SCREENING METHODS FOR DETECTION OF SALINOMYCIN

Velichka Andonova*, Bisera Pilicheva, Petia Peneva, Dimitar Penkov
Medical University – Plovdiv, Faculty of Pharmacy, Department of Pharmaceutical Sciences, 4002, Plovdiv, Bulgaria
Corresponding author: Velichka Andonova, andonova_v@abv.bg

ABSTRACT

Salinomycin (SAL) is a carboxylic polyether ionophore isolated from *Streptomyces albus* that has been widely used as an agricultural antibiotic to prevent coccidiosis in poultry and overdose or use in non-target animal species can result intoxication. SAL intoxication has been described in turkeys, horses, pigs, cats, and calves. SAL has potential pharmacological properties in the human organism. Recently, SAL has been identified as a highly effective chemical in the elimination of CSCs in some types of cancer, such as breast cancer, leukaemia, colorectal cancer, lung cancer, gastric cancer, and osteosarcoma. SAL acts as a potent inhibitor of multidrug resistance protein 1 and induces apoptosis in drug- and apoptosis-resistant human cancer cells. SAL as a polyether ionophore will have pharmacological effects on physiological processes that are sensitive to disrupted ion transport (e.g. the functioning of the heart and nervous system) and may cause toxicity in susceptible species. In this regard the quantitative determination of SAL and its metabolites in biological fluids and tissues of the living body (blood, lymph, tissue), in food products (milk, meat, eggs), and in some natural elements (soil, sediment, fertilizer, plant species, etc.) is essential for human health and the environment.

The purpose of this study is an overview of validated analytical methods for the quantification of SAL in the biological tissues and food products.

***Keywords**: salinomycin, feed additives, maximum residue levels (MRLs), coccidiostats in food, maximum levels (MLs) in feed.*

INTRODUCTION

Nowadays there is no animal food production without using veterinary medicinal products and feed additives (1). This may result in the presence of their residues in the edible tissues and the food of animal origin, like milk and eggs. The residues are small quantity levels of the substances (μg/kg or mg/kg) in the food obtained from the animals after some kind of administration or in the soil, the plants and etc. that have been contaminated with the wastewater and manure (2). Maximum residue levels for the presence of coccidiostats in food (Commission Regulation (EU) No. 124/2009) (3) and maximum levels (MLs) in feed (Commission Regulation No. 574/ 2011) (4) have been set within the EU in order to protect consumers and animals against unintended carry-over, and their monitoring is compulsory.

Salinomycin (SAL) ((2R)-2-[(5S,6R)-6-[(1S,2S,3S,5R)-5-2S,5R,7S,9S,10S,12R,15R)-2-[(2R,5R,6S)-5-ethyl-5-hydroxy-6-methyl-2-tetrahydropyranol]-15-hydroxy-2,10,12-trimethyl-1,6,8-trioxadispiro[4.1.5^{7}.3^{5}]pentadec-13-en-9-yl]-2-hydroxy-1,3-dimethyl-4-oxoheptyl]5-methyl-2-tetrahydropyranyl]butanoic acid) is a monovalent polyether antibiotic with ionophoric properties (2). SAL is synthesized from *Streptomyces albus* (5). It is used as a feed additive for the prophylactic control of the coccidial species *E. acervulina, E. maxima, E. necatrix, E. mitis, E. praecox, E. tenella* and *E. brunetti* affecting chickens for fattening and chickens reared for laying. It is added to the feeds as its sodium salts at levels of 20–70 mg/kg. It is also applied in rabbits for fattening for prevention of coccidiosis caused by *E. coecicola, E. perforans, E. media, E. irresidua, E. magna, E. piriformis, E.exigua, E. intestinalis, E. flavescens, E. stiedai.* SAL also used for improvement of weight gain and feed conversion efficiency in pigs and ruminants with developed forestomachs (calves and lambs) for fattening but it is dangerous for equines and turkeys. SAL administer orally, thoroughly mixed into the feed. The biological activity of SAL is due to its ability to form complexes with cations, particularly its ability to transport potassium ions into and out of

the cell. The resulting disturbance in the parasite's intercellular ion concentration explains the coccidiocidal activity (6).

