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Development of HPLC-Fluorescence Method for the Determination of Ivermectin Residues in Commercial Milk

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Abstract

Objective: A study aimed at the validation of a high performance liquid chromatography (HPLC) method for detection and quantification of ivermectin (IVM) in commercial milk.

Method: After a liquid-liquid extraction with acetonitrile and hexane, a derivatization with a mixture of trifluoroacetic acid and trifluoroacetic anhydride was performed. Chromatography conditions were a C8 column, a fluorescent detector (Ex. 360; Em. 470 nm), a run time of 25 minutes and an isocratic elution at 30°C. The methodology was validated in terms of selectivity, linearity, precision, recovery, limit of quantification (LoQ), limit of detection (LoD), and robustness.

Results: The method showed an adequate linearity and selectivity (r^2=0.999) with an elution time of 13.909 minutes. However, the repeatability and intermediate precision showed RSD values above of 20% and recovery between 37 to 79%. The calculated LoD and LoQ were 2.50 and 5.00 ng/ml respectively. Robustness showed a significant variation on the analytical method with small changes in heating time during the derivatization and flow rate of chromatography system.

Conclusion: The HPLC-fluorescence method showed results partially satisfactory and could be the used for IVM detection in commercial milk samples.

Keywords: Chromatography; Fluorescence; Ivermectin; Macrocyclic lactones; Milk; Validation

Introduction

Ivermectin (IVM) is a macrocyclic lactone, , a class of anti-parasitic drugs derived from the soil microorganism *Streptomyces avermitilis*, used against endectoparasites, especially in bovine, swine and equine production [1]. It was discovered in 1976 but the use related to control of endo-and ectoparasites in animal started only in 1981 [2,3].

The IVM molecule has high liposolubility, this fact is important because their excretion could be by milk [4]. It is recommend a carefull administration and respect of the withdrawal period to avoid IVM residues in milk and milk products. Some countries developed monitoring programs in order to detect the presence of IVM residues in foods of animal origin. In that way, legislation in the United State and Brazil established a tolerance of 2 and 10 ng/ml respectively [5,6]. On the other hand, the European Union determined a zero tolerance for IVM residues, prohibiting its use on animals for human consumption [7].

Although, the IVM concentrations found in milk are very low and aren't related to a serious harm to consumers [8], their presence is important for public health since this product is a food for massive consumption all over the world. For these reason, different methods using chromatography have been described for the analysis of IVM. Shurbaji et al. [9] developed a precise, accurate, and sensitive chromatography method coupled to ultra violet detector (UV) to identify simultaneously triclabendazole and IVM in a pharmaceutical formulation. However, UV did not provide the specificity required because it showed limitations at detection of low levels of IVM in plasma, tissue and milk [10]. Mass spectrometry coupled with liquid chromatography (LC/MS) has also been used for determination of IVM residues in biological matrices for purely confirmatory purposes due to the high cost of equipment [10]. Fluorescence is the most commonly applied because it is a method with low cost and good sensibility [11] being used not only milk, but butter [12], yogurt [13] and cheese [14].

Validation is performed to demonstrate that an analytical methodology is suitable for a determined purpose [12]. Although some working groups developed and validated similar methods, it is preferable that each laboratory does the validation before their implementation because slight variations could affect the final results [15]. The objective of this work was determined if HPLC-fluorescence method is suitable to detect and quantify residues of IVM in commercial milk samples.

Material and Methods

Standard preparation

Stock standard solution of IVM (Sigma-Aldrich, SP, Brazil) was prepared by dissolving 10.0 mg of IVM in 10.0 ml of acetonitrile HPLC- grade (Tedia, RJ, Brazil) resulting in a solution of 1.0 mg/ml. For the preparation of the working standard, an aliquot of 10 μl from the stock solution was taken and added to 9.9 mL of acetonitrile, producing a working standard solution with the concentration of 1000 ng/ml. The validation was performed with different concentrations (640.0; 320.0; 160.0; 80.0; 40.0; 20.0; 10.0; 5.0 and 2.5 ng/ml) using acetonitrile as the solvent. All the standard solutions were stored in a freezer at -20°C until the chromatography injection.

