Journal of Chromatography A, 1331 (2014) 108–116

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Rapid and selective determination of multi-sulfonamides by high-performance thin layer chromatography coupled to fluorescent densitometry and electrospray ionization mass detection

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ARTICLE INFO

Article history:
Received 15 November 2013
Received in revised form 12 January 2014
Accepted 14 January 2014
Available online 24 January 2014

Keywords:
Sulfonamides
Fast screening
HPTLC
Fluorescence densitometry
TLC-MS interface
Mass spectrometry

ABSTRACT

In the European Union (EU), sulfonamides are among the most widely administrated groups of antibiotics in animal husbandry. Therefore, monitoring their residues in edible animal tissues plays an important role in the EU food safety framework. In this work, a simple and efficient method for the rapid screening of twelve prior sulfonamides frequently prescribed as veterinary drugs by high-performance thin-layer chromatography (HPTLC) was established. Sample extracts obtained with acetonitrile were tenfold concentrated and applied to HPTLC without any further cleanup. Following separation and fluram derivatization, sensitive and selective quantitation of the analytes can readily be accomplished with fluorescent densitometry. Limits of detection and quantitation were 15–40 and 35–70 μg/kg, respectively. Additionally, a confirmative detection by HPTLC-electrospray ionization mass spectrometry (HPTLC-ESI/MS) was optimized, offering straightforward identification of target zones. Therefore, the risk of potential false positive findings can efficiently be reduced. The method was validated to meet the enforced commission regulation (EU) No. 37/2010, regarding different matrix complexities (bovine milk, porcine liver and kidney).

1. Introduction

Sulfonamides (SAs) are referred to a group of synthetic compounds characterized by a common *p*-amino-benzene sulfonamide moiety. In the last decade, this drug group was among the most commonly used antibiotics in veterinary medication and to a lesser extent for human prescriptions. According to the data analyses generated from EU surveillance programs of ten major EU member states on the sales of veterinary antibacterial agents, SAs almost were in the second position, right after tetracyclines [1]. It is notable that this type of compounds shows considerable stability, so that they cannot easily be converted to safe degradation products by metabolic processes [2,3]. Due to their well documented adverse effects like acute allergies for instance, strict regulations regarding SAs maximum residues level (MRL) have been established in the EU and many other countries [4–6]. To enforce the administrative demands, most EU member states collaborate and consolidate extensive monitoring and surveillance programs. In these programs, the fundamental question is how to satisfy the demands of high throughput, sensitivity and cost-efficiency in a large-scale screening of SAs residues, which is especially challenging in animal products in view of the vast number of matrix compounds. In Germany, for example, ≥2% of all commercially slaughtered calves and ≥0.5% of all other commercially slaughtered hoofed animals must be officially sampled and analyzed for residues, according to a national regulation [7]. Practically noteworthy, remarkably low rates of non-compliant samples were revealed by the EU monitoring programs. Concerning SAs, for instance, non-compliant results exemplarily only accounted for 0.08% for the categories bovines and pigs, as reported by EU member states in 2010 [8]. Thus, a great effort was undertaken to analyze the huge amount of compliant samples, keeping in mind that only low percentages of samples were positive.

Reviews on the methods dealing with residue analysis of SAs revealed that a large variety of techniques have been reported in this field, each showing advantages and limitations in specific aspects [9–18]. Though displaying remarkable merits, the efficiency of LC-MS methods with SAs screening is far from satisfactory, because they highly demand sample clean-up. On the other hand, microbial growth inhibition assays, generally playing a key role in veterinary drug screening, almost lack in sensitivity, not being able to detect residues of SAs at the tolerance limits. This dilemma leads to the likelihood of misinterpretation and false-negative reports [8,19].

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http://dx.doi.org/10.1016/j.chroma.2014.01.037

Against this background, the potential of the modern HPTLC was deeply underestimated among the reviews. On the contrary, HPTLC has become a full-scale analytical technology and a highly valuable platform for chemical screening, efficiently linked to plate image inspection and sensitive detection methods. Therefore, HPTLC should be highly appreciated for screening purposes. Additionally, direct linking with mass spectrometry provides a powerful analytical tool to substantially expand the scope of detection that can be coupled to HPTLC [20–23]. Especially, the elution-head based TLC-MS interface shows considerable improvements in various aspects. This simple and easily controlled sampling approach can be readily applied in routine screening, offering straight forward identification of zones and, therefore, rapid confirmation of suspected positive-findings [24,25].

In this study, an HPTLC method integrated with fluorescence densitometry (FLD) and elution-head based electrospray ionization mass spectrometry (ESI/MS) was developed and optimized for the preliminary screening of twelve representative SAs at their EU MRLs. In this regard, the aim was to provide timely and reliable screening data from complex matrices, including quantitation and confirmation. The established method was successfully applied to spiked bovine milk and porcine kidney and liver, which are the hot-spots and headaches of traditional LC-MS approaches.

