JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Liquid Chromatography–Tandem Mass Spectrometry for the Determination of Protein-Bound Residues in Shrimp Dosed with Nitrofurans

PAK-SIN CHU* AND MAYDA I. LOPEZ

Center for Veterinary Medicine, U.S. Food and Drug Administration, 8401 Muirkirk Road, Laurel, Maryland 20708

An analytical method was developed for the determination of bound residues of the nitrofuran drugs furazolidone, nitrofurazone, furaltadone, and nitrofurantoin with a sensitivity of 1 ppb in shrimp. In this procedure, shrimp tissue is prewashed with solvents followed by overnight acid hydrolysis, during which the side chains of the bound residues are released and simultaneously derivatized with 2-nitrobenzaldehyde. After liquid−liquid extraction cleanup, the derivatives are detected and quantitated using liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS) with an atmospheric pressure chemical ionization interface. The method was validated using control shrimp fortified with each side-chain analyte at 1, 2, and 4 ppb. Method accuracies were >80% with coefficients of variation of <20% for all four analytes. Tissues from dosed shrimp were assayed to demonstrate the effectiveness of the method for recovering bound residues of nitrofurans. In shrimp dosed with nitrofurans, nitrofurantoin exhibited the lowest level of bound residues.

KEYWORDS: Nitrofurans; shrimp; LC-MS/MS; method; bound residues

INTRODUCTION

Nitrofurans (NFs, **Figure 1**) are broad-spectrum antibacterial drugs commonly used in veterinary medicine for the treatment of protozoan and bacterial infections in a variety of species, including chicken, turkey, swine, cattle, shrimp, and fish (*1*). Among the NFs, furazolidone (FZD), nitrofurazone (NFZ), nitrofurantoin (NFT), and furaltadone (FTD) are the most widely used. Because NFs are mutagenic and carcinogenic, in 1991 the U.S. Food and Drug Administration (FDA) withdrew the approvals of FZD and NFZ for antiprotozoal use in poultry and swine (*2*). The topical uses of NFs, however, were still allowed until 2002 (*3*). Since then, the use of NFs in food-producing animals has been prohibited by the FDA. Nonetheless, illegal use of NFs may exist and result in violative residues in the human food supply. Of greater concern is the possible NF misuse in other countries, which results in food products imported into the United States contaminated with NFs. Analytical methods are therefore needed for monitoring purposes.

Nitrofurans undergo rapid metabolism in animals with the parent drugs rapidly disappearing after drug administration (*4*−*9*). For this reason, parent NFs are not detected in most food products. Methods for determining NFs have to be based on the detection of protein-bound residues (*7*, *9*−*12*). These methods share a common approach involving acid hydrolysis of the bound residues and simultaneous derivatization of the released side chains with 2-nitrobenzaldehyde (2-NBA). In the literature, methods have been described for bound residues as well as for the total bound and free metabolites in edible tissues. These methods also differ in sample cleanup. For example, Conneely et al. (*12*, *13*) described methods for the isolation of nitrofuran bound residues from animal tissues using solid-phase extraction (SPE) and liquid chromatography (LC) with UV and tandem mass spectrometric detection. Leitner et al. (*14*) developed a method for the determination of the total bound and free NF residues in animal tissues using SPE and LC-MS/MS. Although both Conneely's and Leitner's methods involved SPE, others have used liquid−liquid extraction for sample cleanup. Such methods include that reported by Hoogenboom and Polman (*10*). The major differences between these methods are whether the tissue is prewashed with solvents prior to hydrolysis and whether SPE or liquid−liquid extraction is employed for sample cleanup.

Despite the wealth of methods available for nitrofuran residues in terrestrial animal tissues, few were developed for aquatic species. In the literature, most methods for aquatic species were developed for the parent drugs (*15*−*17*). For example, Stehly et al. (*15*) described an LC method for determining the parent FZD in shrimp with UV−vis detection, and Rupp et al. reported an LC method for the determination of the parent NFZ, NFT, and FZD in channel catfish (*17*). Because nitrofuran residues are bound to protein and the parent drugs have short biological half-lives, methods for determining parent NFs are of limited use for monitoring purposes. In this paper, we describe an LC-MS/MS method for the determination

* Author to whom correspondence should be addressed [telephone (301) 210-4583; fax (301) 210-4653; e-mail pak.chu@fda.gov].

