Development and Optimization of a Simple and Sensitive Method for the Determination of Virginiamycin M1 Antibiotic in Aqueous Media by Capillary Electrophoresis

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Development and optimization of a simple
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Abstract

Virginiamycin antibiotic is used in Mexico for animal growth promotion, but has been banned in Europe due to risk of resistance development. Giving that monitoring of antibiotics is critical, the objective of this work was to develop an analytical method based on capillary electrophoresis and liquid-liquid extraction, to quantify virginiamycin antibiotic in livestock wastes. Linearity, precision, bias and extraction parameters were evaluated following ISO/IEC 17025 procedures. Method and extraction validation was satisfactorily, with average values of absolute recovery $AR = 86\%$, extraction efficiency $EE = 87\%$, and matrix effects $ME = -14\%$, $RSD < 15\%$ for concentrations as low as 50 $\mu\text{g/L}$.

Keywords: Capillary electrophoresis, Wastewater, Antibiotics, Monitoring, Extraction, Quantification

1 Introduction

Virginiamycin, an antibiotic belonging to streptogramin class, and mixture of two components: factors M1 and S1, acts individually against *Micrococcus aureus* and *Bacillus subtilis*, but in mixtures becomes highly effective against vancomycin and methicillin-resistant infections in humans, especially from *Enterococcus faecium* and *Staphylococcus aureus* (Hammerum et al, 1998).

Relevant properties and structure for M1 are depicted in Figure 1. Composed of lactone and containing pyrrolidine and oxazole rings and equal to mikamycin A, ostreogrycin A, streptogramin A and pristnamycin IIA (Goffic et al, 1977), M1 factor assembles a lipophilic, neutral, and low-degradable molecule mainly evacuated in excreta without relevant assimilation in animal tissues.

Mexico and the USA have approved virginiamycin as a feedstuff supplement, alone or combined with other agents in cattle, pig and poultry for disease prevention, increased weight growth and fodder efficiency, at doses ranging from 5 g/ton to 25 g/ton of food (Anadón and Martínez-Larrañaga, 1999). These growth promoters are administered for prolonged periods of time at

sub-therapeutic concentrations. However, with such scheme arises an increased risk of inducing antimicrobial resistance in animal pathogens, which could colonize humans and transfer their resistance to other microorganisms (Wegener, 2003). Evidence of adverse human health consequences include infections that probably would not occur, as with fluoroquinolone-resistant *Salmonella* cases (Ventola, 2015). Several researchers argue that resistant infections in clinical settings have been due to migration of genetic determinants, between environmental bacteria and human pathogens (Martinez, 2009b; Wright, 2010). Due to incorrect use of veterinary antibiotics, resistant bacteria populate the environment, animals and food products, increasing pathogen exposure and triggering human infection difficult to treat (Martinez, 2009a).

Europe banned use of virginiamycin for animal growth in 1998, due to concerns about impairing the efficacy of streptogramin to treat human infections due to rise of resistance (Aarestrup et al, 2001). However, Mexico and other countries consider virginiamycin safe and authorize it as a growth promoter. Because most of virginiamycin intake is excreted to environment (de Sanidad Inocuidad y Calidad Agroalimentaria, 2016), the risk of resistance development in environmental bacteria arise, justifying an antibiotic monitoring as part of a surveillance program (Sandegren, 2014; Bellanger et al, 2014; Bengtsson-Palme and Larsson, 2015). In fact, different studies revealed selection of resistant bacteria at low concentrations, like those found in aquatic environments receiving agricultural effluents (Hernando et al, 2006; Martinez, 2009b).

To quantify virginiamycin, scientists have preferred chromatography techniques, such as HPLC and GC, but these methods results in high costs and processing time. Capillary electrophoresis (CE) techniques stand out in monitoring applications due to their high efficiency, economy and low volume sample required. In CE, the analyst could use main techniques: (i) capillary

zone electrophoresis (CZE), and (ii) micellar electrokinetic chromatography (MEKC). In MEKC, micelles and additives dissolves in the sample or background buffer (BGB) to allow massive injection and quantify without loss of resolution (Aranas et al, 2009; Wuethrich et al, 2016). To our knowledge, none CE-based method has been published or validated to quantify virginiamycin factor M1 in waste effluents (Flurer, 2001; Tobback et al, 1999). In consequence, in this work the authors developed an ease and economical MEKC method to quantify virginiamycin M1 in livestock effluents. The specific objectives were to assess the effects on analyte separation from: i; addition of NaCl in samples, ii; injection time and iii; buffer composition. Method was validated according to ISO/IEC-17025 recommendations (Magnusson, 2014).

