Journal of Chromatography A, 1327 (2014) 90–96

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of nitrofuran metabolites in shrimp by high performance liquid chromatography with fluorescence detection and liquid chromatography–tandem mass spectrometry using a new derivatization reagent

Na-Na Du[a], Ming-Ming Chen[a,b], Liang-Quan Sheng[a,b,*], Shui-Sheng Chen[a,**], Hua-Jie Xu[a], Zhao-Di Liu[a], Chong-Fu Song[a], Rui Qiao[a]

[a] College of Chemistry and Chemical Engineering, Fuyang Normal College, Fuyang, Anhui 236041, China
[b] College of Chemistry and Chemical Engineering, Anhui University, Hefei, Anhui 230039, China

ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form
16 December 2013
Accepted 19 December 2013
Available online 28 December 2013

Keywords:
Nitrofuran metabolites
HPLC–FLD
LC–MS/MS
Shrimp

ABSTRACT

A high performance liquid chromatography with fluorescence detection (HPLC–FLD) method for the simultaneous determination of total nitrofuran metabolite residues (furazolidone, furaltadone, nitrofurantoin, and nitrofurazone) in shrimp was developed. The method involves the acid hydrolysis of protein-bound metabolites, followed by the derivatization of the freed metabolites with the new fluorescent derivatization reagent 2-hydroxy-1-naphthaldehyde (HN) and subsequent liquid–liquid extraction (LLE). Separation is achieved on a YMC-Pack Polymer C18 column under alkaline conditions, and the high fluorescence intensity of the derivatives at an emission wavelength E_m = 463 nm (E_x = 395 nm) enables, for the first time, their simultaneous determination in shrimp at concentrations as low as 1 μg/kg by HPLC–FLD. The method was validated using blank shrimp fortified with all four metabolites at 0.5, 1.0 and 2.0 μg/kg. Recoveries were >87% with relative standard deviations of <8.1% for all four metabolites. Furthermore, the results obtained by HPLC–FLD were in very good agreement with those obtained by LC–MS/MS analysis.

1. Introduction

Nitrofurans (NFs), mainly including furazolidone (FZD), nitrofurazone (NFZ), nitrofurantoin (NFT) and furaltadone (FTD), are broad-spectrum antibacterial drugs that were commonly used in veterinary medicine for the treatment of protozoan and bacterial infections [1] before considerable attention was paid to the potential human health risk posed by these compounds because of their carcinogenic and mutagenic potency. In 1993 and 1995, the use of NF drugs (FZD, NFZ, NFT and FTD) was banned in the EU for food animal production because of their negative impact on food safety and international trade [2,3]. However, NFs are still commonly used as veterinary medicines in some developing countries because of their cheapness and effectiveness. Therefore, it is essential to develop sensitive and reliable analytical methods for the detection of the residues of these compounds in edible animal tissues in order to meet the definitive EU minimum required performance level (MRPL), which was set at 1 μg/kg [4].

Previous studies have demonstrated that the parent NFs are rapidly degraded in animal and extensively metabolized to protein-bound metabolites [5,6], and thus methods for the detection of the parent drugs are not practical for most food products. Methods for the determination of NFs must therefore be based on the detection of their metabolites [1-aminohydantoin (AH), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ) and semicarbazide (SEM), respectively] [7]. It should be noted that SEM in foods may be produced by the degradation of NFZ or originate from other sources of contamination [8–11], but SEM often remains the marker residue for NFZ [9], although a more specific cyano metabolite marker for NFZ residues has been discovered [12]. These four stable and persistent tissue metabolites can be released from proteins under mildly acidic conditions, then derivatized in situ with a derivatization reagent [13], enabling detection in various sample matrices, such as pork [14], honey [15], meat [16], egg [17], milk [18], poultry muscle and shrimp [19], and fish feeds [20] using several different analytical techniques. The analytical methods mainly include LC–UV [20,21],

* Corresponding author at: College of Chemistry and Chemical Engineering, Fuyang Normal College, Fuyang, Anhui 236041, China. Tel.: +86 558 2593836.
** Co-corresponding author. Tel.: +86 558 2596249.
E-mail addresses: shenglq@fync.edu.cn (L.-Q. Sheng), chenss@fync.edu.cn (S.-S. Chen).

0021-9673/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.12.065

HPLC–DAD [22], HPLC–ED [23], LC–MS [24], LC–MS/MS [1,14,16], UPLC–MS/MS [25], ELISA [26,27], micellar electro kinetic capillary chromatography [28], immunochromatographic assay (ICA) [29] and thin-layer chromatography [30]. However, it can be difficult to detect residues at the 1 μg/kg level using some of these methods, such as LC–UV.

Despite the extensive availability of analytical methods for the determination of NF metabolites in animal tissues, so far only a few LC–MS/MS methods have been developed for the detection of these compounds in aquatic species [31,32]. Therefore, additional analytical techniques are required for the determination of NF metabolites in aquatic species at the 1 μg/kg level.

It should be noted that the confirmatory methods based on LC–MS/MS for the detection of NF metabolites at the MRPL of 1 μg/kg are in compliance with the regulation established by the EU Commission Decision of 13th March 2003 [4]. Therefore, most analytical methods for the determination of NF metabolites are based on LC–MS/MS [1,14,16]. LC–MS/MS has been widely used because with MS/MS it is possible to rapidly and selectively monitors the intensity of several fragments or transitions during chromatographic separations, however, the cost of such systems causes a real challenge for developing countries. On the other hand, fluorescence detectors (FLDs) are commonly used because of their high sensitivity, rapidity and ease of implementation. In general, the detection limit for a FLD is one to three orders of magnitude more sensitive than that for a diode array detector. Thus, it should be feasible to determine the levels of NF metabolites at the MRPL of 1 μg/kg by a high performance liquid chromatography with a fluorescence detector (HPLC–FLD). To the best of our knowledge, however, there have been no reports till date regarding the simultaneous analysis of the four NF metabolite residues at the 1 μg/kg level by HPLC–FLD.

