DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF NITROFURANS IN WATER

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Abstract

A liquid chromatographic method coupled with UV-Vis and photodiode–array detection for the determination of nitrofuran residues: nitrofurazone, nitrofurantoin, furazolidone, and furaltadone in water was developed and validated. Confirmation of the identity of nitrofurans was performed by liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization interface. The nitrofurans were isolated from water by solid-phase extraction and complete separation was obtained using a C18 analytical column and the mobile phase consisting of acetonitrile and 0.01 M sodium acetate buffer pH=6.0 (250:750, v/v), with the detection at \( \lambda = 376 \) nm. Average recoveries for the compounds ranged from 63% to 90%. The results of the validation show that the method can be successfully used for the official control of nitrofurans in water.

Key words: nitrofurans, water, food analysis, HPLC–UV, HPLC–PAD.

Furazolidone (FZ), furaltadone (FD), nitrofurazone (NZ), and nitrofurantoin (NF) are veterinary drugs that belong to the family of nitrofuran antibacterial compounds, with a molecular structure characterised by the presence of a 5-nitro group. This group has a broad antibacterial and antiparasitic activity and for this reason, nitrofurans are widely used as veterinary drugs, mainly for the treatment of gastrointestinal infections in cattle, pigs, and poultry (9).

The use of nitrofurans, including furazolidone, in veterinary practice, has become questionable in the European Union (EU). In 1993 (1995 for furaltadone), the use of nitrofurans in food producing animals in the EU was banned by listing them in Annex IV of the Council Regulation 2377/90/EEC (2, 4, 5), because of their potentially mutagenic and carcinogenic effects on human health.

Still, the nitrofurans can be illegally applied to food producing animals with feed or water. In animal body, the nitrofurans are quickly metabolised and are not detectable in a few hours after their administration (7). Nitrofuran metabolites however, remain for months bound to tissue proteins and therefore they were established as nitrofuran marker residues (20). For this reason, the nitrofuran metabolites are determined in animal tissues, whereas farm water and animal feeds may be checked for the presence of the nitrofuran parent compounds.

Several methods have been reported for the detection of nitrofuran compounds in feeds and biological matrices. The analysis is performed by thin layer chromatography (TLC) (18), gas chromatography (GC) and liquid chromatography (LC) with electrochemical (8) and UV-Vis detection (11, 15, 17, 19). The last of the mentioned techniques is used most frequently in nitrofuran determination. Some authors used LC–MS to confirm the presence of nitrofurans in the samples (3, 6, 12, 14).

The main difficulty in the determination of nitrofurans, is their instability observed by many authors (13, 17). The compounds are extremely sensitive to daylight; therefore, the methods for their determination should be relatively fast and simple in order to avoid degradation of analytes during the analytical process.

The aim of this research was to develop a fast and simple method for screening (HPLC-UV Vis) and post-screening (HPLC-PAD) of nitrofurans in farm water. The method was validated according to the international criteria (1). We have also investigated the possibility of using LC–MS/MS to confirm the presence of NZ, NF, FZ, and FD residues in water samples.

Material and Methods

Reagents. NZ, NF, and FZ standards were obtained from Sigma–Aldrich Chemical Company (Germany). FD standard was from Riedel–de Haën (Germany). Acetonitrile, methanol, ammonium acetate, 99% acetic acid, and Bakerbond SPE cartridges
(octadecyl C18 500 mg/6 ml, polymeric SDB 200 mg/3 ml, C18 Speedisk 100 mg/3 ml) were from J. T. Baker (Germany). Sodium acetate was from P. O. Ch. Gliwice (Poland). Water was purified using Milli-Q system (Millipore, France).

**Standard solutions.** Stock standard solutions (1 mg/mL), prepared by weighing 10.0±0.1 mg of standard substances and dissolving in 10 ml of acetonitrile, were stable for 6 months when stored at the temperature below -18°C in amber glass. Working standard solutions (100 µg/mL) and mixed standard solutions (10 µg/mL of each compound) prepared by diluting suitable aliquot of stock standard were stable for 1 month when stored at 2-8°C in amber glass. Working standard solutions in mobile phase were prepared on the day of analysis.

