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Determination of nicarbazin and clopidol in poultry feeds by liquid chromatography

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Abstract

A rapid and very effective analytical procedure for the simultaneous liquid chromatographic determination of two coccidiostats, clopidol (CLOP) and nicarbazin (NICA), in poultry feeds was developed and tested. The ground feed samples were extracted using aqueous dimethylformamide after moistening with water. Co-extracted feed constituents were removed with a solid-phase extraction alumina-basic column and the eluates were directly analyzed on an ODS column (250×4.6 mm, 5 μm) with acetonitrile–0.01 *M* acetate buffer (pH 4.6) as eluent. UV detection of CLOP and the 4,4′-dinitrocarbanilide portion of NICA was carried out at 265 and 345 nm, respectively. The mean recovery from NICA spiked samples was 95% with a RSD of 4% in a concentration range of 2–150 mg/kg while for CLOP it was 98% with a RSD of 5% in a concentration range of 5–150 mg/kg. The limits of detection of NICA and CLOP in feed, based on a detector signal-to-noise ratio of 3, were estimated to be 1 mg/kg and 2.5 mg/kg, respectively, and the lowest levels tested in feeds by this procedure were 2 mg/kg and 5 mg/kg, respectively.

Keywords: Coccidiostat; Nicarbazin; Clopidol and poultry feed

1. Introduction

The use of additives in feedingstuff plays a crucial role in intensive livestock production throughout the world. Many finished feeds contain additives licensed in the European Union for inclusion without a veterinary prescription, mainly in the poultry and rabbit industries. Some additives, such as nicarbazin (NICA) and clopidol (CLOP), can be used as prophylatics or therapeutics in the prevention or treatment of coccidiosis. NICA is the generic name for the complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) in a 1:1 molar ratio. CLOP, also called meticlorpindol, is the 3,5-dichloro-2,6-dimethyl-4-pyridinol. Both NICA and CLOP are feed additive drugs used against coccidiosis in poultry [1–4] at a concentration, in finished feeds, ranging between 100 and 125 mg/kg [5].

Italian law [5] permits the use of NICA only for broilers, while CLOP is approved for broilers, chickens reared for laying hens, guinea fowl, turkeys and rabbits. Both are forbidden for use in laying hens in order to prevent the possible persistence of residue in eggs. Likewise, for above-mentioned animal species, law demands a withdrawal period of 9 and 5 days for NICA and CLOP, respectively, prior to slaughter [6,7]. This withdrawal period is suggested to avoid a health hazard. The consumption by humans of small quantities of veterinary pharmacological substances, present as residues in animal food, might cause

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several problems: alteration of the indigenous gut microflora [8], possible involvement in allergic reactions, involvement in the development of antibacterial-resistant strains of bacteria and coccidia in animals and people and interference with starter cultures for fermented food products [9]. Moreover, NICA and CLOP are more dangerous for animal species, especially for those species they are not intended for, because of the possibilities of developing coccidial resistance.

NICA and CLOP are useful drugs when used in a proper way but they may become harmful when administrated in feeds with levels higher than the recommended ones, or when present, also at cross-contamination levels, in feeds used for animal categories for which they were not intended (laying hens and pre-slaughtered animals). Because of the above-mentioned reasons it is quite clear the importance of a regular control of finished feeds for CLOP and NICA levels.

Since the early 1960s, several analytical methods have been published for the determination of NICA and CLOP in feeds. The earlier methods [2,10] are spectrophotometric procedures with poor selectivity and sensitivity while the other published methods [1,3,11] use high-performance liquid chromatography (HPLC) with ultraviolet detection. All the above analytical procedures can be used for the determination of only one of the two coccidiostats in poultry feed. The purpose of this study was to develop a method for the simultaneous determination of NICA and CLOP in finished poultry feeds at their normal added concentration as well as at subtherapeutic levels, as a result of a possible cross-contamination at the point of manufacture. In this way it is possible to analyse at the same time, feeds intended for several and different animal species and categories.

