 μl) of this solution was injected directly into the chromatographic system.

2.6. Chromatographic conditions

The mobile phase of acetic acid (0.2% in water) – methanol – acetonitrile (4:15:31, v/v/v) was pumped at a flow-rate of 1.5 ml/min through a Supelcosil C_{18} column with fluorescence detection at an excitation wavelength of 383 nm and an emission wavelength of 447 nm.

2.7. Calculation and quality control

Calibration graphs for moxidectin in the range 0.1–10 ng/ml were prepared using pooled cow drug-free plasma. Pooled plasma samples were taken through the procedure, and calibration graphs were constructed using the peak area as a function of analyte concentration. Least-squares linear regression analysis was used to determine the slope.

The extraction efficiency of moxidectin was measured by comparing the peak areas from spiked plasma samples with the peak areas resulting from direct injections of standards carried through the derivatization procedure. The inter-assay precision of the extraction procedure and chromatography was evaluated by processing replicate aliquots of pooled cow plasma samples containing known amounts of moxidectin on different days. The within-day precision of the assay was established by the repeated analysis of samples spiked with moxidectin (0.1, 1 and 5 ng/ml).

2.8. Drug disposition study

In order to test the application of the method to in vivo pharmacokinetic studies, moxidectin was administered subcutaneously to a cow at a dose rate of 0.2 mg/kg (Cydectin; American Cyanamid, Paris, France). Blood was withdrawn via the jugular vein at 1, 2, 3, 4, 6, 8, 10, 13, 16, 20, 26, 30, 35, 40, 44, 50, 55, 62, 68, 76, 82, 90, 100 and 110 days after injection into heparinized tubes. The plasma was separated immediately and stored at −20°C until analysis.

3. Results and discussion

3.1. Fluorogenic derivatization

Moxidectin contains hydroxyl groups at C-7 and C-5 (Fig. 1), as does ivermectin. Reaction of moxidectin with acetic anhydride in the presence of a base catalyst (N-methylimidazole) results in acetylation of the two hydroxyl groups [10]. Consequently, this acetylated derivative undergoes dehydration 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 and no sign of significant instability was observed for the fluorescent derivative in the reaction mixture during a period of 4 h. The excitation and emission maxima were 383 and 447 nm, respectively.

3.2. Chromatography

A typical chromatogram is shown in Fig. 2A and displays excellent peak symmetry for a 0.5-ng standard of moxidectin. Under the described conditions, the retention time of moxidectin was around 9.2 min. Fig. 2B shows a chromatogram of a corresponding drug-free plasma sample. The

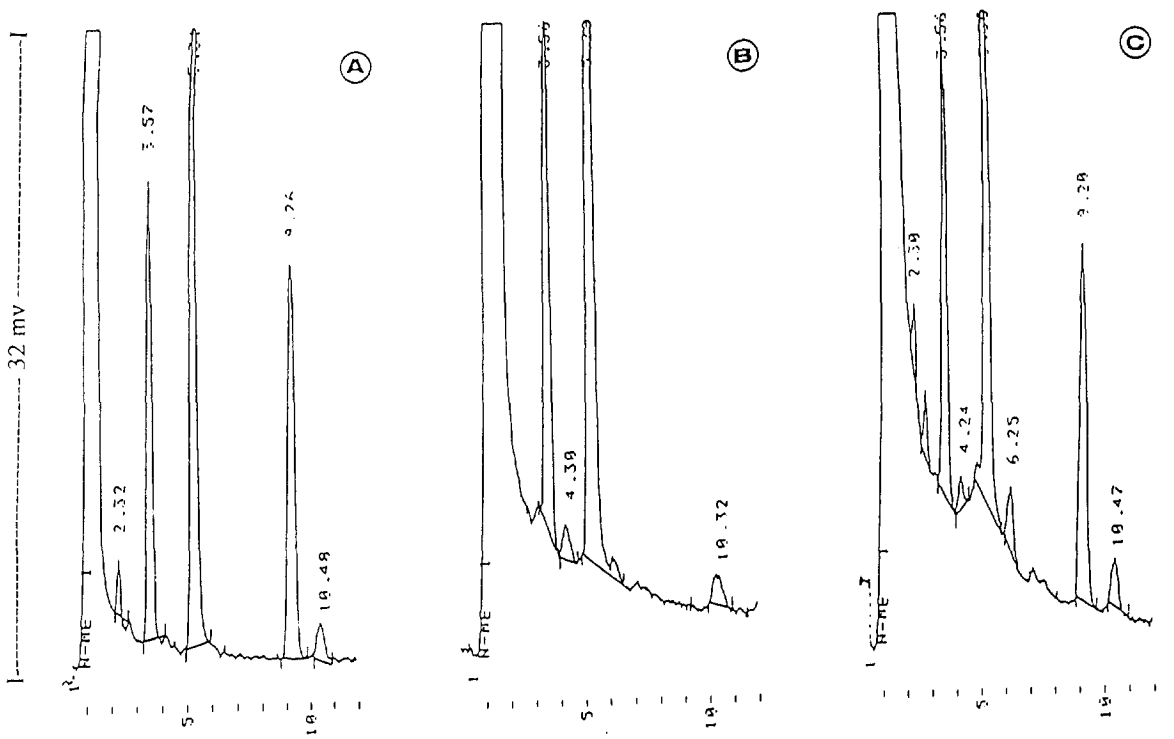

Fig. 2. Typical chromatograms: (A) standard of moxidectin (0.5 ng); (B) blank plasma; (C) plasma sample fortified with 0.5 ng/ml of moxidectin (retention time 9.2 min). Detection sensitivity high, integrator attenuation 5 (32 mV full-scale).

