Journal of Chromatographic Science Advance Access published July 15, 2015

Journal of Chromatographic Science, 2015, 1–9
doi: 10.1093/chromsci/bmv095
Article

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Comparative Study of Different Chromatographic Techniques for the Analysis of Multi-Residues of Some Approved Antimicrobials in Fish Tissues

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Received 31 July 2014; Revised 14 February 2015

Abstract

Two chromatographic methods were developed, optimized and validated for the simultaneous determination of three approved aquaculture antimicrobials, namely sulphadimethoxine sodium, trimethoprim and florphenicol in fish tissues. The developed methods were based on simple liquid extraction technique. The first method employs thin-layer chromatography as a clean-up procedure coupled with densitometric determination for the separated drugs. The second method is an HPLC one using X-Terra™ C_{18} column. Several mobile-phase systems and extracting solvents were tried to optimize the separation and the extraction procedures from fish tissues. The procedures were applied for the analysis of spiked fish tissue samples at three different concentration levels (10, 50 and 100 ppm). A comparative study was conducted between the proposed methods to discuss the advantage of each one. The methods were validated according to the international conference on harmonization guidelines. The proposed methods were successfully applied for the determination of the studied drugs in spiked fish tissues, pure powders and in their veterinary pharmaceutical formulation.

Introduction

The use of antimicrobials in food-producing animals has generated considerable interest. The wide spread administration of these drugs may lead to the development of resistant human pathogens (1). Antimicrobials are used in aquaculture during both production and processing, mainly to prevent (prophylactic use) and treat (therapeutic use) bacterial diseases (2).

The use of antimicrobials in the aquatic environment would cause concern, in terms of both potential environmental impact and potential human health implications (3). Such potential hazards associated with the use of antimicrobials in animal production, results in the recent implementation of more strict regulations on the general prophylactic use of chemicals in aquaculture and their residual limits in fish tissues.

Several international organizations have produced recommendations on the use of antimicrobial agents in veterinary medicine to reduce the overuse or the misuse of antimicrobials in animals (4). The drugs approved for use and their treatment protocols are tightly regulated. The use of specifically banned antibacterial is a violation of regulations. A number of antimicrobials are approved by FDA, to be used safely in aquaculture production from which are sulphadimethoxine sodium (SDM) and trimethoprim (TMP) combination as well as florphenicol (FLOR) and directed to be used as feed additives (http://www.fda.gov/animalveterinary/developmentapprovalprocess/aquaculture/ucm132954.htm).

Sulphadimethoxine sodium (Fig. 1a) is a member of sulphonamides group of drugs. Trimethoprim (Fig. 1b) is one of the most widely used antibacterial additives that act synergistically in combination with sulphonamides. These combinations are commonly used in food-producing animals as growth promoters and as therapeutic and prophylactic drugs (5). The presence of residues of sulfonamides in fish is of toxicological and regulatory concern as some of them could be carcinogenic and cause allergic hypersensitivity reactions and therapeutic ineffectiveness in human beings (6).

Several analytical methods have been reported in the literature for the determination of sulphadimethoxine individually or in combination with other sulphonamides and trimethoprim in different matrices. These methods include HPLC (7–9), LC–MS (10–12), immunoassays (13) and capillary electrophoresis (14).

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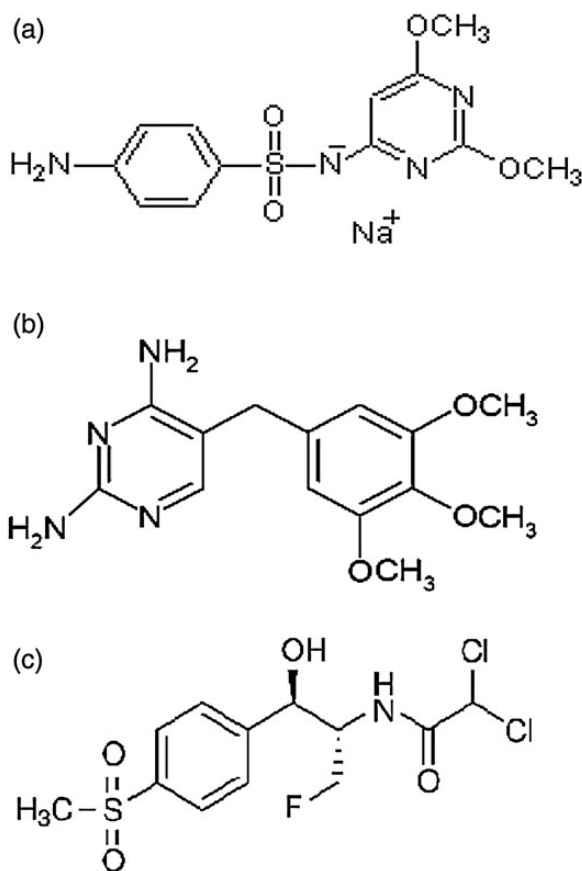

Figure 1. Chemical structure of (a) sulphadimethoxine sodium, (b) trimethoprim and (c) florphenicol.

Florphenicol (Fig. 1c) is a broad spectrum, primarily bacteriostatic, antibiotic with a range of activity similar to that of chloramphenicol. However, florphenicol does not carry the risk of inducing human aplastic anemia that is associated with chloramphenicol. Because of this, chloramphenicol has been banned and florphenicol was permitted as a substitute for use in food-producing animals.

The literature review revealed several analytical methods for quantitative estimation of florphenicol alone or in combination with other drugs in different matrices. These methods include GC (15), HPLC (16, 17), HPTLC (18) and LC–MS (19–21).

