Determination of the Metabolites of Nitrofuran Antibacterial Drugs in Chicken Tissue by Liquid Chromatograph-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Application

Food, Environmental

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Abstract

A liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) method was developed for the simultaneous determination of the metabolites of four nitrofuran antibacterial drugs in chicken tissues: furazolidone, furaltadone, nitrofurazone, and nitrofurantoin. Sample clean-up and analyte enrichment were performed by liquid-liquid extraction with ethyl acetate followed by solvent washing, hydrolysis of the protein-bound drug metabolites, and derivatization with 2-nitrobenzaldehyde (2-NBA). ESI parameters were optimized, and the chromatographic separation of all metabolites was examined. Each metabolite produced a simple mass spectrum containing a strong signal corresponding to $[M+H]^+$. Metabolite calibration curves, in the 0.25 to 1 ng/mL range, exhibited correlation coefficients greater than 0.999. The limit of detection (LOD) for each analyte ranged from 0.02 to 0.06 ng/mL.

Introduction

The four drugs shown in Figure 1, furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, belong to the group of nitrofuran antibacterial drugs. These drugs have been widely used as feed additives to prevent bacterial enteritis by *Escherichia coli* and *Salmonella* in cattle, fish, swine, and poultry. The occurrence of furazolidone residue in edible tissue is a major human health concern. Effective June 1995, these drugs were banned from use in food animal production in the European Union (EU) because of concerns about their carcinogenicity and mutagenicity (Commission Regulation 1442/95).

Nitrofuran antibacterial drugs are characterized by their rapid metabolism, with in vivo half-lives of less than a few hours. Therefore, the detection of parent drugs in animal tissue is not practical. Studies using radioactive-labeled furazolidone have shown that protein-bound metabolites are formed in tissues [1-3]. The tissue-bound metabolites are detectable for several weeks after administration. Hence, the analysis of nitrofuran drugs is based on the detection of the tissue-bound metabolites of the parent drugs.

These tissue-bound metabolites are very small molecules which are not UV absorbing, and they elute too quickly out of a column. To induce UV absorption in the molecule and to be reasonably retained on a column, they are derivatized. It is possible to release these metabolites from the proteins under moderately acidic conditions and derivatize the metabolites with 2-nitrobenzaldehyde (2-NBA) to produce 2-NBA-derivatives for liquid chromatography (LC), UV detection, and mass spectrometry (MS) confirmation. The goal of this study is to develop a routine analytical method to simultaneously detect the target nitrofuran metabolites. Because no maximum residue limit (MRL) has

