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Original Article

Rapid confirmatory analysis of avermectin residues in milk by liquid chromatography tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 18 November 2014
Received in revised form
8 April 2015
Accepted 20 July 2015
Available online 23 October 2015

Keywords:
avermectin
liquid chromatography/tandem mass spectrometry
milk
residue

ABSTRACT

Our study developed a quick method for confirmatory analysis of avermectins (abamectin B_{1a}, doramectin, ivermectin B_{1a}, eprinomectin B_{1a}, and moxidectin) in bovine milk according to the European Commission Decision 2002/657/EC requirements. Avermectins were liquid–liquid extracted with acetonitrile, followed by an evaporation step, and then analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry in the negative ion mode. An in-house method validation was performed and the data reported on specificity, linearity, recovery, limit of detection, limit of quantitation, decision limit, and detection capability. The advantage of this method is that low levels of avermectins are detectable and quantitatively confirmed at a rapid rate in milk.

1. Introduction

Avermectins (AVMs) are widely used as an active substance for endo- and ectoparasites in veterinary medicine. AVMs are isolated from a fermentation broth of the soil actinomyete *Streptomyces avermitilis*. AVMs in commercial use are abamectin (ABA), doramectin (DORA), ivermectin (IVER), emamectin, eprinomectin (EPRI), moxidectin (MOXI), and selamectin. These compounds are registered for use in cattle and other food animals, fish farming, and pet animals [1].

The European Medicines Agency has proposed maximum residue limits (MRLs) of 20 ppb and 40 ppb of EPRI and MOXI for milk, respectively. They are not used in animals from which milk is produced for human consumption for ABA,

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http://dx.doi.org/10.1016/j.jfda.2015.07.004

DORA, and IVER in the Turkish Food Codex based on European Commission Regulations [2,3]. *Codex Alimentarius* (International Food Standards) has proposed MRLs of 10 ppb, 15 ppb, and 20 ppb for IVER, DORA and EPRI, respectively, but not for ABA and MOXI [4].

AVM residues are usually determined using solid phase extraction techniques and liquid chromatography with fluorescence detection [5]. Immunoaffinity chromatography clean-up of AVMs is an alternate method of sample preparation [6]. Mass spectrometry (MS) is the preferred technique for confirmation of suspect residues due to its inherent specificity and sensitivity [1,7,8].

Our study developed a quick method of confirmatory analysis for AVMs (ABA B_{1a}, DORA, IVER B_{1a}, EPRI B_{1a}, and MOXI) in bovine milk, which are liquid–liquid extracted with acetonitrile (ACN), followed by an evaporation step and, finally, analysis by liquid chromatography/electrospray ionization tandem MS (LC/ESI-MS/MS) in the negative ion mode, according to European Commission Decision 2002/657/EC [9].

2. Materials and methods

2.1. Reagents and standards

HPLC-grade methanol and ACN, triethylamine (TEA), and anhydrous sodium sulfate ($NaSO_4$) were purchased from Bilgen Kimya in Istanbul (Turkey). Purified water was obtained from a Milli-Q purifying system (Elga PureLab Prima).

ABA (purity: 92.3%), IVER (99.2%), EPRI (99.3%), DORA (81.1%), MOXI (92.3%), and selamectin (94%; internal standard) were obtained from Sigma–Aldrich (St Louis, MO, USA). An individual standard stock solution (1000 ppm) of AVMs was prepared by dissolving each pure reference compound in methanol. The mixed standard solution was prepared as 0.05 ppm for ABA, DORA, and IVER based on detection limit and 0.5 ppm for EPRI and 1 ppm for MOXI based on MRL limits. The solution of internal standard selamectin was prepared at 3 ppm in methanol. All stock solutions were stored at −20°C stable for 1 year in a freezer. Working standard and internal standard solutions were prepared in a dilution of methanol and stable for 2 months in a refrigerator.

