Method development and validation for simultaneous quantitation of ivermectin, doramectin and moxidectin in muscle by using LC-MS/MS in positive ESI mode

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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 muscle 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. In order to establish the LOD and LOQ, the matrix muscle is spiked with the ivermectin, doramectin and moxidectin (taking into account the sample weight and the dilution factor). The LOD and LOQ are found to be $0.1 \ \mu$ g/kg and $0.2 \ \mu$ g/kg, respectively. The developed method allows the detection, quantitation and confirmation of macrolide endectosides in chicken muscle present at trace levels with high precision, accuracy and sensitivity by using simple extraction procedure.

Keywords: Chicken muscle, Ivermectin, Doramectin, LC-MS/MS, Method development, Moxidectin, Veterinary drug

Veterinary drugs are commonly used in animals as therapeutic agents to increase feed efficiency and to prevent outbreak of disease. In food producing animals, the administration of these drugs may results in the presence of their residues in the food products, such as milk, meat and eggs, derived from these animals. The use of unauthorized drugs or the failure to follow level direction of approve drug may result in unsafe residues in these food products and endanger human health. Therefore, it is necessary to develop and validate analytical methods to monitor drug residues in animal derived food for human consumption.

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 endectocides¹. All the drugs belonging to this class are used for controlling helminthes and ectoparasites in animals^{2,3}. Ivermectin is available in the form of subcutaneous and topical formulations and is used in doses of 0.2 and $0.5 \text{ mg/kg for animals}^{4.5}$. 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 body⁶. Ivermectin residues may be found in various products of animal origin like muscle and meat⁷. In a study in Brazil, ivermectin residues between 2 to 10 ppb were found in 17.8% of muscle samples purchased from retail market⁸. 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 view⁹. 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 muscle¹⁰. A provisional accepted residue (PAR) limit of 20 ppb for ivermectin in the muscle has been proposed in the United States¹¹. European Union has fixed MRL value of 10 ppb for ivermectin in the muscle. The MRL value for moxidectin is 10 ppb in all jurisdictions. The occurrence of drug residues in meat and meat products originating from veterinary treatments has become increasingly noticeable. As all of these compounds are very potent in their anti-parasitic activity and the regulated effective doses are very small, their detection in muscle requires a highly sensitive and specific method. Various methods are available for the simultaneous determination of avermectins¹²⁻¹⁴. 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 muscle 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 in a complex matrix.

Experimental Section

Instruments, apparatus and equipments

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 with readability of 0.01 mg and capacity of 220 g, Mettler Toledo XP-205.Vortex Model-Spinix (Tarsons Products Pvt Ltd), 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 make and model Rapid Vap (Labconco Corporation), 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), disposable 50 mL conical centrifuge tubes with screw caps (Tarsons Products Pvt Ltd) were used for the study.

Materials

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.

Preparation of standard solution

Approximately 5.0±0.01 mg of ivermectin, doramectin and moxidectin reference standards were weighed into individual accurately 100 mL volumetric flasks and dissolved and made to volume using methanol. This gave a stock solution of 50 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 mobile phase to give a standard mix solution of ivermectin, doramectin and moxidectin having a concentration of 1 µg/mL. The solutions were stored at 2 to 8°C.

Preparation of calibration standard solutions

From the standard mix solution having concentration of 1 μ g/mL, appropriate aliquots were taken and further diluted with mobile phase so as to give a series of calibration standard solutions having concentration ranging from 1.0 to 50 ng/mL. All solutions were stored at 2 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 to 8°C.

Preparation of mobile phase

The mobile phase was prepared by mixing two solutions *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 chicken muscle were obtained from local market and were initially tested for the presence of macrolide endectocides before extraction and storage at -20°C. Approximately 5.0±0.1 g of the muscle 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 from all the three extractions was passed through anhydrous sodium sulphate and washed with *n*-hexane saturated with acetonitrile. This solvent was then evaporated to dryness under a stream of nitrogen and the dried extract was redissolved in mobile phase before injecting into LC-MS/MS.

LC-MS-MS conditions

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 was kept as 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 muscle using LC-MS/MS with positive ESI mode. The separation was carried out using X Terra MS C-₁₈ column (2.1 mm × 100 mm; 5 μ m) 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, well resolved peaks for ivermectin, doramectin and moxidectin were obtained with in 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 chicken muscle samples, a

simplified extraction procedure has been developed as compared to the ones in the existing analytical methods reported in the literature^{11,15,16}. The previous method¹¹ 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 like methanol and solvents. acetonitrile. combination of methanol and acetonitrile has been taken for extracting the residues from muscle 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 mobile phase and injected into LC-MS/MS.