The aim of the work was to develop simple analytical methods for the simultaneous multi-residues determination of antimicrobials of different chemical classes in biological fish tissue samples without preliminary separation; employing simple liquid extraction procedure with minimal clean-up steps of the samples.

Experimental

Materials

Pure samples

Standard sulphadimethoxine sodium (SDM) and trimethoprim (TMP) were kindly supplied by Pharma Swede, Cairo, Egypt. Sulphadimethoxine sodium purity was found to be 99.87 ± 0.854 according to the official HPLC method (22). Trimethoprim purity was found to be 100.63 ± 0.777 according to the official nonaqueous titration (22). Florphenicol (FLOR) was kindly supplied by Jiang Su Guo International Group Co. Ltd (China) and its purity was found to be 99.41 ± 0.958 according to a reported HPLC method (23).

Pharmaceutical formulations

Trimethoxin® solution was manufactured by Chemifarma and it was purchased from the Egyptian local market. Each 1 mL of it was claimed to contain 200 mg sulphadimethoxine sodium and 40 mg trimethoprim, batch number (BN): A120009.

Felorex® injectable solution was manufactured by Arabcomed and it was purchased from the Egyptian local market. Each 1 mL of it was claimed to contain 300 mg florphenicol, BN: 0270/12.

Reagents

Acetonitrile, methanol, chloroform, toluene, *n*-hexane and HPLC grade were supplied by Sigma-Aldrich, Germany. Ethyl acetate, glacial acetic acid, ethanol, potassium dihydrogen orthophosphate and analytical grade were purchased from EL-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt. All reagents and chemicals used were of analytical grade.

Instruments

For TLC-spectrodensitometry

Thin-layer chromatographic plates (20 × 20 cm) coated with 0.2 mm silica gel 60 F_{254} were obtained from Sigma-Aldrich, Germany. The samples were applied to the plates using CAMAG Linomat 5 autosampler with Camag micro syringe (100 µL). TLC scanner densitometer model 3 S/N 130319 and Camag TLC scanner model 3 S/N 1302139 with win CATS software for densitometric evaluation was obtained from Camag, Muttenz, Switzerland.

For HPLC

Liquid chromatographic separation was done using Agilent 1100 Series liquid chromatograph consisted of a dual pneumatic pumping system (model G1310A), an ultra-violet variable wavelength detector (model G1314A (and a Rheodyne injector (model 7725 I equipped with 20-µL injector loop, Agilent (Santa Clara, CA, USA). The stationary phase was X-Terra™ C_{18} (250 × 4.6 mm, 5 µm) analytical column, Waters.

For extraction procedure from fish tissues, homogenizer (Heidolph, Diax 900-Germany), vortex (Janke & Kunel- Germany) and shaker (Bibby Srerilin Ltd-UK) were used.

Standard solutions

Stock standard solutions

Stock solutions (each, 1 mg/mL) of SDM, TMP and FLOR were prepared in methanol for TLC-spectrodensitometry and in mobile phase for the HPLC method. This was done by accurately weighing 10 mg of pure powder of each into three separate 10-mL volumetric flasks and dissolving each one in the appropriate solvent.

Working standard solutions

Five milliliters of stock solutions of SDM, TMP and FLOR (each, 1 mg/mL) were separately transferred into three separate 50-mL volumetric flasks and diluted to the volume with methanol for TLC-spectrodensitometry and with the mobile phase for HPLC to get working solutions (100 µg/mL, each).

Procedures

TLC-spectrodensitometry method

Aliquots equivalent to (0.1–1.0 μg, SDM), (0.1–1.0 μg, TMP) and (1.0–10.0 μg, FLOR) were transferred from their working standard solutions (100 μg/mL) and were applied, separately, in the form of compact bands on TLC plates, using Camag Linomat autosampler with micro syringe (100 μL). The band length was 6 mm, dosage speed was 150 nL s^{-1}, and the bands were applied 20 mm apart from each other and 10 mm from the bottom edge of precoated silica gel aluminum plates. Linear ascending development was performed in a chromatographic tank saturated with mobile phase of chloroform : toluene : ethanol : glacial acetic acid (4.5 : 4.5 : 1 : 1, v/v/v/v) for 60 min prior to use at room temperature. The plates were developed over a distance of 8 cm. The developed plates were air dried and scanned at 270 nm for SDM and at 225 nm for TMP and FLOR. The absorbance mode with 3 × 0.45 mm slit dimension and a scanning speed of 20 mm/s were used. The scanning profiles for SDM, TMP and FLOR were obtained. The calibration curves relating the area under the peak to the corresponding concentration were constructed and the regression equations were then computed for the studied drugs and used for determination of unknown samples containing them.

Method validation

Linearity and range. Aliquots equivalent to (0.1–1.0 μg, SDM), (0.1–1.0 μg, TMP) and (1.0–10.0 μg, FLOR) were accurately and separately transferred from their respective working solutions (100 μg/mL) and applied to the TLC plates (20 × 10 cm). The separation was done in the previously described mobile phase. The peak areas were recorded and the calibration curves were constructed by plotting the integrated peak area/10^4 versus the corresponding concentrations (expressed as μg/band), and then the regression equations were computed.

