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Simultaneous multiresidue determination of metronidazole and spiramycin in fish muscle using high performance liquid chromatography with UV detection

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ABSTRACT

An efficient multiresidue method for the simultaneous determination of metronidazole (MET) and spiramycin (SPY) in tilapia fish muscle, based on high performance liquid chromatography with UV detection (HPLC-UV), has been developed. The drugs were extracted with 0.2% orthophosphoric acid–methanol (6:4), and the extracts were cleaned up on a solid phase extraction cartridge, C18 Sep-Pak® light column. The LC separation was performed on a RP stainless-steel C-18 analytical column (150 mm × 4.6 mm, 5 μm) with a gradient elution system of 0.05 M phosphate buffer adjusted to pH 2.4–acetonitrile as the mobile phase at the flow rate of 1.0 ml min^{-1}. A wavelength programming was applied for the UV detection of the analytes. The method not only enabled the determination of the parent drugs, MET and SPY, but also permitted the determination of their metabolites, hydroxymetronidazole (HMET) and neospiramycin (NSPY). The calibration graphs for each drug were rectilinear in the range of 0.005–1.000 μg g^{-1} for MET and HMET and 0.025–1.000 μg g^{-1} for SPY and NSPY. With this method, the cited drugs with their metabolites were determined in fortified fish muscle tissues at levels of 0.025, 0.1 and 1.0 μg g^{-1} with good accuracy and precision. LOD and LOQ obtained for each drug were as follows: 0.002 and 0.005 μg g^{-1} for MET and HMET and 0.005 and 0.025 μg g^{-1} for SPY and NSPY. Utilization of the method to successfully analyze tilapia fish muscle samples incurred with MET and SPY was described.

1. Introduction

Nitroimidazoles are a well-established group of antiprotozoal and antibacterial agents. Metronidazole (MET) is a nitroimidazole derivative primarily used in the treatment of infections caused by Gram negative anaerobic bacteria like *Helicobacter pylori* and protozoans such as *Giardia lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis* [1]. In addition to human medicine, MET has also been used against several parasitic diseases in the veterinary field. Two additional effects have been shown: growth promotion and improvement of feed efficiency [2]. The compound has also shown beneficial effects in the treatment of parasitic infections in farmed fish. 5-Nitroimidazoles are known to be rapidly metabolized (the main metabolites result from oxidation of the side-chain at the C2 position of the imidazole ring) giving 2-hydroxymethyl metabolites [3,4]. Nitroimidazoles are suspected of being genotoxic, carcinogenic and mutagenic, as are their hydroxy metabolites having retained the original nitroimidazole ring (Fig. 1) [3,4]. However, MET is considered to be carcinogenic with sufficient evidence for animal carcinogenicity but inadequate evidence for human carcinogenicity [5]. Previous research indicates that it is considered to be mutagenic and cytotoxic in fish [6]. For this reason, MET, with a number of other nitroimidazoles, has already been banned in Europe by Council Regulation 613/98/EEC for MET [7]. However, irregular use or accidental contamination of feed may result in residues being present in edible tissues.

Macrolide antibiotics are a very important class of antibacterial compounds widely used in medical and veterinary practices. Spiramycin (SPY) belongs to the class of 16-membered macrolide antibiotics (Fig. 1). They are considered to be medium-spectrum antibiotics and are highly active against a wide range of Gram positive bacteria such as *Mycoplasma* and *Chlamydia*. Macrolides are the most effective medicine against diseases produced by *Mycoplasma* [8,9]. These drugs are well absorbed after oral administration and are distributed extensively in tissues, especially lungs, liver and kidneys, with high tissue/plasma ratios [10]. Therefore, these macrolides have been widely used in the rearing of food-producing animals to prevent and treat diseases. The EU has set the maximal residue limits (MRLs) for SPY in livestock products. The MRL in animal muscles is 0.2 ppm [11,12].

