The Analyst

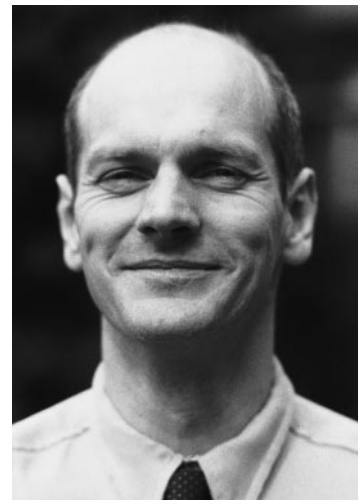
Critical Review

Methods for the detection of polyether ionophore residues in poultry

Christopher T. Elliott*, D. Glenn Kennedy and W. John McCaughey
Veterinary Sciences Division, Stoney Road, Belfast, UK BT4 3SD. E-mail: elliotc@dani.gov.uk

Summary of Contents

Keywords: *Polyether ionophore; residue; health; immunoassay; bioassay; high-performance liquid chromatography; mass spectrometry; reference laboratory; review*



Christopher Elliott is a Principal Scientific Officer in the drug residue laboratory of the Veterinary Sciences Division, Belfast, a European National Reference Laboratory for veterinary drug residue surveillance. He is the leader of a team developing new analytical methods capable of detecting drug residues in both laboratory and on-line environments. Current research is focused on the use of dry chemistry and biosensor based immunoassay techniques. He graduated from the University of Ulster with an M.Sc. in Biomedical Sciences and from the Queen's University of Belfast with a Ph.D. in Veterinary Sciences.

Coccidiosis and coccidiostats

Coccidiosis

Coccidiosis is a parasitic disease, caused by protozoa resident in the intestinal epithelium, which occurs wherever animals arehoused in small areas that are contaminated with coccidial oocysts. Historically, poultry have shown the greatest susceptibility to coccidiosis, because of the intensive nature of most of the poultry industry. In poultry, coccidiosis is caused by *Eimeria* spp., of which eight are known to cause serious clinical disease. A further 22 species cause less severe clinical effects in this species. The symptoms of coccidiosis in poultry may be one or more of the following: bloody diarrhoea, high mortality, reduction in feed and water intake, emaciation and loss of egg production. Much of the economic loss that is associated with coccidiosis is incurred prior to diagnosis. This makes prevention more important than treatment.

Drugs used to combat coccidiosis

A wide range of drugs is available for the prevention and treatment of coccidiosis. However, continuous prophylactic use of coccidiostats can cause a progressive loss of efficacy because of emerging drug resistance in the parasite population. Various

strategies have evolved to cope with this, including changing drugs used in starter and grower feeds and rotation of drugs between successive batches of chickens. The first drugs used to treat coccidiosis were the sulfonamides. Subsequently, a wide range of compounds, such as clopidol, decoquinate and methylbenzoquate; nicarbazin; toltrazuril and diclazuril; robenidine; halofuginone; amprolium and ethopabate have replaced these. However, by far the most widely used compounds are the carboxylic acid ionophores.

Structure of carboxylic acid ionophores

The term 'ionophore' (ion bearer) was coined in 1967[1] to describe this group of naturally occurring compounds. The finding, in 1968, that monensin was effective as an anticoccidial prompted a search for other compounds possessing similar properties. Six members of this family: monensin, narasin, lasalocid, salinomycin, maduramicin and semduramicin (Fig. 1) have become widely used as anticoccidial drugs, particularly in the poultry industry. Chemically, the carboxylic acid ionophores appear to be open-chain molecules consisting of an array of heterocyclic ether-containing rings. When present as deprotonated anions, these compounds form stable, electrically neutral complexes with alkali metal cations. However, the involvement of the ionised carboxyl group is not always essential for metal binding. It is required for metal binding by lasalocid[2] but is not for binding of Na^+ by monensin.[3] The carboxylic acid ionophores differ from each other in their affinity for individual metal ions. However, all of these compounds, with the exception of lasalocid, bind monovalent cations (*e.g.*, Na^+ and K^+). Lasalocid has a tendency to form dimers,[4] and can form complexes with divalent cations such as Mg^{2+} and Ca^{2+}. The formation of metal complexes results in all of these compounds adopting a quasi-cyclic formation consequent to head-to-tail hydrogen bonding (Fig. 2).

Function of carboxylic acid ionophores

In normal cells, the intracellular concentration of Na^+ is low and that of K^+ is high. In the extracellular space, their relative concentrations are reversed. The concentration of Ca^{2+} is similar on both sides of the cell membrane. However, the free intracellular Ca^{2+} concentration is up to four orders of magnitude lower than that outside the cell. All of the carboxylic

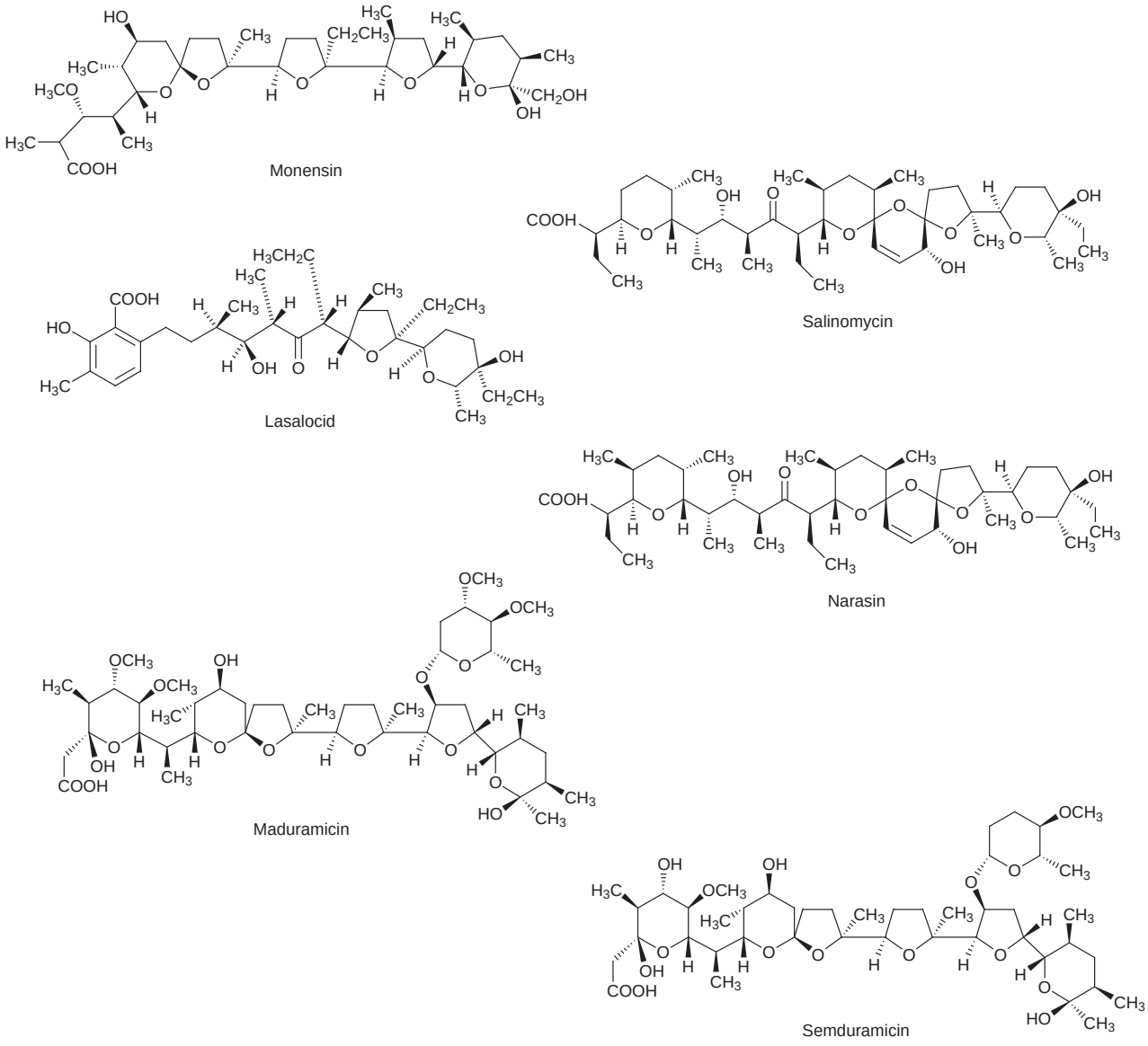


Fig. 1 Structures of the carboxylic acid ionophores.