Precision. Precision of the method was verified by repeatability and intermediate precision studies. The system repeatability was determined by three replicates of the prepared sample solutions. The repeatability of sample application and measurement of peak area of the drugs were calculated by repeating the assay three times at three different levels (0.2, 0.4 and 0.8 μg/band; SDM and TMP) and (2, 4, 6 μg/band; FLOR) for pure samples and at three levels of 10, 50 and 100 ppm for spiked fish tissues, of the three studied drugs, on the same day for intraday precision. Intermediate precision of the method was checked by repeating studies on three different days. Precision was calculated as % RSD.

Specificity. The specificity of the method was ascertained by analyzing standard drug and spiked fish tissue samples. The spots of SDM, TMP and FLOR in fish tissue samples were confirmed by comparing the R_f of the separated residues with that of the standard.

Accuracy. Accuracy of the method was assessed by applying the standard addition technique to blank fish tissues, Trimethoxin® and Felorex® solution, to which known amount of the studied drugs were added separately. After mixing, extraction and analysis were completed as described before.

Robustness. By introducing small changes in different parameters of the developed method, the effects on the results were examined. The variables involved changes in scanning wavelength (± 1 nm), ratios of mobile-phase composition (chloroform : toluene : ethanol : glacial acetic acid), time from spotting to chromatography (0, 30 and 60 min), time from chromatography to scanning (0, 30 and 60 min) and effect of temperature (room temperature ±5°C). For each parameter, six chromatograms of each standard level were run. Robustness of the method was accessed at three different standard levels of each drug and measurements of % recovery was expressed in terms of relative standard deviation (RSD %). Moreover, robustness testing was applied to real samples, and effects of sample preparation and extraction conditions were studied.

HPLC method. Aliquots of SDM, TMP and FLOR were accurately transferred from their respective working standard solutions (100 μg/mL) into three separate series of 10-mL volumetric flasks then completed to the volume with the mobile phase to get a final concentration of 2–100 μg/mL SDM, 1–25 μg/mL TMP and 2–28 μg/mL FLOR.

The samples were then chromatographed using the X-Terra™ C_{18} analytical column and the isocratic mobile-phase methanol : phosphate buffer (pH 3 ± 0.1) in ratio of (40 : 60, v/v). The mobile phase was filtered through EMD Millipore (Billercia, MA, USA) membrane filter and degassed for ~30 min in an ultrasonic bath prior to use. The flow rate was 1 mL/min at ambient temperature with UV detection at 225 nm. The samples were injected using a 25-μL analytical syringe. The analysis was usually performed after passing 50–60 mL mobile phase for conditioning of the stationary phase to reach equilibrium.

The relative peak area ratios were then plotted versus the corresponding concentrations of SDM and TMP (using 2 μg/mL of FLOR as an internal standard), while for FLOR (2 μg/mL of SDM was used as an internal standard) to produce the calibration curves. The corresponding regression equations were then computed. Concentrations of unknown samples of these drugs were determined using the obtained regression equation.

Method validation

Linearity and range

Aliquots of SDM, TMP and FLOR were accurately transferred from their respective working standard solutions (100 μg/mL) into three separate series of 10-mL volumetric flasks, the IS was added, then completed to the volume with the mobile phase to get a final concentration of 2–100 μg/mL SDM, 1–25 μg/mL TMP and 2–28 μg/mL FLOR. The peak areas were recorded and the calibration curves were constructed by plotting the relative peak area ratios versus the corresponding concentrations (expressed as μg/mL), and then the regression equations were computed.

Precision

Precision of the method was verified by repeatability and intermediate precision studies. The repeatability was done by repeating the assay three times at three different levels (5, 10 and 15 μg/mL) for pure samples and at three levels of (10, 50 and 100 ppm) for spiked fish tissues, of the three studied drugs, on the same day for intraday precision. Intermediate precision of the method was checked by repeating studies on three different days. Precision was calculated as % RSD.

Specificity

The specificity of the method was ascertained by analyzing standard drug and spiked fish tissue samples. The peaks of SDM, TMP and FLOR in fish tissue samples were confirmed by comparing the retention times of the separated residues with that of the standard.

Accuracy

Accuracy of the method was assessed by applying the standard addition technique to blank fish tissues, Trimethoxin® and Felorex® solution, to which known amount of the studied drugs were added separately. After mixing, extraction and analysis were completed as described before.

Robustness

By introducing small changes in different parameters of the developed methods, the effects on the results were examined. The variables involved changes in scanning wavelength (±1 nm), ratios of mobile-phase composition changing from methanol : phosphate buffer (pH 3 ± 0.1) in ratio of 40 : 60–45 : 55, v/v), flow rate (0.8, 1 and 1.2 mL/min) and effect of temperature (room temperature ±5°C). For each parameter, six chromatograms of each standard level were run. Robustness of the method was accessed at three different standard levels of each drug and measurements of % recovery was expressed in terms of RSD %. Moreover, robustness testing was applied to real samples, and effects of sample preparation and extraction conditions were studied.

Application to fortified fish tissues

Sample preparation and extraction procedure

Frozen fish tissue samples were thawed overnight in a refrigerator. The muscle samples (100–150 g) were diced into small pieces after being trimmed of skin and bones. Antimicrobials were extracted from fortified fish tissues using simple liquid extraction procedure. The detailed procedure was as follows: 1 g of blank fish tissue samples was weighed in a 20-mL glass centrifuge tube with screw cap and fortified with known amounts of mixed working standard solutions of the studied drugs. The fortified samples were then homogenized using Diax 900 homogenizer (Heidolph, Germany) at 10,000 rpm for 2 min and left to stand for 15 min. A volume of 0.25 mL of 1% aqueous formic acid aqueous solution, 0.5 mL of acetonitrile and 0.5 mL of methanol was added to the fortified samples, and then subjected to vortex for 30 s. Subsequently, the glass tube with sample and solvent was shaken by a vertical shaker for 10 min. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected. The extraction procedure was repeated three times. The four aliquots of the extracting solvent mixture (each, 1.25 mL) were collected quantitatively and pooled in 5 mL-measuring flask. Finally, suitable aliquots were spotted directly on TLC plates under the optimized analytical conditions without the need for further sample clean up.

