A new and rapid method for the determination of nicarbazin residues in poultry feed, eggs and muscle tissue using supercritical fluid extraction and high performance liquid chromatography

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Received 27th October 1998, Accepted 18th February 1999

A simple and rapid method, using supercritical fluid extraction (SFE) with off-line high-performance liquid chromatography for the isolation and determination of nicarbazin, a popular broad-spectrum coccidiostatic drug used principally in poultry, is described. Results show good repeatability with a minimum quantification level of 0.4 μg g^{-1} and mean 'spiked' recoveries of 98%, 100% and 99% using poultry feeds (n = 18), eggs (n = 28) and chicken tissue (n = 20), respectively. SFE using carbon dioxide is proposed as an alternative isolation method to the current Association of Official Analytical Chemists (AOAC) procedure which involves the use of large volumes of a harmful solvent (dimethylformamide) and requires a long tedious separation and clean-up regime (6 h) prior to its determination.

Introduction

Coccidiosis is a contagious amoebic disease affecting livestock, particularly poultry, throughout the world and in particular in warm, humid conditions associated with intensive poultry farming, where it causes intestinal lesions which result in diarrhoea and related health problems in the animals. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class *Sporozoa* and is the most important parasitic disease of poultry.[1–3]

In its acute form coccidiosis causes high mortalities, in its sub-acute form small numbers of oocysts can cause poor weight gain, poor feed conversion and poor egg production in poultry. Affected birds will be depressed, have ruffled feathers, be reluctant to move, and often the feathers around the vent will be soiled with faecal material due to diarrhoea. Infections of coccidiosis also make chickens more susceptible to *Salmonella* infections.[4]

Due to the intensive nature of the poultry industry, it is economically essential to control this disease caused by the *Eimeria* species namely *E. tenella*, *E. acervulina*, *E. maxima* and *E. brunetti*. Preventive medication is very well established and is highly successful in the control of coccidiosis in poultry farming. Since 1979, around 30 drugs have been used for the prevention of coccidiosis in chickens. One of the main factors in coccidiosis management is the ability of feedmills to incorporate these relatively toxic drugs into feeds intended for use as poultry diets.

Nicarbazin was the first drug with a truly broad-spectrum activity against species of *Eimeria* in chicken.[5] It has been used since 1960 and is very active against all the *Eimeria* species. Nicarbazin is a equimolar complex of 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP); both compounds are necessary for the anticoccidial activity. It is used in animal feeds at a dose of 125 ppm to control coccidiosis in broiler and replacement chicken feeds. At this dose in the diet, nicarbazin is primarily a coccidiocidal, acting against the developing second-generation schizont.[6] Nicarbazin is not permitted in feeds for laying and breeding stock as it has an adverse effect on egg production, weight and quality.[7,8]

Several methods have been reported for the determination of nicarbazin from different matrices such as feeds, egg, tissue, liver and litter. All of these methods (below) require solvent extraction for isolating nicarbazin followed by a clean-up stage. Determination is achieved by different types of chromatography, namely, gas-liquid chromatography-mass spectrometry (GLC-MS);[9] liquid chromatography (LC);[10] liquid chromatography-mass spectrometry (LC-MS) [11,12] and matrix solid-phase dispersion combined with liquid chromatography, (MSPD-LC).[13] Several HPLC methods have also been reported.[14–16] Watson[17] used a thin layer chromatograpic (TLC) method for the identification of nicarbazin in feeding stuffs by Dragendorff's spray reagent. Other methods include voltametric analysis by the Osteryoung square-wave technique[18] and differential pulse polarography.[19,20]

The AOAC method [21] which is only specific for feeds uses a 30 g alumina column (500 × 22 mm id) with dimethylformamide (DMF) and alcohol as solvents for extraction followed by a clean-up procedure and finally spectrophotometric determination at 430 nm. The method is not ideal as it takes a long time (6 h), using large volumes of a potentially harmful solvent (DMF) and can only detect niacarbazin in feed (not eggs or muscle tissue) at levels of 25–125 ppm. It is also not specific as feed additives such as furazolidone, nitrofurazone and ninhydrazone are known to interfere with the determination.

