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Detecting nitrofuran metabolites in animal products using LC/MS/MS

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Introduction

Before 1995, antibiotics of the nitrofuran family were widely used as feed additives and for the prophylactic and therapeutic treatment of diseases such as gastrointestinal infections in cattle, pigs, poultry, rabbits and fish. They were extremely effective at preventing enteritis and promoting growth, but further research showed that they could cause birth defects and cancer in humans, and their use was subsequently banned in food-producing animals in the European Union (EU). The use of some nitrofurans, including nitrofurantoin and nitrofurazone, is still authorised under specific conditions in non-food production animals, for example in the treatment of urinary and skin infections in pet animals. Legitimate use of nitrofurans should not therefore lead to residues in EU produced and imported food. However, nitrofuran contamination still occurs, whether through deliberate and direct misuse of the drug, contaminated animal feed or from environmental sources, and methods are being continually updated to quickly identify their presence in food.

Nitrofuran antibiotics, which include furazolidone, nitrofurantoin, furaltadone and nitrofurazone (Figure 1) are rapidly metabolised and have *in vivo* half-lives of only a few hours. Their metabolites, however, are highly stable and have significant potential for genotoxicity. These compounds include 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD).[1]

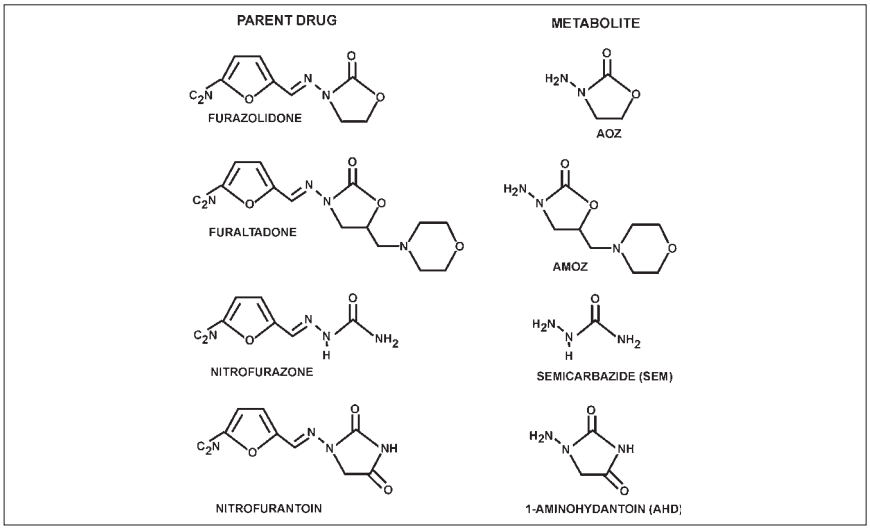

Figure 1. Structure of the nitrofuran antibiotics and their metabolites.

The sample matrices investigated tend to be complex, so a highly selective and sensitive bioanalytical method is needed to detect the metabolites (currently 1 ppb for each metabolite is achievable).

Liquid chromatography tandem mass spectrometry (LC/MS/MS) is currently the best method for detection of multiple components. Alternative detection methods include immunoassays, gas chromatography mass spectrometry (GC/MS) and liquid chromatography (LC) with diode array detection (DAD). Immunoassay detection methods are normally based on the compound class, so cannot determine which compound within the class is detected; being only able to detect one compound, they are costly and time-consuming. Additionally, immunoassays have a high risk of false positive data due to cross-reactions, and testing kits available limit the compounds that may be detected. GC/MS detects long chain aliphatic compounds and very low mass volatile material better than LC/MS, but requires compounds to be volatile to be ionised. Most compounds need to be derivatised for GC/MS, and traditionally GC/MS only uses the electron impact source while the source can be changed with LC/MS. GC/MS is still widely used for very polar compounds. LC/MS is ideal for analysing multiple components, it being possible to detect multiple compounds of different classes in one run. However, both LC/MS and LC/DAD require baseline separation between compounds, requiring HPLC method development and long run times.

