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Short Communication

High-performance liquid chromatographic method for the determination of the dinitrocarbanilide component of nicarbazin in eggs with on-line clean-up

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ABSTRACT

A high-performance liquid chromatographic method for the determination of the dinitrocarbanilide component of the anti-coccidial drug nicarbazin has been developed. The drug was extracted from egg with acetonitrile. The extract was evaporated to dryness and taken up in water–acetonitrile (80:20, v/v). The extract was then injected onto a reversed-phase precolumn. After clean-up with 20% aqueous acetonitrile for 5 min, the precolumn was eluted onto a Chromspher C_{18} cartridge column with 0.01 *M* potassium dihydrogenphosphate pH 4.0–acetonitrile (50:50, v/v). Detection was by ultraviolet at 343 nm. Average recoveries at five levels from 0.005 to 0.500 mg kg^{-1} were >80%. The limit of determination was 0.005 mg kg^{-1}.

INTRODUCTION

Nicarbazin is a mixture of two compounds: N,N′-bis(4-nitrophenylurea) (dinitrocarbanilide) and 4,6-dimethyl-2(1*H*)-pyrimidinone (Fig. 1) in the ratio 1:1–2:1. It is used for the prophylaxis of coccidiosis in poultry at a feeding level of 125 g per tonne feed [1]. The persistence of nicarbazin in chicken liver [2] and eggs [3] has been studied. Broilers were fed nicarbazin at 125 g per tonne feed for 28 days and then fed nicarbazin-free feed. Levels of nicarbazin in the liver decreased from 1.5–1.8 mg kg^{-1} on day 1 of the withdrawal period to 0.009–0.022 mg kg^{-1} on day 9 (the recommended withdrawal period before consumption of the tissue). When layers were given feed containing nicarbazin at 125 g per tonne for 70 or

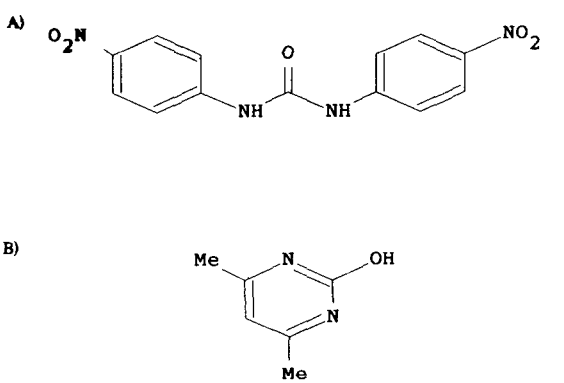

Fig. 1. Structures of (A) dinitrocarbanilide and (B) 4,6-dimethyl-2(1*H*)-pyrimidinone.

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100 days from birth and then nicarbazin-free feed until laying, no residues of nicarbazin were detected in the eggs. However, when birds were given feed containing nicarbazin at 0.45–1.1 g per tonne continuously from birth, levels of 0.25 mg kg^{-1} were detected in eggs. At feeding levels of 0.05–0.1 g per tonne, nicarbazin residues were not detected in eggs. A further study [4] demonstrated that nicarbazin was detectable after 10 days in tissues and after 28 days in eggs after feeding at 200 g per tonne for 2–6 weeks. Nicarbazin has been shown to have an adverse effect on eggshell pigmentation, egg weight and hatchability [5].

It has been shown that chickens excrete the dinitrocarbanilide component of nicarbazin more slowly than the pyrimidinone component [6], hence methods of analysis have focused on the former compound. There are a number of procedures in the literature for the determination of dinitrocarbanilide in poultry tissues and eggs. Almost all the procedures utilise HPLC as the final determinative procedure with either UV or electrochemical detection. The most common solvent used to extract dinitrocarbanilide from poultry tissues is acetonitrile [7–11]. Acetonitrile containing a small amount of acetic acid [12], ethyl acetate [13], methanol [14] and chloroform–ethyl acetate–dimethyl sulphoxide (50:50:0.8) [15] have also been used. Clean-ups by liquid–liquid extraction [7] or solid-phase extraction (SPE) have been used. A number of different SPE columns have been used, including silica [11,12], alumina [13,15], alumina followed by C_{18} [8] and cation exchange followed by Florisil [14].

This paper reports a simple and rapid procedure for the determination of dinitrocarbanilide in eggs based on on-line reversed-phase clean-up.

EXPERIMENTAL

Chemicals

Analytical-grade anhydrous sodium sulphate, potassium dihydrogenphosphate and orthophosphoric acid were obtained from BDH (Poole, UK). HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, UK). Standard nicarbazin was a gift from Merck, Sharp and Dohme (Hoddesdon, UK). The standard purity was 96.8% and contained 69.1% dinitrocarbanilide and 27.7% 4.6-dimethyl-2(1*H*)-pyrimidinone.

