DEPLETION STUDY ON NICARBAZIN AND NARASIN IN TISSUES AND EGGS OF HENS HOUSED IN DEEP LITTER

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Received: 25 October, 2011   Accepted: November 22, 2011

Abstract

The experiment was performed to define the depletion of dinitrocarbanilide (DNC, marker of nicarbazin residue) and narasin residues in hen tissues and eggs after administration of Maxiban (nicarbazin and narasin, 80 mg kg^{-1}). A flock of 50 hens were kept in deep litter and fed coccidiostats for 14 d following 22 d of withdrawal. Randomly selected birds were slaughtered after the withdrawal of anticoccidial-containing feed. Samples of eggs, liver, and breast muscles were collected and analysed using validated LC-MS/MS method. The concentration of dinitrocarbanilide in the liver on day 0 of the withdrawal was 4,440 ± 569 µg kg^{-1} and quickly dropped to reach plateau level after 5 d. Long persistence of dinitrocarbanilide at plateau level and its presence in gastric contents during the withdrawal suggest the recycling of DNC with litter. The concentration of narasin in tissues and eggs was low even at the beginning of withdrawal period, which confirms the low probability of occurrence of narasin residues in food, even in the case of off-label use of this coccidiostat.

Key words: hens, tissues, eggs, narasin, nicarbazin, dinitrocarbanilide, residues.

Coccidiostats are widely used in animal production, especially as feed additives for poultry. According to European Commission, there is no efficient and cost-effective alternative to the prophylactic use of anticoccidial feed additives (9). In the European Union, 11 anticoccidial active substances are authorised as feed additives and allowed to be used in chicken, turkeys, and rabbits for fattening and for birds reared for layers. The off-label use of coccidiostats (especially not respecting withdrawal times) or feeding non-target animals (laying hens, animals at withdrawal periods) with cross-contaminated feeds can result in occurrence of residues of coccidiostats in food of animal origin. Some authors suggest that the type of animal husbandry (wire floor versus deep litter system housing) is also important because recycling of some compounds (especially nicarbazin) with litter can also play a role in the residue occurrence (2).

Among all registered coccidiostats, ionophore antibiotics (including narasin, NAR) and nicarbazin are used most frequently. Nicarbazin is an equimolecular mixture of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-hydroxypryrimidine (DHP). The studies performed on the kinetics of these compounds show higher persistence of DNC in animal tissues and eggs (18). Therefore, this compound was chosen as the marker residue of nicarbazin and maximum residue limits were set at 15,000 µg kg^{-1} and 4,000 µg kg^{-1} for broiler liver and muscles, respectively (4). Narasin is approved for use in broiler chickens alone or in the mixture with nicarbazin. In the EU, the maximum residue limit (MRL) was established at 50 µg kg^{-1} for all wet tissues of chickens (6).

Since 2009, the regulations concerning the maximum levels (ML) of coccidiostats in food of animal origin, resulting from unavoidable carry-over of these substances in non-target feed, have been implemented in the European Union (5). The ML values for nicarbazin are 100 µg kg^{-1} for liver and eggs and 25 µg kg^{-1} for muscles. The MLs for narasin are the following: 50 µg kg^{-1}, 5 µg kg^{-1}, and 2 µg kg^{-1} for liver, muscles, and eggs, respectively.

The aim of the presented study was to investigate the depletion of residues of two coccidiostats often used together in poultry production – nicarbazin (determined as its marker residue – dinitrocarbanilide) and narasin in hen tissues and whole eggs. The experiment was designed to verify the role of recycling in the persistence of dinitrocarbanilide residues.

