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Rapid screening method for halofuginone residues in poultry eggs and liver using time-resolved fluorometry combined with the all-in-one dry chemistry assay concept

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Abstract

The present study describes the development and validation of an immunoassay for the screening of coccidiostat halofuginone in poultry eggs and liver. The power of time-resolved fluorometry is utilised with a novel all-in-one dry chemistry assay concept, in which all the reagents needed for the competitive immunoassay are built into a single microtiter well in a dry, stable form. The entire immunoassay is performed by an automated immunoanalyser and the total assay time is only 18 min. The assay protocol is simple: the extracted sample is added to the well and after the 15 min sample incubation and wash steps are completed, the fluorescence signal is measured directly from the surface of the dry well in a time-resolved manner. The analytical limit of detection has been calculated as 0.02 ng ml^{-1} ($n = 12$) and the functional limit of detection as 1.7 and 1.0 ng g^{-1} for egg ($n = 6$) and liver ($n = 6$) samples, respectively. If needed, the assay sensitivity can be further improved simply by adjusting the dilution factor of the samples. The mean recovery has been determined as 90.5% for egg at concentration levels of 15 and 60 ng g^{-1} , and as 106.0% for liver at concentrations of 7.5 and 30 ng g^{-1} . The intra-assay variations have typically been below 10% and interassay variations have ranged between 7.7 and 11.6%. The halofuginone immunoassay has also been validated according to Commission Decision 2002/657/EC. © 2004 Elsevier B.V. All rights reserved.

Keywords: Halofuginone; Residues; Screening; Immunoassay; Time-resolved fluorometry; Dry chemistry

1. Introduction

Coccidiosis is a contagious parasitic infection causing bloody diarrhoea, emaciation and loss of egg production in poultry. In order to prevent and control the spread of this disease, coccidiostat drugs are used in the commercial poultry production. Since coccidiostats only arrest the growth of the parasite, they are needed throughout the bird's life span. Consequently, a broad range of coccidiostats has been introduced to prevent the emerging drug resistance in the parasite popu-

lation. However, the extensive usage of coccidiostats has led to the occurrence of coccidiostat residues in poultry products [1,2].

In general, the lack of rapid and inexpensive analysis methods has complicated the mandatory implementation of coccidiostat residue monitoring to the national residue programmes [3]. In order to be cost-efficient, monitoring schemes have usually adopted a two-tier testing system consisting of screening and confirmatory tests. Screening tests are typically perceived as rapid, low-cost and high throughput tests, which classify a substantial number of samples as compliant or potentially non-compliant. Accordingly, confirmatory methods, which are very specific, but generally also slow, high-cost and of low volume, are utilised to re-analyse the potentially non-compliant samples. There are several methods

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available for the analysis of halofuginone residues in different matrices. However, these methods are typically based on liquid chromatography analysis with expensive detection systems, lengthy extraction and cleanup procedures or high consumption of solvents [4–8]. Therefore, they tend to be more suited to confirmatory purposes than to screening. In addition, an electrophoresis method for halofuginone residues in feedstuffs has been described [9]. An ELISA protocol for residues in chicken liver has also been reported [10]. However, the sensitivity of the ELISA is not adequate for screening purposes, and the assay procedure is quite tedious. Thus, there is a definite need for a rapid and sensitive screening method for coccidiostat halofuginone.

Time-resolved fluoroimmunoassays (TR-FIA) [11,12] have been in use since the 1980s mainly in human diagnostics, but several TR-FIA methods have been reported in the field of food and residue analysis [13–19] as well. TR-FIAs utilise fluorescent lanthanide chelates for detection. These chelates have special characteristics, which enable the fluorescence to be measured in a time-resolved manner, thereby omitting the unspecific background signal. Previously TR-FIAs have been based on a dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA) format, but recently a novel all-in-one dry chemistry assay concept has been introduced [20]. The all-in-one dry chemistry immunoassays are simple to perform: all the assay specific reagents are pre-dispensed into a single microtiter well in a dry, stable form and the assay is started simply by adding the sample to the well. Moreover, the signal measurement mode is developed one step further in the dry chemistry assay concept: with intrinsically fluorescent lanthanide labels the time-resolved fluorescence can be measured directly from the dry surface of the well, thus making the use of a separate signal generation step obsolete. Consequently, the surface measurement is faster to perform and also more reliable, since the additional liquid handling step vulnerable to contamination from external lanthanide ions from the surroundings is eliminated.

Here we report the development and validation of a rapid screening method for halofuginone residues in poultry eggs and liver utilising TR-FIA combined with the all-in-one dry chemistry concept. The assay is easy to perform: the sample is extracted according to a simple protocol and all the other assay steps are automated.