Fortification of commercial milk samples

The fortification step was conducted by spiking individual samples, adding a specific aliquot from the working standard solution (1000 ng/ml) to obtain the same exact concentrations described previously.

Sample preparation

A modified protocol proposed by Gianetti et al. was developed employing a liquid-liquid extraction. In brief, 4 mL of fortified milk and 4 mL of acetonitrile (ACN) were mixed into 15 ml polypropylene conical tubes (Kasvi, São Paulo, Brazil) and vortexed (Certomat® MV, B. Braun Biotech International, Melsungen, Germany) for 10 s. Then, the mixture was submitted to ultrasonic bath (Cleaner USC 2800 A, São Paulo, Brazil) for 10 min. Afterwards the solution was placed on a shaker table (Biomixer, model TS-2000A VDRL, USA) for 10 min. Later on, the tubes were centrifuged (Sorvall ST16R, Thermo Scientific, Germany) at 1,465 g for 10 min at 25°C. The supernatant was separated into clean 15 ml polypropylene conical tubes and the residue was re-extracted following the same steps previously described. After the second centrifugation, the supernatant was mixed with the one previously separated and vortexed for about 10 s. From this mixture, an aliquot of 10 ml was extracted and mixed with 2 ml of hexane (Tedia, RJ, Brazil) followed by vortexing for 15-20 s. After that, the solution was divided in two phases and 1 ml was carefully extracted from the superior phase, avoiding the inferior phase, and placed in glass vials (Tedia, RJ, Brazil). Evaporation was achieved by placing the vials under gentle nitrogen (N2) stream (Sample Concentrator Techne®, Cambridge, UK) at room temperature.

The derivatization step was performed following the method proposed by Danaher et al. (2006). The dried extract was recovered with 100 μl of acetonitrile:1-methylimidazole (1:1), 50 μl of trifluoroacetic acid (Sigma-Aldrich, SP, Brazil), 150 μL of acetonitrile:Trifluoroacetic anhydride 99% (2:1), 50 μl of trifluoroacetic acid and 1000 μl of acetonitrile. The vials were heated at 70°C for 30 min, followed by 10 minutes at room temperature for cooling down and injected in the HPLC system.

Chromatographic conditions

The chromatographic system consisted of a Prominence UFLC apparatus (Shimadzu, Kyoto, Japan) equipped with a DGU-20A5 degasser, a SIL-20AC auto sampler, a LC-20AD quaternary pump, a CTO-20A column oven, a RF-20Axs fluorescence detector, and a CBM-20A communication bus module. IVM separations were performed on a Waters SunFire C8 column (150 × 4.6 mm, 5 μm,

Waters, USA) connected to a guard column (12.5 × 4.6 mm, 5 μm). The mobile phase was a mixture of ACN (A) and Milli-Q water (B). The flow rate was 1.2 ml/min constructed for the identification of the IVM peak. The gradient elution was set up as followed: starting at 88% A, applying a linear gradient to 97% A for 8 min and kept at 97% from 8-15 min. From there a linear gradient to 100% A until 25 min and afterwards, returning to a gradient of 88% A. The total run time was 25 min. After that, an isocratic elution of 10 min to 88% A at 1.2 ml/min was performed to clean and stabilize the chromatographic system. The column temperature was 30°C and the injection volume was 20 μl. The excitation and emission wavelengths were 365 nm and 470 nm respectively. The solvents were degassed in an ultrasonic bath prior to use. IVM residues were identified by their specific retention time and quantified by peak area using external standards.

Validation parameters

The method for the identification of IVM in milk was validated in terms of the analytical parameters of linearity, selectivity, accuracy, recovery, limit of quantification (LoQ), limit of detection (LoD), and robustness following conventional protocols from international guidelines [16,17]. In brief, linearity was determined using seven concentrations of IVM standard (2.5, 5, 10, 20, 40, 80 and 160 ng/ml) injected three times into the chromatography system. Linear calibration curve was constructed, and both regression equation and the regression coefficient ($r2$) were calculated. Selectivity was performed by injecting different concentrations (10, 20, 40 and 160 ng/ml) of IVM standard and comparing with a solution of milk spiked with the IVM standard in the same concentration previously specified.