For HPLC method, an additional clean-up step was performed by evaporating 2 mL of the extract to dryness with an Eppindorf's evaporator at 45°C followed by reconstitution with 1 mL of the mobile phase. Two milliliters of *n*-hexane were added to de-fat the residues. Vortex was done to the mixture for 30 s to allow efficient de-fatting. Centrifugation at 10,000 rpm for 5 min was then carried out. A volume of 500 µL of the bottom layer, that contain the drugs, was drawn and filtered through a 0.45-µm nylon filter (Agilent). Finally, 20 µL was injected under the optimized analytical conditions.

Application to pharmaceutical formulations

Appropriate dilutions were carried out separately from both of Trimethoxin® solution and Felorex® injectable solution in methanol for TLC-spectrodensitometry and in the mobile phase for HPLC. The procedures mentioned under the two methods were followed. By applying the corresponding regression equations, the concentrations of SDM, TMP and FLOR and then the mean recoveries were calculated.

Results

TLC-spectrodensitometry

A simple and selective TLC-spectrodensitometric method was described for the simultaneous determination of SDM, TMP and FLOR in fish tissues; employing thin-layer chromatography as a clean-up procedure and in their veterinary pharmaceutical formulations. Several solvent systems were tried for the separation of the targeted drugs from the fish tissue co-extractives. Satisfactory results were obtained by using a mobile phase composed of chloroform : toluene : ethanol : glacial acetic acid (4.5 : 4.5 : 1 : 1, v/v/v/v). Compact and well-separated bands were obtained when the chamber was saturated with the mobile phase for 60 min at room temperature.

The instrumental conditions for the densitometric measurement including the scan mode and the wavelength detection were optimized. The scanning wavelengths 225, 270 and 288 nm were investigated; a better band shape and sensitivity were observed at 270 nm for SDM and at 225 nm for both TMP and FLOR with minimum interference from the matrix co-extractives. SDM, TMP and FLOR were well resolved from each other as shown in Figure 2 and their R_f values were 0.63, 0.16 and 0.48, respectively. This allowed the quantitative determination of the drugs without any interference. The TLC plates (20 × 10 cm, coated with 0.25 mm silica gel 60 F_{254}) were used as it gave best separation with no tailing.

The linearity was confirmed by plotting the measured peak area per 10^4 at 270 nm versus the corresponding concentrations over a range of 0.1–1 µg/band for SDM and at 225 nm over a concentration range of 0.1–1 µg/band and over a range of 1–10 µg/band for TMP and FLOR, in order. Results were best represented by a polynomial fit, as it gave random distribution of the residuals with no outline. The scanning profile of different concentrations of the three drugs is shown in Figure 3.

The regression equations were computed and found to be

$$P_{\mathrm{SDM}} = -0.3882C^2 + 1.4203C + 0.0934, \quad r = 0.9997,$$
$$P_{\mathrm{TMP}} = -0.0785C^2 + 0.6023C + 0.0853, \quad r = 0.9996,$$
$$P_{\mathrm{FLOR}} = -0.0044C^2 + 0.1543C + 0.2596, \quad r = 0.9997,$$

where P is the peak area per 10^4, C is the concentration in µg/band and r is the correlation coefficient.

HPLC method

The main goal of the proposed method was to determine the antimicrobial residues in a single run. A simple isocratic HPLC method was developed for the simultaneous determination of SDM, TMP and

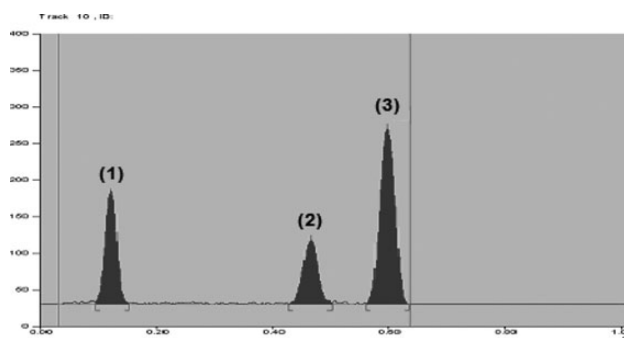

Figure 2. TLChromatogram of a resolved mixture (1) TMP (R_f = 0.16), (2) FLOR (R_f = 0.48) and (3) SDM (R_f = 0.63).

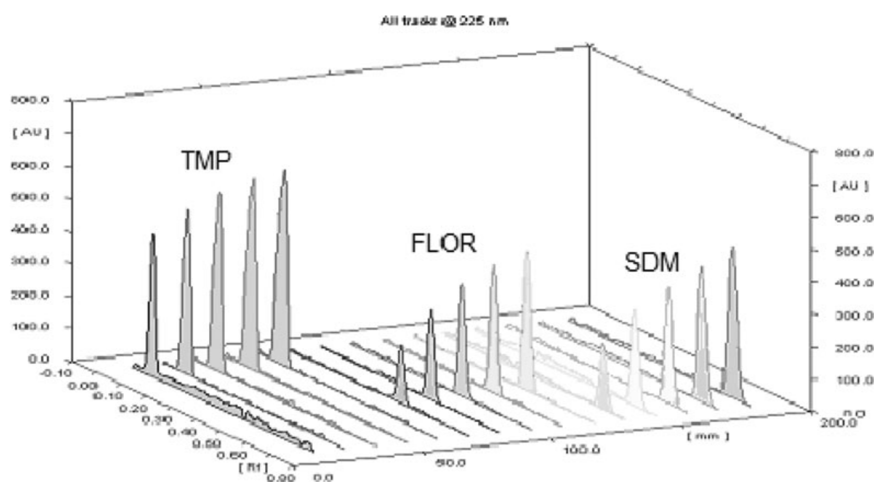

Figure 3. Scanning profile of TLChromatogram illustrating separation of different concentrations of TMP, FLOR and SDM.