Material and Methods

Animals, experiment design, and sample collection. The experiment was performed in compliance with the Animal Experimentation Ethics Committee. A flock of 50 laying hens was kept in deep litter system and given water and feed ad libitum. After
DNC and 50
Nicarbazin was below 0.5 mg kg
formic acid were obtained from Sigma (Germany). SPE
JT Baker (Germany). Dimethylsulfoxide (DMSO) and
(MeCN) and methanol (MeOH), both LC-MS grade, and
obtained from Sigma (Germany) and DNC-d8 was
(DNC), narasin (NAR), and nigericin (NIG) were
from POCh (Poland), centrifugal filters 0.2
N Plus ("Plus long" format, 1710 mg, 1.2 ml) were
columns Oasis HLB 60 mg/3 mL and Sep-Pak Alumina
µ
prepared in DMSO (DNC, DNC-d8) or acetonitrile
formate, pH 4.0). The mass spectrometer (API 3000,
842.0
57x176]DNC and 50
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Table 1
Selected validation parameters of the LC-MS/MS method for the determination of dinitrocarbanilide (DNC) and narasin (NAR) in chicken tissues and hen eggs (15, 16) and the verification of the performance of the procedure adopted for higher concentrations

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Matrix</th>
<th>LOD, µg kg(^{-1})</th>
<th>LOQ, µg kg(^{-1})</th>
<th>Spiking level, µg/kg</th>
<th>Recovery, % (n=6)</th>
<th>Repeatability, CV, % (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNC</td>
<td>Liver</td>
<td>2.12</td>
<td>5.38</td>
<td>200</td>
<td>101</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2,000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.95</td>
<td>2.17</td>
<td>200</td>
<td>101</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,000*</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2,000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eggs</td>
<td>0.33</td>
<td>0.92</td>
<td>5.0</td>
<td>101</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2,000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAR</td>
<td>Liver</td>
<td>0.43</td>
<td>1.13</td>
<td>25</td>
<td>128</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.21</td>
<td>0.58</td>
<td>50</td>
<td>101</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eggs</td>
<td>0.92</td>
<td>2.92</td>
<td>5.0</td>
<td>104</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*concentration levels that were verified for the purpose of this experiment

Table 2
Results of the determination of dinitrocarbanilide (DNC) and narasin (NAR) in the breast muscles and gastric contents of hens

<table>
<thead>
<tr>
<th>Withdrawal time (days)</th>
<th>Concentrations in muscles (µg kg(^{-1}))</th>
<th>Concentrations in gastric contents (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNC</td>
<td>NAR</td>
</tr>
<tr>
<td></td>
<td>[DNC]</td>
<td>[NAR]</td>
</tr>
<tr>
<td></td>
<td>Concentrations in muscles (µg kg(^{-1}))</td>
<td>Concentrations in gastric contents (mg kg(^{-1}))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>415 ± 47.6</td>
<td>10.9 ± 8.44</td>
</tr>
<tr>
<td>1</td>
<td>45.2 ± 13.95</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>2</td>
<td>10.9 ± 7.54</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>3</td>
<td>9.6 ± 8.25</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>4</td>
<td>2.6 ± 2.22</td>
<td>&lt; 0.21</td>
</tr>
<tr>
<td>6</td>
<td>1.8 ± 2.18</td>
<td>&lt; 0.21</td>
</tr>
</tbody>
</table>

The DNC was found in all tested samples in relatively high concentrations, in liver and eggs even after 22 d of withdrawal. In contrast, the residues of NAR in hen tissues decreased rapidly despite the fact that this compound was still applied to hens during the withdrawal period at relatively high concentration (5.13 mg kg\(^{-1}\)). Only in eggs, NAR was detected above LOQ in all samples during the whole experiment.

At the beginning of the withdrawal period, DNC was detected in the liver at 4,440 ±569 µg kg\(^{-1}\). At the end of the experiment it was still measurable with concentration of 30.5 ±32.40 µg kg\(^{-1}\) (Fig. 1A). Subsequently, until the 4th d of withdrawal, the concentration of DNC was dropping quickly but after 5 d of the withdrawal the level of DNC reached a plateau level of 56.1 ±51.38 µg kg\(^{-1}\).

NAR depleted in the liver very rapidly (Fig. 1B). Since the second day of withdrawal, its residues in the liver were found at the levels close to the method LOQ. The concentrations measured within the period of 2 to 22 d of withdrawal did not differ significantly, reaching average 1.65 ±1.144 µg kg\(^{-1}\). After 4 d of withdrawal, the concentration of NAR was below LOD (0.43 µg kg\(^{-1}\)) in some individual samples.

