DETERMINATION OF RIFAXIMINE IN MILK OF DAIRY COWS USING HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Detección de Rifaximina en la Leche de Vacas Lecheras Utilizando la Cromatografia Líquida de Alta Presion (HPLC)

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ABSTRACT

The objective of this work was to develop and validate a High Performance Liquid Chromatography (HPLC) method for the detection of rifaximine in milk of dairy cows. HPLC grade methanol and ammonium acetate as the Mobile phases, a C18 column and a UV detector were used. Validation of the method was carried out using standards of pure rifaximine dissolved in methanol. It was observed that linearity was above 0.99 of r^2. Average recovery was above 54%. A high specificity was evident and interfering substances did not affect the results. The minimum limit of detection was 30 ng/mL and the quantification limit was 150 ng/mL. For the kinetic analysis the antibiotic (100 mg) was infused into the udder of two lactating cows. For one cow a coefficient of depuration of 0.0122, with a half life of 24.6 hours and 8.65 g/mL*h (* = multiplied by), Area Under the Curve (AUC) was observed. For the second cow the coefficient of depuration was 0.00749 with a half life of 23.9 hrs and 7.75 µg/mL*h was detected. In the field rifaximine (100 and 200 mg) was infused into the udder of 20 cows at the end of the lactation period; thereafter samples were obtained from treated cows and it was observed that rifaximine was not detected in calostrum and milk of treated cows, using the described HPLC method. It was concluded that the use of HPLC is a reliable method to detect rifaximine in milk and colostrum of cows treated with the antibiotic.

Key words: Rifaximine, udder, cow, detection, milk, colostrums.

RESUMEN

El objetivo de este trabajo fue desarrollar y validar un método por cromatografía líquida de alta resolución (HPLC) para la detección de Rifaximina en leche de vacas**.** Se utilizó metanol y acetato de amonio grado HPLC como fases móviles, con columna C18 y detector ultravioleta. La validación del método se realizó mediante estándares de sal pura de Rifaximina disuelta en metanol. Se observó una linealidad superior al 0,99 de r^2; en la precisión se observaron desviaciones relativas menores al 5%. El porcentaje de recuperación promedio fue superior al 54%; se obtuvo una alta especificidad al evitar sustancias que interfirieran con los resultados. El límite mínimo de detección resultó de 30 ng/mL y su límite de cuantificación de 150 ng/mL. Para el estudio de la cinética de la Rifaximina se aplicó el antimicrobiano por infusión intramamaria (100 mg) en dos vacas en lactación. En este estudio, en una primera vaca, se identificó un coeficiente de depuración de 0,0122, una vida media de 24,6 horas, y un área bajo la curva (AUC) de 8.65 µg/mL*h. Para la segunda vaca el coeficiente de depuración fue de 0.00749 con una vida media de 23.9 horas, y un AUC de 7.75 µg/mL*h. En campo, en dos hatos lecheros con 800 vacas Holstein se aplicó el antimicrobiano por el orificio del pezón (100 y 200mg) en vacas que finalizaron la lactación; obteniéndose muestras de calostro y leche de 20 vacas después del parto. Estas muestras resultaron negativas a Rifaximina al ser analizadas por el método de HPLC. La aplicación de este método puede considerarse aceptable para la detección de Rifaximina en leche.

Palabras clave: Rifaximina, pezón, vaca, detección leche, calostro.

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INTRODUCTION

Success in a milking program is based on the following factors: Optimal function of the milking machines, hygiene of the milking process, control of mastitic cows and effective therapy of lactating and non lactating cows.

Furthermore, sampling of non lactating treated cows that were medicated with antibiotics for prevention and control of mastitis at the beginning of the drying off period; should be monitored systematically [1, 6].

During the drying off period, it is important to know the elimination time of antibiotics to avoid their residual presence in the milk capable of affecting its quality at the beginning of their lactation period.