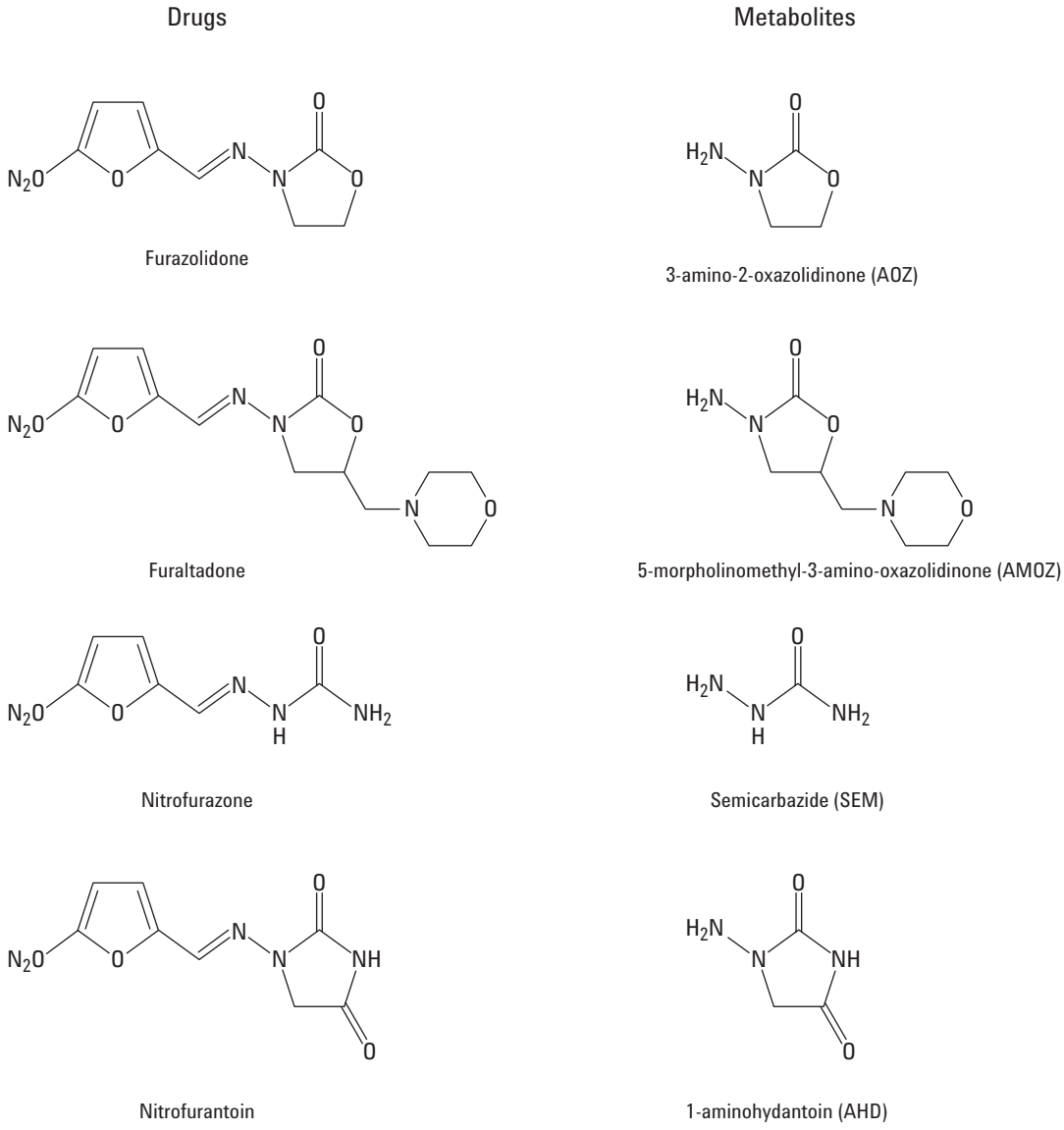

Figure 1. Structure of the nitrofuran antibacterial drugs and their metabolites .

been set by any regulatory agency, the goal of the analytical method was to estimate the lowest possible detection limit.

Experimental

Chemicals and Solvents

Three metabolites: 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), and 1-aminohydantoin (AHD) were purchased from Sigma Aldrich Japan (Tokyo, Japan). The purity of these compounds was greater than 99%. The 2-NBA derivatives of these metabolites were prepared by the Livestock Department in Thailand (Palm Thani, Thailand) using the procedure described by Leitner [4]. Stock solutions of these three 2-NBA derivatives were prepared in methanol at 1000 ng/mL and stored in the dark at 4 °C. The stock solution was diluted to the desired concentration just prior to its use for the optimization of ESI parameters.

Acetonitrile, ethyl acetate, formic acid, and dimethyl sulfoxide (DMSO) were supplied by Wako Chemical (Osaka, Japan). Hydrochloric acid and 2-NBA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Water was purified with a Milli-Q system (Millipore, Tokyo, Japan).

Sample Preparation

Sample preparation procedures included solvent wash and acid extraction by homogenization and derivatization with 2-NBA. Chicken muscle and liver were prepared by the Livestock Department in Thailand.

Calibration curves for the four nitrofuran metabolites (from the Livestock Department) were constructed in the range 0.25 to 1.0 ng/mL. The derivatization and sample preparation procedures used by the Livestock Department are the following:

1. The four metabolite solutions in water, 12.5, 25.0, 37.5, and 50 µL at 100 ng/mL, were transferred to separate 40 mL glass vials with screw caps.
2. A solution of 10 mL HCl (125 mM in water) and 200 µL 2-NBA (50 mM in DMSO) were added to each vial.
3. The reaction mixtures were kept in a water bath at 37 °C for 16 hours.
4. The solutions were cooled to room temperature.
5. The pH was adjusted to about 7.4 by adding 0.1 M aqueous $KHPO_4$ or 0.8 M aqueous NaOH.
6. A 5-mL measure of ethyl acetate was added to each reaction mixture, and shaken for 2 min.
7. Each ethyl acetate phase was transferred to a separate glass vial and evaporated under a stream of nitrogen.
8. Finally, each residue was reconstituted in 5 mL of 1:1 methanol:water (V/V).