Mass spectrometery

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 muscle. 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 showed a molecular mass of 892.7 instead of 874.5 as per the molecular structure and the same was 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 detected fragment ions 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 1.

Method performance characteristics

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

Specificity and selectivity

The chromatographic interferences from the muscle samples were investigated by comparing the chromatograms of blank and the spiked samples. For this purpose the chromatographic conditions were optimized to get good peak shape and sensitivity of the analytes. No significant interfering peaks from the endogenous compound were observed at the retention time of ivermectin, doramectin and moxidectin, thus, providing reliability of the method. Total chromatographic run time was 10 min. The shorter run time make the method more productive. Figure 1 shows the typical MRM chromatograms of the blank muscle sample (Free of analytes) and Figure 2 is chromatograms of the sample.

Linearity

Six calibration standards evenly spread over the concentration range of interest and encompassing the concentration levels reflecting regulatory limits were analyzed. The calibration standards were run in triplicate and average correlation coefficient values have been reported. The matrix matched calibration curve was found to be linear in the range of 1.0-50 ng/mL with correlation coefficient of 0.9992, 0.9988 and 0.9989 for ivermectin, doramectin and moxidectin, respectively. The matrix effect was investigated by comparing standards in solvents with matrix matched standards at different

Table 1-MRM setting for positive ion MS/MS analysis of ivermectin, doramectin and moxidectin Compound Retention Time Parent Ion ESI (mode) Product Ion C.E. (v) Capillary voltage Cone Voltage Dwell Time (min) (Da) (Da) (KV) (v) (milli second) Ivermectin 892.7 569.6 18 3.5 40 100 3.63 +ve 551.5 Doramectin 2.96 916.88 593.83 18 3.5 40 100 +ve 331.40 Moxidectin 3.20 199.03 22 3.5 20 100 640.85 +ve498.61



Fig. 1-MRM chromatogram of blank in muscle sample showing absence of ivermectin, doramectin and moxidectin



Fig. 2-MRM chromatogram of spiked sample of muscle showing ivermectin, doramectin and moxidectin

concentration levels. The matrix effect was found to be small for doramectin, whereas, the matrix suppressed the signal response considerably for ivermectin and moxidectin. No single calibration point was dropped during validation. Results indicated that the method was accurate and precise within the analytical range (Table 2).

Precision and accuracy

The precision and accuracy studies carried out for both intra-day and inter-day repeatability and reproducibility by analyzing six replicates of spiked sample of muscle at five different concentrations are shown in Tables 3, 4 and 5. The intra-day precision for the spiked samples of ivermectin, doramectin and moxidectin were ranged from 0.501-1.950, 0.403-1.689 and 0.459-1.895, respectively. The interday precision for the spiked samples of ivermectin, doramectin and moxidectin ranged from 1.170-1.367, 0.783-1.312 and 1.042-1.582, respectively.

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 muscle was spiked with the ivermectin, doramectin and moxidectin (taking into

Nominal concentration (ppb) 1	2.5	5			
		3	10	25	50
Observed concentration (ppb) 0.977	2.402	4.837	9.599	23.997	50.603
SD 0.022	0.049	0.074	0.216	0.114	0.087
% RSD 2.219	2.045	1.539	2.245	0.477	0.171
% Accuracy 97.17	96.10	96.74	95.99	95.99	101.21
	Doramectin				
Nominal concentration (ppb) 1	2.5	5	10	25	50
Observed concentration (ppb) 1.001	2.449	4.817	9.578	23.774	50.719
SD 0.031	0.035	0.130	0.408	0.339	0.259
% RSD 3.058	1.424	2.689	4.255	1.428	0.510
% Accuracy 100.08	97.95	96.34	95.78	95.09	101.44
	Moxidectin				
Nominal concentration (ppb) 1	2.5	5	10	25	50
Observed concentration (ppb) 1.009	2.450	4.952	9.883	24.698	50.181
SD 0.025	0.044	0.088	0.360	1.448	0.802
% RSD 2.509	1.786	1.785	3.644	5.863	1.599
% Accuracy 100.92	97.99	99.05	98.83	98.79	100.36

Table 2—Summary of matrix match calibration (MMC) for ivermeetin, dorameetin and moxidectin in muscle sample

Table 3-Intra-day and inter-day precision and accuracy data of ivermectin in muscle

