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Note

Separation and detection of monensin, lasalocid and salinomycin by thin-layer chromatography/bioautography

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Monensin, lasalocid and salinomycin are three compounds (Fig. 1) known as carboxylic-polyether antibiotics[1]. These structures are ionophores, which cause ionic fluxes and altered membrane potential[2]. The high molecular weights and multiple functionalities of these ionophores hinder analysis[3]. These compounds are presently used primarily as anticoccidial agents for poultry[4]. They are also being used in other species (such as beef cattle) as growth stimulants and to improve the efficiency of feed utilization[3].

Several reports have appeared recently on the toxic effects of each of these compounds[5,6]. Toxicities arise primarily from three avenues[7]: first, from feeding a dose higher than the recommended level of the ionophore; second, from the inadvertent inclusion of the ionophore in a feed presented to a species for which it was not intended; and third, from an adverse interaction between the ionophore and a simultaneously administered alternate ionophore or drug.

It is believed that the increasing use of these compounds in animal husbandry may induce bacterial resistance, leave residues in tissues and lead to environmental pollution[8–12]. In addition, owing to the potential for toxicity from low level mixtures of these ionophores, alone and in combination with other drugs, a precise assay that will separate and quantify each individual ionophore is essential.

Multiresidue methods for the separation of these three ionophores have been developed, using a variety of chromatographic technology. Asukabe *et al.*[1] have achieved this separation using high-performance thin-layer chromatography (HPTLC), Blanchflower *et al.*[7] by high-performance liquid chromatography (HPLC) and Martinez and Shimoda[13] by LC methods. The method described here is believed to be the first to attempt such a separation using TLC/bioautography. This method is based on one currently available for the quantitative analysis of monensin in poultry tissues[14].

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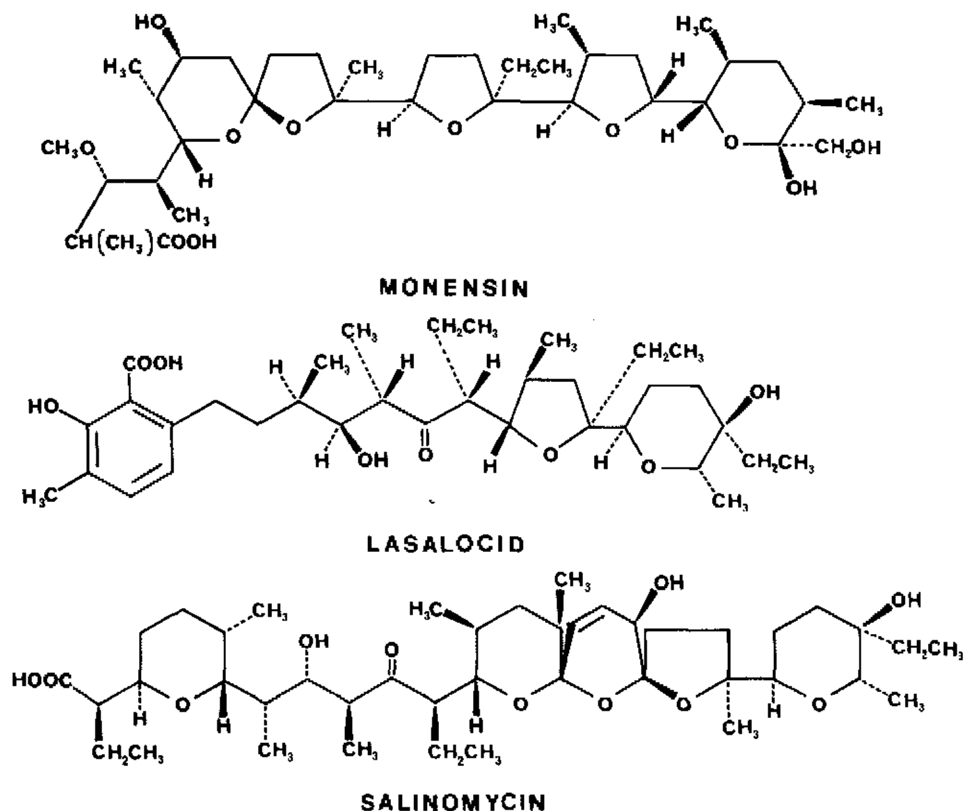

Fig. 1. The chemical structures of the monocarboxylic polyether ionophores monensin, lasalocid and salinomycin.

EXPERIMENTAL

Apparatus and reagents

For a complete listing of the apparatus and reagents involved see VanderKop and MacNeil[14].

Preparation of tissue extracts

All experimental glassware must be silanized before use in order to increase ionophore recovery. The preparation of tissue extracts is as described by VanderKop and MacNeil[14]. Briefly, the method involves obtaining 10 g of frozen tissue, dicing, homogenizing for 20 s, adding methanol, mechanically shaking for 15 min, centrifuging for 10 min at 1700 *g* then collecting the supernatant. This supernatant is transferred to a round-bottomed flask, extracted with 90 ml carbon tetrachloride, rotary evaporated, collected with 8 ml hexane, reevaporated by nitrogen flow, and then the final residue is dissolved in 1 ml methanol.