The calibration curve was based on the metabolite concentration in clean solvent and derivatization using 2-NBA. Previous studies done by the Livestock Department showed that recovery of all metabolites from chicken extracts was above 80%. Therefore, the amounts of metabolite in chicken extract can be calculated by comparing the responses of 2-NBA derivatives from the samples against the calibration curve.

Instrument and Experimental Conditions

An Agilent 1100 series LC, with a solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, and a column thermostat, was used for separation. An 1100 series diode array detector (DAD) was connected in line with an 1100 MSD for detection and confirmation. The column and MS conditions are described in Table 1.

Table 1. Instrument Parameters

LC:	Agilent 1100 series
Column:	Inertsil ODS3, 150 mm × 2.1 mm, 5 µm (GL Science, Tokyo, Japan)
Solvent A:	Acetonitrile
Solvent B:	Aqueous 0.5% formic acid
Gradient:	20/80 A/B to 70/30 A/B in 20 min
Column temp	20 °C
Sample volume	30 µL
Flow rate:	200 µL/min
MS:	Agilent 1100 MSD, SL
Ionization:	ESI (Positive)
Scan range:	100–500 *m/z* for optimization
SIM ion:	Base peak for quantitation
Drying gas:	Nitrogen, 10 L/min at 350 °C
Nebulizer gas:	Nitrogen, 50 psi
Fragmentor:	120 or 140 V
V_{cap}	2000 V

Quantitative analysis was carried out using selective ion monitoring (SIM) of the base peak ions according to the program shown in Table 2. To confirm the presence of the target analytes in chicken extract, the sodium adduct ions (qualifier ions) of all target analytes were also monitored.

Table 2. SIM Program

Group	Time window min	Analyte(s)	Target ion	Qualifier ion	Dwell time msec	Fragmentor voltage, V
1	0–6	2-NBA-AMOZ	335	357	500	140
2	6–12.5	2-NBA-SEM and 2-NBA-AHD	209 and 249	231 and 271	250 and 250	120 and 140
3	12.5–14	2-NBA-AOZ	236	258	500	140

System Optimization

Positive ion mass spectra were acquired over the scan range *m/z* 100–500 using a step size of 0.1 amu and a scan rate of 2 seconds per scan for the optimization of fragmentor voltage. Ion lens voltages in the MS were automatically optimized using a Calibrant Delivery System and the AutoTune program.

Using the analytical column and three 2-NBA derivatives (AOZ, SEM, and AHD) at 100 ng/mL, instrument performance was optimized by adjusting the four major ESI parameters: the capillary voltage, fragmentor voltage, the nebulizer gas pressure, and the drying gas flow rate. However, significant variation in the intensity of analytes was not observed when the drying gas flow rate and nebulizer gas pressure were varied from 4 L/min to 13 L/min and 20 psi to 60 psi, respectively.

Capillary and fragmentor voltages applied to the inlet and exit end of the capillary affected the ion transmission significantly. Fragmentor voltage also affected the fragmentation of sample ions. In general, higher fragmentor voltage helps the transmission of ions through the relatively high-pressure region between the exit of the capillary and the entrance of the skimmer. High fragmentor voltage can cause fragmentation to occur which provides structural information of the ion. For compounds that do not fragment easily, higher fragmentor voltage often results in better ion transmission. Optimal fragmentor voltage is compound dependent. Evaluation of the fragmentor voltages for the three 2-NBA-metabolites was done under the same chromatographic conditions as the analysis. Mass spectra of three 2-NBA-metabolites are shown in Figure 2. Each mass spectrum exhibited $[M+H]^+$ as the base peak. Adducts ions $[M+NH4]^+$ and $[M+Na]^+$ were observed at lower fragmentor voltage (120 V) and some fragment ions (*m/z*=178 and 192) were observed at higher fragmentor voltage (180 V). Interestingly, the $[M+NH_4]^+$ ion was not observed at