The aim of this study was to develop an alternative method that was rapid, robust and therefore easily applicable to different matrices such as feeds, eggs and chicken tissue. Such a method would prove useful for rapid screening as nicarbazin has been approved by the USA Food and Drug Administration for the

prevention of coccidiosis in chicken,[22] and a tolerance of 4 ppm was established in uncooked chicken muscle, liver, skin and kidneys.[23]

The monitoring and surveillance of drugs at different stages in food production using new, rapid and reliable methods would be of immense benefit in the control and use of drug residues in foods, particularly where existing methods cannot routinely be used as they are tedious, expensive and require the use and disposal of large volumes of harmful solvents.

Experimental

Reagents and materials

Poultry feed free from coccidiostats was obtained from the Charnwood Milling Co. (Framlingham, Suffolk, UK). The feed was homogenised and stored at room temperature. Fresh eggs were bought from the local supermarket, they were pooled together by mixing (beaten) and stored in the refrigerator. The pooled liquid egg was checked for the presence of nicarbazin by the HPLC method described in the study. Fresh chicken was bought from the local butcher. The tissue was homogenised and stored in the freezer. The tissue was checked to be free from nicarbazin as above.

Reagents. *Solvent.* Acetonitrile: HPLC grade; methanol: HPLC grade; ethanol: absolute (Rathburns, Walkerburn, UK); dimethylformamide: 99% (Aldrich, Gillingham, Dorset, UK).

Modifier. Ethanol–water, (90 + 10 v/v).

Sodium sulfate. Analytical grade, (Aldrich).

Alumina. Aluminium oxide, 90 active, activity 1, 70–230 mesh ASTM, neutral for column chromatography (Merck, Poole, Dorset, UK).

Nicarbazin analytical standard. Eli Lilly & Co (Windelsham, Surrey, UK).

Nicarbazin standard solution. A 50 mg portion of the reference standard was weighed into a 50 ml volumetric flask, and dissolved in approximately 15 ml of DMF with the aid of gentle heat. The solution was cooled and diluted to volume with DMF, mixed well and stored protected from light.

Spiking of samples. Poultry feed: 1 g samples were spiked with 25, 50, 75, 100 and 125 μl of the above standard solution representing 25–125 ppm of nicarbazin dosed onto the control feed sample. Eggs: 1 g samples were spiked with 40, 50, 100, 150 and 200 μl of a ten-fold diluted solution of the above standard solution with methanol, representing 4–20 ppm of nicarbazin dosed onto the control egg samples. Chicken tissue: 1 g samples were spiked with 40, 100, 200 and 300 μl of a hundred-fold diluted solution of the above standard solution with methanol, representing 0.4–3 ppm of nicarbazin dosed onto the control chicken tissue. In each of the above cases, the sample following the addition of a spike was allowed to equilibrate for a minimum of 12 h prior to recovery extractions.

Apparatus. *HPLC*. Mobile phase of acetonitrile–water (55 + 45 v/v), flow rate of 1 ml min^{-1}; UV detection wavelength of 344 nm; column, APEX ODS 5 μm, (150 × 4.6 mm id), Jones Chromatography (Hengoed, Mid Glamorgan, UK), injection volume of 20 μl; detector, Spectra Physics (Hemel Hempstead, Herts., UK) Analytical UV 1000; integrator, Shimadzu C-R5A, chart speed of 10 cm min^{-1}.

SFE. The supercritical fluid extractor used was an ISCO model 260 D, marketed by Jones Chromatography. A number of the SFE parameters were optimised prior to the extraction of nicarbazin. These included (i) pressure (3000–6000 psi), (ii) extraction time 10–90 min in static and dynamic and combination of both modes, (iii) temperature (40–90 °C) and (iv) choice of modifier, methanol, acetonitrile, butanol, acetone, propanol, and other solvents. The final optimised SFE conditions used throughout the study for the extraction of nicarbazin were (i) extraction temperature of 85 °C, (ii) pressure (constant) of 4000 psi, (iii) restrictor temperature of 100 °C and (iv) carbon dioxide flow rate of 2 ml min^{-1}.

