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# Improved LC method to determine ivermectin in plasma

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**Abstract**

A simple, rapid and sensitive high-performance liquid chromatographic (HPLC) method has been developed to quantify Ivermectin (IVM) in plasma using an isocratic system with fluorescence detection. The method included a fast liquid phase extraction using cold methanol. HPLC separation was carried out by reversed phase chromatography with a mobile phase composed of methanol:acetonitrile:water with 0.2% acetic acid (45:50:5 v/v/v), pumped at flow rate of 2 ml min$^{-1}$. Fluorescence detection was performed at 365 nm (excitation) and 475 nm (emission). The calibration curve for IVM was linear from 0.25 to 100 ng ml$^{-1}$. The validation method yielded good results regarding linearity, precision, accuracy, specificity and recoveries. The values of the limit of detection (LOD) and limit of quantification (LOQ) were 0.032 and 0.167 ng ml$^{-1}$, respectively.
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*Keywords:* Ivermectin; High-performance liquid chromatography; Liquid phase extraction; Fluorescence detection; Validation

## 1. Introduction

Ivermectin (IVM) is macrocyclic lactone that has been known as a potent, effective and safe antiparasitic drug for 20 years [1–3]. It is widely used as an antiparasitic agent in domestic animals and is considered the drug of choice for lymphatic filariasis and river blindness (onchocerciasis) in humans. IVM is a member of the Avermectins; this group includes natural compounds produced by fermentation of the soil-dwelling actinomycete *Streptomyces avermitilis.* IVM, a semi-synthetic derivative of avermectin $B_1$, consists of an 80:20 mixture of the equipotent homologous 22, 23 dehydro $B_{1a}$ and $B_{1b}$ [4]. The structures of IVM and Doramectin (DRM) are shown in Fig. 1.

Chromatographic detection of IVM has been performed by De Montigny et al. [5] and has been used in the determination of the drug in plasma [6,7] and other tissues [8]. In this method, the extraction protocol is based on the precipitation of protein with acetonitrile (at laboratory temperature) and solid-phase extraction.

The methodology evaluated in this article is based on the use of cold methanol for protein precipitation with the aim of avoiding the solid-phase extraction process. Thus, the purpose of this work was to elaborate a simple and sensitive

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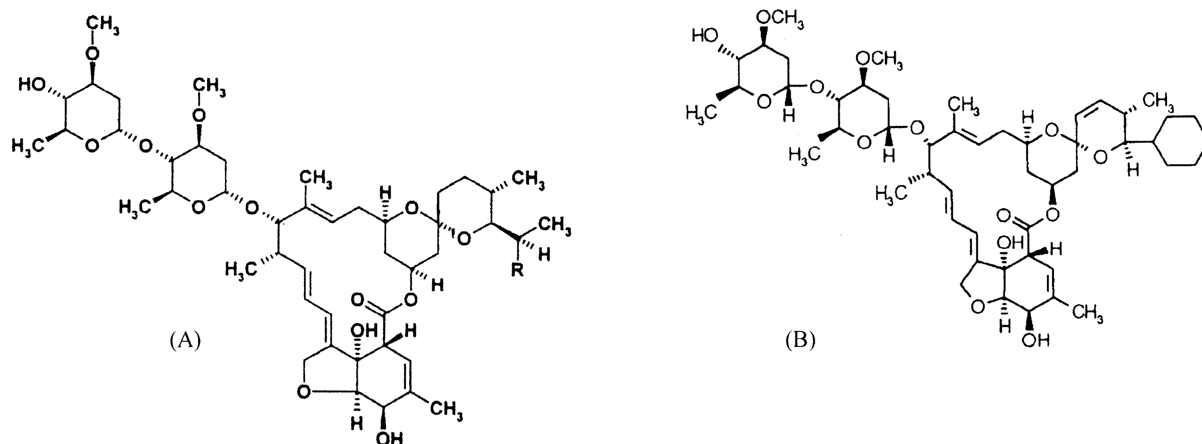


Fig. 1. Structure of IVM (A) and DRM (B).

analytical method, not involving solid-phase extraction, able to determine the concentration of IVM in plasma, using DRM as internal standard (IS) (Fig. 1) with an HPLC isocratic system, fluorescence detection and the establishment of validation parameters under the new chromatographic conditions.