2.2. Sample preparation

Milk obtained for use as a negative control was separated into 50 mL aliquots and stored at −20°C. The milk was dissolved in a water bath at 45°C and then mixed by gentle shaking. A 5 mL milk sample was pipetted into 50 mL polypropylene centrifuge tubes. Mixed standard solutions were added at 100 μL, 200 μL, 300 μL, and 400 μL and the internal standard at 100 μL to all tubes and then this sample was added to 10 mL of ACN. The tubes were capped, vortexed briefly and left for 15 minutes in a Multi Reax (multiple vortex). Next, 5 g of $NaSO_4$ was added, vortexed, and centrifuged at 4000 g at −4°C for 15 minutes. The 5-mL supernatant was removed using a pipette and transferred into a 15 mL graduated tube. The organic fraction was evaporated to full dryness under a stream of nitrogen in a water bath at 45°C. The dry residue was redissolved in 0.5 mL of ACN (0.1% TEA). The sample was filtered using a 0.45 μm syringe filter to place it into an autosampler vial.

2.3. Instrumentation

Chromatographic analysis was performed on an LC-MS/MS; equipment consisted of a Thermo Electron TSQ Quantum Discovery Max and mass spectrometer controlled by Xcalibur 1.4 software.

Chromatographic separations were achieved on an Agilent Extend C18 column (100 mm × 2.0 mm, 5 μm) and protected with a C18 guard column from Agilent. The isocratic mobile phases used 0.1% TEA in water (20%) and acetonitrile (80%) at a flow rate of 0.3 mL/min and with an injection volume of 50 μL. Detection of the analytes was carried out in the negative ESI-MS-MS ion mode.

2.4. MS

MS/MS parameters and precursor–product ions of each compound were tuned by direct infusion in the selective reaction-monitoring mode at a 0.3 mL/min flow rate mobile phase.

Desolvation temperature was 350°C; capillary voltage 5500 V; sheath gas pressure (air) 40 (arbitrary units); auxiliary gas pressure (air) 10 (arbitrary unit); collision gas pressure 10 (arbitrary units) and collision-induced dissociation source voltage 5 eV. The collision gas was argon and the desolvation gas was nitrogen. MS/MS parameters and precursor–product ions of each compound are given in Table 1.

3. Results and discussion

Method validation was performed with consideration of the criteria and recommendations of European Commission Decision 2002/657/EC and implementation of Council Directive 96/23/EC.

3.1. Specificity/selectivity

Specificity/selectivity were evaluated via analysis of blank matrix samples fortified separately with mixed benzimidazole

Table 1 – Liquid chromatography–tandem mass spectrometry parameters for the analytes.

Compounds	Precursor ion *m/z*	Product ion *m/z*	Collision energy	Retention time (min)
Abamectin	871.4	565.1*	26	3.12
		228.9	36	
Doramectin	897.6	591.1*	26	3.84
		228.9	30	
İvermectin	873.4	567.1*	20	5.43
		228.9	30	
Eprinomectin	912.5	565.1*	26	2.42
		269.9	30	
Moxidectin	638.5	528.2*	26	4.12
		236.0	35	

* Confirmative ion.

and nitroimidazole standards. According to the analysis, neither significant peaks with an signal:noise ratio of 3 or more nor chromatographic interference were observed at the retention times of the targeted AVMs using LC/ESI-MS/MS, as shown in Fig. 1.

3.2. Linearity

The calibration curves at four levels were: 1.0 ppb, 2.0 ppb, 3.0 ppb, and 4.0 ppb for ABA, DORA, and IVER; 10.0 ppb, 20.0 ppb, 30.0 ppb, and 40.0 ppb for EPRI; and 20.0 ppb, 40.0 ppb, 60.0 ppb, and 80.0 ppb for MOXI, according to minimum required performance limit (MRPL) and MRL levels. For each compound, we made three matrix calibration curves for 3 days. Linearity was good for all the tested concentrations. No significant differences were found between the curves ($r^2 > 0.9720$).

3.3. Limit of detection, limit of quantitation, decision limit, and detection capability

For each compound, limit of detection, limit of quantitation, decision limit (CC_α), and detection capability (CC_β) were calculated from the linearity study and are summarized in Table 2.

The CC_α and CC_β were calculated using calibration curves (at four levels: 1.0 ppb, 2.0 ppb, 3.0 ppb, and 4.0 ppb for ABA, DORA, and IVER; 10.0 ppb, 20.0 ppb, 30.0 ppb, and 40.0 ppb for EPRI; and 20.0 ppb, 40.0 ppb, 60.0 ppb, and 80.0 ppb for MOXI) from 3 different days, using different analysts and according to the European Commission Decision 2002/657/EC [9] and ISO 11843 [10].