**Extraction and clean up.** A 100 ml volume of water was centrifuged at 3 500 g for 10 min. A fifty millilitre sample was cleaned up by the SPE procedure. In the method, optimalisation cartridges with following sorbents were used: C18 500 mg/6 ml, polymeric SDB 200 mg/3 ml, and C18 Speedisk 100 mg/3 ml. The optimal results were obtained using SPE C18 500 mg/3 ml, and the cartridges were conditioned with 3 ml of methanol and 3 ml of water. Nitrofurans were eluted with 5 ml of methanol and evaporated to dryness at 45°C under nitrogen. Residues were re-dissolved in mobile phase and destined to chromatographic analysis.

**LC–UV analysis.** The instrumental analysis was performed using Varian Prostar HPLC system, equipped with quaternary pump, autosampler, column oven, and UV-Vis (λ=376 nm) or PAD detector (λ=250-400 nm), controlled by Galaxie Workstation software. The separation was performed on Luna (Phenomenex) C18 column (5 µm, 250 mm x 4.6 mm) with mobile phase consisting of acetonitrile and 0.01M sodium acetate, pH=6.0 (250:750, v/v) at 1.0 mL/min flow rate.

**LC–MS/MS analysis.** Confirmation was performed using Agilent 1100 HPLC system coupled with ThermoFinnigan LCQDuo mass spectrometer with APCI interface and Xcalibur workstation. The chromatographic conditions were: Luna Phenylhexyl column (3 µm, 150 mm x 2.1 mm), acetonitrile – ammonium acetate, pH=6.0 (300:700, v/v) at 0.15 mL/min flow rate.

**Validation.** Standard calibration curve was prepared by injection of mixed standard solutions on five levels over the range 0.05–1.00 µg/mL and the working range was established. Water samples were spiked with NF, NZ, FZ, and FD at levels of 0.5, 1.0, and 1.5 µg/L in order to evaluate matrix-matched calibration curves.

Precision (repeatability and reproducibility) of the assay was determined as the coefficient of variation calculated for three concentration levels. Detection limits of the method were calculated on the base of signal to noise ratio (S/N = 3 for limit of detection - LOD and S/N = 10 for limit of quantitation - LOQ). Recoveries were calculated by comparing peak areas of fortified samples to the corresponding standards. Decision limit (CCα) and detection limit (CCβ) were evaluated according to ISO (10).

**Results**

Typical HPLC UV-Vis chromatograms of control water sample and water sample spiked with four nitrofurans are shown in Fig. 1. The analytes were well separated in 15 min as sharp and symmetrical peaks. Retention times were 6.12, 7.24, 10.27, and 11.71 for NZ, NF, FZ, and FD, respectively. Blank control samples did not elute peaks at the retention times of the nitrofurans.

Fig. 2 shows the UV-Vis spectra of the nitrofuran standards and spiked samples. The maxima of absorbance of fortified samples were congruent with the ones of nitrofuran standards spectra.

Confirmation of the identity of the nitrofurans tested was performed by the LC–MS/MS using an atmospheric pressure ionisation source (APCI). The analysis was performed in selected reaction monitoring (SRM) mode with the following transitions monitored: m/z 197 to 124, m/z 237 to 152, m/z 243 to 226, m/z 325 to 281, 252 for NZ, NF, FZ, and FD, respectively. The SRM chromatograms of blank and spiked samples are presented in Fig. 3.

In the extraction and clean up step, the use of C18 cartridges and elution of the analytes with a methanol enabled the best recovery. The recoveries for the tested cartridges varied in the range of 24%–60%, 36%–65%, and 60%–90% for SDB, Speedisk, and C18, respectively. The statistical evaluation of the validation data obtained for nitrofuran determinations in spiked water samples is presented in Table 1.
Fig. 2. Typical UV spectra of nitrofuran standard and spike sample of NF, NZ, FZ, FD monitored from $\lambda=250$ nm to $\lambda=400$ nm.