Accuracy was considered at two levels: (1) repeatability: it was established with two different concentrations of IVM standard (5 and 20 ng/ml), injected three times and expressed as the mean, standard deviation (SD), and relative standard deviation (RSD); and (2) intermediate precision, performed with the same concentrations injected three times. This procedure was repeated on two consecutive days and expressed in the same way for repeatability.

Recovery was evaluated by analyzing milk samples spiked with four different concentrations of IVM standard (10, 20, 40 and 160 ng/ml). The following equation, $R=[(C-A)/B]\times100$, was used with milk samples (A), standard of IVM (B), and milk samples fortified with IVM standard (C), prepared and injected in triplicate. LoD and LoQ were determined from baseline noise. For this purpose, smaller concentrations were injected in a decreasing sequence until the chromatographic signal reached an area that could be visually differentiated from the signal noise (baseline) at the lowest attenuation. When this area was identified, three injections of each solution were performed for confirmation. Finally, robustness was performed applying small variations in the heating time of derivatization step (20, 30 and 40 minutes) and the flow rate (1, 1.2 and 1.3 ml/minutes) used in the chromatographic system.

Statistical analysis

Data collected in this study were analyzed using GraphPad Prism® 5.00 package [18] for windows by one-way ANOVA, and the means were compared with Tukey test (P<0.05).

Results and Discussion

The linearity (Figure 1) showed a regression equation (y=23.093x-31.868) and regression coefficient ($r2$=0.999) which are

compatible with an optimal setting. The construction of the calibration curve was possible with seven levels of IVM standards, which showed a best-fit linear regression model. According the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) recommends at least five concentration levels for the construction of calibration curves [17]. This result is consistent with Brazilian legislation, which considers 0.99 and 0.90 excellent r^2 values (INMETRO 2011; ANVISA 2003). Other researchers show an optimal data adjustment for the regression coefficient. Lobato et al. [1] showed an r^2 value of 0.9991 for IVM in commercial milk using six levels of IVM standards. Same linearity was reported by Souza et al. [15] who found r^2 values between 0.98 and 0.99 in milk samples spiked with IVM.
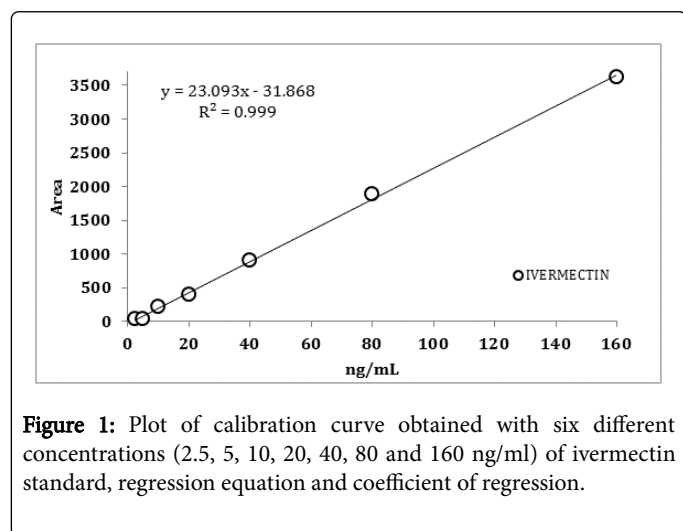

Figure 1: Plot of calibration curve obtained with six different concentrations (2.5, 5, 10, 20, 40, 80 and 160 ng/ml) of ivermectin standard, regression equation and coefficient of regression.

Curves of IVM standard and milk samples spiked with the IVM standard were compared (Figure 2). The curve of IVM showed a perfect match with all concentration (r^2=1). However, milk samples spiked with the IVM standard did not show the same trend (r^2=0.9841), this result suggest slight interference of the matrix in the chromatography method. In fact the components present in the extracts of food samples may affect the performance of chromatography method, increasing or decreasing the signal detector [19].