## 2. Materials and methods

Determination of IVM (purchased from SIGMA S.A., Madrid, Spain) was accomplished using DRM as IS (obtained from Pfizer S.A. Madrid, Spain), the regaents for derivatization, obtained from SIGMA Chemical Co, were of analytic grade. The solvents for the mobile phase were HPLC grade and were purchased from MERCK S.A. (Barcelona, Spain). The method included chromatographic measurements that were carried out with a Waters HPLC system, equipped with Waters 600 controller pumps, a Waters™ 474 scanning fluorescence detector, and a Waters 717 plus autosampler. Chromatography data acquisition was carried out using the MILLENIUM[32] software package (Waters Chromatography S.A., Madrid, Spain). Separation was performed at 35 °C on a LiChrospher 100 RP 18 column (125 × 4 mm, 5 μm particle size) by reversed phase chromatography [9–11]. The components of the mobile phase were the same as those used in other methodologies [6,12,13], but the percentages were modified to a mixture of methanol:acetonitrile:water with 0.2% acetic acid, (45:50:5 v/v/v). The solution was degassed with helium and pumped isocratically at a flow rate of 2 ml $min^{-1}$. Spectrofluorimetric detection was carried out at 365 nm (excitation) and 475 nm (emission), in agreement with previous works [8,14,15].

### 2.1. Sample preparation procedure

A first standard solution of IVM was prepared by dissolving an appropriate amount of the substance in methanol. Fifty microliters of the standard solutions were added to 950 μl of control bovine plasma to obtain a concentration range of 0.25 and 100 ng $ml^{-1}$.

The final solution of IS (0.1 μg $ml^{-1}$) used in the extraction process was prepared from a stock solution: DRM in dimethylsulfoxide (DMSO), at 1 mg $ml^{-1}$, adding a mixture of DMSO:$H_2O$ (1:1 v/v).

The extraction of IVM from plasma was begun by adding 100 μl of plasma and 16 μl of IS to a 1.5 ml reaction tube. The mixture was vortexed vigorously for 30 s, and 500 μl of methanol at −30 °C were added. This method for protein precipitation with an organic solvent was developed by Hames, 1981, cited in [16]. Extraction was carried out over 30 s by shaking the reaction tube

and incubating at −30 °C for 10 min. The organic layer was separated by centrifugation at 16 000 × *g* for 12 min at 4 °C and the organic phase was transferred to an Eppendorf reaction tube and evaporated at 45–50 °C under a nitrogen atmosphere. The dry residue was resuspended in 100 μl of a mixture of *N*-methylimidazole solution in acetonitrile (1:1, v/v) and subsequently 150 μl of a solution of trifluoroacetic anhydride in acetonitrile (1:2, v/v) was added [7,17]. After completing the reaction by vortexing, 100 μl of this solution was injected into the HPLC system.

### 2.2. Validation method

The method was subjected to a validation procedure. Linear regression analysis using a least-squares fit was performed. The calibration curve was obtained from standard plasma samples with the following ten concentrations: 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20, 50, 75 and 100 ng ml$^{-1}$. Five injections of each concentration were used daily for the calibration curve during five different days ($n = 250$).

Determination of Limits of detection (LOD) and quantification (LOQ) was performed according the method described by Quattrocchi [18] where:

$$\mathrm{LOD} = \frac{Y_{\mathrm{bl}} + 3\mathrm{S.D.}_{\cdot\mathrm{bl}}}{b} \times 100$$

$$\mathrm{LOQ} = \frac{Y_{\mathrm{bl}} + 10\mathrm{S.D.}_{\cdot\mathrm{bl}}}{b} \times 100$$

$Y_{\mathrm{bl}}$ is the blank response, calculated as the intercept of the linear regression curve (concentration vs. ratio) constructed with three different concentrations lower than the lowest concentration of the calibration curve (0.10, 0.15 and 0.20 ng ml$^{-1}$) with three replicate samples of each one.

