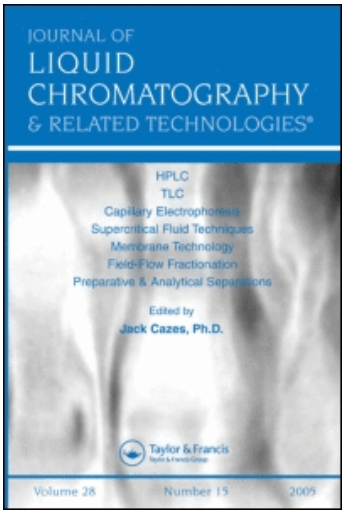

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A New Improved High Performance Thin Layer Chromatography (HPTLC) Method for the Detection of Ionophore Antibiotics in Feeds and Animal Tissues

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ABSTRACT

Ionophore antibiotics are widely used in veterinary medicine as anticoccidial drugs for poultry and as growth promoters for farm animals. The ionophores, despite their utility, possess a narrow range of safety; several species (horses in particular) appear highly susceptible to the toxic effects of these compounds, compared with other animal species. The purpose of this study was to apply a quantitative high performance thin layer chromatography (HPTLC) method to identify monensin and lasalocid residues in feeds. We then applied this method to the determination of liver residues. Our method, slightly modified from literature data, appears

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to be specific, linear ($r^2 > 0.99$), repeatable and reproducible, sensitive (limit of detection of 0.2 μg/g in liver for monensin, 1 μg/g for lasalocid), convenient, and simple to use.

Key Words: HPTLC; Antibiotics; Ionophores.

INTRODUCTION

The ionophore antibiotics (polyether compounds) are extensively used in veterinary medicine in the treatment and prophylaxis of coccidiosis, but also sarcocystosis and abortion induced by *Toxoplasma gondii*[1] in the poultry industry and for increasing feed efficiency in ruminant animals. Coccidiosis is a continuing problem in the intensive chicken industry and in chicken meat; infection with coccidia (*Eimeria* or *Isospora* spp.) causes high morbidity, reduced weight gain and, less frequently, destruction of the intestinal epithelium, which may lead to mortality.[2,3] As premixes, ionophores antibiotics are also used to increase food conversion and weight gain in growing and fattening cattle. This is achieved by improving the efficiency of rumen fermentation and altering the proportions of volatile fatty acids in the rumen. Decrease in acetic acid and butyric acid are accompanied by an increase in propionic acid, which is the most efficiently utilised of the ruminal fatty acids,[4] and an inhibition of methane production. Ionophores reduce energy loss associated with formation of volatile fatty acids and possess ideal characteristics for preventing acidosis, a major disease of feedlot cattle unadapted to high carbohydrate rations.[5,6]

The ionophores have a wide spectrum of activity, but a narrow range of safety; several species, appear highly susceptible to the toxic effects of these compounds. Horses, especially, do not tolerate high doses of ionophores and may present severe clinical disorders after accidental exposure to one of the compounds.[7–10] This greater sensitivity of the equine species to intoxication by ionophores is of particular concern, because of the tendency to share habitats and similar dietary needs that commonly prevail between horses and cattle; inadvertent consumption of ionophores by horses may result in mortality. In animals, lesions associated with ionophore toxicosis include cardiac and skeletal muscle degeneration and necrosis, with secondary lesions from acute cardiac failure or chronic cardiovascular insufficiency.

In our study, we consider two ionophores; monensin (a monovalent polyether antibiotic produced by a natural strain of *Streptomyces cinnamonensin*) and lasalocid (a divalent polyether antibiotic produced from fermentation by *Streptomyces lasaliensis*).[11] Both compounds are carboxylic ionophores,

capable of creating a flux of ion transport across lipid membranes; several reports describe incidents of accidental poisoning by monensin- and lasalocid-containing feed.[12–16]

The diagnosis of ionophore poisoning is difficult because none of the clinical signs are pathognomonic. Moreover, many methods of detection are limited because of the complex chemical and biological properties of these compounds.[17–19] The following, describes a rapid, sensitive, non-expensive analytical method, based on high performance thin layer chromatography (HPTLC), to identify monensin and lasalocid residues in feeds to confirm animal exposure. This method will be applied to the determination of liver residues in accidental poisoning cases.

