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Rapid Time-Resolved Fluoroimmunoassay for the Screening of Narasin and Salinomycin Residues in Poultry and Eggs

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Anticoccidial drugs are extensively used in the poultry industry to control the infection of the single-cell protozoa of the genus *Eimeria*. The most commonly used coccidiostats in poultry are the polyether ionophores such as narasin and salinomycin. This paper presents a rapid and simple method for the screening of residues of these two coccidiostatic compounds in poultry and eggs. The method is based on time-resolved fluoroimmunoassay. Sample preparation of eggs consists only of one extraction and evaporation step, and a solid phase extraction step is needed only for the muscle sample preparation. Mean recoveries were 91.0% from muscle tissue and 81.1% from eggs for both narasin and salinomycin. The performance of the assay was evaluated only for narasin because salinomycin had a cross-reactivity of 100% in the assay, and the recoveries of the compounds were not significantly different ($P > 0.05$). The limits of detection [mean + 3 × standard deviation (SD)] of narasin were 0.56 and 0.28 μg/kg, and the limits of quantification (mean + 9 × SD) were 1.80 and 0.57 μg/kg for muscle and eggs, respectively. The coefficients of variation (CV) of the interassay precision of the method, evaluated by five replicate analyses of muscle samples spiked with 2 μg/kg of narasin and egg samples spiked with 1 μg/kg of narasin, were 4.1 and 6.4%, respectively. The CVs of intra-assay precision tests, determined by 10 replicate analyses at the above-mentioned concentration levels, were 3.8 and 4.5%, respectively.

KEYWORDS: Narasin; salinomycin; time-resolved fluoroimmunoassay; screening

INTRODUCTION

Narasin and salinomycin (**Figure 1**) are structurally very similar polyether ionophores. These ionophores are widely used coccidiostats for the prevention of coccidiosis in the poultry industry (*1*). Coccidiosis is one of the most common and costly diseases in poultry. Infection is caused by a number of different species of single-cell protozoas of the genus *Eimeria*. The anticoccidial activity of narasin and salinomycin is based on their ionophoric properties to form complexes with polar cations on the cell membrane of parasites and their effects on the function of membranes (*2*, *3*).

Polyether ionophores have potential pharmacological properties and may cause toxicity in susceptible species. These compounds increase 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 (*4*). Despite the fact that European Union (EU) legislation demands residue monitoring of anticoccidial compounds (*5*), there is a evidence that some coccidiostat residues are present in poultry tissue and eggs and that the consumer is not being given adequate protection. This creates a need for simple and rapid methods for the screening of these compounds in food.

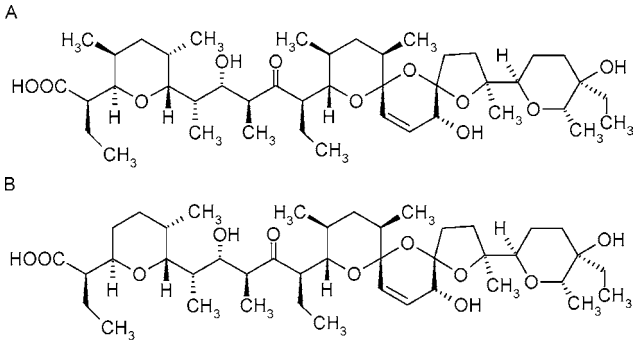

Figure 1. Chemical structure of narasin (A) and salinomycin (B).

The most common techniques utilized recently in the area of coccidiostat analysis are thin-layer chromatography (TLC) and liquid chromatography combined with mass spectrometry (LC-MS) (*1*). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) application is also reported (*6*). The chromatographic techniques for the residue analysis of narasin and salinomycin in poultry tissue samples are generally based on complex and time-consuming extraction, cleanup, and derivatization procedures followed by separation on a reverse phase chromatographic column (*1*, *7*). Recently,

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procedures based on solid phase microextraction (SPME) coupled with LC-MS have been proposed (*1*, *8*) with a gain in terms of sample workup and sensitivity. However, those methods are too complex and expensive for routine screening of samples.