$\mathrm{S.D.}_{\cdot\mathrm{bl}}$ is the S.D. of the blank response, calculated as the intercept of the curve: concentration (0.10, 0.15 and 0.20 ng ml$^{-1}$) versus S.D., $b$ is the slope of the calibration curve (concentration vs. ratio).

The other validation parameters were: Precision (as repeatability and reproducibility from calibration curve). Repeatability was determined by the analysis of five samples within a single run and Reproducibility was determined by analysing samples on five different days by different analysts to evaluate the run-to-run variation in the method. Accuracy was determined with the data from the repeatability assays (intra-assay variation). The extraction Recovery of IVM from plasma was determined at low (2.5 ng ml$^{-1}$), medium (10 ng ml$^{-1}$) and high (50 ng ml$^{-1}$) concentrations by comparing the ratios of standards in bovine plasma after extraction (standard samples) with the ratios of standard spiked samples (standards in methanol, considered as 100% recovery).

The specificity of the method was determined by comparing the chromatograms of drug-free bovine plasma samples with the chromatograms of plasma with IVM, plasma with DRM, and plasma with IVM and DRM. Additionally, we studied the effect of freezing-thawing cycles on the stability of IVM, since the samples were frozen and thawed at different times. This study was performed with three replicate samples at three control concentrations (1, 10 and 75 ng ml$^{-1}$). Relative standard deviation (R.S.D.) values were calculated to measure the stability of IVM, according to the expression:

$$\mathrm{R.S.D.} = \frac{\text{Standard deviations (S.D.)}}{\text{Mean value}} \times 100$$

## 3. Results and discussion

IVM was seen to remain stable after several freezing and thawing procedures. The RSD values obtained for each concentration level (1, 10, 75 ng ml$^{-1}$) were 0.107, 0.015, and 0.021%, respectively. These results show that freezing and thawing processes have no effect on the determination of IVM in plasma.

### 3.1. Validation method

The validation method used here revealed that analytical method used to extract, derivatize and

Table 1
Precision of the HPLC method for IVM determination, measured as repeatability and reproducibility

| Concentration of IVM (ng $ml^{-1}$) | Repeatability (intra-assay) | | Reproducibility (inter-assay and inter-analyst) | |
|---|---|---|---|---|
| | Mean ratio ± S.D. ($n = 5$) | R.S.D. (%) | Mean ratio ± S.D. ($n = 25$) | R.S.D. (%) |
| 0.25 | 0.0253 ± 0.00078 | 3.07 | 0.0249 ± 0.0010 | 3.86 |
| 0.5 | 0.0489 ± 0.00294 | 5.99 | 0.0495 ± 0.0035 | 7.09 |
| 1.0 | 0.0990 ± 0.00528 | 5.87 | 0.1000 ± 0.0059 | 5.91 |
| 2.5 | 0.2482 ± 0.01053 | 4.24 | 0.2500 ± 0.0063 | 2.51 |
| 5.0 | 0.4835 ± 0.01183 | 2.44 | 0.4838 ± 0.0225 | 4.65 |
| 10 | 0.9679 ± 0.02822 | 2.91 | 1.0039 ± 0.0654 | 6.51 |
| 20 | 1.9323 ± 0.04239 | 2.19 | 2.0044 ± 0.0951 | 4.74 |
| 50 | 4.9651 ± 0.11006 | 2.21 | 4.9991 ± 0.2349 | 4.69 |
| 75 | 7.5292 ± 0.13396 | 1.77 | 7.4928 ± 0.1998 | 2.66 |
| 100 | 10.0830 ± 0.13205 | 1.31 | 9.8762 ± 0.3198 | 3.24 |

