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Short Communication

High-performance liquid chromatographic method for the determination of the anthelmintic nitroxynil in cattle muscle tissue with on-line anion-exchange clean-up

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ABSTRACT

A high-perfomance liquid chromatographic method for the determination of the anthelmintic nitroxynil has been developed. The drug was extracted from cattle muscle tissue with 1% triethylamine in acetonitrile. The extract was evaporated to dryness and taken up in 0.1 *M* ammonium acetate–acetonitrile (50:50, v/v). The extract was then injected onto a polymeric anion-exchange precolumn. After clean-up with 0.1 *M* ammonium acetate–acetonitrile (50:50, v/v) for 5 min, the precolumn was eluted with 1% aqueous trifluoroacetic acid–acetonitrile (50:50, v/v) onto a PLRP-S polymer column and chromatographed with a mobile phase of 0.01 *M* phosphate pH 7–acetonitrile (80:20, v/v). Detection was by ultraviolet at 273 nm. Average recoveries at four levels from 0.005 to 1.000 mg kg^{-1} were >88%. The limit of determination was 0.005 mg kg^{-1}.

INTRODUCTION

Nitroxynil (Fig. 1) is a 2,4,6-trisubstituted phenol anthelmintic. It is used for the treatment of liver fluke and gastro-intestinal round worm (*Haemonchus* sp.) in cattle and sheep and gapeworm in game birds [1]. Nitroxynil is active both against adult liver fluke and, in higher dosages against immature fluke. Dosage in cattle and sheep is by subcutaneous injection at 10 mg kg^{-1} body weight and in game birds by addition to drinking water. A withdrawal period of 30 days prior to slaughter has been recommended. Milk from treated animals should not be used for human consumption.

As far as is known, there are only three published procedures for the determination of nitroxynil in animal tissues and fluids. Two of these refer to the analysis of nitroxynil in milk and one in cattle tissues. Kazacos and Mok [2] carried out the analysis of nitroxynil in milk. Milk was defatted at alkaline pH with 1:1 acetone–hexane and

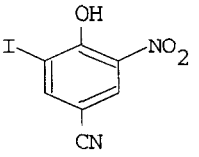

Fig. 1. Structure of nitroxynil.

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then acidified and extracted with diethyl ether. The residue was derivatized with diazomethane and nitroxynil determined by GLC with electron-capture detection. The limit of determination was 10 $\mu g\ l^{-1}$. Takeba *et al.* [3] also carried out the analysis of nitroxynil in milk. Extraction was with acetone and acetonitrile, followed by liquid–liquid extraction as clean-up. Final determination was by HPLC with electrochemical detection. A limit of detection of 0.7 $\mu g\ l^{-1}$ was claimed. Blanchflower and Kennedy [4] carried out the analysis of nitroxynil in cattle muscle, liver and kidney tissues. Extraction was with diethyl ether, followed by liquid–liquid clean-up. Final determination was by HPLC–thermospray MS. A limit of detection of 0.002 mg kg^{-1} was claimed.

This laboratory had a requirement to analyse for nitroxynil in cattle muscle tissue. The non-availability of LC–MS as a practical screening tool necessitated the development of an alternative method. This paper reports a simple and rapid procedure for the determination of nitroxynil in cattle muscle tissue based on on-line anion-exchange clean-up.

EXPERIMENTAL

Chemicals

Analytical-grade anhydrous sodium sulphate, ammonium acetate, ethylene glycol, potassium dihydrogenphosphate, 1 *M* aqueous sodium hydroxide and HPLC-grade triethylamine were obtained from BDH (Poole, UK). Trifluoroacetic acid was obtained from Aldrich (Gillingham, UK). HPLC-grade acetonitrile was obtained from Rathburn (Walkerburn, UK). Standard nitroxynil was a gift from Rhone Poulenc Rorer (Dagenham, UK).