Immunoassays provide a useful alternative for the estimation of coccidiostat levels due to their sensitivity, easy sample pretreatment, and affordable instrumentation. Some enzyme-linked immunoassay (ELISA) methods have been reported for the analysis of salinomycin in poultry products (*9*−*12*). However, these procedures have been developed and validated to detect residues only in liver and muscle. ELISA methods have also some disadvantages because they are very sensitive to matrix interferences caused by, for example, naturally occurring enzymes in the sample matrix and the signal is very dependent on physical and chemical conditions during the substrate incubation.

Time-resolved fluorescence immunoassays (TR-FIAs) based on lanthanide chelates are well established in the screening of contaminants and drug residues in food (*13*−*15*). This technology is based on the unique fluorescent properties of the chelates as characterized by narrow-band emission lines, long Stokes shifts, and long fluorescence lifetimes. The long emission duration is utilized in the delayed measurement of specific fluorescence, which is distinct from the short-lived background signal and it is not so sensitive to interferences (*16*).

In the present paper we report the application of competitive TR-FIA to the determination of narasin and salinomycin in poultry and eggs.

MATERIALS AND METHODS

Reagents. All reagents, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO), and salinomycin was obtained from Fluka (Steinheim, Switzerland). Bovine serum albumin (BSA) was supplied by Intergen (Chicago, IL). The goat anti-sheep IgG was obtained from Biospacific (Emeryville, CA). The polyclonal antiserum against narasin, which was produced in sheep, was a gift from Dr. Christopher Elliott (Queen's University of Belfast, Veterinary Sciences Division, Belfast, Northern Ireland, U.K.). Low Fluorimax polystyrene microplates were from Nunc (Roskilde, Denmark). A Sephadex G-25 DNA-grade columns and a Superdex 200 HR 10/30 gel filtration column were supplied by Amersham Biosciences (Uppsala, Sweden). Bond Elut solid phase extraction (SPE) cartridges were supplied by Varian (Walnut Creek, CA). Delfia Wash solution and Delfia Enhancement solution were purchased from Perkin-Elmer Life Sciences (Wallac, Turku, Finland).

Preparation of Europium-Labeled Narasin—Apotransferrin Conjugate. Narasin was activated by using modified *N*-hydroxysuccimide active ester method (*17*). Narasin (6.6 μmol) was incubated overnight at room temperature with 7.3 μmol of *N*-hydroxysuccinimide and 7.3 μmol of dicyclohexylcarbodiimide in 300 μL of dimethylformamide. A 100 times molar excess of the product was combined with 1 mg of apotransferrin in 0.5 mL of 50 mM phosphate buffer (pH 7.0). The solution was allowed to stand overnight at room temperature, and the buffer was changed to 50 mM carbonate buffer (pH 9.2) by using a Sephadex G-25 column. A 30 times molar excess of the europium labeling reagent [2,2′,2″,2‴-({4-[(4-isothiocyanatophenyl)ethynyl]-pyridine-2,6-diyl}bis(methylenenitrilo))tetrakis(acetato)]europium (III) (*18*) was coupled to the hapten−apotransferrin conjugate in 1 mL of 50 mM carbonate buffer (pH 9.2). The reaction was carried out overnight at room temperature. The Eu-labeled narasin−transferrin conjugate was purified by gel filtration with 50 mM Tris-HCl (pH 7.75) as elution buffer.

Preparation of Immunosensitive Microplate Wells. Polystyrene microplate wells were coated with 200 μL of the goat anti-sheep IgG at a concentration 5 μg/mL in 0.2 M NaH_2PO_4. After an overnight incubation at 37 °C, the wells were washed two times with washing buffer and then blocked with 300 μL of saturation buffer (50 mM NaH_2PO_4, 85 μM $CaCl_2$, 0.9% NaCl, 6% sorbitol, 0.05% Germall II, 0.1% BSA, and 4 μM EDTA). After an overnight reaction at room temperature, the wells were aspirated. The coated microwell plates were sealed in aluminum bags with desiccant and stored at 4 °C until use. The polyclonal antiserum against narasin and salinomycin was immobilized into the wells before the assay. An appropriate concentration of the antibody (50 μL) was added to the wells in assay buffer. After 1 h of incubation in a shaker at room temperature, the wells were washed four times with washing buffer.

