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Short communication

Determination of furazolidone in animal feeds using liquid chromatography with UV and thermospray mass spectrometric detection

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Abstract

A method is presented for the detection of the nitrofuran antibacterial, furazolidone in animal feeds. Following solvent extraction of feed samples, the extracts were purified on an alumina column. The column eluents were separated by HPLC using a reversed phase column and detected either by UV monitoring at 365 nm, or by thermospray mass spectrometry, monitoring the ion at m/z 243. The LC–UV procedure was used to quantify furazolidone at medicated levels (200 mg/kg) and at contamination levels of 5 and 20 mg/kg. The limit of detection was 1 mg/kg. Overall mean recoveries from fortified samples were, 93.4, 98.2 and 98.0% at concentrations of 5, 20 and 200 mg/kg, respectively. The mass spectrometric procedure was used to analyse sample extracts at concentrations <5 mg/kg. The overall recovery from feed samples fortified at 1 mg/kg was 93.8%. The assay is therefore capable of the determination of furazolidone in animal feeds at both high medicated concentrations and at low contamination levels.

Keywords: Furazolidone

1. Introduction

The antibacterial drug, furazolidone is often added to feeds to stimulate growth and prevent and control a number of diseases in animals. Depending on the intended use, the concentrations used vary from 8 to 400 mg/kg. For example, when fed to pigs at 100 mg/kg, furazolidone can help prevent bacterial enteritis due to *Salmonella* spp. Chickens and turkeys may be fed continuously at the rate of 8–10 mg/kg to stimulate growth and improve feed conversion.

All permitted veterinary medicinal products in the European Union (EU) must have an established MRL [1]. Compounds for which no MRL can be established, because of concerns about their possible effects on human health have been added to Annexe IV of EC Regulation 2377/90. This means that, with effect from 1 January 1997, the use in animal production of all of the compounds listed in Annexe IV will be prohibited within the EU. Furazolidone was added to Annexe 4 of Regulation 2377/90 on 26 June 1995 [2]. This decision was taken because furazolidone residues, at whatever limit, in foodstuffs of animal origin constitute a hazard to the health of the consumer. Furazolidone is known to be a genotoxic carcinogen and insufficient data are available concerning the identity and toxic potential of compounds released from bound furazolidone res-

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idues [3]. Furazolidone therefore joins the other nitrofuran drugs; growth promoting hormones, such as diethylstilboestrol and trenbolone; and other antibacterial compounds, such as chloramphenicol, which are banned in the EU.

The detection of residues of furazolidone in animal tissues has presented analysts with great difficulty. Furazolidone is very rapidly metabolised in vivo [4] and furazolidone residues are very unstable in vitro [4,5]. More recently, it has become possible to detect tissue bound residues of furazolidone that contain a 2-amino-3-oxazolidinone (AOZ) moiety [6,7]. A previous report from this laboratory has shown that up to 17% of porcine kidneys produced in Northern Ireland contain detectable residues of AOZ [7]. However, the concentrations of these residues were consistent with proper withdrawal periods having been observed. Residues of AOZ are stable, and may be detected for prolonged periods after withdrawal of medication. Therefore, analysis of animal tissues for AOZ presents an excellent method for regulatory laboratories to use to detect possible furazolidone abuse after the introduction of the ban on furazolidone. However, the recently issued directive on residues testing within the EU [8], places considerable emphasis on the monitoring for banned substances on samples collected on-farm. Analysis of feedstuffs for furazolidone therefore may provide an effective method for monitoring for abuse on the farm.

Previously reported methods for the determination of furazolidone in feeds have employed either thin-layer chromatography (TLC) [9], colorimetric determination [10] or HPLC [11]. These methods have limits of detection of around 50 mg/kg and were therefore unsuitable for the determination of trace levels for our research programme [12], supporting and underpinning the statutory testing programmes carried out in this laboratory. The assay presented is capable of measuring furazolidone in both medicated feeds and in feeds with low level contamination. Following solvent extraction and purification on an alumina column, furazolidone is detected and quantified using HPLC with either UV or MS detection. The described HPLC method may be used, after the introduction of the ban, to screen samples for the possible presence of furazolidone and the LC–MS method may be used to confirm any positives.