2 Methods

2.1 Reagents and Solutions

Antibiotic standard solutions were prepared weekly using virginiamycin M1 (Sigma Aldrich, purity 95 %, CAS 21411-53-0) with methanol (J. T. Baker) as diluent and stored at 275.15 K. Working solutions were prepared diluting standard solutions with NaCl (J. T. Baker) 0.5% w/w. Background buffer (BGB) was prepared mixing quantities of sodium dodecyl sulfate solutions (SDS, CE grade), phosphoric acid (85% w/w), monosodium phosphate, and β -cyclodextrin (Sigma Aldrich). All solutions and samples were filtered using 0.45 μ m nylon filters (Millipore).

2.2 Capillary Electrophoresis Procedures

An MDQ P/ACE Capillary Electrophoresis System (Beckman Coulter) was used to separate samples. A capillary tube filled with the sample and BGB

are subjected at a constant voltage and current, separating molecules at different electrophoretic velocities and read in a capillary window by a diode array detector (DAD). The analytical method is based on Micellar Electrokinetic Chromatography (MEKC), with BGB consisting of SDS, methanol, phosphates and β -cyclodextrin as partition modifier. The MEKC-CE technique was introduced by [Terabe et al \(1984\)](#) to analyze neutral compounds typically impossible to separate by capillary zone electrophoresis (CZE). The conditions and compositions depend on analyte and capillary selected. To obtain the best results, buffer and instrument conditions were optimized and validated using different approaches, as summarized in Figure 2.

A bare-fused silica capillary covered with polyamide (Polymicro Technologies) was used for separation using negative voltages. For this study, capillary length-to-window of 50 cm and diameter of 50 μ m was used to enhance resolution. However, different lengths of capillary tubes could be used, resulting in variable migration times but with no significant loss of efficiency at total lengths greater than 40 cm. Capillary conditioning was required for precision, with 2 min rinses of HCl 0.5 M, NaOH 0.5 M and BGB between runs. After conditioning, up to $\approx$ 15 cm of sample plug was injected. Both ends of capillary were placed in buffer vials and a negative voltage from 12 kV to 25 kV was applied. Temperature was held constant in the range of 293.15 K to 298.15 K. The instrument injects using as low as 5 μ L of sample volume, generally by hydrodynamic pressure. Samples and calibrators were diluted with NaCl solution from 0.25 to 1.0% w/w and 10% acetonitrile. Absorbance measures at the capillary window was read at 220 nm, recording an electropherogram with analyte and sample components signals. To quantify the analyte, a standard curve was built, correlating concentration against analyte peak area. Working range was selected as the linear portion of calibrated curve. Finally, assessment

of separation efficiency was realized comparing values of the number of theoretical plates N , resolution R_s and peak height H . While H is given directly, N and R_s were calculated according to equations 1 and 2, where t_m stands for migration time and w_h for peak width at half height.

$$N = 5.545 \left(\frac{t_m}{w_h} \right)^2 \quad (1)$$

$$R_s = \frac{t_{m2} - t_{m1}}{0.5 \times (w_{h1} + w_{h2})} \quad (2)$$

2.3 Development of Analytical Method

To determine the most significant factors of running buffer that affects analyte separation, an experimental design approach was chosen, as it enables lower time consumption and best data analysis. A screening method based on a 2^{4-1} reduced factorial design was set-up to identify the most significant buffer variables at two levels: A-pH; B-SDS; C- phosphate and D- β -cyclodextrin, as shown in Table 1. Response variables H and N were computed using Design Expert 7.0 software. Other variables, were optimized modifying one variable at a time with a constant running buffer. The selected variables were: *A-NaCl* percent added to standard; *B-separation voltage*; *C-injection time* and *D-cartridge temperature*. Selection of the best conditions was done comparing electropherograms and peak efficiencies.