POTENTIAL PHARMACOLOGICAL PROPERTIES

SAL has potential pharmacological properties in the human organism and may cause toxicity in susceptible species. It increases coronary flow by dilating coronary vessels, and the induced dilation may be a risk for victims of coronary artery disease whose blood flow is already maximized (7). Thus the insufficiently precise control of residues in foodstuffs could lead to severe consequences to the human health. Despite the fact that European Union (EU) legislation demands residue monitoring of anticoccidial compounds (3, 4, 8), there is evidence that some coccidiostat residues are present in poultry tissue and eggs and that the consumer is not being given adequate protection. SAL is not intended for layer hens, but nonetheless, residues of SAL in poultry products, especially eggs, may occur if laying hens receive a feed contaminated with broiler feed that contains SAL. Eggs are very important nutriment component. They are highly nutritious, commonly available, and inexpensive, and also they can be consumed in the primary form or as intermediate products (dried eggs, egg powder, blend of bakery or protein powder). On the other hand, poultry meat also is one of the most commonly consumed meats in many regions. This creates a need for simple and rapid methods for the screening of SAL in food. MRLs for the presence of coccidiostats in food (3) and MLs in feed (4) are presented in Table 1.

In recent decades cytotoxic and anti-proliferative activities of SAL have thoroughly investigated (9). In this context the depth study of the various analytical methods for qualitative and quantitative determination of SAL is essential.

The purpose of this study is an overview of validated analytical methods for the quantification of SAL in the biological tissues and food products.

SCREENING METHODS CLASSIFICATION

According to the guidelines for the validation of screening methods for residues of veterinary medicines (14) the screening methods can be classified either according to the principle of detection or according to whether they are qualitative or (semi-)quantitative (Fig.1). A screening method has to discriminate suspicious (noncompliant) samples from the total amount of samples. Setting up the cut-off level implies the finding of the appropriate balance between the false-positive (false noncompliant) and false-negative (false-compliant) measurements. According to the Commission Decision (8), the false-negative rate should be less than 5 %.

Elliott *et al.* (10) have presented a broad overview of the analytical methods for ionophore residue detections used until then. The authors have paid special attention to the chemical methods for the detection of ionophore residues as HPLC and mass spectrometric methods. As screening methods they have considered enzyme-linked immunosorbent assay (ELISA) and a TLC–bioautographic assay. But these methods have some drawbacks that make them not so attractive for routine testing. It should be noted that there are requirements for lengthy incubation periods in *in vitro* assay procedures and for TLC–bioautographic procedures rely heavily on lengthy solvent-based extraction systems and the sensitivities achieved vary widely.

Bienenmann-Ploum *et al.* (11) have described single-laboratory validation of a multiplex flow cytometric immunoassay for the simultaneous detection of six coccidiostats in eggs and five of them in feed including SAL. The cut-off levels authors have determined by three methods, a statistical assessment and two rapid estimations. The cut-off levels have obtained with one of rapid method for narasin/SAL in eggs has been 60 (%B/B0). In feed, this level has been 70, 64, 72 and 78 (%B/B0). These levels have been almost identical with the statistical (reference) approach and the other rapid approach. Furthermore, a very good correlation (r ranging from0.994 to 0.9994) has been observed between the two used different analysers, FlexMAP 3D® and MAGPIX®, demonstrating adequate transferability.