0 114		Measured concentration of ivermectin (ppb)							
sample (ppb)	Run	Intra-day precision and accuracy (n=6)				Inter-day precision and accuracy (n=18)			
		Mean	SD	RSD (%)	Accuracy (%)	Mean	SD	RSD (%)	Accuracy (%)
	1	0.995	0.007	0.716	99.47				
1	2	0.993	0.008	0.782	99.25	0.990	0.013	1.303	99.02
	3	0.983	0.019	1.950	98.33				
	1	2.461	0.017	0.700	98.44				
2.5	2	2.508	0.028	1.135	100.31	2.491	0.033	1.313	99.62
	3	2.503	0.031	1.227	100.12				
	1	4.910	0.052	1.059	98.20				
5	2	4.955	0.052	1.042	99.09	4.912	0.067	1.367	98.25
	3	4.873	0.077	1.577	97.45				
	1	9.946	0.085	0.855	99.46				
10	2	9.811	0.127	1.294	98.11	9.870	0.115	1.170	98.70
	3	9.854	0.103	1.045	98.54				
	1	20.116	0.350	1.741	100.58				
20	2	19.913	0.145	0.730	99.56	19.973	0.237	1.186	99.86
	3	19.890	0.100	0.501	99.45				

account the sample weight and the dilution factor). The LOD and LOQ were found to be 0.1μ g/kg and 0.2μ g/kg for ivermectin and doramectin, respectively. For moxidectin, the LOD and LOQ were found to be 0.2μ g/kg and 0.5μ g/kg, respectively.

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 μ g/kg, 5.0 μ g/kg and 20 μ g/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).

Quality control sample (ppb)		Measured concentration of doramectin (ppb)							
	Run	Intra-day precision and accuracy (n=6)				Inter-day precision and accuracy (n=18)			
		Mean	SD	RSD (%)	Accuracy (%)	Mean	SD	RSD (%)	Accuracy (%)
	1	0.993	0.011	1.146	99.28				
1	2	1.006	0.014	1.358	100.58	0.997	0.013	1.306	99.65
	3	0.991	0.010	1.012	99.08				
	1	2.463	0.021	0.839	98.53				
2.5	2	2.469	0.024	0.970	98.77	2.462	0.019	0.783	98.48
	3	2.453	0.010	0.403	98.12				
	1	4.973	0.053	1.069	99.46				
5	2	4.957	0.046	0.925	99.14	4.956	0.050	1.010	99.12
	3	4.938	0.053	1.082	98.76				
	1	9.893	0.079	0.795	98.93				
10	2	9.906	0.123	1.237	99.06	9.927	0.128	1.286	99.27
	3	9.984	0.169	1.689	99.84				
	1	19.761	0.329	1.666	98.80				
20	2	19.767	0.116	0.586	98.83	19.770	0.259	1.312	98.85
	3	19.783	0.327	1.651	98.51				

Table 4—Intra-day and inter-day precision and accuracy data of doramectin in muscle

Table 5-Intra-day and inter-day precision and accuracy data of moxidectin in muscle

		Measured concentration of Moxidectin (ppb)								
Quality control sample (ppb)	Run	Intra-day precision and accuracy (n=6)				Inter-day precision and accuracy (n=18)				
		Mean	SD	RSD (%)	Accuracy (%)	Mean	SD	RSD (%)	Accuracy (%)	
1	1	0.999	0.11	1.074	99.85					
1	2	0.997	0.019	1.895	99.68	0.998	0.014	1.449	99.76	
	3	0.998	0.015	1.544	99.75					
2.5	1	2.461	0.021	0.870	98.42					
	2	2.433	0.011	0.459	97.31	2.447	0.025	1.042	97.88	
	3	2.448	0.034	1.392	97.91					
	1	4.926	0.075	1.518	98.52					
5	2	4.930	0.078	1.588	98.61	4.918	0.078	1.582	98.36	
	3	4.898	0.090	1.840	97.96					
10	1	9.927	0.131	1.315	99.27					
	2	9.859	0.095	0.964	98.59	9.896	0.121	1.224	98.96	
	3	9.903	0.145	1.464	99.03					
	1	19.742	0.302	1.531	98.71					
20	2	20.034	0.214	1.070	100.17	19.950	0.313	1.571	99.75	
	3	20.073	0.344	1.712	100.37					

Under all of these conditions, the analytical values of the spiked samples were not affected and it was in accordance with the actual values.

Conclusion

The developed method using positive ESI ,LC-MS/MS allows the detection, quantitation and confirmation of ivermectin, doramectin and moxidectin in muscle present at trace levels with high precision, accuracy and sensitivity by using a simple

extraction procedure. The method can be used for the routine analysis of these residues in muscle with added advantages of speed and economy.

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