HPLC mobile phases

Mobile phase 1a: 0.1 *M* ammonium acetate–acetonitrile (50:50, v/v); mobile phase 1b: 1% aqueous trifluoroacetic acid–acetonitrile (50:50, v/v); mobile phase 2a: 0.01 *M* phosphate pH 7–acetonitrile (80:20, v/v); mobile phase 2b: acetonitrile.

HPLC columns

The precolumn was an Anagel-TSK DEAE-5PW (diethylaminoethyl-bonded hydrophilic polymeric support), 10 μm, 10 mm × 6 mm I.D. supplied by Anachem (Luton, UK). The analytical column was a Polymer Labs. PLRP-S (styrene–divinylbenzene copolymer), 5 μm, 150 mm × 4.6 mm I.D. with PLRP-S 5-μm, 5 mm × 3 mm I.D. guard column supplied by Jones Chromatography (Hengoed, UK).

Apparatus

Homogeniser (Ultra-Turrax, Janke & Kunkel), nitrogen blowdown apparatus with hot-block (Grant), vortex mixer (Fisons), ultrasonic bath (L&R 140S), centrifuge (Jouan CR4.22) and disposable syringe filters (Millipore Millex-HV_{13}, 0.45 μm, 13 mm) were used. The HPLC system consisted of an LKB binary gradient pump system (two LKB 2150 pumps plus LKB 2152 gradient controller) (Pharmacia LKB Biotechnology, Uppsala, Sweden), a Gilson binary gradient pump system (Gilson 305 master pump, Gilson 306 slave pump, Gilson 805 manumetric module and Gilson 811C dynamic mixer) (Anachem), a Gilson 231 autosampler with 401 dilutor (Anachem), an Anachem Universal electronic column-switching unit (Anachem), a Spectra-Physics Spectrasystem UV1000 UV detector and a Spectra-Physics SP4400 Chromjet integrator (Spectra-Physics, Hemel Hempstead, UK).

Extraction

Thinly sliced cattle muscle (2 g) and 1% triethylamine in acetonitrile (10 ml) were homogenised for 1 min, anhydrous sodium sulphate (5 g) was added, followed by rehomogenisation for 1 min and centrifugation for 5 min at 4200 *g*. The supernatant was decanted into a test-tube, ethylene glycol (10 μl) added and blown down under nitrogen at 45–55°C. While the first extract was being blown down, the solid residue was rehomogenised for 1 min with 1% triethylamine in acetonitrile (10 ml), centrifuged for 5 min at 4200 *g* and decanted into the same test-tube. The combined supernatants were then blown down to dryness. The residue was taken up in mobile phase 1a (2

ml) by vortex-mixing for 15 s and ultrasonication for 3 min, centrifuged for 5 min at 1860 *g* and filtered (Millipore Millex-HV_{13}, 0.45 μm, 13 mm).

High-performance liquid chromatography

Extracts in mobile phase 1a (1 ml) were injected (Gilson 231/401 fitted with a 5-ml loop) onto a weak anion-exchange precolumn (Anagel-TSK DEAE-5PW guard). Mobile phase was mobile phase 1a. Mobile phase was maintained at 1.0 ml min^{-1} (LKB binary gradient pump system) for 5 min. At this point, the flow-rate was reduced to 0.5 ml min^{-1} and the mobile phase switched to mobile phase 1b. After 1 min, the eluate from the precolumn was switched onto the analytical column using an Anachem Universal electronic column-switching unit. The analytical column consisted of Polymer Labs. PLPR-S 5-μm, 150 mm × 4.6 mm I.D. column plus PLRP-S guard column installed across the column-switching unit. After 2 min the eluate from the precolumn was switched back to waste and nitroxynil was eluted from the analytical column with mobile phase 2a. This mobile phase was maintained at 1.0 ml min^{-1} (Gilson binary gradient pump system). Detection was by UV at 273 nm (Spectra-Physics Spectrasystem UV1000). Data capture and peak-height calculation was undertaken using a Spectra-Physics SP4400 Chromjet. Quantitation was by reference to replicate injections (1 ml) of standard nitroxynil. After elution the analytical column was cleaned with mobile phase 2a–mobile phase 2b (60:40) for 5 min. The analytical column was then reequilibrated with mobile phase 2a while the next sample was undergoing clean-up on the precolumn. Loading and elution of the precolumn and initiation of the precolumn and analytical column mobile phase switches were all automated under the control of the autosampler. In between batches, the precolumn was cleaned by injecting 1.0 *M* sodium hydroxide (1 ml) bracketed on each side by water (0.5 ml). The configuration of the system is shown in Fig. 2.