2. Experimental

2.1. Chemicals

Hipersolv acetonitrile was obtained from BDH (Poole, UK). Acetic acid (100%) was purchased from Prolabo (Fontenay s/Bois, France). Ammonium acetate was purchased from Carlo Erba (Milan, Italy). Dimethylformamide was obtained from J.T. Baker-Schilling (Milan, Italy). Isolute solid-phase extraction (SPE) Al-B columns, each with 1 g of stationary phase and a 6-ml volume (manufactured by IST, Hengoed, UK) were purchased from StepBio (Bologna, Italy). The SPE columns were connected to a Supelco SPE vacuum manifold block, from Supelco (Milan, Italy). Water for HPLC analysis was prepared with a Barnstead Nanopure Ultrapure water system from International PBI (Milan, Italy).

2.2. Standards and spiked samples

NICA was obtained from Sigma–Aldrich (Milan, Italy), while CLOP was generously donated by Filozoo (Carpi, Italy). The stock standard solutions of each coccidiostat were prepared in dimethylformamide at the concentration of 1 mg/ml for NICA and of 0.5 mg/ml for CLOP. The standard curve solutions, containing both coccidiostats, were prepared by diluting the stock standard solutions to: 0.4–2–10–20–50 μg/ml in a mixture of water–acetonitrile (1:1). The solutions were kept at room temperature in dark test-tubes.

For the recovery studies, feed samples spiked with NICA and CLOP over a concentration range of 2–150 mg/kg and 5–150 mg/kg, respectively, were prepared on the day of use by fortifying aliquots of blank control feeds with the stock standard solutions. Samples were processed as described in Section 2.4.

2.3. Instrumentation

The LC system consisted of a Hewlett-Packard (HP) 1100 Series quaternary pump, a HP 1100 Series diode array detection (DAD) system, a HP 1100 Series autosampler all controlled by a Vectra VE Serie 8 computer using HP Chemstation software.

The HPLC separation was performed at room temperature on a 5 μm Supelcosil LC-18 (250×4.6 mm, 5 μm) column equipped with a Supelguard LC-18 (20×4.6 mm, 5 μm) guard column (both columns from Supelco, Bellefonte, PA, USA). HPLC eluent A was pure acetonitrile; HPLC eluent B was 0.01 *M* ammonium acetate buffer (pH 4.6). The gradient was initiated with 0% eluent A continued with a linear increase to 30% eluent A over 10 min

followed by a linear increase to 80% eluent A over 5 min and constant 80% eluent A for 4 min. The system was returned to 0% eluent A in 1 min and was re-equilibrated for 4 min before the next injection. The flow rate was 1.2 ml/min and the injection volume was 10 μl. The wavelength was 265 nm (CLOP detection) between 0 and 12 min and 345 nm (detection of the DNC component of NICA) from 12 min to the end of the chromatography run.

2.4. Sample preparation

The feed sample was pulverised using a domestic grinder to obtain a homogeneous powder. A portion (10 g) was weighed into a 250-ml glass jar and was moistened, for 1 min, with 5 ml of water before adding 45 ml of the extraction solution water–dimethylformamide (5:95, v/v). The mixture was shaken with a horizontal shaker for 1 h. About 10 ml of supernatant was centrifuged for 5 min at 3000 rpm.

Five ml of supernatant was pipetted onto a non-pretreated SPE Al-B column. The elution was quite slow (about 1 drop each second). The first ml of the eluate was discarded while the next 2 ml were collected in a HPLC vial. Ten μl of the purified eluate were injected into the HPLC–DAD system.

3. Results and discussion

3.1. SPE clean-up and chromatographic separation

In order to determine which kind of alumina column, among the acid, neutral and basic ones, had to be used in the SPE purification step, we analysed the eluates of extraction mixture spiked with NICA and CLOP. We found that both coccidiostats were not retained by acid and basic alumina columns, while CLOP was partially retained by the neutral ones. Blank finished feeds for broilers, laying hens, turkeys and guinea fowl were extracted according to the method described and the purification step was performed on acid and basic alumina columns. The chromatographic analysis of the purified eluates showed that the basic alumina columns gave better purification than the acid ones.

Fig. 1a shows the chromatogram of a blank feed for broilers. Fig. 1b shows the chromatogram of the same blank feed spiked with both coccidiostats at final concentrations of 5 mg/kg.

As shown in Fig. 1a, some interfering peaks were observed on the chromatogram but none of these were present at the retention times of CLOP and NICA. Fig. 1b indicates that this method lends itself to the quantification of these additives at sub-therapeutic levels in feeds, as a result of possible cross-contamination at the point of manufacture.

The chromatographic profiles of laying hens, guinea fowl and turkeys feed extracts were practically identical to those shown for broiler feed extract.

