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Efficient HPLC method for the determination of nicarbazin, as dinitrocarbanilide in broiler liver

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Abstract

A simple, fast and reliable HPLC-UV method has been developed for the determination of dinitrocarbanilide residues in broiler liver. Liver samples (2 g) were extracted with two portions of acetonitrile (10 and 5 ml), defatted with hexane and evaporated to dryness under nitrogen. Extracts were reconstituted in acetonitrile–water (70/30, v/v, 500 μl), loaded onto C_{18} solid phase (SPE) cartridges and eluted with acetonitrile–water (70/30, v/v, 2.5 ml) into clean test-tubes. Extracts were evaporated to dryness and reconstituted in acetonitrile–water (80/20, v/v, 500 μl). An aliquot of the extract was assayed by high performance liquid chromatography (HPLC) with UV detection at 350 nm. The method was validated according to EU guidelines using liver tissues fortified at levels of 100, 200 and 300 μg/kg, with dinitrocarbanilide. The decision limit (CCα) and the detection capability (CCβ) were calculated from the within laboratory repeatability data to be 228 and 266 μg/kg, respectively. The mean recovery was typically >70% and the limits of quantitation was 12.5 μg/kg (based on the lowest standard on the calibration curve).

Keywords: Dinitrocarbanilide; Nicarbazin; Broiler liver; C_{18} SPE; HPLC-UV

1. Introduction

Nicarbazin is an equimolar mixture of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). It is administered to poultry in feed for the prophylactic treatment of coccidiosis. Coccidiosis is an infectious disease caused by a microscopic protozoan parasite, which damages the intestinal tract of the bird (or other animal host), causing illness and sometimes death. Intensively reared broilers are particularly susceptible to the disease, owing to the warm and humid conditions of broiler houses. The disease is not as common when birds are raised under extensive conditions. The licence for feed premixes containing nicarbazin as a single active ingredient were withdrawn under Commission Regulation 2205/2001/EC [1]. However, nicarbazin continues to be marketed, together with the feed additive ionophore narasin as the combined product Maxiban®.

Nicarbazin is classified as a feed additive and not as a veterinary drug. As a result, no MRLs have been set for nicarbazin in the European Union. Nicarbazin depletion studies have been conducted in broilers showing that DNC is a more persistent residue than the HDP component in edible tissues [2]. As a result, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) has established a maximum residue limit (MRL) of 200 μg/kg for DNC, as the marker residue, in edible tissue (liver and meat). DNC residues have been found in eggs and poultry liver [3–6]. Therefore, there is a particular need for simple and reliable residue methodology for the determination of DNC residues in poultry tissues. It has been demonstrated in residue depletion studies that higher concentrations of DNC occur in liver

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tissue, compared to muscle [7]. Therefore, it would appear more effective to monitor DNC residues in liver than muscle tissue. A number of residue methods have been developed for the determination of DNC in eggs [8–15], muscle [14–17] and liver [12–17]. Published methods for determination of DNC in liver are LC–MS [11,13,14,16], HPLC [8–10,15,17] and Biacore™ biosensor [12] based methods. The available HPLC methods involve liquid–liquid partitioning using large volumes of organic solvent [8,15] or are MSPD (matrix solid phase dispersion) based and lack sensitivity [17].

This paper reports a simple procedure for the determination of DNC in poultry liver based on a single C_{18} clean-up step with detection by HPLC-UV. The method was validated as described in Commission Decision 2002/657/EC [18].

2. Experimental

2.1. Reagents and chemicals

4,4′-Dinitrocarbanilide standard material was from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (both HiPerSolv grade), dimethylsulphoxide and *n*-hexane (Analar grade) were obtained from BDH (Merck, Poole, Dorset, UK). Bond Elut™ cartridges (C_{18}, 500 mg, 3 ml) were from Varian (Harbor City, CA, USA). The primary standard stock solution (1 mg/ml) was prepared in dimethylsulphoxide. Secondary standard stock solutions (100 and 10 μg/ml) were prepared by dilution of the primary standard stock in methanol. Working standards were prepared by diluting the 10 μg/ml solution in acetonitrile–water (80/20, v/v). The primary stock standard solution was prepared every 3 months and was stored in a glass test-tube covered in aluminium foil at room temperature in the dark. Storage at room temperature prevented solidification of the stock solution. Intermediate and working standard solutions for HPLC were prepared monthly in methanol and stored at 4 °C.