Thin-layer chromatography

The adsorbent zone of a 20 cm × 20 cm pre-scored 19-channel silica gel TLC

plate with pre-adsorbent spotting zone (Whatman LK6D) is preheated by placing it over a heating strip attached to a variable autotransformer, set to provide a temperature of 20°C. Using a micro-syringe, 30 μl of the extract are spotted onto the preheated TLC plate and allowed to dry for half an hour. The plate is then developed for 35 min in a sealed glass tank containing 200 ml of ethyl acetate–acetonitrile (50:50, v/v), that had been allowed to equilibrate for at least 2 h. The TLC plate is removed from the chamber and the solvent front marked immediately with a pencil. The TLC plate is air-dried for 2 h in a fumehood.

Bioautography

The bioautography is as reported by Salisbury *et al.*[15], with the exception of adjusting the SAM-3 medium pH to 5.5 by adding either 0.1 *M* citric acid or 0.1 *M* sodium hydroxide, as required, and using 0.6 ml of *Bacillus subtilis* spore suspension to seed the media.

RESULTS AND DISCUSSION

This method is based on one developed specifically for the detection and determination of monensin in poultry tissues[14]. In the original method, using a developing system of chloroform–methanol–acetone–glycerol, the three ionophores could not be chromatographically resolved. Lasalocid could be identified by the characteristic "teardrop" shape of its zone of inhibition. Monensin and salinomycin, however, both produced circular zones of inhibition and could not be distinguished by R_F.

To permit ionophore identification, a variety of solvent developing systems were tested (Table I). A minimum of ten replications per system was done. The data for the R_F values (within each solvent system) were analyzed statistically using an analysis of variance with the level of significance set at 0.05 (ref. 16). The relationship between tissue concentration of ionophore and the size of the zone of inhibition

TABLE I

SEPARATIONS SEEN ON BIOAUTOGRAPHIC MEDIA BETWEEN MONENSIN, LASALOCID AND SALINOMYCIN WHEN USING A VARIETY OF SOLVENT SYSTEMS

R_F values are means of ten replications.

Solvent system, 200 ml	*R_F value*		
	Monensin	*Lasalocid*	*Salinomycin*
Chloroform–methanol–acetone–glycerol (98:60:40:2)	0.80^a	0.80^a	0.80^a
Ethyl acetate	0.15^a	0.57^b	0.21^a
Cyclohexane–2-propanol (150:50)	0.48^a	0.50^a	0.58^b
Ethyl acetate–acetonitrile (100:100)	0.12^a	0.34^b	0.21^c

$^{a-c}$ Letters represent significant differences in R_F values between the three ionophores within a solvent system ($p > 0.05$). For each solvent system tested, R_F values with different superscripts are significantly different, while those with the same superscript do not differ significantly.

produced on the bioautographic media was measured by simple correlation[16]. Monensin and salinomycin were separated from lasalocid using 100% ethyl acetate in the developing system. However, monensin and salinomycin were not well separated from each other. Since lasalocid can be identified as distinct by its zonal shape, it is only critical that it have a different R_F value when it is suspected to occur in combination with either monensin or salinomycin. Through further experimentation it was observed that lasalocid could be well separated from salinomycin, but not from monensin, by using a developing system with cyclohexane–2-propanol (150:50, v/v). Similarly, the solvent system originally developed by Martinez and Shimoda[17] for monensin residues in feeds and subsequently adapted to tissue was reported to give similar R_F values for monensin and lascalocid[13]. The best solvent developing system for the complete chromatographic separation of monensin, lasalocid and salinomycin tested in this research was one combining ethyl acetate and acetonitrile in a 50:50 ratio (see Fig. 2).

Problems with non-specific inhibitory substances (*i.e.* enzymes) also present in the tissue matrices were not observed. These substances migrate at different rates than do the ionophoric compounds, and leave a characteristic inhibitory pattern on the bioautographic medium. This mark is typically seen as a faint line running in the middle of the channel on the TLC plate.

Thus far, this method has only been tested in chicken liver and kidney tissues. Using the method as listed, the following detection limits were achieved: monensin

Fig. 2. Bioautogram showing TLC separation of monensin (R_F = 0.12), salinomycin (R_F = 0.21) and lasalocid (R_F = 0.34) on pre-scored 19-channel silica gel plate.

0.45 μg/g, lasalocid 1 μg/g and salinomycin 1 μg/g. A linear relationship between inhibition zone size and concentration was observed for monensin (r = 0.98), but poor linearity (r = 0.60) was found for both lasalocid and salinomycin, using linear regression analysis.

The methodology provides a simple screening test to distinguish qualitatively between residues of the three ionophores, monensin, lascalocid and salinomycin in tissue, and permits an estimation of the concentration range of the residue. For laboratories with access to more sophisticated equipment, quantitation by HPLC, and mass spectral confirmation, as described by Martinez and Shimoda[13] may be attempted. However, the latter approach does involve extensive derivatization with a reagent of limited availability and stability and thus may not be practical for laboratories performing occasional analyses in support of toxicological investigations. The HPLC separation of the ionophores described by Blanchflower *et al.*[7] is specifically for the analysis of residues in feeds and has not been tested for residues in animal tissue.

CONCLUSIONS

A method was developed for the separation of monensin, lasalocid and salinomycin, three ionophore antibiotics. A linear response (inhibition zone size *versus* concentration) was observed for monensin, but linearity was poor for lasalocid and salinomycin. The TLC/bioautography approach is useful as it is a simple method of relatively low cost and skill-level. Screening methods used in slaughter plants for antibiotics in animal tissues are based on bioassay, so this technique enables separation between those ionophores while confirming the initial bioassay response.

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