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# DEVELOPMENT AND VALIDATION OF AN HPLC METHOD TO DETERMINE LASALOCID IN RAW MILK SAMPLES FROM DAIRY COWS

## Desarrollo y validación de un método por HPLC para determinar Lasalocida en muestras de leche cruda de vacas lecheras

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**ABSTRACT**

The ionophore lasalocid has been approved for use as a ruminant feed additive in many countries. This paper describes a simple and rapid method for the determination of lasalocid in cow milk using high performance liquid chromatography with ultraviolet detection. The method is specific for the analysis of lasalocid in raw milk following a liquid-liquid extraction and was validated for the drug in bovine raw milk. The standard curve was linear from 0.5 to 3.0 µg/mL Intra (7.2%) and inter (7.0%) day (d) precision and accuracy (84%) of the method were within the limits proposed by Food and Drug Administration in the Bioanalytical Method Validation Guidance. The stability of lasalocid was established during three freeze-thaw cycles and long-term stability at -30°C for 79 d. The sensitivity and simplicity of the method make it suitable for control of lasalocid residues in raw milk of dairy animals.

**Key words:** Ionophores, chromatography, raw milk, bioanalytical.

**RESUMEN**

El ionóforo lasalocida ha sido aprobado para su uso en rumiantes en varios países. Este artículo describe un método simple y rápido para la determinación de lasalocida en leche de vaca utilizando cromatografía líquida de alta eficiencia con detección ultravioleta. El método es específico para el análisis de lasalocida en leche cruda bovina, con una extracción líquido-líquido y fue validado para este fármaco en la leche. La curva de calibración fue lineal de 0,5 a 3,0 µg/mL. La precisión intra (7,2%) e inter (7,0%) día (d) y la exactitud (84%) estuvieron dentro de los límites propuestos por la Food and Drug Administration en la Guía de Validación de Métodos Bioanalíticos. La estabilidad de la lasalocida fue establecida en tres ciclos de congelamiento-descongelamiento y la estabilidad a largo plazo, la temperatura fue de -30°C, se desarrolló durante 79 d. La sensibilidad y sencillez del método lo hacen adecuado para el análisis de residuos de lasalocida en muestras de leche cruda bovina.

**Palabras clave:** Ionóforos, cromatografía, leche cruda, bioanalítico.

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## INTRODUCTION

Ionophores are polyether compounds used in veterinary applications as cocciodiostats in poultry and livestock and as growth promoters in ruminants to increase food conversion efficiency [15].

Lasalocid belongs to a group of compounds called carboxylic ionophores with a molecular structure characterized by a polyether composition (FIG. 1). Ionophores are complex high molecular weight molecules, produced by various Streptomyces species (*Streptomyces lasaliensis* in the lasalocid case) [17]. The molecule of lasalocid have a free carboxylic and an aromatic groups. Carboxylic ionophores move ions across lipid by layers binding the ions from one side of the cell membrane, moving the resulting complex across the layer, and releasing the ions on the other side of the cell membrane [14]. Consequently, the gradients of $Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$ are diminished, causing death cell [1, 27]. Lasalocid is a divalent ionophore compound [1, 25]. It transports bivalent ions such as

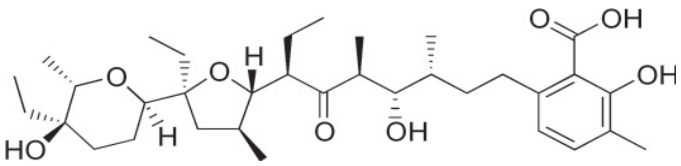


**FIGURE 1. MOLECULAR STRUCTURE OF LASALOCID.**

$Ca^{2+}$ and $Mg^{2+}$ [1, 18], and monovalent ions as $K^{+}$ [1, 2]. The ionophore compounds are active against Gram-positive organisms and mycoplasmas [1]. The cell wall of most Gram-negative bacteria does not permit the passage of hydrophobic molecules with molecular weights above 600 g $mol^{-1}$ not being susceptible to the action of ionophores [1, 27].