2. Experimental

2.1. Chemicals and solvents

All chemicals and solvents used were of analytical reagent grade and HPLC grade, respectively. Furazolidone (Sigma, Poole, UK) was dissolved in acetonitrile–methanol (1:1, v/v) to give a stock solution of 250 μg/ml. Working standards of 12.5, 1.0 and 0.5 μg/ml were prepared by serial dilution of the stock with acetonitrile–methanol (1:1, v/v) and water (35:65, v/v). Sodium acetate solution (pH 6.0) was prepared by dissolving 0.82 g of sodium acetate in water, adjusting pH to 6.0 with 10% (v/v) acetic acid, and diluting to 1 l.

2.2. HPLC–UV system

The HPLC system comprised a Merck–Hitachi (Darmstadt, Germany) L6000 pump, a Merck-Hitachi AS2000 autosampler and a Waters (Millipore, Milford, MA, USA) μBondapak, 10 μm, C_{18} reversed-phase column, 300×3.9 mm I.D.. The column was interfaced to a Merck–Hitachi L4200 UV–visible variable-wavelength detector, set to monitor at 365 nm.

2.3. LC–MS system

The LC system was comprised of a Hewlett–Packard (Cheadle, UK) series1050 pump, a series 1050 automatic sampler and a Merck LiChrocart (E. Merck, Darmstadt, Germany) reversed-phase column, 125×4 mm I.D., containing LiChrospher RP-18 (end-capped) 5 μm and fitted with an RP18 4-4 guard column. The column was interfaced to a Hewlett–Packard HP5989A Engine mass spectrometer fitted with a thermospray source. Instrument control was via an HP ChemStation.

2.4. Sample extraction

A 5 g sample of ground feed was placed in a plastic bottle and 15 ml of water was added. Fortified samples were prepared by adding an appropriate volume of furazolidone standard to the feed prior to the addition of water. The mixture was allowed to stand for 5 min before the addition of 35 ml of

acetonitrile–methanol (1:1). The bottle was capped tightly and shaken on a mechanical shaker for 30 min. The solution was then filtered, under gravity, through a glass fibre paper. The filtrate was passed down a dry-packed column containing 4 g of neutral alumina (Sigma, UK). The first 4 ml of eluent were discarded and the following 8 ml were collected. This solution was used for the HPLC and LC–MS analyses.

2.5. HPLC–UV analysis

The mobile phase consisted of 0.01 *M* sodium acetate pH 6.0 and acetonitrile (8:2). The rate of flow was 1.5 ml/min and the sample injection volume was 20 μl. The run time was 10 min. Quantitative results were obtained by comparing peak areas of samples with those of standards.

2.6. LC–MS analysis

The HP Engine was calibrated using a solution of polypropylene glycol (PPG) 425 and PPG 725 in methanol–water (25% v/v) containing 0.1 *M* ammonium acetate. The mobile phase consisted of 0.1 *M* ammonium acetate–acetonitrile (65:35, v/v). For sample analysis the instrument was operated in the positive ion mode with filament assisted ionisation. The MS source was maintained at a temperature of 200°C with a typical thermospray stem temperature of 125°C. The electron multiplier and high energy dynode voltages were typically 2000 and 8000 V, respectively. For maximum sensitivity, samples were analysed using selected-ion monitoring (SIM) of the ion *m/z* 243 with a dwell time of 500 ms. The rate of flow of mobile phase was 1 ml/min and sample injection volume was 50 μl. Results were quantified by comparing peak areas of samples with those of standards.

3. Results and discussion

HPLC–UV chromatograms of a furazolidone standard (1 μg/ml), a blank feed extract and feed extracts fortified at 5 and 20 mg/kg are shown in Fig. 1. Furazolidone elutes at 7.2 min. The linearity of the assay was checked by running a series of

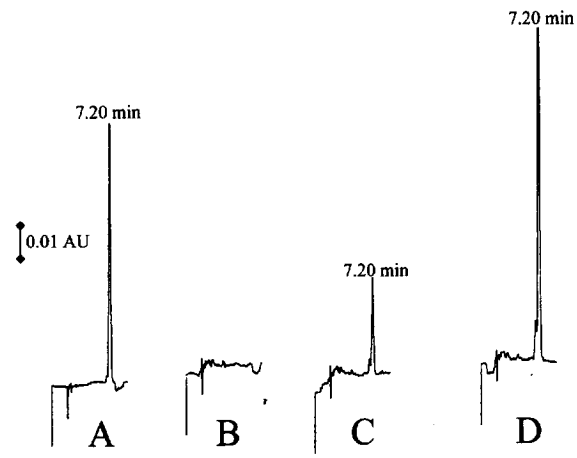

Fig. 1. HPLC–UV chromatograms of (A) 1 μg/ml furazolidone standard; (B) blank feed extract; (C) blank feed fortified with furazolidone at 5 mg/kg; (D) blank feed fortified with furazolidone at 20 mg/kg. The run time was 10 min. Furazolidone eluted at approximately 7.2 min. Full scale deflection was set at 0.1 absorbance units (AU).

