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An All-In-One Dry Chemistry Immunoassay for the Screening of Coccidiostat Nicarbazin in Poultry Eggs and Liver

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An automated immunoassay for the detection of nicarbazin residues in poultry eggs and liver was developed. The assay was based on a novel all-in-one dry chemistry concept and time-resolved fluorometry. The analyte specific antibody was immobilized into a single microtiter well and covered with an insulation layer, on top of which the label was dried in a small volume. The extracted sample was added automatically to the dry microtiter well, and the result was available within 18 min. Due to the rapidity and simplicity, the quantitative immunoassay could also be used as a high throughput screening method. The analytical limit of detection for the assay was calculated as 0.1 ng mL^{-1} (n = 12) and the functional limit of detection as 3.2 ng g^{-1} for egg (n = 6) and 11.3 ng g^{-1} for liver (n = 6) samples. The sample recovery varied from 97.3 to 115.6%. Typically, the intra-assay variations were less than 10%, and interassay variations ranged between 8.1 and 13.6%.

KEYWORDS: Coccidiostat; nicarbazin; residues; screening; dry chemistry; time-resolved fluorometry

INTRODUCTION

Coccidiosis is a parasite-derived disease, which affects especially poultry. It is caused by protozoa, which belong to the genus *Eimeria* in the class *Sporozoa* and are resident in the intestinal epithelium of the bird. The symptoms of coccidiosis, decreased growth rate and increased mortality, have a substantial economical impact on the poultry industry. Therefore, a variety of coccidiostats has been developed during the years to control this disease.

Coccidiostat nicarbazin, an equimolar mixture of 4,4′-dinitrocarbanilide (DNC) (**Figure 1**) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), has been commonly in use since the 1960s. Despite the emerging drug resistance in the parasite population, nicarbazin has maintained its effectiveness against all *Eimeria* species. However, the EU-wide authorization for pure nicarbazin was withdrawn in May 2002, mainly due to incomplete toxicological data (*1*). Despite this, it is still allowed in combination with another coccidiostat, narasin. Although the European Union has not set a maximum residue limit (MRL) for nicarbazin, the Veterinary Medicines Directorate in the UK has defined a differential action limit (DAL) of 100 ng g^{-1} for DNC, the marker residue for nicarbazin, in eggs (*2*), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has elaborated an MRL of 200 ng g^{-1} for DNC in chicken liver (*3*).

Because the European Union's agricultural policies aim to ensure food safety and provide consumer protection, testing

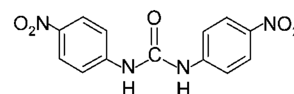

Figure 1. Chemical structure of DNC.

foodstuffs for veterinary drug residues has become quotidian practice. The mandatory control of coccidiostat residues since January 1st, 1998 (*4*) is justified by indications that these residues can be found in poultry eggs and liver (*2*, *5*−*10*). In general, the monitoring of coccidiostat residues has been difficult in the absence of rapid, high volume screening tests. Several methods, typically based on liquid chromatography combined with different detection systems, have been developed for the determination of nicarbazin residues from matrixes such as feed, tissue, liver and eggs (*11*−*21*). Although these methods are specific, they tend to be quite laborious, expensive, and of low volume. Therefore, they are not applicable to use as rapid screening assays.

On the other hand, immunoassays, which are rapid and simple to perform, are ideal for screening purposes. With immunoassays, a high sample throughput can be achieved, which is a prerequisite for an efficient and inexpensive screening method. Time-resolved fluoroimmunoassays (TR-FIAs) employ fluorescent lanthanide chelates with characteristics such as long Stokes shift, narrow-band emission lines, and long fluorescent lifetimes. The time-resolved measuring mode enables the specific fluorescence to be measured after the disappearance of the unspecific, short-lived background fluorescence (*22*−*24*). Nowadays, the conventional dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) protocol has been extended to a novel all-in-one dry chemistry concept, in which all the

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reagents needed for the assay are dry coated into a single microtiter well (*25*). Unlike in the DELFIA, the intrinsically fluorescent label allows the time-resolved fluorescence detection directly from the surface of the dry well without any separate enhancement step. Therefore, the all-in-one dry chemistry immunoassays are even simpler to perform than the traditional fluoroimmunoassays.