Purification of dinitrocarbanilide

Pure dinitrocarbanilide was obtained by dispersing standard nicarbazin (0.25 g) in water (100 ml) and extracting with ethyl acetate (150 ml followed by 75 ml). The organic layers were combined and evaporated to dryness. Recovery was 94.2% and the estimated purity based on the composition data for nicarbazin supplied by Merck, Sharp and Dohme was 96.8%.

Apparatus

Magnetic stirrer (Janke & Kunkel, supplied by Merck, Poole, UK), hot-block and nitrogen blow-down apparatus (Grant, supplied by Merck), vortex mixer (Fisons, Loughborough, UK), ultrasonic bath (L&R 140S, Kearny, NJ, USA) and disposable syringe filters (Millipore Millex-HV_{13}, 0.45 μm, 13 mm) were used.

Extraction

Homogenised egg (1 g) and acetonitrile (2.5 ml) were vortex-mixed to ensure thorough mixing and then stirred for 4 min. Anhydrous sodium sulphate (0.3 g) was added and stirring continued for a further 2 min. The extract was then centrifuged for 2 min at 1860 *g* and the supernatant decanted. Acetonitrile (2.5 ml) was added to the solid residue, vortex-mixed to break up the solid and stirred for 3 min. The extract was then centrifuged again for 2 min at 1860 *g* and the supernatant decanted. The combined organic layers were evaporated to dryness under a stream of nitrogen on a hot-block at 50–60°C, taken up in water–acetonitrile (80:20, v/v) (1 ml) by vortex-mixing for 15 s and ultrasonication for 3 min and filtered (Millipore Millex-HV_{13}, 0.45 μm, 13 mm).

High-performance liquid chromatography

Samples (250 μl preceded and followed by 100 μl air) were injected (Gilson 231 autosampler with 401 dilutor fitted with a 5-ml loop, Anachem, Luton, UK) onto a reversed-phase precolumn (Chrompack, London, UK) 10 mm × 2.1 mm

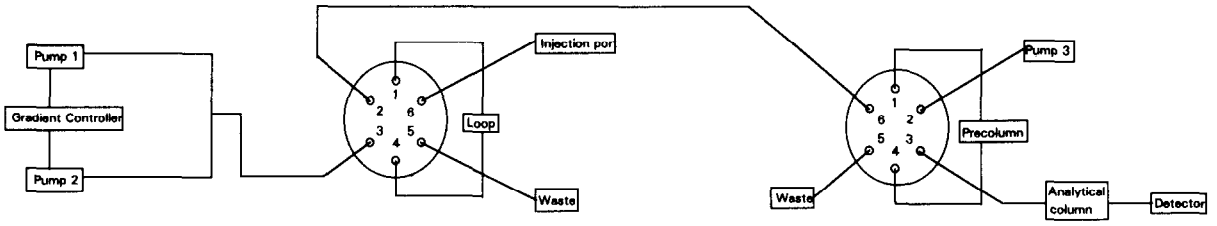

Fig. 2. Configuration of automated HPLC system.

I.D. installed across a column-switching unit (Anachem Universal). Mobile phase was water–acetonitrile (80:20, v/v). Mobile phase was maintained at 0.4 ml min^{-1} (2152 gradient controller. plus two 2150 pumps, Pharmacia LKB Biotechnology, Uppsala, Sweden). After 5 min the precolumn was eluted with 0.01 *M* potassium dehydrogenphosphate pH 4.0–acetonitrile (50:50, v/v) at 0.4 ml min^{-1} (Gilson 307 pump) onto a Chromspher C_{18} (Chrompack) 5 μm, 100 mm × 3 mm I.D. column protected by integral guard column, 10 mm × 2.1 mm I.D. (pellicular reversed-phase). The precolumn was eluted for 1 min prior to switching back to a cleaning and reequilibration procedure (see below), while the analytical column continued to be eluted with its mobile phase. The configuration of the system is shown in Fig. 2. Detection was by UV at 343 nm (Applied Biosystems 783 programmable, Anachem). Data capture and peak-height calculation was undertaken using a SP4400 Chromjet (Spectra-Physics, Hemel Hempstead, UK). Quantitation was by reference to replicate injections (250 μl) of standard dinitrocarbanilide.

After elution the precolumn was cleaned by passing water–acetonitrile (80:20, v/v) at 1.0 ml min^{-1} for 1 min, followed by acetonitrile at 2.0 ml min^{-1} for 13 min. The precolumn was then reequilibrated for use by passing water–acetonitrile (80:20, v/v) at 2.0 ml min^{-1} for 5 min.