10.1021/jf051615o
Published on Web 10/21/2005

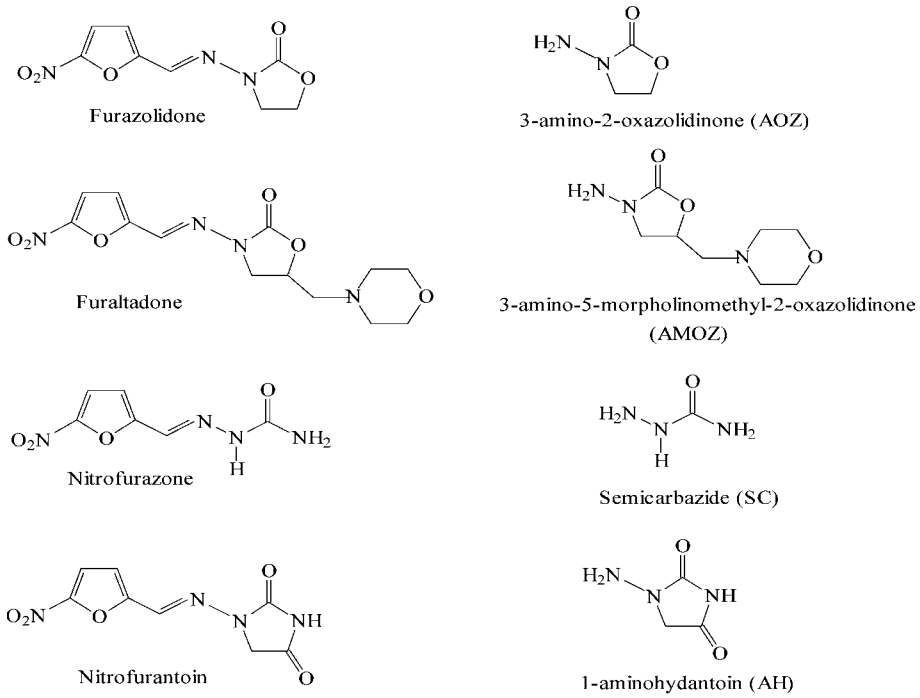

Figure 1. Structures of nitrofurans and side chains of bound metabolites.

of NF bound residues in shrimp and report on their levels in shrimp dosed with NFs.

MATERIALS AND METHODS

Reagents. LC grade water was purified in-house with a Milli-Q Plus water system and was used in the preparation of all solutions. Ammonium acetate, potassium phosphate dibasic anhydrous, and sodium hydroxide were of ACS reagent grade (Sigma-Aldrich, Milwaukee, WI). 1-Aminohydantoin hydrochloride (AH), semicarbazide hydrochloride (SC), and 2-nitrobenzaldehyde (2-NBA) were purchased from Sigma-Aldrich. 3-Amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 2-nitrobenzaldehyde-semicarbazone (NPSC), 5-morpholin-4-ylmethyl-3-[(2-nitrobenzylidene)-amino]oxazolidin-2-one (NPAMOZ), 3-[(2-nitrobenzylidene)amino]-oxazolidin-2-one (NPAOZ), and 1-[(2-nitrobenzylidene)amino]imidazolidine-2,4-dione (NPAH) were obtained from WITEGA (Berlin, Germany). Methanol (MeOH), hexane, and ethyl acetate (EtOAc) were high-purity solvents obtained from Burdick & Jackson (Muskegon, MI). Reagent alcohol (200 proof ethanol containing 5% isopropyl alcohol) and dimethyl sulfoxide (DMSO) were high-purity OmniSolv solvents obtained from EM Science (Gibbstown, NJ). Sodium chloride (granular) was obtained from Mallinckrodt (Paris, KY). The HPLC mobile phase consisted of 55% MeOH and 45% 20 mM NH_4Ac. The 20 mM NH_4-Ac solution was prepared by dissolving 1.54 g of NH_4Ac in 1 L of water. The HPLC mobile phase was prepared by measuring 550 mL of MeOH and 450 mL of the 20 mM NH_4Ac solution followed by mixing and filtering through a 0.45 μm filter.

Standard Solutions. *Primary Stock Solutions (100 μg/mL).* On the basis of the purity and composition, the amount of AOZ, SC, AH, and AMOZ needed to prepare 100 mL of the individual 100 μg/mL standard solution was calculated. All standards are expressed as the un-ionized moiety equivalent. Each standard was weighed to the nearest $\pm$0.1 mg into respective 100 mL volumetric flasks and brought to the mark with MeOH. These solutions were used immediately for the preparation of the solution below.

Intermediate Stock Solution (2 μg/mL). An appropriate volume ($\sim$2 mL) of each stock standard solution (100 μg/mL) was pipetted into a 100 mL volumetric flask and brought to the mark with MeOH. This solution was stored at $-$20 °C.

Stock Solution (0.2 μg/mL). One milliliter of the intermediate stock solution (2 μg/mL) was pipetted into a 10 mL volumetric flask and brought to the mark with MeOH. This solution was stored at $-$20 °C.