Here we describe the development of a rapid and quantitative analysis method for nicarbazin using the all-in-one dry chemistry assay concept and time-resolved fluorometry as the detection technology.

MATERIALS AND METHODS

Reagents. Goat anti-rabbit IgG-coated single wells, intrinsically fluorescent europium chelate [2,2′2″2‴-({4-[(4-isothiocyanatophenyl)-ethynyl]pyridine-2,6-diyl}bis(methylenenitrilo))tetrakis(acetato)] europium (III) (*26*) and Buffer Concentrate were supplied by Innotrac Diagnostics (Turku, Finland). Delfia Wash Solution was purchased from PerkinElmer Life Sciences (Turku, Finland). Polyclonal nicarbazin antibody was raised in rabbits as part of the Poultry-check project (*27*). Hapten (4′-nitrosuccinanilic acid), standard (4,4′-dinitrocarbanilide = DNC), ovalbumin and D(+)trehalose were obtained from Sigma (St. Louis, MO). Dry *N*,*N*-dimethylformamide (>99.5%) and dimethyl sulfoxide were supplied by Fluka (Steinheim, Switzerland) and *N*-hydroxysuccinimide (>98%) and *N*,*N*′-dicyclohexylcarbodiimide (99%) by Acros Organics (Geel, Belgium). CHAPSO, (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), was purchased from Calbiochem (San Diego, CA). Methanol, acetonitrile, and hexane (all of HPLC grade) were obtained from J. T. Baker (Phillipsburg, NJ).

Labeling of Ovalbumin with Europium Chelate. Typically, 0.6 mg of ovalbumin was coupled with 60× molar excess of europium (Eu) chelate. The reaction was carried out overnight at room temperature in 50 mM carbonate buffer (pH 9.8) in a volume of 500 μL. The Eu-labeled ovalbumin was purified by passing it through a NAP5-column (Amersham Biosciences, Uppsala, Sweden) with 50 mM phosphate buffer (pH 7.0) as elution buffer.

Activation of Hapten. The hapten 4′-nitrosuccinanilic acid was conjugated to the labeled ovalbumin via a modified NHS-enhanced carbodiimide -mediated coupling reaction (*28*). The activation of the hapten was done with 1.1× molar excess of *N*,*N*′-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The reaction was started by dissolving *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and 4′-nitrosuccinanilic acid in dry *N*,*N*-dimethylformamide. The reagents were combined to fill up a total volume of ∼200 μL and evaporated to dryness. The dried mixture was resuspended to ∼50 μL of dry *N*,*N*-dimethylformamide.

Preparation of Conjugate. The activated hapten was conjugated to the labeled ovalbumin by using 200× molar excess of the activated hapten. The reaction was carried out overnight at room temperature in 50 mM phosphate buffer (pH 7.0). The volumes used in the conjugation were critical: To protect the protein structure, the volume of *N*,*N*-dimethylformamide was not to exceed 10% of the total reaction volume. The conjugate was purified by a NAP5-column with 50 mM TRIS-HCl (pH 7.0), 0.9% NaCl, and 0.05% NaN_3 as elution buffer and by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) with the same elution buffer.

Preparation of Dry Chemistry Wells. The nicarbazin antibody was diluted to a suitable working titer with 50 mM TRIS-HCl buffer (pH 7.75) containing 0.9% NaCl, 0.5% BSA, 0.05% NaN_3, 0.01% Tween 40, 0.05% bovine γ-globulin, 20 μM diethylenetriaminepentaacetic acid, and 20 μg mL^{-1} Cherry Red. The diluted antibody was incubated in anti-rabbit IgG-coated single microtiter wells in a volume of 50 μL for 1 h at room temperature, after which the wells were washed four times with Delfia Wash Solution. The insulation layer was prepared by adding 50 μL of 50 mM phosphate buffer (pH 7.2) containing 0.1% NaCl, 0.05% NaN_3, 0.001% CHAPSO, 6% trehalose, and 0.2% casein to the wells. An overnight incubation in a climate chamber (+35 °C, 5% relative humidity) was used to dry the insulation layer to the bottom of the well. A suitable amount of the conjugate was applied on top of the insulating layer in a volume of 1 μL and immediately dried by blowing warm air into the well. The dry wells were stored at +4 °C in sealed packages with desiccant.