Ionophores were used in veterinary medicine until 16 years after their discovery due to their high toxicity. Toxicity studies have shown that the primary target organs of ionophore are cardiac and skeletal muscles as well as peripheral nerves. Many cases of severe poisoning in poultry, horses (*Equus caballus*) and other animals have been reported, however, fewer numbers of reports affecting cattle (*Bos taurus*) have been described [11, 14]. The increasing administration of these agents in veterinary medicine may represent a potential hazard to humans, as residues of these drugs may be present in tissues or fluids used for human consumption.

Lasalocid has been used extensively in the beef industry and, in some countries, in lactating dairy cattle [15]. According to the European Union (EU) [8] and the Food and Drug Administration (FDA) [14] regulations, lasalocid is not authorized for use in lactating dairy cows.

Various methods have been described in order to determine the presence of ionophores used in veterinary medicine. Analytical techniques such as high performance liquid chromatography (HPLC) with ultraviolet (UV) detection [3, 5, 6, 20, 21, 26], fluorescence detection [9, 16] and mass spectrometric detection [7, 12, 13, 22-24] have been used for this purpose. For this methods, sample preparation includes precolumn or postcolumn derivatization and solid phase extraction. The quantification limits (LOQ) in HPLC-based methods are mainly higher than 1 mg/kg, with some exceptions for lasalocid and narasin (LOQ of 0.5 mg/kg) [19]. Liquid chromatography-mass spectrometry (LC-MS) has been also used to determine polyether ionophores in different matrices. The use of a mass spectrometer detector minimizes sample preparation and increases the sensitivity of the method [19]. However, the equipment necessary is expensive compared with other detectors used for chromatography. It would be of interest to develop a simple and efficient method to determine lasalocid in raw milk to maintain control milk quality produced for human consumption. The objective of this work was to develop and validate a screening HPLC with UV detection method of lasalocid in bovine raw milk samples according to Bioanalytical Method Validation Guidance set by the FDA [10].

## MATERIALS AND METHODS

### Chemicals and Reagents

All chemicals used were purchased from Merck (Darmstadt, Germany), and included methanol and dichloromethane (HPLC grade), sodium chloride (analytical grade) and trifluoroacetic acid (reagent grade). Water was generated by an ELGA purification system (HPLC grade) and nitrogen gas was supplied by AGA Chile (Santiago, Chile). Lasalocid sodium salt (95.1%) was supplied by Alpharma Chile. All the solutions were filtered before HPLC injection using PTFE filters, 0.45 µm from Merck (Darmstadt, Germany).

### Chromatographic conditions

High performance liquid chromatography (HPLC) was performed using a Shimadzu Prominence HPLC system (Shimadzu, Tokyo, Japan) consisting of a LC-10AT pump, and SCL-10A system controller, an degasser DGU-20A5, an SIL-20A auto injector, and a SPD-M20A detector. Chromatographic data were acquired and analyzed using LC-Solution Chromatography Data System (Shimadzu, Tokyo, Japan).

Working wavelength in the detector was set at 315 nm. Separation was carried out on a C18 column, (250 x 4.6 mm, 5 µm), Kromasil, (Sweden), using methanol: water: trifluoroacetic acid 0.45% 90:10:3 v/v as an isocratic mobile phase, at a flow rate of 1.2 mL/min, at 25°C. The mobile phase was filtered through a nylon membrane filter (0.45 µm pore size) and ultrasonically degassed prior to use. Total time for analyse was 14 min. One hundred µL of standard solutions and analytical samples were injected to the LC system in order to increase sensitivity. The peak area for response was measured.

### Standard stock and working solutions

All stock and working solutions of sodium lasalocid were diluted in methanol and prepared under low light intensity. A primary stock solution (0.5 mg/mL) and secondary working stock solutions of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg/mL were prepared.

### Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared for each run by adding the individual working stock solutions to 1 mL of pooled blank bovine raw milk, and vortex-mixed for 30 seconds. Quality control samples were prepared on the first day of validation using stock solutions at 0.5, 1.5 and 3.0 µg/mL. All stock and working solutions were stored at 4°C and used to obtain the standard curve on the day (d) of analysis. Quality control (QC) samples were aliquoted and stored until analysis under low light intensity.