EXPERIMENTAL

Reagents

The ionophores selected were of the highest purity available: monensin was 98% pure (Acros) and lasalocid was 97% pure (Sigma).

For sample extraction, silica-gel cartridges (Sep Pak®) are used.

The solvents used were methanol, chloroform, hexane, ethyl acetate (Merck, HPLC grade). Silica gel HPTLC plates (Si 60 Merck®) 10×20 were used for chromatographic separation.

Apparatus and Chromatographic Conditions

The HPTLC system (CAMAG Basel, Switzerland) used is composed of three parts: an automatic sampler (TLC3, version 2.05), a scanner (TLC Scanner II) equipped with a deuterium and tungsten lamp for UV and visible light detection (absorbance mode), driven by the WinCats® software (version 1.1.1).

Each sample or standard solution is sprayed automatically with high purity pressurized nitrogen gas (Air Liquide, Vénissieux, France) on a glass HPTLC plate, coated with silica gel (granulometry 60), 10×20 cm, with a film thickness of 0.25 mm and with fluorescence indicator (Merck, Nogent-sur-Marne, France).

For each standard or sample solution, a volume of 10 μL is sprayed on the plate under high Nitrogen pressure (6000 hPa) at 8 mm of the lower edge of the plate. The solutions are sprayed as 6×0.5 mm bands. Samples bands are separated by a 10–12 mm space. Plates are eluted in a mixture of ethyl acetate/hexane 8/3 (v/v) for 10–15 min or until the solvent front reaches 7 cm.

After drying, the plates are revealed with vanillin spray (vanillin 5% in methanol and sulfuric acid 99.5/0.5). The plates are heated at 90°C for 5–15 min. Monensin appears as a thin pink band, while lasalocid appears as an orange band on a light yellow plate. Monensin color is stable for several hours, but lasalocid fades away after 1 hour. Plates are read at 500 nm.

Sample Preparation

Feeds

5 g are extracted with 20 mL methanol, mixed for 30 min, and centrifuged at 3000 rpm for 10 min. This extraction is repeated twice. Both supernatant fractions are collected in a flask and evaporated to dryness in a rotary evaporator. Five mL methanol are added in the flask and stirred with 5 mL hexane and 5 mL water. Sodium chloride is added (0.1 g) to prevent emulsification and the hexane layer is removed for further purification on a silica gel cartridge.

Liver

1 g liver is extracted twice after grinding with a Thurax® apparatus with 5 mL methanol. After centrifugation and evaporation to dryness as described above, 1 mL methanol is added and this fraction is used directly for HPTLC analysis.

Cartridge Purification

The silica-gel cartridge is activated with 4 mL chloroform. The hexane fraction for analysis is poured. The cartridge is rinsed with 10 mL chloroform and elution is performed with 10 mL chloroform/ methanol 95/5 (v/v). This fraction is collected and evaporated to dryness. The dry residue is dissolved in 5 mL methanol. This final fraction is used for analysis.

Validation of the Analytical Procedure

Specificity is determined on feeds and liver of unexposed animals. For each compound, five standard solutions at 5 μg/mL, five spiked samples, and five blank samples were prepared.

Based on the noise level estimated on the blank samples, the limit of detection (noise + 3 standard deviation, SD) and the limit of quantification (noise + 10 SD) were determined theoretically.

Spiked samples were prepared and analyzed to obtain peaks corresponding to the above limits.

Linearity is evaluated on 5 points (μg/mL) and three consecutive days on both standard and spiked samples.

A Student's *t*-test is performed to check the *Y*-axis intercept and confirm that it is not significantly different from zero.

Homogeneity of variances within groups is verified based on a Cochran's *C* test. *Y*-axis intercepts and slopes are compared statistically. The existence of a significant slope and validity of the regression line is determined by means of analysis of variance.

Percent recovery for monensin and lasalocid are determined at each concentration.

Repeatability and reproducibility of the extraction procedure are determined on 6 spiked samples. This procedure is repeated over three days.

Variance homogeneity is verified by means of a Cochran's *C* test, and coefficient of variation of repeatability (*CVr*) and of reproducibility (*CVR*) are computed.[20]

RESULTS AND DISCUSSION

Specificity

Specificity was checked on feeds and on liver samples. After post-chromatographic derivatization, we did not detect any interference within the ranges of linearity tested. Therefore, we consider that the method is specific. A densitogram of blank samples is presented in Fig. 1 (liver samples). Interestingly, the presence of pigments was more significant in feeds, which was the reason for a more sophisticated extraction and purification procedure; and we did not attempt to analyze very low concentrations in feeds. However, at the lowest concentration tested (40 μg/g in feeds) interferences did not occur.

