Spectrochimica Acta Part A 100 (2013) 127–130

Contents lists available at SciVerse ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

An alternative derivatization reaction to the determination of doramectin in bovine milk using spectrofluorimetry

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ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 17 February 2012
Accepted 22 February 2012

Keywords:
Doramectin
Risk to human health
Derivatization reaction
Spectrofluorimetry
Determination in milk

ABSTRACT

The doramectin (DOR), which belongs to the avermectins group (AVM), has a high antiparasitic activity and so it has been widely used in food-producing animals. The DOR shows low fluorescence quantum efficiency and as a consequence, chemical derivatization reactions are necessary to produce derivatives with improved luminescent properties before its determination by fluorimetry. As the presence of this compound in food represents a risk to human health, an easy, clean and low cost derivatization reaction, which is alternative to those usually employed and that enables its spectrofluorimetric determination in milk samples, was developed. Ethanolic solutions of DOR, containing sodium hydroxide at a final concentration of 0.25 mol L^{-1}, after 60 min of heating at 50 °C, produced fluorescent signals 1000 times higher than the original ethanolic solution. Using these optimized conditions, a linear response range that extended from 50.00 to 1000 μg L^{-1}, with a value of (R^2) equal to 0.9970, was obtained. Average recovery of DOR was 92.5 ± 1.5% (n = 3) in bovine milk fortified samples submitted to a liquid–liquid extraction at low temperature and pre concentration process, indicating the usefulness and effectiveness of the proposed method. The proposed spectrofluorimetric method is an alternative to high-performance liquid chromatography (HPLC) based methods, allowing rapid and simple detection of doramectin in milk samples.

1. Introduction

Avermectins (AVM) are a series of 16-membered macrocyclic lactones produced by *Streptomyces avermitilis* with potent anthelmintic and insecticidal activity [1]. They are effective agricultural pesticides and antiparasitic agents, which are widely employed in the agricultural, veterinary, and medical fields. Avermectins are composed of structurally related compounds, and the doramectin (DOR) is a representative compound of this group (Fig. 1) [2].

The DOR is an endectocide compound with exceptional potency and a broad antiparasitic spectrum of activity against nematodes and arthropods [3]. This compound is largely used worldwide to control endo- and ectoparasites in livestock animals [4]. DOR is a highly lipophilic compound, which has been shown to extensively distribute from plasma to different tissues, particularly those with the highest fat content [5].

The DOR also is believed to act as an agonist of the neurotransmitter gamma-aminobutyric acid (GABA), thereby disrupting GABA-mediated central nervous system (CNS) neuro synaptic transmission provoking the paralysis of the parasites [6].

Although it presents a moderate toxicity [7], the use of DOR for lactating animals is not recommended for use, because pharmacokinetic studies have shown that milk taken from a number of species of treated animals shows long-term persistence of the drug residues [8,9].

As the presence of this compound in food represents a risk to human health, the development of alternative methodologies for its determination becomes essential. Only a few analytical methods are described in the literature for the determination of doramectin. Most of them employ high-performance liquid chromatography (HPLC) with ultraviolet (UV), fluorescence or mass spectrometry detection [10–14]. However, the detection in some methods is not sensitive enough for residue determination in food. The most accepted and sensitive analytical method for the detection of DOR is HPLC with fluorescence detection, after derivatization reaction of DOR with trifluoroacetic anhydride (TFAA) in the presence of catalytic amounts of N-methylimidazole [10]. This reaction requires a long time to occur, produces by-products that are potential interferents to the chromatographic determination and toxic residues into the environment, and it is also affected by minimum concentrations of residual water [10]. The liquid chromatography/mass spectrometry technique can be used for the determination of the avermectins in foods, but this technique requires complex sample preparation procedures and needs expensive equipment [14].

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1386-1425/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.saa.2012.02.084

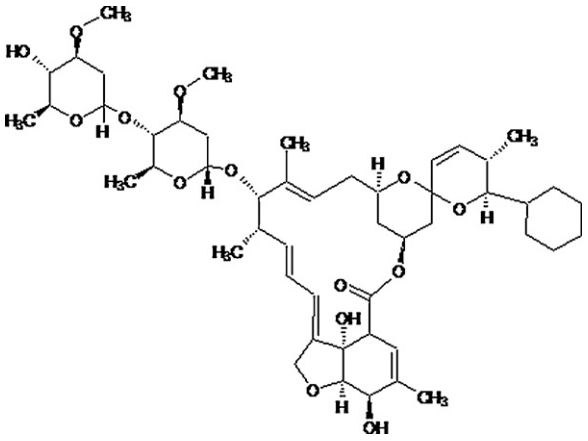

Fig. 1. Structure of doramectin.

In this work, the fluorescent characteristics of DOR were studied in order to identify simple, fast and low cost experimental conditions, which do not produce toxic residues and that are alternatives to chemical derivatization reactions usually employed, enabling its determination in milk samples by spectrofluorimetry.

2. Materials and methods

2.1. Instrumentation

Fluorescence measurements were performed using a Varian luminescence spectrophotometer (Cary Eclipse model). Fluorescence spectra were acquired on a PC using the SW Eclipse® Bio Pack V 1.1 (XP WIN2000) software. Quartz cuvettes (1 cm optical path length), 20 nm spectral band pass and 1200 nm s^{-1} scan velocity was used. DOR fluorescence intensities were measured in its maxima wavelengths of excitation (362 nm) and emission (466 nm).