Fortification Standard. The stock solution (0.2 μg/mL) was diluted with MeOH to prepare a 0.04 ng/μL fortification solution, which was used for sample fortification and for preparing a four-point standard curve at the following concentrations: 0.5, 1, 2, and 5 ng/g (ppb). This solution was stored at $-$20 °C.

Liquid Chromatograph. The LC-MS conditions were adapted from those of Leitner et al. (*14*). The liquid chromatography system consisted of two Perkin-Elmer (Norwalk, CT) series 200 micropumps and a Perkin-Elmer series 200 autosampler equipped with a 100 μL loop. The LC column was an Inertsil ODS-3 5 μm, 150 $\times$ 2.0 mm, with a guard column of the same packing (Ansys Technologies, Inc., Lake Forest, CA). An optional precolumn filter (Upchurch, Oak Habour, WA) was installed between the autosampler and the guard column. A TurboVap LV evaporator (Zymark, Hopkinton, MA) was used for sample concentration.

Mass Spectrometer. An Applied Biosystems (Foster City, CA) Sciex API 2000 triple-quadrupole mass spectrometer with atmospheric pressure chemical ionization (APCI) source in the positive ion mode was used. The protonated molecules $[M + H]^+$ at *m/z* 249 (NPAH), 209 (NPSC), 236 (NPAOZ), and 335 (NPAMOZ) were selected as the precursor ions for collision-induced dissociation (CID). The following product ions were identified for selected reaction monitoring (SRM) LC-MS/MS analysis: NPAH, *m/z* 249 $\rightarrow$ 134, 104, 178; NPSC, *m/z* 209 $\rightarrow$ 166, 192, 134; NPAOZ, *m/z* 236 $\rightarrow$ 134, 104, 149; NPAMOZ, *m/z* 335 $\rightarrow$ 291, 262, 128. The dwell time for each monitored transition was 150 ms. Both Q1 and Q3 were set at unit resolution. The source temperature and ion spray voltage were set at 350 °C and 5500 V, respectively. Quantitation was computed by summing the areas of product ions of the respective analytes using PE-Sciex Analyst version 1.3 software.

Shrimp Dosing. Dosing of shrimp was performed at the University of Arizona. Live shrimp (*Litopenaeus vannamei*), each weighing 6$-$10 g, were obtained from Molokai Seafarms (Kaunakaki, Molokai, HI). Each treatment group consisted of sufficient shrimp to provide 100 g of tail tissue for each depletion time sample. Each aquarium typically contained 30$-$35 animals. They were acclimated for at least 2 days prior to initiation of the study. Because nitrofurans are not soluble in water, triethylene glycol was used as the carrier. After acclimation, shrimp were placed in separate 37 L aquaria containing either 0.25 mg/L (ppm) of FZD, 0.25 ppm of NFZ, or a mixture of 0.25 ppm of FTD and 0.25 ppm of NFT. The NFT bound residue level resulted from this dose was too low to be useful for method validation.

Therefore, additional shrimp were treated with 0.75 ppm of NFT. Eight hours after drug exposure had begun, half of the water in each aquarium was removed and replaced with clean seawater containing the dose. This was needed to maintain water quality. After 16 h, the treated shrimp were removed from the exposure aquaria, rinsed with clean seawater, and transferred to unmedicated tanks. Shrimp were taken out at 6, 24, 48, and 72 h after drug withdrawal. After the head and shell were removed, shrimp collected at the same time point were pooled and homogenized with dry ice in a blender. The homogenized shrimp tissues were stored at −20 °C for a few days to sublime the dry ice and thereafter stored at −80 °C until analysis. Control shrimp used for method development were either purchased from commercial suppliers or obtained from the University of Arizona.

Extraction Procedure. The extraction procedure was a modification of that reported by Hoogenboom and Polman (*10*). Shrimp tissues (2.0 ± 0.2 g) were weighed into 50 mL polypropylene centrifuge tubes. To these were added 5 mL of 50% aqueous MeOH followed by polytron homogenization for 1 min. The tubes were vortex-mixed for 15 s or until the pellets dispersed. After centrifugation at 15 °C for 5 min at 3000 rpm (∼1840 g), the supernatants were decanted and discarded. The remaining pellets were sequentially washed with 5 mL each of EtOAc and EtOH by vortex-mixing and centrifugation. The supernatants were decanted and discarded.