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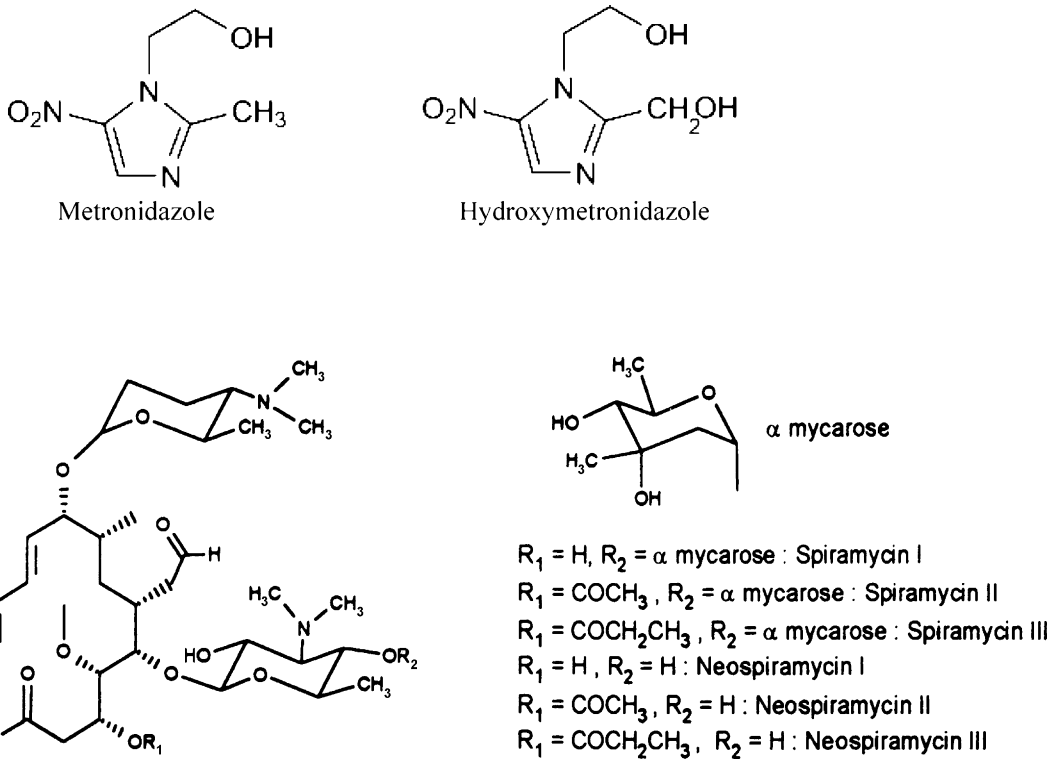

Fig. 1. Chemical structures of metronidazole (MET), spiramycin (SPY) and their main metabolites, hydroxymetronidazole (HMET) and neospiramycin (NSPY).

Aquaculture has expanded globally in the past 30 years and the industry has developed comprehensive plans to manage fish health; however, the need for antimicrobial therapeutants remains unavoidable in certain instances [13]. Macrolide antibiotics and nitroimidazole antiprotozoal drugs are classes of compounds which could potentially be used in fish. A combination of MET and SPY can be used in farmed fish to get benefit of their synergistic antibacterial action to treat diseases or as feed additives to promote growth.

Concerns about food hygiene have arisen regarding the presence of drug residues in livestock products [14]. Antibiotic residues may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or may indirectly cause problems through the induction of resistant strains of bacteria. Therefore, efficient analytical methods are required to monitor these drug residues in the edible tissues of livestock animals including fish tissues. High performance liquid chromatography (HPLC) is by far the most widely applied quantitative technique for the analysis of MET [15] or SPY residues [16,17] in fish tissues.

Multiresidue methods which will simultaneously determine more than one class of veterinary drugs in any matrix are still rare and are largely restricted to either microbiological, electrochemical, conductometric screening assays [18–20] or liquid chromatography–mass spectrometry (LC–MS) methods [21–23]. LC–MS methods are capable of identifying individual antibiotics within a class but involve relatively expensive and complex instrumentation, which may not always be available for routine monitoring [24]. HPLC with fluorescence detection has been applied for the simultaneous multiresidue determination of tetracyclines and fluoroquinolones in catfish muscle [24].

A combination of MET and SPY have been previously determined in human plasma, saliva and gingival crevicular fluid by LC–MS/MS [25]. Review of the literature reveals that no multiresidue methods have been reported until now for the simultaneous determination of both drugs in fish tissues. In addition, to our knowledge, SPY residues have not been determined in actual dosed fish (incurred) samples, only fortified tissue samples were used in previous studies [16,17]. Since incurred samples are closer to what would be found in an actual monitoring situation than samples which have only been fortified, this work is considered beneficial in this respect.

The goal of this work was to develop a validated simple and reliable HPLC-UV method for the simultaneous multiresidue determination of MET and SPY in tilapia muscle tissues. The proposed method not only enabled the determination of the parent drugs, MET and SPY, but also permitted the determination of their metabolites, hydroxymetronidazole (HMET) and neospiramycin (NSPY), without any matrix interference. This work is significant as it would provide an important alternative approach for multiresidue determination of members of more than one class of antibiotics in fish simultaneously. This efficient approach would be particularly useful in cases where multiclass LC–MS methods are not available, where an LC–MS instrument is not available for routine monitoring, or where quantitation alone is required [24].