The residues of both coccidiostats in breast muscle samples were much lower than those observed in the liver. After 2 d of withdrawal, they dropped below ML value (25 µg kg\(^{-1}\)) for DNC and limit of detection of NAR (Table 2). Therefore, only samples collected during the first six samplings were analysed in the experiment.

The highest concentrations of residues were found in eggs. The plateau level during the Maxiban feeding period came to 5,140 ±1,390 µg kg\(^{-1}\) and 119 ±47.2 µg kg\(^{-1}\) for DNC and NAR, respectively. At the beginning of the withdrawal period, the concentration of both coccidiostats in eggs depleted rapidly, but after 22–24 d of the experiment, other plateau levels were established averaging 54.2 ±45.77 µg kg\(^{-1}\) and 9.32 ±3.049 µg kg\(^{-1}\) for DNC and NAR, respectively (Fig. 2).

Discussion
The residues of dinitrocarbanilide in eggs and tissues. The reasons of the occurrence of DNC residues in eggs were studied and discussed comprehensively (1, 13). Most of the researchers, however, checked the extent of carry-over of DNC from cross-contaminated feed. Mortier et al. (13) determined DNC in eggs after experimental administration of feed containing 40 mg kg\(^{-1}\) and 2 mg kg\(^{-1}\) of the compound.
Fig. 1. Nicarbazin (A) and narasin (B) concentrations in hen liver during 22 d of withdrawal. Data points represent mean ± standard error of the mean (SEM). The EU maximum levels, MLs for non-target animals (5) are shown for comparison.

In both cases, a plateau level was achieved averaging respectively 6,500 µg kg\(^{-1}\) and 350 µg kg\(^{-1}\). The concentration of DNC in eggs obtained in our study was also above 5,000 µg kg\(^{-1}\) after feeding 80 mg kg\(^{-1}\) of narbazin, confirming its tendency to cumulate in high fat content tissues.

Studies on narbazin depletion kinetics in tissues of chickens have been previously carried out on several occasions (2, 18). All the authors have detected DNC in high concentrations, especially in the liver. In the presented experiment, the concentration of DNC also exceeded 4,000 µg kg\(^{-1}\) in the liver and 400 µg kg\(^{-1}\) in muscles. Cannavan and Kennedy (2) found DNC in the liver of broiler chickens at a concentration of 223.8 µg kg\(^{-1}\) after 9 d of withdrawal of feed containing 100 mg kg\(^{-1}\) of NAR. The levels of DNC in muscles were approximately 15 times lower. These authors observed a significant difference between the content of the coccidiostat in tissues of birds housed in the deep litter and wire floor systems, which can be caused by the DNC recycling.

The recycling of dinitrocarbanilide from litter. In the presented study, DNC did not deplete completely during the 22 d of withdrawal, in both liver and eggs, despite of the absence of DNC in the withdrawal feed. The relatively high concentrations of...
DNC in eggs and high persistence of its residues in the liver may be an indication of slow depletion of DNC, but it can also be, as suggested previously (2, 12, 18), the result of its recycling with litter.

To verify this thesis, the samples of hens' gastric contents were tested to determine whether the birds consumed nicarbazin with litter during the withdrawal period. Concentrations of NAR and DNC in gastric contents determined immediately after the withdrawal of Maxiban containing feed represented about 40%-50% concentration in the feed. Already at the 2nd d of withdrawal, the concentration of DNC in the gastric contents dropped below 0.3 mg kg\(^{-1}\) but in individual samples taken even 6 d after the withdrawal, traces of the coccidiostat were still detected (Table 3). Assuming that the concentration of coccidiostats in the gastric contents can be used to estimate the dose received by the birds, consumption of DNC with litter was comparable to the consumption of feed containing about 0.5-1 mg kg\(^{-1}\) of nicarbazin.