Protocol

For method validation, samples were analysed in batches of five to nine spikes and one blank. Time of preparation for a batch of ten samples was approximately 2 h.

RESULTS AND DISCUSSION

The chromatography of dinitrocarbanilide on reversed-phase columns was first established. Suitable conditions were found using a Chromsep Chromspher C_{18} 100 mm × 3 mm I.D. column (Chrompack) with integral reversed-phase precolumn. Initially 50% aqueous acetonitrile was used as mobile phase. Sharper peaks were obtained using 0.01 *M* phosphate pH 4–acetonitrile (50:50, v/v). Evaluation of the UV (diode array) spectra indicated a λ_{max} at 343 nm, which was used for all subsequent determinations.

The chromatographic behaviour of dinitrocarbanilide on Chrompack reversed-phase precolumns was also examined. Using water–acetonitrile (80:20, v/v) as mobile phase, dinitrocarbanilide was retained on the precolumn for 8 min before breakthrough occurred. The mobile phase for the analytical column [0.01 *M* phosphate pH 4–acetonitrile (50:50, v/v)] eluted dinitrocarbanilide efficiently from the precolumn.

An automated system by which dinitrocarbanilide could be loaded onto the reversed-phase precolumn and then eluted and chromatographed on the Chromspher C_{18} column was set up (Fig. 2). The effect of the length of the column-switch was examined, using a constant 2-min clean-up with water–acetonitrile (80:20, v/v). Recovery of dinitrocarbanilide did not start to decrease until the elution time was decreased to less than 0.5 min. The effect of the length of the clean-up time on recovery was also examined, keeping the elution time constant at 0.5 min. Recovery was found to start dropping after a clean-up time of 8 min. In view of these findings a

clean-up of 5 min and elution period of 1 min of the precolumn was adopted. The standard curve for dinitrocarbanilide was linear (correlation coefficient $r = 0.9999$) from 0 to 125 ng on column (equivalent to 0–0.500 mg kg^{-1}).

The extraction procedure of Vertommen *et al.* [12] was used with the modification that no acetic acid was added. After extraction, the combined acetonitrile layers were evaporated to dryness and the residue taken up in water–acetonitrile (80:20, v/v) ready for HPLC analysis. The procedure was validated at five levels: 0.005, 0.010, 0.050, 0.100 and 0.500 mg kg^{-1} (Table I). Homogenised egg (1 g) was spiked with 100 μl of standard in acetonitrile at the appropriate concentration (0.05, 0.10, 0.50, 1.0 and 5.0 $\mu g\ ml^{-1} = 0.005$, 0.010, 0.050, 0.100 and 0.500 mg kg^{-1}, respectively). Single batches were performed at 0.005, 0.010, 0.100 and 0.500 mg kg^{-1} and three batches at the 0.050 mg kg^{-1} level. Recovery ranged from 81.0 to 94.0% with coefficients of variation (C.V.) of 11.3% at 0.005 mg kg^{-1} down to 2.4% at 0.500 mg kg^{-1}. Typical chromatograms of standard dinitrocarbanilide equivalent to 0.050 mg kg^{-1}, blank egg extract and blank egg extract spiked at 0.050 mg kg^{-1} are shown in Fig. 3.

TABLE I

RECOVERIES OF DINITROCARBANILIDE FROM EGG

Batch	Recovery (mean ± S.D.) (%)	C.V. (%)	*n*
0.005 mg kg^{-1}			
1	81.0 ± 9.7	11.3	8
0.010 mg kg^{-1}			
1	89.7 ± 5.6	6.2	8
0.050 mg kg^{-1}			
1	91.8 ± 4.7	5.2	8
2	84.8 ± 5.9	6.7	9
3	83.0 ± 2.7	3.2	9
Overall	86.3 ± 5.6	6.5	26
0.100 mg kg^{-1}			
1	94.0 ± 4.0	4.3	9
0.500 mg kg^{-1}			
1	81.3 ± 1.9	2.4	5

CONCLUSION

A method has been developed for the determination of dinitrocarbanilide in eggs by HPLC with on-line sample clean-up. The procedure has