Another common feature of the most widely employed analytical methods for the determination of NF metabolites is the use of derivatizing agents. A sample is first subjected to acid hydrolysis to release the protein-bound metabolites, which are then derivatized by a reaction of the released side chains with a derivatizing agent. Sample cleanup is then often achieved using liquid–liquid extraction (LLE) with the adjustment of pH, sometimes followed by solid-phase extraction (SPE) [1,14,33], a process that often requires a full day to complete.

The choice of derivatizing agent directly affects the sensitivity and selectivity of analytical methods. Derivatizing agents for amine analyses mainly include ortho-phthalaldehyde (OPA) [34], 2,3-naphthalene dialdehyde (NDA) [35], fluorescein isothiocyanate (FITC) [36], 6-oxo-(N-hydroxysuccinimidyl acetic acid ester)-9-(2′-methyloxocabonyl) fluorescein (SAMF) [37], etc. Separating effects on column were investigated by using OPA and FITC as derivatizing agents of NF metabolites by our group. However, OPA does not react with the NF metabolites, and the FITC derivatives of the metabolites cannot be effectively separated by LC because of the bulky group of FITC. In fact, only 2-nitrobenzaldehyde (2-NBA) and 2-naphthaldehyde (2-NTA) are commonly used as derivatizing agents for NF metabolites. These compounds cannot be used as fluorescent derivatizing agents for HPLC–FLD analysis, however, because of their low quantum yields. Thus, a novel derivatizing agent, 2-hydroxy-1-naphthaldehyde (HN), was developed. Generally speaking, the conjugated naphthalene ring of HN molecule is a good chromophoric group while the substituted hydroxyl group is strong auxochromic group because this functional group attached to the naphthalene ring can share the lone pair electrons of O atom with the chromophore. More importantly, the strong fluorescence intensity of HN will be improved owning to the strong electron-donating effect if phenolic hydroxyl is deprotonated to —O— anion. The strong fluorescence intensity of HN makes it suitable for detection by HPLC–FLD. One the other hand, the four derivatives of NF metabolites (AH–HN, SEM–HN, AOZ–HN and AMOZ–HN) are easily formed and much stable, which were previously synthesized and reported by our group [38–40].

With respect to sample cleanup, LLE without the adjustment of the pH and further SPE were adopted, enabling more rapid analyses and a reduction in lab turnaround times. While the procedure seems to be slightly complicated for quantitative analysis, it is easier to implement for real samples than previously reported procedures [1,14,33].

Using the newly developed method, all four derivatized metabolites are well separated on a YMC-Pack Polymer C18 column, thus enabling for the first time their simultaneous determination at the 1 μg/kg level by HPLC–FLD.

2. Experimental

2.1. Chemicals and materials

The derivatizing agent HN was purchased from HBC Chem. Inc. (United States), and AH–HCl, SEM–HCl, AMOZ and AOZ were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The purity of these compounds was greater than 99%. Boric acid (pro analysis grade) and potassium dihydrogen phosphate (pro analysis grade) were purchased from Qinye Chemical Reagent Factory (Shanghai, China). Ammonium formate and sodium hydroxide (pro analysis grade) were provided by Jingchun Chemical Reagent Factory (Shanghai, China). Methanol (HPLC gradient grade), acetonitrile (HPLC gradient grade), ethyl acetate (HPLC gradient grade), and formic acid (pro analysis grade) were supplied by Tianjin Jindong Tianzheng Precision Chemical Reagent Factory (Tianjin, China). Distilled deionized water was generated in-house using a Milli-Q Plus water system.

An Allsheng model D10 sample concentrator (Hangzhou Allsheng Instruments Co. Ltd., Hangzhou, China) was used for sample concentration. A Feige model 6L-20G centrifuge (Shanghai, China) equipped with a swing-out rotor with 10 and 50 mL centrifuge tube holders was used for centrifugation. Nylon syringe filters (13 mm and 0.2 μm) were obtained from Shanghai Peninsula Industrial Co. Ltd.

Live shrimp were purchased from eight supermarkets in the Anhui Province, China. The heads and shells of the undosed shrimp were removed. Next, the shrimp were ground in a mincing machine and stored at −20 °C until analysis. Dosing of the remaining shrimp was performed according to a previously reported procedure [41]. The shrimp were first acclimated for 7 days and then placed in separate aquaria containing 0.25 mg/L of NFZ. Eight hours after initiation of drug exposure, half of the water in each aquarium was removed and replaced with clean water containing the dose of NFZ to maintain the water quality. After 16 h, the treated shrimp were removed from the aquaria, rinsed with clean water and transferred to unmedicated aquaria for 6 h. The heads and shells were then removed, and the treated shrimp were collected, pooled and homogenized in a mincing machine. The homogenized shrimp tissues were stored at −20 °C until analysis.

The compounds with derivatized side chains are denoted with HN (e.g. AMOZ vs. AMOZ–HN).

2.2. Preparation of standard solutions

Standard stock solutions (400 μg/mL) of AOZ, SEM, AH and AMOZ were prepared by separately dissolving 10 mg of each compound in 25 mL of methanol. These solutions were immediately used to prepare an intermediate mixed standard solution containing 40 μg/mL analyte by dilution with methanol. Working standard solutions (2.0 μg/mL) were prepared by diluting the