2.2. HPLC conditions

The HPLC system consisted of a model 600 HPLC pump with a model 717 autosampler and model 484 UV detector (set at 350 nm), all from Waters (Milford, MA, USA). The separation was carried out on a stainless-steel analytical column (250 mm × 4.6 mm i.d) equipped with a Securiguard™ pre-column, both packed with Hypersil BDS C_{18} material (Phenomenex, Cheshire, UK). The column temperature was maintained at 40 °C. The mobile phase, consisting of water–acetonitrile (55/45, v/v), was pumped at 1 ml/min. Under these conditions the retention time of DNC is approximately 13.5 min. A Waters 746 data processing module was used for recording and processing chromatograms.

2.3. Sample preparation

For preparation of fortified liver samples, 2 g portions of negative control broiler liver (not containing any detectable DNC residues) were weighed into 30 ml polypropylene tubes. For routine analysis, negative control samples were fortified at levels of 25 and 250 μg/kg by adding 50 μl portions of 1 and 10 μg/ml standard solutions, respectively. After fortification, samples were held for 15 min prior to extraction. Acetonitrile (10 ml) were added and samples were homogenised using a Polytron™. The homogeniser probe was washed with acetonitrile (5 ml), which was retained. Samples were vortexed (2 min), sonicated (3 min) and shaken (15 min), before centrifugation (2500 rpm, 10 min, 4 °C). The supernatant was transferred to a clean polypropylene tube and the sample was re-extracted as before using the acetonitrile (5 ml) previously used to wash the homogeniser probe, plus water (1 ml). The supernatants were combined and defatted using hexane (2 ml × 10 ml) by vortex mixing, centrifugation and removal of the hexane layer. The acetonitrile layer was evaporated to dryness under nitrogen (60 °C) and reconstituted in acetonitrile–water (70/30, v/v, 500 μl). Samples extracts were passed through C_{18} SPE cartridges (preconditioned with 2.5 ml acetonitrile and 2.5 ml acetonitrile–water (70/30, v/v)) and eluted with 2.5 ml acetonitrile–water (70/30, v/v). The eluate from the cartridge was collected in a clean glass test-tube. Extracts were evaporated to dryness under nitrogen (60 °C) and reconstituted in acetonitrile–water (80/20, v/v, 500 μl). Extracts were allowed to sit for 15 min prior to transfer to HPLC vials; a phase separation may occur in some samples and care is taken not to transfer this lower oily layer into the HPLC vials. A 25 μl portion of the final sample extract was injected onto the HPLC system.

2.4. Calibration

Standards were prepared at concentrations of 0, 50, 100, 250, 500 and 1000 ng/ml in acetonitrile–water (80/20, v/v). Calibration curves were prepared by plotting peak area as a function of DNC concentration (0–1000 ng/ml). Recovery was measured from the peak areas obtained for fortified sample extracts, as calculated from the calibration curve.

2.5. Method validation

For the validation study, 2 g portions of negative control broiler liver were fortified at levels of 100, 200 and 300 μg/kg ($n = 6$, each level) by adding 20, 40 and 60 μl portions of a 10 μg/ml stock solution. This study was performed on three separate occasions. Performance characteristics were determined following the procedures described in Commission Decision 2002/657/EC [18]. Further evaluation of the method was carried out through applying the method in routine testing of broiler liver samples. Recovery data from ten analytical runs were used to determine performance of the method at low (25 μg/kg) and high (250 μg/kg) fortification levels.