Methods

Extraction of poultry feed using the supercritical fluid (carbon dioxide). A 1 g portion of the feed sample was placed on a bed of 3 g of alumina (used to retain extracted lipids from the sample[24,25]) in a 10 ml SFE cartridge. The void above the sample was loosely filled with tissue paper and 5 ml of modifier was added to the SFE cartridge. The feed was extracted for 30 min, the first 10 min in the static mode and the next 20 min in the dynamic mode. This extraction was repeated once more (10 min in the static mode and 20 min in the dynamic mode) with the addition of a further 5 ml of modifier to the cartridge. The combined extract was collected in a 20 ml volumetric flask containing 5 ml of methanol. After both extractions, the extract was made to the mark in the volumetric flask with methanol and injected into the HPLC.

Extraction of egg by the supercritical fluid method. A 3 g portion of alumina was weighed into an extraction cartridge, 2 g of anhydrous sodium sulfate was then placed on the top of the alumina layer and 1 g of liquid egg was applied directly to the sodium sulfate layer. The contents of the cartridge were then mixed with a small spatula to form a paste. Sodium sulfate was used as a drying agent to prevent restrictor blockage due to the extracted water forming ice during decompression and also allowed for sample dispersion within the SFE cartridge.[24, 25] The spatula was washed with 5 ml of the modifier solution into the cartridge. Each extraction was carried out as for the feeds, *i.e.*, 10 min static SFE followed by 20 min dynamic SFE carried out twice. The combined extract following both extractions (static and dynamic) was collected in a 20 ml volumetric flask containing methanol and made up to the mark with methanol. The solution was then filtered through a 0.45 μm filter prior to quantification by HPLC.

Extraction of chicken tissue by the supercritical fluid method. A 3 g portion of alumina was weighed into an extraction cartridge. A 2 g portion of anhydrous sodium sulfate was then placed on the top of the alumina layer and 1 g of homogenised chicken meat was applied directly on top of the sodium sulfate layer. A further 1 g of anhydrous sodium sulfate was applied on top of the chicken to sandwich the sample. The meat was mixed with the sodium sulfate with a small spatula to give a paste and the same extraction procedure followed as for the egg sample.

Results and discussion

Table 1 shows the actual mean and percentage recoveries of nicarbazin from feeds, eggs and muscle tissue while Table 2 summarises the results from the previous table. Each of the above samples reported a linear standard curve with no significant interferences. These results show (Figs. 1 and 2) that nicarbazin was successfully extracted using the SFE approach. The mean recoveries of nicarbazin by the SFE method for poultry feed, egg and chicken with a range of added nicarbazin are summarised in Table 2 and were 98, 100 and 99%, respectively. The consistent average recoveries of nicarbazin from a wide range of added nicarbazin from these matrices indicate the versatility of this method. Other benefits of the SFE

Table 1 Recovery of nicarbazin from poultry feed, eggs and tissue using SFE (n = 2 for each nicarbazin spike level)

Added nicarbazin (ppm)	Mean nicarbazin recovered (ppm)			Mean recovery (%)		
	Feed total n = 18	Egg total n = 28	Tissue total n = 20	Feed total n = 18	Egg total n = 28	Tissue total n = 20
0.4	—	0.39	0.40	—	98	100
1	—	1.0	0.98	—	100	98
2	—	2.0	2.0	—	100	100
3	—	3.1	3.1	—	103	103
4	—	4.1	4.0	—	103	100
5	5.1	5.0	4.9	102	100	98
10	9.9	10.1	9.8	99	101	98
15	14.7	15.2	14.6	98	101	97
20	19.5	19.7	19.7	98	99	99
25	25.5	25.0	24.9	102	100	10
50	49.7	49.6	—	99	99	—
75	72.5	74.1	—	97	99	—
100	93.5	99.5	—	94	100	—
125	119.5	124.3	—	96	99	—

Table 2 Summary of results

Matrix	Number of samples (n)	Added nicarbazin range (ppm)	Mean recovery (%)	Range	Standard deviation	RSD (%)
Poultry feed	18	5–125	98	93–104	2.81	2.87
Eggs	28	0.4–125	100	95–104	2.30	2.30
Chicken tissue	20	0.4–25	99	95–110	4.16	4.20