The only limitation may be with respect to lasalocid, since the yellow/orange colors fades away rapidly. If the plates are read within 10 min, this problem should not be a critical issue.

Limit of Detection and Limit of Quantification

Based on the analysis of blank liver samples, the limit of detection was determined at 0.2 μg/g for monensin and 1.0 μg/g for lasalocid in animals. We

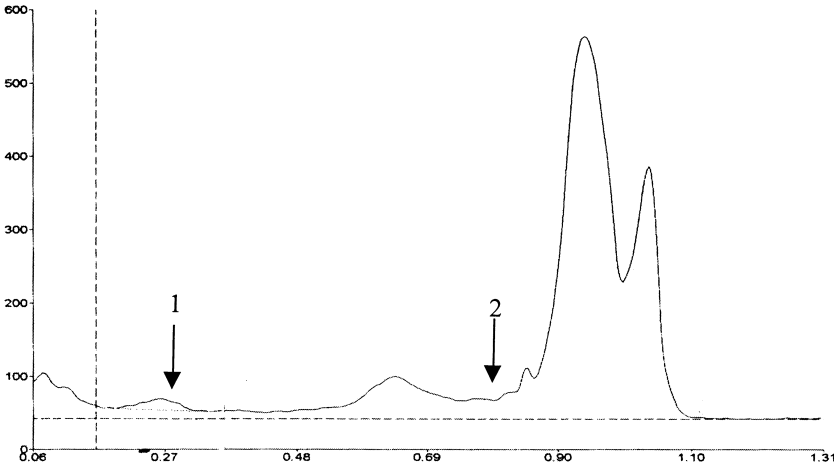

Figure 1. Densitogram of a blank liver sample showing the Rf of monensin (1) and lasalocid (2).

did not determine the limit of detection in feeds, since the lowest concentration tested was high enough to check horse feed. Indeed, a concentration of 100 μg/g is considered toxic to horses.[21] Based on the blank samples tested, however, we could estimate a routine limit of detection around 10 μg/g.

Limits of quantification were determined at 0.4 μg/g and 1.5 μg/g for monensin and lasalocid, respectively. These values are acceptable to monitor liver residues of horses poisoned with these compounds since, for instance, monensin residues as high as 7.7 μg/g have been detected.[22] So far, most cases of ionophore poisoning are diagnosed only on the basis of case history, clinical findings and histopathological findings.[10,17] Therefore, it is still difficult to estimate liver residues in horses and ascertain that our method will detect 100% of poisoned animals. It is, however, an interesting step since it will provide more definite conclusions about many suspected cases.[15,17]

Linearity—Percent of Recovery

As depicted in Fig. 2, linearity was excellent for both compounds in feeds ($r^2 > 0.99$) and in spiked liver samples ($r^2 > 0.98$). The ranges of concentration tested were 40–200 μg/g in feeds and 1–5 μg/g in liver samples. The percent recovery was consistently around 80% (78–84%) for monensin in feeds and liver extracts. It reached 82% for lasalocid in feeds but was only