Fig. 3. Typical SRM chromatograms of nitrofurans: a) Blank water sample, b) Water sample spiked with nitrofurazone, nitrofurantoin, furazolidone, and furaltadone at the level of 1.0 $\mu$g/L.

Table 1

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Nitrofurazone</th>
<th>Nitrofurantoin</th>
<th>Furazolidone</th>
<th>Furaltadone</th>
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<tbody>
<tr>
<td>LOD, $\mu$g/L</td>
<td>0.10</td>
<td>0.09</td>
<td>0.15</td>
<td>0.14</td>
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<tr>
<td>LOQ, $\mu$g/L</td>
<td>0.21</td>
<td>0.18</td>
<td>0.30</td>
<td>0.27</td>
</tr>
<tr>
<td>Calibration curve</td>
<td>$y = 20.18x + 55.06$ $R^2 = 0.9942$</td>
<td>$y = 19.18x + 72.79$ $R^2 = 0.9845$</td>
<td>$y = 14.38x + 65.36$ $R^2 = 0.9982$</td>
<td>$y = 13.12x + 38.04$ $R^2 = 0.9960$</td>
</tr>
<tr>
<td>Linearity range, $\mu$g/mL</td>
<td>0.05-1.0</td>
<td>0.05-1.0</td>
<td>0.05-1.0</td>
<td>0.05-1.0</td>
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<tr>
<td>Repeatability, CV, % (n=6)</td>
<td>0.5 $\mu$g/L</td>
<td>4</td>
<td>6</td>
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<td></td>
<td>1.0 $\mu$g/L</td>
<td>5</td>
<td>7</td>
<td>5</td>
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<td></td>
<td>1.5 $\mu$g/L</td>
<td>2</td>
<td>5</td>
<td>2</td>
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<tr>
<td>Repeatability, CV, % (n=18)</td>
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<td>7</td>
<td>18</td>
<td>7</td>
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<td></td>
<td>1.0 $\mu$g/L</td>
<td>9</td>
<td>15</td>
<td>9</td>
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<tr>
<td></td>
<td>1.5 $\mu$g/L</td>
<td>14</td>
<td>12</td>
<td>6</td>
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<tr>
<td>Reproducibility, CV, % (n=18)</td>
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<td>81</td>
<td>63</td>
<td>75</td>
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<td></td>
<td>1.0 $\mu$g/L</td>
<td>86</td>
<td>65</td>
<td>79</td>
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<tr>
<td></td>
<td>1.5 $\mu$g/L</td>
<td>81</td>
<td>79</td>
<td>86</td>
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<tr>
<td>Recovery, % (n=18)</td>
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<td></td>
<td>1.0 $\mu$g/L</td>
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<td>0.88</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>1.5 $\mu$g/L</td>
<td>0.66</td>
<td>0.88</td>
<td>0.66</td>
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</table>
Discussion

Animal feeds and drinking water are very convenient matrices for residue control as they can be taken at the farm level. Many procedures of liquid chromatography with ultraviolet detection (LC-UV) available for nitrofurans control were usually applied to the determination of a single nitrofuran compound (8, 12), but they were not suitable for multi-component screening and confirmation procedures. Most of them were developed for animal feeds and involved extraction with organic solvents (6, 11, 19). Only very few methods for nitrofurans determination in farm water were published (21).

In the present study, the analytical parameters of four nitrofurans were optimised using reversed-phase liquid chromatography with UV detection. The best separation was obtained by isocratic mobile phase, which consisted of a 250:750 (v/v) acetonitrile/sodium acetate buffer (pH=6.0) at a flow rate of 1 mL/min. The detection limit in the water samples ranged between 0.09 and 0.15 µg/L, and it was lower in comparison to 0.21-0.31 µg/L reported in literature (20). The time was short (15 min) and the resolution was good for analytes of interest.

In conclusion, this study proved that the applied analytical method proved to be sensitive, selective, and rapid for the determination of NZ, NF, FZ, and FD in farm water. The method was validated according to European Commission criteria (1) and could be applied to the control of the nitrofurans in farm water.

References