An antibiotic residue is defined as any antibiotic substance or mixture of antibiotics in food for man or animals resulting from the therapeutic use of an antibiotic and includes any specified derivatives, such as degradation and conversion products, metabolites, reaction products, and impurities that are considered to be of toxicological significance

The resulting effects of the use of antibiotics gives way to three possibilities: toxicity, bacterial resistance and the economical impact of their presence; examples of toxicity for humans are the hypersensitive reactions, frequently present in individuals that are sensitive to β-lactamic antibiotics and some pathologies caused by carcinogenic or mutagenic drugs such as nitrofurazone [2, 6].

Different Health institutions in different countries regulate the presence of residues of drugs in foods and animal products to protect health and well being of the consuming public. The Food and Agriculture Organization (FAO) [3], the European Union [2] and the World Health Organization [12] are some of the International organizations that establish limits on the use of new drugs. In Mexico, the general law for Health of 1998, dictates similar requirements [10].

Rifaximin is a semi-synthetic non systemic antibiotic with good bactericidal activity, belonging to the family of naphthalene-ringed ansamycins (rifamycins group). It possesses a broad spectrum of activity against Gram-positive and Gram-negative bacterias. Rifaximin has a minimal absorption of <1% of its oral dose due to low aqueous solubility, low permeability, and P-glycoprotein efflux [3]. This results in the majority of the drug residing in the gastrointestinal tract, with more than 96% excreted in the feces, almost entirely as unchanged drug [4]. The latter properties of rifaximine indicates that this antibiotic can be used for localized infections such as mastitis in dairy cattle. It was reported that when infused in to the udder of dairy cows, no residues were detected in plasma of treated cows, and concentrations of 4 µg/g were present in the lactiferous sinus, 1.6 µg/g in the superficial parequima and 0.8 µg/g in the dip parenchima of the udder [1, 8]. Furthermore, it was observed that after udder infusion of rifaximine in 10 dry cows, concentrations of the antibiotic was 0.25 µg/g and 0.14 µg/g, respectively, in samples collected 28 and 42 hours after medication [5].

For the treatment of mastitis, the recommended dose of rifaximine is 100 mg for each quarter and the maximal permited residual presence of the antibiotic in milk for human consumption stablished by the Committee for Veterinary Products of the European Union is 60 µg/kg [2].

Liquid chromatography has become a universal methodology and is highly specific for the detection of rifaximine and other antibiotics in milk, blood, faeces and other fluids [5].

The justification of this work was based on the need of a high sensitivity method for the detection of rifaximine in cow's milk. Objectives of this work were to implement and validate the use of High Pressure Liquid Chromatography (HPLC) for the study of the kinetics of rifaximine in the milk of dairy cattle, and to study the presence of the antibiotic in colostrum and milk of cows treated with rifaximine at the beginning of the dry period.

MATERIALS AND METHODS

This work was carried out in two phases: first, mayor absorbance of rifaximine was studied using spectrophotograhy, maximum absorption through uv scan was found at 455 nm. A Shimadzu – 1700 Double Beam UV- Visible Spectrophotometer, (Japan) 1 cm matched Quartz cells were used for all absorbance measurements.

Thereafter, validation of the liquid chromatography method was carried out using a UV detector for the identification of pure rifaximine, the variables validated were: linearity, repeatability, specificity, precision, minimum detection limit, quantification limit and stability.

In the HPLC method the following elements were used:

Binary pump, movil phase A (molar solution of ammonium acetate), movil phase B (methanol HPLC grade), a C_{18} column of 150 mm and 4 µ particle size, automatic sampler Perkin Elmer 200, UV detector Perking Elmer 785A and the computer package Total Chrome as a integrator of the analysis (PowerChrom 280 System Model ER280. eDAQ Pty. Ltd. USA).

A pure rifaximine standard was prepared with methanol (0.0513g in 50 mL of methanol), standards were stored deep frozen in a Whirpool W8TXNWFWT house refrigerator,Mexico, until assayed.

Three standard dilutions were made in methanol (1/100, 1/200 and 1/400), to obtain a final concentration of 513, 256, 128 ng/ 50µL (50 µL= injection volume). The duration of the analysis per sample was of 7 minutes running time (4 minutes from 50 to 100%, and 3 minutes to return to initial condition).