Comparison of concentrations obtained in the experiment with the calculations of other authors confirmed that recycling with litter could be the source of nicarbazin – the plateau level of DNC in eggs (54.2 ±45.77 µg kg\(^{-1}\)) was very close to 50 µg kg\(^{-1}\) equivalent to 1 mg/kg in feed according to Cannavan et al. (1).

Recycling of coccidiostat in litter could be also an explanation for the plateau level of DNC residues (17.8-127 µg kg\(^{-1}\) in the liver) established in the second phase of the experiment. According to Cannavan and Kennedy (2), 1 mg kg\(^{-1}\) nicarbazin in the feed corresponds to the DNC levels of 75 µg kg\(^{-1}\) and 2.5 µg kg\(^{-1}\) in the liver and muscle, respectively.

At the same time, it should be noted that the concentrations of DNC in the gastric contents, eggs, and liver during withdrawal period were very diverse. This may be related to different degree of litter consumption by individual animals, and indirectly confirms the possibility that recycling can be the cause of the persistence of DNC residues.

The residues of narasin in eggs and tissues. The described experiment on NAR residues in eggs can be regarded in two aspects: the depletion of NAR after feeding feed containing NAR at levels intended for broiler chickens, and the carry-over from cross-contaminated feed. Most of the available data concern rather the latter case, which has a greater practical importance. Mortier et al. (13) found 90 µg kg\(^{-1}\) and 6 µg kg\(^{-1}\) of NAR in eggs after its administration in feed at a concentration of 40 mg/kg and 2 mg kg\(^{-1}\), respectively. In another study (19), the determined plateau level of NAR was about 7 µg kg\(^{-1}\) in egg yolk after feeding hens 2.5 mg kg\(^{-1}\) of the coccidiostat. The obtained results - NAR concentration approximately 100 µg kg\(^{-1}\) and 9 µg kg\(^{-1}\) at the time of administration Maxiban 80 mg kg\(^{-1}\) and 5.13 mg kg\(^{-1}\) contaminated feed, respectively, are very similar to the above results.

Concentrations of NAR in tissue samples were significantly lower than the residues of DNC - immediately after the cessation of Maxiban feeding they were 22.5 µg kg\(^{-1}\) and 10.9 µg kg\(^{-1}\) for the liver and muscle samples, respectively. This confirms a low probability of occurrence of residues of this ionophore in animal tissues. Peippo et al. (17) found that the levels of NAR residues in breast muscles of chickens receiving this coccidiostat were very similar to those obtained in this study – 2.1–2.3 µg kg\(^{-1}\) in birds given 70 mg/kg of NAR, and 0.6–1.3 µg kg\(^{-1}\) when animals were fed NAR contaminated diet (3.5 mg kg\(^{-1}\)). In the second experiment, 319 µg kg\(^{-1}\) and 40 µg kg\(^{-1}\) of NAR equivalents were detected in the liver and muscles of chickens given 50 mg kg\(^{-1}\) of NAR for 5 d, respectively (20). This corresponds to 16 µg kg\(^{-1}\) and 2 µg kg\(^{-1}\) of NAR considering the 5% of marker residue - total residue ratio (14).

The low possibility of occurrence of NAR residues is confirmed by the results of the depletion of other ionophore antibiotics in chickens. Most of the data indicate that these compounds are rapidly metabolised and probably slightly absorbed. Salinomycin, the compound most similar in chemical structure and properties to NAR, was determined under similar conditions, and the analysis showed its presence in the liver at concentration around 14 µg kg\(^{-1}\) (11). The residues of monensin in all tissues other than fat were also low and depleted rapidly (10).

The results of the presented study confirm the low probability of the occurrence of residues of NAR in chicken tissues, as well as low levels of its residues in eggs when layers are exposed to anticoccidial-containing feed. In contrast, the depletion profile of DNC causes high residue levels in both tissues and eggs. The recycling of nicarbazin in the conditions of deep litter housing can cause the longer persistence of its residues. Since DNC is a compound of low toxicity, its residues have high regulatory limits. Therefore, the violation of lately introduced EU MRL and ML levels (4, 5) is also not probable for this compound.

References