dilute standards in the concentration range between 5 and 125 mg/kg of furazolidone in feed. The equation of the best fit line was $y=190.4x-18.7$ ($r=0.9999$). When concentrations in feed were >125 mg/kg,

Table 1
Recovery of furazolidone from fortified negative pig feed using HPLC–UV

Fortified concentration	Day 1	Day 2	Day 3	Overall
200 mg/kg				
Mean	197.6	197.2	193.2	196.0
s	2.5	3.5	3.7	3.7
s_r	1.2	1.8	1.9	1.9
Recovery (%)	99	99	97	98
n	6	6	6	18
20 mg/kg				
Mean	19.4	19.2	20.4	19.6
s	1.0	0.7	0.4	0.9
s_r	5.3	3.8	2.2	4.5
Recovery (%)	97	96	102	98
n	6	6	6	18
5 mg/kg				
Mean	4.8	4.6	4.7	4.7
s	0.2	0.5	0.2	0.3
s_r	3.8	9.9	5.2	6.6
Recovery (%)	95	91	93	93
n	6	6	6	18

Table 2
Recovery of furazolidone from fortified (1 mg/kg) negative pig feed using LC–MS

	Day 1	Day 2	Day 3	Overall
Mean	0.83	0.99	0.99	0.94
s	0.02	0.09	0.08	0.10
s_r	2.3	9.22	8.2	10.9
Recovery (%)	83	99	99	94
n	6	6	6	18

samples were diluted with water and run against an appropriate working standard.

The HPLC assay was validated by measuring the recovery of furazolidone from a known negative pig feed fortified with furazolidone at concentrations of 5, 20 and 200 mg/kg on three separate occasions (Table 1). Overall, the mean recoveries were 93.4, 98.2 and 98% at the 5, 20 and 200 mg/kg levels, respectively. The limit of detection of the assay, defined as the concentration of analyte which yields a peak-to-peak signal-to-noise ratio of at least 3:1, was determined by running a series of 10 negative feed extracts. A value of 1 mg/kg was calculated.

The LC–MS assay was validated at a fortified level of 1 mg/kg (Table 2). The overall recovery was 94% and the approximate limit of detection was 0.1 mg/kg. A full scan LC–MS spectrum of a 250 μg/ml solution of furazolidone is shown in Fig. 2. The molecular mass of furazolidone is 225, however the base peak observed is the M+18 $(M+NH_4)^+$ ion at m/z 243. This spectrum is typical for those produced in thermospray LC–MS systems. A smaller peak at m/z 284 is due to the formation of a furazolidone–acetate ion adduct $(M+59)^+$. The intensity of this peak is influenced greatly by source and vapour temperatures and consequently is not always observed. Single ion mass chromatograms of a 0.5 μg/ml standard, equivalent to 5 mg/kg in feed; and a negative pig feed fortified with 1 mg/kg, are shown in Fig. 3.

The HPLC assay described is both simple and rapid to perform. Furazolidone can be measured in medicated feeds with high levels of recovery and good precision. At low levels, the assay is useful down to 5 mg/kg, which is considerably lower than previously described methods. Recovery at this concentration is 93.4% and precision is 6.6%. For concentrations below 5 mg/kg it is recommended that the presence of furazolidone in feed be confirmed using the MS procedure. This provides unequivocal identification of the drug since mass related data are produced. The same sample extracts can be used. Recovery and precision are 93.8 and 10.9%, respectively at the 1 mg/kg level.

Following the introduction of the ban on furazolidone, any occurrence of residues in animal tissues will be attributable to one of two sources.