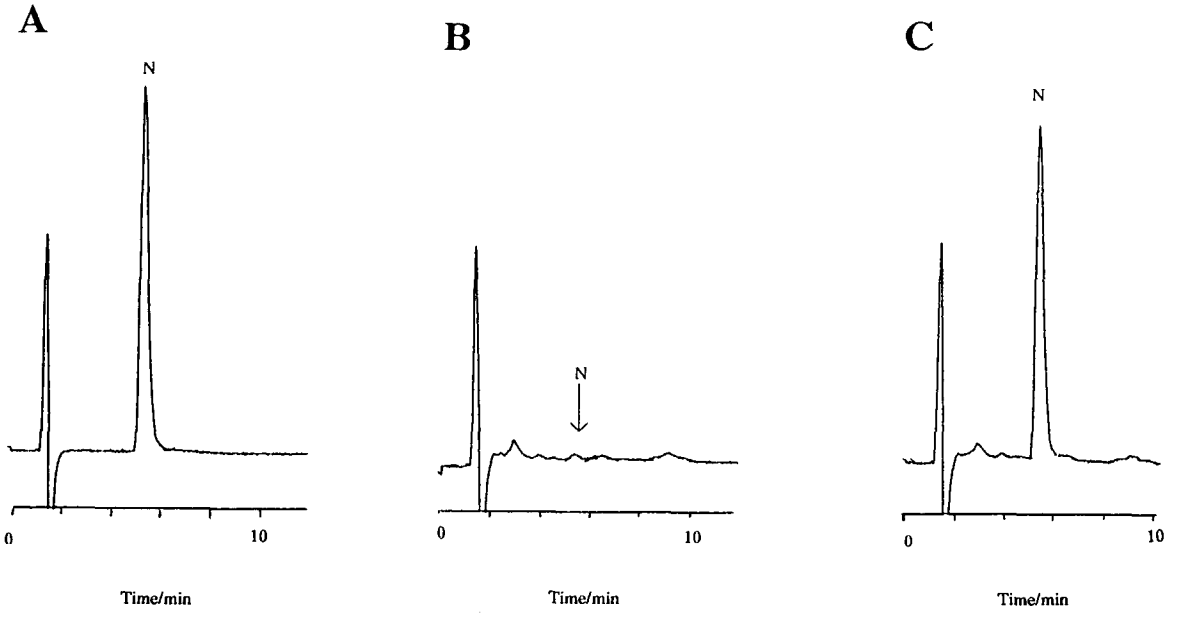

Fig. 3. Chromatograms of (A) standard dinitrocarbanilide equivalent to 0.050 mg kg^{-1}, (B) blank egg and (C) blank egg spiked at 0.050 mg kg^{-1} (0.02 a.u.f.s.) (N = position of nicarbazin).

been validated for the range 0.005–0.500 mg kg^{-1}. The limit of determination of 0.005 mg kg^{-1} is equal to or better than the literature procedure cited in the Introduction. In addition, the procedure is rapid, a batch of ten samples taking approximately 2 h to prepare. Because of the format of the extraction procedure, it is possible to increase the size of the batch without an equivalent increase in the preparation time.

REFERENCES

1 Y. Debuf (Editor), *The Veterinary Formulary*, The Pharmaceutical Press, London, 1988, p. 97.
2 A. Grassetelli, E. Pierdominici, A. Macri and S. Castelli, *Ind. Aliment. (Pinerolo, Italy)*, 29 (1990) 237.
3 Y. Oishi and T. Oda, *Shokuhin Eiseigaku Zasshi*, 30 (1989) 542.
4 N. Nose, Y. Hoshino, Y. Kikuchi, H. Masaki, S. Horie and S. Kawasaki, *Shokuhin Eiseigaku Zasshi*, 23 (1982) 246.
5 B. L. Hughes, J. E. Jones, J. E. Toler, J. Solis and D. J. Castaldo, *Poult. Sci.*, 70 (1991) 476.
6 C. C. Porter and J. J. GilFillan, *Poult. Sci.*, 34 (1955) 995.
7 M. L. Cortesi, F. De Giovanni, G. Catellani and A. Lucisano, *Ind. Aliment. (Pinerolo, Italy)*, 27 (1988) 999.
8 Y. Hori, *Shokuhin Eiseigaku Zasshi*, 24 (1983) 447.
9 Y. Komiyama, E. Mochizuki, I. Yamada and A. Nakayama, *Yamanashi-ken Eisei Kogai Kenkyusho Nenpo*, 32 (1988) 13.
10 G. Knupp, G. Bugl-Krieckmann, C. Commichau, T. Schmidt and H. Buening Pfaue, *Z. Lebensm.-Unters. Forsch.*, 185 (1987) 472.
11 Y. Suzuki, *Ishikawa-ken Eisei Kogai Kenkyusho Nenpo*, (1990) 399.
12 M. H. Vertommen, A. van der Laan and H. M. Veenedaal-Heselman, *J. Chromatogr.*, 481 (1989) 452.
13 J. L. Lewis, T. D. Macy and D. A. Gartiez, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 577.
14 N. Nose, Y. Hoshino, Y. Kikuchi and S. Kawauchi, *Shokuhin Eiseigaku Zasshi*, 23 (1982) 176.
15 O. W. Parks, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 778.