Protocol

Samples were analysed in batches of five to seven spikes and one blank. Time of preparation for a batch of eight samples was approximately 2.5 h.

RESULTS AND DISCUSSION

The chromatography of nitroxynil on reversed-phase columns was first established. Polymer-based columns were investigated since it was anticipated that mobile phases used to elute on-line anion-exchange clean-up precolumns would involve pH values beyond the normal range of silica-based columns. Using a Polymer Labs. PLRP-S (styrene–divinylbenzene copolymer) 5-μm, 150 mm × 4.6 mm I.D. column, a mobile phase of 0.01 *M* phosphate pH 7–acetonitrile (80:20 or 70:30, v/v) was found to be suitable. Evaluation of the UV (diode array) spectra indicated a λ_{max} of 272 nm. This was used for all subsequent determinations. It was found that lowering the pH of the buffer component of the mobile phase to 2 resulted in considerably increased retention.

The chromatographic behaviour of nitroxynil on anion-exchange precolumns was also investigated. A polymer-based strong anion-exchange precolumn (Hamilton PRP-X100 guard cartridge

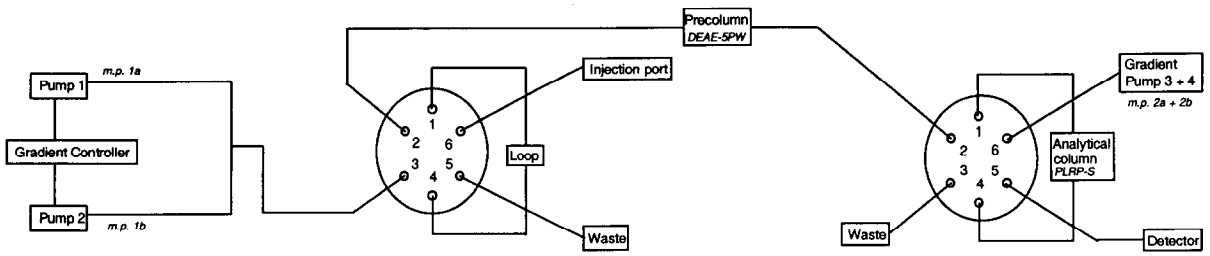

Fig. 2. Configuration of automated system for on-line anion-exchange clean-up.

column) was used for the initial work. It was found that nitroxynil was retained on the column at neutral or basic pH, but could be eluted using a mobile phase containing 1% trifluoroacetic acid (TFA) in the aqueous component. A mobile phase composition of 1% aqueous TFA–acetonitrile (50:50, v/v) was found to be suitable. A number of other acids were also investigated, but were not found to improve on results obtained using TFA. When tissue extracts were being processed through this column, a problem was encountered with reducing peak heights of standards. It was thought that this might be due to blocking of active sites on the column by tissue components. Attempts to remove this problem by cleaning of the precolumn failed. An alternative precolumn, Anagel-TSK DEAE-5PW (diethylaminoethyl groups bonded onto a hydrophilic polymeric phase), was used. Since this column is a weak anion exchanger, it has the advantage that it can be cleaned between batches by flushing with 1 ml of 1 *M* aqueous sodium hydroxide. To avoid precipitation of the sodium hydroxide, injection onto the precolumn needs to be preceded and followed by a small volume of water. A mobile phase of 0.1 *M* ammonium acetate–acetonitrile (50:50, v/v) was found to give the best results for loading of the precolumn. The column was eluted with 1% aqueous TFA–acetonitrile (50:50, v/v).

