Development and validation of LC-MS/MS method to determine the residue of veterinary drugs ivermectin, doramectin and moxidectin in milk

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A comparatively simple, sensitive and rapid analytical method has been developed and validated to determine the residues of avermectins, such as ivermectin, doramectin and moxidectin in milk using LC-MS/MS in positive ionization mode. The MRM transitions 892.71 >569.6, 892.71 >551.5 for ivermectin, 916.88 >593.83, 916.88 >331.40 for doramectin and 640.85 >199.03, 640.85 >498.61 for moxidectin have been used for the purpose of quantification and evaluation of other parameters of the method. The limits of detection of the method have been determined as 0.1 µg/kg, 0.1 µg/kg and 0.2 µg/kg for ivermectin, doramectin and moxidectin, respectively. The limits of quantitation have been calculated as 0.2 µg/kg, 0.2 µg/kg and 0.5 µg/kg for ivermectin, doramectin and moxidectin, respectively. The developed method allows the detection, quantitation and confirmation of macrolide endectocides in raw milk present at trace levels with high precision, accuracy and sensitivity by using simple extraction procedure. In spite of using a simplified extraction procedure, no interferences have been observed from the matrix components during the determination of drug residues. The method can be used for the routine analysis of macrolide endectocides in milk with added advantages of speed and economy. The method can also be tried for other animal products like meat and poultry. All the three avermectins can be determined in a single run. The response observed in positive ionization mode has been found to be better as compared to negative ionization mode. The use of positive ionization mode enables detection of all the three ivermectin, doramectin and moxidectin to very low levels.

Keywords: LC-MS/MS, method development, validation, ivermectin, doramectin, moxidectin, veterinary drug

The avermectins, ivermectin, doramectin and moxidectin are veterinary drugs commonly used for animal husbandry. The drugs are available in the form of oral, topical or injectable solutions. The use of these drugs may cause accumulation of their residues into the animal tissues which ultimately find their ways into food products derived from animal origin. The avermectins, the members of macro cyclic lactones and the broad spectrum antiparasitic are isolated from the naturally occurring fungus Streptomyces avermetilis. Macro cyclic lactones are strong pesticides for cow, sheep, pig and horse. They show good efficacy in killing interior nematodes and are named as endectocides. All the drugs belonging to this class are used for controlling helminthes and ectoparasites in animals. Ivermectin is available in the form of subcutaneous and topical formulations and is used in doses of 0.2 and 0.5 mg/kg for animals. Doramectin and moxidectin are available in the form of injectable and pour on solutions which are applied topically and all are highly lipophilic and tend to accumulate in fat tissues. The fat tissues act as a reservoir, contributing to the long term persistence in the body. Ivermectin residues may be found in various products of animal origin like milk and meat. In a study in Brazil, ivermectin residues between 2 to 10 ppb were found in 17.8% of milk samples purchased from retail market. Since the residue of ivermectin is responsible for several health hazards, it therefore becomes essential that the residue be strictly regulated from food safety point of view. Ivermectin and doramectin are used to treat a variety of food producing mammals in Canada, the United States and the European Union. The limits for maximum residue levels for the endectocides in food producing animals have been established by various regulatory authorities. Joint expert committee of food additives and contaminants (JECFA) has recommended a temporary MRL of 10 ppb for ivermectin in milk. A provisional accepted residue (PAR) limit of 20 ppb for ivermectin in the milk has been proposed in the United States. European Union has fixed MRL value.
of 10 ppb for ivermectin in the milk. The MRL value for moxidectin is 10 ppb in all jurisdictions. Milk is known as a nutritious wholesome food consumed globally and inexpensive source of protein and calcium essential for promontory growth in children. Milk contaminated with residue of veterinary drugs in concentration above the MRL is considered adulterated and inappropriate for consumption representing risk to public health and commercial risk to the drug industry. The occurrence of drug residues in food and food products originating from veterinary treatments has become increasingly noticeable. Residue of these drugs are responsible for several health hazards. It therefore becomes essential that the residues be strictly regulated from food safety point of view. As all of these compounds are very potent in their anti-parasitic activity and the regulated effective doses are very small, their detection in milk requires a highly sensitive and specific method. Various methods are available for the simultaneous determination of avermectins\textsuperscript{12-14}. However, extraction procedure in several methods is complicated and lengthy. The present paper describes an analytical method developed for simultaneous determination of residue of ivermectin, doramectin and moxidectin in milk using LC-MS/MS with ESI positive ionization mode. The aim was to develop a method that involved a simple and less time consuming extraction procedure.