Fortification of tissue samples and calibration standards were performed at this step. A four-point calibration standard curve at 0.5, 1, 2, and 5 ppb (with reference to 2 g of tissue) was prepared by adding 25, 50, 100, and 250 μL of the 0.04 ng/μL fortification solution to blank 15 mL centrifuge tubes. Tissue samples for method accuracy determination were fortified at 1, 2, and 4 ppb by adding 50, 100, and 200 μL of the 0.04 ng/μL fortification solution to the prewashed pellets, respectively.

To the tissue samples and calibration standards were added 10 mL of 0.125 M HCl and 400 μL of freshly prepared 2-NBA solution (50 mM in DMSO). The samples were vortex-mixed for 15 s and placed in a 37 °C water bath overnight (∼14−16 h) with gentle shaking. The next day, 1 mL of 0.1 M K_2HPO_4 was added to the samples. The pH was adjusted to 7.1−7.5 by adding 0.8 M aqueous NaOH. The tissue samples (but not the calibration standards) were centrifuged at 3000 rpm for 5 min at 4 °C. The supernatants were decanted into respective 20 mL reservoirs equipped with filtering frits and attached to an SPE manifold. A slight vacuum was needed to start the flow. The filtrates were collected into new 50 mL polypropylene tubes. To avoid analyte loss, the remaining pellets were washed twice with 3 mL of water. The tubes were vigorously vortex-mixed to break up the pellets and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatants were decanted into their respective reservoirs and filtered. All samples (including the calibration standards) were adjusted to 20−22 mL by adding Milli-Q water. The actual volume could vary from set to set; however, all samples within the set should be made up to the same volume.

One-fourth of a teaspoon of NaCl and 10 mL of hexane were added. The samples were gently hand-mixed and centrifuged at 4 °C for 10 min at 3000 rpm to effect phase separation. The top hexane layers were removed and discarded. The remaining aqueous layers were partitioned three times with 4 mL of EtOAc by hand-mixing. After centrifugation for 5 min at 4 °C and 3000 rpm, the EtOAc layers were transferred into 15 mL polypropylene centrifuge tubes. The combined EtOAc layers were washed twice with 1 mL of Milli-Q water. The bottom aqueous layers were pipetted out and discarded. The remaining EtOAc layers were evaporated to dryness using a Zymark evaporator at 40 °C. To prevent analyte loss on the tube wall, EtOAc or MeOH (2 mL) was added to the samples followed by vortex-mixing and evaporation to dryness. The residues were dissolved in 200 μL of the HPLC mobile phase. After vortex-mixing, sonication for 2 min, and vortex-mixing again, the final extracts were filtered through 0.2 μm Gelman nylon filters into autosampler vials with 300 μL inserts.

Chromatographic Conditions. Shrimp extracts were analyzed for bound residues of NFs using the following isocratic LC conditions: mobile phase, 55% MeOH/45% 20 mM NH_4Ac; flow rate, 200 μL/min. A typical injection sequence was as follows: a water blank to equilibrate the LC system, 40 μL of each calibration standard, a sample set, and the standards again. At the end of each day's analyses, the analytical and the guard columns were flushed with water/MeOH (10+90, v/v) to remove retained materials.

Quantitation. A calibration curve of peak area versus concentration (ppb) was plotted for each analyte. The least-squares regression parameters were calculated for the calibration curve, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y = peak area and X = concentration of standard in ppb. Correlation coefficients for each of the calibration curves were routinely >0.99. For quality control, a control and a fortified control were processed along with each set of unknown or incurred samples.

RESULTS AND DISCUSSION

The method described here was a modification of that developed by Hoogenboom and Polman for the determination of bound residues in pig hepatocytes (*10*). Their method was modified in sample preparation and in the detection system for use in shrimp. Prewashing steps were modified from four MeOH and two EtOH washes to once each with 50% aqueous MeOH, 100% EtOAc, and 100% EtOH, and the centrifugation time decreased from 10 to 5 min, so as to shorten the sample preparation time. An additional hexane partitioning was included in our procedure to remove lipid materials and excess 2-NBA. On the basis of our observations, it is critical not to vortex-mix samples during hexane partitioning; otherwise, a gel-like emulsion layer forms. To further reduce emulsion formation, NaCl was added to each sample. However, the residual NaCl remaining in the final extract caused a thin white coating on the APCI ionization needle, which led to a gradual drop in sensitivity across a sample set. This problem was resolved by washing the EtOAc layer twice with 1 mL of water, which also provided an additional sample cleanup.