### Milk sample preparation

Fresh raw bulk milk was collected directly from the milk tank at the experimental dairy farm of the Universidad Austral de

Chile. Dairy herd was based on 62 lactating Holstein Frisian cows fed with a controlled ration based in pasture, grass silage and free of additives concentrate (Iansagro®, Chile). Milk sample were kept in glass containers at 4°C and used within 48 hours. One mL of a representative well-mixed full milk sample was added to a 15 mL centrifuge tube along with 6 mL of methanol and sonicated in an Ney 104 H ultrasonic bath (Ney Ultrasonics, Bloomfield, EUA), for 30 seconds. Additionally, samples were blend for 30 seconds and let at room temperature (± 20°C) for 10-15 min. Finally, samples were centrifuged at 600 g for 10 min until a pellet was produced.

Lasalocid was extracted by a liquid-liquid procedure. Supernatants were decanted into a 250 mL separator flask. Then, six mL of NaCl solution (10% w/v) were added. The lasalocid contained in the aqueous methanol supernatant was extracted with 12 mL of dichloromethane (DCM). The lower phase (DCM) was collected and dried under a nitrogen stream.

**Validation method**

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation in the matrix raw milk for this case [10]. The following validation parameters were assessed: selectivity, linearity, sensitivity, precision, accuracy, recovery, and stability.

The selectivity of the method was assessed by the absence of any interference in the elution times of lasalocid. Pooled milk samples were analysed after being pre-treated as described above.

Linearity was quantified using the peak area of the analyte. Peak areas were plotted associated with the analyte concentration of six calibration standards ranging from 0.5 to 3.0 µg/mL including the LLOQ (Lower limit of quantification). The standard curve were in the form $y = a + bx$. Concentrations of lasalocid were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µg/mL, respectively. The calibration curve used to determine the concentration of lasalocid in experimental samples was set at a correlation coefficient (r) of 0.995 or better.

The limit of detection (LOD) was defined as the lowest detectable concentration with a signal:noise ratio of three The lower limit of quantification (LLOQ) was defined at least five times as the analyte response compared to the blank response. At this point, the precision was set on 20% and the accuracy on the 80-120% range.

Accuracy and precision were determined by analysis of quality control standards of lasalocid (0.5, 1.5 and 3.0 µg/mL) with six replicates in the same day and one on each of three different days, respectively. Accuracy should be within 75-115% and precision, expressed as coefficient of variation (CV) should less than 15%.

The recovery of lasalocid from milk was determined following liquid-liquid extraction with methanol and dichloromethane at three concentrations of the drug: 0.5, 1.5 and 3.0 µg/mL. Recovery was assessed comparing post-extracted raw milk samples with unextracted samples (in the reconstituting solvent) at the same concentrations. Post-extracted samples were prepared by the addition of adequate volume of working solutions into 1 mL of raw milk and processed as described in section “Milk sample preparation”. Recovery of lasalocid was determined by comparing each peak area of extracted QC standards with the peak area of unextracted standard solutions containing the corresponding concentration of lasalocid dissolved in a methanol:water (90:10) medium. Recoveries were performed in triplicate.

Stability of lasalocid in milk was tested using QC standards for three freeze-thaw cycle and long term stability. In each freeze-thaw cycle, the quality control samples were frozen at -30°C for 24 h and thawed at room temperature (± 20°C). Long-term stability was evaluated by storing the quality control samples at -30°C during 79 d. The results were compared with freshly prepared QC samples.

## RESULTS AND DISCUSSION

The present study describes a specific, isocratic, and reliable as well as low-cost HPLC-UV method for the determination of lasalocid in milk samples. The free acid or the salt form of lasalocid adopt a cyclic structure [26] where the carbonyl group is directed inward and participates in ion metal chelation. In contrast with other ionophores, lasalocid cannot be derivatized because of steric hindrance. Fortunately, lasalocid detection is possible in a non derivatized form by UV absorption of the benzenic group at 315 nm [6]. An internal standard (I.S.) was not used because other polyether ionophores must be derivatized for HPLC analysis with UV detection. Calibration standards were run daily to maintain a control of the procedure.