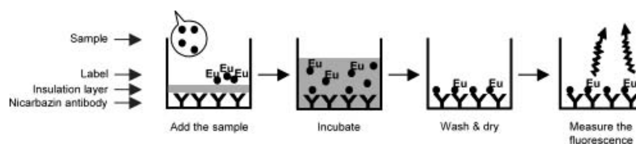

Figure 2. Principle of the all-in-one dry chemistry assay used in a competitive immunoassay format. All the assay reagents were dry coated into a single microtiter well in advance. All the assay steps were performed automatically by an immunoanalyzer.

Preparation of Standards. A standard stock solution was prepared by dissolving 5 mg of DNC, the marker residue for nicarbazin, in 10 mL of dimethyl sulfoxide to give a final concentration of 0.5 mg mL^{-1}. The stock solution was stable at least 3 months when stored at room temperature. The working standards were prepared daily by diluting the standard stock with deionized water.

Egg Extraction. A nicarbazin-free egg was homogenized and divided into 1-g samples. The samples were fortified (25 μL) with various concentrations of DNC and allowed to stand for 5 min before extraction. A 4-mL aliquot of acetonitrile was added to the samples, which were mixed for 15 s and placed in an ultrasonic bath for 2 min. The samples were centrifuged at 1230*g* for 10 min at +10 °C. The supernatants were collected and evaporated to dryness under N_2-flow (+65 °C). The dried samples were reconstituted in 200 μL of methanol and shaken for 15 s, after which 800 μL of deionized water was added, and the samples were shaken for another 15 s.

Liver Extraction. The beginning of the liver extraction was performed similarly to the egg extraction. However, after the evaporation step, the sample preparation was continued with a hexane wash. A 1-mL aliquot of hexane was added to the samples, which were mixed for 15 s. Then, 1 mL of methanol/deionized water (3:1, v/v) was added, and the samples were shaken for 15 s. The samples were placed in a water bath (+37 °C) for 10 min and centrifuged (1230*g*, 10 min, +10 °C). The aqueous bottom layer (1 mL) was evaporated to dryness under N_2-flow (+65 °C). The dried samples were reconstituted in the same way as the egg samples.

Dry Chemistry TR-FIA Protocol. To adjust the assay sensitivity to a suitable range, the extracted samples were further diluted, egg 100-fold and liver 200-fold, with deionized water. All the following assay steps were performed automatically by an immunoanalyzer (Innotrac Diagnostics, Turku, Finland): A sample volume of 50 μL was added in duplicate to the dry microtiter wells. After 15 min incubation at +36 °C, the wells were washed six times with diluted Buffer Concentrate and dried by blowing warm air into the wells. The fluorescence signal was measured directly from the solid surface of the well.

RESULTS AND DISCUSSION

The competitive time-resolved fluoroimmunoassay in combination with the all-in-one dry chemistry concept (**Figure 2**) provided a rapid and user-friendly method for the analysis of nicarbazin residues in poultry eggs and liver. Because all the analyte specific reagents were already present in the single microtiter well in a dry form, only the addition of the sample was needed to start the assay. After the short incubation period and wash steps were completed, the time-resolved fluorescence was measured directly from the surface of the dry well, and the result was available within 18 min.

The production and characterization of the nicarbazin antibody done as a part of the Poultry-check project is described elsewhere (*27*). To obtain a rapid assay without compromising the sensitivity or the precision, the assay kinetics were studied (data not shown), and a 15 min incubation time was found to be feasible. A typical standard curve for nicarbazin in liver is shown in **Figure 3**. The analytical limit of detection (mean +