The CC_α and CC_β for banned substances were calculated with the application of the following formulae:

$$CC_\alpha = C_1 + 2.33 \times SD_{wIR}$$

where C_1 is the lowest concentration level of the validation study (MRPL) and SD_{wIR} is the standard deviation from within-laboratory reproducibility; and:

$$CC_\beta = CC_\alpha + 1.64 \times SD_{wIR,CC\alpha}$$

where $SD_{wIR,CC\alpha}$ is the standard deviation of CC_α concentration.

The CC_α and CC_β for MRL substances were calculated with the application of the following formulae:

$$CC_\alpha = C_2 + 1.64 \times SD_{wMRL}$$

where C_2 is the second concentration level of the validation study (MRL concentration) and SD_{wMRL} is the standard deviation from within-laboratory reproducibility (at the MRL);

$$CC_\beta = MRL + 3.28 \times SD_{wMRL}$$

where $SD_{wIR,MRL}$ is the standard deviation of the MRL concentration.

3.4. Recovery

The recoveries were calculated by analyzing fortified milk spiked samples with: 1.0 ppb, 2.0 ppb, 3.0 ppb, and 4.0 ppb for ABA, DORA, and IVER, according to MRPLs; 10.0 ppb, 20.0 ppb, 30.0 ppb, and 40.0 ppb for EPRI; and 20.0 ppb, 40.0 ppb, 60.0 ppb, and 80.0 ppb for MOXI, according to MRLs.

According to the results, recovery was observed in the acceptable range of 70–110%. All the data relating to method recovery and precision are summarized in Table 3; mean recoveries ranging and coefficient of variable percentage values were satisfactory as required by the European Commission Decision 2002/657/EC.

3.5. Ruggedness

The robustness of the method performed on conscious/unconscious changes across the study verified the reliability of the analysis method. Studies were evaluated using the Youden [9] plan of factorial design. In the Youden approach, the terms of experimental conditions require the simultaneous combination of seven factors that promote multiple variations of a factorial experimental design.

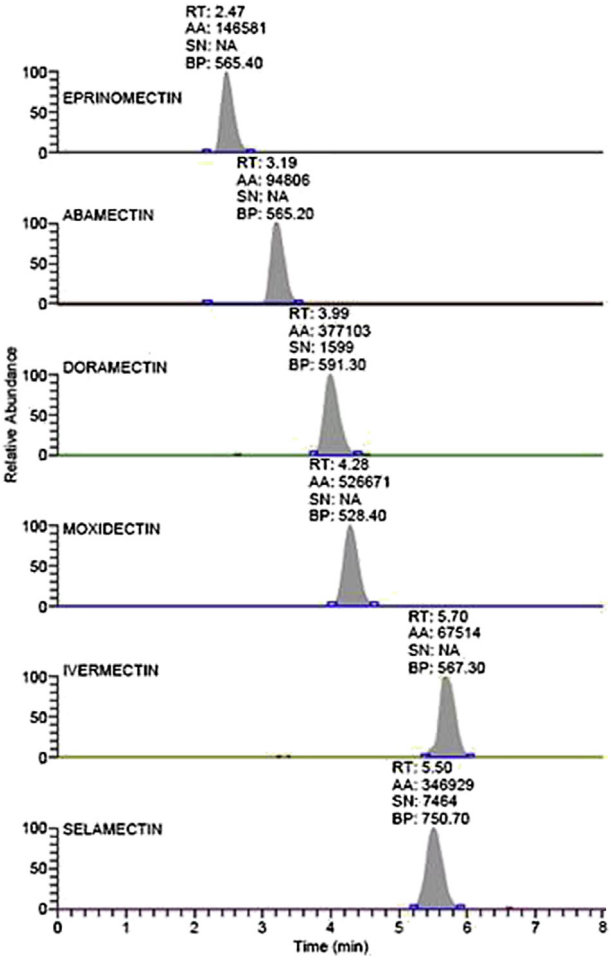

Fig. 1 – Chromatogram of a spiked milk sample with 1.0 ppb for abamectin, doramectin, and ivermectin; 10 ppb for eprimectin; and 20 ppb for moxidectin.