For the detection system, the Hoogenboom and Polman method (*10*) utilized an HPLC with UV−vis. To meet our sensitivity requirement of 1 ppb, a more sensitive tandem mass spectrometer was used. The LC-MS/MS conditions were adapted from those of Leitner et al. (*14*), except that an atmospheric pressure chemical ionization (APCI) interface was used instead of an electrospray (ESI). In our laboratory, we found the APCI interface more sensitive and more rugged toward matrix effects than the ESI source. At the outset, we attempted Leitner's method for determining the total free and bound residues in shrimp. However, our method accuracy for AH was >150%. The high AH method accuracy might be due to (1) matrix effects at the MS interface, which caused standards and tissue extracts to be ionized differently, or (2) matrix effects on the SPE columns, which caused standards and tissue extracts to exhibit different recovery for NPAH. Matrix effect is a common problem for LC-MS/MS, as unwanted matrix components compete with the analytes during ionization in the interface. To avoid such problems, the shrimp tissues were prewashed with several solvents, as outlined in the method of Hoogenboom and Polman (*10*). The prewashing steps removed not only the unbound parent drugs and metabolites but also the unwanted matrix. To circumvent matrix effects on the SPE, a liquid−liquid extraction was substituted for the SPE step. With the above changes, we were able to attain a method with good accuracy and precision without the use of matrix-matched calibration curves and internal standards, which with the exception of AMOZ-d5 were not available at the time of this study.

Early studies have shown that parent nitrofurans are light sensitive (*18*). As a precaution, all of our initial experiments

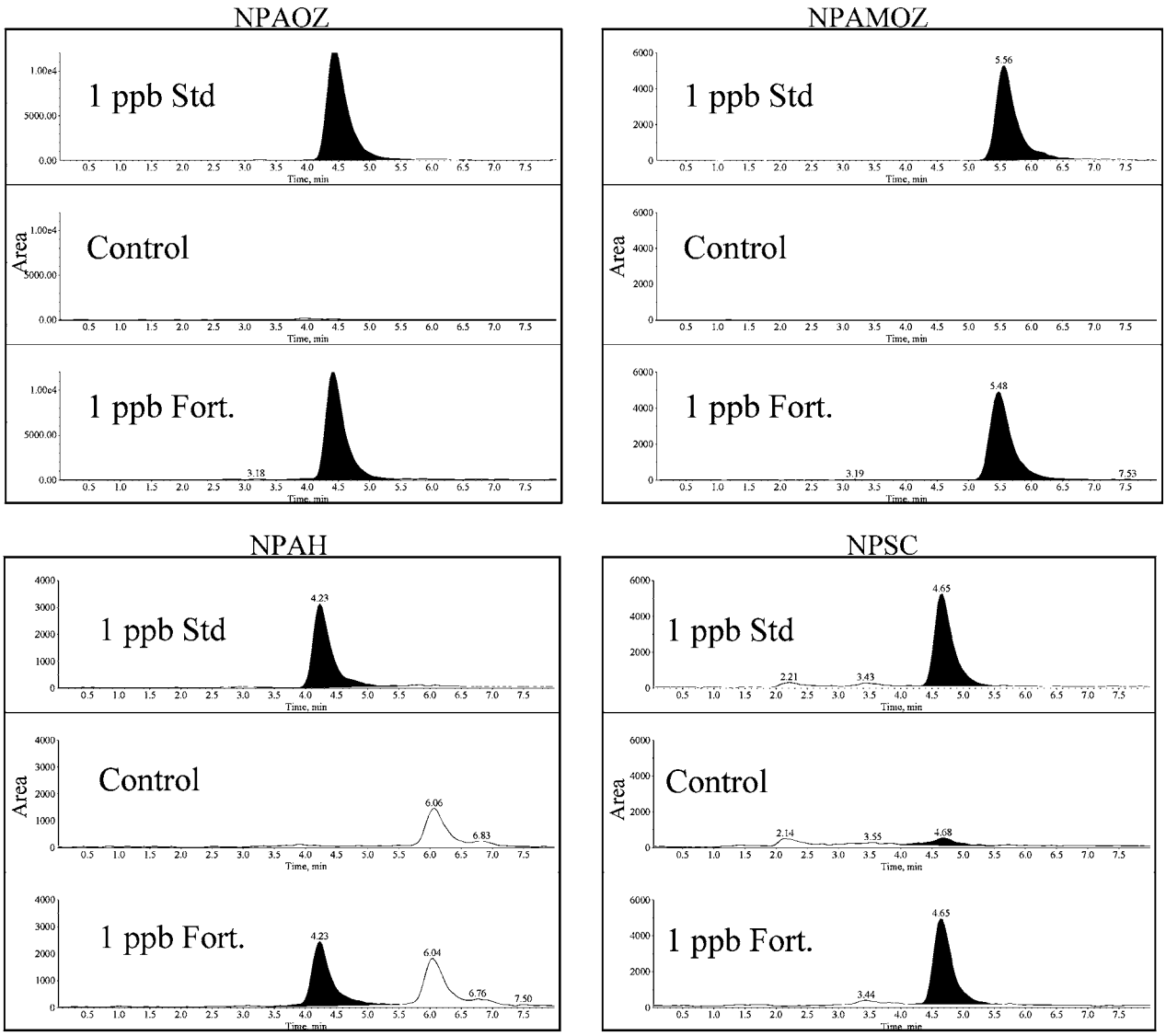

Figure 2. Chromatograms of NF bound residues extracted from shrimp: 1 ppb mixed standard; control; control fortified at 1 ppb.