2. Experimental

2.1. Materials and reagents

Metronidazole (99.7%) and spiramycin (4413 I.U./mg) were kindly supplied by Pharonia Pharmaceuticals, New Borg El-Arab City, Alexandria, A.R.E. Hydroxymetronidazole was obtained as a special gift through personal communications. Neospiramycin, the demycarosyl residue of spiramycin, was easily obtained from spiramycin in acidic media (pH, 2) [26,27]. All reagents used were of analytical grade, namely: acetonitrile (HPLC grade, Panreac Co., E.U.), methanol (BDH Chem. Ltd, Poole, England), sodium dihydrogenphosphate, phosphoric acid and sodium hydroxide (BDH, Poole, England). The water for HPLC was double glass distilled.

2.2. Instrumentation

The HPLC system (Thermo Sepatation® Products) consisted of SCM1000 vacuum degasser (Spectra SYSTEM®, USA), P 2000 pump (Spectra SYSTEM®, USA), UV 2000 variable-wavelength UV–VIS detector (Spectra SYSTEM®, USA) and AS 3000 autosampler fitted with a 200-μl sample loop (Spectra SYSTEM®, USA). HPLC separations were performed on a RP stainless-steel C-18 analytical column (150 mm × 4.6 mm) packed with 5 μm diameter particles (Alltech Associates, Inc., USA). Data were processed using EZ Stat® Software, (Spectra SYSTEM®, USA), on an OMEGA compatible PC connected to a Laser printer. For solid phase extraction, C 18 Sep-Pak® light column (Waters Sep-Pak®Vac, USA) was used.

2.3. Preparation of standards

Stock solutions (200 μg ml^{-1}) of MET, HMET and SPY were prepared in water. NSPY stock solution (100 μg ml^{-1}) was prepared from acid hydrolysis of SPY as follows: 1.0 ml volume of SPY (1000 μg ml^{-1}) was transferred into 10.0 ml volumetric flask, 3.0 ml volume of 0.2% orthophosphoric acid was added, the solution was mixed well and left at room temperature for 1 h. The solution was then neutralized by a pre-determined amount of 5 M sodium hydroxide aqueous solution then completed to the mark with dist. water. Regarding the stability of solutions of the cited drugs [4,9], stock solutions were freshly prepared and stored in a refrigerator in amber glass vessels. A fortification mixture of MET, HMET, SPY and NSPY (4 μg ml^{-1}) in water was prepared from these stock solutions on the day of an experiment. When needed for lower fortification levels, an additional dilution (1 μg ml^{-1}) was made on the day of an experiment.

2.4. Preparation of Nile tilapia muscle

2.4.1. Control fish tissue

Farm-raised adult tilapia (body wt. 90 ± 5 g) were obtained from Barseek Fish Farm, Behera province. Prior to the experiment, the fish were acclimatized for 15 days in three glass aquaria (90 cm × 60 cm × 50 cm/each) filled with de-chlorinated tap water under laboratory conditions (natural photoperiod 11.58–12.38 h and temperature of 25.8 ± 1.8 °C). Continuous aeration was maintained in each aquarium using an electric air pump. A commercial feed containing 25% crude protein was provided daily at 3% of body weight [28]. Dissolved oxygen, pH and electric conductivity of the tap water used in the experiment were determined by Hack Method (Sigma Laboratory) according to WHO [29]. The mean values for test water qualities were as follows: dissolved oxygen 6.5 mg l^{-1}, pH 7.1, electrical conductivity 219 ± 2 μmhom^{-1} cm^{-1}, temperature 29 ± 2 °C, alkalinity 124 mg l^{-1}, hardness 150 mg l^{-1} as $CaCO_3$ and no free chlorine. Tilapia muscle was cut into small pieces and blended with a food processor to a homogeneous consistency. The homogenized fish samples were stored at −80 °C until analysis.

2.4.2. Preparation of medicated diet

2.4.2.1. Preparation of MET medicated diet. MET was incorporated into a commercial production feed ration to provide 60 mg MET kg^{-1} fish per day when fish were fed 3% of their body weight. The commercial diet was ground to less than 0.5 mm. Ration with the drug added was thoroughly blended in a blender for 15 min. Dry ingredients were then mixed with distilled water in a commercial food mixer until a uniform mixture was obtained. The moistened mixture was passed through a meat grinder equipped with a 3-mm die to obtain uniform pellets. The same method described was used to prepare basal diet (no drug added). Pelleted diets were air-dried for 12–24 h at room temperature and then frozen at −20 °C until needed. Small quantities of diet were thawed and refrigerated at 4 °C until fed [30].

2.4.2.2. Preparation of SPY medicated diet. SPY was incorporated into a commercial production feed ration in the same manner as described above to provide 40 mg kg^{-1} fish when fish were fed 3% of their body weight per day.

2.4.2.3. Preparation of MET and SPY medicated diets. The two drugs were incorporated into the same commercial production feed ration using the same method as described above to provide 30 mg MET and 20 mg SPY kg^{-1} fish when fish were fed 3% of their body weight.