Table 2 – Summary of validation data.

Analyte	Calibration range (ppb)	Linearity r^2	CC_α (ppb)	CC_β (ppb)	LOD (ppb)	LOQ (ppb)	MRL (ppb) (EU and Turkish Codex)	MRL (ppb) (*Codex Alimentarius*)
Abamectin	1–4	0.9944	1.13	1.21	1.17	1.55	*	—
Doramectin	1–4	0.9842	1.14	1.24	1.17	1.58	*	15
İvermectin	1–4	0.9720	1.27	1.47	1.35	2.16	*	10
Eprinomectin	10–40	0.9915	11.38	12.23	11.77	15.89	20	20
Moxidectin	20–80	0.9894	23.79	26.32	24.86	36.21	40	—

CC_α = decision limit; CC_β = detection capability; LOD = limit of detection; LOQ = limit of quantitation; MRL = maximum residue limit.
* Not for use in animals from which milk is produced for human consumption.

Table 3 – Recovery of avermectins: Between-day repeat measures.

Compounds	Amount added (ppb)	Mean amount calculated (ppb)	SD	Mean recovery %	RSD	Ion ratio low/high
Abamectin	1	1.05	0.055	97	5	0.78
	2	2.02	0.107	97	5	0.71
	3	3.04	0.250	98	8	0.74
	4	3.96	0.196	97	5	0.86
Doramectin	1	1.01	0.058	91	6	0.35
	2	1.83	0.169	85	9	0.47
	3	3.38	0.206	111	6	0.45
	4	3.79	0.205	86	5	0.52
Ivermectin	1	0.96	0.116	80	12	0.14
	2	1.73	0.241	78	14	0.16
	3	2.60	0.205	82	8	0.10
	4	4.44	0.158	107	4	0.08
Eprinomectin	10	10.49	0.589	108	6	0.37
	20	20.76	0.822	94	4	0.41
	30	29.48	1.827	92	6	0.38
	40	39.89	1.644	106	4	0.36
Moxidectin	20	21.79	1.621	102	7	0.74
	40	42.49	5.187	92	12	0.76
	60	62.55	3.638	88	6	0.75
	80	76.40	3.266	80	4	0.80

RSD = Relative Standard Deviation; SD = standard deviation.

In this study, the Youden approach was applied by changing the following factors: different extraction (Strata X-C18 cartridges and liquid–liquid extraction), an acetonitril extraction volume (10–15 mL) and 5 g NaCl and 5 g $NaSO_4$. The final solution (0.1% TEA in ACN and ACN–0.1% TEA in water, 70:30), column temperature (40–50°C), flow rate (0.3–0.4 mL/min), and mobile phase composition (ACN–0.1% TEA in water; 70:30 and ACN–0.1% TEA in water; 85:15) were tested and checked. A significant difference was not observed between the factors.

3.6. *Stability*

Individual stock solutions were stable for 1 year stored in a freezer, the standard mix from the individual stock solution was stable for 6 months in a refrigerator and working standard solutions were stable for 2 months in a refrigerator [7].

4. Conclusion

To measure AVMs using the selective reaction-monitoring mode, full scan, and product ion spectra of the analytes were investigated under the LC conditions described in mass spectrometry. AVMs could be detected under the negative ionization mode ESI-MS conditions following 0.1% TEA in the mobile phase of water/acetonitrile.

Inoue et al [7] measured AVMs using the positive ionization mode ESI-MS. However, it seems possible that the $[M + Na]^+$ adduct ions are in the positive ionization mode. Our study already used $NaSO_4$ for extraction; therefore, the negative ionization mode was preferred in terms of selectivity and sensitivity of method.

Consequently, this LC/ESI-MS/MS method allows the simultaneous determination of five AVM residues in bovine milk. The method avoids the use of clean-up by Solid Phase Extraction (SPE) and should be performed quickly. The obtained validation results indicate accordance with the method of the European Commission Decision 2002/657/EC. The repeatability and within-laboratory reproducibility (precision) of the method were <14% for all analytes. This new analysis method has been used for rapid confirmatory analysis of AVM residues in milk samples.

Conflicts of interest

The authors declare no conflicts of interest.

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