Table 1. Method Performance on Fortified Shrimp ($n = 6$)

analyte	fortification level (ppb)	accuracy (%)	CV (%)
AOZ	1	89	4
	2	90	1
	4	88	3
SC	1	98	6
	2	94	2
	4	90	3
AH	1	102	11
	2	94	11
	4	91	10
AMOZ	1	99	6
	2	98	3
	4	97	4

Table 2. Levels of Nitrofuran Bound Residues Found in Treated Shrimp ($n = 5$)

analyte	incursion level	ppb found	CV (%)
AOZ	1	1.7	9
	2	2.7	8
SC	1	1.4	6
	2	3.0	5
AH	1	1.2	10
	2	2.6	17
AMOZ	1	1.3	8
	2	2.0	13

were performed under yellow sodium light. Then the entire assay was repeated under regular light to compare the difference in analyte responses. Little difference in peak response was found between regular and yellow sodium lights. All validation data presented in this paper were therefore generated under normal laboratory conditions without protection from light.

To increase the number of samples assayed per batch, we attempted to replace the metabolite standards with the synthetic NPAH, NPAOZ, NPSC, and NPAMOZ standards (referred to as the derivative standards). However, the method performed well only for the first two trials. Thereafter, the method accuracies were found to be >110%. The chromatograms of all four derivative standards showed the appearance of an extra peak, the intensity of which increased over time. It is possible that the derivative standards when stored at 4 °C might have degraded over time. For this reason, only the metabolite standards derivatized along with the samples should be used for quantitation.

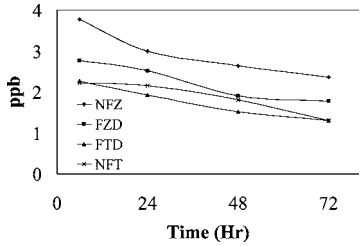

Figure 3. Depletion of bound residues of nitrofurans in shrimp after exposure of 0.25 ppm of FZD, 0.25 ppm of NFZ, 0.25 ppm of FTD, and 0.75 ppm of NFT.

The method was validated using control shrimp fortified with AH, AOZ, SC, and AMOZ at 1, 2, and 4 ppb for each analyte. Results of these analyses are presented in **Table 1**. Method accuracies of the four bound residues were >80% with CVs of <20%. Typical total ion chromatograms of control and fortified control shrimp are shown in **Figure 2**. No major interference peaks were detected in the retention times of NPAH, NPAOZ, and NPAMOZ. However, a small interference peak was detectable at the retention window of NPSC. Multiple sources of control shrimp were assayed, and the interference peak levels ranged from 0.1 to 0.3 ppb. As such, the limits of detection (LODs) were calculated as defined in the U.S. Pharmacopeia−National Formulary (*19*) and estimated to be 0.1, 0.3, 0.2, and 0.1 ppb for AOZ, SC, AH, and AMOZ, respectively. The limits of quantitation (LOQs) were estimated to be 0.1, 0.5, 0.4, and 0.1 ppb for AOZ, SC, AH, and AMOZ, respectively.

With the method validated for fortified shrimp, our next endeavor was to apply the method to shrimp treated with the four NFs. There is little literature on incursion studies that describes the levels of bound residues in shrimp. Stehly et al. (*15*) published a paper on FZD in shrimp that measured the parent FZD, not the bound metabolites. Using Stehly's dose as a starting point, we performed a pilot incursion study with a dose of 5 ppm of FZD in a water bath for 16 h. Six hours after drug withdrawal, the FZD bound residues level found was too high (~100 ppb). For method validation, the U.S. FDA Center for Veterinary Medicine guideline requires testing of the method on dosed animal tissues at the proximity of the target concentration. With trial and error, a dose of 0.25 ppm was found to be appropriate for FZD, NFZ, and FTD. However, the NFT bound residue level resulting from this dose was too low to be useful for method validation. Therefore, we increased the dose for NFT to 0.75 ppm, to achieve the desired target concentration. The method validation data for incurred residues are shown in **Table 2**. The depletion curves of the bound residues of the four nitrofurans over 72 h are shown in **Figure 3**. The bound residue levels of NFZ are the highest, followed by FZD and FTD, with NFT being the lowest. In a previous study, Paul et al. (*1*) demonstrated that the extent of urinary excretion in rat varied considerably with nitrofurans and that the percent of dose excreted in urine was trace for FZD, 3.4% for FTD, 4.6% for NFZ, and 52% for NFT. In channel catfish, Stehly and Plakas (*20*) reported that ~21% of the oral dose of radioactivity (predominantly as the unchanged NFT) was eliminated in the urine in 24 h. In another study, Plakas et al. (*21*) reported that ~41% of the oral dose of radioactivity (parent FZD below their limit of determination of 5 ng/g) was eliminated in urine of channel catfish in 24 h. The observed lower levels of NFT bound residues in our shrimp study correlate well with these studies in which NFT exhibits disposition characteristics different from those of the other three NFs.