FLOR in fish tissues and in their veterinary pharmaceutical formulations. In order to develop and optimize a suitable mobile phase for separation of SDM, TMP and FLOR, the chromatographic behavior was examined using mobile phase mixtures of different polarity in various proportions and different pH values. A better separation was obtained using a mobile phase formed of methanol : phosphate buffer (pH 3 ± 0.1) in ratio of (40 : 60, v/v). The UV detection at 225 nm was chosen to give the best sensitivity for both TMP and FLOR with acceptable sensitivity for SDM, which allowed the analysis of the three drugs in a single run. The best resolution, peak symmetry and elution time were obtained by adjusting the flow rate at 1 mL/min. By using the optimized conditions, a good separation of the three suggested drugs was obtained as shown in Figure 4. The chromatographic system in this method allows complete baseline separation of the three drugs. Calibration curves were obtained by plotting the peak area ratios of drug to that of internal standard versus corresponding concentrations of the drugs. The linearity range was found to be 2–100 μg/mL for SDM, 1–25 μg/mL for TMP and that of FLOR was found to be 2–28 μg/mL using the following regression equations:

$$P_{SDM} = 0.354C - 0.109, \quad r = 0.9999,$$
$$P_{TMP} = 1.3813C + 0.1064, \quad r = 0.9999,$$
$$P_{FLOR} = 0.7994C + 0.068, \quad r = 0.9999,$$

where P is the relative peak area, C is the concentration in μg/mL and r is the correlation coefficient.

Optimization of the extraction procedure from fish tissues:

The effective extraction of compounds of very different physicochemical properties, as in case of the examined analytes, is a significant challenge in multi-class residue method development due to the different recoveries of the substances when extracted simultaneously.

In this work, simple liquid extraction procedure was carried out. TLC technique acts as a clean-up step that separates the studied drugs from the fish tissues co-extractives spot as shown in Figure 5. However, severe overlap of the fish tissues co-extractives was observed with the TMP peak in the HPLC separation as shown in Figure 6. It was better to carry out a de-fatting step using n-hexane to remove the interference from the fish tissues matrix. Better separation was observed as shown in Figure 7.

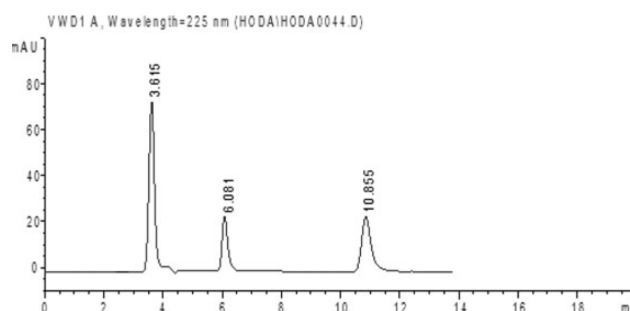

Figure 4. HPLChromatogram of a resolved mixture of pure TMP (10 μg/mL, average $R_t = 3.6$ min), FLOR (10 μg/mL, average $R_t = 6.1$ min) and SDM (10 μg/mL, average $R_t = 10.9$ min).

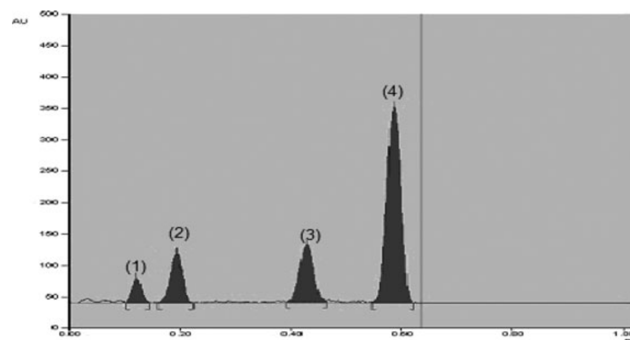

Figure 5. TLChromatogram of a spiked fish tissue extract without purification showing resolved peaks of (1) trimethoprim, (2) fish tissues co-extractives, (3) florphenicol and (4) sulphadimethoxine.

Several solvents were tried separately to achieve the best recoveries of extraction for each drug as shown in Figure 8. Acetonitrile was found to be the best extracting solvent for SDM with average recovery of 97.37%, whereas a mixture of methanol: 1% aqueous formic acid (4 : 1, v/v) was the best extracting solvent system for TMP with average recovery of 98.02% and ethyl acetate was the best extracting solvent for FLOR average recovery of 99.69%. The optimum extracting

solvent mixture was found to be acetonitrile : methanol : 1% aqueous formic acid (2 : 2 : 1, v/v/v) that allowed a single-step extraction procedure and satisfactory recoveries for the studied drugs from the fish tissues matrices. This mixed solvent system was advantageous in terms of both de-proteinization (removal of >99% of protein and fat) and recovery.