2.4.3. Preparation of incurred fish

Three flow-through tanks with supplemental aeration were used, each stocked with 20 fish (body wt. 90 ± 5 g). The fish were allowed to acclimate in a 15-day period, which was followed by a 5-day medication period then a 5-day withdrawal period. During the 5-day medication period the fish were given MET medicated diet for tank 1, SPY medicated diet for tank 2 or a combination of MET and SPY medicated diet for tank 3 (fed at 3% body weight), while during the acclimation and withdrawal periods the fish were given basal diet, drug-free, at the same rate (3%). Water quality parameters were measured every 8 days and maintained at the levels mentioned under Section 2.4.1.

2.4.4. Sampling of incurred fish

Immediately prior to the first feeding of the medicated diet, for each of the three tanks, three fish were randomly sampled as controls from each tank and the rest of the fish were weighed to calculate the weight of diet to be fed. This first sampling was followed by two samplings during the 5-day medication period (after days 3 and 5 of medication) and three samplings during the 5-day withdrawal period (after days 6, 8 and 10 from the medication, i.e. days 1, 3 and 5 of withdrawal). At each sampling, three fish were removed from each tank, (three replicates per sampling), and weighed. All fish sampled were filleted and the fillet was frozen at −80 °C. After each tank sampling, the amount of feed administered was adjusted to account for the new body weight of the group.

2.4.5. Fortification and extraction of drugs from fish muscle

Homogenized fish muscle samples (2.0 g) were placed in 50 ml screw-capped polypropylene centrifuge tubes. Appropriate volumes of MET, HMET, SPY and NSPY fortification mixtures or their dilutions (Section 2.3) were added to control fish muscle to generate the desired fortification levels. In place of a fortification mixture or its dilution, an equal volume of water was added to control and incurred samples for a given experiment. Samples were homogenized at high speed for 2 min with 50 ml of 0.2% orthophosphoric acid–methanol (6:4, v/v) used as the deproteinizing extractant. After centrifugation (5 min, 3716 × *g*), the supernatant was filtered through a 0.45-μm membrane filter. The filtrate was passed through a C18 Sep-Pak® light column used as a solid phase extraction cartridge, previously conditioned with 5.0 ml methanol followed by 5.0 ml water. The cartridge was then eluted with 5 ml methanol. The eluate was evaporated to dryness under reduced pressure and the residue was re-suspended in 1.0 ml water. Portions of 20 μl volume were then injected into the HPLC system.

2.5. HPLC conditions

A gradient elution program was used with solvent A (0.05 M phosphate buffer adjusted to pH 2.4) and solvent B (acetonitrile) as follows: 15% B (3.5 min), 15–50% B (2 min), 50% B (4.5 min) then

50–15% B (5 min). The system was conditioned with 15% solvent B for 15 min before the next injection. The mobile phase was degassed and filtered by passing through a 0.45-μm pore size membrane filter (Millipore, Milford, MA, USA) prior to use. The samples were also filtered using 0.45-mm disposable filters. The flow rate was 1 ml min^{-1}. All determinations were performed at ambient temperature with an injection volume of 20 μl. The solutions were analyzed by a UV detector with wavelength programming. During the first 4.0 min of the analysis, the wavelength was set at 319 nm then shifted to 232 nm for 11.0 min.

3. Results and discussion

3.1. Method development

3.1.1. Indicator component

Macrolides are mostly produced as complex mixtures of related components [8]. SPY consists of three components, spiramycin I (SPY-I), II and III; SPY-I is the major component. It is difficult to monitor all the components of SPY in animal tissues. When the residual level is high enough to detect each component, it is preferable to monitor each component. However, the residual drug level in animal tissues is actually too low. Therefore, it is thought to be a practical measure to assume the main component of the drug to be an indicator to evaluate the residual level [9,17]. Accordingly, SPY-I was an indicator for SPY. It is known that SPY is metabolized to neospiramycin, NSPY in animals [17,31]. The primary metabolite NSPY has an estimated antimicrobial activity of ca. 90% compared to SPY [17,31]. Therefore, NSPY-I, or simply NSPY, was also simultaneously analyzed.

3.1.2. Sample extraction and clean-up

Tissue samples contain many diverse compounds in addition to the possible traces of the target analytes. So it is very important to extract as much as possible of the determined analytes and also to exclude interfering substances present in biological matrices. Different extraction and cleanup methods have been developed [9,15,17].

First, the extracting solvents were examined. Horie et al. [9] extracted SPY from muscle samples with a mixture of 0.3% metaphosphoric acid–methanol (7:3). However, when this extracting solvent was used, the pH of the extracts was 3.9–4.5. Generally, macrolide antibiotics are unstable in acidic media [16]. Therefore, later on, the same author used a mixture of 0.2% metaphosphoric acid–methanol (6:4) for the sample extraction [17]. When this extracting solvent was used, the pH of the extracts was 4.5–5.0. In addition, the extraction with 0.1% metaphosphoric acid–methanol (6:4) was not effective enough with regard to deproteinization [17]. Consequently, in this work, a mixture of 0.2% orthophosphoric acid–methanol (6:4) was used for sample extraction since it enabled satisfactory determination of both MET and SPY, together with their metabolites.