LC/MS/MS generally does not require derivatisation; however, it is required for nitrofurans because their molecular weight is so small. Several research

studies have shown that HPLC coupled to tandem mass spectrometry is a highly sensitive and selective technique but it is less useful when sample matrices are complicated. LC/MS/MS is able to detect specific compounds based upon column retention time, parent mass and structure. LC/MS/MS methods may also screen for more than one compound class per assay. LC/MS/MS is 20–100 times more sensitive than LC/MS when screening for multiple compounds. The second filtering process of LC/MS/MS makes the technique far more specific than LC/MS, resulting in less background interference from the sample and meaning that shorter columns may be used so run times are quicker. The reduced background interference also dramatically improves the quality of data produced.

There are various different systems available for small molecule quantitative analysis with LC/MS/MS, including ion trap, quadrupole time-of-flight (QqToF); Q TRAP® and triple quadrupole (QQQ) systems. All of these systems are capable of LC/MS/MS analysis and separate ions based on their mass/charge ratio; each has its advantages and disadvantages. The ion trap, QqToF and Q TRAP systems are ideal for identification, the QqToF system has better mass accuracy and resolution compared with Q TRAP and QQQ systems. The Q TRAP system's linear range is identical to that of a QQQ and both these systems are suited for quantification, having higher sensitivity and linearity for quantitative analysis than ion trap and QqToF systems, and are capable of simultaneous multiple component analysis and multiple reaction monitoring (MRM).

In addition, there are different types of source available for LC/MS/MS systems, including electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI). The suitability of the source depends on the polarity of the compounds for detection.

Optimising LC/MS/MS for nitrofurans

All nitrofuran metabolites have molecular masses between 75 and 201 g mol^{-1}, a range where there is abundant spectrometric background noise. This can lead to non-selective fragmentation and consequently false positive results, particularly if there is inter-sample variation present. However, research investigations have shown that the derivatisation of the free amino group of the target analytes using 2-nitro-benzaldehyde (2-NBA) gives improved sensitivity in a number of studies,[2] and improving the sample preparation or LC separation steps can also help to eliminate background noise. A simultaneous hydrolysis of the tissue-bound metabolites and derivatisation is preferable, followed by a one step liquid–liquid extraction. Finally, the careful choice of specific fragment ion pairs for MRM during method development is also desirable.

As a consequence of these observations, we devised a method of sample preparation that involved taking one gram of homogenised sample and adding AMOZ D5 as an internal standard. The nitrofuran metabolites were then derivatised with 2-NBA at 37°C for 16 h, the pH adjusted to 7.4 and the sample then centrifuged. The metabolites were extracted using ethyl acetate and an Extrelut® column (Merck). Prior to injection, the sample was dried and reconstituted in a mobile phase. Liquid chromatography was then performed on an Agilent® 1100 System using a C18 Luna® column (150 × 3 mm, 3 µm, Phenomenex).

Our testing revealed that it is absolutely crucial which type of buffer system is used. We found that we could only achieve sufficient sensitivity when using methanol instead of acetonitrile (ACN) and ammonium acetate buffer instead of the more commonly used acetic acid, especially for the metabolite AHD. The optimised eluent A was 0.5 mM ammonium acetate and eluent B methanol. The flow rate was set to 200 µL min^{-1} and the gradient run isocratically with a 50/50 split for 14 min and then increased to 100% methanol to remove the remaining matrix from the column.

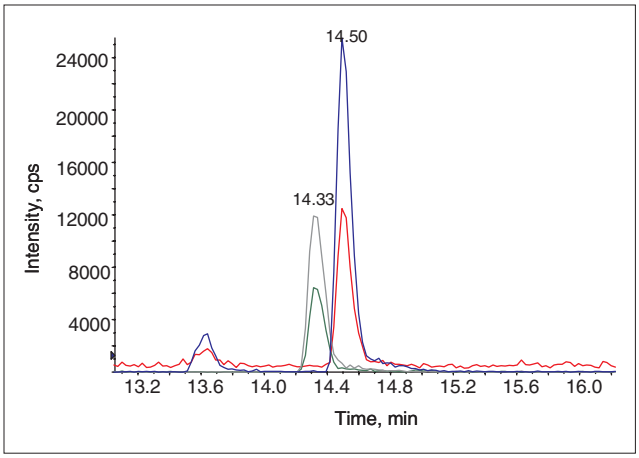

Figure 2. API 4000 system; standard with 100 pg absolute: 2-NBA-SEM 209/166 (blue), 209/192 (red), 2-NBA-AHD 249/178 (green), 249/134 (grey).