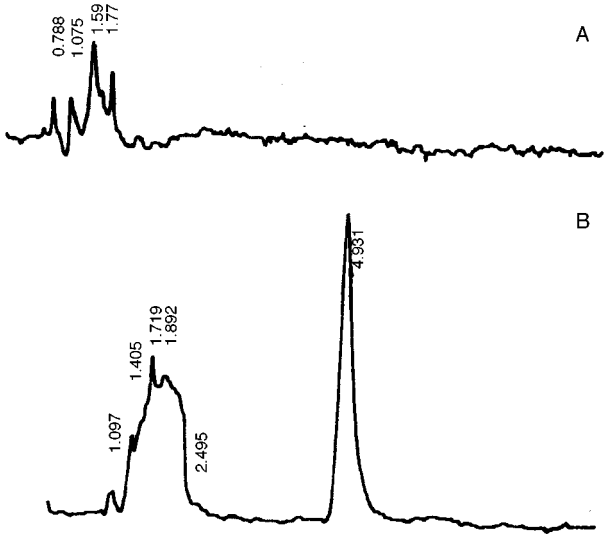

Fig. 1 HPLC chromatograms following SFE extraction of: (A) egg sample (control); (B) egg sample spiked with 10 ppm of nicarbazin.

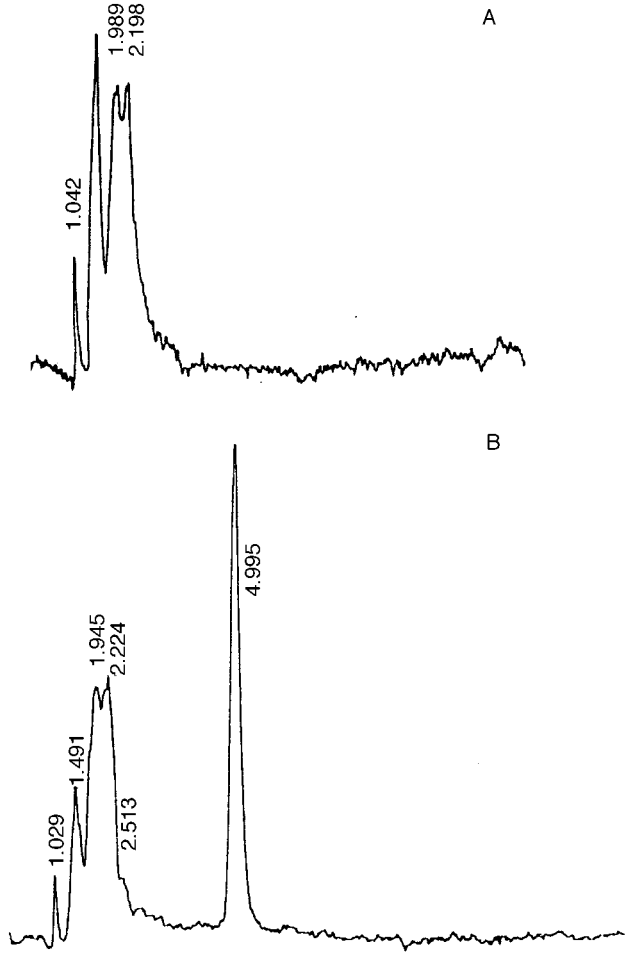

Fig. 2 HPLC chromatograms following SFE extraction of: (A) feed sample (control); (B) feed sample spiked with 10 ppm of nicarbazin.

method include a significantly reduced overall analysis time (90 min) which is made possible as the SFE extract does not require any further clean-up prior to HPLC analysis (conventional extraction methods require a lengthy column chromatography step). In addition, the use of minimum solvent for the complete extraction of nicarbazin (10 ml of 90% ethanol and a final volume made up to 20 ml with methanol) would represent a significant economic saving as well as significantly reduce the exposure risk to solvents.

Conclusion

This study shows that the SFE method can be used as an alternative to the classical extraction methods used for the isolation of nicarbazin from poultry matrices. The SFE method has the advantage over the conventional extraction procedures in that it functions both as an extraction and clean-up procedure. This is therefore a simple and rapid method, which can be used

for the routine monitoring or surveillance of nicarbazin in poultry feed, eggs or muscle tissue.

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Paper 8/08327D