The kinetics of rifaximine in the milk of the mammary gland was calculated considering the total milk produced by

the gland and the no diffusion characteristics of rifaximin. The depuration coefficient of the antibiotic infused in each quarter was studied using two healthy Holstein cows near drying off, in a design of 2 X 2 with two repetitions.

A 100 mg dose of rifaximine was infused in two quarters of each mammary gland and the two remaining quarters were used as control. In a second trial, 200 mg of rifaximine were infused in two quarters of the mammary gland.

Milk samples were collected before the antibiotic infusion, and the following samples were collected for 7 consecutive days before milking was carried out. Samples were refrigerated (Whirpool, W8TXNWFWT house refrigerator, Mexico) and there after transported to the Animal Nutrition Laboratory of the Faculty of Veterinary Medicine, Universidad Nacional Autónoma de Mexico, Coyoacan, Distrito Federal, Mexico for the HPLC determination of rifaximine.

Analysis of milk samples

Five samples of 1 mL each were extracted with 5 mL of methanol and centrifuged at 3,400 rpm (2 g) for 15 minutes (Cole Palmer centrifuge EW-17305.00).

Once samples were extracted and were analized using the described HPLC validated method. Once the minimum detectable quantity was determined together with the maximum concentrations, a regression curve was made and the concentration of rifaximine was determined using the reference signal from the detector with the same retention time.

Results were evaluated with the statistic package PKa Analyst (IBM SPSS Statistics 20) for the values of Area under the Curve (AUC), elimination constant and half life.

For the second phase of the study, 10 cows from two different milk producing units were selected at random, and the dose of rifaximine used was as follows:

At the end of the milking period, and using the California Mastitis Test (CMT), a 100 mg of rifaximine were infused into the trial udders and graded with the CMT as negative, traces, one and two grades. When the CMT was grade 3, rifaximine was infused at a dose of 200 mg.

There was a period of 60 to 95 days for the dry period, and samples of colostrum and first milk were collected at 1, 2, 3, 4 and 5 days of lactation. Samples were deep frozen until studied to detect rifaximine using HPLC.

RESULTS AND DISCUSSION

Validation of the chromatographic method

First, as previously described. the physicochemical characteristic of rifaximine were identified by spectrometric scanning, it was observed that the maximum absorption of the wave length using the ultraviolet light was 455 nm.

To validate the HPLC method for the identification of rifaximine. the following variables were considered:

Linearity, established through the regression index using three standards: 128, 256 and 513 ng, where the size of the signal should be proportional to injected antbiotic. And the linearity is given by the value of the area of r^2 in a regression line of all three concentrations was 0.992 (FIGS. 1-3 y TABLE I).

Repeatability of these three concentrations (128, 256, 513ng/50µg) was determined after 5 repetitions of each one. Relative deviation for 128 ng was 3.303%, for 256 ng was1.19% and for 513 ng was 3.32%. The standard deviation for all three samples was 2.603% (TABLE II).

Precision of the method was evaluated by the identification of the average and the deviation of the relative area and the retention time after repetitions of all three concentrations (128, 256, 513 ng/50 L). At 128 ng, the average area was 527605 µV*s, average retention time was 0.52 min, giving the area relative deviation value of 3.97%, and for retention time 1.71% (FIG. 3).

With 256 ng concentration the average area was 1316516 µV*s with an average retention time of 0.519 minutes, giving the area relative deviation value of 4.43%, time or retention was 1.92% (FIG. 2).

The average area obtained for the 513 ng concentration was 2358419 µV*s, with an average retention time of 0.527 minutes, the resulting relative deviations were 4.25% for the area and 2.07% for retention time (TABLES II, IV, FIG. 1).

The exactitude of the method was obtained through the recuperation of rifaximine when added to the milk sample (1 mL of the standard 1/10, 4 mL of milk and 5 mL of methanol) after 4 repetitions, giving average values of recuperation of 54.17% with a relative deviation of 4.5% (TABLE VI).

Specificity was obtained after the injections of the standard and the added milk with rifaximine, an interfering signal was not found when considering time of retention similar or equal to rifaximine, that is, no elements other than rifaximine were observed with a similar wavelength as that of the antibiotic here studied.