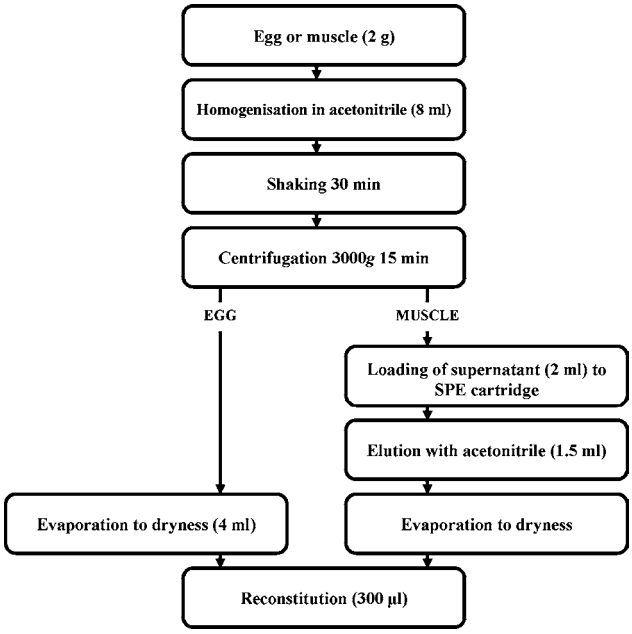

Figure 2. Flowchart for the sample pretreatment of eggs and poultry muscle.

Sample Pretreatment. Samples of chicken muscle tissue and eggs from different suppliers were collected in Finland in 2002 and stored at −20 °C until analyzed. The flowchart for the sample pretreatment of eggs and poultry muscle is presented in **Figure 2**.

Egg. Well-mixed egg yolk and white (2 g) was homogenized in acetonitrile (8 mL) in a centrifuge tube. Homogenized egg was shaken for 30 min in a horizontal shaker at room temperature and centrifuged at 3000*g* at 10 °C for 15 min. An aliquot of the supernatant (4 mL) was moved to a glass tube and reduced to dryness on a heating block (60 °C) under stream of nitrogen. The residue was reconstituted in 300 μL of 50 mM Tris-HCl buffer (pH 7.75) containing 0.5% casein, 0.01% Tween 40, and 20 μM diethylenetriaminepentaacetic acid (DTPA).

Muscle. Well-chopped muscle (2 g) was homogenized in acetonitrile (8 mL) in a centrifuge tube. Homogenized muscle was shaken for 30 min in a horizontal shaker at room temperature and centrifuged at 3000*g* at 10 °C for 15 min. An aliquot of the supernatant (2 mL) was loaded onto a SPE silica cartridge previously equilibrated with acetonitrile (2 mL). The cartridge was eluted with acetonitrile (1.5 mL), and the solution was collected into a glass tube. The eluate was reduced to dryness in a heating block (60 °C) under nitrogen stream. The residue was reconstituted in 300 μL of 50 mM Tris-HCl buffer (pH 7.75) containing 0.5% casein, 0.01% Tween 40, and 20 μM DTPA.

TR-FIA. Standards prepared in the reconstitution buffer and the reconstituted sample extracts were applied in duplicate (25 μL) to the microtiter wells containing the antibody. A suitable dilution of Eu-labeled narasin−transferrin conjugate in reconstitution buffer was added in 25 μL volume into each well. The plate was shaken for 30 min at room temperature before the wells were washed four times with wash solution. The enhancement solution (200 μL) was added to each well, and the plate was shaken for 20 min at room temperature. The time-resolved fluorescence was measured by using a Victor 1420 multilabel counter with Wallac 1420 workstation software and MultiCalc online data reduction software (Perkin-Elmer Life Sciences, Turku, Finland).

Validation. The specificity of the assay was assessed by measuring the cross-reactivity of the related ionophores. Cross-reactivity was expressed as the percentage ratio of the amounts of narasin and the tested substance needed to cause 50% displacement of tracer. The accuracy of the salinomycin and narasin TR-FIA was studied by comparing fortified sample concentrations to their determined values. The recoveries from eggs and muscle were assessed at 1 and 2 μg/kg concentrations, respectively. The limit of detection (LOD) and limit of quantification (LOQ) of the assay were defined as the concentrations corresponding to the mean signal of replicates of blank samples (n = 6) plus 3 times and 9 times the standard deviation (SD), respectively. The intra-assay repeatability was determined by repeated analysis (n = 10) of spiked blank sample with two levels of narasin. The interassay reproducibility was studied with repeated analyses (n = 5) of the same samples.