An automated system by which nitroxynil could be loaded onto an anion-exchange precolumn and then eluted and chromatographed on a PLRP-S analytical column was set up (Fig. 2). The mobile phase for the analytical column was 0.01 *M* phosphate pH 7–acetonitrile (80:20, v/v). The effects of the length of the column-switch and of the delay time prior to column-switching were investigated. Using the Hamilton PRP-X100 as precolumn, a delay time of 0.5 min and an elution time of 1.5 min were required. Using the Anagel-TSK DEAE-5PW precolumn, a delay time of 1.0 min and a column-switching time of 2.0 min was found to be satisfactory. Standard nitroxynil could be held on the precolumn for at least 15 min without breakthrough occurring. The standard curve for nitroxynil was linear (correlation coefficient $r = 0.9998$) for 2–1000 ng on column (equivalent to 0.002–1.000 mg kg^{-1}).

Initially, acetonitrile was used to extract nitroxynil from cattle muscle. The acetonitrile extract was evaporated to dryness and taken up in 2 ml of mobile phase 1a. Recovery could be increased and consistency improved by using 1% triethylamine in acetonitrile as the extracting solvent. Recovery was also improved by carrying out two extractions and by the addition of a keeper (ethylene glycol) prior to evaporation. Centrifugation and filtration were necessary after dissolution of the residue in mobile phase 1a to remove some insoluble material. A clean-up time of 15 min on the precolumn was used initially. However, this could be reduced to 5 min with no increase in interferences.

The run times used gave rise to interferences from long-running components from previous runs. These were not eliminated by increasing the clean-up time to 15 min. The possibility of attempting to eliminate these interferences by increasing the time between runs was rejected, as the time required would have been excessively long. The interferences were removed by flushing the analytical column with mobile phase 2a–acetonitrile (60:40, v/v) for 5 min at the end of the chromatographic run. The column was then re-equilibrated with mobile phase 2a while the next sample was undergoing clean-up on the precolumn. A schematic representation of the method is shown in Fig. 3.

The procedure was validated at four levels: 0.005, 0.010, 0.100 and 1.000 mg kg^{-1} (Table I). Muscle tissues (2 g) were spiked with 100 μl of standard in acetonitrile at an appropriate concentration (0.1, 0.2, 2 and 20 $\mu g\ ml^{-1}$ = 0.005, 0.010, 0.100 and 1.000 mg kg^{-1}, respectively). Single batches were performed at 0.005, 0.100 and 1.00 mg kg^{-1} levels and three batches at the 0.010 mg kg^{-1} level. Recovery at the 0.005 mg kg^{-1} level was 88.7 ± 6.2% (coefficient of variation, C.V. 7.0%) ($n = 6$). Overall recovery at the 0.010 mg kg^{-1} level was 90.6 ± 5.8% (C.V. 6.4%) ($n = 18$). Recovery at the 0.100 mg kg^{-1} level was 93.6 ± 1.6% (C.V. 1.7%) ($n = 7$). Recovery at the 1.000 mg kg^{-1} level was 90.7% ±