Materials and Methods

Reagents and chemicals: Reference standards of ivermectin, doramectin and moxidectin with purity of $>99\%$ were purchased from Sigma Aldrich. Acetonitrile, water and methanol (liquid chromatographic grade) were purchased from Merck Specialties Private Limited. Ammonium formate (analytical reagent grade) was purchased from Loba Chem Private Limited and formic acid (analytical reagent grade) was purchased from S.D. Fine Chem Limited.

LCMS/MS Instrumentation and Chromatographic conditions

LC-MS/MS system: Waters 2695 series Alliance quaternary liquid chromatographic system (Waters, USA) with a Triple Quadrupole Mass Spectrometer, Quattro micro API (Micro mass, UK) equipped with an electro spray interface and masslynx 4.1 software (Micro mass) for data acquisition and processing was used. The instrument was provided with a 120-vial capacity sample management system.

Balance: Balance with readability of 0.01 mg and capacity of 220 g, Mettler Toledo XP-205.

Vortex: Model-Spinix (Tarsons Products Pvt Ltd).

Syringe filter: Syringe filters were of pore size 0.2 µm and 0.45 µm, with diameter of 25 mm (Advanced Micro devices Private Limited).

Nitrogen evaporator: Rapid Vap (Labconco Corporation).

Centrifuge: The extracts were centrifuged by using a high-speed refrigerated centrifuge, The rotor head was suitable for eight tubes of 50 mL size (Remi Sales and Engineering Ltd).

Centrifuge tubes: Disposable 50 mL conical centrifuge tubes with screw caps (Tarsons Products Pvt Ltd).

Preparation of standard solution: Approximately 10.0 mg ± 0.01 mg of ivermectin, doramectin and moxidectin reference standards were accurately weighed into individual 100 mL volumetric flasks and dissolved and made to volume using methanol. This gave a stock solution of 100 µg/mL for ivermectin, doramectin and moxidectin each. From all the three stock solutions 1 mL of aliquot was taken in a 100 mL volumetric flask and made to volume using methanol to give a standard mix solution of ivermectin, doramectin and moxidectin having a concentration of 1 µg/mL. The solutions were stored at 2°C to 8°C.

Preparation of calibration standard solutions: From the standard mix solution having concentration of 1 µg/mL, appropriate aliquots were taken and further diluted with methanol so as to give a series of calibration standard solutions having concentration ranging from 1.0 ng/mL, to 100 ng/mL. All solutions were stored at 2°C to 8°C.

Preparation of matrix-matched calibration standard solutions: Matrix-matched calibration standard solutions were prepared at the same concentration levels as the solutions of calibration standards by adding appropriate aliquots of mixed standard solution. All the solutions were stored at 2°C to 8°C.

Preparation of mobile phase: The mobile phase was prepared by mixing two solutions \textit{i.e.} A and B in the ratio of 12:80 (A:B) and filtered through 0.45-micron filter membrane using the Millipore filtration unit. Solution A: ammonium formate 5 mM in water and solution B: 0.1% formic acid in methanol.

Preparation of sample: Samples of milk were obtained from local milk processors and were initially
tested for the presence of macrolide endectocides before processing and storage at −20°C. Approximatively 5.0±0.1 g of the liquid milk sample equilibrated at room temperature was taken in the centrifuge tube and extracted with 10 mL of 50:50 mixture of acetonitrile and methanol using vortex mixer. The solution was then centrifuged at ambient temperature for 10 min at 7000 rpm followed again by centrifugation at 4°C at 7000 rpm for further 10 min. The supernatant layer was collected in a dry separating funnel. The residue was extracted using the same process twice. The combined organic solvent was evaporated to dryness under a stream of nitrogen and the dried extract was redissolved in methanol before injecting into LC-MS/MS.

LC-MS-MS conditions

Column: The separation was carried out using X Terra MS C-18 column (2.1 mm × 100 mm; 5 μm) and mobile phase comprising of A: 5 mM ammonium formate; B: 0.1% formic acid in methanol; (A:B-20:80 in the isocratic mode). The LC column was set at 50°C.

ESI Interface: Optimal parameters of the ESI interface were optimized by infusing 250 ng/mL standard solution of ivermectin, doramectin and moxidectin one by one in the mobile phase using a Harvard syringe pump. LC-MS/MS determination was performed by operating the mass spectrometer in positive ionisation mode. Nitrogen used as a neutralisation gas and dissolvation gas was delivered at a flow rate of 750 L/hr.