RESULTS AND DISCUSSION

TR-FIA was found to be successful for the screening of narasin and salinomycin residues in poultry muscle and eggs. Narasin is accumulatated in the liver (*19*), and the previously reported screening methods have usually applied to the poultry liver screening (*9*, *10*). However, there is a need to monitor residues also in muscle and eggs. Although coccidiostats are not allowed to be used for egg-laying hens, lasalocid residues have been found in eggs in national residue examination in Finland in 2002 (*20*). Narasin was detected in half of the 24 egg samples analyzed in the Swedish national surveillance scheme for drug residues in 1999 (range = 0.2−11 μg/kg) (*8*). Similar findings have been reported from Northern Ireland, where residues of narasin and salinomycin have been detected in eggs in a national survey (*21*). These findings underscore the need for an efficient screening method for narasin and salinomycin in eggs.

ELISA methods are usually based on colorimetric measurement; they offer an alternative in the field of nonchromatographic residue screening, but the sensitivity of the assay may be limited by matrix interference. This interference can be avoided by using lanthanide chelates as labels and time-resolved fluorometry for measuring the signal of the immunoassay (*16*). The label components used in competitive TR-FIA methods can be synthesized either by attaching the lanthanide chelate directly to the hapten (*22*) or by coupling both the hapten and the chelate label to a carrier molecule, generally protein (*13*, *15*). In the present method the latter option is useful for increasing the labeling degree and, thus, signal level of the label. The conjugation reactions for haptens and lanthanide chelates are usually done sequentially, which complicates the label preparation process and makes the control of coupling ratios difficult (haptens/lanthanide chelates per carrier molecule). Also, the byproducts of the conjugation reactions may have a negative effect on the performance of competitive immunoassay. Europium-labeled narasin−apotransferrin conjugate was purified by gel filtration, which separates molecules by molecular weight and removes low molecular weight compounds such as unreacted haptens and Eu-chelate from the final product. However, in the current method a small amount of DTPA in the assay buffer was needed to bind an unidentified compound interfering with the biospecific reaction. DTPA is a strong chelator, and it binds with free lanthanides, which stabilizes the assay.

A typical narasin standard curve and the precision of the assay are demonstrated in **Figure 3**. The precision profile was demonstrated as concentration coefficient of variation (CV) of the assay, because it is more informative compared to signal CV, when the curve is not linear. The signal from the blank samples was close to the signal of reconstitution buffer without

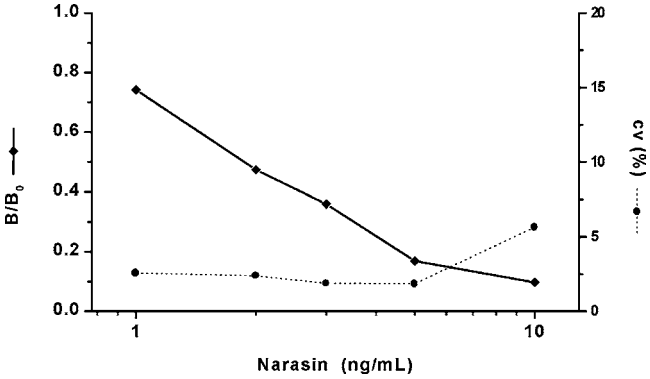

Figure 3. Standard curve and the profile of concentration CV of narasin TR–FIA (n=10).

Table 1. Recovery of Added Narasin and Salinomycin from Eggs and Poultry Muscle (n = 5)

sample	compound	added (μg/kg)	recovery[a] (%)	CV (%)
egg	narasin	1	83.2 a	6.4
	salinomycin	1	79.0 a	9.1
muscle	narasin	2	89.6 b	4.1
	salinomycin	2	92.4 b	4.5

[a] Recoveries followed by a common letter are not significantly different at the 5% level by paired t test.

narasin, and the recovery was acceptable throughout the detection range. It is evident that matrix interference is minimal in eggs and poultry muscle samples, and the results can be determined by using a buffer-based calibration curve. This means that time-consuming spiking of the standard matrix is avoided.

