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Micro high-performance liquid chromatography for the determination of nicarbazin in chicken tissues, eggs, poultry feed and litter

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Abstract

A micro high-performance liquid chromatography (HPLC) method has been developed for the determination of the anti-coccidial drug nicarbazin in chicken tissues, eggs, poultry feeds and litter. The 4,4'-dinitrocarbanilide (DNC) component of nicarbazin was extracted from foods, feeds and litter with acetonitrile. The extracts were purified by liquid–liquid partitioning, evaporated to dryness and taken up in methanol–acetonitrile–water (50:30:20, v/v). Micro HPLC of the 4,4'-dinitrocarbanilide (DNC) portion of nicarbazin is performed isocratically using a small bore column (1 mm I.D.) packed with reversed-phase and a UV detector set at 340 nm.

The average recoveries of nicarbazin added to muscle, liver and egg were 92.8, 84.3 and 85.2%, respectively, 95.9% in poultry feed and 76.8–95.9% in different litters. The limit of detection was 25 pg, based on a detector signal-to-noise ratio of 3. These results were achieved with a simplified step extraction without the solid-phase extraction used by various researchers. This method offers a sensitive, selective, rapid, and less expensive alternative to conventional HPLC for such evaluations.

1. Introduction

In recent years increasing attention has been given to the use of micro HPLC in a variety of food analysis applications [1–5]. This interest is due to several advantages that micro HPLC has compared to conventional HPLC [6–8]. Of these, the most important are low consumption of the mobile and stationary phases, an increase in mass sensitivity, compatibility with small samples and easy coupling to mass spectrometers and secondary chromatographic systems.

Conventional liquid chromatographic analysis of the anti-coccidial drug nicarbazin in poultry and eggs has been investigated by many researchers. Nicarbazin, an equimolar complex of 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), is used as a feed additive at a maximum level of 125 mg/kg for the prevention of coccidiosis in poultry [9]. Italian and European law demands a 9-day suspension of nicarbazin treatment prior of slaughter in order to reach a detectable residual level of zero in chicken tissues.

The contamination and persistence of nicarbazin in eggs [10] and in chicken liver [11] has been studied and it has been found that chickens excrete the DNC component more slowly than the HDP component [12]. For this reason most analyses for residues in tissue have focused on

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the DNC component. However, it has also been shown that litter is the main source of DNC contamination for broilers of slaughtering age [13]. Hence, it is of great importance to determine the DNC component in litter.

Almost all the procedures developed to date utilise conventional HPLC with either UV detection in chicken tissues [14–17] and eggs [18,19], electrochemical detection in tissues [20] or thermospray mass spectrometry for confirmation analysis [17,21].

Owing to the advantages of micro HPLC, especially the reduced mobile-phase consumption and obvious economic advantages in routine analysis, we hypothesized and tested the possibility of employing micro HPLC for the separation and quantification of the DNC component of nicarbazin in chicken tissues, eggs and poultry feed. We also tested this method on litter.

2. Experimental

2.1. Reagents and standards

All the solvents were LC grade, unless otherwise stated. Acetonitrile, methanol, *n*-hexane and dichloromethane were obtained from Farmitalia Carlo Erba (Rodano, Italy). Acetic acid glacial, sodium acetate anhydrous, sodium chloride and sodium sulphate anhydrous (p.a. quality) were from Farmitalia Carlo Erba. N,N-dimethylformamide (DMF) was purchased from BDH (Poole, UK). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Standard nicarbazin, a complex of a 1:1 molar ratio of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), was purchased from Merck, Sharp and Dohme Research (Rahway, NJ, USA). The standard purity was 97.4%.

The standard stock solution (1000 μg/ml) in DMF was obtained by dissolving the nicarbazin standard by mixing on a magnetic stirrer with heating at 75°C. The solution is stable for at least 1 month at room temperature.