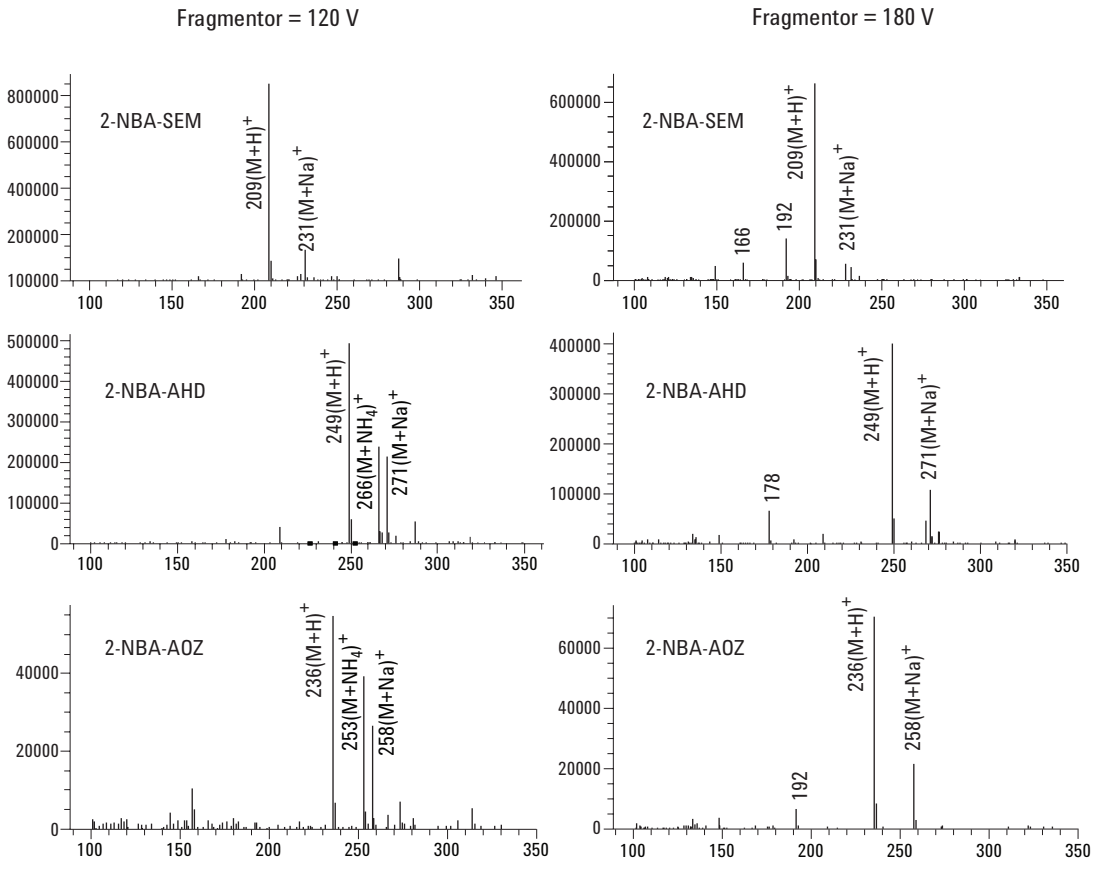

Figure 2. Mass spectra of 2-NBA-SEM, 2-NBA-AHD, and 2-NBA-AOZ from two ESI fragmentor voltages.

180 V fragmentor voltage due to its stability. As seen in Figure 3, in order to ensure the best sensitivity, the fragmentor voltage for 2-NBA-SEM was set to 120 V and that of 2-NBA-AHD and 2-NBA-AOZ was set to 140 V for the analysis. Although 2-NBA-AMOZ was not examined, fragmentor voltage of this compound was set to 140 V because of its structural similarity to 2-NBA-AOZ. For the capillary voltage varied between 1500 and 4500 V, the optimal voltage was found to be 2000 V for all three metabolites.

Linearity, Detection Limits, and Precision

In order to achieve optimal sensitivity, all quantitation experiments were carried out under SIM conditions, and the $[M+H]^+$ ions were monitored for all 2-NBA-metabolites. To evaluate the linearity of the calibration curves, various metabolite solutions ranging from 0.25 ng/mL to 1 ng/mL were derivatized and then analyzed. As shown in Table 3, the linearity was very good for all 2-NBA-metabolites with correlation coefficients (r^2) greater than 0.999.