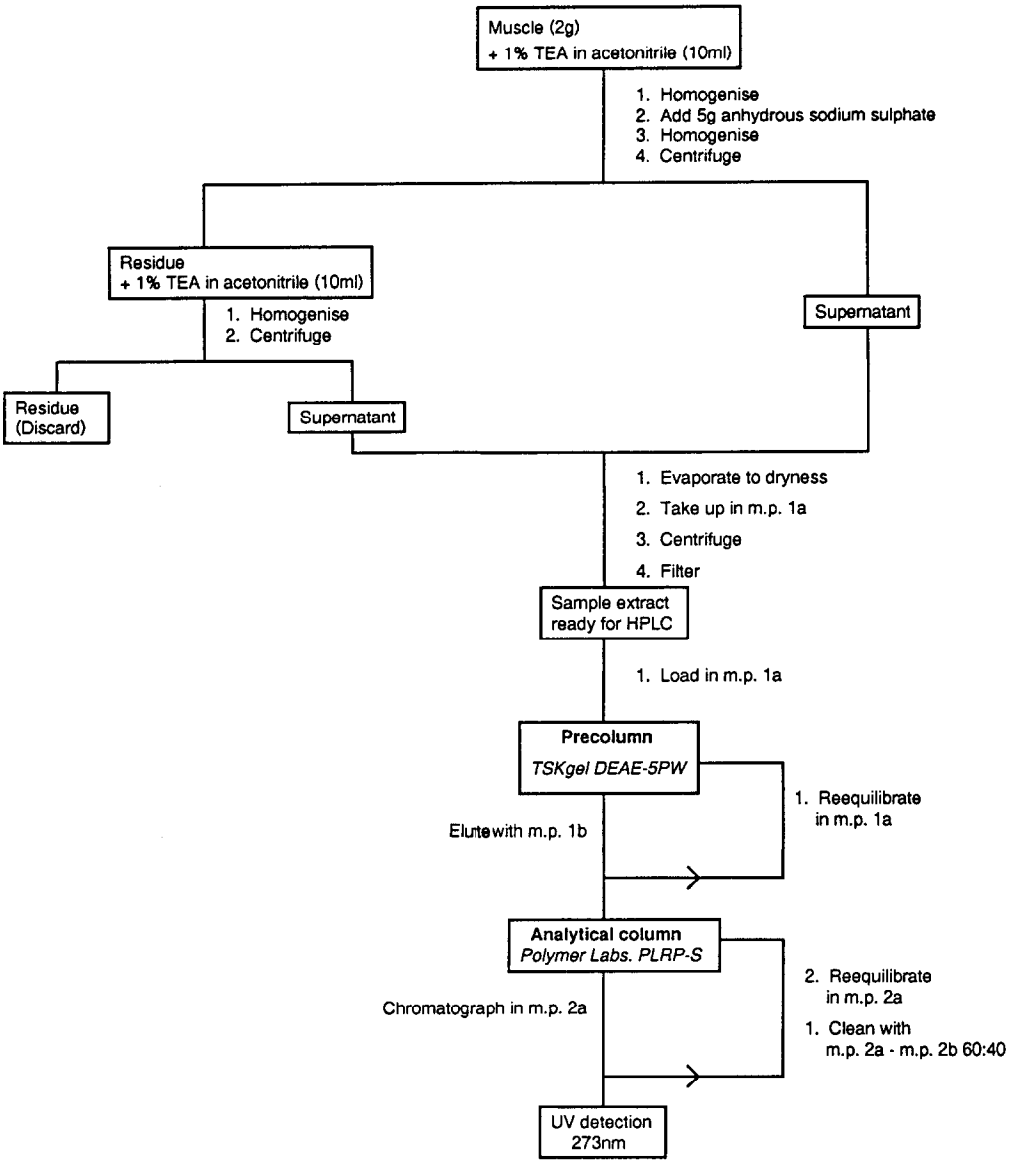

Fig. 3. Schematic representation of the method.

TABLE I

RECOVERIES OF NITROXYNIL FROM CATTLE MUSCLE TISSUE

Batch	Recovery (mean ± S.D.) (%)	C.V. (%)	*n*
0.005 mg kg^{-1}			
1	88.7 ± 6.2	7.0	6
0.0010 mg kg^{-1}			
1	89.9 ± 6.8	7.5	5
2	89.3 ± 4.2	4.7	7
3	92.6 ± 5.9	6.4	6
Overall	90.6 ± 5.8	6.4	18
0.100 mg kg^{-1}			
1	93.6 ± 1.6	1.7	7
1.000 mg kg^{-1}			
1	90.7 ± 1.5	1.6	7

1.5% (C.V. 1.6%) (n = 7). Typical chromatograms of standard nitroxynil equivalent to 0.100 mg kg^{-1}, blank cattle muscle extract and blank cattle muscle extract spiked at 0.100 mg kg^{-1} are shown in Fig. 4.