Table 2
Accuracy of HPLC method for IVM determination from 0.25 to 100 ng $ml^{-1}$ ($n = 5$)

| Concentration of IVM (ng $ml^{-1}$) | Measured concentration (ng $ml^{-1}$) | Accuracy (%) | R.S.D. (%) |
|---|---|---|---|
| 0.25 | 0.2803 | 112.11 | 3.37 |
| 0.5 | 0.5263 | 105.25 | 6.69 |
| 1.0 | 1.0116 | 101.62 | 7.51 |
| 2.5 | 2.5411 | 101.64 | 2.46 |
| 5.0 | 4.9446 | 98.89 | 6.22 |
| 10 | 9.9694 | 99.69 | 5.39 |
| 20 | 19.7573 | 98.78 | 2.20 |
| 50 | 49.7271 | 99.45 | 2.00 |
| 75 | 76.1164 | 101.48 | 1.00 |
| 100 | 99.3056 | 99.30 | 2.58 |

quantify IVM in plasma by chromatographic analysis using a fluorescence detector is correct. The calibration curve for IVM was constructed by plotting the concentration versus area ratio (IVM area, DRM area) and disclosed a functional linear relationship between the concentration of analyte and the area ratio.

The linear regression equation obtained for the proposed HPLC method as calibration curve was $Y = (0.0993 \pm 0.0022)X + (0.006 \pm 0.0194)$ ($r = 0.999$, $n = 250$), where $Y$ is the area ratio and $X$ is the analyte concentration in ng $ml^{-1}$.

The LOD is defined as the lowest analyte concentration in a sample that can be detected, but not necessarily quantified, by the method of analysis. The LOD of IVM was 0.033 ng $ml^{-1}$. The LOQ is defined as the lowest analyte concentration in sample that can be determined under the stated operational conditions of the method. The method was sensitive enough to quantify 0.167 ng $ml^{-1}$ of IVM. Our results are in agreement with the data reported by other authors [19–21].

Precision is the degree of repeatability and reproducibility of an analytical method and is usually expressed as the percent R.S.D. for a statistically significant number of samples. The R.S.D. values of the repeatability and reproducibility assays for the different concentrations used are shown in Table 1. The R.S.D. in the repeatability assay (intra-day variation) was less than 6%