The chromatogram analysis for IVM standard, milk sample spiked with IVM standard and milk samples (Figure 3) suggested that separation of IVM from other components in the milk sample was effective. In addition, the mean retention time (minutes) was defined in 13.909 for the present method. This fact can be explained by the separation ability of the C8 column (150 × 4.6 mm, 5 μm). Lobato et al. [1] established a retention time of 11.5 min for IVM using a C18 column and fluorescence detector. Errors of determination in samples can be considered a problem of analyte detection or low efficiency in the extraction. The selectivity of this method was confirmed for the absence of interfering peaks coeluting with the analytes in blank chromatograms of different unspiked samples.
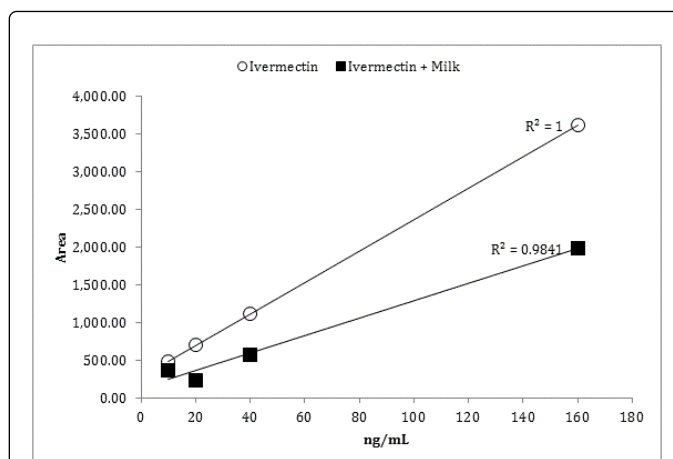
Figure 2: Effect of matrix: Comparison of calibration curves of IVM standard solution (10, 20, 40 and 160 ng/ml) and commercial milk samples spiked with IVM (same concentrations).
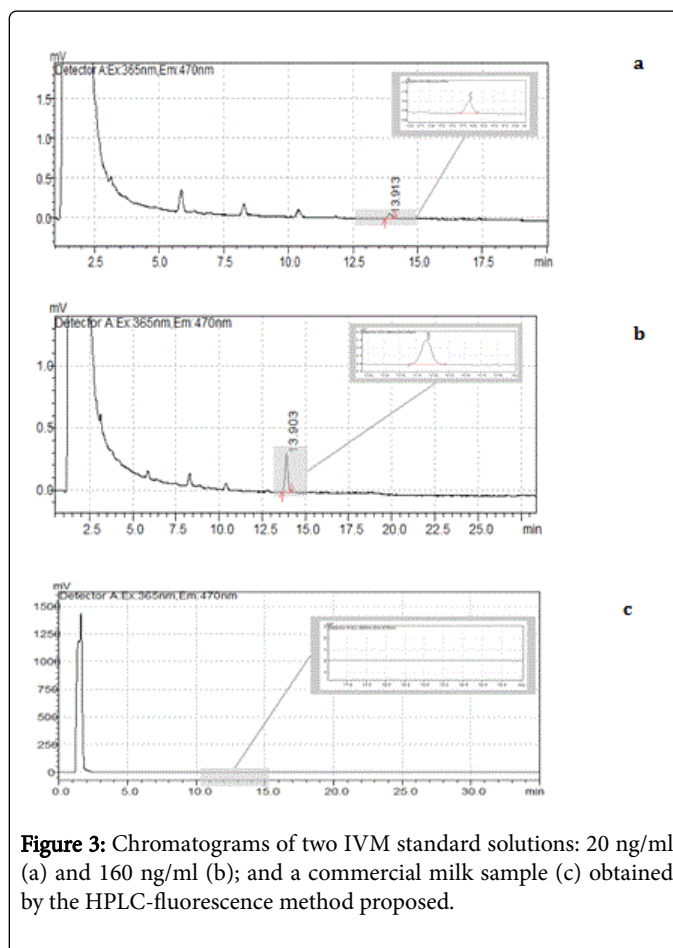
Figure 3: Chromatograms of two IVM standard solutions: 20 ng/ml (a) and 160 ng/ml (b); and a commercial milk sample (c) obtained by the HPLC-fluorescence method proposed.