3. Results and discussion

3.1. Development of extraction and clean-up procedure

At the commencement of this work, extraction and clean-up procedures from published literature [8,12] were evaluated for compatibility with liver samples and HPLC-UV determination. Using these methods, late eluting peaks interfered with subsequent sample analysis. Chromatograms of samples showed the occurrence of non-polar interference peaks at up to 70 min retention time. This indicated that a C_{18} SPE clean-up procedure might be developed that would remove this matrix interference. The principle of this approach was to selectively elute DNC using an acetonitrile–water mixture, while leaving the more non-polar interferences retained on the SPE cartridge.

Preliminary extraction of DNC from liver was obtained with acetonitrile and a hexane defatting step was included to remove some of the non-polar matrix components prior to the C_{18} clean-up. Initial experiments concentrated on identifying a suitable solvent for reconstitution of the sample extracts prior to application onto the SPE cartridges. It was found that an appropriate composition of acetonitrile–water (70/30 or 80/20, v/v) allowed solubilisation of DNC in a small volume of solvent (200–500 μl).

The effect of different SPE parameters on the recovery of DNC is shown in Table 1. An SPE application solvent of 80/20 acetonitrile–water (v/v) gave higher recovery than 70/30 acetonitrile–water (v/v) for both 500 and 100 mg C_{18} SPE cartridges. However, in both cases it was found that 80/20 acetonitrile–water (v/v) also caused an increase in the presence of matrix interference in chromatograms. Consequently, it was decided to apply extracts onto the SPE cartridges using 70/30 acetonitrile–water (v/v). A similar situation was observed with the elution solvent; 70/30 acetonitrile–water (v/v), used as elution solvent, was found to reduce the presence of matrix interference in chromatograms while giving reasonable recovery. Elution solvents containing higher proportions of organic solvent were found to result in matrix interference in the final extracts. A lower strength elution solvent of 60/40 acetonitrile–water (v/v) gave lower recovery; increasing the volume of the 60/40 acetonitrile–water (v/v) elution solvent, from 2.5 to 3.0 ml, gave only a slight increase in recovery.

The robustness of the SPE step was evaluated by applying the method to 100 mg C_{18} SPE cartridges. The optimum volumes of elution solvent for 500 and 100 mg SPE cartridges were 2.5 and 1.0 ml, respectively. It was found that 100 and 500 mg C_{18} SPE cartridges gave similar recovery. However, it was decided to validate the method using 500 mg cartridges because these cartridges were likely to give more reproducible results because of the larger volumes of solvent used. In addition, there was less chance of the SPE cartridge blocking because of the larger surface area of the 500 mg/3 ml cartridge compared to the 100 mg/1 ml cartridge.

The extraction and clean-up procedure that was developed in this work offers advantages over previous HPLC-UV methods for determination of dinitrocarbanilide in liver [8,15]. Malisch developed an early method for isolation of a range of drugs from tissues using labour intensive liquid–liquid partitioning clean-up procedures [8]. Draisci et al. later developed a method to isolate DNC residues from broiler tissues, eggs, feed and litter using large volumes of solvent, which required the use of rotary evaporation systems. The sample preparation procedure that is employed in this newly developed method is much simpler than those described. The method has been scaled down, allowing extraction and purification of up to 24 samples in a single batch. Simpler sample preparation procedures have been developed by other groups but require the application of more selective and expensive LC–MS/MS detection systems [13,14]. The technology used in this method is available in most residue analysis laboratories. It has been proposed that the method is suitable for confirmation of the presence of nicarbazin residues in broiler liver samples that have been screened positive using an alternative detection system, such as Biacore™ biosensor. This screening and confirmatory approach has been applied in the authors' laboratory for the effective analysis of nicarbazin residues in broiler liver samples.

3.2. Method validation

3.2.1. Sensitivity

The method can quantitatively determine DNC residues in poultry liver samples at 12.5 μg/kg, based on the lowest standard on the calibration curve, and can detect DNC residues in liver to 5 μg/kg or lower. Since the JECFA MRL for DNC in liver tissue is 200 μg/kg, the method is suitably sensitive for the determination of DNC in broiler liver.