Detection of a drug in biological media is a complex procedure, mainly associated to matrix characteristic, because of this, different extractions using solvents were employed to effectively separate drug components from endogenous interferences. The ultimate sensitivity and selectivity of the method may be limited to the clean-up procedures. To avoid losses associated to the clean-up procedure and to increase the recovery of the analyte the number of clean up steps in a sample preparation should be kept to a minimum [26]. In this way, sample preparation was based in the solubility of lasalocid in solvents like methanol and dichloromethane [9]. The latter showed no satisfactory results due to the adsorption of lasalocid to the polypropylene container. The best extraction results were obtained with methanol/NaCl (10% w/v)/DCM (6:6:12). The liquid-liquid extraction was time-consuming but the chromatographic requirements and inexpensive procedure for sample preparation made this method widely suitable for routine monitoring.

The chromatographic parameters (retention time, peak shape and peak resolution) were tested under several experi-

mental conditions. A series of mobile phases with different proportions of organic solvents and pH values were evaluated. A pure sample of lasalocid and standard solutions prepared in methanol were suitable for chromatographic conditions. Lasalocid was separated from the milk components on a C18 chromatographic column. The peak of lasalocid was well resolved under the conditions described and showed good shape with adequate asymmetry factor. Moreover, the mobile phase allowed the compromise between resolution and analysis time. An advantage of this method was the application of an isocratic mobile phase with a simpler compared to the complex compositions of mobile phase and/or gradient elution used in other HPLC methods [4]. The retention time of lasalocid was 11.33 ± 0.2 min (FIG. 2) under the chromatographic conditions employed here. Chromatograms showed a clear and excellent separation between lasalocid and endogenous interferences from milk, with absence of interfering peaks at the retention time of the lasalocid.

Data for the parameters used to validate the method were according to those described by FDA guideline for bioanalytical methods and well within the set limits.

The linearity of the matrix-assisted calibration curve was tested using a least-square method, accordingly the determination of lasalocid in the matrix was linear in the range of 0.5 – 3.0 µg/mL, with a correlation coefficient of 0,996.

Accuracy was higher than 77.6% (mean of 84.5%) and precision for the three concentrations of analyte were in the 4.8 to 13.3% range (TABLE I).

LOD and LLOQ values were 0.03 and 0.5 µg/mL, respectively. The recovery rate was larger to 80% in six repetitions. On the other hand, samples with lasalocid in cow milk proved to be stable during 79 d stored at -30°C and through three freeze and thaw cycles.