The minimum detection limit was computed identifying the size of area response considering twice the response at the limit of the signal coming from the detector. For rifaximine was 30 ng/mL. The Minimum quantification level was estimated multiplying the minimum detection level with volume of the injection and divided between the volume of the sample. In this case was in the order of 150 ng of rifaximine.

The standard for rifaximine remained stable when kept under at 4°C; all samples were kept frozen in a refrigerator (Whirpool, W8TXNWFWT house refrigerator, Mexico) until studied. The stability was corroborated injecting the standards three times with a 30 day interval. The relative deviation obtained for 128 ng was 3.22%, for 256 ng was 1.24% and for 513 ng was 1.75% (TABLE VII).

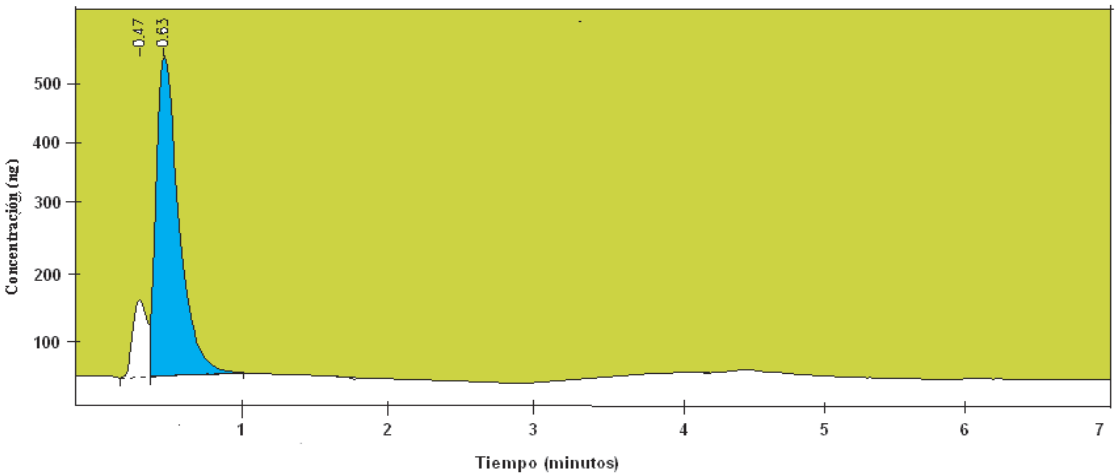

FIGURE 1. RIFAXIMINE ANALYSIS (513 NG CONCENTRATION). THE BLUE PEAK REPRESENTS THE ANTIBIOTIC. THE GRAPH IS THE OUTPUT OF THE TOTAL CHROME PROGRAMME.