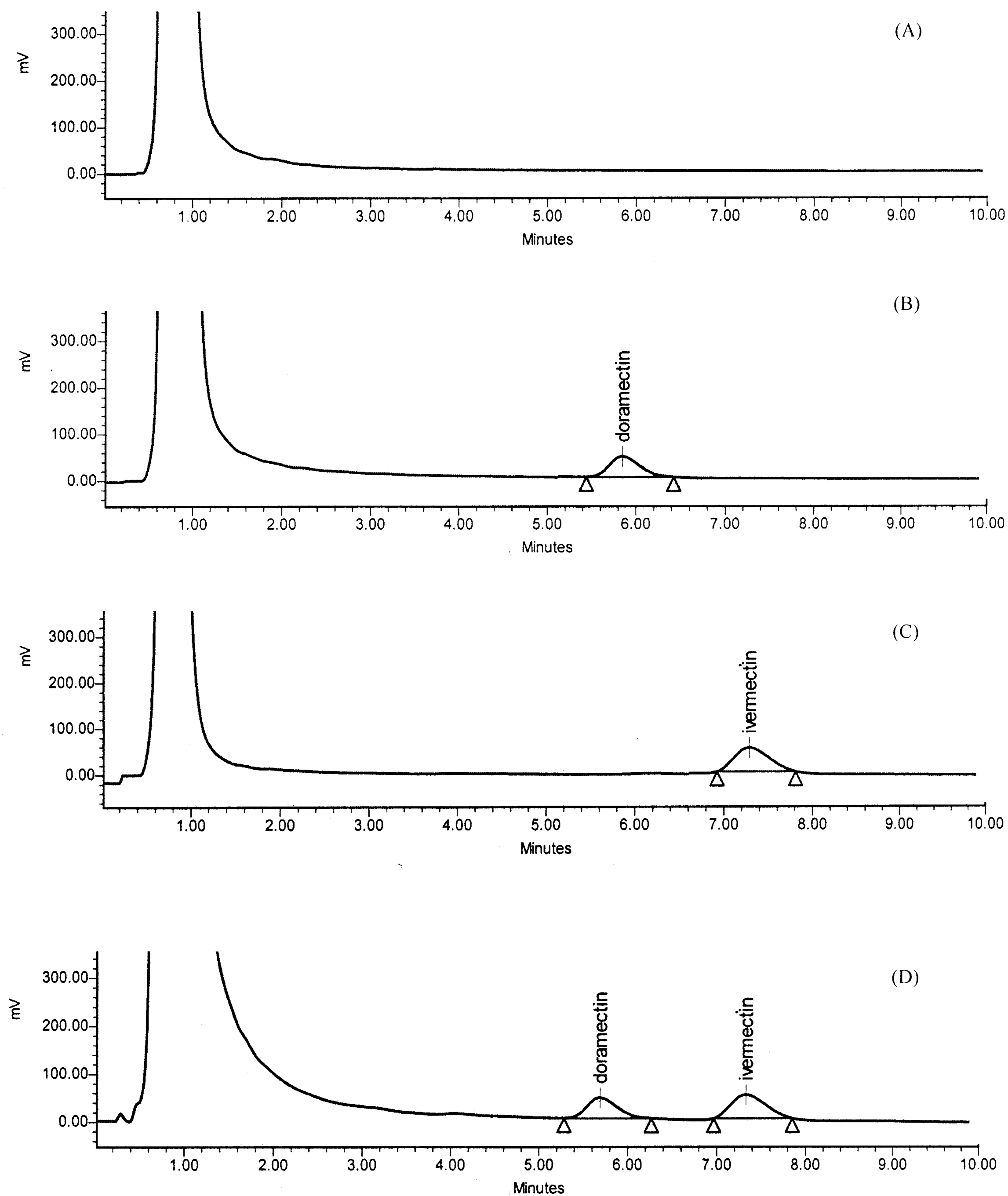


Fig. 2. Representative chromatograms of IVM and Doramectin as Internal Standard (DRM) in bovine plasma (1 ml). (A) Control plasma blank; (B) plasma sample spiked with IVM; (C) control plasma spiked with DRM; (D) plasma sample spiked with DRM and IVM.

and for the reproducibility assay (inter-day and inter-analyst variation) was less than 7.1% in all the concentrations.

The accuracy of the method is the closeness of the agreement between the theoretical value (which is accepted either as a conventionally true or an accepted reference value) and the value found. The percentage of variation between the true value and the value found of IVM ranged between 98.78 and 112.11%. The R.S.D. values were $<10\%$ for all cases, implying that the method is accurate. The results are shown in Table 2.

Recovery is measured as the analyte recovery percentage in the extraction procedure. The percentage of IVM after extraction from plasma was 78.1; 80.2 and 92.8% in 2.5; 10 and 50 ng ml$^{-1}$ samples.

Specificity, according to United States Pharmacopeia (USP), is the ability to measure accurately and specifically the analyte of interest in the presence of the other components that might be expected to be present in the sample matrix. The chromatograms represented in Fig. 2 obtained by fluorescence detection showed the high specificity of this method.

The mean retention time of IVM was $7.6 \pm 1$ min. The drug peak did not show any interference due to any of the components of plasma, including the IS (mean retention time: $5.5 \pm 1$ min). The chromatograms in the Fig. 2 clearly show that the method is sufficiently specific to quantify IVM in plasma using DRM as IS. For the method developed by Asbakk et al. [22], the authors report a retention time for IVM of 7.3 min and for Abamectin, used as IS, of 4.9 min. These values, obtained with similar chromatographic conditions to ours, are in agreement with the results observed here, but that method included three previous extractions with organic solvents. Harrison et al. [23] studied the chromatographic detection of DRM using IVM ($B_{1a}$) as IS and reported retention times of 12 and 14 min, respectively, using solid-phase extraction and a mobile phase composed by a mixture of acetonitrile, water and tetrahydrofuran.

The results reported here showed that the method used for the chromatographic detection of IVM is good, precise, accurate and specific, permitting—with an autosampler—30–40 analyses per day. Moreover, very low concentrations can be quantified (0.167 ng ml$^{-1}$). The sample preparation and extraction procedure is simple and fast. The method developed simplifies extraction since it only requires a single liquid–liquid procedure using a minimum number of steps.

## 4. Conclusion

Our validated chromatographic method to quantified IVM in plasma samples is based in using methanol at low temperature for protein precipitation and extraction of the drug.

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