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Journal of Chromatography B, 772 (2002) 185–189

JOURNAL OF CHROMATOGRAPHY B

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Short communication

# Sensitive liquid chromatographic–mass spectrometric assay for norfloxacin in poultry tissue

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Received 28 August 2001; received in revised form 10 January 2002; accepted 28 January 2002

**Abstract**

A highly sensitive and specific method for the determination of norfloxacin in poultry tissues by LC–MS was developed and validated. An extract of the sample was separated on a $C_{18}$ reversed-phase column and analyzed by LC–MS. The mobile phase was gradiently flowed with 2% acetic acid and acetonitrile. The limit of detection and limit of quantitation were 1 and 5 ng/g, respectively. Mean recoveries from various spiked tissues were 87.2% (ranging from 82.5 to 92.7%) for norfloxacin. The method has been successfully applied to determine norfloxacin in poultry muscle. 

*Keywords:* Norfloxacin

## 1. Introduction

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid, NFX] is a fluoroquinolone antibacterial, which exhibits high antimicrobial activity in vitro against a wide variety of gram-negatives and gram-positives, including gentamicin-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* [1,2]. NFX has a remarkably broad spectrum of activity and excellent pharmacokinetics allowing for once-daily dosing. Because of this, it has found increased use in animal production.

There is now a strict legislative framework controlling the use of some quinolones, with the aim of minimizing the risk to human health associated with consumption of their residue. Therefore, to ensure human food safety, the European Union (EU) has set tolerance levels for these compounds as maximum residue limits (MRLs). Recently, MRLs have been fixed for several quinolones, but no MRL set for NFX which are currently used [3]. Only a few studies on the kinetics and residue depletion of NFX in animals have been published [4–9].

Several HPLC methods have been suggested for the determination of NFX in various biological matrices [10–14]. Some methods achieved only relatively high detection limits in the range of several hundred μg/kg or μg/l. They were not suitable to determine low levels of NFX in biological fluid. A sensitive analytical technique is needed to determine NFX in animal tissues.

In this study, we developed a rapid and sensitive

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1570-0232/02/$ – see front matter 
PII: S1570-0232(02)00066-1

method to determine NFX in poultry tissues with LC–MS and evaluated residue depletion times after multiple-dose oral administration.

## 2. Experimental

### 2.1. Chemical

NFX was from Deasung Microbials (Seoul, South Korea). HPLC grade water, methanol, acetonitrile, acetic acid, and hexane were purchased from TEDIA (OH, USA).

### 2.2. Instrumentation and chromatographic conditions

Samples were analyzed on a Hewlett-Packard LC–MSD system. It consisted of a G1322A degasser, a G1312A binary pump, a G1315A photodiode-array detector, 59987A electrospray interface and 5989B mass spectrometer. Separation was achieved on Nova-Pak $C_{18}$ reversed-phase column (4 μm, 3.9 mm×150 mm I.D., Waters, USA). Flow-rate was operated at 0.4 ml/min. The mobile phase consisted of water–acetic acid (98:2, v/v, A) and acetonitrile (B). Gradient runs were programmed as follows: 100% A for 10 min, increase from 0 to 90% B in 8 min, 90% B for 2 min, re-equilibration with 100% A for 5 min, until the next sample injection.

The pressure of nebulizer was 45 p.s.i. and heated to 350 °C. The quadrupole was heated to 100 °C. The mass spectrometer was run in the positive mode and selective ion monitoring mode. Since $[M+H]^+$ forms gave the strongest signals, the mass spectrometer was focused on $m/z=320.3$ with peak width of 0.12 ms per ion (Fig. 1).