lack of interferences in the separation suggests a high specificity of this method. Fig. 2C depicts a chromatogram obtained from a plasma sample fortified with 0.5 ng/ml of moxidectin.

3.3. Extraction recovery

According to other reports [10,13,14], the use of solid-phase extraction for ivermectin and related compounds results in a clean extract with good recovery. The extraction recoveries were 85.5, 77.4 and 75.5% at moxidectin concentrations of 0.1, 1.0 and 10.0 ng/ml, respectively.

3.4. Linearity

The linearity of the analytical procedure was tested at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml in quintuplicate at each concentration. The linear regression obtained between peak area and analyte concentration was best described by the equation $y = ax + b$, where x is the concentration of moxidectin (ng/ml), a the slope, y the area of the peak and b the intercept. The correlation coefficient generally exceeded 0.990.

3.5. Precision

As shown in Tables 1 and 2, the reproducibility of the method, expressed by the coefficient of variation (c.v.), ranged from 5.21 to 7.72%.

3.6. Quantification limit

The limit of detection of the assay was defined as the lowest calibration standard that would have a C.V. $< 20\%$. It was found to be 0.1 ng/ml (C.V. = 6.95).

Table 1
Inter-assay precision of the method for the determination of moxidectin in plasma ($n = 5$)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Recovery (%)	R.S.D. (%)
0.20	0.207 ± 0.016	103.5	7.72
1.00	1.01 ± 0.060	101.0	5.94
2.00	2.11 ± 0.11	105.5	5.21
10.00	10.01 ± 0.63	100.1	6.29

Table 2
Within-day precision of the HPLC method for the determination of moxidectin in plasma ($n = 5$)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)
0.5	0.477 ± 0.027	5.74
1.0	0.996 ± 0.057	5.72
5.0	4.862 ± 0.376	7.7

3.7. Preliminary kinetic study

The moxidectin plasma concentration is depicted in Fig. 3. The peak plasma concentration of moxidectin in cow occurred after 1.5 days. Thereafter, the plasma concentration decreased progressively with a distribution half-life of 4.04 days and a terminal half-life of 25.4 days.

4. Conclusion

An improved derivatization procedure associated with a solid-phase extraction has been developed for the determination of moxidectin. This method allows the rapid conversion of moxidectin into a fluorescent derivative by the same mechanism as for ivermectin and is an improvement on previously proposed procedures [8].

The automatic sample preparation procedure using the Benchmate II system gives better reproducibility (see Tables 1 and 2) owing to the precise control of flow-rates (cartridge loading, rinsing, elution) and volumes. Further, the

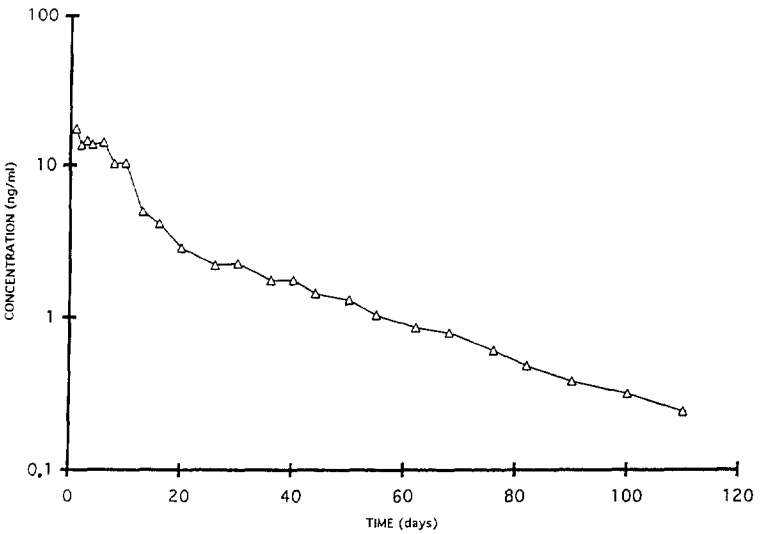

Fig. 3. Plasma concentration–time curve for a cow given a 0.2 mg/kg subcutaneous dose of moxidectin.

Benchmate II performs a gravimetric control of every preparation step, which can be reviewed as an extra control procedure.

The protocol described here provides a reliable method to detect moxidectin in animal plasma at low concentrations (0.1 ng/ml) compared with detection limit of 1.0 ng/ml for a previously described technique [8].

The method is currently being used as a routine technique for the determination of moxidectin in cattle plasma.

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