Table 1: MRLs for the presence in food (Commission Regulation (EU) No. 124/2009) and MLs in feed (Commission Regulation No. 574/ 2011) for SAL (sodium salt)

Salinomycin sodium	***Products intended for animal feed***	***Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %***
Authorized feed additives in non-target feed following unavoidable carry-over	Feed materials Compound feed for: — equine species, turkeys, laying birds and chickens reared for laying (> 12 weeks), — chickens for fattening, chickens reared for laying (< 12 weeks) and rabbits for fattening for the period before slaughter in which the use of salinomycin sodium is prohibited (withdrawal feed), — Other animal species.	0.7 0.7 0.7 2.1
	Foodstuffs	***Maximum content in µg/kg (ppb) wet weight***
Maximum levels in foodstuffs	Food of animal origin from animal species other than chickens for fattening and rabbits for fattening: — eggs; — liver; — other food.	 3 5 2

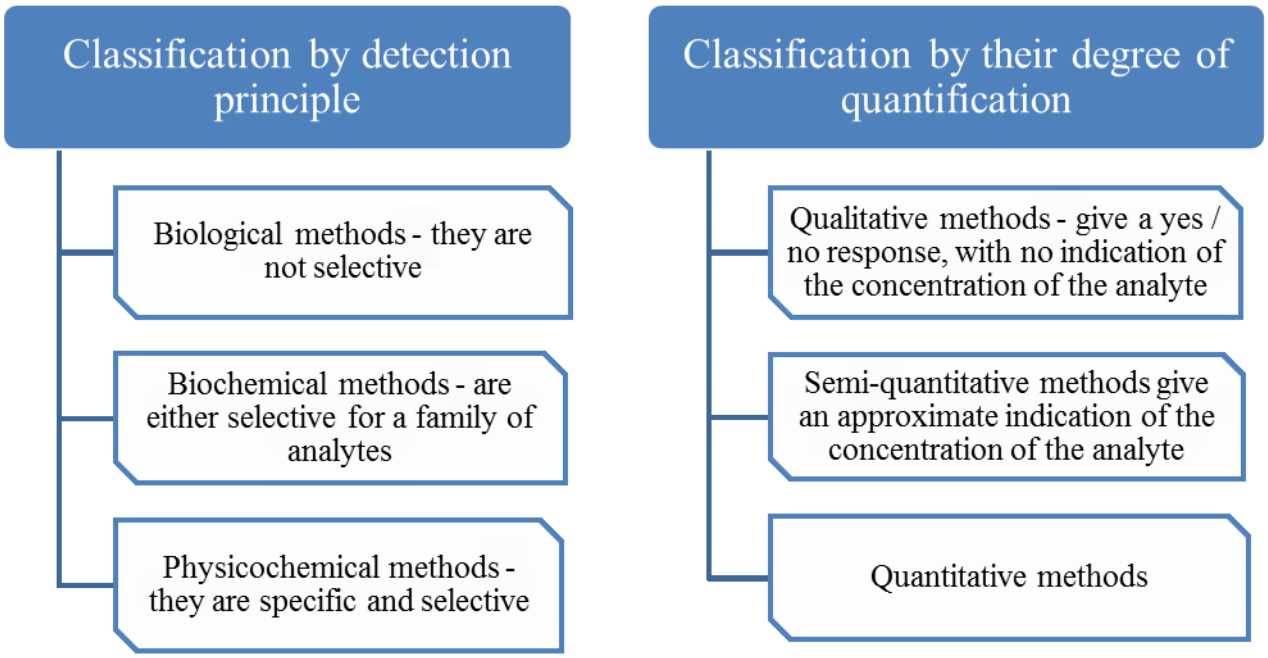

Fig. 1: Screening Methods classification.

A screening method based on rapid time-resolved flour-immunoassay (TR-FIA) has been described by Peippo *et al.* (12) for the screening of SAL in muscle and eggs. The limit of detection (LOD) for SAL has been 0.56 and 0.28 µg/kg for muscle and eggs, respectively. Heller and Nochetto (13) have described a screening method based on Ion-Trap LC-MS/MS for several non-polar residues in eggs. The LOD of this method has been 1 µg/kg for SAL in eggs.