### 2.3. Sample preparation

Each 1 g muscle sample was added to 2 ml of extraction solution, homogenized, and then shaken for 10 min. Extraction solution consisted of methanol and acetic acid (98/2, v/v). The samples were centrifuged at 1300 *g* for 10 min, the supernatants being transferred into another tube and evaporated to dryness at 30 °C under a stream of nitrogen. The residue was reconstituted with 1 ml of the extraction solution and added to 2 ml of saturated hexane with acetonitrile and vortexed for 5 min. The samples were centrifuged at 1300 *g* for 10 min, and the upper layer discarded. The lower phase was transferred into the other tube and evaporated to dryness at 30 °C under a stream of nitrogen. The residue was reconsti-

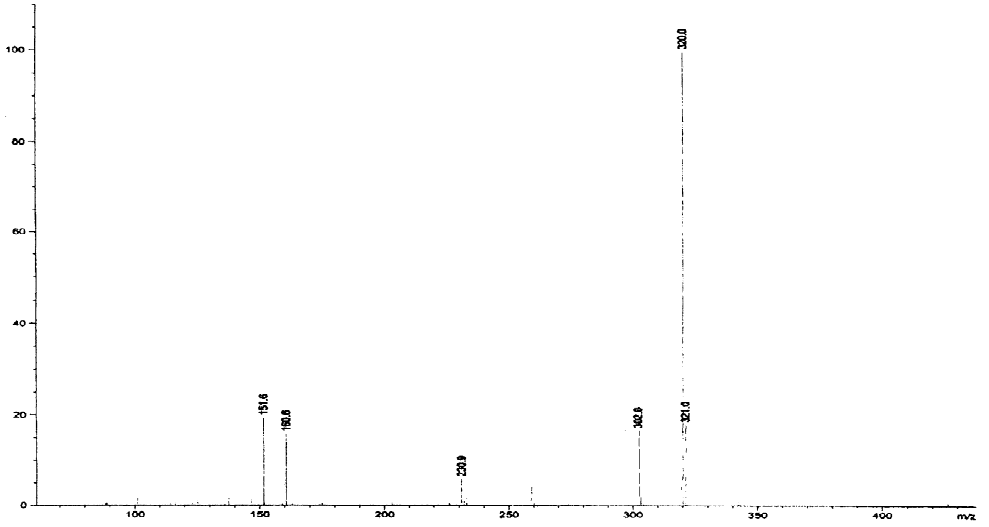


Fig. 1. Representative mass spectrum of NFX (as scan mode from $m/z$ 100 to $m/z$ 500).

tuted with 1 ml of the extraction solution and vortexed for 30 s. A 10-μl aliquot was injected after filtration with a 0.4-μm filter.

### 2.4. Treatment of data

Concentrations of NFX in various poultry tissues were calculated from the standard curves constructed by plotting area of NFX against the working standard concentrations of NFX (10, 1, 0.1, 0.01, 0.001 μg/g). Quantification was carried out using a five-level external standard calibration curve. A freshly prepared set of calibration samples and recovery samples were run before determination of samples, along with blank tissues extracts. Results are presented as mean±standard deviation (SD).

### 2.5. Accuracy and precision

The accuracy is defined as the percentage deviation from the added concentration (RSD) and the precision was determined by calculating the coefficient of variation (C.V.). The recovery of NFX was assessed in triplicate determinations at spiked muscles. The responses from the spiked sample were compared with those from the blank muscle sample.

### 2.6. Application of depletion-kinetic studies

The proposed method was applied to the determination of NFX in poultry for depletion kinetic studies. The experiment was conducted in farms housing broilers of around 1 kg body weight. NFX was given for 7 days as a mixed feed at a daily dose rate of 0.2 and 0.8 g/kg feed. Six broilers were taken at random and killed before the start of the experiment and 0, 1, 3, 5, 7, 10 days after the last dose. Samples of liver, kidney, muscle, adipose tissue, and serum were collected and stored in a freezer at −20 °C and allowed to thaw at room temperature before processing.

## 3. Results and discussion

A highly sensitive and specific method for the determination of NFX in poultry tissues by LC–MS has been established.

Various methods available for quinolones assay have been reported. Thin-layer chromatography, capillary electrophoresis, high-performance lipid chromatography, liquid chromatography–mass spectrometry, and gas chromatography–mass spectrometry have been developed to analyze biological samples such as serum or urine [10–14]. Some methods lack identity confirmation of drugs and require a long time for chromatographic separation and method development. In addition, they were not suitable to determine low levels of NFX in biological fluid. The limit of detection and limit of quantitation of NFX in the present study were 1 and 5 ng/g, respectively. These values satisfied the acceptance criteria of the limit of detection and limit of quantitation. The LOQ of this method is more sensitive than other HPLC methods previously reported [10–14].