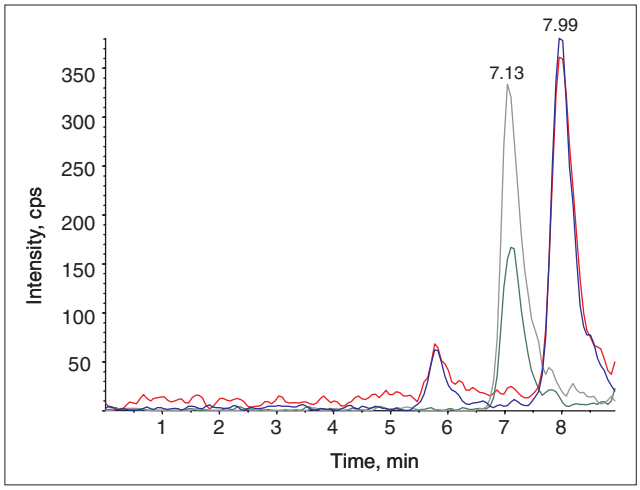

Figure 3. API 2000 system: standard with 100 pg absolute: 2-NBA-SEM 209/166 (blue), 209/192 (red), 2-NBA-AHD 249/178 (green), 249/134 (grey).

The metabolite detection was performed on either an API 2000™ or API 4000™ LC/MS/MS system (Applied Biosystems) and two fragment ions per analyte were acquired to meet EU regulations. Both these instruments have fast scan times and are suitable for high throughput quantitation, although the API 4000 system has the advantage of a tenfold increase in sensitivity compared with the API 2000 system, which still meets Maximum Residue Limit (MRL) requirements.

Despite their small molecular weight, the derivatised metabolites could be quantitified over several orders of magnitude with high accuracy. On the API 4000 a calibration curve from 0.5 pg to 750 pg was created for all metabolites and the linear regression coefficients (weighed by 1/x) were determined to be 0.999 and better. Similar coefficients were found on the API 2000 System in a concentration range of 50 pg to 5 ng. Figures 2 and 3 show the excellent sensitivities of the two instruments for AHD and SEM.

The sample preparation has been demonstrated to be appropriate for different matrices, including egg powder and animal tissue (data not shown); SEM and AOZ, for example, could be effectively quantified to levels of 0.6 $\mu g\,kg^{-1}$ in egg powder, a matrix known for its complexity.

The recovery of the extracts was found to be around 70% for SEM and AHD, 40% for AOZ and 80% for AMOZ. The quantification limits using the API 4000 were found to be 0.02 $\mu g\,kg^{-1}$ for AOZ, 0.01 $\mu g\,kg^{-1}$ for AMOZ, 0.05 $\mu g\,kg^{-1}$ for SEM and 0.1 $\mu g\,kg^{-1}$ for AHD. With the API 2000 the quantification limits were found to be higher: 0.5 $\mu g\,kg^{-1}$ for AOZ and AMOZ, and 1 $\mu g\,kg^{-1}$ for SEM and AHD. The quantitation limits were based on a signal-to-noise (S/N) ratio of 10 and the detection limit on a S/N ratio of three.

In conclusion, LC/MS/MS was found to be a highly sensitive method for detecting nitrofurans in animal tissue and other matrices, such as eggs, shrimp and animal tissue, using the presence of its metabolites as an indicator. The combination of a one-step hydrolysis and derivatisation process, solid-phase or liquid–liquid sample extraction clean-up and careful selection of fragment ions can significantly improve the poor MS detection sensitivity of small and polar molecules of the drug residues.

References.

1. A. Conneely, A. Nugent and M. O'Keeffe, *Analyst* **127(6),** 705–709 (2002).
2. A. Leitner. P. Zöllner and W. Lindner, *J. Chromatogr. A* **939(1–2),** 49–58 (2001).