acid ionophores, including lasalocid, promote perturbations in the intracellular cation balance: Na^+ increases and K^+ decreases. Given the intracellular Na^+ and K^+ concentrations, the relative change in the intracellular concentration of Na^+ is greater than that of K^+. The increase in the intracellular concentration of Na^+ is believed to result in Na^+ : Ca^{2+} exchange, leading to a dramatic increase in the intracellular free Ca^{2+} concentration.[5]

Use of carboxylic acid ionophores as coccidiostats

The carboxylic acid ionophores can dramatically reduce the mortality associated with coccidiosis. For example, in a floor pen trial,[6] salinomycin, fed to broilers at a concentration of 100 mg kg^{-1}, resulted in mortality decreased to a rate of 0.1% compared with a 20.5% mortality rate found in an infected group that received no salinomycin. In the same birds the feed conversion ratio (ratio of feed consumed to liveweight increase) was 2.36 in the untreated, infected birds and 2.14 in the salinomycin-treated, infected birds. The feed conversion ratio is of major importance to poultry producers as it combines their greatest profit element (bird weight) with their greatest cost element (feed). Any reduction in the feed conversion ratio is inextricably linked to an increase in net income. This has produced the incentive for the manufacturers of veterinary pharmaceuticals to develop and market new carboxylic acid ionophores as coccidiostats.

Of all of the coccidiostats developed to date, the carboxylic acid ionophores have proved to be remarkably free from problems of drug resistance, perhaps as a result of their rather non-specific mechanism of action. There has, however, been a gradual reduction in the sensitivity of coccidia to these compounds, indicating that resistant strains may be beginning to emerge. Unfortunately, there is as yet, no clear alternative to the carboxylic acid ionophores.

Coccidiostat residues and human health

Pressman and Fahim[7] reviewed this area in 1983. Carboxylic acid ionophores are potent pharmacological agents, exerting marked cardiovascular effects in experimental animal systems. Most of these effects have been characterised using monensin as the model for the whole group. The principal effect is an increase in coronary flow, indicative of coronary dilatation. It has been estimated that a threshold dose for increased coronary flow in the dog, following injection of monensin, is 1.0 μg kg^{-1}. The threshold dose in man, following oral administration of monensin, in food, will inevitably exceed 1.0 μg kg^{-1}. In normal individuals, coronary dilatation is unlikely to have any adverse effect. However, it has been suggested[7] that victims of coronary artery disease may be at an increased risk. In ischaemic areas of cardiac tissue, blood flow is already maximised in an attempt to maintain optimum perfusion of these areas. Induced dilatation, by an ionophore, of normal coronary vessels would tend to reduce further the perfusion of the partially occluded myocardium, an effect known as 'coronary steal'. Any instances of adverse reactions to monensin would, inevitably, be swamped by the spontaneous occurrence of hypoxia in victims of coronary artery disease. Nonetheless, the possibility that monensin or other carboxylic acid ionophores in food could exacerbate the condition of affected individuals remains.

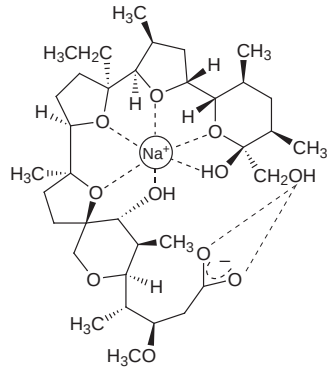


Fig. 2 Binding of sodium by monensin.

Legislative background to residue testing

The control of residues of veterinary medicines in cattle, sheep and pigs has been a cornerstone of the European Union's (EU's) agriculture policies to ensure consumer protection and promote even competition for markets for many years. Member States are required to monitor food animals for a range of legal and illegal compounds to provide assurance to consumers about the safety and wholesomeness of their food (Council Directive 96/23/EC). Currently, the EU is assessing the safety of all pharmacologically active compounds that are administered to food-producing animals, and is attempting to set a legally binding maximum residue limit (MRL) for each compound. With effect from January 1, 2000, only those compounds listed in Annexes I, II and III of Council Regulation 2377/90/EC can be used in food-producing animals. Annexe I lists compounds with an established MRL, Annexe II lists those compounds that are generally recognised as safe and therefore need no MRL, and Annexe III lists those compounds with a provisional MRL. No MRLs have yet been set by the EU for any of the carboxylic acid ionophores.

The EU legislation on the control of residues in poultry meat (Directive 96/23/EC) came into effect on July 1, 1997, and will be implemented by Member States from January 1, 1998. Member States will include coccidiostats in their national residues testing programmes. For cost-effective detection of most other classes of veterinary drugs, Member States have adopted, wherever possible, a two-tier testing system of: screening and confirmatory tests (Commission Decision 93/256/EEC). Screening tests are rapid, high volume, low cost tests that are designed to select samples for confirmatory analysis. They are biased to produce no false negatives, but may produce a low level of false positives. In effect, they classify large numbers of samples as being either 'negative' or 'potentially positive'. All samples in the latter category are then subjected to a confirmatory test. These are low volume, high cost tests geared to produce no false positives, and a mimimal rate of false negatives. Taken together, this combination of screening and confirmatory tests provides an efficient and cost-effective means to control veterinary drug residues in food-producing animals.

In anticipation of the forthcoming inclusion of carboxylic acid ionophores in national residue testing programmes, this review assesses available screening and confirmatory methods and the pharmacokinetics of these compounds.

Analytical methods for ionophore residue detection

Screening methods for the detection of ionophores

The development of an individual analytical method, with the complex and interrelated requirements of a screening procedure (fast, reliable, broad spectrum, sensitive, inexpensive), poses a formidable challenge. The method developed will relate to the specific needs of the end-user and the analytical technologies available at the time.

The analytical techniques used to detect ionophore residues not surprisingly date back to the time of the discovery of monensin. Despite the variety and depth of coccidiostat screening assays, the topic has not been reviewed since 1985

when Weiss and MacDonald[8] summarised detailed information on the bioassays available at that time. As with many types of screening assays for drug residues there has been a change in developmental emphasis from bioassays to immunoassays, a trend that is likely to continue.

In vitro *assays*

Amongst some of the earliest methods designed to detect the presence of ionophore drugs were cell culture procedures. Strout and Ouellette[9] and McDougald and Galloway[10] described similar techniques whereby the effects of the presence of anticoccidial agents, including the ionophores, on the growth of *Eimeria tenella* in cell culture were monitored. Kidney cells in culture were inoculated with sporozoites and incubated for 72 h. After this time the cultures were fixed and stained and viewed under a light microscope. In untreated controls, normal coccidia development was noted whereas in cultures with ionophores added there was a predominance of abnormal sporozoites. Detection limits of the order of 10 000 μg l^{-1} for monensin were claimed.

In a similar assay, the detection method was altered to allow a more quantitative measure of anticoccidial activity.[11] An ELISA (enzyme-linked immunosorbent assay) was performed following the cell culture stage of the procedure. This allowed the detection of mature *Eimeria tenella* antigens present in culture supernatants. The inhibition of growth elicited by the ionophore compounds caused a reduction in the presence of these antigens. Ionophore concentrations as low as 0.5 μg l^{-1} could be detected by this technique.

In summary, these *in vitro* assays were able to differentiate between many classes of anticoccidial drugs and, in particular, ionophores. This was achieved by observing the particular effects on the growth inhibition detected during culture. All the studies mentioned performed the assays with drugs in buffer solutions and were primarily designed to define the nature of the anticoccidial activity of various drugs. In addition, it should be noted that there are requirements for lengthy incubation periods in these procedures, making them less attractive for routine analytical testing. There is, however, great scope to use refined versions of these assays to develop their potential to be used as receptor-based assays for ionophore residue presence in complex biological samples. Such assays would have the capability of being able to detect multi-ionophore residues at high sensitivity.

Screening by thin-layer chromatography–bioautography (Table 1)

The ionophores, being antibiotics, inhibit the growth of certain bacteria. The earliest ionophore residue detection methods developed used this property.[17] However, the basic microbiological assay technique was unsuccessful in reliably detecting monensin residues, owing to the interference of sample components, even after extensive sample extraction, and the use of thin-layer chromatography (TLC) as a clean-up procedure became the accepted method by many workers. In their review article of TLC methods for monensin residue testing, Weiss and MacDonald[8] detail the evolution of this methodology. Only minor changes appear in later published techniques.