Different volumes of this mixed solvent system were tried with applying the extraction procedure. The extracts were analyzed by the proposed method under the specified chromatographic conditions. Maximum percentage recoveries were obtained by using 5 mL of this mixed solvent system, a further increase in the volume did not affect the percentage recoveries.

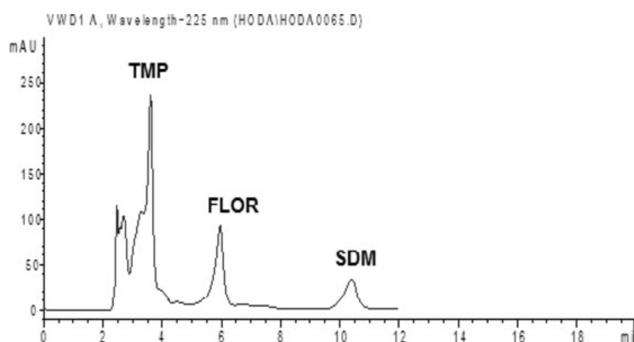

Figure 6. HPLChromatogram of TMP, FLOR and SDM from a fortified fish tissues extract without purification.

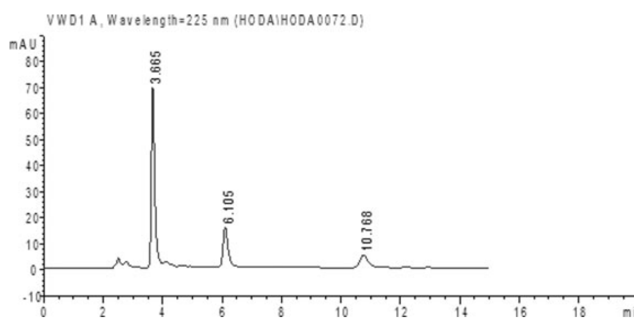

Figure 7. HPLChromatogram of TMP (R_t = 3.665), FLOR (R_t = 6.105) and SDM (R_t = 10.768) from a fortified fish tissues extract after purification.

Blank fish tissue samples were fortified in three levels of 10, 50 and 100 ppm. To determine the recovery of each fortification level, the recorded peak area at this level was compared with that of working standard solution injected under the same conditions, where acceptable recoveries for the studied drugs were obtained as shown in Table I.

Method validation

The methods were validated for linearity, accuracy, specificity, interday and intraday precision in accordance with the international conference on harmonization (ICH) guidelines (24) for the proposed methods.

Table II shows the assay validation sheet of the proposed chromatographic methods for the simultaneous determination of pure SDM, TMP and FLOR. The results of the repeatability and intermediate precision experiments in fish tissues are shown in Table III. The developed method was found to be precise as indicated by the small % RSD.

Accuracy of the proposed methods was assessed by applying the standard addition technique by spiking different known concentrations of pure drugs to the blank fish tissues and the veterinary formulations. Good recoveries were obtained revealing no interference from the excipients, additives or other matrices co-extractives and good accuracy of the proposed method as shown in Tables I and IV.

Specificity of the method can be demonstrated by the resolution of the three components as shown in chromatograms in Figures 2 and 4. Figures 5 and 7 show the applicability of the proposed methods for the determination of the studied drugs in fish tissues with no interference from the matrix co-extractives by comparing the R_f of the spots and the elution times with that of the standard.

System suitability was checked by calculating capacity factor (K'), selectivity factor (α), resolution (R) and tailing factor (T), column efficiency (N) and the plate height equivalent to theoretical plates, where the system was found to be suitable as shown in Tables V and VI.

Robustness of the proposed methods was assessed with respect to the effect of small but deliberate variation in chromatographic conditions. The variables involved changes in scanning wavelength (λ_{max} ±1 nm), ratios of mobile phase composition, time from spotting to chromatography (0, 30 and 60 min), time from chromatography to scanning (0, 30 and 60 min), flow rates and effect of temperature (room temp. ±5°C). Robustness of the method was accessed at three

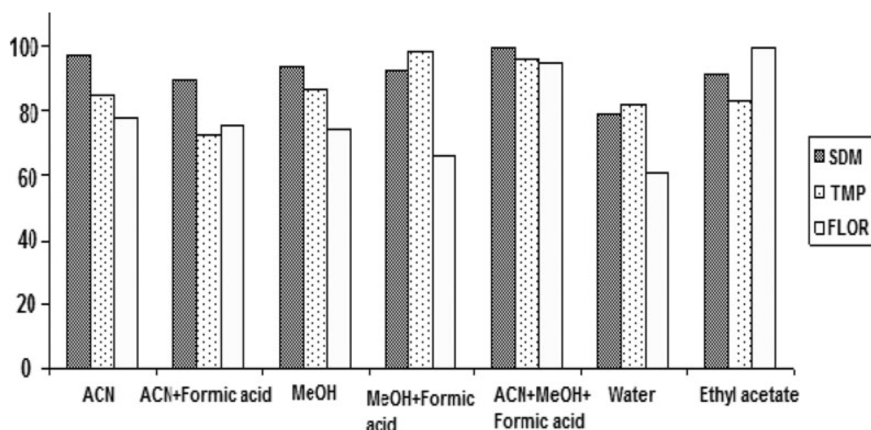

Figure 8. Effect of different solvents used for extraction on the recovery of SDM, TMP and FLOR in fish tissues.