According to the guidelines for the validation of screening methods for residues of veterinary medicines (14) the most common techniques utilized recently in the area of coccidiostat analysis are thin-layer chromatography (TLC) and liquid chromatography combined with mass spectrometry (LCMS) (15). The chromatographic techniques for the residue analysis of salinomycin in poultry tissue samples are generally based on complex and time-consuming extraction, clean-up, and derivatization procedures followed by separation on a reverse phase chromatographic column.

A multi-residue quantitative method based on LC-MS/MS has been described by Mortier *et al.* (15) for analysis of four coccidiostats, including SAL, in eggs. For SAL, the decision limit (CCα) has been 1 μg/kg. Finally, an LC-MS/MS multi-residue method has been described by Rokka and Peltonen (16) for the quantitative detection of four coccidiostats (lasalocid, monensin, SAL and narasin) in eggs and chicken meat. With this method, the CCα values for SAL have been 0.9 and 2.5 μg/kg in eggs and muscle, respectively.

Recently, procedures based on solid phase microextraction (SPME) coupled with LC-MS have been proposed (17) with a gain in terms of sample workup and sensitivity.

For determination of SAL in eggs Šinigoj-Gačnik *et al.* (18) have been used a method of high-performance thin-layer chromatography (HPTLC) with chemical detection using p-anisaldehyde as a derivative reagent. The intensity of coloured spots has been measured by densitometry at 510 nm. The detection limit (LOD) have been 10 $\mu g \cdot kg^{-1}$ and 7.5 $\mu g \cdot kg^{-1}$ for egg yolk and albumen, respectively. For the determination of SAL in muscle and liver tissue the drug has been extracted by isooctane and solid phase extraction (SPE) has been followed on silica column. Thin-layer chromatography (TLC) by bioautographic identification with the test organism *B. subtilis* has been used for identification and quantification in order to achieve low detection limits. For muscle and liver tissues ethyl acetate-water (97+3) has been used as the mobile-phase, while for the fat, chloroform-methanol-ammonium hydroxide (95+5+0.5) has been preferred. Quantitative evaluation has done by measuring the size of inhibitory zones of *B. subtilis* growth. The LOD have been 7.5 $\mu g \cdot kg^{-1}$, 10 $\mu g \cdot kg^{-1}$ and 20 $\mu g \cdot kg^{-1}$ for the muscle tissue, liver and fat, respectively. However, those methods are too complex and expensive for routine screening of samples.

Galarini *et al.* (19) have described a method for determination of eleven coccidiostats including SAL in eggs. The sample has been extracted with acetonitrile, defatted with hexane and cleaned-up on a silica SPE cartridge. The analytes have been identified and quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The results have proved that the method enables the confirmation of regulated coccidiostats in eggs at the levels required in the official control of residues (CCα in the range of 2.2–174 $\mu g.kg^{-1}$, depending on the coccidiostat). The repeatability (CV r in the range of 1.1–19%) and within-laboratory reproducibility (CV Rw in the range of 1.8–30%) have been also acceptable. The procedure has been successfully verified in the proficiency test and has been implemented in the national residue control plan.

Wide variations in the concentrations of SAL in eggs and edible tissues have found between these studies which reported. The differences could be explained by the different analytical methods used in the studies as well as the various apparatuses in the same method.

CONCLUSION

In conclusion, SAL as an ionophore drug has been, and continues to be, used extensively in the control of coccidiosis in poultry. Tests for daily routine analysis should be fast, easy, accurate and cheaply. Used up to now methods for the determination of SAL in animal tissues and food products is labour-intensive, time-consuming and expensive. These methods are not suitable enough for daily routine analysis. Given that the use of SAL as an additive in poultry feed will continue, the demand and the development of extremely precise, express and cheap tests to ensure human safety is crucial.