Unlike the previously reported approaches, which involve complex extraction procedures (*10*, *11*), the current method involves only a simple extraction with acetonitrile. The SPE cleanup step could be omitted in the sample preparation protocol of eggs. In the case of muscle samples an extra purification step was needed due to matrix interference. Sample purification by means of solvent partitioning has been used in most of the previously reported methods. However, in our case the hexane cleanup step was not enough for cleaning the fat that remained in some muscle samples. The SPE was also preferred because the solvent partitioning is prone to operator-dependent errors and the SPE step is easier to automate.

The cross-reactivity of the antibody was tested with four structurally related ionophores. The cross-reactivity was 100% for both narasin and salinomycin, but it was <0.1% for lasalocid and monensin, which demonstrates the high specificity of the antibody.

In eggs the mean recovery was 81.1% for both narasin and salinomycin (**Table 1**). The SPE cleanup step improved the recovery of compounds in the muscle tissue by minimizing the matrix interference. The mean recovery in muscle was 91.0% for both narasin and salinomycin. Recoveries were similar with previously reported immunoassay methods (*11*, *12*) for the screening of narasin and salinomycin. Because these two molecules differ by only the presence of a methyl group at the position close to the site of the linkage between carrier protein, it is not surprising that in our tests narasin and salinomycin produced practically identical standard curves and a cross-reactivity close to 100%. The recovery data also show that the assay is capable of similar detection of these compounds in eggs

Table 2. Precision of Narasin and Salinomycin TR-FIA for Eggs and Poultry Muscle

sample	added (μg/kg)	CV (%) intra-assay ($n = 10$)	CV (%) interassay ($n = 5$)
egg	1	4.5	6.4
	3	5.0	10.3
muscle	2	3.8	4.1
	3	3.2	6.4

and poultry muscle. Recoveries were compared using a two-tailed paired *t* test, and statistical analysis showed that no significant differences ($P > 0.05$) were detected between the recoveries of narasin and salinomycin from eggs and poultry muscle.

The LODs of narasin were 0.56 and 0.28 μg/kg, and the LOQs were estimated to be 1.80 and 0.57 μg/kg for muscle and eggs, respectively. The LODs and LOQs reported above can be adjusted to a suitable screening level by diluting sample wells or decreasing sample size in the extraction procedure. The sensitivity of this assay is better than in previously reported studies employing chromatographic methods (*1*) and slightly lower than the ELISA procedure developed for the analysis of poultry muscle (*11*). In the study of Sweeney et al. (*19*) chickens were fed with 50 mg/kg narasin concentrated feed for 5 days, and the mean residual concentration of narasin in muscle samples was found to be ~40 μg/kg, which is easily detected with the current method.

The results for the precision studies are shown in **Table 2**. The average coefficients of intra- and interassay variation in egg measurements were 4.8 and 8.4%, respectively. The respective coefficients in muscle analysis were 3.5 and 5.3%. The TR-FIA procedure used in our study showed good precision regardless of sample type.

The power of a screening method is generally associated with high sample throughput. TLC is simple and has high throughput but is relatively insensitive. HPLC connected to MS is sensitive and specific but requires expensive equipment and trained staff and is therefore most suited to reference laboratories and confirmatory purposes. HPLC methods also typically involve lengthy extraction and cleanup procedures, which limits sample throughput. Most of the current protocols for high-throughput analysis are based on 96-well microtiter plate technology, whereby a large number of samples can be processed in parallel. With this TR-FIA method the potential throughput is 42 samples per hour, but as in most of the cases in food residue analysis, the sample preparation inevitably slows the analysis. The sample preparation employed in the current method is very simple; when it is connected to a rapid TR-FIA system, the screening capacity of the combination is very good. Samples can also be pooled, which decreases the total amount of work needed for sample pretreatment.

In conclusion, we have described a novel method for the screening of narasin and salinomycin residues in eggs and poultry muscle. To our knowledge this is the only application for the screening of narasin and salinomycin based on time-resolved fluorescence. The method presented here has satisfactory analytical performance, and the sample preparation is simple enough for rapid routine analysis. This application is a very useful tool for laboratories monitoring ionophoric drug residues.

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