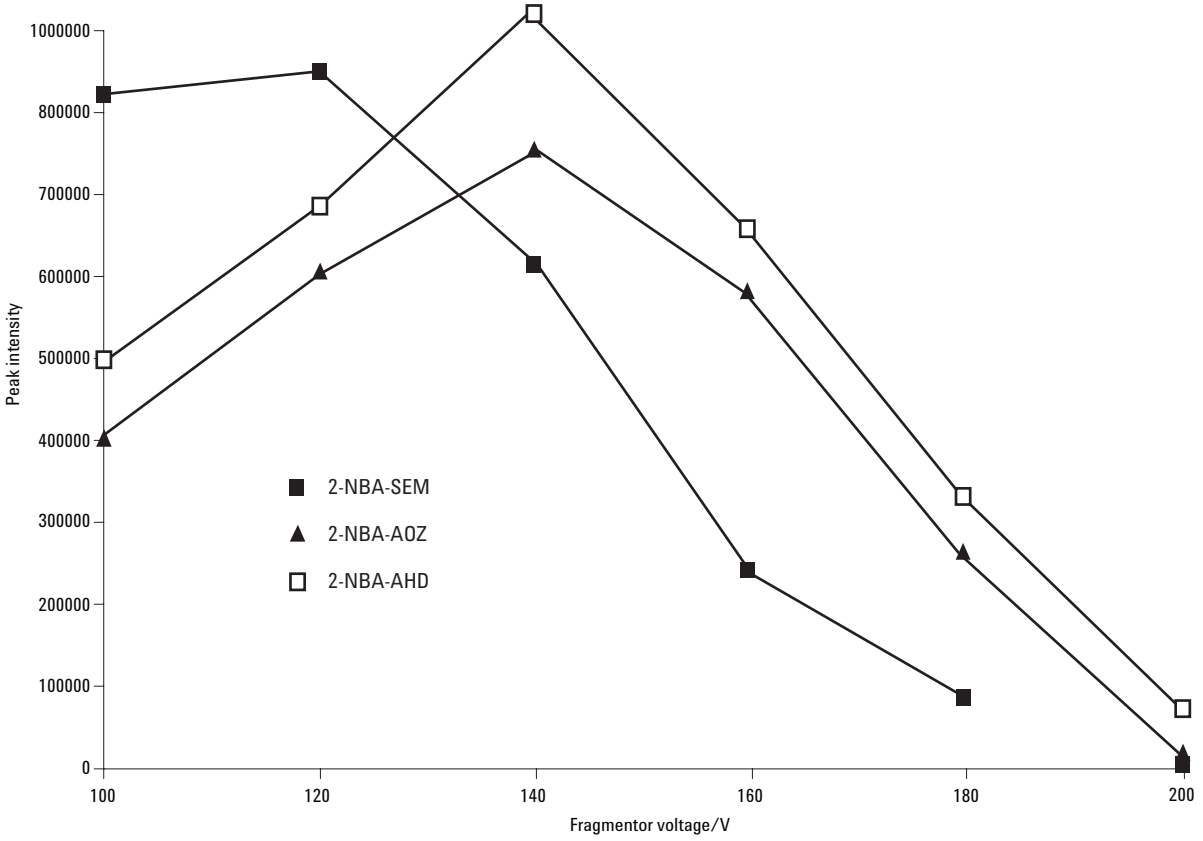

Figure 3. Effect of fragmentor voltage on peak intensity. Mobile phase, 20% acetonitrile/80% water 0.1% formic acid; Analyte concentration, 100 ng/mL.

The LOD for all 2-NBA-metabolites was estimated by extrapolating to a signal-to-noise ratio (S/N) of 3 using the signal from the standard solution at 0.25 ng/mL. These SIM chromatograms are shown in Figure 4. The LODs of the metabolites were in the range of 0.02 ng/mL to 0.06 ng/mL. These LODs were lower than those of the LC/MS/MS method developed by Leitner [4]. The intraday instrument precision (repeatability) was determined by injecting aqueous standard solutions containing all of the 2-NBA-metabolites at 0.5 ng/mL five times during a working day. The interday instrument precision (reproducibility) was evaluated by analyzing the same sample three times over 3 working days. The precision for all analytes ranged from 3.1% to 8.2%, as seen in Table 3.