The first description of a TLC–bioautographic assay and the model for many to follow appeared in 1967.[12] Tissue samples (muscle, liver and kidney) were sequentially extracted into methanol and carbon tetrachloride before spotting onto silica gel thin-layer plates. Fat samples required additional purification prior to analysis by means of solid-phase columns packed with silica gel. The bioautography was performed by melting agar over the surface of the TLC plate seeded with *Bacillus subtilis* innoculum. Following an overnight incubation, the sizes of zones of inhibition were measured to determine monensin presence. The sensitivity of this procedure was determined as being about 25 μg kg^{-1}.

Modifications to this assay were devised by Okada *et al.*[18] These included a more refined sample clean-up, improvement in the agar and the use of an alternative developing solvent. The net result was to achieve improved chromatography of monensin and enhanced zones of inhibition, leading to improved detection limits of about 10 μg kg^{-1} in muscle, liver, kidney and fat. Further improvements to the TLC–bioautography assay were introduced by Vanderkop and MacNeil,[13] who identified the silylation of glassware used in extractions as an important means of increasing the amount of monensin recovered. In addition, further optimisation of the TLC solvent system employed and bioassay conditions resulted in a relatively fast and simple method capable of routine use.

Salinomycin residues have also been successfully detected by TLC–bioautography.[14] An extraction procedure based on acetone and ethanol partitioning and zone visualisation following TLC separation utilising the organism *B. stearothermophilus* resulted in a procedure capable of detecting down to 10 μg kg^{-1} salinomycin. Vanderkop and MacNeil[15] developed a multi-TLC–bioautography method that allowed the identification of monensin, salinomycin and lasalocid residues. This

Table 1 TLC–bioassay for ionophore residues

Ionophore*	Matrix†	Extraction	Clean-up	TLC conditions	Organism	LOD‡/ μg kg^{-1}	Ref.
Mo	M,L,K,F	MeOH and CCl_4	Silica-based SPE§ on fat extracts only	Silica gel plates developed in carbon tetrachloride–benzene–methyl cellosolve (80 + 10 + 5)	*Bacillus subtilis*	≈ 50	12
Mo	Unspecified poultry tissues	MeOH and CCl_4	None	6D silica gel developed in chloroform–methanol–acetone–glycerol (98 + 60 + 40 + 2)	*Bacillus subtilis*	250	13
Sa	M,L,K,F	Acetone and light petroleum	None	Unspecified plates developed in hexane–diethyl ether–methanol–acetic acid (70 + 30 + 4 + 0.5)	*Bacillis stearothermophilis*	≈ 10	14
Mo, Las and Sa	L, K	MeOH and CCl_4	None	Silica gel developed in ethyl acetate–acetonitrile (50 + 50)	*Bacillus subtilis*	45–1000	15
Mo	M,L,K,F	MeOH and CCl_4	Silica SPE§ except for Mo	Silica gel 60 plates developed in ethyl acetate	*Bacillus subtilis*	10–12.5	16

* Mo = Monensin, Sa = salinomycin, Las = lasalocid. † M = muscle, L = liver, K = kidney, F = fat. ‡ LOD = Limit of detection. § SPE = Solid-phase extraction.

differed from their previous method[13] for monensin by altering the developing solvents to ethyl acetate and acetonitrile (50 + 50). Sensitivities ranging from 450 to 1000 μg kg^{-1} in chicken liver and kidney extracts were achieved. In another procedure, capable of simultaneously detecting monensin and lasalocid residues, samples (serum and lung) were extracted using a chloroform-based procedure.[19] The organism *B. stearothermophilus* was used to determine residue presence. The detection levels of the assay were not given.

In summary, there are TLC–bioautographic procedures capable of detecting most of the ionophore residues in tissue. These methods rely heavily on lengthy solvent-based extraction systems and the sensitivities achieved vary widely.

Ionophore residue screening by immunoassay

Antibody production. Owing to the requirement for extensive clean up prior to analysis by TLC–bioassay workers sought methodologies that would act as alternative ionophore screening tests. As with many other forms of veterinary drug screening analysis, attention focused on the use of immunoassays.

The prerequisite analytical tool for this methodology is an antibody that recognises the ionophore in a sensitive and specific manner. To produce such antibodies the ionophore must be linked to a suitable carrier protein to enable the complex to be recognised as foreign and thus evoke a humoral immune response in immunised animals. Ionophore conjugates have been successfully produced by a variety of synthetic approaches (Table 2). Heitzman *et al*.[20] converted monensin salt to an acid and conjugated this to ovalbumin (OVA) by means of a mixed anhydride reaction.[30] Rabbits were immunised with 1 mg aliquots of the conjugate and a polyclonal serum was harvested after several months. Pauillac *et al*.[21] also used the mixed anhydride conjugation method to couple a succinylated monensin derivative to both OVA and bovine serum albumin (BSA) to prepare polyclonal (rabbit) and monoclonal (mouse) antibodies.

Mount and Failla[22] used an alternative means of preparing a rabbit polyclonal serum to the same compound. In this procedure monensin salt was converted to a reactive bromoacetate derivative which spontaneously coupled to BSA. In a more recent study, Godfrey *et al*.[24] coupled the carboxylic acid form of monensin to haemocynanin and thyroglobulin using the reactive *N*-hydroxysuccinamide.

In summary, antibodies (both polyclonal and monoclonal) have been raised to most of the ionophores. These tend to be highly specific and, with the exception of salinomycin/narasin, antibodies, do not have significant cross reactivity profiles. It should be noted, however, that little data were produced to show the cross reactivities against ionophore metabolites.

Extraction procedures used in immunoassay procedures (Table 3). Only a limited number of the ELISA procedures have been developed and validated to detect ionophore residues in poultry samples.[23,24,27–29,31] The matrix most often chosen in these assays has been liver. The extraction procedures have generally relied on solvent partitioning. However, one excep-

Table 2 Methods and procedures employed in the production of monoclonal and polyclonal antibodies to ionophores

Ionophore	Derivative produced	Conjugation procedure	Carrier protein	Species	Cross-reactivity	Ref.
Monensin	Monensin acid	Mixed anhydride	Ovalbumin	Rabbit	*O*-Desmethylmonensin (42%)	20
	Monosuccinyl monensin	Mixed anhydride	BSA*	Rabbit and mouse	None found	21
	Monensin bromoacetate	Direct	BSA	Rabbit	None found	22
	Monensin acid	Active ester	Keyhole limpet haemocyanin	Rabbit	None found	23
	Monensin acid	Mixed anhydride	Transferrin	Rabbit	None found	24
Salinomycin	Salinomycin hemisuccinate	Mixed anhydride	BSA	Mouse	Narasin (> 100%)	25
	Salinomycin hydrazide	Active ester	Not stated	Mouse		26
	None	Carbodiimide	Human serum albumin	Rabbit	Narasin (100%)	27
Lasalocid	None	Carbodiimide	Human serum albumin	Sheep	None found	28
Maduramicin	None	Carbodiimide	Human serum albumin	Rabbit	None found	29

* BSA = Bovine serum albumin.

Table 3 Extraction procedures applied to ELISA methods for poultry sample analysis for ionophore residues. Assay performance data are also outlined for individual methods

Ionophore	Matrix*	Extraction	Clean-up	RSD† (%)	LOD‡/μg kg^{-1} or μg l^{-1}	LOQ§/μg kg^{-1} or μg l^{-1}	Ref.
Monensin	L	Water–acetonitrile	Hexane–diethyl ether	8.5–19.6	2.9	4.6	24
	M, L, K, F, S	Proteolytic digest	Immunoaffinity	8.7–16.3	0.09–1.99	No data	23
Salinomycin	L	Water–methanol	Dichloromethane	19.9	No data	≈ 50	31
	Se, M, L	Water–acetonitrile	hexane	11–31	0.016–0.09	0.028–0.15	27
Lasalocid	Se, M, L	Water-acetonitrile	Hexane	5–34	0.1–0.18	0.16–0.29	28
Maduramicin	Se, M, L	Ethyl acetate or water–acetonitrile	Diethyl ether	5–33	0.01–0.02	No data	29

* Se = Serum, M = muscle, L = liver, K = kidney, F = fat, S = skin. † RSD = Relative standard deviation. ‡ LOD = Limit of detection. § LOQ = Limit of quantification.

tion to this is the method of Godfrey *et al.*,[23] who utilised the technique of immunoaffinity chromatography. This procedure greatly reduced the volumes of organic solvents required in the extraction process, allowing for the possibility of some form of automation to be introduced into the process. The disadvantage of this technique was the requirement to perform a proteolytic digest on the sample material prior to extraction.

Sensitivities of immunoassays for ionophores (Table 3). In general, the ELISA procedures developed to detect ionophore residues were significantly more sensitive compared with the earlier TLC–bioautography based screening assays.