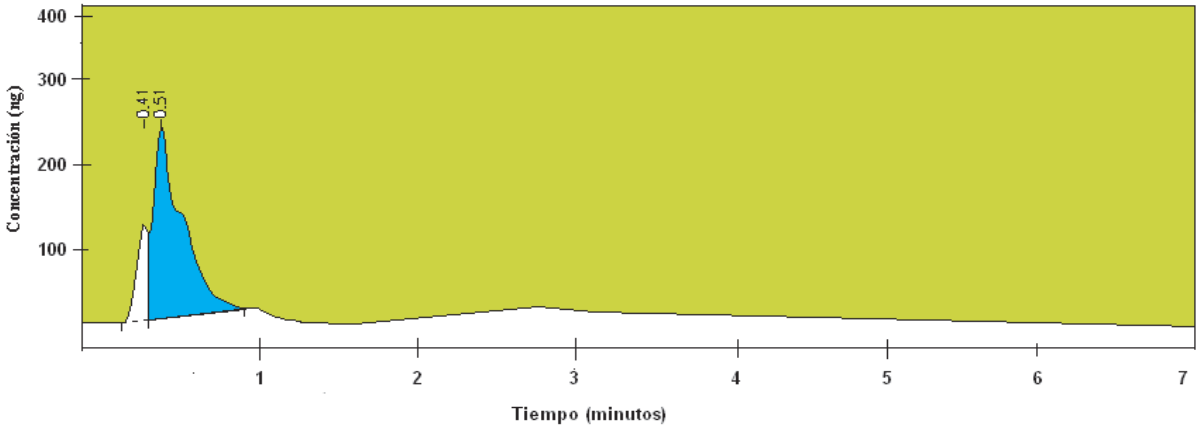

FIGURE 2. RIFAXIMINE ANLYSIS (256 NG CONCENTRATION) THE BLLUE PEAK IS THE ANTIBIOTIC.

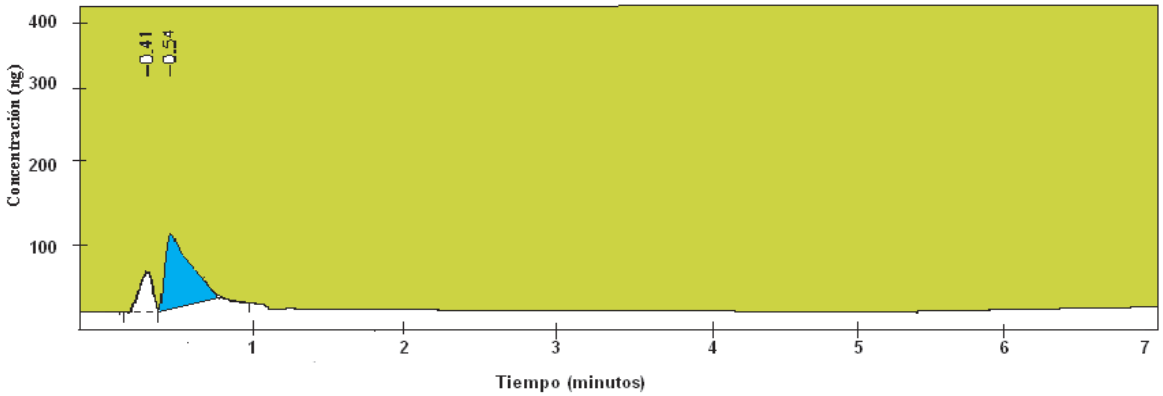

FIGURE 3. RIFAXIMINE ANALYSIS (128 NG CONCENTRATION). THE BLUE PEAK IS THE ANTIBIOTIC.

Kinetics of Rifaximine in milk

Using two of the rifaximine medicated cows, the antibiotic was detected present for 5 days after the intrammamary infusion with concentrations that fluctuated between 1684.2 ng/mL and up to 1112.2 ng/mL in the first cow. In the second cow concentrations of the antibiotic fluctuated between 3373.927 ng/mL on the fourth day. On the sixth day rifaximine was not detected (TABLE VIII).

To study first order kinetics the formula $C_R=C_i^{*eat}$ was used, where C_R represents the residual concentration (716 ng/mL); C_I initial concentration (3370 ng/mL); **e** is naperian

TABLE I
CONFIRMATION OF LINEARITY (R^2) FOR RIFAXIMINE STANDARDS (RIFA).

Assay	Dilution	mg/mL	ngRifa/50µL	Area	r^2
1	0.01	0.01026	513	2364994	0.992
2	0.01	0.01026	513	2423337	
3	0.02	0.00513	256	1368923	
4	0.02	0.00513	256	1335423	
5	0.04	0.00257	128	500364	
6	0.04	0.00257	128	568163	

r^2=coeficiente de correlació. r^2=correlation coeficiente.

TABLE II
RESULTS OF STANDARDS STUDY OF RIFAXIMINE TO OBTAIN REPEATABILITY.

ng/50µL	Area µV*s	Area µV*s	Area µV*s	Area µV*s	Area µV*s	Prom µV*s	Desv. relativa
513	2364994	2423337	2314141	2219911	2291076	2322692	3.303
256	1368923	1335423	1371355	1344997	1367111	1357562	1.19
126	550364	551105	558389	568173	519390	549484	3.32

Desv relativa= Relative deviation µV*s= milivolts per second.