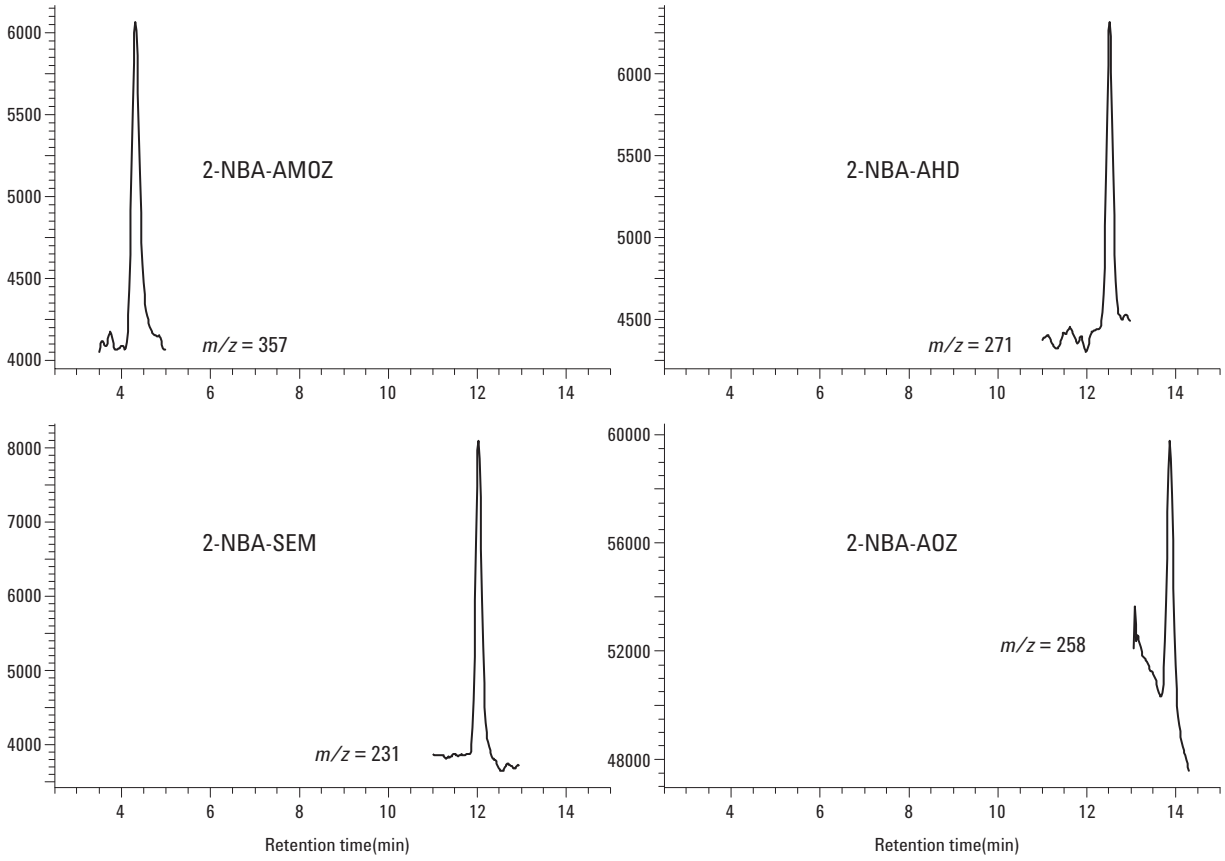

Figure 4. SIM chromatograms of aqueous 2-NBA nitrofuran metabolites solution at 0.25 ng/mL.