Table I. Percentage Recovery of SDM, TMP and FLOR from Fish Tissues at Three Different Fortification Levels Using the Proposed Chromatographic Methods

Fortification levels (ppm)	% Recovery ± RSD[a]					
	TLC-spectrodensitometry			HPLC method		
	SDM	TMP	FLOR	SDM	TMP	FLOR
10	96.24 ± 1.221	97.83 ± 2.646	97.63 ± 1.339	90.43 ± 2.451	91.42 ± 1.673	94.22 ± 0.991
50	98.87 ± 1.173	95.03 ± 1.758	98.69 ± 1.092	96.16 ± 1.583	96.18 ± 0.385	92.86 ± 1.903
100	95.43 ± 2.054	93.83 ± 2.340	99.92 ± 1.245	94.00 ± 0.034	105.27 ± 1.762	97.04 ± 1.322

[a]Three fortified samples at the same amount were analyzed.

Table II. Assay Validation Sheet of the Proposed Chromatographic Methods for the Simultaneous Determination of Pure SDM, TMP and FLOR

Parameter	TLC-spectrodensitometric method			HPLC method		
	SDM	TMP	FLOR	SDM	TMP	FLOR
Accuracy (mean ± SD)	99.61 ± 1.457	99.43 ± 1.551	100.25 ± 1.720	99.65 ± 1.516	99.41 ± 1.343	100.18 ± 1.265
Precision						
Repeatability[a]	0.981	1.453	1.364	1.380	1.464	1.762
Intermediate precision[b]	1.132	1.215	0.933	1.532	1.041	0.751
Robustness[c]	0.764	0.523	0.978	0.912	1.453	1.490
Linearity						
Coefficient 1[d]	−0.3882	−0.0785	−0.0044	0.354	1.3813	0.7994
Coefficient 2[d]	1.4203	0.6023	0.1543			
Intercept	0.0934	0.0853	0.2596	−0.109	0.1064	0.068
Correlation coefficient (r)	0.9997	0.9996	0.9997	0.9998	0.9999	0.9999
Range	0.1–1 μg/band	0.1–1 μg/band	1–10 μg/band	2–100 μg/mL	1–25 μg/mL	2–28 μg/mL

[a]The intraday (n = 3), average of three concentrations (0.2, 0.4 and 0.8 μg/band; SDM and TMP) and (2, 4 and 6 μg/band; FLOR) for TLC and (5, 10 and 15 μg/mL; SDM, TMP and FLOR) for HPLC repeated three times within the day.

[b]The interday (n = 3), average of three concentrations (0.2, 0.4 and 0.8 μg/band; SDM and TMP) and (2, 4 and 6 μg/band; FLOR) for TLC and (5, 10 and 15 μg/mL; SDM, TMP and FLOR) repeated three times in 3 days.

[c]Robustness (n = 3), average of three concentrations (0.2, 0.4 and 0.8 μg/band; SDM and TMP) and (2, 4 and 6 μg/band; FLOR) for TLC and (5, 10 and 15 μg/mL; SDM, TMP and FLOR) for HPLC analyzed in different conditions mentioned before.

[d]Coefficients 1 and 2 are the coefficients of X^2 and X, respectively. Following a polynomial regression $A = ax^2 + bx + c$, where A is the peak area per 10^4, x is the concentration of SDM, TMP and FLOR (μg/band), a and b are coefficients 1 and 2, respectively, and c is the intercept.

Table III. Intra- and Interday Precision Results for SDM, TMP and FLOR in Fish Tissues by the Proposed Chromatographic Methods

Value	% RSD					
	TLC-spectrodensitometry			HPLC method		
	SDM	TMP	FLOR	SDM	TMP	FLOR
Intraday precision						
Fish tissues[a]						
10 ppm	1.221	2.646	1.339	2.451	1.673	0.991
0 ppm	1.173	1.758	1.092	1.583	0.385	1.903
00 ppm	2.054	2.340	1.245	0.034	1.762	1.322
Interday precision						
Fish tissues[a]						
10 ppm	2.127	1.908	1.590	0.943	1.521	2.102
50 ppm	1.970	2.432	1.756	1.134	0.872	1.353
100 ppm	1.823	2.619	2.223	1.835	0.684	1.482

[a]n = 3.

different standard levels of each drug and measurements of % recovery was expressed in terms of RSD %. Moreover, robustness testing was applied to real samples, and effects of sample preparation and extraction conditions were studied.

The developed methods are robust as by small variations done in a number of variables The R_f-values and the elution times were slightly modified; however, the areas and peaks symmetry were conserved.

Discussion

Sample preparation is a critical step in the simultaneous multi-residues determination of antimicrobials residues of different chemical classes in biological fish tissue samples, due to the high protein and fat content in the matrix, which can interfere with analytical procedures.

The goal of this work was to establish sensitive, selective, and accurate analytical methods for the simultaneous quantification of SDM, TMP and FLOR in fish tissues without preliminary separation; employing simple liquid extraction procedure with minimal clean-up steps of the samples.

In this work, efficient simple liquid extraction made it possible to get clean extracts instead of using the multistep, sophisticated and time-consuming solid-phase extraction procedures. Several solvents were tried separately to achieve the best recoveries of extraction for each drug in fish tissues. The optimum extracting solvent mixture was found to be acetonitrile : methanol : 1% aqueous formic acid (2 : 2 : 1, v/v/v) that allowed satisfactory recoveries for the studied drugs from the fish tissues matrices.