The method described in this paper is capable of detecting low levels of SC. However, its detection in edible tissues may not constitute proof of illegal use of NFZ, as recent findings have linked the formation of SC with azodicarbonamide (*22*−*24*) and hypochlorite (*25*). The possible contamination of SC in packaged food was traced to the use of azodicarbonamide, a blowing agent, in the manufacture of foamed plastic gaskets used in glass jars (*24*). In a more recent paper, Pereira et al. (*22*) reported that azodicarbonamide is responsible for the SC contamination in processed chicken coated with flour. In light of these new findings, we fortified control shrimp tissues with SC prior to the prewashing steps and then processed the samples following the above method. After assay, ~5% of the fortified SC was found in the final extracts, indicating that ~95% of the unbound SC was removed in the prewashing steps. Although the prewashing steps markedly decrease the chance of false positive samples, the assay could not rule out the possibility that a positive sample is an outcome of environmental contamination. Research in the identification of a better marker compound is needed to address this issue.

This paper describes an LC-MS/MS method for the determination of bound residues of nitrofurans in shrimp. The method is sensitive to 1 ppb and suitable for regulatory purposes. It can be applied for research purposes in studying the depletion of bound residues in shrimp dosed with nitrofurans.

ABBREVIATIONS USED

FZD, furazolidone; NFZ, nitrofurazone; NFT, nitrofurantoin, FTD, furaltadone; AH, 1-aminohydantoin hydrochloride; SC, semicarbazide hydrochloride; 2-NBA, 2-nitrobenzaldehyde; AOZ, 3-amino-2-oxazolidinone; AMOZ, 3-amino-5-morpholinomethyl-2-oxazolidinone; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; MeOH, methanol; EtOAc, ethyl acetate; LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry.

ACKNOWLEDGMENT

We thank Rodney Williams of the University of Arizona for providing the incurred shrimp.

LITERATURE CITED

(1) Paul, M. F.; Paul, H. E.; Bender, R. C.; Kopko, F.; Harrington, C. M.; Ells, V. R.; Buzard, J. A. Studies on the distribution and excretion of certain nitrofurans. *Antibiot. Chemother.* **1960**, *10*, 287−302.

(2) *Federal Register*. Nitrofurans; Withdrawal of Approval of New Animal Drug Applications; Final Rule; Final Decision Following a Formal Evidentiary Public Hearing. *Fed. Regist.* **2005**, *56*, 41902−41912.

(3) *Federal Register*. Topical Nitrofurans; Extralabel Animal Drug Use; Order of Prohibition. *Fed. Regist.* **2002**, *67*, 5470−5471.

(4) Vroomen, L. H.; Berghmans, M. C.; Hekman, P.; Hoogenboom, L. A.; Kuiper, H. A. The elimination of furazolidone and its open-chain cyano-derivative from adult swine. *Xenobiotica* **1987**, *17*, 1427−1435.

(5) Vroomen, L. H.; Berghmans, M. C.; van Bladeren, P. J.; Groten, J. P.; Wissink, C. J.; Kuiper, H. A. *In vivo* and *in vitro* metabolic studies of furazolidone: a risk evaluation. *Drug Metab. Rev.* **1990**, *22*, 663−676.

(6) Hoogenboom, L. A.; van Kammen, M.; Berghmans, M. C.; Koeman, J. H.; Kuiper, H. A. The use of pig hepatocytes to study the nature of protein-bound metabolites of furazolidone: a new analytical method for their detection. *Food Chem. Toxicol.* **1991**, *29*, 321−328.

(7) Hoogenboom, L. A.; Berghmans, M. C.; Polman, T. H.; Parker, R.; Shaw, I. C. Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs. *Food Addit. Contam* **1992**, *9*, 623−630.

