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Determination of nitroxynil in cow milk by reversed-phase high-performance liquid chromatography with dual-electrode coulometric detection

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ABSTRACT

A sensitive and specific method is described for the determination of nitroxynil (fasciolicide) residues in cow milk. The milk samples were extracted with acetone and acetonitrile, following clean-up using a simple liquid–liquid extraction step. Nitroxynil was separated from the matrix peaks by reversed-phase high-performance liquid chromatography and detected using dual-electrode coulometric detection. The mobile phase was a mixture (20:80, v/v) of acetonitrile and 0.05 *M* potassium dihydrogenphosphate with the pH adjusted to 4.0. The flow-rate was 1 ml/min at 40°C. The applied potentials of detectors 1 and 2 were maintained at −0.7 and +0.2 V, respectively. Average recoveries ($n = 5$) of nitroxynil from milk samples spiked at concentrations of 0.01 and 0.1 μg/ml were found to be 92.0% and 97.0% with coefficients of variation of 6.2% and 2.2%, respectively. The detection limit of nitroxynil in milk was 0.7 ng/ml. During the analysis of 30 raw cow milk and 140 market milk samples, nitroxynil was detected at a level of 4 ng/ml in one raw cow milk sample.

INTRODUCTION

Nitroxynil (4-hydroxy-3-iodo-5-nitrobenzonitrile) is an anthelmintic that controls fascioliasis caused by *Fasciola hepatica* in cattle. When compared with other fasciolicides, this compound shows high activity against both adult and immature liver flukes [1–4]. Infected cattle are injected subcutaneously with 10 mg/kg nitroxynil in the form of an aqueous solution of its N-alkylglucamine salt.

However, this compound is known to be excreted as a long-lasting residue in milk [5–11]. In Japan, milk for human consumption must not be taken from cows which have been fed or injected with medicine that has an effect on milk during the period when the medicine remains in the milk [12]. This has led to the need to developing a simple, sensitive and specific method of monitoring residual levels of nitroxynil in cow milk.

Most of the methods reported for determining residual levels of nitroxynil in milk involve polarography [5,10,13], gas chromatography [6,8,9,14] and high-performance liquid chromatography (HPLC) [11]. In this paper, a simple, sensitive and specific method for the determination of nitroxynil in milk using reversed-phase HPLC with electrochemical (dual-electrode coulometric) detection is described.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Model LC-6A pump (Shimadzu, Kyoto, Japan) with a 20-μl sam-

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ple loop injector (Rheodyne, Cotati, CA, USA), a series of Model 870-UV UV monitors (Jasco, Tokyo, Japan), a Model Coulochem 5100A coulometric detector comprising a solid-state analytical cell (Model 5010), containing dual coulometric working electrodes made from porous graphite, and a guard cell (Model 5020) (ESA International, Bedford, USA). The applied potentials of the guard cell, detector 1 and detector 2 of the coulometric detector were maintained at −0.7, −0.7 and +0.2 V, respectively, at ambient temperature. The guard cell was directly connected to detector 1. The sensitivity of detector 2 was maintained with gain 10 × 40 and response time 10 s. The UV monitor was set at a wavelength of 270 nm with 0.08 a.u.f.s. LiChroCART LiChrospher 100 RP-18 (5 μm particle size, 250 × 4 mm I.D.) was used as an analytical column. The mobile phase consisted of acetonitrile–0.05 *M* potassium dihydrogenphosphate (20:80, v/v), adjusted to pH 4.0 with 10% (w/v) phosphoric acid. The flow-rate of the recycled mobile phase was 1.0 ml/min at 40°C with bubbling by nitrogen gas throughout the HPLC analysis.

Standard and chemicals

Nitroxynil was obtained from Rhone-Poulenc-Rorer (Dagenham, UK) through Kyowa Hakko Kôgyo (Tokyo, Japan). The structure of the compound is shown in Fig. 1. Nitroxynil was dissolved in acetonitrile and the working standard was diluted with the mobile phase. All other reagents and organic solvents used were of special and HPLC grade.