2.4 CE Method Validation

The CE method was validated by evaluating precision, linear range, limit of detection (LOD), limit of quantitation (LOQ) and bias. An Analysis of Variance (ANOVA) was used to compare variation within groups at 95 percent confidence. The LOD is the lowest concentration that could be detected with

enough confidence; it was approximated by diluting a calibrator solution until signal of analyte is similar to noise, then analyzed ten times and calculated as $LOD = 3 \times S'_o$, where $S'_o = \frac{S}{\sqrt{n}}$ explains data dispersion. The limit of quantification (LOQ) was then estimated as $LOD \times 5$.

To quantify virginiamycin, a linear regression of measured absorbance against concentration was built using seven calibrators determined by triplicates at analyte concentrations of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/L. Precision was evaluated analyzing single and intra-day repeatability of the smaller calibrator along with two midpoints, running five and three repetitions respectively. For inter-day repeatability, analysis was done at three days at constant conditions. One-factor ANOVA was run to test for variability. Repeatability limit (r) was calculated as $r = 2.8 \times S_r$, where S_r is the standard deviation of repeatability. Intermediate precision was obtained by the square root of the sum of the squares of within-group and between-group precision. Then standard deviation of the repeatability S_r , was obtained by calculating the square root of the within-group mean square term, which represents the within-group variance as $S_r = \sqrt{MS_i}$. Contribution to the total variance of the clustering factor S_i is also obtained from the ANOVA test: $S_i = \sqrt{\frac{MS_e - MS_i}{n}}$. The intermediate precision S_I was calculated by combining both terms: $S_I = \sqrt{S_r^2 + S_i^2}$. Finally, bias was measured in relative terms as a percentage of recovery $R' = \frac{\bar{x}' - \bar{x}}{x_{add}} \times 100$ using blank and spiked samples, where $\bar{x}'$ is the average spiked value, $\bar{x}$ is the average value with the added concentration and x_{add} is the added standard (Magnusson, 2014).

2.5 Sample Treatment and Extraction Validation

Immediately after sampling, waste water was acidified to pH 4 with sulfuric acid 85% w/w, filtered and stored at 275.15 K. Antibiotic extract was obtained

using a liquid-liquid biphasic systems as described by [Díaz-Quiroz et al \(2020\)](#). Such procedure is based on the formation of an aqueous two-phase system (ATPS) comprised of citrate salt, acetonitrile and water. The system produce an organic layer rich on acetonitrile in top phase to which virginiamycin migrates. The bottom phase forms an aqueous phase rich in citrate salt, where majority of interferences are removed from the sample. For the ATPS sample clean up, a diagram phase and tie line at constant pH and temperature was used at an appropriate composition. Selected system was prepared at 298.15 K with 800 μL of sample volume, 0.6 g of citrate salt and 150 μL of acetonitrile. To form the two phases, the substances in the vials were dissolved aided by a sonication bath for 5 min. Then, vials were centrifuged for 3 minutes and an aliquot of 10 μL was transferred to a microvial. Finally, 90 μL of diluent was added, vortextured and ready for injection in the CE system. Fortified samples were prepared in the same fashion, but adding antibiotics to diluent. Performance was verified using the parameters of extraction efficiency EE , matrix effects ME and recovery AR , as disclosed in following equations:

$$EE = \left(\frac{C}{B} \right) \quad (3)$$

$$ME = \left(\frac{B}{A} - 1 \right) \quad (4)$$

$$AR = \left(\frac{C}{A} \right) \quad (5)$$

Each variable represents the peak area of virginiamycin M1 in the conditions: A , in the standard solution; B , spiked in sample after extraction; C , spiked in sample before extraction. Matrix effects indicate if suppression of

signal occurs (negative values), or if the signal has been enhanced (positive values).

3 Results

3.1 Method development

Initially, the MEKC method was initiated with the injection of a standard virginiamycin M1 solution dissolved in distilled water. A typical electropherogram for the separation is presented in Figure 3 at a concentration of 20 mg/L, at t_m around 13 min.