Results of accuracy represent by repeatability and intermediate precisions were showed in table 1. For repeatability, RDS values were between 20 and 29%, these values resulted not satisfactory because the literature determined that an RSD equal to or less than 5% is an acceptable value for bioanalytical methods [20], and up to 20% can be considered in methods for quantifying trace elements [21].

Standard concentration of Ivermectin (ng/ml)	Repeatability		Intermediate precision			
			Day 1		Day 2	
	Mean ± SD	RDS	Mean ± SD	RDS	Mean ± SD	RDS
5	6.52 ± 1.91 a	29.2	8.63 ± 2.85 a	33.06	9.88 ± 1.83 a	18.55
20	10.52 ± 2.16 a	20.52	21.00 ± 15.43 a	73.5	24.00 ± 10.60 a	44.16

Table 1: Results of accuracy (Repeatability and Intermediate precision) for the chromatographic method evaluated with two concentration of IVM standard. Different letters in column for repeatability and in rows for intermediate precision represent significantly different averages (P ≤ 0.05). SD=standard deviation; RSD=relative standard deviation.

Values of recovery showed a high variability (79, 37, 52 and 55%) according the IVM standard concentrations (10, 20, 40 and 160 ng/ml) respectively. These variations may be related to changes in the methodology such as filters with smaller diameter pores, derivatization substances, different concentrations, gradient systems, and other factors. Pérez et al. [8] determined values of recovery ranged in 73 to 79% using solid phase extraction (SPE cartridges).

Limit of detection (LoD) and quantification (LoQ) were determined by the injection of serial dilutions from the lowest concentration used in the calibration curve and were set at 2.50 and 5.00 ng/ml respectively. These values are slightly higher than Lobato et al. [1], Berendsen et al. [11] and Pérez et al. [8] who found values of 0.6 ng/ml (LoD) and 2 ng/ml (LoQ), 0.1ng/ml (LoD), and 0.042 ng/ml (LoD) respectively in chromatography methods with fluorescence detector for milk samples. Berendsen et al. [11] suggested that fluorescence detection is superior to LC-MS/MS detection with respect to the LoD and LoQ. Although, the use of fluorescence detection requires a derivative agent to generate fluorescence, it provided a greater sensitivity and selectivity than UV detection [10].

Parameter	Standard concentration of Ivermectin (ng/ml)			
	5	10	20	40
Heating time of derivatization (min)				
30	6.52 ± 1.91 a	8.04 ± 1.01 b	10.52 ± 2.16 c	21.43 ± 4.76 c
20	9.72 ± 0.33 a	16.82 ± 3.28 a	32.12 ± 0.94 a	61.35 ± 1.43 a
40	6.89 ± 0.09 a	12.70 ± 1.09 a	22.06 ± 0.06 b	55.23 ± 0.97 b
Flow rate (ml/min)				
1.2	13.62 ± 0.02 a	13.58 ± 0.01 a	13.60 ± 0.01 a	13.59 ± 0.01 a
1	13.40 ± 0.12 a	13.32 ± 0.03 a	13.34 ± 0.02 a	13.33 ± 0.01 a
1.3	11.33 ± 0.02 b	11.30 ± 0.01 b	11.32 ± 0.01 b	11.32 ± 0.01 b

Table 2: Results of robustness for variations in heating time of derivatization and flow rate. Results expressed as mean ± SD. Different letters in columns represent significantly different averages (P ≤ 0.05). Normal parameters used in the proposed method: heating time of derivatization=30 min, and flow rate=1.2 ml/min.

The results of robustness are shown in table 2. Changes in heating time of derivatization did not modify results for the lowest IVM concentration. However, the rest of concentrations showed a significant difference (P ≤ 0.05), with superior values compared with the temperature standard (30 min). Another parameter evaluated was flow rate. This variable did not showed differences between 1.0 and 1.2 ml/min, but when the flow rate increases to 1.3 ml/min, the values showed significant differences (P ≤ 0.05).

Conclusion

The results suggest the applicability of the HPLC-Fluorescence method to identify IVM residues in milk samples and are according with the maximum residue limit of ivermectin (10 ng/ml) established by the Brazilian Ministry of Agriculture. However, some procedures must be reevaluated and/or changed for improvement of the method. It is suggested further testing to define the optimal validation of this methodology.

Acknowledgements

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