In summary, a number of immunoassays have been developed which can detect trace levels of ionophore residues. The extraction procedures required for these assays are similar to those of the TLC procedures. However, in contrast to the TLC methods, which were capable of being multi-ionophore residue detection methods, the ELISAs normally detect only one, or occasionally two ionophore compounds. The other important difference between these two methodologies relates to the detection of ionophore metabolites. The TLC–bioautographic technique relies on biological activity of the ionophores and is unlikely to measure significant amounts of biologically inactive metabolites. The ELISA methods, owing to unavailability of metabolite reference standards, are largely untested for the cross reactivity profiles of the antibodies against them, *i.e.*, it is not known if the ELISAs also detect the presence of these metabolites present in samples. However, examples of structural similarities which exist between parent ionophores and metabolites which have been documented[32] are recognised by the antibodies to at least some degree.

In summary, a wide variety of screening assays have been developed to detect residues of ionophores. The choice of method used in a laboratory will often be dictated by the availability of suitable expertise, suitable equipment and the ability to purchase appropriate commercial products.

Chemical methods for the detection of ionophore residues

During the last decade, there has been a dramatic increase in the number of published HPLC and mass spectrometric methods for the detection of carboxylic acid ionophores. However, there are still relatively few sensitive and specific methods from which regulatory analysts can choose. The chemical analysis of the carboxylic acid ionophores has been reviewed on two previous occasions, in 1985[8] and 1995.[33]

HPLC methods

Lasalocid, alone among the carboxylic acid ionophores, has a fluorescent chromophore. All of the HPLC-based assays that have been developed to determine the other carboxylic acid ionophores in tissue require derivatisation to introduce a suitable chromophore.

Methods requiring derivatisation (*Table 4*)

9-Anthryldiazomethane (*ADAM*) *derivatisation*. ADAM reacts with carboxylic acid groups to form a highly fluorescent derivative. It was first used for the analysis of monensin residues in bovine muscle in 1985.[34] Monensin, extracted from beef liver, was first acetylated with acetic anhydride to form acetyl esters from the two hydroxyl groups in monensin. It is not clear why this step was needed. This derivative was then reacted with ADAM to form monensin–9-anthryldiazomethane. An additional silica clean-up was required prior to HPLC analysis. No sample traces were presented. Since then, three other methods have been described[35–37] that have used ADAM as a derivatising agent. The method described by Hoshino *et al.*[37] was similar to the method described above,[34] although the acetylation step was not included. No traces from negative tissues were presented. Martinez and Shimoda subsequently described a modification of their original assay[34] that was capable of detecting monensin, salinomycin, narasin and lasalocid.[36] Acetylation of sample extracts was apparently necessary for the determination of monensin, narasin and salinomycin. However, they claimed that lasalocid did not form an ester derivative following treatment with acetic anhydride, and so this step could be omitted when attempting to determine lasalocid. Using their method, narasin and salinomycin were incompletely resolved. Furthermore, lasalocid eluted on the tailing edge of a very major matrix peak. Most of the methods that use ADAM derivatisation suffer from poor sensitivity (limit of quantification >100 μg kg^{-1}),[37], or poor recoveries[35] ($<60\%$) or both.[36] In addition, with one exception,[37] clean-up procedures are lengthy. However, the simpler clean up procedure described by Hoshino *et al.*[37] was accompanied by very poor sensitivity. All of these methods required an

Table 4 HPLC methods for carboxylic acid ionophores requiring derivatisation

Analyte*	Derivatisation mode	Derivatising agent	Extraction	Clean-up	Detection method†	LOQ‡/ μg kg^{-1}	Recovery (%)	Ref.
Mo	Off-line	Anthryldiazomethane	MeOH–H_2O	Alumina/CH_2Cl_2/LH-20/acetylate	Fl 365/418	50	71–96	34
Mo	off-line	Anthryldiazomethane	MeOH–H_2O	$CHCl_3$/silica/$CHCl_3$	Fl 365/412	10	46–78	35§
Las, Ma, Na, Sa	Off-line	Anthryldiazomethane	MeOH–H_2O	Alumina/CH_2Cl_2/LH-20/acetylate	Fl 365/418	150	57–90	36¶
Mo	Off-line	Anthryldiazomethane	Orthophosphoric acid	$CHCl_3$	Fl 365/412	500	68–82	37
Mo	Post-column	Vanillin	MeOH–H_2O	CCl_4/silica	VIS 520	25	82–96	38
Se	Post-column	Vanillin	MeOH–ammonia	C_8/silica	VIS 522	40	82–107	39
Las, Mo Na, Sa	Post-column	Vanillin	Isooctane–ethyl acetate	Silica	VIS 520	3–10	71–94	40‖
Sa	Post-column	Vanillin	MeOH–H_2O	CH_2Cl_2	VIS 520	100	89	31, 41
Sa	Post-column	Vanillin	Acetone	Light petroleum	VIS 520	10	83–98	42
Sa	Post-column	Dimethylaminobenz-aldehyde	Ethanol–propan-2-ol	Microwave	VIS 592	10	87–100	43
Mo, Sa	Off-line	1-Bromoacetylpyrene	Acetonitrile	Ethyl acetate/silica/derivatise/ Florisil	Fl 360/450	100	66–96	44
Sa	Off-line	Pyridinium dichromate	MeOH	CCl_4/silica/C_{18}/derivatise/silica	UV 225	100	95–102	45

* Mo = Monensin, Sa = salinomycin, Las = lasalocid, Ma = maduramicin. † Fl = Fluorescence, VIS = visible, UV = ultraviolet; all values in nm. ‡ LOQ = Limit of quantification. § Recoveries very low (46 and 56%) at 10 and 100 μg g^{-1}. ¶ Acetylation not needed for lasalocid. ‖ Lasalocid not derivatised in muscle. Separate extraction used for lasalocid in liver.

additional clean-up step, using silica gel, following derivatisation to remove unreacted ADAM, increasing the complexity of the method.

Aromatic aldehyde derivatisation. Vanillin and dimethylaminobenzaldehyde react with hydroxyl groups in the Komarowsky reaction. These post-column reagents, prepared in methanolic sulfuric acid, react with the carboxylic acid ionophores at elevated temperatures. The ionophores decompose in a poorly understood reaction, to form coloured products. This property was first utilised in the determination of carboxylic acid ionophores in 1973, when a colorimetric method for monensin in feeds, based on the reaction between vanillin and monensin, was reported.[46] Although subsequently applied to the determination of monensin, narasin and salinomycin in animal feeds in 1985,[47] the use of vanillin as a derivatising agent for carboxylic acid ionophore residues in tissues has only been reported recently.[33,38–42] These methods offer a significant improvement over the earlier ADAM-based assays. Much simpler one- or two-step clean-up procedures are possible coupled with, in most cases, improved sensitivity. Typically, the extraction step consists of either a liquid–liquid[3,41,42] or a silica gel solid-phase extraction.[40] This method, for lasalocid, monensin, narasin and salinomycin, claimed limits of quantification of between 3 and 10 ng g^{-1}, with excellent recoveries. However, the detection of lasalocid required a significant alteration to be made to the extraction procedure, diminishing the claim that the four main carboxylic acid ionophores are included. Derivatisation with vanillin has also been applied to the determination of semduramicin, in an assay that employed sequential C_8 and silica gel solid-phase clean-up steps. Chromatograms were very clean and free from possible interfering matrix components. This is the only chemical method that has been described for this compound, to date, and has a limit of quantification of 40 μg kg^{-1} One promising method for salinomycin[43] combines a very simple extraction procedure with derivatisation with dimethylaminobenzaldehyde. Samples are mixed with ethanol and propan-2-ol and salinomycin is extracted by a brief pulse of microwave energy from a domestic microwave oven. This assay has a limit of quantification of 10 μg kg^{-1} and recoveries range from 87 to 100%. However, the authors did not compare their novel extraction procedure with more conventional approaches using incurred positive samples. Such derivatisation/degradation methods are limited by the fact that other hydroxyl-containing compounds in the sample extract will also yield coloured products. This may unacceptably increase the false positive rate of the assay.