For the standard curve solutions a series of standard solutions of nicarbazin containing 0.05–20 μg/ml were prepared by dilution of 1000 μg/ml stock solution with methanol–acetonitrile–water (50:30:20, v/v).

Standard fortification solution of nicarbazin containing 20 μg/ml was prepared in the same way as the standard curve solutions.

2.2. Apparatus

Meat grinder/mincer (Farmitalia Carlo Erba, Rodano, Italy), rotary vacuum evaporator VV 2000 (Heidolph-Elektro, Kelheim, Germany), magnetic stirrer (PBI, Milan, Italy), centrifuge 4237 (ALC, Milan, Italy), homogenizer H04 (Büler, Tubingem, Weilheim), S and S filter paper, No. 588 (Sleicher and Schuell, Keene, NH, USA), and FEP-teflon centrifuge tubes (10 ml) (Nalgene, Rochester, NY, USA), were used.

2.3. Chromatographic equipment

An HPLC system with a Model 420 pump equipped with pump head S (flow range 10–2000 μl/min (Kontron, Munich, Germany) and a capillary detector Model 433 equipped with a capillary flow cell of 90 nl (22 mm optical pathlength) (Kontron) were used. The system was interfaced via a Multiport to a Data-450 personal computer (Kontron). Chromatographic software DS-450 (Kontron) was used for data acquisition and instrument control. Samples were injected using a Model 7520 micro sample injector (0.5 μl, Rheodyne, Cotati, CA, USA) and separated on a 300 mm × 1 mm I.D. Supelcosil LC-18 (5 μm particle size) small bore column (Supelco, Bellefonte, PA, USA).

Stainless-steel tubing (0.1 mm I.D. × 1/16 in. O.D.) was employed between the injector and the separation column (4 cm in length). The tubing between the separation column and flow cell of the detector was fused-silica capillary of 50 μm I.D.

2.4. Preparation of samples

The samples of muscle, liver, egg and poultry feed were obtained from a farm under study. The samples of litter were made up of either

straw or wood shavings, used for a period of 28 days in the coops of broilers fed with nicarbazin-free feed.

Before extraction, samples of liver, muscle, feed and litter were carefully minced in a meat grinder/mincer. Samples of whole egg without shell were homogenized by shaking.

2.5. Spiking of liver, muscle, egg, poultry feed and litter samples for recovery

Before recovery all the samples were tested in order to verify the absence of nicarbazin. To determine recovery, 0.1, 0.25, 0.5 and 1 ml of the standard nicarbazin fortification solution (20 μg/ml) were added to 25.0 g of blended tissues (liver, muscle), blended litter and shaken egg. This resulted in samples containing 0.08, 0.2, 0.4 and 0.8 μg/g of nicarbazin in tissue, egg and litter. For poultry feed 1, 2.5, 5 and 12.5 ml of standard nicarbazin fortification solution were added to 5.0 g of blended feed to obtain 4, 10, 20 and 50 μg/g of nicarbazin.

2.6. Extraction of liver, muscle, egg and litter

An amount of 25.0 g of homogenised sample was accurately weighed into a 150-ml glass homogenising cup. Samples were blended at low speed (2–3 min) with 50 ml of acetonitrile. The extract was centrifuged (1000 *g* for 10 min) and the supernatant transferred to a separatory funnel. The sediment in the centrifuge tube was re-extracted with 30 ml of acetonitrile. After centrifugation at 1000 *g* for 10 min, the supernatant was combined with the first extract in the funnel and shaken well. After decanting, the lower layer was discarded and the organic layer was saved in the funnel. To the extract in the funnel 80 ml of dichloromethane and 3 g of sodium chloride were added and shaken well. After decanting, the lower layer was discarded and the organic layer was saved in the funnel. To this 3 g of sodium sulphate anhydrous was added and shaken well. Finally, the extract was filtered through filter paper and transferred to a 250-ml round bottom flask and evaporated to dryness using a rotary vacuum evaporator with a temperature controlled bath (45–50°C).