Typical MS settings: Capillary voltage (kV): 3.5; Cone voltage (V): 30; Source Temperature (°C): 100; Dessolvation Temperature (°C): 450.

Results and Discussion

Liquid chromatographic separation: A comparatively simple, sensitive and accurate method has been developed for the determination of ivermectin, doramectin and moxidectin residues in raw milk using LC-MS/MS with positive ESI mode. Using the chromatographic conditions as mentioned above, well resolved peaks for ivermectin, doramectin and moxidectin were obtained within 4 min of the injection. Optimum separation was achieved using 5 mM ammonium formate (A) and 0.1% formic acid in methanol (B) in the ratio 20:80 as mobile phase.

Extraction procedure: For the extraction of ivermectin, doramectin and moxidectin from the raw milk samples, a simplified extraction procedure has been developed as compared to the ones in the existing analytical methods reported in the literature. The previous method has reported the use of tris buffer and SPE techniques for sample cleanup which makes the sample preparation cumbersome. Based upon the past experience of the authors, the extraction method was thereby simplified as has been described above. Since ivermectin, doramectin and moxidectin are soluble in solvents like methanol and acetonitrile, a combination of methanol and acetonitrile has been taken for extracting the residues from milk samples. Any fat components which might have been coextracted along with the residues were washed off with n-hexane saturated with acetonitrile. The extract was dried off under nitrogen and the dried extract was dissolved in methanol and injected into LC-MS/MS.

Mass spectrometry: For the purpose of evaluating the fragment ions and the intensity of the signals, the reference standard solutions of all the three, ivermectin, doramectin and moxidectin were infused one by one using both positive and negative ESI mode of the mass spectrometer detector. The results showed that the signals for both positive and negative modes were comparable and either of the modes could be used for the purpose of development of the method for determination of residue of all three drugs in milk. When the conditions were optimized using liquid chromatography, although all the ions were distinctly observed in both the modes, the signal response was poor in negative mode as compared to positive mode. Hence the method was developed using ammonium formate buffer in positive ionization mode which produced highly intense signals so that the residue of all three drugs were detectable at very low concentrations. In the mass spectra (ESI-MS and ESI-MS/MS mode) of ivermectin, doramectin and moxidectin the parent components of ivermectin shows a molecular mass of 892.7 instead of 874.5 as per the molecular structure and the same is observed in the case of doramectin, the mass obtained in this case was 916.88 instead of 899.11. The explanation lies in the fact that the parent component gets ammoniated in the presence of ammonium formate used in the mobile phase. Here it may be noted that the fragment ions detected match exactly with the reported data for ivermectin and doramectin in the positive ionization mode. For the purpose of
developing and validating the method the most distinct ions used are tabulated in Table I.

Method performance characteristics

The method was validated as per the International Union of Pure and Applied Chemistry (IUPAC) and Eurachem guidelines.\textsuperscript{17,18}

**Linearity:** Six calibration standards evenly spread over the concentration range of interest and encompassing the concentration levels reflecting EU regulatory limits\textsuperscript{19} were analyzed. The calibration standards were run in triplicate. The matrix matched calibration curve was found to be linear in the range of 1.0 ng/mL -100 ng/mL with correlation coefficient of 0.9990, 0.9995 and 0.9993 for ivermectin, doramectin and moxidectin, respectively. The matrix effect was investigated by comparing standards in solvents with matrix matched standards at different concentration levels. The matrix effect was found to be small for doramectin, whereas the matrix suppressed the signal response considerably for ivermectin and moxidectin. For calculation purpose MMC (matrix matched calibration) was used as this corrects the matrix effects.

**Specificity:** The chromatographic interferences from the milk samples were investigated by comparing the chromatograms of blank and the spiked samples. For this purpose, samples were prepared using the same procedure as mentioned earlier and the specificity of the method was measured. It was found that the presence of interferences did not have any effect on the quantitative results of the analyte of interest, thus providing reliability of the LC-MS/MS method for determination of ivermectin, doramectin and moxidectin.

**Precision:** Precision studies were carried out for both inter-day and intra-day repeatability and reproducibility. Three spiked samples of milk at different concentration levels (Table II) were injected seven times on the same day and the same number of times on three subsequent days by three different analysts. The low %RSD value obtained for inter-day and intra-day variation within the acceptable norms showed that the proposed method is precise and can be adopted for analysis.