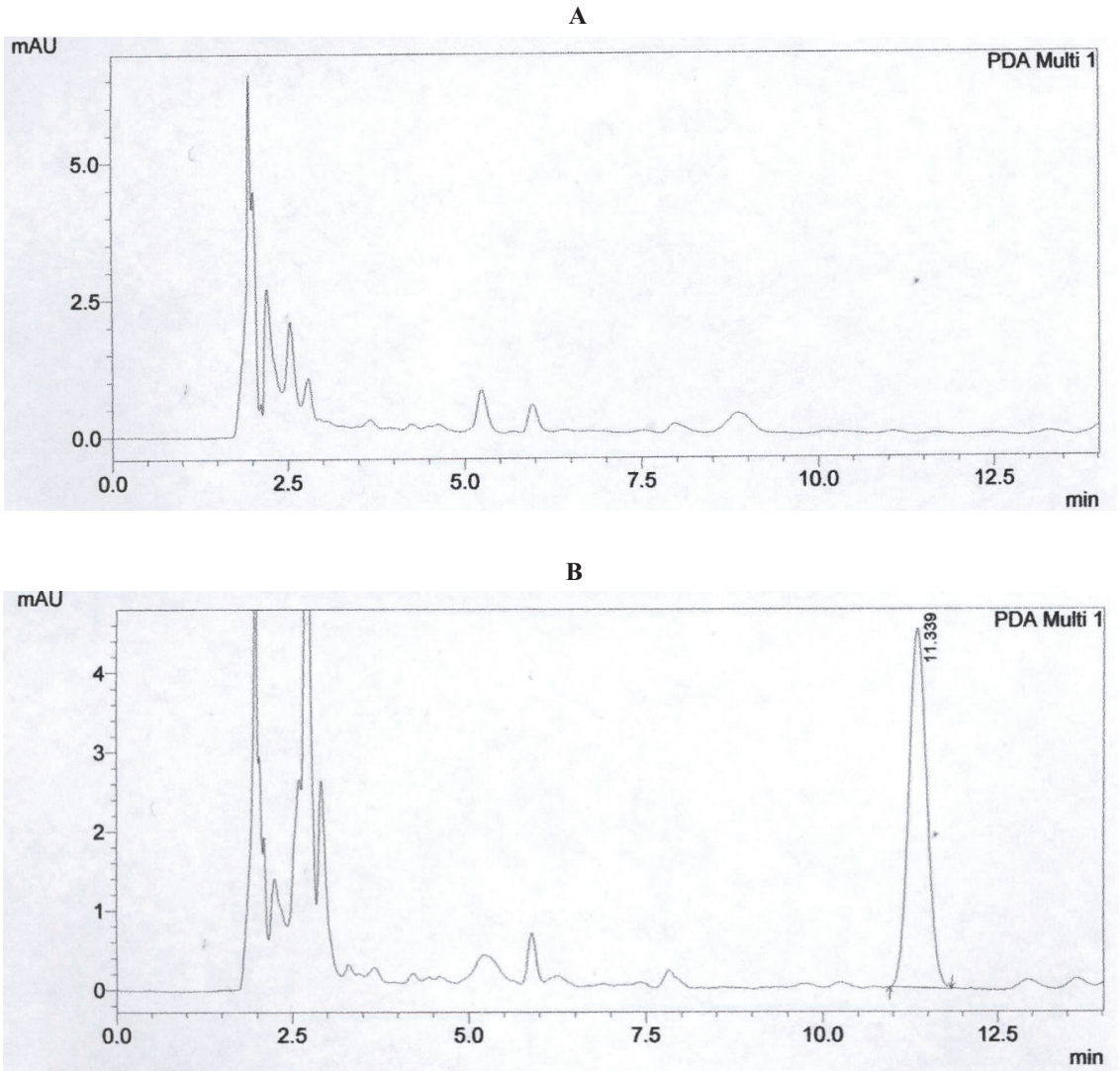


**FIGURE 2. CHROMATOGRAMS OF A BLANK MILK (A) AND SODIUM LASALOCID (3.0 µg/mL) IN A MILK SAMPLE (B).**

*TABLE I*
**PRECISION AND ACCURACY FOR LASALOCID DETERMINED BY A HPLC-UV METHOD IN COW RAW MILK**

| Quality control (QC) | Analysis day | Observed concentration | Precision (a) | Accuracy (b) |
|---|---|---|---|---|
| | | Mean ± SD(µg/mL) | **%** | **%** |
| | 1 | 0.4799 ± 0.0033 | 8.0 | 96.3 |
| 0.5 µg/mL | 2 | 0.4038 ± 0.0339 | 5.6 | 80.8 |
| | 3 | 0.4043 ± 0.0177 | 4.8 | 80.9 |
| | 1 | 1.4200 ± 0.0861 | 6.4 | 94.7 |
| 1.5 µg/mL | 2 | 1.2416 ± 0.0513 | 8.1 | 82.8 |
| | 3 | 1.1668 ± 0.0969 | 4.3 | 77.8 |
| | 1 | 2.5744 ± 0.1698 | 7.2 | 85.8 |
| 3.0 µg/mL | 2 | 2.3269 ± 0.1275 | 5.5 | 77.6 |
| | 3 | 2.3759 ± 0.3149 | 13.3 | 79.2 |

(a) Expressed as coefficient of variation percentage. (B) Calculated as (mean determined concentration/nominal concentration) x 100%. (N = 6).

According with the data obtained in the validation process, this technique and protocol show adequate selectivity and linearity, acceptable accuracy and precision and efficient recovery and stability of the sample. The utility of this analytical method for the determination of lasalocid in cow milk has a potential application in quality control system in the dairy industry with a detection limit of 0.03µg/mL and quantification limit of 0.5µg/mL.

## CONCLUSIONS

The results indicate that the developed HPLC-UV method is adequate to detect and quantify lasalocid in bovine raw milk samples in concentrations larger than 0.03 µg/mL and 0.5 µg/mL respectively.


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