2.7. Extraction of feed

An amount of 5.0 g of ground sample was accurately weighed into a 50-ml glass homogenising cup. Feed was homogenised at low speed (2–3 min) with 25 ml of acetonitrile. The extract was centrifuged (1000 *g* for 10 min) and the supernatant transferred to a 100-ml round bottom flask. The sediment in the centrifuge tube was re-extracted with 25 ml of acetonitrile. After centrifugation at 1000 *g* for 10 min, the supernatant was combined with the first extract in the funnel. Finally, the extract was filtered through filter paper and transferred to a 100-ml round bottom flask and evaporated to dryness using a rotary vacuum evaporator with a temperature controlled bath (45–50°C).

2.8. Purification of samples

After extraction as described above, the residue was quantitatively transferred from the round bottom flask to a 10-ml centrifuge tube with 2 ml of methanol–acetonitrile–water (50:30:20, v/v) and 1 ml of *n*-hexane. The flask was then rinsed four times with 1 ml portions of acetonitrile. The solution was then evaporated to dryness under nitrogen, using a water bath at 50°C. Finally, the residue in the centrifuged tube was redissolved with 2 ml of methanol–acetonitrile–water (50:30:20, v/v) and 0.5 ml of *n*-hexane. After centrifugation (10 min, 4000 rpm), methanol solution was injected into HPLC. The supernatant was generally the hexanic layer, but it depended on tissue type. For feed samples methanol solution was diluted with methanol–acetonitrile–water to yield peak responses within the range of the standard curve.

2.9. Chromatographic conditions

The mobile phase was acetonitrile–acetate buffer (0.1 *M*, pH 4.8) (70:30, v/v). A flow-rate of 30 μl/min was used and the DNC component

of nicarbazin was detected at 340 nm using a UV capillary detector, sensitivity 0.1 AUFS.

3. Results and discussion

3.1. Extraction and chromatographic separation

Various works that use conventional HPLC with extraction and purification by SPE, have shown the presence of interference in the determination of the DNC component in chicken tissues [20,21] and in eggs [18] deriving from the solvents and/or the solid-phase extraction (SPE) column.

In order to eliminate this type of interference, we cut out clean-up by SPE according to Cortesi et al. [22]. This simplified the extraction procedure and should also avoid losses in recovery [21]. However, we still found interference in the determination of the DNC component both in egg and muscle samples and in litter (straw and wood shavings). This was not eliminated when different mixtures of acetonitrile–water, methanol–water or acetonitrile–methanol–water were used as the mobile phase. To separate the DNC component from the interference found in the various matrixes, it was necessary to use a mixture of acetonitrile and acetate buffer as mobile phase (0.1 *M*, pH 4.8) (70:30, v/v). DNC was retained for 9.5 min in this way. The reproducibility of the retention time was less than 1% for 5 injections.

Representative chromatograms of blank control and fortified liver, muscle, eggs and litter (straw) are shown in Figs. 1 and 2. This micro