3.2. Linearity

Nicarbazin concentration was calculated by assaying DNC component and assuming that NICA is a 1:1 molar ratio of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) [11]. The linearity of the photometric detector response to NICA and CLOP was verified over the range 0.4–50 μg/ml by injecting 10 μl of each of the standard curve solutions. All the responses, over the concentration range 4–500 ng injected (2–250 mg coccidiostat per kg of feed according to this method) were found to be linear with a correlation coefficient of 0.999 or greater.

3.3. Recovery

To evaluate the method, blank samples of broiler, turkey, guinea fowl and laying hen feeds were spiked in a concentration range of 2–150 mg/kg for NICA and 5–150 mg/kg for CLOP and processed, under repeatability condition, as described in Section 2.4.

Both coccidiostats were quantified using the external standard method and the results obtained are summarized in Tables 1 and 2 for NICA and CLOP, respectively.

The mean recovery of NICA from spiked feed samples was 95% with a RSD of 4%, while for CLOP the average recovery was 98% with a RSD of 5%.

The described method permits to achieve a detection limit ($S/N=3$), estimated from the chromatograms of blank feeds, of 1 mg/kg and 2 mg/kg for

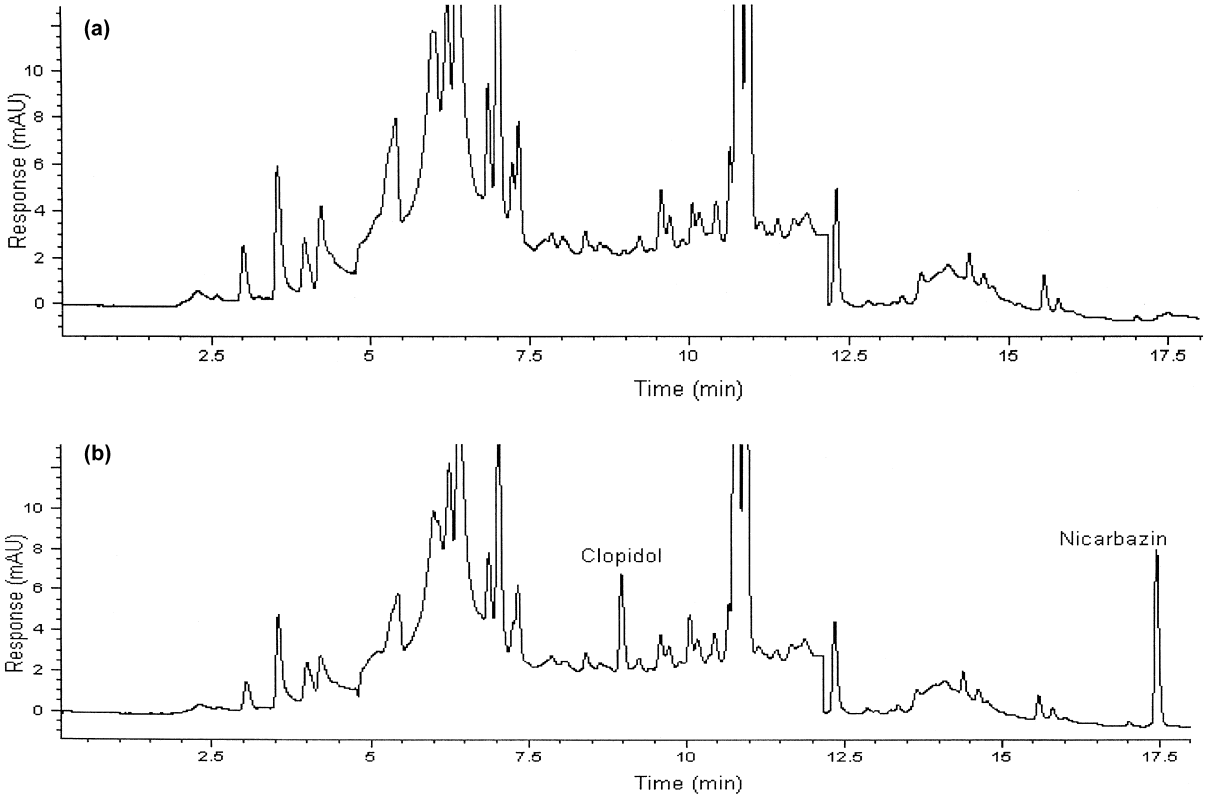

Fig. 1. HPLC chromatograms of broiler feed. (a) blank; (b) blank spiked with both NICA and CLOP at 5 mg/kg.