A pH meter (Digimed, model DM-22, São Paulo, Brazil) and a 0.01 mg analytical balance (AND, Kyoto, Japan) were employed for the preparation of the working and sample solutions.

Chromatographic analysis was performed by high-performance liquid chromatography with fluorescence detection (HPLC-Fluo). The HPLC system was controlled by an Agilent ChemStation and consisted of a quaternary pump, an automated injector and a column oven interfaced to a fluorescence detector (Model G1321A) (all Agilent 1100 Series, USA). The determination of the DOR derivative was carried out using the wavelengths of maximum excitation (λ_{exc} = 362 nm) and emission (λ_{em} = 466 nm) to allow the largest sensitivity. Separation was achieved in a Waters SunFire C18 (150 × 4.6 mm × 5 μm) column connected to a Zorbax Eclipse Plus C18 (12.5 × 2.1 mm × 5 μm) pre-column. The column was kept inside an oven set at 30 °C.

A water bath (Kacil, model BM02, Recife, Brazil) with temperature control was used to heat the solutions before the measurements in the luminescence spectrophotometer. A vortex mixer (Fisatom, model 771, São Paulo, Brazil) and a shaker table (Biomixer, model KJ-YL-KJMR-II, São Paulo, Brazil) were used to homogenize the samples.

2.2. Reagents

Acetone, isopropanol, acetonitrile and anhydrous ethanol (HPLC grade, Tedia Brazil, RJ, Brazil) were employed. Ultra purified water (resistivity of 18.2 MΩ cm) was prepared through a simplicity system (Millipore, EUA) following distillation. The reagents employed for the derivatization (namely, N-methylimidazole, triethylamine, acetic acid, trifluoracetic acid and trifluoracetic anhydride) and the solid standard of doramectin were purchased from Sigma Chemical Co. (WI, USA) and were of the best purity available.

Analytical grade sodium hydroxide and sodium chloride (Vetec, RJ, Brazil) were employed without further purification. Nitrogen (99.99%) was from Alta Pressão Rio Comércio de Gases (Rio de Janeiro, Brazil).Commercial UHT milks of different brands and lots were purchased in local supermarkets of Niterói and Rio de Janeiro cities, Rio de Janeiro State, Brazil. Representative samples were obtained after homogenizing the whole content of the packages in the shaker table.

2.3. Procedures

2.3.1. Preparation of standard solutions and samples

Standard stock solutions of DOR were freshly prepared after weighing the solid compound and diluting it to 1000 mg L^{-1} using ethanol. Sodium hydroxide stock solutions (10 mol L^{-1}) were prepared in ultra purified water. Standard solutions with lower concentrations were prepared by further dilution of the stock solutions with ethanol containing NaOH at a final concentration of 0.25 mol L^{-1}. The final volume of water was kept and fixed at 10% of the total volume of the solution to avoid the precipitation of the lipophilic DOR. Before signal measurement, the DOR solutions were heated in a water bath at 50 °C during 40 min.

For optimization and validation of the method, analytical curves were obtained in triplicates, at a concentration range from 50 to 1000 μg L^{-1}. Recovery studies were carried out using bovine milk samples. Appropriated aliquots of the DOR stock solution (1000 mg L^{-1}) were added to polypropylene flasks and made up to a final volume of 25 mL with the milk sample.

The extraction of DOR was performed using the liquid–liquid extraction procedures with low temperature purification as described below. The mixture was vortex-mixed for 30 s and then left in refrigerator for 12 h. After that, 12.5 mL of acetonitrile were added to the polypropylene flask and vortex-mixed for 30 s in order to prevent the coprecipitation of macrocyclic lactones with milk proteins. This procedure was repeated for more three times and the final volume was completed up to 50 mL using acetonitrile. Then, the solution was stirred for 20 min in a shaker table at 180 rpm. Finally, 1.5 g of NaCl were added (to promote the salting out effect), followed by shaking until dissolution of the salt in a shaking table at 180 rpm during 10 min, and centrifugation at 2500 rpm during 10 min. The upper phase was transferred to another polypropylene flask and kept in the freezer for 12 h at −20 °C. After this time, 100 mL of ultra purified water were added to the remaining liquid phase and this solution was passed through a Strata-X cartridge (3 mL; 60 mg; Phenomenex, USA) previously conditioned with 5 mL of ethanol followed by 5 mL of ethanol:water (3:7, v/v) and 5 mL of water at a constant flow rate of 1 mL min^{-1}. After sample loading, the SPE cartridge was washed with 5 mL of ethanol:water (3:7, v/v), and finally, the content of the SPE cartridge was slowly eluted with 5 mL of an ethanolic solution of NaOH (0.25 mol L^{-1}). This eluted solution was heated at 50 °C during 40 min and filtered using a 0.45 μm filter, before measuring of the intensity of fluorescent signal.

In order to compare the recoveries achieved by fluorimetry, samples were analyzed by HPLC using the method proposed by Carbonell-Martin et al. [15], in which the mobile phase was obtained by mixing three components: methanol/water 95%/5% (v/v), acetonitrile/water 95%/5% (v/v) and water 100%. The mobile phase at 1.2 mL min^{-1} was pumped in the isocratic mode with 48% methanol/water, 50% acetonitrile/water and 2% water for 2.0 min. Then an elution gradient was applied by increasing the proportion of water from 2 to 5% and a decrease of methanol/water from 48 to 45% in 2.0 min. Then the proportion of acetonitrile/water increased from 50 to 60% followed by the decreasing of methanol/water from