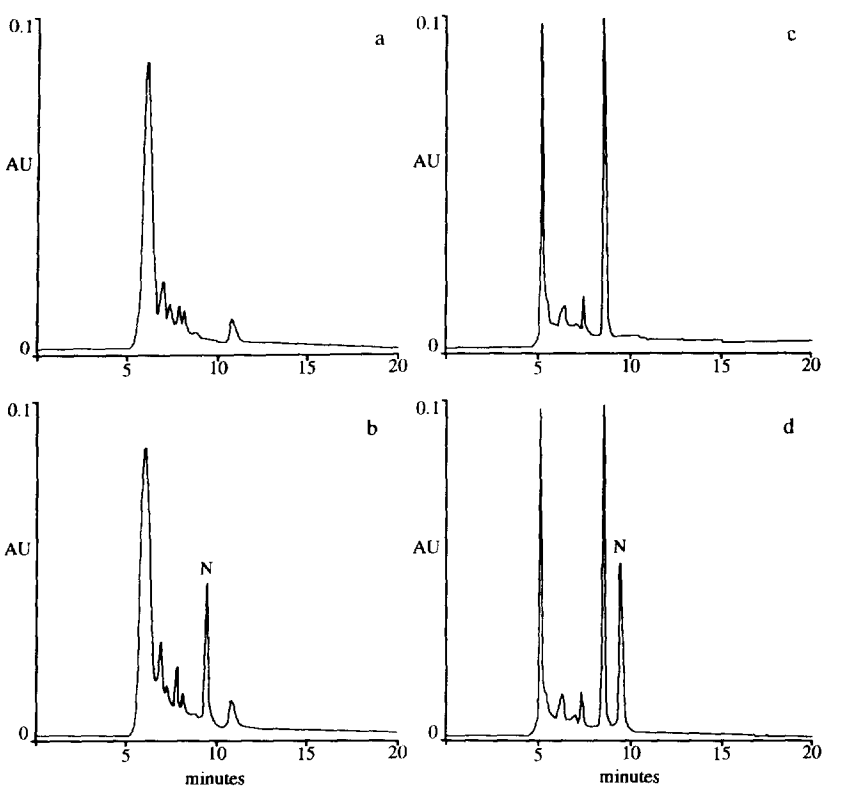

Fig. 1. Micro HPLC chromatograms of: (a) control chicken liver; (b) liver fortified with 0.4 μg of nicarbazin (N) per g sample; (c) control chicken muscle; (d) muscle fortified with 0.4 μg of nicarbazin (N) per g sample. Column, Supelcosil LC-18 (300 × 1 mm I.D., 5 μm). Isocratic elution with mobile phase, acetonitrile–sodium acetate buffer (0.1 *M*, pH = 4.8), (70:30, v/v). Flow-rate, 30 μl/min. Loop, 0.5 μl. UV detection, 340 nm.