CONCLUSIONS

A method has been developed for the determination of nitroxynil in cattle muscle tissue by HPLC with on-line anion-exchange chromatography as sample clean-up. The procedure has been validated for the range 0.005–1.000 mg kg^{-1}. The procedure is rapid, a batch of eight samples taking approximately 2.5 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 preparation time.

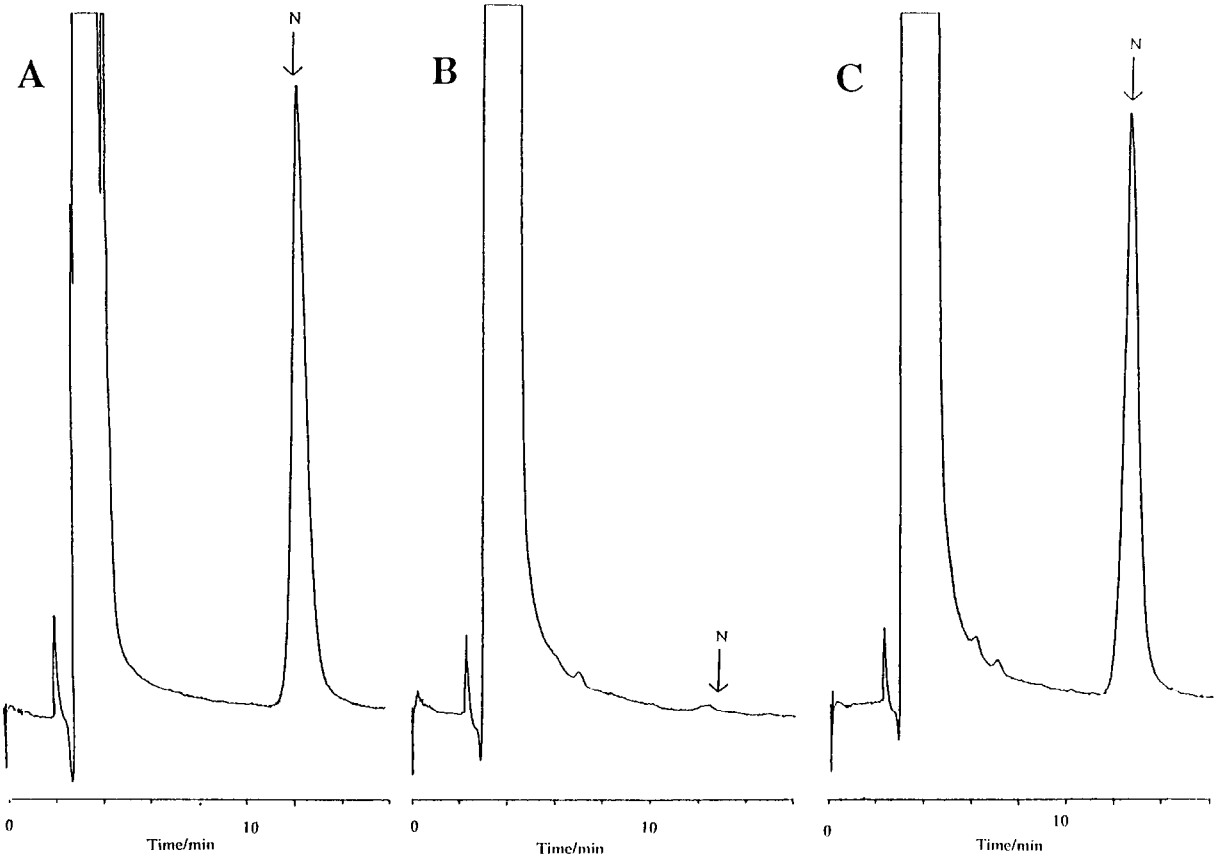

Fig. 4. Chromatograms of (A) standard nitroxynil equivalent to 0.100 mg kg^{-1}, (B) blank cattle muscle extract and (C) blank cattle muscle spiked at 0.100 mg kg^{-1} prior to extraction (0.01 a.u.f.s.) (N = position of nitroxynil).

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