Sample preparation

A 10-ml sample of cow milk was mixed with 10 ml of acetonitrile and 10 ml of acetone in a 50-ml glass centrifuge tube. The mixture was shaken well and centrifuged at 2000 *g* for 10 min. The supernatant was transferred into a 100-ml separation funnel and extracted with 20 ml of dichloromethane. The organic phase was evaporated to dryness under reduced pressure. The wet residue was dissolved in 5 ml of 1% (w/v) sodium hydrogencarbonate and acidified by the addition of 1 ml of concentrated hydrochloric acid, to a final pH of 2–3. The acidified solution was transferred into a 100-ml separation funnel and extracted with 10 ml of dichloromethane. The organic phase was washed with 10 ml of distilled water. The extract was evaporated to dryness. A 1-ml aliquot of the mobile phase was added to the residue. This solution was filtered through a 0.2-μm membrane filter, and 5 μl of the sample were injected into the HPLC system.

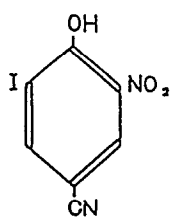

Fig. 1. Structure of nitroxynil (4-hydroxy-3-iodo-5-nitrobenzonitrile).

RESULTS AND DISCUSSION

To develop a sensitive and specific method for determining nitroxynil, electrochemical detection was evaluated because nitroxynil has a phenol group in its structure, as shown in Fig. 1. Although nitroxynil was oxidized above a potential of +0.8 V, this potential gave almost the same sensitivity as UV detection at 270 nm. In order to improve the sensitivity, the electrochemical behaviour of the nitro group at the reductive and oxidative area was investigated.

The effects of the applied potentials on the electrochemical response of nitroxynil at different pH values of the mobile phase are shown in Fig. 2, and the relationship between the capacity factor (k') and the pH is shown in Fig. 3. The applied potential of the guard cell and detector 1 was fixed at −0.7 V, and that of detector 2 was varied from +0.7 V to lower voltages.

The higher the applied potential of detector 2 and the lower the pH of the mobile phase, the higher the electrochemical response obtained. Nitroxynil in a mobile phase of pH 3.0 showed the highest electrochemical response, while the nitroxynil capacity factor (k') was 15.8 (retention time *ca.* 24 min), unsuitable for HPLC analysis. Therefore, from this current–voltage curve, the mobile phase was adjusted to pH 4.0 ($k' = 5.7$). The applied potential of detector 2 was adjusted to +0.2 V, resulting in the most stable baseline.

Secondly, the effect of the reductive applied potential of the guard cell and detector 1 on the elec-

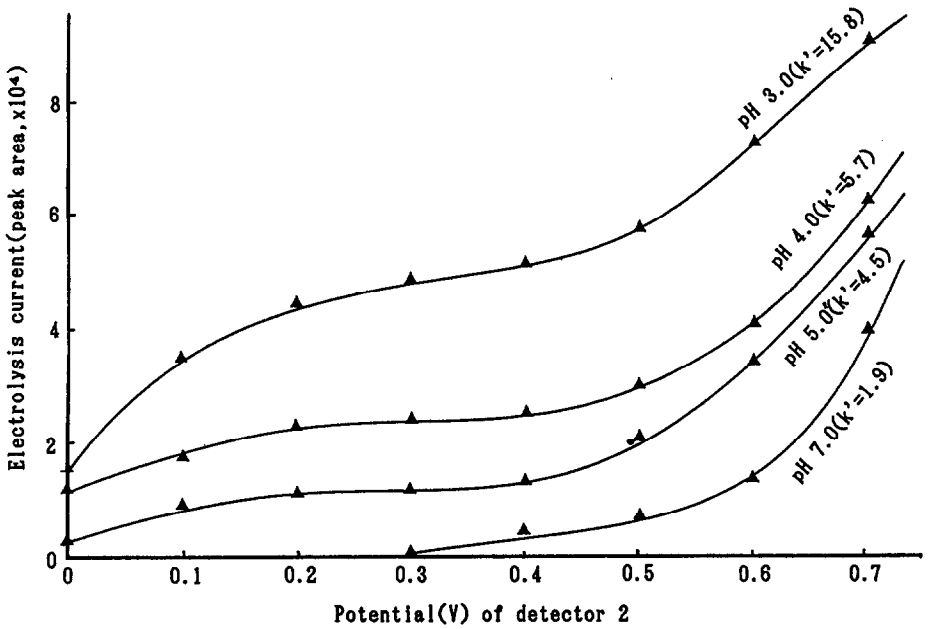

Fig. 2. Effects of the potential of detector 2 on the electrolysis current determining nitroxynil at different pH values (3.0, 4.0, 5.0, 7.0) of the mobile phase. Conditions of coulometric detection: guard cell, −0.7 V, detector 1, −0.7 V; detector 2, +0.7–0.0 V; gain, 10 × 40; response time, 10 s.