Table 1
Effects of different SPE conditions on the recovery of DNC from broiler liver fortified at a level of 200 μg/kg ($n = 2$)

Treatment	SPE application solvent	Volume (μl)	Elution solvent	Volume (ml)	SPE cartridge (C_{18}) (mg)	Mean recovery (%)
1	ACN–H_2O (80/20)	500	ACN–H_2O (60/40)	2.5	500	58.6
2	ACN–H_2O (80/20)	500	ACN–H_2O (70/30)	2.5	500	75.0
3	ACN–H_2O (80/20)	500	ACN–H_2O (60/40)	3.0	500	61.8
4	ACN–H_2O (80/20)	500	ACN–H_2O (70/30)	2.5	500	79.2
5	ACN–H_2O (70/30)	500	ACN–H_2O (70/30)	2.5	500	73.6
6	ACN–H_2O (80/20)	200	ACN–H_2O (70/30)	1.0	100	84.1
7	ACN–H_2O (70/30)	200	ACN–H_2O (70/30)	1.0	100	81.3

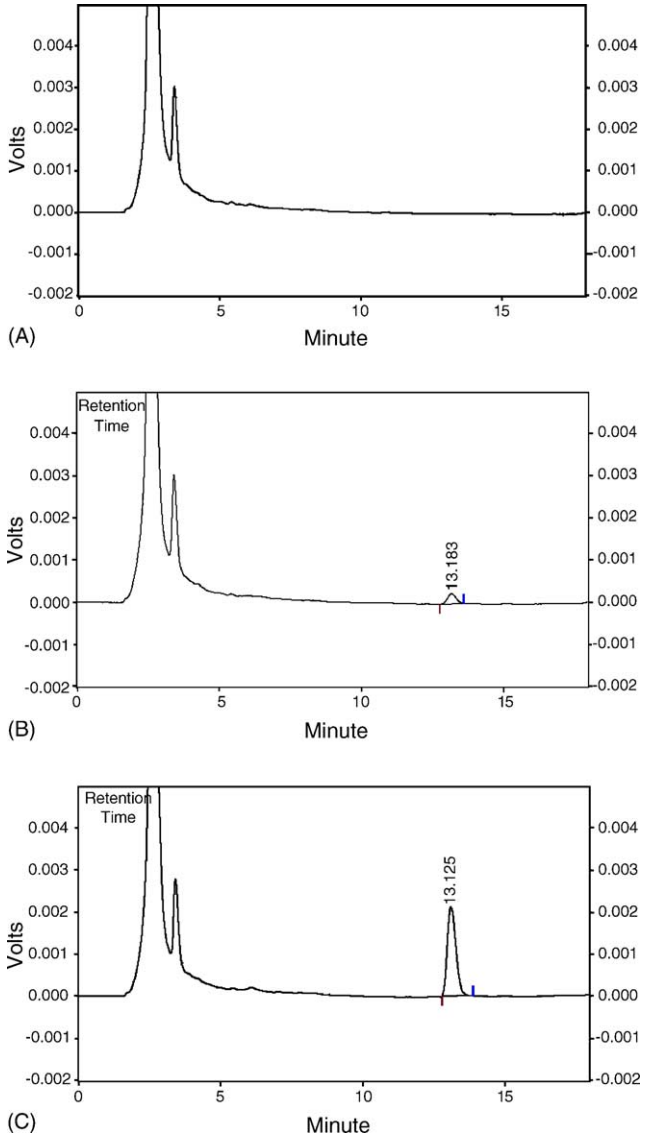

Fig. 1. Chromatograms of a negative control broiler liver sample fortified with DNC at levels of 0 μg/kg (A), 25 μg/kg (B) and 250 μg/kg (C).