1-*Bromoacetylpyrene* (*BAP*) *derivatives*. The Kryptofix K222-catalysed formation of pyrenacyl derivatives of carboxylic acid ionophores has been the subject of study by Asukabe's group. In 1984, they reported the formation of fluorescent ester derivatives of salinomycin and monensin in standard solutions.[48] Subsequent studies showed that this technique could also be used for lasalocid and narasin.[49] Although this group went on to develop a method for the determination of lasalocid, monensin and salinomycin in animal feeds,[50] another Japanese group described the use of the same derivative for the analysis of residues of monensin and salinomycin.[44] Their method produced well-resolved peaks, with no interfering peaks at the retention time of either compound. This method, with a limit of quantification of 100 μg kg^{-1} was not, however, particularly sensitive. Recoveries ranged from 66 to 96%.

Pyridinium dichromate oxidation. Dimenna *et al*.[45] described a method that gave good recoveries, but poor sensitivity, for salinomycin in chicken skin/fat. Following extraction with methanol, and clean up by partitioning into carbon tetrachloride, followed by silica gel and C_{18} solid-phase extraction, salinomycin was derivatised using pyridinium dichromate. This oxidises the allylic hydroxyl group to form an α,β-unsaturated ketone, with strong UV absorbance at 225 nm. However, the limit of quantification of this method was poor, at 100 μg kg^{-1}.

Hydrazone derivative. Another derivatising reagent has been applied to the determination of salinomycin in standard solutions.[51] It relied on the formation of a hydrazone derivative, with maximum absorbance at 419 nm. However, this reagent has not been applied to the analysis of carboxylic acid ionophore residues in tissues.

Methods requiring no derivatisation (*Table 5*)

The only intrinsically fluorescent carboxylic acid ionophore is lasalocid. If excited at λ = 308–315 nm, lasalocid fluoresces at 400–430 nm. This intrinsic fluorescence of lasalocid is highly pH-dependent. At pH 8.3 the intensity of emission is some two orders of magnitude greater than that at pH 3.0. However, the corrosive nature of such an alkaline pH towards silica columns has limited the applicability of this property.

Weiss *et al*.[52] developed a method for the determination of lasalocid in tissues that was an extension of a similar method developed for the determination of lasalocid in blood.[57] Lasalocid was extracted into acetonitrile, and defatted using hexane. An aliquot was dried and residues were reconstituted in water, saturated with HPLC mobile phase. The lasalocid was then extracted into the mobile phase, which was a complex mixture of tetrahydrofuran, methanol, hexane and ammonia. Lasalocid was, however, incompletely resolved from a matrix component by the use of two silica columns (Whatman Partisil 10) connected in series. Despite this, a limit of quantification of 25 μg kg^{-1} was achieved, with recoveries averaging 72%. Traces of negative samples were not presented. This method was subsequently found to be robust in two multi-laboratory studies using chicken fat[58] and bovine liver[59] as the test matrix. A similar extraction procedure, column and mobile phase was used by Kozak and Wisniewska-Dymytrow,[53] a method that had a comparable limit of quantification (20 ng g^{-1}) and recovery (85%). No traces were presented. Ishikuro[54] developed a method that involved extraction with acetonitrile and defatting with hexane. In this case, however, a silica Sep-Pak cartridge was used to effect clean-up. Reversed-phase HPLC (Unisil Pack $5C_{18}$) was used to resolve lasalocid. The pH of the mobile phase was 3.0. The limit of quantification was 50 μg kg^{-1}, although the detection limit was approximately 5 μg kg^{-1}. The recovery achieved ranged from 85 to 92%. Blank tissue traces were not presented.

Table 5 HPLC methods for lasalocid residue detection requiring no derivatisation

Ionophore	Extraction	Clean-up	Detection method*	LOQ†/μg kg^{-1}	Recovery (%)	Ref.
Lasalocid	Acetonitrile	Hexane wash/dry/extract into basic mobile phase	Fl 310/430	25	72	52
Lasalocid	Acetonitrile	Hexane wash/dry/extract into basic mobile phase	Fl 310/440	20	85	53
Lasalocid	Acetonitrile	Hexane wash/silica Sep-Pak	Fl 310/420	50	85–92	54
Lasalocid	Methanol	Extract with CCl_4/silica Sep-Pak	Fl 310/420	400	86–100	55
Lasalocid	Acetonitrile	Extract with CCl_4/silica Sep-Pak	Fl 310/425	2	66-76	56

* Fl = Fluorescence; all values in nm. † LOQ = Limit of quantification based on lowest concentration at which the method was validated.

Horii *et al.*[55] described a method in which liver was extracted with methanol, and then extracted with carbon tetrachloride. Following silica Sep-Pak clean-up, samples were run on a Nucleosil 100 C_{18} reversed-phase column in a mobile phase at pH 7.0. Although these workers claimed a limit of quantification of 10 ng g^{-1}, validation data were produced only at 400 and 2000 ng g^{-1}. Blank tissue traces were not shown.

Tarbin and Shearer[56] presented a well-validated method in which lasalocid was extracted from tissues and eggs using acetonitrile. The extract was partitioned, with salting-out, into carbon tetrachloride–acetonitrile and dried. The extract was further cleaned up using a silica solid phase column. HPLC was carried out in a basic mobile phase using either a polymeric PLRP-S column or a porous graphitic carbon column (Hypercarb), both of which were stable over a wide pH range. The polymeric column proved unsuitable for the determination of lasalocid in eggs, because of the presence of an interfering peak. However, the porous graphitic column, which required a mobile phase containing 5% tetramethylguanidine in acetonitrile to achieve good peak shape, was unaffected by this interference. The limit of quantification of the assay was 2 μg kg^{-1} in tissue and 10 μg kg^{-1} in egg. Recoveries ranged from 66 to 72% (egg) and 76% (tissue).

HPLC methods: summary

No truly multi-residue HPLC method for all of the carboxylic acid ionophores has yet been developed. Monensin, narasin and salinomycin can readily be combined in a single analytical procedure. To include lasalocid residue detection requires alterations to the extraction procedure.[36,40] Derivatisation with vanillin, with UV/VIS detection, appears to offer the best sensitivity and simplest extraction procedure. For lasalocid, however, HPLC with fluorescence detection is the method of choice. The great simplicity of the microwave extraction procedure developed by Akhtar and Croteau[43] merits further examination, given the limit of quantification and recoveries obtained, particularly if the assay is as effective in measuring incurred residues of salinomycin as it is in the analysis of salinomycin spikes. Some of the more sensitive HPLC methods that have been described rely on the use of solvents such as chloroform and carbon tetrachloride.[24,38,56] These methods may be of limited value in the modern analytical laboratory, given the increasing costs of waste disposal.

Mass spectrometric methods (*Table 6*)

Increasingly, legislative demands are requiring analysts, wherever possible, to use mass spectrometric detection for the confirmation of residues of veterinary drugs. However, few mass spectrometric methods have been published for the determination of carboxylic acid ionophores in tissues in the last 15 years. The lack of published methods reflects, in part, the instability and relative insensitivity of these compounds in GC systems. Most of the published methods have relied on the emerging technology of LC–MS.

The earliest methods for the mass spectrometric determination of these compounds did not have the advantages offered by LC coupled to MS. As a consequence, these tended to be cumbersome. In 1983, Weiss *et al.*[60] described a pyrolysis GC–mass spectrometric method for lasalocid in bovine liver. Samples were extracted and cleaned up using semi-preparative HPLC. Following pyrolysis of a ditrimethylsilyl derivative, two GC peaks were produced for lasalocid following a retroaldol cleavage. For the retroaldol aldehyde, which contained both trimethylsilyl (TMS) groups, two ions (*m/z* 381, MH^+; and *m/z* 291, $MH^+ - TMS$) were monitored. The MH^+ ion of the retroaldol ketone could not be measured because of matrix interference, but ions corresponding to the loss of water (*m/z* 337) and to a second decomposition product (*m/z* 211) were monitored. Validation data were not presented, but the method was capable of detecting lasalocid at a concentration of 51 μg kg^{-1} in an incurred liver sample. A fast atom bombardment (FAB) MS method, capable of measuring 10 μg kg^{-1} monensin in chicken fat, was described by Blomquist *et al.*[61] In addition to $[M+H]^+$, ions corresponding to a sodium adduct and to loss of water could be detected. Validation data were not presented.

Takatsuki *et al.*[35] described the determination of a trimethylsilyl ester of monensin using GC–MS. However, the method was very insensitive, being capable of detecting monensin only at concentrations above 5000 ng g^{-1}. Validation data were not presented. Monensin is, however, not well suited to GC–MS because of its high molecular mass and their low volatility. Since then, the increasing availability of LC–MS has enabled sensitive methods for the determination of such compounds to be developed. Horii *et al.*[62] described a thermospray LC–MS–MS method that could detect 60 μg kg^{-1} lasalocid in chicken liver. The molecular ion was not a significant ion. Instead, a fragment at *m/z* 337 was chosen as the parent ion, and daughter ions at *m/z* 319, 281 and 237 were measured. Validation data were not presented. The determination of maduramicin in chicken fat using thermospray LC–MS and thermospray LC–MS–MS has also been reported.[63] Fat was extracted with acetonitrile and partitioned with hexane. The extract was cleaned up using an alumina column prior to analysis. This clean-up procedure was insufficient to permit analysis using LC–MS. However, the increased selectivity offered by tandem mass spectrometry permitted the detection of maduramicin at concentrations down to approximately 50 μg kg^{-1}.