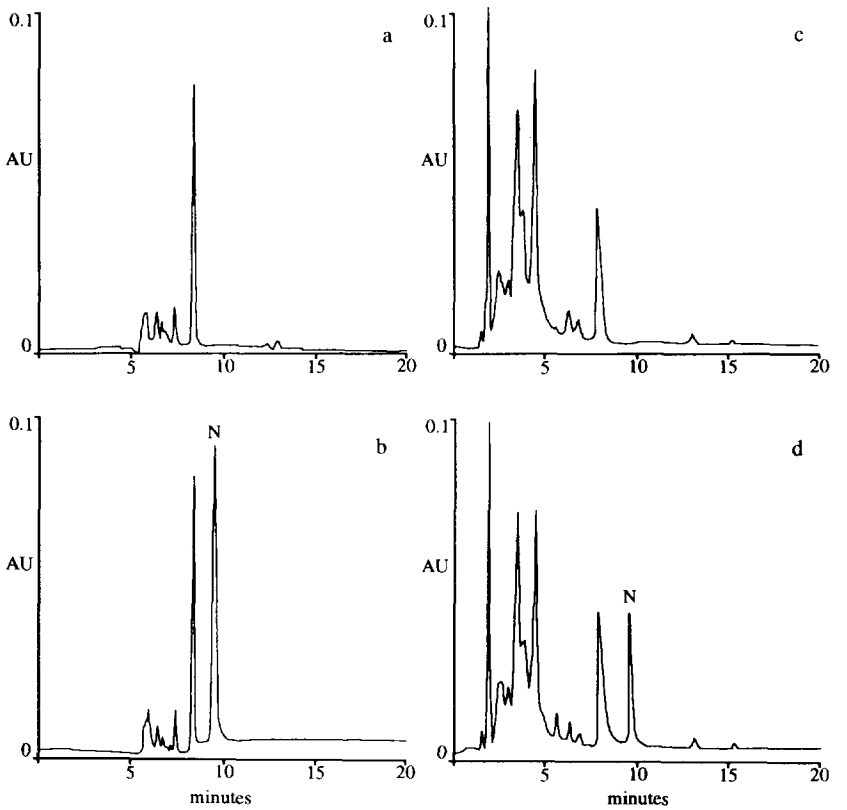

Fig. 2. Micro HPLC chromatograms of: (a) control egg sample; (b) egg fortified with 0.8 μg of nicarbazin (N) per g sample; (c) control chicken litter (straw); (d) litter fortified with 0.4 μg of nicarbazin (N) per g sample. Column, Supelcosil LC-18 (300 × 1 mm I.D., 5 μm). Isocratic elution with mobile phase, acetonitrile–sodium acetate buffer (0.1 *M*, pH = 4.8), (70:30, v/v). Flow-rate, 30 μl/min. Loop, 0.5 μl. UV detection, 340 nm.

HPLC method has also been evaluated for possible interference with certain compounds used in broiler poultry such as narasin.

A chromatogram of poultry feed containing 50 μg/g of nicarbazin with narasin is shown in Fig. 3. No interference was observed around the DNC retention time in blank control tissues (liver and muscle), egg, litter and in poultry feed samples.

3.2. Linearity range and limit of detection

Linearity of the photometric detector response to nicarbazin in standard solution was verified in the range 0.05–20 μg/ml (0.025–10 ng injected). A correlation coefficient of 0.998 was obtained. Nicarbazin concentration can be calculated by assaying DNC and assuming that nicarbazin is a 1:1 molar ratio of the DNC and HDP [15]. The lowest detectable amount of nicarbazin with micro HPLC was found to be 25 pg at a signal-to-noise ratio of 3:1. The limit of detection is about 5 times lower than that found by Vertommen et al. [18] with conventional HPLC. The limit of detection was satisfactory both for monitoring residue for health safety and for checking animal rearing facilities. In fact, the level of nicarbazin found in broiler livers 9 days after the suspension of treatment can often be much higher (ca. 200 ng/g, corresponding to ca. 1.05 ng injected) than the detection level in cases where the litter is not completely changed after treatment. The limit of detection obtained permits the checking of the litter effect, which is the principal cause of nicarbazin residue persistence in chicken tissues [13,23].

3.3. Recovery

The average recoveries and standard deviations for the fortified samples are indicated in Table 1. Recovery and precision data for this method were generated each day for 2 days from the analysis of duplicate control samples which were fortified at 0.08, 0.2, 0.4 and 0.8 μg/g nicarbazin for tissues, egg and litter and 4, 10, 20 and 50 μg/g nicarbazin for poultry feed.

Average recoveries were consistently above 90% for the muscle, feed and litter (wood shavings) with a mean recovery of 92.8% for muscle, 95.9% for poultry feed and 95.9% for litter. Recoveries were above 80% for liver and eggs; only in the litter (straw) sample was the average recovery less than 80% (76.8%).

The precision is also satisfactory at all levels with coefficients of variation less than 3.7% for liver, 3.3 for muscle, 4.5 for eggs and 6.3 for poultry feed. The increased variability (C.V. = 6.8–10.9%) for straw litter is probably due to difficulties in obtaining homogeneous samples.

3.4. Economic considerations

The economic advantages of using micro HPLC was evaluated from two points of view; purchase of a new system already configured for micro HPLC and conversion from conventional

Table 1
Recovery of nicarbazin from fortified chicken tissues, eggs, poultry feed and litter