The mass spectra of NFX showed that $[M+H]^+$, $m/z$ 320, was the predominant ion (Fig. 1). $[M+H]^+$ ion produce two fragment ions, $m/z$ 302 due to the loss of water and $m/z$ 259 due to the loss of water and $C_2H_5N$. The loss of CO from the quinolone ring, $m/z$ 276, showed low intensity in the mass spectrum. $[M\text{-}H_2O\text{-}C_2H_5N]^+$ ion, $m/z$ 231, exhibited fragments arising from loss of CO, pyrrolidine from the quinolone ring. These fragment ions were only detected with fragmentation voltage of 100 V; attempts to increase the abundance of these ions with even high fragmentation voltages resulted in lower molecular mass fragments.

As a result of analysis of blank samples, matrix interference was not detected (Fig. 2). NFX eluted from the analytical column with a retention time of 18.7 min and increased in proportion to concentrations. The linear regression line for NFX in the range of 1–10 μg/g showed high correlation coefficients ($r$) of 0.999. A calibration curve described by the equation $y = mx + b$, where $y$ represents the response value of peak area, and $x$ the concentration of NFX, was generated. Precision and accuracy are shown in Table 1. The C.V. at 10 and 1000 ng/ml ranged from 8.5 to 11.1%, and the RSD ranged from 9.1 to 12.1% for various tissues. The recovery of NFX in the poultry tissues ranged from 85.3±9.3% to 92.7±8.9% for 1000 μg/kg samples, and from 82.5±9.1% to 87.5±9.1% for 10 μg/kg samples.

Various tissue concentrations of NFX after treatment declined in the course of time and were not

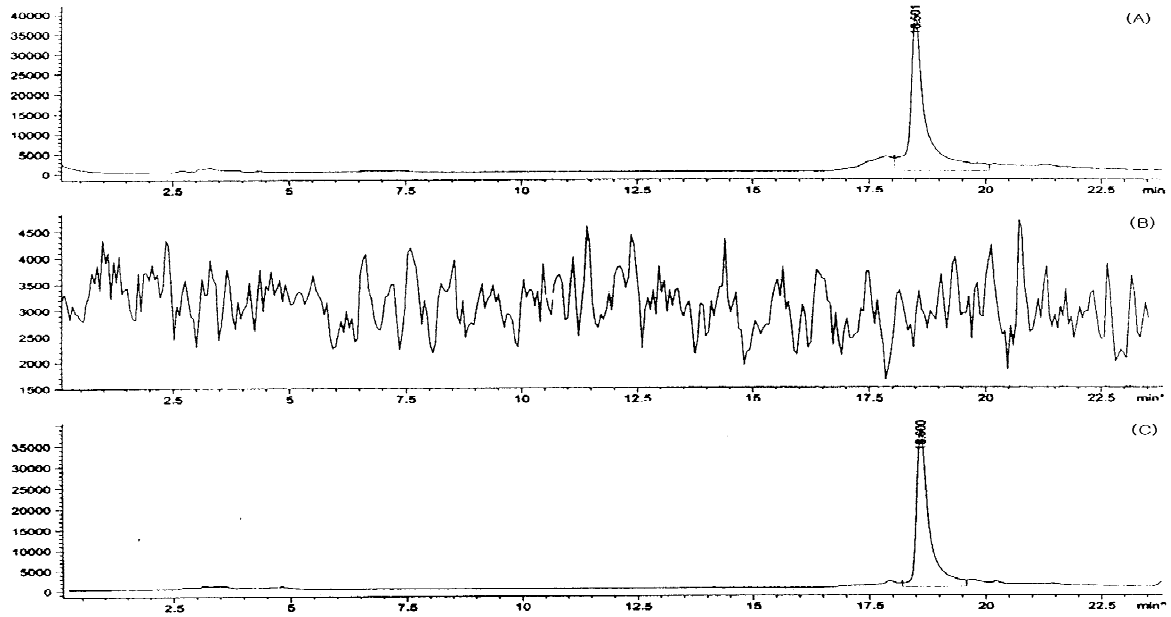


Fig. 2. Total ion chromatograph (TIC) for the $[M+H]^+$ ion of NFX ($m/z$ 320.0) as a selected ion monitoring mode; (A) 100 ppb spiked standard; (B) blank muscle tissue; (C) 100 ppb spiked muscle tissue.

detected 10 days after treatment (Tables 2 and 3). The method has been successfully applied to determine NFX concentration in poultry tissues for depletion-kinetic studies.