TABLE III
HPLC PRESISSION FOR RIFAXIMINE (ESTÁNDAR CONCETRATION = 128 NG/mL).

Injection No	Area µV*s	Retention time
1	551496	0.51
2	506983	0.52
3	521009	0.53
4	548963	0.53
5	511236	0.53
Media (Mean)	527605	0.52
Desv estándar (SD)	20995	0.00894
Desv relativa (Relative D	3.98%	1.71%

Desv estándar= desviación estándar. Desv relativa=desviación relativa. µV*s=milivolts por segundo.

TABLE IV
HPLC PRESISSION FOR RIFAXIMINE (STANDARD CONCENTRATION = 256 ng/mL).

Injection No	Area µV*s	Retention Time
1	1368923	0.53
2	1335423	0.52
3	1371355	0.51
4	1244997	0.51
5	1267111	0.53
Media (Mean)	1316516	0.519
Desv estándar	58441	0.01
Desv relativa	4.43%	1.92%

Desv estándar= desviación estándar. Desv relativa=desviación relativa. µV*s=milivolts por segundo.

logarithm; **a** represents the depuration coefficient that was 0.0122 and the half life of 24.6 hours; the area under the curve was 8.65 µg/mL*h obtained with the PK analyst computer program (FIG. 1). Using the same formula for the second cow, depuration coefficient was 0.00749 with a half life of 23.9 hours and AUC of 7.75 µg/mL*h.

Field study

From samples of the 20 selected cows of the two productions units and medicated at the beginning of the drying off period with 100 and 200 mg of rifaximine samples collected were negative for rifaximine (FIG. 4).

The results obtained for the validation of the HPLC method were considered satisfactory. When linearity was studied, a high correlation of 0.992 was observed, similar results were found with the requirements of the European Union. Correlation value for repetitivity of the method was 0,997 that is above of Bacteriology Analytical Manual suggestion, [11] that established that the ideal value was >0.98. Results of 4.25 and 4.43% on the precision of the areas, and of 2.07 and 1.92% were considered to be out of the area; when comparing with

TABLE V
HPLC PRESISSION FOR THE DETECTION OF RIFAXIMINE (STANDARD CONCENTRATION = 513 ng/mL).

Injection number	Area µV*s	Time of Retention (min)
1	2364994	0.52
2	2423337	0.54
3	2314141	0.52
4	2219911	0.52
5	2480822	0.54
Media (Mean)	2358919	0.527
Desv estándar	100449	0.01
Desv relativa	4.25%	2.07%

Desv. estándar= desviación estándar. Desv. relativa=desviación relativa. µV*s=milivolts por segundo.

TABLE VI
RECUPERATION ANALISYS OF RIFAXIMINE.

Número de ensayo	ng Rifa/50µL	Cantidad recuperada	%
1	205.2	109.967	53.59
2	205.2	114.781	55.93
3	205.2	104.583	50.96
4	205.2	115.688	56.37
		Media	54.17
		Desv. estándar	2.48
		Desv. relativa	4.59

Desv. estándar= desviación estándar. Desv. relativa=desviación relativa. µV*s=milivolts por segundo.

TABLE VII
MEASURING THE STANDARD STABILITY.

ngRifa/50µL	Area µV*s	Area µV*s	Area µV*s	Prom µV*s	Desv. relativa
513	2364994	2423337	2342689	2377007	1.75
256	1368923	1335423	1348693	1351013	1.24
128	527163	510388	544369	527306.7	3.22

Desv. estándar= desviación estándar. Desv. relativa=desviación relativa. µV*s=milivolts por segundo.

TABLE VIII
CONCENTRATION OF RIFAXIMINE IN MILK OF LACTATING COWS TREATED WITH THE ANTIBIOTIC.