Secondly, sample cleanup procedures were studied. In previous work [9,17] describing the simultaneous determination of macrolide antibiotics in animal tissues, the samples were extracted with metaphosphoric acid–methanol and the cleanup procedure was performed using different types of solid phase extraction (SPE) cartridges, e.g., a pre-packed cation exchange resin cartridge [9] or C18 (ODS) cartridge [17]. Also analytical methods based on SPE technique have been reported for the determination of MET and HMET in fish tissues where C 18 cartridges were used for the cleanup procedure [15]. Since the latter is the most widely used solid-stationary phase for extraction and cleanup, a reversed phase C18 Sep-Pak® light column (Waters Sep-Pak®Vac, USA) which is an octadecyl silane bonded to silica was used in this work together with methanol as eluting solvent.

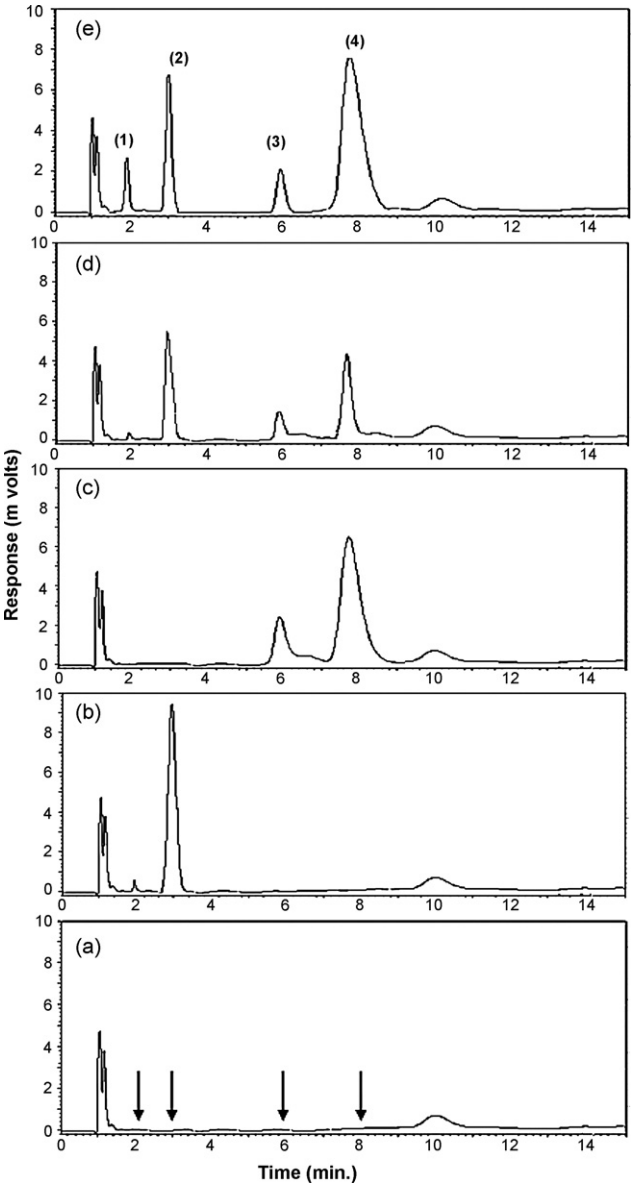

Fig. 2. HPLC chromatogram of tilapia fish muscle extract: (a) control, (b) MET-incurred, (c) SPY-incurred, (d) MET and SPY-incurred and (e) control muscle extract after fortification with 0.15 μg g^{-1} HMET: (1), 0.4 μg g^{-1} MET, (2), 0.2 μg g^{-1} NSPY and 0.8 μg g^{-1} SPY. Samples were taken on day 5 dosing. Arrows indicate retention times of the analytes.

As a result, fish tissue samples were extracted with a mixture of 0.2% orthophosphoric acid–methanol (6:4) followed by a cleanup procedure using a reversed phase C18 extraction cartridge. This was found optimal for the determination of both MET and SPY, together with their metabolites, with a good recovery without any interference from coexisting substances (Fig. 2).

3.1.3. Optimization of chromatographic conditions

MET and SPY have relatively strong UV absorption [32], so that UV detection is sufficiently sensitive in the determination of these drugs. In the HPLC-UV analysis of chemicals, it is extremely important that an appropriate detection wavelength is to be selected without any interference from contaminants. The maximum absorption wavelength of the compound to be analyzed is

generally employed as the wavelength of detection [9]. Maximum absorbances were detected for MET, HMET and SPY, NSPY aqueous solutions at 319 and 232 nm, respectively. Therefore, for maximum sensitivity, a wavelength programming was applied. The UV detector was first adjusted at 319 nm for 4.0 min, sufficient for the elution of MET and HMET, then shifted to 232 nm for 11.0 min through which SPY and NSPY were determined.