Schneider *et al.*[64] were the first to apply electrospray LC–MS to the determination of carboxylic acid ionophores. They described a method that could detect semduramicin at concentrations of approximately 30 μg kg^{-1} in chicken liver. In addition to the $[M+Na]^+$ ion at *m/z* 895, daughter ions at *m/z* 852 and 834, corresponding to the sequential loss of CO_2 and H_2O, were detected.

Table 6 Mass spectrometric methods for the determination of carboxylic acid ionophores

Ionophore	Extraction	Clean-up	Detection method*	LOQ†/μg kg^{-1}	Recovery (%)	Ref.
Las	Acetonitrile	Hexane wash/dry/extract into basic mobile phase	Pyrolysis GC–MS	> 50	NS‡	60
Mo	Acetonitrile	Hexane wash/dry/extract into basic mobile phase	FAB–MS	> 10	NS	61
Mo	Chloroform	Silica gel	GC–MS	> 5000	NS	35
La	NS	NS	Thermospray LC–MS	> 60	NS	62
Ma	Acetonitrile	Partition with hexane/alumina	Thermospray LC–MS	250	61–80	63
Se	Methanol	C_8 and silica-solid phase cartridges	Electrospray LC–MS	NS	NS	64
Las	Acetonitrile	Hexane–toluene	Electrospray LC–MS	5	77–88	65
Mo. Sa, Na	Methanol	Hexane–toluene	Electrospray LC–MS	5	85–117	66

* Mo = Monensin, Sa = salinomycin, Las = lasalocid , Ma = maduramicin, Se = semduramicin, Na = narasin. † LOQ = Limit of quantification. ‡ NS = Not stated.

More recently, this laboratory has published methods for the determination of lasalocid[65] and monensin, salinomycin and narasin[66] in biological matrices using electrospray LC–MS. In both instances, the extraction procedures were relatively simple. For lasalocid,[65] acidified sample homogenates were extracted into acetonitrile, and then cleaned-up by liquid–liquid extraction into hexane–toluene (1 + 1, v/v). For monensin, salinomycin and narasin,[66] samples were homogenised with methanol, and the drugs extracted into hexane–toluene (1 + 1, v/v) under basic conditions. These simple extraction procedures, combined with the compound-specific selectivity offered by electrospray LC–MS, resulted in the production of very clean traces. The limit of quantification in each assay was 5 μg kg^{-1}. In both assays, a pseudo-molecular ion, corresponding to $[M + Na]^+$, was the most prominent ion. It is often possible to adjust the amount of analyte fragmentation by increasing the skimmer cone voltage. However, for all four of these compounds, increasing the cone voltage had only a limited effect on the degree of fragmentation obtained.

Mass spectrometric methods: summary

Electrospray LC–MS appears to offer the best possibility for the confirmation of low levels of the carboxylic acid ionophores. The published methods are very sensitive, and require very simple sample extraction and clean-up steps. However, as with the HPLC methods described above, no truly multi-residue method has yet been developed.

In summary, as with the screening assays for ionophore residues, a wide variety of chemical procedures have been developed. The choice of method used in a laboratory will be dictated by the availability of suitable expertise and equipment.

Pharmacokinetics of ionophores

An important aspect of all drug incorporation in an animal's diet is the pharmacokinetic characteristics displayed; *i.e.*, the absorption, distribution, metabolism and clearance of the drug from the treated animal. These parameters all play a part in determining the matrix most suitable to monitor a particular drug residue. In addition, MRL legislation also determines whether or not the parent drug, metabolite(s) or both must be measured. However, MRLs for ionophores have not yet been established and the interpretation of pharmacokinetic information in this review will be restricted to distribution and clearance aspects.

A number of pharmacokinetic studies have been performed on ionophores in poultry. Some of the data generated have given conflicting results with regards to residue concentrations detected. The most likely explanations for these variations lie in the differences in analytical techniques used and, specifically, in wide variations in method sensitivities, extraction recoveries and the ability to differentiate metabolites from the parent compound.

Monensin studies

The earliest reported pharmacokinetic study of monensin was that of Herberg and Van Duyn.[67] Following administration of [^{3}H]monensin orally at a concentration of 360 mg kg^{-1} for two days, chickens were slaughtered at zero, two and four days after withdrawal. The radioactivity present in a wide range of samples was measured and converted to monensin equivalents. The highest residue concentrations were found in liver samples (7660 μg kg^{-1}) taken without withdrawal from the drug. Residues were still detectable in liver after the fourth day of withdrawal. (Table 7). From this study it was clear that the main excretion route for monensin was faecal as opposed to urinary (99 : 1 ratio). A similar study was performed by Donoho[68] with the exception that a more typical monensin medication regime was employed, *i.e.*, 120 mg kg^{-1} for 2 weeks. Substantially lower residue concentrations were detected in this latter study but residues in liver at day 0 (495 μg kg^{-1}) remained the highest concentrations found (Table 7). In contrast to these radiolabelled studies, pharmacokinetic experiments performed using

Table 7 Pharmacokinetic data on monensin residue concentrations present in poultry samples

				Matrix/sample type				
				Mean concentration detected/ μg kg^{-1} or μg l^{-1}				
Medication level/mg per kg of feed	Duration	Withdrawal period/d	Analytical assay	Serum	Fat	Liver	Muscle	Ref.
360	2 d	0	Radiolabel	1840	2320	7660	2160	67
		2		630	440	1470	1120	
		4		340	140	720	700	
120	1week	0	TLC–bioassay		110	39	29	18
		1			17	ND*	ND	
		2			ND	ND	ND	
120	2 weeks	0	TLC–bioassay		106	60		68
		1			ND	ND		
		0	Radiolabel		100	495	10	
		1			12	272	11	
		2			66	222	ND	
		3			50	149	ND	
		5			50	106	ND	
120	2 weeks	0	TLC–bioassay	1320	900	1300	700	69
		1		ND	100	180	ND	
		2		ND	ND	ND	ND	
100–120	30 d	0	ELISA		79	15	26	23
120	2 weeks	0	ELISA			93		
		1				18		24
		2				111		
		3				ND		

* ND = Not detected.

TLC–bioassay and ELISA as the means of residue quantification[18,24,69] gave more consistent results (Table 7). A noticeable exception to this was the results shown by Godfrey *et al*.,[23] who found lower concentrations of monensin residues in liver compared with fat and muscle. This finding may be due to the longer medication given during this study (30 d).

A study employing administration of [^{3}H]monensin gave clear evidence of extensive drug metabolism. Donoho *et al*.[70] concluded that only 70 and 7% of the radioactivity found in fat and liver samples, respectively, was due to the presence of the parent form of monensin. Analysis of liver extracts by chromatography revealed that many monensin metabolites were present.

In summary, monensin residues were readily detected in liver, fat and muscle for between zero and two days post drug withdrawal with liver generally giving the highest concentrations. After this time only the more sensitive methods were able to detect the presence of monensin residues. These data suggest that the withdrawal periods recommended by monensin manufacturers of 3 d are adequate to deliver meat with low residue content, provided good farm management is employed.

Salinomycin (Table 8)

In all salinomycin pharmacokinetic studies reviewed, a 2 week medication period at doses ranging from 30 to 132 mg kg^{-1} was given. Wide variations in the concentrations found between these studies were reported. Atef and co-workers[69,71] reported 1100 μg kg^{-1} salinomycin in the liver of chickens given no withdrawal; this compared with 3 μg kg^{-1} found by Kennedy *et al*.[27] under similar conditions. Such extensive differences may be best explained by the different analytical methods used in these studies.

The salinomycin data (Table 8) clearly show that after a one day withdrawal from the drug, the concentrations present in the edible tissues of the animals are very low and barely detectable.

Lasalocid and maduramicin (Table 9)

Pharmacokinetic data for lasalocid and maduramicin residues in poultry were available only in two and one studies, respectively. However, from this limited information it appears that lasalocid residues persist in edible tissues for longer than either salinomycin or monensin and may require a substantially longer withdrawal period than any of the other ionophore compounds studied to date.