2. Experimental

2.1. Reagents

Goat anti-rabbit IgG-coated single microtiter wells, intrinsically fluorescent europium chelate [2,2',2'',2'''-(4-{4-isothiocyanatophenyl}ethynyl)pyridine-2,6-diyl}bis(methylenetrinitro))tetrakis(acetato)]europium(III) [21] and buffer concentrate were obtained from Innotrak Diagnostics (Turku, Finland). Delfia Wash Solution was purchased from Perkin-Elmer Life Sciences (Turku, Finland). Hapten (halofuginone

succinimide) and standard (halofuginone hydrobromide) were kindly provided by the Queen's University Belfast, Northern Ireland, UK. Ovalbumin and D(+)-trehalose were supplied by Sigma (MO, USA). Dry *N,N*-dimethylformamide was purchased from Fluka (Steinheim, Switzerland) and *N*-hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide from Acros Organics (Geel, Belgium). CHAPSO, (3-[(3-cho-lamidopropyl)dimethylammonio]-2-hydroxy-1-propanesul-fonate) and casein were supplied by Calbiochem (CA, USA). Methanol, acetonitrile and hexane (all of HPLC grade) were purchased from J.T. Baker (NJ, USA). Polyclonal halofugi-none antibody was raised in rabbits as described by Rowe et al. [22].

2.2. Preparation of labelled hapten–protein conjugate

The protein was labelled with a 60-fold molar excess of europium chelate. Typically, 1 mg of ovalbumin was used in the reaction, which was carried out overnight at room temperature in 50 mmol l⁻¹ carbonate buffer (pH 9.2). For the following conjugation reaction carbonate buffer was changed to phosphate buffer (50 mmol l⁻¹, pH 7.0) with a NAP5-column (Amersham Biosciences, Uppsala, Sweden). The hapten, halofuginone succinimide, was conjugated to the labelled ovalbumin by using a modified *N*-hydroxysuccinimide active ester method [23]. The hapten was activated with a 1.1-fold molar excess of *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The activation was carried out overnight at room temperature with shaking in dry *N,N*-dimethylformamide. The conjugation of the hapten to the labelled ovalbumin was performed with a 100-fold molar excess of the activated hapten. The conjugation was let to proceed overnight at room temperature with slow shaking in 50 mmol l⁻¹ phosphate buffer (pH 7.0). The conjugate was purified through a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences, Uppsala, Sweden) by using 50 mmol l⁻¹ Tris–HCl (pH 7.0), 0.9% NaCl, 0.05% NaN₃ for elution.

2.3. Preparation of all-in-one dry chemistry wells

Tris–HCl buffer (50 mmol l⁻¹, pH 7.75) containing 0.9% NaCl, 0.5% BSA, 0.05% NaN₃, 0.01% Tween 40, 0.05% bovine γ -globulin, 20 μ mol l⁻¹ diethylenetriamine-pentaacetic acid and 20 μ g ml⁻¹ Cherry Red was used to dilute the halofuginone antibody to an appropriate working titre. The diluted antibody (1/4000, 50 μ l/well) was incubated in anti-rabbit IgG-coated single wells for 1 h at room temperature with slow shaking, after which the wells were washed four times with Delfia Wash Solution. The insulation layer, composed of 50 mmol l⁻¹ phosphate buffer (pH 7.2) containing 0.9% NaCl, 0.05% NaN₃, 0.01% CHAPSO, 8% trehalose and 0.2% casein, was applied to the wells in a volume of 50 μ l and was left to dry in a climate chamber (o/n, 35 °C, 5% relative humidity). The labelled conjugate was carefully dispensed (1 μ l) on top of the dry insulation layer and dried by

blowing warm air into the well. The single microtiter wells were packed into pens (12 wells/pen) and stored at 4 °C in sealed packages with desiccant.

2.4. Standards

The standard, halofuginone hydrobromide, was dissolved in a small volume of *N,N*-dimethylformamide and diluted to a final concentration of 1 mg ml⁻¹ with methanol. The working solutions were prepared daily by diluting the standard stock with de-ionised water.

2.5. Sample pre-treatment

2.5.1. Egg samples

Samples of homogenised egg (2.00 ± 0.01 g) were weighed and spiked with appropriate concentrations of halofuginone hydrobromide. The samples were allowed to stand for 5 min prior to extraction with acetonitrile (8 ml), after which the samples were vortexed for 15 s and sonicated for 2 min. After a centrifugation step (1230 × g, 10 min, 10 °C), the supernatants were collected and dried off at 65 °C under a stream of nitrogen. The dried samples were reconstituted in 2 ml of de-ionised water.