(8) Hoogenboom, L. A.; Polman, T. H.; Lommen, A.; Huveneers, M. B.; van Rhijn, J. Biotransformation of furaltadone by pig hepatocytes and *Salmonella typhimurium* TA 100 bacteria, and the formation of protein-bound metabolites. *Xenobiotica* **1994**, *24*, 713−727.

(9) McCracken, R. J.; Kennedy, D. G. Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography-thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland. *J. Chromatogr. B: Biomed. Sci. Appl.* **1997**, *691*, 87−94.

(10) Hoogenboom, L. A. P.; Polman, Th. T. H. Simultaneous detection of protein bound residues of the nitrofuran drugs furazolidone, furaltadone, nitrofurazone and nitrofurantoin. In *Proceeding of Euroresidue II Conference*; Haagsma, N., Ruiter, A., Czedik-Eysenberg, P. B., Eds.; Veldhoven, The Netherlands, 1993; pp 376−381.

(11) McCracken, R. J.; Blanchflower, W. J.; Rowan, C.; McCoy, M. A.; Kennedy, D. G. Determination of furazolidone in porcine tissue using thermospray liquid chromatography−mass spectrometry and a study of the pharmacokinetics and stability of its residues. *Analyst* **1995**, *120*, 2347−2351.

(12) Conneely, A.; Nugent, A.; O'Keeffe, M.; Mulder, P. P. J.; van Rhijn, J. A.; Kovacsics, L.; Fodor, A.; McCracken, R. J.; Kennedy, D. G. Isolation of bound residues of nitrofuran drugs from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection. *Anal. Chim. Acta* **2003**, *483*, 91−98.

(13) Conneely, A.; Nugent, A.; O'Keeffe, M. Use of solid-phase extraction for the isolation and clean-up of a derivatised furazolidone metabolite from animal tissues. *Analyst* **2002**, *127*, 705−709.

(14) Leitner, A.; Zollner, P.; Lindner, W. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography−tandem mass spectrometry. *J. Chromatogr. A* **2001**, *939*, 49−58.

(15) Stehly, G. R.; Plakas, S. M.; el Said, K. R. Liquid chromatographic determination of furazolidone in shrimp. *J. AOAC Int.* **1994**, *77*, 901−904.

(16) Rupp, H. S.; Munns, R. K.; Long, A. R. Simultaneous determination of nitrofurazone and furazolidone in shrimp (*Penaeus vannamei*) muscle tissue by liquid chromatography with UV detection. *J. AOAC Int.* **1993**, *76*, 1235−1239.

(17) Rupp, H. S.; Munns, R. K.; Long, A. R.; Plakas, S. M. Simultaneous determination of nitrofurazone, nitrofurantoin, and furazolidone in channel catfish (*Ictalurus punctatus*) muscle tissue by liquid chromatography. *J. AOAC Int.* **1994**, *77*, 344−350.

(18) Kumar, L.; Toothill, J. R.; Ho, K. B. Determination of nitrofuran residues in poultry muscle tissues and eggs by liquid chromatography. *J. AOAC Int.* **1994**, *77*, 591−595.

(19) *U.S. Pharmacopeia−National Formulary 1990*; U.S. Pharmacopeia Convention: Rockville, MD, 1989; p 1711.

(20) Stehly, G. R.; Plakas, S. M. Pharmacokinetics, tissue distribution, and metabolism of nitrofurantoin in the channel catfish (*Ictalurus punctatus*). *Aquaculture* **1993**, *113*, 1−10.

(21) Plakas, S. M.; el Said, K. R.; Stehly, G. R. Furazolidone disposition after intravascular and oral dosing in the channel catfish. *Xenobiotica* **1994**, *24*, 1095−1105.

(22) Pereira, A. S.; Donato, J. L.; De Nucci, G. Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products. *Food Addit. Contam.* **2004**, *21*, 63−69.

(23) Pereira, A. S.; Pampana, L. C.; Donato, J. L.; De Nucci, G. Analysis of nitrofuran metabolic residues in salt by liquid chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2004**, *514*, 9−13.

(24) Stadler, R. H.; Mottier, P.; Guy, P.; Gremaud, E.; Varga, N.; Lalljie, S.; Whitaker, R.; Kintscher, J.; Dudler, V.; Read, W. A.; Castle, L. Semicarbazide is a minor thermal decomposition product of azodicarbonamide used in the gaskets of certain food jars. *Analyst* **2004**, *129*, 276−281.

(25) Hoenicke, K.; Gatermann, R.; Hartig, L.; Mandix, M.; Otte, S. Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse? *Food Addit. Contam* **2004**, *21*, 526−537.

Received for review July 6, 2005. Revised manuscript received September 2, 2005. Accepted September 2, 2005.

JF051615O