Table 3. Linearity, LOD, and Instrument Precision of Metabolites in Aqueous Solutions

			Instrument precision (%RSD)	
Metabolites	**r^2**	**LOD* (ng/mL)**	**Repeatability****	**Reproducibility*****
AMOZ	0.9999	0.04	5.0	7.3
SEM	0.9998	0.02	4.7	8.1
AHD	0.9989	0.06	4.9	7.9
AOZ	0.9997	0.06	3.1	8.2

*Detection limit is LOD defined as S/N = 3 for standard solution at 0.25 ng/mL

**Repeatability was calculated based on five replicates at 0.5 ng/mL within 1 day

***Reproducibility was calculated based on once per day for 3 days at 0.5 ng/mL

Evaluation of Chromatographic Separation

Several reverse-phase columns were evaluated for HPLC performance. In terms of minimizing the inherent matrix suppression effects on the ESI process, Inertsil ODS3 column provided the best separation between analytes and the majority of the matrix components with the given mobile phase. Further, the linear solvent gradient gave the best compromise between short analysis time and sufficient matrix and analytes separation. Figure 5 shows individual SIM chromatograms for the four metabolite derivatives in spiked chicken muscle at 0.2 ng/g. No interference peaks were observed for 2-NBA-AMOZ and 2-NBA-AOZ, but it was difficult to separate 2-NBA-SEM and 2-NBA-AHD from the interfering matrix peaks. However, these peaks could still be identified by comparison with the blank sample, and the analyte amounts could then be calculated.

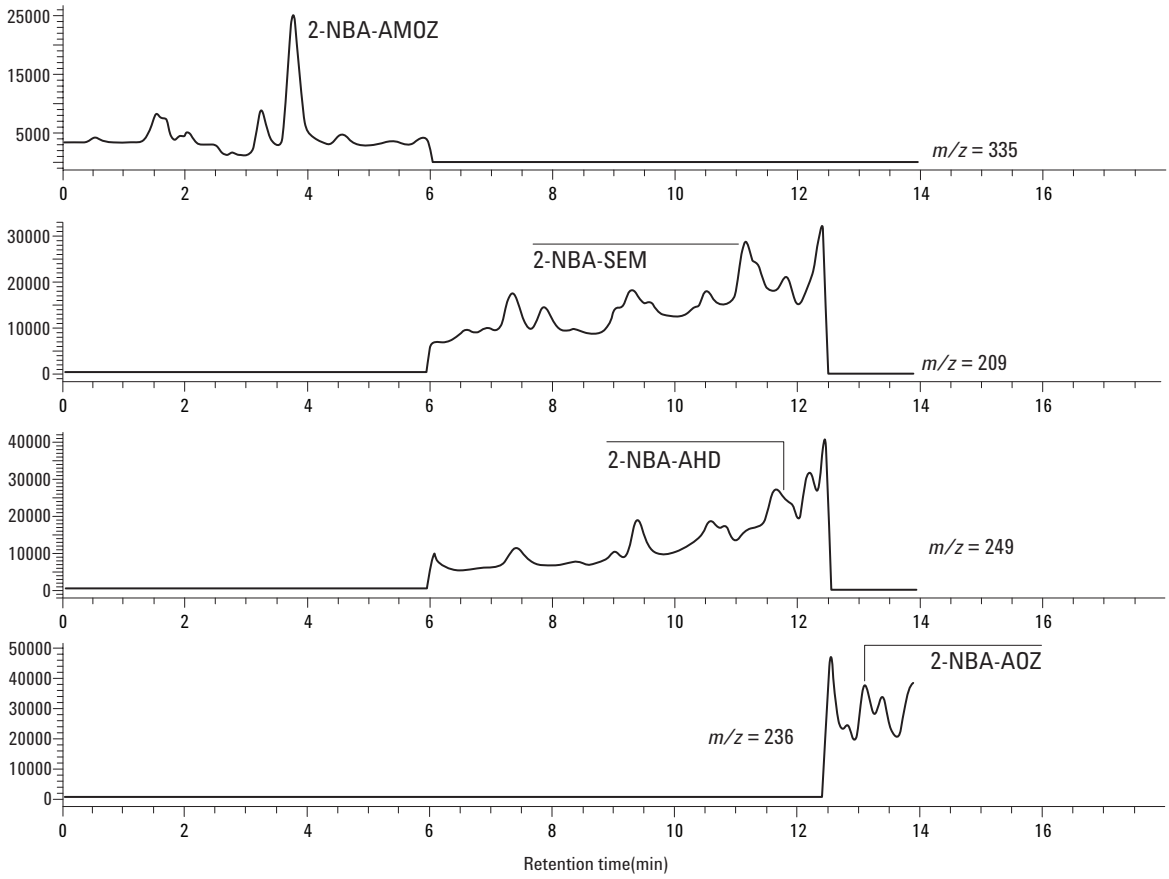

Figure 5. SIM chromatograms of a spiked chicken muscle tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Application of the Method to Chicken Liver Samples