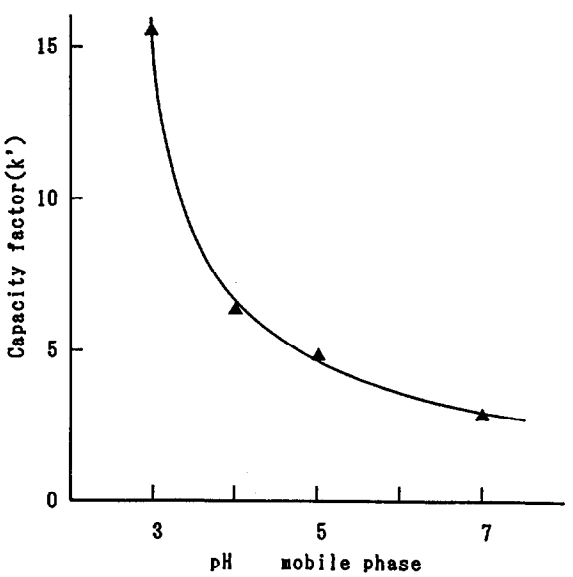

Fig. 3. Effects of the pH of the mobile phase on the capacity factor (k') of nitroxynil. Conditions of coulometric detection: guard cell, −0.7 V; detector 1, −0.7 V, detector 2, +0.7–0.0 V; gain, 10 × 40; response time, 10 s.

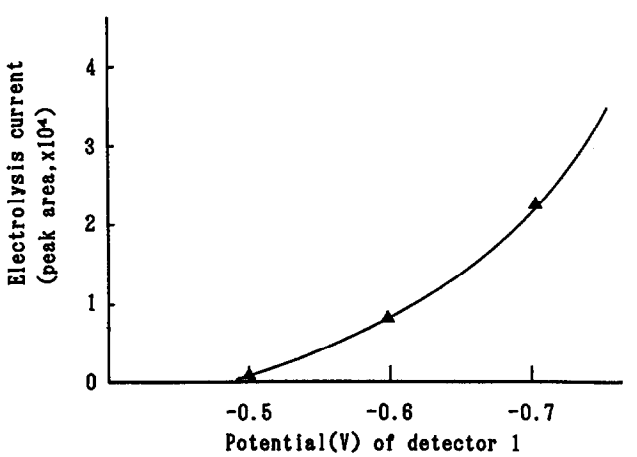

Fig. 4. Current–voltage curve of nitroxynil. Conditions of coulometric detection: guard cell, −0.7–0.5 V; detector 1, −0.7–0.5 V; detector 2, +0.2 V; gain, 10 × 40; response time, 10 s.