Sample	Nicarbazin added (μg/g)	Recovery[a] (%)	Standard deviation	C.V. (%)
Liver	0.08	82.7	2.7	3.3
	0.2	86.0	3.2	3.7
	0.4	80.9	2.5	3.1
	0.8	87.5	2.6	3.0
Muscle	0.08	90.7	2.7	3.0
	0.2	93.3	3.1	3.3
	0.4	92.6	2.0	2.2
	0.8	94.7	2.2	2.3
Eggs	0.08	81.9	3.7	4.5
	0.2	86.4	3.9	4.5
	0.4	88.0	1.8	2.0
	0.8	84.5	2.3	2.7
Feed	4	90.7	5.7	6.3
	10	95.5	5.3	5.5
	20	99.8	5.2	5.2
	50	97.7	4.9	5.0
Litter (straw)				
	0.08	75.2	8.2	10.9
	0.2	78.0	5.3	6.8
	0.4	72.0	6.5	9.0
	0.8	81.9	6.9	8.4
Litter (wood shavings)				
	0.08	91.6	4.8	5.2
	0.2	95.9	4.1	4.3
	0.4	98.6	3.5	3.5
	0.8	97.5	3.8	3.9

[a] Each value is the average of 4 extractions.

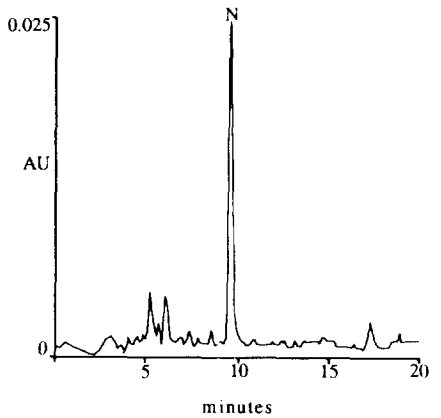

Fig. 3. Micro HPLC chromatogram of poultry feed containing 50 μg of nicarbazin (N) per g sample. Extract was diluted 1:50 before injection. Column, Supelcosil LC-18 (300 × 1 mm I.D., 5 μm). Isocratic elution with mobile phase, acetonitrile–sodium acetate buffer (0.1 *M*, pH = 4.8), (70:30, v/v). Flow-rate, 30 μl/min. Loop, 0.5 μl. UV detection, 340 nm.

HPLC. In either case, the pump and the detector would be the same. The difference in cost would only regard the detector cell, the injection valve, the column and the connecting tubes. These costs were then compared to saving in mobile phase consumption. In our case, we purchased a system already configured for micro HPLC. The additional cost over a conventional HPLC system was ca. US$ 800. On the other hand, if we had converted from a conventional HPLC system, the overall cost of modification would have been ca. US$ 3200. To calculate the saving in mobile phase consumption, we estimated likely conventional HPLC consumption and compared it to ours.

It has been demonstrated that for two columns with the same porosity and different diameters, the ratio between flow-rates required in order to produce the same mobile phase linear velocity is proportional to the square of the ratio of the internal diameters. In our case, the flow-rate of 30 μl/min with the small bore column corresponds to a flow-rate of ca. 0.6 ml/min with a conventional column. This means that with micro HPLC, the daily consumption (8 h) of mobile phase was ca. 14.4 ml compared to ca. 290 ml that would have been necessary with conventional HPLC. This consumption is about 20 times less than that of conventional HPLC. In our laboratory, the saving on mobile phase consumption, calculated on 4000 sample injections (average column lifetime) was ca. US$ 2170. An additional economic advantage is that the quantity of waste solvent is reduced.

In the light of these economic considerations, we calculated that sample injections in excess of 5870 make the cost of conversion viable and that only 1475 injections would cover the relative increase in the cost of a new system.

4. Conclusion

The procedures described represent an easy and relatively inexpensive alternative to conventional HPLC for the determination of nicarbazin in different food samples (liver, muscle, eggs) and also litter and poultry feed.

This method is particularly useful for monitoring involving a large number of samples and for routine checks where repetitive analysis is required. It is a valid procedure for checking animal rearing facilities and food, where residues could have an effect on health. In addition, considering the low flow-rates that make the direct coupling of LC to mass spectrometry possible, the use of small bore columns could be particularly advantageous in LC–MS confirmation analysis, recently tested [17,21] to confirm the presence of nicarbazin in chicken tissues.

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