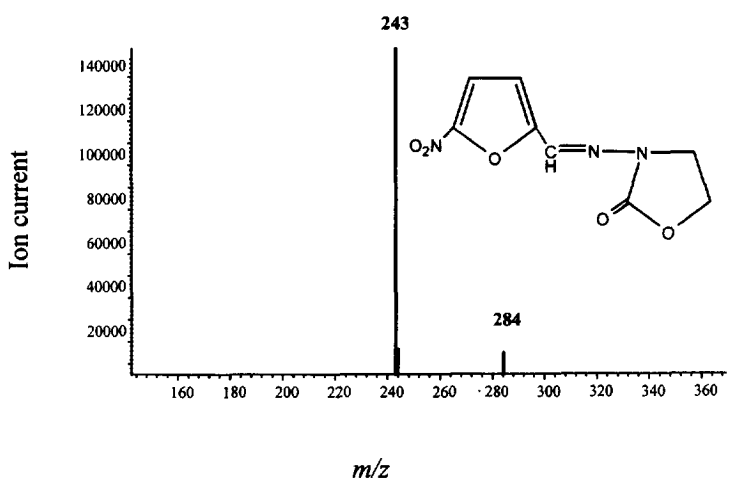

Fig. 2. Positive ion full scan spectrum of a 250 μg/ml solution of furazolidone in acetonitrile–methanol (1:1, v/v).

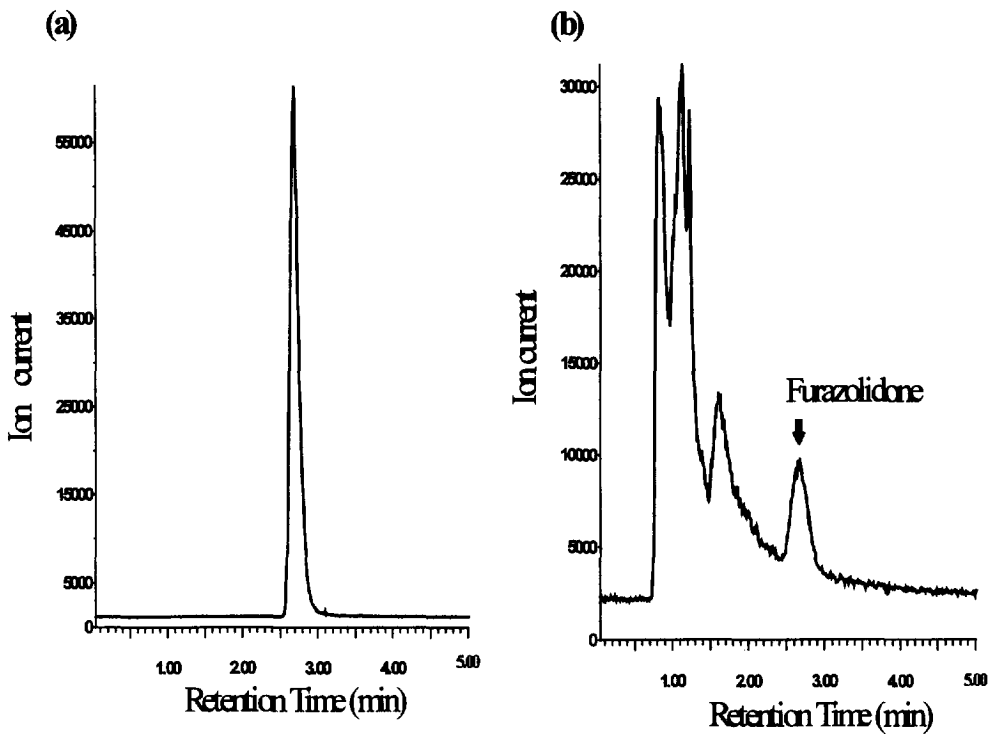

Fig. 3. Single ion chromatograms (normalised) m/z 243 of 50 μl injections of (a) furazolidone standard 0.5 μg/ml (equivalent to 5 mg/kg in feed); (b) blank feed fortified with 1 mg/kg furazolidone.

Either animals will have been treated with furazolidone illegally or unmedicated animals will have come into contact with illegally treated animals. This might occur during transport or during temporary holding at meat plants. It has been shown previously [12] that unmedicated pigs, exposed to a furazolidone contaminated environment for as little as two hours can result in detectable concentrations, in their tissues, of the AOZ metabolite. With zero tolerance for furazolidone residues, detection of AOZ in tissues would be sufficient to condemn the carcass. However, a positive tissue result gives no indication of whether the animal was therapeutically treated or inadvertantly contaminated with furazolidone. At present there is no way of distinguishing between deliberate abuse of the drug and inadvertent contamination. However, the detection of furazolidone in feeds, collected on-farm, may provide an effective method. The HPLC–UV procedure may be used to screen feed samples for medicated concentrations of furazolidone. MS detection may then be used to confirm any positives. Therefore, analysis of tissues together with the associated feed samples will provide a more complete picture which may be used to account for the presence of furazolidone residues in edible tissues.

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