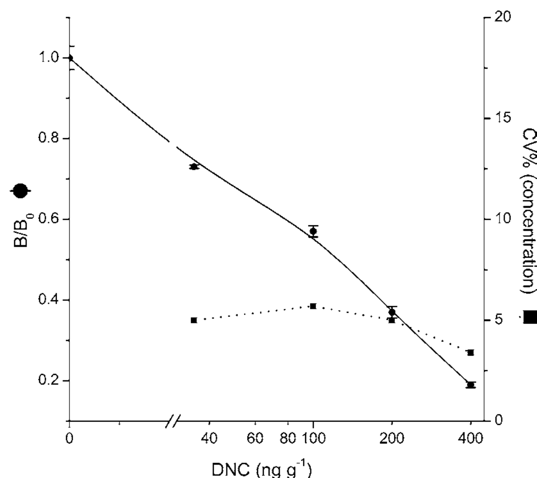

Figure 3. Standard curve (●) and precision profile (■) for nicarbazin in liver ($n = 4$). The *y* error bars in the standard curve present the coefficient of variation (CV%) between replicates calculated from fluorescence signals. The CV% in the precision profile is based on calculated concentrations.

Table 1. Interassay Variation of the Nicarbazin Immunoassay Using Fortified Egg Samples

	fortification	
	35 ng g^{-1}	70 ng g^{-1}
day 1	28.7	60.1
	27.8	60.3
day 2	35.3	76.2
	29.8	82.3
day 3	30.3	75.0
	38.9	87.1
day 4	26.1	66.1
	32.1	70.9
avg	31.1	72.3
CV%	13.5	13.6

3 × SD) for nicarbazin was determined by repeating the analysis of a zero calibrator ($n = 12$) and calculated as 0.1 ng mL^{-1}. To reduce the risk for false compliant results in screening, the ED$_{50}$-value of the assay was adjusted to the region of 0.5 × DAL for eggs and 0.5 × MRL for liver, that is to say 50 and 100 ng g^{-1}, respectively. Therefore, egg samples were diluted 100-fold and liver samples 200-fold before analysis. The functional limit of detection (mean + 3 × SD) was determined by analyzing nicarbazin-free egg ($n = 6$) and liver ($n = 6$) samples and calculated as 3.2 ng g^{-1} and 11.3 ng g^{-1}, respectively. The lowest limit of quantification, defined as a sample giving ~85% inhibition of the measured signal and having a CV% (concentration) less than 15, was established as 10 ng g^{-1} for egg (CV%(concentration) = 9.9) and 35 ng g^{-1} for liver (CV%(concentration) = 3.9). The intra-assay variation was determined with two blank samples fortified with DNC using four replicates. For egg, the fortification was done at concentration levels of 25 and 50 ng g^{-1}, and the variation ranged from 2.3 to 8.1%. For liver, concentrations of 50 and 100 ng g^{-1} were used, and the variation was 5.5 and 1.6%, respectively. The interassay variation for both matrixes was determined by analysis of two fortified samples in duplicate over 4 days (**Tables 1** and **2**). The recovery was assessed by extracting two samples fortified with DNC at concentrations of 35 and 70 ng g^{-1} for egg and 75 and 300 ng g^{-1} for liver. The fortified samples were kept overnight at +4 °C before extraction, and the recoveries were calculated as 115.6 and 97.3% for egg and 103.6 and 98.4% for liver.

Table 2. Interassay Variation of the Nicarbazin Immunoassay Using Fortified Liver Samples

	fortification	
	75 ng g^{-1}	300 ng g^{-1}
day 1	69.8	300.3
	66.1	297.6
day 2	82.6	332.7
	74.2	293.5
day 3	79.1	243.5
	76.3	299.5
day 4	81.7	312.4
	71.3	312.4
avg	74.7	299.0
CV%	8.1	8.6

Table 3. Comparative Analysis of Incurred Egg and Liver Samples Using an LC-MS-MS Method and the Nicarbazin Immunoassay

	incurred eggs		incurred liver	
	LC-MS-MS (ng g^{-1})	immunoassay (ng g^{-1})	LC-MS-MS (ng g^{-1})	immunoassay (ng g^{-1})
sample 1	16	17	101	125
sample 2	86	112	479	580
sample 3	158	174	195	295