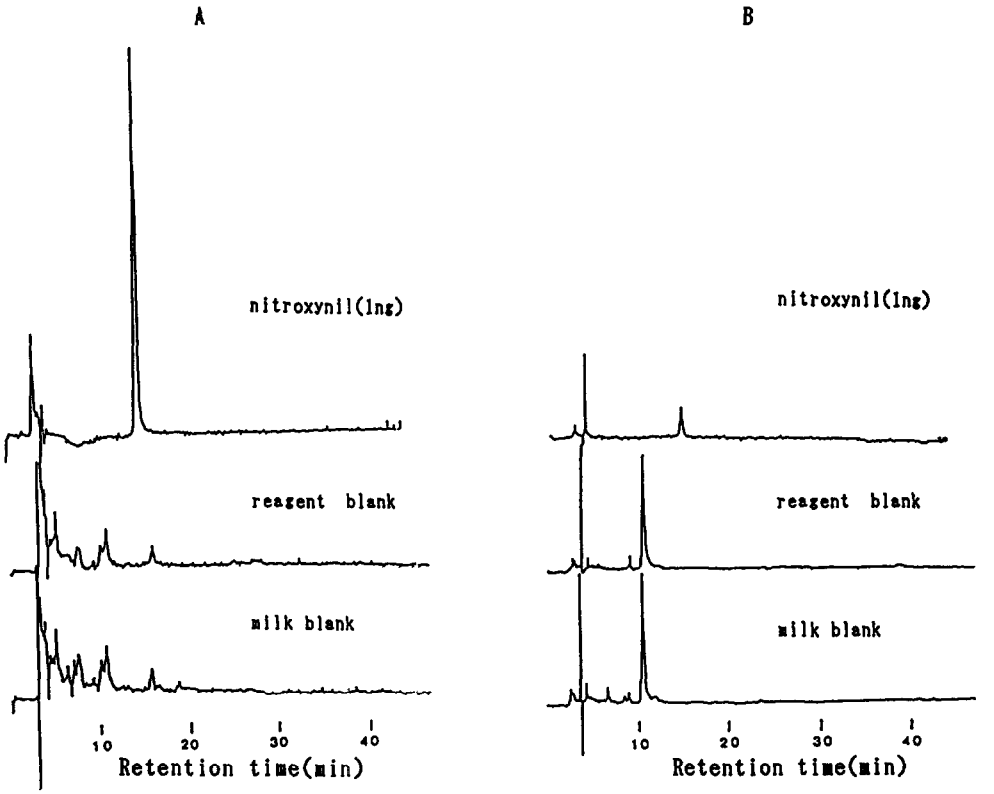

Fig. 5. High-performance liquid chromatograms of nitroxynil with electrochemical (A) and ultraviolet spectrophotometric (B) detection. HPLC conditions: column, LiChrospher 100 RP-18 (5 μm, 250 × 4 mm I.D.); mobile phase, acetonitrile–0.05 *M* potassium dihydrogenphosphate (20:80, v/v), pH 4.0; flow-rate, 1 ml/min at 40°C. Coulometric detection: guard cell, −0.7 V; detector 1, −0.7 V; detector 2, +0.2 V; gain, 10 × 40; response time, 10 s. UV: 270 nm; 0.08 a.u.f.s.

trochemical response of nitroxynil was evaluated. The current–voltage curve is shown in Fig. 4. The applied potential of detector 2 was fixed at +0.2 V and that of the guard cell and detector 1 was varied from −0.7 V to lower voltages. As is evident from Fig. 4, the applied potential of the guard cell and detector 1 at −0.7 V resulted in the highest electrochemical reduction. The applied potential of the guard cell and detector 1 was adjusted to −0.7 V.

Fig. 5 shows typical chromatograms of nitroxynil, a cow milk blank and a reagent blank. There are no peaks interfering with the matrix peaks. The detection limit of electrochemical determination was 15-fold higher than the UV detection limits.

The calibration curve of nitroxynil was linear in the range 1.0–10.0 ng. Average recoveries (n = 5) of nitroxynil from milk samples spiked at concentrations of 0.01 and 0.1 μg/ml were found to be 92.0% and 97.0% with coefficients of variation of 6.2% and 2.2%, respectively.

The recovery and detection limits with a concentration of nitroxynil as low as 0.7 ng/ml at a signal-to-noise ratio of 3:1 were satisfactory for the monitoring of residual nitroxynil in milk. This resulted in a 3- to 140-fold increase in the detection limit compared with other reported methods [5,10–12].

When the method was applied to 30 raw cow milk and 140 market milk samples, nitroxynil was detected at a level of 4 ng/ml in one sample, as shown in Fig. 6.

In conclusion, the proposed method is sensitive and specific and is applicable to the routine analysis of nitroxynil in milk.

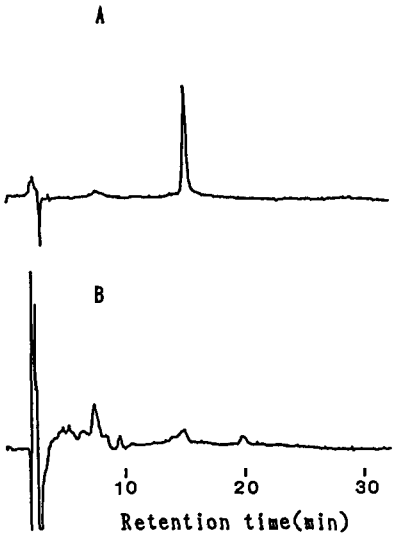

Fig. 6. High-performance liquid chromatograms. (A) Nitroxynil standard; (B) nitroxynil in raw milk sample. HPLC conditions: column, LiChrospher 100 RP-18 (5 μm, 250 × 4 mm I.D.); mobile phase, acetonitrile–0.05 *M* potassium dihydrogenphosphate (20:80, v/v), pH 4.0; flow-rate, 1 ml/min at 40°C. Coulometric detector: guard cell, −0.7 V; detector 1, −0.7 V; detector 2, +0.7 V; gain, 10 × 40; response time, 10 s.

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