Narasin and semduramicin

No pharmacokinetic information was found on either of these compounds. However, owing to structural similarities between narasin and salinomycin and semduramicin and maduramicin, similar residue profiles may be anticipated.

Summary

The concentrations of the various ionophores added to poultry feed in the studies reviewed varied in concentration from low to high ppm levels; the duration of medication ranged from a few days to a few weeks. There did not, however, seem to be simple correlations between the residue content found in samples when compared with either the size of dose or duration of treatment. This may be, in part, due to the various types of analytical techniques used to quantify the residue content and, in part, to differences in the procedures used to medicate and house the experimental animals.

What is clear, however, is that following withdrawal of medicated feed, residue concentrations fall quickly and that the time periods specified in manufacturer's guidelines will normally ensure that high residue levels do not reach the food chain. This is assuming that the correct level of medication has been given for the correct time period and that possible sources of cross-contamination of the drug have been removed. If any of these factors are not adequately controlled, then the likelihood of high residue concentrations in edible tissues increases sharply.

One important area for drug residue depletion studies that appears to have been overlooked is that of eggs. Reports have shown[72,73] that in both caged and free-range birds a significant number of positives have been found in eggs. This is

Table 8 Pharmacokinetic data on salinomycin residue concentrations present in poultry samples

Medication level/mg per kg of feed	Duration/ weeks	Withdrawal period/d	Analytical assay	Matrix/sample type: Mean concentration detected/ μg kg^{-1} or μg l^{-1}				Ref.
				Serum	Fat	Liver	Muscle	
60	2	0	TLC	700	900	1100	670	71
		1		ND*	110	100	ND	
		2		ND	ND	ND	ND	
30	2	0	HPLC		73	9	ND	42
		1			37	ND	ND	
		3			ND	ND	ND	
60	2	0	HPLC		162	13	ND	
		1			62	ND	ND	
		3			ND	ND	ND	
90		0	HPLC		201	24	60	
		1			73	ND	ND	
		3			ND	ND	ND	
60	2	0	ELISA	8		3	1	27
66	2	0	ELISA			203		33
		1				ND		
132		0				351		
		1				ND		

* ND = Not detected.

Table 9 Pharmacokinetic data on lasalocid and maduramicin residue concentrations present in poultry samples

Ionophore	Medication level/ mg per kg of feed	Duration/ weeks	Withdrawal period/d	Analytical assay	Matrix/sample type: Mean concentration detected/ μg kg^{-1} or μg l^{-1} Serum	Lung	Liver	Muscle	Ref.
Lasalocid	90	2	0	ELISA	250		450	1	28
			1		90		80	5	
			2		30		70	1	
			3		10		60	1	
Lasalocid	75	1	0	Bioassay	1360	6.9			19
Maduramicin	5	2	0	ELISA	70		106	28	29
			1		30		60	6	
			2		20		20	3	
			3		10		20	3	

particularly true of lasalocid. Work in this matrix is at an early stage.

Current position of ionophore residue testing in European national reference and control laboratories

A survey of all EU National Reference Laboratories (NRLs) and Control Laboratories (CLs) associated with drug residue analysis was performed, by the authors of this review, between May and September, 1997. This exercise was intended to provide an overview of the extent of coccidiostat residue analysis in Europe and a breakdown of the analytical methods currently employed. Of the 28 laboratories surveyed, replies were received from 23. All EU member states with the exception of Germany which carried out the survey provided a written response. In total 15 of the laboratories were actively involved in coccidiostat residue analysis; a further three had an interest in the subject and provided comments and opinions on the subject. This review will only deal with the array of information provided that was relevant to ionophore residue testing. A full report of the survey findings will appear elsewhere.

Range of substances monitored

Assays for monensin and salinomycin were performed in ten of the laboratories surveyed, eight of which also included narasin in their monitoring programmes. Only four laboratories had analytical methods to detect lasalocid residues; only one had methods for maduramicin, and semduramicin residue analysis was not performed in any laboratory.

At the time of this survey, none of the European laboratories that replied was able to measure residues of the whole group of ionophores. Furthermore, only one laboratory predicted that it would have the analytical capability to detect five of the six compounds in 1998.

Analytical methods utilised

The most frequently used analytical method employed to determine the presence of ionophore residues was undoubtedly HPLC. Many laboratories indicated that this technique was used as both a screening and confirmatory tool (49 and 35%, respectively). The second most popular analytical methodology was that of TLC and TLC–bioassays; these again were used in screening and confirmatory modes (27 and 39%, respectively). The other form of screening test used was immunoassay (15%). The more widespread use of this method was hampered by the lack of commercial test-kit availability. A small proportion of laboratories also indicated that LC-MS screening and confirmatory assays were available (9 and 26%, respectively).

Target matrices used for residue surveillance

The majority of laboratories chose to perform ionophore residue analysis on either muscle or liver samples. A smaller proportion of those surveyed also tested feed, egg and kidney samples for these substances. In only a single case was urine tested and no laboratory indicated that serum analysis was performed.

Comments and suggestions from survey

The recipients of the survey were asked for their opinions on the subject of the current state of coccidiostat residue analysis, what future changes are likely to occur within their own establishments and what progress they would like to see on a European scale.

There was virtually unanimous agreement that there was a great need for improvement in the field of coccidiostat monitoring. Whilst some laboratories thought that their current array of analytical methods was satisfactory, it was clearly felt that there was wide scope for the development of methods, both screening and confirmatory, which would offer greater sensitivity and be multi-residue in nature.

A second important aspect of the survey was to determine the interest in organising interlaboratory studies and a workshop on the theme of coccidiostat residue analysis. There was widespread approval of these two suggestions and a general feeling was conveyed that an organised provision of literature and analytical methods would be of considerable help to laboratories.

In summary, the area of coccidiostat monitoring in European residue testing laboratories has apparently been given a lower priority than other classes of veterinary drugs. This is most likely because there has not previously been a requirement under National Surveillance Schemes to include coccidiostat monitoring. There also appears to be a realisation that this will change in 1998 and many laboratories are looking for guidance and help with the implementation of satisfactory residue monitoring programmes which will enable them to comply with European legislative requirements.

In conclusion, the ionophore drugs have been, and continue to be, widely used in the control of coccidiosis in poultry. Analytical methods have been developed to detect trace levels of ionophore residues in treated birds. There has been a move towards ELISA for screening tests and LC–MS for confirmatory procedures. However, despite these improvements there is a lack of fast and reliable multi-residue methods available. With a requirement for coccidiostat residue monitoring appearing in new European Directives there is a clear need to move in this direction.

The authors thank Dr. Sabine Hahnau of the BGVV, Berlin, for assisting in the preparation and subsequent analysis of informa-

tion provided in the survey of European residue testing laboratories.