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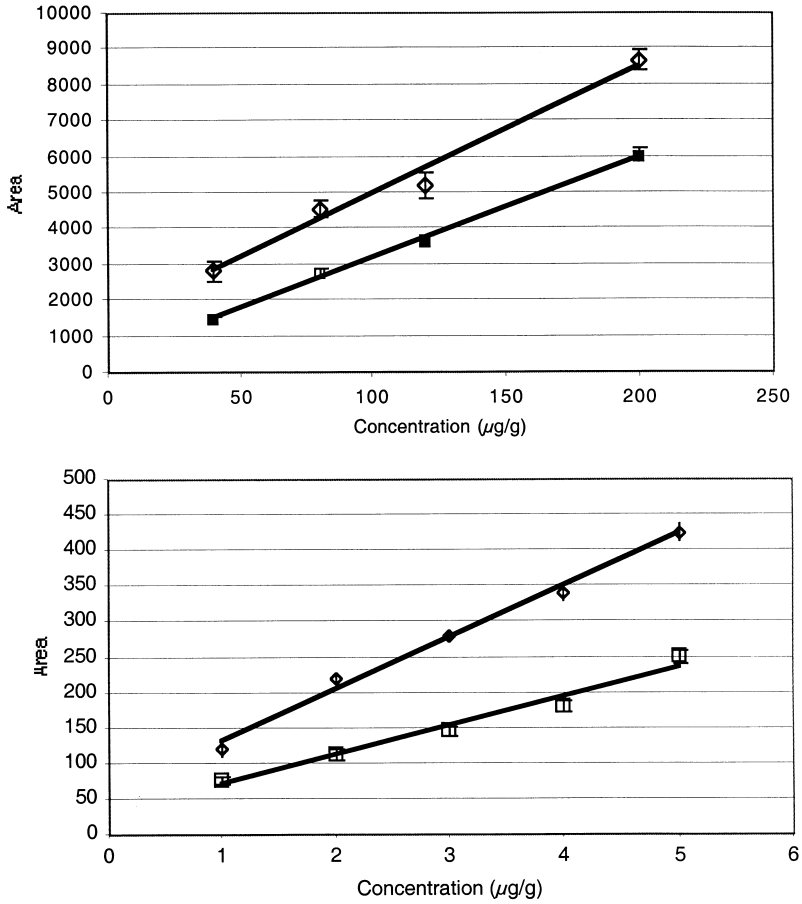

Figure 2. Linear relationship for monensin (◆) and lasalocid (■) in spiked feeds (top) and in spiked liver samples (bottom). Each curve was obtained after three replicates of each point.

65% in liver samples. These values are quite acceptable when compared with other routine techniques, especially when considering the fact that our method is one of the few quantitative techniques for the determination of ionophore antibiotics in both feeds and biological samples. Indeed, the screening method developed by the AOAC[18] is only quantitative.

Repeatability—Reproducibility

Concentration of monensin or lasalocid is determined in each sample based on the following formula:

$$\text{Concentration in } \mu g/g = \frac{\text{Area sample} \times (C) \text{ standard} \times (V) \text{ final dilution}}{\text{Area standard} \times \text{sample amount}}$$

Area sample or standard: area of the corresponding peak on the densitogram

(C) standard = concentration of ionophore standards.

(V) dilution: final volume of dry sample in mL.

Sample amount in g.

Variances obtained over the three study days were homogeneous (Cochran's C test table value 0.05, 3, 6: 0.707, computed values <0.5 for all four groups: monensin/feeds, monensin/liver, lasalocid/feeds, lasalocid/liver).

All coefficients of variations (CV) were $<10\%$ in feeds and $<15\%$ in liver samples. These values are in good agreement with recommendations of the European Union.[20]

Analysis of Feeds in a Suspected Poisoning Case

A suspected poisoning case was referred to the laboratory. The veterinarian was not aware that liver samples could be analyzed, since the technique was being developed at the time. Therefore, only food samples could be tested.

The case history was as follows: horse feed was produced in a factory where poultry and cattle feeds were also regularly produced. The use of monensin in poultry feeds was acknowledged and, despite strict cleaning procedures, the absence of ionophore residues in horse feed produced after poultry feeds was not certain. Based on this hypothesis, the local veterinarian suspected a potential contamination of feeds.

A sample of feeds was submitted for analysis (*ca* 500 g). After homogenization 3 separate samples were taken for analysis. The results were negative for all three samples ($<10\,\mu g/g$). The batch analyzed was the only batch and the only bag open at the time of poisoning. The two animals displayed a severe myositis with elevated creatine phospho-kinase and trans-

aminase. However, they did not show any cardiac sign of ionophore toxicity. Therefore, the hypothesis of monensin poisoning was definitively rejected.

CONCLUSION

From our laboratory data, we can conclude that our modified method should be applicable in cases of suspected ionophore poisoning, especially for monensin and lasalocid. It is specific, sensitive, quantitative, and repeatable in feeds and in the liver. When HPTLC material is available, the method is not expensive and sufficient to confirm the suspected poisoning case.

Moreover, our results confirmed the validity of the analytical process in negative cases. In order to improve this method, a separate study is planned to investigate liver residue levels in animals fed 100 ppm feeds and ascertain the validity of our method in field situations.

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