2.5.2. Liver samples

The initial stages of the liver extraction were carried out according to the egg extraction protocol. However, after the evaporation step the liver samples were dissolved in 1 ml of hexane followed by a 1 ml aliquot of methanol/de-ionised water (3:1, v/v). After a 10 min incubation in a water bath (37 °C) and a centrifugation step (1230 × g, 10 min, 10 °C), the aqueous bottom layer (1 ml) was dried off at 65 °C under a stream of nitrogen. The dried samples were reconstituted similarly to the egg samples.

2.6. TR-FIA protocol

Egg and liver samples were diluted 10-fold with de-ionised water prior to analysis. Pens containing the microtiter wells were identified with appropriate barcodes and loaded into an immunoanalyser (Innotrac Diagnostics, Turku, Finland). Every subsequent assay step was performed automatically by the immunoanalyser. In brief, the samples were applied to the wells in duplicate 50 µl aliquots and incubated at 36 °C under continuous mixing for 15 min. The wells were washed six times with diluted buffer concentrate and dried under a stream of hot dry air. The time-resolved fluorescence was measured directly from the surface of the dry wells. The total assay time was 18 min.

3. Results and discussion

One of the cornerstones of the European Union's agricultural policies is to assure food safety. Recently, the presence

of veterinary drug residues in food has evoked public concern. Therefore, the Commission has laid down principles that strive for a common purpose: to guarantee a high level of food safety [24]. In the EU, the use of certain coccidiostats is already prohibited [25] and maximum residue limits (MRL) for those remaining will be set in the future [26]. The European Medicines Agency (EMA) has set MRLs for halofuginone residues in bovine muscle, fat, kidney and liver [27]. However, for poultry there are no official MRLs. Since halofuginone is not even licensed for use in egg-laying birds, eggs should, in principle, be free from halofuginone residues. Therefore, an MRL for halofuginone residues in poultry eggs is not very likely to be established at all. However, there is evidence that, for instance, feed contamination can result in the accumulation of halofuginone residues in eggs [28]. A review by Kennedy et al. focuses in more detail on the contamination problem as a cause of residues in food [29].

Consequently, we decided to use the EMA MRL for bovine liver (30 ng g⁻¹) as a guideline in the assay validation for both matrices, poultry eggs and liver. In general, screening assays should produce no false compliant results and preferably only a low level of false non-compliant results. Samples giving false compliant results are not eliminated from the food chain, and therefore may pose a threat to consumers. Hence, to avoid false compliants in screening, the assay sensitivity was adjusted approximately to the region of 0.5 × MRL by diluting both egg and liver samples 10-fold with de-ionised water prior to analysis. However, if the MRL-values were to be set or modified in the future, the present assay sensitivity could be revised simply by adjusting the dilution factors of samples.

The aim of this research was to develop a rapid screening method for halofuginone residues in poultry eggs and liver. A traditional competitive time-resolved fluoroimmunoassay was combined with a novel all-in-one dry chemistry concept, which together provided the basis for an automated, rapid and user-friendly assay system. In the all-in-one dry chemistry immunoassays single microtiter wells were used, in which all the assay specific reagents were pre-dispensed and dried (Fig. 1). The microtiter well was first coated with anti-rabbit IgG, after which the analyte specific antibody was

Fig. 1. All-in-one dry chemistry single microtiter well, which contains all assay specific reagents in a dry, stable form.

immobilised to the bottom of the well. The insulation layer in the well provided the buffer components for the reaction and separated the antibody from the label. The components of the layer, mainly proteins, carbohydrates and detergents, influenced the non-specific binding, sensitivity and signal level of the assay. It was also observed that a premature contact between the antibody and label would distort the equilibrium of the immunoassay. Hence, the insulation layer had to be very stable. Several reagents were tested (data not shown) before a proper insulation layer was achieved with optimised concentrations of casein, trehalose and CHAPSO. Moreover, the addition of the label on top of the insulation layer had to be performed carefully, in order to avoid the premature solubilisation of the layer. The importance of the optimisation of the insulation layer has also been discussed by Lövgren et al. [20] and Tuomola et al. [18].

A universal test format would be an advantage, if a number of tests for various contaminants were to be built into one immunoanalyser. Therefore, we aimed to integrate the present halofuginone immunoassay protocol as well as possible with another immunoassay, which we had previously developed for coccidiostat nicarbazin [30]. In addition to the immunoassay parameters, the sample extraction protocols for both analytes, halofuginone and nicarbazin, were designed to be similar. Typically, in food residue analysis the sample pre-treatment is the most time-consuming step. We tried to develop a simple extraction protocol for both sample matrices, in order to save time and also reduce the volumes of organic solvents. However, the extraction was found to be greatly dependent on the sample matrix. For egg, a relatively simple procedure with only one extraction step using acetonitrile was sufficient, but for liver, an additional step, a hexane wash, was necessary, in order to remove fat from the samples.