Vaca	Día	Area µV*s	Concentración ng/mL	Vaca	Día	Area µV*s	Concentración mg/mL
46	1	*	*	102	1	296797	1102.415
46	2	806895	3373.927	102	2	427463	1684.282
46	3	434615	1716.13	102	3	299726	1115.458
46	4	205794	697.17	102	4	252270	904.132
46	5	*	*	102	5	299009	1112.265
46	6	*	*	102	6	*	*
46	7	*	*	102	7	*	*

µV*s=milivolts por segundo.

different works using HPLC, they reported variations coefficients of 3, 10.8 and 9.1% [8] these variations show that the operator´s skill and laboratory facilities are important factors that can influence final results.

The average percent of recovery (54%) appears to be in the low range when compared with values mentioned in other works [9] and in the laboratory manuals, in which it is established a 90% recovery [11]. There is a need to do at least two extractions of the same sample with the solvent in order to have a complete 90% extraction, quantity acceptable during the validation of the method.

The resulting specificity allows for exact measurements of the area and the height of the peak that corresponds to rifaximine in the chromatogram. The peak produced by the solvent front is given before the peak of rifaximine and it is easily discarded using time of retention given by the standard or by the addition of a known concentration, and that can be used as a reference.

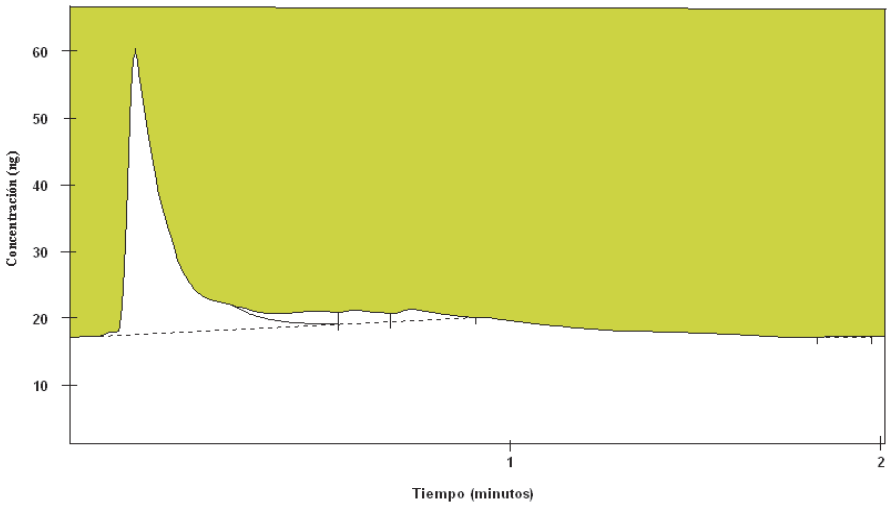

FIGURE 4. COLOSTRUM (FIRST DAY OF LACTATION). NO ANTIBIOTIC SIGNAL WAS DETECTED.

With the established method it is possible to do the corresponding measurements of the minimum required quantities demanded by the European Union (60 ng/mL), it should be recognized that European Union establishes very strict levels for quality control of their products. Other alternatives for this work are fluorescence mass detectors [1].

This proposed method, developed and validated can be used in accordance to the level of technology of each country, and must be used to improve the quality of animal and agricultural products for human consumption.

There are no reports about the kinetics of rifaximine in lactating cows because the antibiotic is formulated only for use in dry cows. However, there are some reports of the determination of rifaximine in rats and humans [7, 13, 14].

The undetectable levels of rifaximine in colostrums after intra mammary infusion at the end of lactation corroborate results reported by the European Union and others [1, 10]. Some reports showed concentrations of 4 µg/g in gland tissue and on the sinus lactiferous, with no presence in plasma of the studied cows [8, 9].

CONCLUSIONS

The described method is in accordance with standards related to the identification of a chromatographic method for the determination of rifaximine in milk and colostrums of dairy cattle.

Rifaximine does not persist in colostrums and milk when used in a mastitis control program for cow's at the beginning of the dry period.

When rifaximine is infused in lactating cows, the antibiotic presence persisted for 5 days with concentration that varied from 697.17 ng/mL to 1112.2 ng/mL

The use of HPLC for the detection of rifaximine in the milk of dairy cows is adequate and it is possible to adapt to daily checks to avoid the presence of the antibiotic in milk for human consumption.

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