Then, a factorial design was employed to determine which BGB conditions affects H and N . The results are presented in Table 2, where H and N differ significantly for several treatments. All experiments were performed at 298.15 K, -14 kV and standard injection of 60 s at 13.79 kPa (2 psi), corresponding to an injection of about 15 cm of capillary length. Enhanced behavior at lower pH probably is due to reduction of electroosmotic flow at $\text{pH} < 4$. Likewise, micelle properties depend on pH; hydronium ions decrease electrostatic repulsion of the charge heads by decreasing the charge density on the surface of the micelle, thus increasing hydrophobic affinity for virginiamycin and granting efficient separations. Regarding buffer composition, the treatments with lower levels (–) of: sodium phosphate, SDS and β -cyclodextrin, allowed better separations. Behavior of the different variables are better understood using a 3D plot, as in Figure 4. Reviewing those surfaces, clearly β -cyclodextrin addition proved detrimental to separation and therefore optimum experimental conditions closely fluctuate around pH 2, 35 mM monosodium phosphate and 60 mM SDS.

Statistical information to estimate error and significance was done with aid of ANOVA. It was performed only for peak height H , because it is not

affected by small variations of t_m or capillary length. According to Table 3, the model has statistical significance and pH sets the main influence on response. Goodness of fit test also suggests adequate model adjustment and precision, with $Adj.R^2 > 0.8$ and $RSD < 15\%$. Regarding optimization of NaCl added to sample and injection volume, addition of NaCl from 0.1% to 1% to standard solution increased N massively; as for the standard peak N rises up to 7×10^5 . In this MEKC method, online concentration was based on salt addition to samples, in a manner ionic strength scale up two to three times higher than BGB (Palmer et al, 1999). It causes an analyte sweeping at the interphase of sample plug and BGB, allowing increased sample injection without affecting negatively the analyte separation. The salt comprised NaCl or KLi, enabling injection of sample plug up to 15 cm. Interferences from samples will affect the volume injected, reducing N and H but in the method conditions, injection length up to 15 cm did not affect the separations. However, injections higher than 15 cm resulted in gradual peak broadening and loss of N . Considering typical injection in CE fluctuates around 1 cm, then online concentration up to 15 folds of analyte could be attained. Finally, temperature between 295.15 K and 230.15 K and voltages between 12 kV and 20 kV performed satisfactorily.

3.2 Method Validation

Validation parameters evaluated were precision, working range, bias, LOD and LOQ . Linear regression and goodness of fit are presented in Table 4. Working range was validated from 0.05 mg/L to 3.2 mg/L. Repeatability and intermediate precision were 9.57% and 11.57% RSD respectively along the working range. Bias was calculated as $R\% = -8.51$ and $RSD\% = 12.16$, which represent acceptable values. $LOD \approx 0.093$ mg/L and $LOQ \approx 0.047$ mg/L were also estimated.

3.3 Extraction Validation

Figure 5 shows an electropherogram for blank and spiked sample at 5 mg/L. As noted, not major signal suppression appears at 1:9 weight ratio dilution, though spiked samples have to be analyzed for every run. Further, resolution $R_s \geq 1.5$ seems reasonable to detect and quantify virginiamycin M1. To verify the identity of analyte, Figure 6 shows EG's of samples fortified before and after extraction, at 50 µg/L and 500 µg/L respectively. Virginiamycin peak appears labeled and their vicinity is spanned in the bottom EG, showing the identity of virginiamycin unambiguously. Extraction data is presented in Table 5 for three concentrations. The computed parameters were: $EE = 0.87$, $ME = -0.14$ and $AR = 0.86$. Since signal suppression stand low, no significant matrix effects reduce analyte signal. Also, average values of EE and AR found satisfactory, allowing most analyte recovered from extraction procedure.

4 Conclusions

Data confirms extraction of virginiamycin as an efficient process, with satisfactory recovery and minimal matrix effects at a 1:9 sample dilution ratio. The method proves fast and reliable analysis for concentrations as low as 50 µg/L.

5 Tables

Table 1 Two-levels screening factorial design

Factor	(-1)	(0)	(+)
A: pH	2	3	4
B: Sodium dodecyl sulphate	35	50	70
C: Sodium phosphate ¹	30	60	90
D: β -cyclodextrin	0	5	10

Except for pH, treatment levels are concentrations expressed in mM

¹Sodium phosphate monohydrate

Table 2 Results of screening factorial design. Dependent variables, peak height H and theoretical plate numbers N