As the structures of MET, HMET and SPY, NSPY are different (Fig. 1), it was not possible to resolve them in biological matrices in the same run using isocratic conditions [25]. Consequently a gradient elution was used. The optimum gradient system was obtained with solvent A (0.05 M phosphate buffer, adjusted to pH 2.4) and solvent B (acetonitrile) as follows: 15% B (3.5 min), 15–50% B (2 min), 50% B (4.5 min) then 50–15% B (5 min). It was noticed that the capacity factor (k) significantly decreased for MET, HMET and SPY, NSPY as the acetonitrile concentration rose. However, acetonitrile content of more than 20% in the mobile phase would prevent the resolution of the hydroxy metabolite of MET (HMET) from its parent drug (MET). After MET and its metabolite had been completely eluted, acetonitrile % was increased to fasten the elution of SPY and its metabolite, NSPY, with acceptable peak shape. The gradient elution method described above was then adopted in this experiment.

It is known that a disadvantage of HPLC of basic substances on silica-based reversed phases is peak tailing due to interaction with residual silanols on the silica gel. Similar to a number of other macrolide antibiotics, SPY, which is a basic molecule containing amino sugars in its structure, is strongly affected by silanol groups in the column packing material [9,17]. The choice of adequate conditions for the HPLC procedure is governed by the ionizable groups, amino sugar(s), of the macrolide antibiotics. Consequently, the pH of the aqueous phase has a drastic effect on the peak shape and retention time of SPY and NSPY. The simple aqueous acetonitrile mobile phase has a pH of around 7 which is thought to dissociate any residual silanols to weakly anionic species that strongly retain the basic macrolide antibiotic, SPY. To prevent this, the mobile phase pH was adjusted to be acidic [9,17]. The asymmetry and retention time of SPY and NSPY peaks increased significantly with increasing the pH in the range (2.0–5.5). Generally, macrolide antibiotics are unstable in acidic solutions [9,17]. However, as found before [9,17], investigations of the stabilities of 2.0 $\mu g\,ml^{-1}$ of SPY in the mobile phase, 0.05 M phosphate buffer pH 2.4–acetonitrile (50:50), at room temperature for 30 min demonstrated that they were relatively stable under these conditions. Consequently, the use of an acidic HPLC mobile phase did not present a problem.

A liquid chromatogram illustrating the separation of the analytes in a fortified fish muscle extract sample is shown in Fig. 2e. Comparison to a chromatogram of control fish muscle extract in Fig. 2a shows no significant interferences present in the control sample.

Under these conditions, The chromatographic characteristics of the mixture are summarized in Table 1 which indicates that the proposed HPLC method permitted adequate resolution of the mixture's components (good resolution and selectivity values) within reasonable run-time, HMET being eluted at 1.95 min, MET at 3.12 min, NSPY at 6.01 min and SPY at 7.81 min (suitable capacity factors). In addition, high column efficiency was indicated from the large number of theoretical plates. The degree of peak asymmetry was also evaluated using the tailing factor which did not exceed the critical value (1.2) indicating acceptable degree of peak asymmetry.

The gradient elution adopted in the present study has a relatively lower repeatability than isocratic elution [9]. Thus, the repeatability of the retention time and of the peak area of MET, HMET and SPY, NSPY was examined (Table 2). Satisfactory results were obtained under these conditions: relative standard deviations (R.S.D.%) for retention time of 0.25% or less, and R.S.D.% of peak area of 0.56–1.89%. The peaks obtained were sharp and had clear baseline separation.

Table 1
Chromatographic characteristics of HMET, MET and NSPY, SPY using the proposed HPLC method.

Compound	t_R	N	k	α	R_s	T_f
HMET	1.95	1394	1.00			1.035
				2.14	4.00	
MET	3.12	1240	2.14			1.100
				2.40	8.07	
NSPY	6.01	4058	5.14			1.151
				1.36	2.68	
SPY	7.81	1024	7.00			1.025

t_R: retention time, in minutes. N: number of theoretical plates. k: capacity factor. α: selectivity, between each two successive peaks. R_s: resolution, between each two successive peaks. T_f: tailing factor.

Table 2
Repeatability of retention time and peak area of HMET, MET and NSPY, SPY using the proposed chromatographic conditions.

Drug	Retention time (R.S.D.%)[a]	(R.S.D.%)[a] of peak area
HMET	1.95 (0.21)	1.89
MET	3.12 (0.08)	0.56
NSPY	6.01 (0.25)	0.66
SPY	7.81 (0.19)	1.67

[a] $n = 5$.