NICA and CLOP, respectively. The lowest levels tested (quantification limit) in fortified feed samples were 2 mg/kg and 5 mg/kg for NICA and CLOP, respectively.

Rabbit feeds have more pigments and interfering contaminants than the poultry feeds, probably due to the high fibre content. Our clean-up step reduces these interferences but does not completely eliminate them. We tested our method and concluded that it can be used in rabbit feeds only for the determination

Table 1
NICA recovery from replicates ($n=6$) of spiked samples of blank control poultry feeds

NICA added (mg/kg)	Broiler		Laying hen		Turkey		Guinea fowl	
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
2			96	1	98	4	102	1
5	94	1	95	1	97	3	93	5
10			95	2	97	2	93	3
50	98	2						
100	93	1						
150	91	1						

Table 2
CLOP recovery from replicates ($n=6$) of spiked samples of blank control poultry feeds

CLOP added (mg/kg)	Broiler		Laying hen		Turkey		Guinea fowl	
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
5	99	1	91	4	99	5	93	3
10	101	2	91	4	95	4	95	4
50	101	1						
100	99	2			101	2	99	3
150	102	3			101	3	101	2

Table 3
Assay of CLOP and NICA in commercial poultry feeds

Sample number	NICA decl. amount (mg/kg)	CLOP decl. amount (mg/kg)	Concentration found (mg/kg)	RSD (%)	Number of assays
12822	125		124	6	6
10922	125		120	5	6
15918		100	96	4	6
11395		100	103	5	6

of CLOP and NICA above 20 mg/kg and 5 mg/kg, respectively.

3.4. Test on commercial samples.

The method proposed were tested on four commercial poultry feed submitted from different manufacturers to our routine laboratory for analysis. The result are given in Table 3. The agreement between the declared amount of the two coccidiostats and the found concentrations are very good and the RSDs are below 6%. Fig. 2 shows the chromatogram of one of the commercial poultry feed.

4. Conclusions

The data reported indicate that the above method

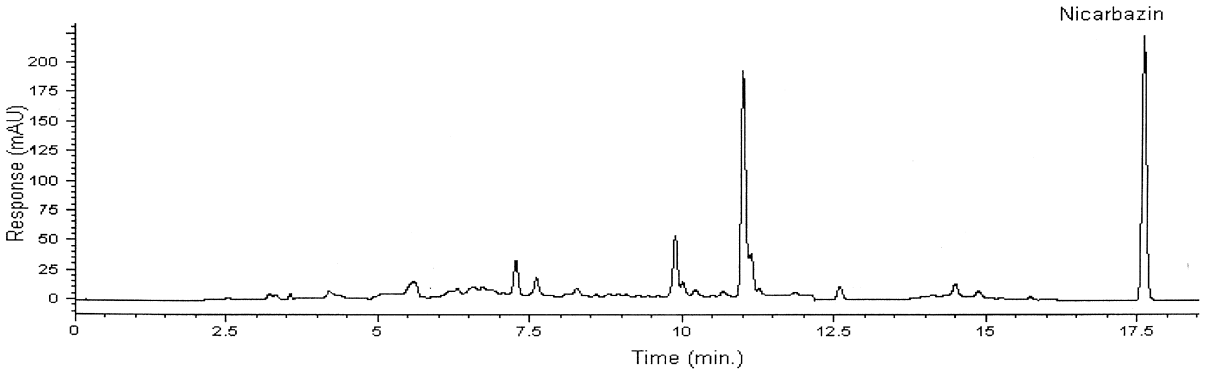

Fig. 2. HPLC of a commercial poultry feed (sample number 12822).

for the analysis of NICA and CLOP in finished poultry feeds, can achieve good recovery, specificity and repeatability. The described assay offers a number of significant advantages over previously published methods in terms of simplicity, time and cost of analysis.

These factors indicate that this method is suitable for routine analysis of NICA and CLOP in poultry feeds at concentrations ranging from the added legally permitted level down to cross-contamination ones; it can also be applied to the determination of the two coccidiostats in rabbit feeds at the added legally permitted concentration.

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