Run	A	B	C	D	H	N
1	3	90	50	5	4152	61134
2	4	120	70	10	2014	12314
3	4	60	30	10	1661	5639
4	3	90	50	5	4315	60415
5	4	120	30	0	2779	35468
6	3	90	50	5	3833	54890
7	3	90	50	5	3476	46119
8	2	120	70	0	4521	60757
9	2	60	70	10	4177	58972
10	2	60	30	0	5586	70000
11	4	60	70	0	2430	27863
12	2	120	30	10	4278	56884
13	3	90	50	5	5000	6000

Table 3 ANOVA data for screening factorial design of analyte peak height H

Source	df	F-value	p-value
Model	5	10.97	0.0056
A	1	47.55	0.0005
B	1	0.0349	0.8581
C	1	0.6855	0.4394
D	1	5.15	0.0637
AB	1	1.41	0.2801
Curvature	1	6.56	0.0428
Lack of Fit	2	0.265	0.7797
Adeq. Precision	R^2	$Adj.R^2$	RSD%
9.9766	0.9014	0.8192	13.38

Table 4 Virginiamycin standard calibration in dH₂O and extract from wastewater samples. Addition of ten percent v/v acetonitrile and 0.25 percent w/v NaCl was added to all calibrators as diluent

Calibration	Best-Fit Values		Std Error		Goodness of Fit	
	Slope	Y-intercept	Slope	Y-intercept	R squared	$Sy.x^1$
dH_2O	4075	-109.1	117.2	54.04	0.9955	98.9
Extract ²	3618	-189.1	149.53	86.63	0.9904	123.86

$$^1Sy.x = \sqrt{\frac{\sum(residual^2)}{n-k}}$$

²The extract was reconstituted with diluent in a 1:9 weight ratio.

Table 5 Validation of Virginiamycin M1 extraction using external standard calibration. Values are average of three replicates

$Y(\mu g/L)$	$\hat{Y}(\mu g/L)$	$RSD \times 100$
50	44.29	14.39
250	221.1	2.09
500	506	5.96

6 Figures

Fig. 1 Virginiamycin M1 molecule. Relevant properties calculated by OPERA: $\text{Log}P = 2.49$, $\text{Solubility} = 2.37 \text{ E-3 mol/L}$, $\text{Biodegr.HalfLife} = 3 \text{ days}$

Fig. 2 Flowchart of the general method development of Virginiamycin M1 analyzed by capillary electrophoresis in porcine wastewater

Fig. 3 Typical electropherogram of virginiamycin (1) standard analyzed by CE-MEKC at 20 mg/L. Conditions: 15 kV, 25 °C, capillary 40 cm length and 50 μ m ID, reverse polarity. Sample: 0.25 percent w/v NaCl in distilled water. Buffer: SDS 35 mM, sodium phosphate 30 mM, methanol 20 percent v/v, pH 2 acidified with HCl
Software: Karat 32

Fig. 4 Response surface plots for the reduced factorial design in the analysis of viginiamycin M1. Independent variables are pH and molar concentrations of SDS, sodium phosphate and β -cyclodextrin. Dependent variable is analyte peak height H . Concentrations of phosphate and cyclodextrin corresponds to following plots, coded in parenthesis: A, 35 mM phosphate without β -cyclodextrin ($-$, $-$); B, 70 mM phosphate and 10 mM β -cyclodextrin ($+$, $+$); C, 70 mM phosphate without β -cyclodextrin ($+$, $-$) and D, 30 mM phosphate with 10 mM β -cyclodextrin ($-$, $+$)

Software: Design-Expert 12

Fig. 5 Electropherogram (EG) of virginiamycin extraction in wastewater by MEKC. Bottom EG is from a blank sample and top EG is a spike added after extraction
Software: Karat 32

Fig. 6 Electropherogram (EG) of virginiamycin (1) extraction in wastewater by MEKC. Bottom EG is at 50 $\mu\text{g/L}$ and top EG is at 500 $\mu\text{g/L}$
Software: Karat 32

Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author C.Díaz-Quiroz, upon reasonable request.

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1083 Statements and Declarations

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1093 6.2 Competing Interests

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6.3 Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juan Francisco Hernández-Chávez and Jesús Fernando Robles-Castro. The first draft of the manuscript was written by Carlos Abraham Díaz-Quiroz. Gabriela Ulloa-Mercado and Carlos Díaz contributed to original concept, funding, supplied reagent and manuscript revisions and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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