It has been reported that AOZ concentrations in liver tissue are several times higher than in muscle tissue [1,2]. This indicates that detection of nitrofuran metabolites in liver would be possible over an even longer period of time. Since the nature of the liver matrix is considered to be different from muscle and more difficult to separate target compounds from the interfering matrix, the developed LC/MS muscle method was also tested for the applicability to liver matrix. Figure 6 shows individual SIM chromatograms of the four metabolite derivatives in spiked chicken liver tissue. AMOZ, SEM, and AOZ derivatives were identified unambiguously and quantified down to 0.2 ppb. However, the AHD derivative overlapped with the matrix component and was difficult to quantify.

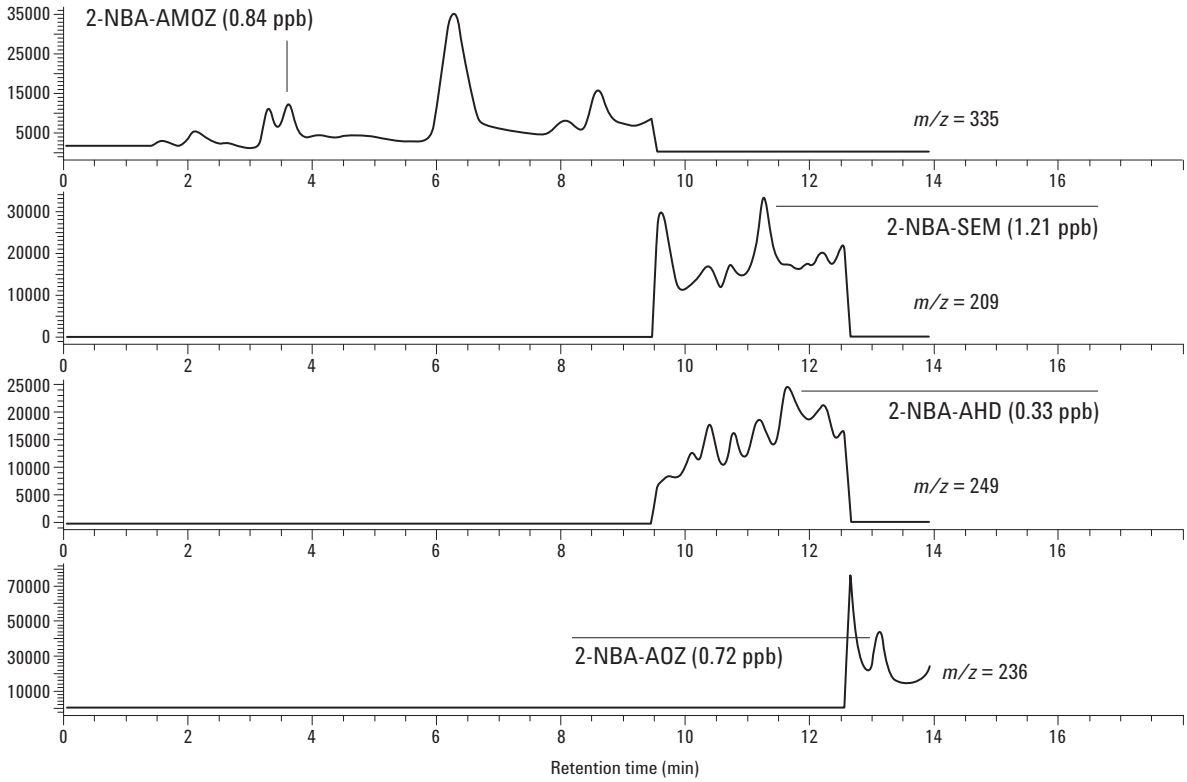

Figure 6. SIM chromatograms of a spiked chicken liver tissue sample containing 0.2 ng/g of each of the four 2-NBA nitrofuran metabolites.

Conclusion

The development of a routine and sensitive LC/MS method allows for the simultaneous detection of four nitrofuran metabolite derivatives. The detection limit of each analyte ranges from 0.05 to 0.2 ng/g in chicken muscle and liver tissues.

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