A typical standard curve ($n = 4$) for halofuginone hydrobromide in poultry eggs is presented in Fig. 2. The cut-off

Fig. 2. Standard curve for halofuginone using spiked egg samples. The dotted line represents the cut-off value of the assay, which is set to $0.5 \times \text{MRL}$ and used to discriminate samples as compliant or non-compliant.

value ($0.5 \times \text{MRL}$), which was used to classify samples as compliant or non-compliant in the assay validation, is indicated by a dotted line. The assay validation was performed according to the EU guidelines on the validation of qualitative screening methods [31]. In the validation process 20 blank samples and 20 spiked samples at the MRL concentration of 30 ng g^{-1} were analysed in duplicate. In addition, a negative control containing no halofuginone residues and a positive control spiked with halofuginone hydrobromide at a concentration of $0.5 \times \text{MRL}$ for egg and $0.25 \times \text{MRL}$ for liver were used in the validation. A sample was classified as compliant, if the fluorescence signal was higher than the signal from the positive control, and non-compliant, if less than that. An example of the validation data for egg samples is presented in Table 1. For both matrices, all the blank samples were screened as compliant and all the spiked samples (at the MRL concentration) as non-compliant. Accordingly, the detection capability ($\text{CC}\beta$) of the assay was determined as less than 30 ng g^{-1} for both matrices.

The analytical limit of detection, based on the mean plus three standard deviations of the measured concentrations of a zero calibrator ($n = 12$), was calculated as 0.02 ng ml^{-1} . The functional limit of detection (mean + 3 S.D.) for the assay was assessed by analysing halofuginone-free egg ($n = 6$) and liver samples ($n = 6$) and calculated as 1.7 and 1.0 ng g^{-1} , respectively. The limit of quantification (LOQ) was defined as a sample giving $\sim 80\%$ inhibition of the measured signal and having a coefficient of variation (CV), based on calculated

Table 1
Qualitative screening method validation according to Commission Decision 2002/657/EC using blank and spiked (30 ng g^{-1}) egg samples

Sample	Fluorescence (counts)	
	Blank samples (0 ng g^{-1})	Spiked samples ($\text{MRL} = 30 \text{ ng g}^{-1}$)
1	24235	4126
2	21949	4127
3	27406	4785
4	24411	3546
5	22554	4112
6	20530	4043
7	25833	3197
8	22138	3524
9	24599	2939
10	21182	3658
11	24385	3866
12	23485	3620
13	22485	3407
14	20223	3301
15	24651	3055
16	23257	3571
17	25182	3232
18	21476	3297
19	20630	3522
20	22554	3224
Negative control (blank = 0 ng g^{-1})	23977	
Positive control ($0.5 \times \text{MRL} = 15 \text{ ng g}^{-1}$)	6479	

Table 2
Intra- and interassay variation of the halofuginone immunoassay using poultry egg and liver samples spiked at various concentration levels

Sample	Added (ng g^{-1})	CV (%)	
		Intra-assay	Interassay
Egg	15 ($= 0.5 \times \text{MRL}$)	5.9	11.0
	60 ($= 2 \times \text{MRL}$)	3.7	11.6
Liver	7.5 ($= 0.25 \times \text{MRL}$)	3.2	8.5
	30 ($= \text{MRL}$)	9.2	7.7

Table 3
Recovery data for halofuginone in poultry egg and liver samples ($n = 3$)

Sample	Added (ng g^{-1})	Recovery (%)	CV (%)
Egg	15 ($= 0.5 \times \text{MRL}$)	86.7	10.9
	60 ($= 2 \times \text{MRL}$)	94.3	3.5
Liver	7.5 ($= 0.25 \times \text{MRL}$)	114.7	3.1
	30 ($= \text{MRL}$)	97.3	11.9

concentrations, less than 20%. The LOQs were determined to be 2.5 ng g^{-1} for egg and 2.0 ng g^{-1} for liver with CVs of 16.7 and 9.7%, respectively. The intra- ($n = 4$) and interassay variations ($n = 3$) were determined by analysing two spiked samples at various concentration levels (Table 2). The slightly better interassay variation with liver samples might be explained by the more thorough sample preparation. The recoveries were established with two blank samples, which were spiked with halofuginone hydrobromide and allowed to stand overnight at 4°C prior to analysis (Table 3). The mean recovery was calculated as 90.5% for egg and 106.0% for liver.

4. Conclusions

The described all-in-one dry chemistry immunoassay for halofuginone is based on a new trend in time-resolved fluorometry utilising intrinsically fluorescent lanthanide chelates, a surface measurement mode and a fully automated immunoanalyser. The only manual step is the extraction of the sample. The assay can be used to determine halofuginone residues in poultry eggs and liver in a qualitative or quantitative mode. The same assay concept has already been used for the analysis of other coccidiostat residues and can easily be applied to other contaminants as well.

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