Table IV. Determination of SDM, TMP and FLOR in Their Veterinary Pharmaceutical Formulations by the Proposed Methods and Application of Standard Addition Technique

Product	Drug	Standard addition							
		TLC-spectrodensitometry				HPLC			
		Recovery %[a] ± SD	Taken (μg/band)	Added (μg/band)	Recovery %[a]	Recovery %[a] ± SD	Taken (μg/mL)	Added (μg/mL)	Recovery %[a]
Trisulmix® solution (BN): A120009	SDM	101.79 ± 0.513	0.4	0.2	100.82	99.46 ± 1.1349	50	5	100.82
				0.4	100.46			10	101.78
				0.6	97.48			25	101.34
			Mean ± RSD		99.59 ± 1.84		Mean ± RSD		101.31 ± 0.474
	TMP	98.38 ± 0.667	0.4	0.2	99.56	100.75 ± 0.678	10	5	98.56
				0.4	97.56			10	99.76
				0.6	100.25			15	99.31
			Mean ± RSD		99.56 ± 1.77		Mean ± RSD		99.21 ± 0.611
Felorex® injectable solution (BN): 0270/12	FLOR	100.17 ± 1.931	3	2	99.26	101.13 ± 0.198	12	6	99.75
				3	100.02			10	99.59
				6	101.18			12	99.93
			Mean ± RSD		100.15 ± 0.96		Mean ± RSD		99.76 ± 0.171

[a]Average of three determinations.

Table V. System Suitability Parameters of the Proposed TLC-Densitometry Method

Parameters	TMP	FLOR	SDM
Retardation factor (R_F)[a]	0.16	0.48	0.63
Capacity factor (K')[a]	7.33	1.08	0.59
Resolution (R)[b]	—	7.20	2.50
Selectivity factor (α)[b]	—	6.79	1.83
Tailing factor (T)[a]	1.08	1.10	1.02

[a]Tailing factor and capacity factor determined for individual peak.
[b]Selectivity factor and resolution factor determined between TMP and FLOR peaks and between FLOR and SDM peaks.

Table VI. System Suitability Parameters of the Proposed HPLC Method

Parameters	TMP	FLOR	SDM
Retention times (min)	3.615	6.081	10.855
Capacity factor (K')[a]	2.61	5.08	9.85
Resolution factor (R_s)[b]	—	12.33	15.91
Selectivity factor (α)[b]	—	1.94	1.94
Tailing factor (T)[a]	1.05	1.25	1.33
Column efficiency (N)[c]	5227	14791	11805
Height equivalent to theoretical plates	0.0048	0.0017	0.0021

[a]Tailing factor and capacity factor determined for individual peak.
[b]Selectivity factor and resolution determined between TMP and FLOR peaks and between FLOR and SDM peaks.
[c]Column efficiency expressed as number of theoretical plates for SDM, TMP and FLOR.

A TLC-spectrodensitometric method is described for the simultaneous determination of three approved aquaculture antimicrobials: SDM, TMP and FLOR in fish tissues. Optimization of different parameters was done for efficient separation of bands. Several solvent mixtures were tried to develop and optimize the mobile phase used for separation of the suggested drugs from the matrices co-extractives. Satisfactory results were obtained by using the described mobile phase. Thin-layer chromatography acts as a clean-up step; separating the fish tissues co-extractives spot from those of the targeted drugs.

A simple isocratic high-performance liquid chromatographic method was developed for the determination of SDM, TMP and FLOR in fish tissues using X-Terra™ C_{18} column. The mobile phase was chosen after several trials to reach the optimum stationary/mobile-phase matching. It was better to carry out a de-fatting step using *n*-hexane to remove the interference from the fish tissues matrix.

The procedures were applied for the analysis of spiked fish tissue samples at three different concentration levels (10, 50 and 100 ppm). The methods were validated according to the ICH guidelines. The proposed methods were successfully applied for the determination of the studied drugs in spiked fish tissues, pure powders and in their veterinary pharmaceutical formulation.

Stability of the studied drugs was checked in spiked fish tissue samples extracts using the proposed methods. The studied drug solutions exhibited no chromatographic changes for at least 6 h when kept at room temperature (25°C) and protected from direct light, and for at least 12 h when stored at 4°C.

Overall, the developed methods are simple to be applied for routine analysis and particularly suitable for screening of SDM, TMP and FLOR residues in fish tissues. These drugs are commonly used in aquaculture practice throughout the world. The proposed methods provide much data about the extraction efficiency and possible interference from the complex matrices that could be of high value for further investigations regarding this topic. The extraction procedure attempted in this study was rapid and simple with no need for complicated extraction and clean-up steps. Although the detection levels achieved were still higher than the concentrations of real samples, the manuscript provides a comparative study of different chromatographic techniques for the analysis of some approved antimicrobials in fish tissues.

Conclusion

The two proposed methods provide sensitive, accurate, and reproducible means for the simultaneous multi-residues analysis of SDM, TMP

and FLOR in fish tissues employing simple liquid extraction procedure. Simultaneous extraction of all antimicrobials in the fish tissues was performed in a single procedure, in spite of the different chemical natures of the analytes. A comparative study was conducted to show the advantages of the proposed methods which showed that the TLC-spectrodensitometric method was simpler, more sensitive and economic, while RP-HPLC method was more precise. TLC-spectrodensitometric method has the advantage of minimizing the cost of reagents and time of analysis. It also utilizes the merit of applying several sample bands on TLC plate. The matrix interference was removed satisfactory by making use of the power of thin-layer chromatography as a clean-up procedure, while for HPLC method a post-extraction clean-up step with *n*-hexane was carried out. The proposed HPLC method is environmentally friendly and easily performed. In addition, simultaneous analysis was accomplished with high sensitivity.

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