3.2. Method validation

3.2.1. Linearity

Five point calibration curves were prepared for each analysis day. Quantitation was based on the peak area for each analyte. The calibration curves were found to be linear in the range of 0.005–1.000 $\mu g\,g^{-1}$ muscle tissues ($R = 0.9993, 0.9991$) for MET and HMET and 0.025–1.000 $\mu g\,g^{-1}$ muscle tissues ($R = 0.9992, 0.9995$) for SPY and NSPY, respectively.

3.2.2. Accuracy

Accuracy of the method was tested by fortification of control fish muscle samples with the four analytes at three different known levels (0.025, 0.5, and 1.0 $\mu g\,g^{-1}$), extraction, analysis, and determination of the recovery for each analyte. Data for these experiments are shown in Table 3. Good results were obtained, with average recoveries ranging from 69.5 to 93.8%. It is interesting to note that fish muscle samples did not require matrix matched calibration

Table 3
Recoveries of HMET, MET and NSPY, SPY in fortified tilapia fish muscle.

Fortification conc. ($\mu g\,g^{-1}$)	Recovery% (R.S.D.%)			
	MET	HMET	SPY	NSPY
0.025[a]	92.4 (5.7)	79.2 (3.5)	84.5 (2.5)	71.3 (4.5)
0.025[a]	93.1 (2.0)	82.4 (1.8)	85.8 (2.9)	77.8 (6.8)
0.025[a]	82.2 (4.3)	80.3 (3.1)	92.1 (1.2)	69.5 (4.4)
Average[b]	89.2 (6.1)	80.6 (1.6)	87.5 (4.1)	72.9 (4.4)
0.5[a]	81.8 (4.6)	77.1 (2.9)	73.6 (3.2)	83.5 (3.2)
0.5[a]	90.8 (6.5)	80.0 (4.2)	78.5 (2.5)	77.1 (4.2)
0.5[a]	87.5 (2.8)	81.0 (1.7)	84.7 (2.1)	75.9 (2.5)
Average[b]	86.7 (4.6)	79.4 (2.0)	78.9 (5.6)	78.8 (4.1)
1.0[a]	93.8 (1.8)	85.5 (3.2)	81.2 (1.4)	77.6 (1.8)
1.0[a]	90.5 (1.2)	70.4 (2.2)	91.8 (3.2)	82.5 (3.8)
1.0[a]	88.5 (2.8)	74.5 (2.9)	77.8 (1.9)	81.7 (4.1)
Average[b]	90.9 (2.7)	76.8 (7.8)	83.6 (7.3)	80.6 (2.6)

[a] $n = 5$.
[b] $n = 15$.

Table 4
Analysis of MET and SPY-incurred tilapia fish muscle.

	Measured ($\mu g\,g^{-1}$) (R.S.D.%)[a]				Dilution	Actual ($\mu g\,g^{-1}$)			
	HMET	MET	NSPY	SPY		HMET	MET	NSPY	SPY
1. MET-incurred fish									
Day 3 dosing	0.014 (1.6)	0.713 (2.1)	–	–	1:5	0.071	3.566	–	–
Day 5 dosing	0.012 (2.7)	0.564 (1.5)	–	–	1:10	0.118	5.640	–	–
Day 1 withdrawal	0.012 (4.4)	0.587 (3.4)	–	–	1:5	0.059	2.933	–	–
Day 3 withdrawal	0.013 (1.9)	0.617 (3.1)	–	–	1:2	0.026	1.233	–	–
Day 5 withdrawal	0.015 (2.4)	0.524 (1.7)	–	–	–	0.015	0.524	–	–
2. SPY-incurred fish									
Day 3 dosing	–	–	0.265 (1.9)	0.741 (2.6)	1:5	–	–	1.325	3.705
Day 5 dosing	–	–	0.225 (1.5)	0.640 (2.6)	1:10	–	–	2.251	6.400
Day 1 withdrawal	–	–	0.261 (2.4)	0.754 (1.7)	1:5	–	–	1.311	3.770
Day 3 withdrawal	–	–	0.295 (1.9)	0.846 (1.4)	1:2	–	–	0.592	1.691
Day 5 withdrawal	–	–	0.221 (2.8)	0.630 (2.0)	–	–	–	0.221	0.630
3. MET- and SPY-incurred fish									
Day 3 dosing	0.006 (3.6)	0.355 (1.9)	0.135 (2.1)	0.377 (1.4)	1:5	0.032	1.775	0.675	1.885
Day 5 dosing	0.006 (1.7)	0.279 (2.7)	0.130 (1.4)	0.395 (1.2)	1:10	0.064	2.794	1.300	3.952
Day 1 withdrawal	0.005 (1.9)	0.290 (1.4)	0.084 (1.9)	0.401 (2.3)	1:5	0.025	1.450	0.422	2.005
Day 3 withdrawal	0.007 (2.6)	0.311 (3.5)	0.155 (2.6)	0.466 (1.7)	1:2	0.014	0.622	0.312	0.932
Day 5 withdrawal	0.008 (2.6)	0.266 (1.9)	0.105 (3.3)	0.320 (2.9)	–	0.008	0.266	0.105	0.320

[a] $n = 5$.

curves to compensate for difficulties in measuring peak areas of the determined analytes at low concentrations, making more rapid analysis possible [24].