**Accuracy:** The recoveries (Table III) of all the three drugs in spiked samples were calculated to study the effect of matrix on the determination of ivermectin, doramectin and moxidectin. The recovery studies were carried out at five different concentrations. For this, five different portions of pre-analyzed milk samples were spiked with 0.5 µg/kg, 1.0 µg/kg, 2.5 µg/kg, 10 µg/kg and 20 µg/kg, respectively in triplicate on three different days and then extracted and determined by the same method as mentioned earlier. The recoveries were within the range of 92.0 -

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**Table I — MRM setting for positive ion MS/MS analysis of macrolide endectosides**

<table>
<thead>
<tr>
<th>Compd</th>
<th>Parent Ion (Da)</th>
<th>ESI (mode)</th>
<th>Product Ion (Da)</th>
<th>C.E. (v)</th>
<th>Cone Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivermectin</td>
<td>892.7</td>
<td>+ve</td>
<td>569.6</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>551.5</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>Doramectin</td>
<td>916.88</td>
<td>+ve</td>
<td>593.83</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>331.40</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>Moxidectin</td>
<td>640.85</td>
<td>+ve</td>
<td>199.03</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>498.61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Table II — Intra-day and inter-day precision data for the proposed method for drug residues in milk**

<table>
<thead>
<tr>
<th>Concentration of Drug (µg/kg\textsuperscript{-1})</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean peak area</td>
<td>% RSD</td>
<td>Mean peak area</td>
<td>% RSD</td>
<td>Mean peak area</td>
</tr>
<tr>
<td>Concentration of ivermectin (µg/kg\textsuperscript{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>719.28</td>
<td>3.34</td>
<td>721.1</td>
<td>3.49</td>
</tr>
<tr>
<td>2.5</td>
<td>1622.15</td>
<td>2.57</td>
<td>1629.38</td>
<td>2.87</td>
</tr>
<tr>
<td>5.0</td>
<td>3324.1</td>
<td>2.42</td>
<td>3411.5</td>
<td>2.50</td>
</tr>
<tr>
<td>Concentration of doramectin (µg/kg\textsuperscript{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>511.28</td>
<td>3.24</td>
<td>519.1</td>
<td>2.49</td>
</tr>
<tr>
<td>2.5</td>
<td>1332.15</td>
<td>2.27</td>
<td>1329.38</td>
<td>2.17</td>
</tr>
<tr>
<td>5.0</td>
<td>5124.17</td>
<td>2.76</td>
<td>5411.5</td>
<td>2.10</td>
</tr>
<tr>
<td>Concentration of moxidectin (µg/kg\textsuperscript{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>319.28</td>
<td>3.54</td>
<td>321.1</td>
<td>3.59</td>
</tr>
<tr>
<td>2.5</td>
<td>922.15</td>
<td>2.23</td>
<td>929.35</td>
<td>2.31</td>
</tr>
<tr>
<td>5.0</td>
<td>1535.1</td>
<td>2.27</td>
<td>1516.5</td>
<td>2.30</td>
</tr>
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</table>
100.4% for ivermectin, 96.2 - 102.0%, for doramectin and 95.6 - 102.0% for moxidectin.

Robustness: Robustness of the method was determined by analyzing the same set of spiked samples (i.e. samples spiked at concentration levels of 1.0 µg/kg, 5.0 µg/kg and 20 µg/kg) under different parameters; such as same column chemistry from different manufacturers, different analysts and different injection volumes. The method was found to be robust even with small changes in analytical conditions: change in flow rate (± 0.05 mL/min), a change in column temperature (± 5°C), use of same column from different manufacturer (Waters C18 column and Varian C-18). Under all of these conditions, the analytical values of the spiked samples were not affected and it was in accordance with the actual values.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD was determined by considering signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise obtained from the blank sample whereas LOQ was determined similarly by considering signal to noise ratio (S/N) ratio of 10:1. In order to establish the LOD and LOQ the matrix milk was spiked with the macroclide endectocides (taking into account the sample weight and the dilution factor). The LOD was found to be 0.1 µg/kg, 0.1 µg/kg and 0.2 µg/kg for ivermectin, doramectin and moxidectin, respectively. The LOQ was found to be 0.2 µg/kg, 0.2 µg/kg and 0.5 µg/kg for ivermectin, doramectin and moxidectin, respectively.

References
1. The Merck Index, ivermectin, 2006, 5248, 908.