To further test the quantitative potential of the assay, three incurred egg and liver samples produced in the Poultry-check project were analyzed with two separate methods, the nicarbazin immunoassay and an LC-MS-MS method (*12*). The LC-MS-MS analysis was performed at Queen's University of Belfast, Belfast, UK. It can be seen from **Table 3** that the immunoassay gave constantly slightly higher concentrations than did the LC-MS-MS method, but the results were in good agreement. The observed difference in the concentrations might be due to minor fluctuations in the homogeneity of different sample batches, or the antibody might also recognize the metabolites of DNC. In addition, some of the incurred samples contained very high concentrations of DNC, while the immunoassay was set to accommodate the area around 0.5 × DAL/MRL. Naturally, the target area of the immunoassay could be scaled up or down simply by adjusting the dilution factor of the samples and standards.

The nicarbazin immunoassay was also validated in accordance with the Commission Decision 2002/657/EC, which defines methods for calculating the detection capability of a screening assay. Blank samples and fortified samples (20 each) at the DAL/MRL concentration of 100 ng g^{-1} for egg and 200 ng g^{-1} for liver were analyzed in duplicate. A negative control, which was a blank sample, and a positive control, which was fortified with DNC at a concentration of 0.5 × DAL/MRL (50 ng g^{-1} for egg and 100 ng g^{-1} for liver), were also used in the validation. All samples were classified as compliants or noncompliants by comparing them to the positive control. If the recorded fluorescence signal was higher than that generated by the positive control, the sample was regarded as compliant, and vice versa. For both matrixes, all the blank samples were screened as compliant and all the fortified samples (at the DAL/MRL concentration) as noncompliant. Therefore, according to the Commission Decision 2002/657/EC the CCβ-value of the assay was defined as less than the level of fortification (100 ng g^{-1} for egg and 200 ng g^{-1} for liver).

In addition to traditional TR-FIAs, there are already some dry chemistry TR-FIA methods available for residue analysis. Tuomola et al. (*29*) reported a TR-FIA method for zeranol in

bovine urine samples, and Crooks et al. (*30*) described a screening method for the analysis of coccidiostat monensin in poultry plasma samples. The dry chemistry concept was used in both assays, but at the time, the concept could not be fully exploited. Although all the assay reagents were in a dry, stable form, the assay steps were still carried out manually on a microtiter plate, and an enhancement step was needed for the signal measurement. Tuomola et al. explored the dry measurement mode in the measurement stage, but without automation, the results were found to be too operator-dependent. The present assay protocol was developed one step further: The nicarbazin assay was based on the use of single microtiter wells, to which samples were added automatically by an immunoanalyzer and on the measurement of the fluorescence directly from the surface of the dry wells. The inherent fluorescent properties of the lanthanide chelates (*26*) enabled the utilization of the dry measurement mode and the omission of the separate, time-consuming signal development step. Furthermore, the automation of the assay reduced the overall analysis time and minimized human errors. The immunoanalyzer could be used in a random or continuous access mode. The conveyor system with a capacity of 88 samples enabled the continuous loading of the samples and new samples could be added to the wells every 70 s. The microtiter wells were packed into pens (12 wells/pen), which were marked with specific barcodes. Because the only analyte specific components were the dry coated wells, one sample could in principle be used for a multiresidue analysis simply by inserting pens with particular analyte specificity in the immunoanalyzer.

The present study describes a competitive immunoassay for the analysis of nicarbazin residues in poultry eggs and liver. It is evident that although the immunoassay was originally developed to function as a simple screening tool, classifying samples as compliant or noncompliant, quantitative results can also be obtained. The assay technology is based on the dry chemistry concept and time-resolved fluorometry. The assay is rapid (the immunoanalyzer has a throughput of 50 samples per hour) and simple to perform, which makes it particularly suitable for screening purposes. The all-in-one dry chemistry assay concept may easily be extended also to other analytes of interest in the field of residue analysis.

ABBREVIATIONS USED

DNC, 4,4′-dinitrocarbanilide, marker residue for nicarbazin; MRL, maximum residue limit; DAL, differential action limit; TR-FIA, time-resolved fluoroimmunoassay; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; NHS, *N*-hydroxysuccinimide; ED_{50}-value, effective dose giving 50% inhibition of the measured signal.

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