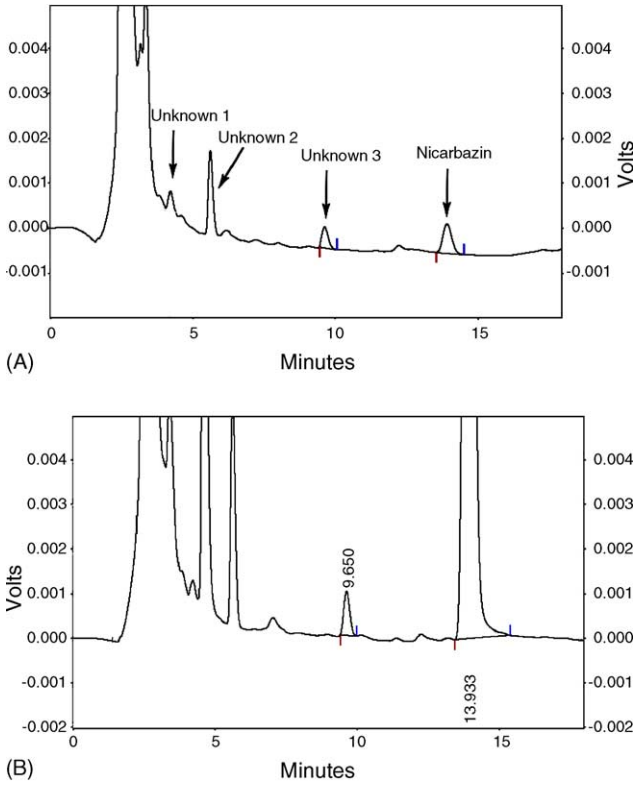

Fig. 2. Chromatograms of incurred broiler liver samples containing (A) low (43 μg/kg) and (B) high (3642 μg/kg) levels of DNC.

3.2.2. *Specificity*

Chromatograms of fortified and negative control broiler liver samples are shown in Fig. 1. It can be seen from the chromatograms that the DNC peak (13.5 min) is clearly resolved from any matrix peaks, which typically elute at less than 10 min. Chromatograms of broiler liver samples containing high and low incurred levels of DNC are shown in Fig. 2. It can be seen from the chromatogram of the high incurred positive that there are two large additional peaks (at 4 and 6 min, approximately). Unpublished work indicates the existence of two main metabolites of DNC, which are acetylated amines arising from the reduction of one or both nitro groups [19]. A third minor metabolite results from the cleavage of the carbanilide group followed by reduction and acetylation of the nitro group. Liver residues consist mainly of about 80% DNC, less than 12% acetylated metabolites and some 3.3% of unidentified matter. These additional peaks are normally observed in the chromatograms of broiler liver samples (and not other species), and broilers are routinely treated with nicarbazin. The fact that two of the peaks are at elevated levels in the highly positive sample, in comparison to the low positive sample, is an indication that these peaks may be DNC metabolites. It may be seen in the chromatograms of samples A and B (Fig. 2) that there are differences in the relative sizes of the additional peaks to each other. Samples A and B were collected from poultry slaughter plants as part of an industry survey. Residues in the two samples may have resulted from different factors, or combinations of factors, such as insufficient withdrawal period, exposure to contaminated feed or recycling from litter. It is proposed that these factors might contribute to the relative differences in the peak areas.

The HPLC assay has been used to confirm positive results for samples that were screened by a Biacore™ biosensor assay. A comparison between the two methods, based on approximately 350 routine samples, showed good agreement between the methods for both negative and positive samples. An additional inter-laboratory study was carried out, where the results were compared with those by an LC–MS/MS method. These results are described in detail elsewhere [20].