REFERENCES

1. Regulation (EC) (2003) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 L 268.
2. Piątkowska, M., P. Jedziniak, J. Żmudzki, 2012. Residues of veterinary medicinal products and coccidiostats in eggs – causes, control and results of surveillance program in Poland, Polish Journal of Veterinary Sciences, 15(4): 803-12.
3. Commission Regulation (EC) (2009) No 124/2009 of 10 February 2009 L40/7.
4. Commission Regulation (EU) (2011) No 574/2011 of 16 June 2011 L159.
5. Yurkovich, M. E., P. A. Tyrakis, H. Hong, Y. Sun, M. Samborskyy, K. Kamiya, P. F. Leadlay, 2011. A Late-Stage Intermediate in Salinomycin Biosynthesis is Revealed by Specific Mutation in the Biosynthetic Gene Cluster, ChemBioChem, 13(1): 66–71.
6. INTERNATIONAL REGISTRATIONS. Anticoccidials. SALINOPHARM® 12% PREMIX (salinomycin sodium).
7. Pressman, B.C., M. Fahim, 1983. Cardiovascular toxicity of ionophores used as feed additives. AdV. Exp. Med. Biol., 161, 543-61.
8. European Union Law, Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/ EEC and 91/664/EEC. Off. J. Eur. Communities 1996, L125, 10-32.
9. Naujokat, C., R. Steinhart, 2012. Salinomycin as a Drug for Targeting Human Cancer Stem Cells, Journal of Biomedicine and Biotechnology, ID 950658, 17 pages.
10. Elliott, C. T., D. G. Kennedy, W. J. McCaughey, 1998. Methods for the detection of polyether ionophore residues in poultry, Analyst, 123, 45R-56R.
11. Bienenmann-Ploum, M. E., U. Vincent, K. Campbell, A.-C. Huet, W. Haasnoot, P. Delahaut, L. (A.)A.M. Stolker, C. T. Elliott, M. W. F. Nielen, 2013. Single-laboratory validation of a multiplex flow cytometric immunoassay for the simultaneous detection of occidiostats in eggs and feed, Anal Bioanal Chem, 405: 9571–7.
12. Peippo, P., V. Hagren, T. Lövgren, M. Tuomola, 2004. Rapid time-resolved fluoroimmunoassay for the screening of narasin and salinomycin residues in poultry and eggs. J Agric Food Chem, 52(7): 1824-8.
13. Heller, D.N., C.B. Nochetto, 2004. Development of multiclass methods for drug residues in eggs: silica SPE cleanup and LC-MS/MS analysis of ionophore and macrolide residues. J Agric Food Chem, 52(23): 6848-56.
14. Community Reference Laboratories Residues (CRLs) (2010) Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer). January 2012.
15. Mortier, L., E. Daeseleire, C. Van Peteghem, 2005. Determination of the ionophoric coccidiostats narasin, monensin, lasalocid and salinomycin in eggs by liquid chromatography/tandem mass spectrometry, Rapid Commun Mass Spectrom, 19(4): 533-9.
16. Rokka, M., K. Peltonen, 2006. Simultaneous determination of four coccidiostats in eggs and broiler meat: validation of an LC-MS/MS method, Food Addit Contam, 23(5): 470-8.
17. Rosen, J., 2001. Efficient and sensitive screening and confirmation of residues of selected polyether ionophore antibiotics in liver and eggs by liquid chromatography-electrospray tandem mass spectrometry, Analyst, 126, 1990-5.
18. Šinigoj-Gačnik, K., O. Zorman Rojs, 2008. Salinomycin Concentration in Eggs and Tissues of Laying Hens, Acta Vet. Brno, 77: 423–9; doi:10.2754/avb200877030423.
19. Galarini, R., L. Fioroni, S. Moretti, L. Pettinacci, G. Dusi, 2011. Development and validation of a multi-residue liquid chromatography-tandem mass spectrometry confirmatory method for eleven coccidiostats in eggs, Anal Chim Acta, 700: 167- 176.