References

1 Pressman, B. C., Harris, E. J., Jagger, W. S., and Johnson, J. H., *Proc. Natl. Acad. Sci. USA*, 1967, **58**, 1949.
2 Reed, P. W., *Methods Enzymol*., 1979, **55**, 435.
3 Pressman, B. C., *Annu. Rev. Biochem*., 1976, **45**, 501.
4 Johnson, S. M., Herrin, J., Liu, S. J., and Paul, I. C., *J. Am. Chem. Soc*., 1970, **92**, 4428.
5 Pressman, B. C., and Fahim, M., *Annu. Rev. Pharmacol. Toxicol*., 1982, **22**, 465.
6 Danforth, H. D., Ruff, M. D., Reid, W. M., and Johnson, J., *J. Poultry Sci*., 1977, **56**, 933.
7 Pressman, B. C., and Fahim, M., *Adv. Exp. Med. Biol*., 1983, **161**, 543.
8 Weiss, G., and MacDonald, R., *J. Assoc. Off. Anal. Chem*.,1985, **68**, 971.
9 Strout, R. G., and Ouellette, C. A., *Exp. Parasitol*., 1973, **33**, 477.
10 McDougald, L. R., and Galloway, R. B., *Exp. Parasitol.*, 1973, **34,** 189.
11 Gilbert, J. M., Fuller A. L., and McDougald, A., *Colloq. INRA*, 1989, **49**, 253.
12 Donoho, A. L., and Kline, R. M., *Antimicrob. Agents Chemother*., 1967, 763.
13 Vanderkop, R. A., and MacNeil, J. D., *J. Assoc. Off. Anal. Chem*., 1989, **72,** 735.
14 Heil, K., Fleur, P., and Cieleszky, S., *J. Agric. Food Chem*., 1984, **32**, 997.
15 Vanderkop, R. A., and MacNeil, J. D., *J. Chromatogr*., 1990, **508**, 386.
16 Kondo, F. J., *Food Prot.*, 1980, **51**, 786.
17 Kline, R. M., Stricker, R. E., Coffman, J. D., and Bikin, H., *J. Assoc. Off. Anal. Chem*., 1970 , **53**, 49.
18 Okada, J., Higuchi, I., and Kondo, S., *J. Food Hyg. Soc*., 1980, **21**, 177.
19 Stipkovits, L., and Juhasz, S., *Acta Vet. Hung.*, 1987, **35**, 237.
20 Heitzman, R. J., Carter, A. P., and Cottingham, J. D., *Br. Vet. J.*, 1986, **142**, 516.
21 Pauillac, S., Halmos, T., Labrousse, H., Antonakis, K., and Avrameas, S. J., *Immunol. Methods*, 1993, **164**, 165.
22 Mount, M. E., and Failla, D. L., *J. Assoc. Off. Anal. Chem*., 1986, **70,** 201.
23 Godfrey, M. A. J., Luckey, M. F., and Kwasowski, P., *Food Addit. Contam.*, 1997, **14**, 281.
24 Crooks, S. R. H., Traynor, I. M., Elliott, C. T., and McCaughey, W. J., *Analyst*, 1997, **122**, 161.
25 Miller, G. A., Clark, R. C., and Jessee, W., *Hybridoma*, 1986, **5**, 353.
26 Elissalde, M. H., Beier, R. C., Rowe, L. D., and Stanker, L. H., *J.Agric. Food Chem*., 1993, **41**, 2167.
27 Kennedy, D. G., Blanchflower, W. J., and O'Dornan, B. C., *Food Addit. and Contam.*, 1995, **12,** 93.
28 Kennedy, D. G., Blanchflower, W. J., and O'Dornan, B. C., *Food Addit. Contam.*, 1995, **12**, 83.
29 Kennedy, D. G., Blanchflower, W. J., and O'Dornan, B. C., *Food Addit. Contam.*, 1995, **14**, 27.
30 Erlanger, B. F., Borek, F., Beiser, S. M., and Lieberman, S., *J. Biol. Chem.*, 1957, **228**, 479.
31 Muldoon, M. T., Elissalde, M. H., Beier, R. C., and Stanker, L. H., *J. Agric. Food Chem.*, 1995, **43**, 1745.
32 Herberg, R., Manthey, J., Richardson, L., Cooley, C., and Donoho, A., *J. Agric. Food Chem.*, 1978, **26,** 1087.
33 Asukabe, H., and Harada, K. I., in: *Chemical Analysis for Antibiotics used in Agriculture*, ed. Oka, H., Nakazawa, H. Harada, K. I., and MacNeil, J. D., AOAC International, Arlington, VA, 1995, p. 121.
34 Martinez, E. E., and Shimoda, W., J. Assoc. Off. Anal. Chem.,1985, **68**, 1149.
35 Takatsuki, K., Suzuki, S., and Ushizawa, I., *J. Assoc. Off. Anal. Chem*., 1986, **69**, 443.
36 Martinez, E. E., and Shimoda, W., *J. Assoc. Off. Anal. Chem*., 1986, **69**, 637.
37 Hoshino, Y., Horie, M., Nose, N., and Iwasaki, H., *J. Food Hyg. Soc. Jpn*., 1985, **26**, 585.
38 Moran, J. W., Rodewald, J. W., Donoho, A. L., and Coleman, M. R., *JAOAC Int.*, 1994, **77**, 885.
39 Ericson, J. F., Calcagni, A., and Lynch, M. J., *JAOAC Int.*, 1994, **77**, 577.
40 Gerhardt, G. G., Salisbury, C. D. C., and Campbell, H. M., *Food Addit. Contam.*, 1995, **12**, 731.
41 Muldoon, M. T., Elissalde, M. H., Beier, R. C., and Stanker, L. H., *Immunoassays for Residue Analysis*, Am. Chem. Soc., Symp. Ser., vol. 621, 1996, ch. 9, p. 109.
42 Akhtar, M. H., El-Sooud, K. A., and Shehata, A. A., *Food Addit. Contam.*, 1996, **13**, 897.
43 Akhtar, M. H., Croteau, L. G., *J. Environ. Sci. Health*, 1996, **B31**, 117.
44 Miyakawa, H., Horii, S., and Kokubo, Y., *J. Food Hyg Soc.*, 1995, **36**, 725.
45 Dimenna, G. P., Creegan, J. A., Turnbull, L. B., and Wright, G. J., *J. Agric. Food Chem.*, 1986, **34**, 805.
46 Golab, T., Barton, S. J., and Scrogs, R. E., *J. Assoc. Off. Anal. Chem*., 1973, **56**, 171.
47 Blanchflower, W. J., Rice, D. A., and Hamilton, J. T. G., *Analyst*,1985, **110**,1283.
48 Asukabe, H., Sasaki, T., Harada, K.-I., and Suzuki, M., *J. Chromatogr.*, 1984, **295**, 453.
49 Asukabe, H., Murata, H., Harada, K.-I., and Suzuki, M., *J. Chromatogr. A*, 1993, **657**, 349.
50 Asukabe, A., Murata, H., Harada, K.-I., Suzuki, M., Oka, H., and Ikai, Y., *J. Agric. Food Chem.*, 1994, **42**, 112.
51 Mathur, A. K., *J. Chromatogr. A*, 1994, **664**, 284.
52 Weiss, G., Felicito, N. R., Kayakaty, M., Chen, G., Caruso, A., Hargroves, E., Cowley, C., and MacDonald, A., *J. Agric. Food Chem*., 1983, **31**, 75.
53 Kozak, A., and Wisniewska-Dymytrow, H., *Med. Weter*., 1995, **51**, 160.
54 Ishikuro, E., *Eisigaku Zasshi*, 1989, **30**, 160.
55 Horii, S., Miyahara, K., and Momma, C., *J. Liq. Chromatogr*., 1990, **13**, 1411.
56 Tarbin, J. A., and Shearer, G., *J. Chromatogr*., 1992, **579**, 177.
57 Kayakaty, M., and Weiss, G., *J. Agric. Food Chem*., 1983, **31**, 81.
58 Frank, L. R., and Barnes, C. J., *J. Assoc. Off. Anal. Chem*., 1989, **72**, 584.
59 Newkirk, D. R., and Barnes, C. J., *J. Assoc. Off. Anal. Chem*., 1989, **72**, 581.
60 Weiss, G., Kaykaty, M., and Miwa, B., *J. Agric. Food Chem*., 1983, **31**, 78.
61 Blomquist, G. B., Jansson, M., Ryhage, E. R., and Osterdahl, B.-G., *J. Agric. Food Chem*., 1986, **34**, 274.
62 Horii, S., Miyahara, K., and Maruyama, T., *Shokuhin Eisigaku Zasshi*, 1991, **32**, 30.
63 Stout, S. J., Wilson, L. A., Kleiner, A. I., da Cunha, A. R., and Francl, T. J., *Biomed. Environ. Mass Spectrom.*, 1989, **18**, 57.
64 Schneider, R. P., Lynch, M. J., Ericson, J. F., and Founda, H. G., *Anal. Chem*., 1991, **63**, 1789.
65 Blanchflower, W. J., and Kennedy, D. G., *Analyst*, 1995, **120**, 1129.
66 Blanchflower, W. J., and Kennedy, D. G., *J. Chromatogr*. 1996, **675**, 225.
67 Herberg, R. J., and Van Duyn, E., *J. Agric. Food Chem*. 1969 **17**, 853.
68 Donoho, A. L., *J. Anim. Sci*., 1982, **58**, 1528.
69 Atef, M., Ramadan, A. and Abo-El-Sooud, K., *Br. Poultry Sci*., 1993, **34,** 195.
70 Donoho, A. L., Herberg R. J., Zornes, L. L., and Van Duyn, E., *J. Agric. Food Chem*., 1982, **30**, 909.
71 Atef, M., Ramadan, A., Youssef, S. A. H., and El-Sooud, A., *Res. Vet Sci.*, 1993, **54,** 179.
72 *Medicines Act Veterinary Information Service*, Veterinary Medicines Directorate, UK, 21st edn., John C. Alborough Ltd., Ringshall, Suffolk, UK, January, 1997.
73 Kennedy, D. G., Blanchflower, W. J., Hughes, P. J., and McCaughey, W. J., *Food Addit. Contam*., 1996, **13**, 787.

Paper 7/08698I
Received December 2, 1997
Accepted February 23, 1998