3.2.3. *Recovery*

The method was validated as described in Commission Decision No. 2002/657/EC [18]. Intra- and inter-assay repeatability was determined by extracting negative liver

Table 2
Intra-and inter-assay repeatability for the determination of DNC in fortified liver tissue

DNC added (μg/kg)	Parameter	Day 1	Day 2	Day 3	Overall
100	DNC measured (μg/kg)	88.8	92.4	82.4	87.9
(0.5 × MRL)	*s*	3.11	6.49	6.90	6.89
	R.S.D. (%)	3.50	7.03	8.37	7.84
	Recovery (%)	88.8	92.4	82.4	87.9
	n	6	6	6	18
200	DNC measured (μg/kg)	176.7	176.7	168.6	174
(1.0 × MRL)	*s*	10.94	19.67	6.09	13.24
	R.S.D. (%)	6.19	11.13	3.61	7.61
	Recovery (%)	88.3	88.3	84.3	87.0
	n	6	6	6	18
300	DNC measured (μg/kg)	265.0	262.3	237.1	254.8
(1.5 × MRL)	*s*	7.28	14.24	30.10	22.50
	R.S.D (%)	2.75	5.43	12.69	8.85
	Recovery (%)	88.3	87.4	79.0	84.9
	n	6	6	6	18
	CCα (μg/kg)				228
	CCβ (μg/kg)				266

samples, fortified at levels of 100, 200 and 300 μg/kg ($n = 6$, each level) on three different occasions by a single analyst. Mean recovery for samples fortified at levels above 10 μg/kg of an analyte are required to be in the region of 80 to 110% recovery. Mean recovery from this validation study ranged between 79 and 93%, with an overall mean recovery of 87% (Table 2).

Table 3
Recovery of DNC from broiler liver samples fortified at levels of 25 and 250 μg/kg

Assay no.	Recovery (%)		Calibration curve, correlation coefficient (R^2)
	Control liver + 25 μg/kg DNC	Control liver + 250 μg/kg DNC	
1	73.2	73.0	0.999
	87.1	81.2	
2	124.5	100.8	0.999
	103.1	92.1	
3	92.8	81.7	0.999
	77.8	77.3	
4	88.4	78.8	1.000
	79.9	86.7	
5	85.2	88.3	1.000
	102.1	90.3	
6	104.4	88.2	0.999
	95.4	91.3	
7	93.5	110.8	0.999
	86.9	108.5	
8	82.2	102	1.000
	102.5	99.9	
9	86.1	102.6	1.000
	125.5	98.0	
10	91.8	85.3	0.999
	96.5	79.1	
Mean	94	91	
s	13.7	10.8	
R.S.D.	14.6	11.9	

3.2.4. Within-laboratory repeatability

The within laboratory repeatability ranged between 7.6 and 8.8% (Table 2). These values were within the acceptable limits as calculated by the Horwitz equation for a fortification level of 200 μg/kg.

3.2.5. Decision limit (CCα) and detection capability (CCβ)

The decision limit (CCα) and detection capability (CCβ) of the method were calculated using the within-laboratory repeatability results. Using this approach, the CCα and CCβ performance factors were calculated to be 228 and 266 μg/kg, respectively.

3.2.6. Additional studies

An additional validation of the method was carried out while applying it to the routine analysis of liver samples (Table 3). Mean recovery ($n = 10$) for samples fortified at levels of 25 and 250 μg/g were 94 and 91%, respectively. The relative standard deviations for the recovery were less than 15%. R^2 values for the calibration curve showed good linearity and were ≥ 0.999.

4. Conclusions

A simple and reliable method has been developed for the determination of DNC, the marker residue for nicarbazin, in poultry liver. The method allows quantitative determination of DNC residues in liver down to 12.5 μg/kg (based on the lowest standard in the calibration curve). The method has been validated according to 2002/657/EC guidelines using broiler liver samples fortified at levels of 100, 200 and 300 μg/kg. The performance factors CCα and CCβ were calculated to be 228 and 266 μg/kg, respectively. The method is being routinely applied to confirm DNC residues in samples

that have been screened positive using a Biacore™ biosensor assay. HPLC-UV (single wavelength) is suitable for confirmation of DNC in animal tissues if a second independent detection system (in this case a Biacore™ biosensor assay) is used [18]. This approach has been evaluated through application to testing of more than 350 samples in the laboratory.

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