3.2.3. Precision

The method exhibited excellent precision, as shown in Table 3. Fortification/recovery experiments resulted in low intraday relative standard deviations (R.S.D.%) for all analytes ($n = 5$, R.S.D.% < 7%). A comparison of fortification/recovery experiments conducted on three different days ($n = 15$) also displayed low inter-day R.S.D.% (<8%), confirming the excellent reproducibility of the method.

3.2.4. Limits of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal to noise ratio (S/N) of 3 and 10, with a R.S.D. value less than 10%, respectively. LOD and LOQ were obtained for each drug and were as follows: 0.002 and 0.005 $\mu g\,g^{-1}$ for MET and HMET and 0.005 and 0.025 $\mu g\,g^{-1}$ for SPY and NSPY.

3.2.5. Assay specificity

The specificity of the method was demonstrated by comparing chromatograms of blank and fortified samples. Fig. 2a and e demonstrates that no interferences were detected from endogenous substances with the analytes.

3.3. Analysis of MET- and SPY-incurred fish

It is important, whenever possible, to test a method for detection of veterinary drug residues using actual dosed animal (incurred) samples as these samples are closer to what would be found in an actual monitoring situation than samples which have only been fortified [15,24]. This was accomplished, and the results obtained are summarized in Table 4. For all cases, four of the five incurred samples contained high enough levels of MET and/or SPY that required dilution of the samples with control muscle tissue prior to extraction and analysis in order for the drug levels to fall within the range of the calibration curve for the method. The "actual" values for MET, HMET and SPY, NSPY, after correction for the dilution, are shown in the last columns of the table. As done in previous work [30], the data obtained were based on pooled muscle samples, each from three incurred tilapia fish, to average out differences in fish feeding behavior.

3.3.1. MET-incurred fish

Results from analysis of MET-incurred fish muscle samples are shown in Table 4, and a sample chromatogram from day 5 of dosing (1:10 dilution) is shown in Fig. 2b. The method also enabled the determination of the 2-hydroxy metabolite (HMET) along with MET, being eluted at 1.95 and 3.12 min, respectively. Relatively high levels of MET are present in the muscles during dosing; these levels decrease during withdrawal, as might be expected. It is interesting to note that the levels observed in this study are similar to those in which MET-dosed catfish muscle samples were analyzed by another method [15]. The fraction of HMET to MET was 2% or less, as was also observed in previous work [15].

3.3.2. SPY-incurred fish

Results from analysis of SPY-incurred fish muscle samples are shown in Table 4, and a sample chromatogram from day 5 of dosing (1:10 dilution) is shown in Fig. 2c. The metabolite neospiramycin-I (NSPY) was detected along with the parent SPY, being eluted at 6.01 and 7.81 min, respectively. The concentrations of both SPY and its metabolite in treated samples are shown in Table 4. Again, the pattern shows high levels of SPY and NSPY during dosing, and decreasing levels during withdrawal.

3.3.3. MET and SPY-incurred fish

Samples from fish subjected to a combination of MET and SPY medication were analyzed using the proposed chromatographic conditions as mentioned above. Both MET and SPY along with their main metabolites, HMET and NSPY, were detected. A sample chromatogram from day 5 of dosing (1:10 dilution) is shown in Fig. 2d. The results shown in Table 4 indicate that high levels of drugs and their metabolites are found during dosing and then decrease throughout the withdrawal.

4. Conclusion

MET and SPY are widely used in veterinary medicine to treat diseases or as feed additives to promote growth. The use of these compounds can result in the appearance of residues in livestock products. The residues can result from incorrect use of the drugs and lack of withdrawal times and pharmacokinetic data, and may have toxic effects on consumers. An efficient method for the simultaneous determination of MET and SPY, together with their

metabolites, HMET and NSPY, in tilapia fish muscle, using high performance liquid chromatography with UV detector, has been developed. Good recoveries (69.5–93.8%) and excellent R.S.D. (<8%) were obtained, with low limits of quantitation, 0.005 $\mu g\,g^{-1}$ for MET, HMET and 0.025 $\mu g\,g^{-1}$ for SPY, NSPY. The utility of the method was further shown by its determination of drug levels in incurred tilapia fish muscle samples. This method provides a valuable approach for the simultaneous determination of members of more than one class of antibiotics in fish muscle.

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