In conclusion, LC–MS is a simple, rapid and effective technique for the determination of NFX in poultry tissues. The precision and accuracy developed in this method are suitable and sensitive to

Table 1
Accuracy, precision and recovery of NFX

| Tissue | Concentration added ($\mu g\ kg^{-1}$) | Concentration detected ($\mu g\ kg^{-1}$) | Recovery (%) | Precision (C.V., %) | Accuracy (RSD, %) |
|---|---|---|---|---|---|
| Serum | | | | | |
| | 1000 | 937.8±89.9 | 92.7±8.9 | 8.5 | 9.1 |
| | 10 | 8.7±0.9 | 87.5±9.1 | 10.7 | 6.0 |
| Liver | | | | | |
| | 1000 | 866.4±81.0 | 86.6±8.1 | 9.0 | 9.7 |
| | 10 | 8.5±1.6 | 85.0±16.1 | 17.0 | 12.1 |
| Kidney | | | | | |
| | 1000 | 877.8±84.7 | 87.8±8.5 | 8.9 | 9.1 |
| | 10 | 8.5±0.5 | 85.0±5.1 | 6.8 | 11.6 |
| Muscle | | | | | |
| | 1000 | 902.8±110.3 | 90.3±11.0 | 11.6 | 15.4 |
| | 10 | 8.8±0.9 | 87.5±9.1 | 9.6 | 12.1 |
| Adipose tissue | | | | | |
| | 1000 | 852.8±92.0 | 85.3±9.3 | 11.1 | 17.3 |
| | 10 | 8.3±0.9 | 82.5±9.1 | 10.7 | 12.1 |

Table 2
Mean concentrations of NFX in poultry tissue after medicated feed as a dose of 0.2 g/kg

| Time (day) | Tissue concentration (μg/g) | | | | |
|---|---|---|---|---|---|
| | Serum | Liver | Kidney | Muscle | Adipose tissue |
| 0 | 0.66±0.291 | 1.18±0.251 | 1.05±0.490 | 0.68±0.261 | 0.67±0.27 |
| 1 | 0.33±0.223 | 0.50±0.354 | 0.45±0.134 | 0.31±0.121 | 0.31±0.12 |
| 3 | 0.09±0.072 | 0.11±0.083 | 0.12±0.032 | 0.10±0.042 | 0.09±0.05 |
| 5 | 0.01±0.002 | 0.03±0.031 | 0.03±0.014 | 0.01±0.004 | 0.02±0.01 |
| 10 | – | – | – | – | – |

–, Not detected or under LOQ.

Table 3
Mean concentrations of NFX in poultry tissue after medicated feed as a dose of 0.8 g/kg

| Time (day) | Tissue concentration (μg/g) | | | | |
|---|---|---|---|---|---|
| | Serum | Liver | Kidney | Muscle | Adipose tissue |
| 0 | 0.98±0.45 | 2.54±1.00 | 2.19±0.95 | 1.14±0.32 | 0.96±0.48 |
| 1 | 0.68±0.18 | 0.99±0.36 | 0.95±0.37 | 0.73±0.24 | 0.78±0.15 |
| 3 | 0.16±0.12 | 0.36±0.21 | 0.38±0.18 | 0.19±0.11 | 0.23±0.12 |
| 5 | 0.01±0.00 | 0.07±0.05 | 0.08±0.05 | 0.02±0.01 | 0.05±0.02 |
| 10 | – | – | – | – | – |

−, Not detected or under LOQ.

determine the concentration of NFX for depletion-kinetic studies. These results could be applied to establish withdrawal times of NFX in poultry.

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