2. Experimental

2.1. Chemicals and materials

Analytical standards of studied SAs (sulfadoxin SDX, sulfadiazine SDZ, sulfamethazine SMZ, sulfanilamide SNMD, sulfamethiozole SMTZ, sulfachloropyridazine SPDZ, sulfathiazole STAZ, sulfapyridine SPD, sulfamerazine SMRZ, sulfisoxazole SIXZ, sulfaquinoxaline SQXL, sulfacetamide SCTD), all with a purity >95%, and acetonitrile, ethyl acetate, and methanol, all HPLC grade, and magnesium sulfate of analytical purity were purchased from Sigma-Aldrich (Steinheim, Germany). Fluram of 98% purity, ammonium hydroxide solution (28%), ammonium formate, and anhydrous sodium acetate of analytical purity were from Fluka (Darmstadt, Germany). Ultra pure water was prepared by a Synergy system (Millipore, Schwalbach, Germany).

HPTLC silica gel 60 F_{254} plates (20 cm × 10 cm) No.1.05641.0001 were supplied by Merck (Darmstadt, Germany). Before using, all plates were washed by pre-developing with methanol, dried in an oven at 120 °C for 20 min, wrapped in aluminum foil, and stored in a glass container to prevent contamination.

2.2. Standard solutions

Separate stock solutions of each SA (0.05 mg/mL) were prepared in methanol and stored at -20 °C. Working solutions for spiking and calibration were freshly prepared by equally mixing 200 μL of the stock solutions within the same sub-group (group 1: SPD, SMRZ, SIXZ, STAZ, SQLX, SMTZ; group 2: SNMD, SDZ, SDX, SPDZ, SMZ, SCTD), resulting in concentrations of 0.01 mg/1.2 mL.

2.3. Sample preparation

Blank bovine milk, porcine kidney and liver samples of organic sources were purchased in a local supermarket. Kidney and liver samples were manually sliced before extraction. For extraction, 10 g food samples were homogenized with 10 mL water in a MediFASTH 2 homogenizer (Sam-Sol, Bahlingen a.K., Germany) for 2 min. Artificial contamination of blank homogenates was achieved by adding 0.5, 1 and 2 μg analytes (namely, 60, 120, or 240 μL working solutions, respectively), resulting in 0.5-, 1-, or 2-folds the MRL (100 μg/kg). The homogenates were transferred into 50-mL polyethylene centrifuge tubes (Sarstedt, Germany) and extracted with 10 mL acetonitrile. After vigorously shaking by hand for 1 min, 4 g anhydrous magnesium sulfate and 1 g sodium acetate were added. The tubes were immediately shaken for another min and subjected to centrifugation at 4000 × *g* for 5 min, while the temperature was controlled at 15 °C. The supernatants were pipetted into ampoules and evaporated at room temperature under a stream of nitrogen. The final residues were redissolved with 1 mL acetonitrile, followed by nylon membrane filtration (0.45 μm) prior to HPTLC sampling.

2.4. HPTLC

Appropriate volumes of standard mix solutions resulting in 5, 15, 25, 40 and 50 ng/band and sample extracts (20 μL) were applied as 6-mm bands by the Automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). The sampling started 15 mm from the left side and 8 mm from the bottom of plates with the automatically setting of band distance. Application conditions: filling speed 10 μL/s, dosage speed 150 μL/s, rinsing (with methanol) vacuum time 4 s, filling vacuum time 1 s, and rinsing cycles 1. The plates were developed with a mobile phase consisting of 8 mL ethyl acetate, 2 mL methanol and 0.1 mL 28% ammonium hydroxide solution. An automatic developing chamber (ADC 2, CAMAG) was used with the following settings: 30 s pre-drying, 1 min humidity control (33% relative humidity with saturated magnesium chloride), 5 min tank saturation with mobile phase, 5 min plate pre-conditioning, 60 mm migration distance, 3 min post-chromatography drying. To remove residual ammonia completely, the plates were dried at 100 °C on a TLC Plate Heater III (CAMAG) for 5 min, and then cooled to room temperature for 2 min. Post-chromatographic derivatization was performed by dipping the plate into a solution of fluram (10 mg in 100 mL acetone) using a TLC Immersion Device III (CAMAG) with a vertical speed of 2 cm/s and 2 s immersion time. Thereafter, the plate was heated at 100 °C for 5 min on the plate heater.

Digital documentation of the developed plates was carried out with a TLC Visualizer (CAMAG) both before and after derivatization under 254 and 366 nm, respectively. Images of 0.10 mm/Pixel resolution were captured by a Baumer Optronic DXA 252 digital camera. Then, the derivatized plates were densitometrically evaluated with a TLC Scanner 4 (CAMAG) in fluorescence mode with the general settings: slit dimension 3.00 × 0.30 mm (Micro), optical system for maximum light, scanning speed 20 mm/s, data resolution 50 μm/step. Parameters for fluorescence excitation spectrum recording: deuterium/wolfram lamp, scanning range 250–450 nm, optical filter K540; parameters for quantitation: mercury lamp, λ_{ex} 400 nm, optical filter K400. Data acquisition and processing was done winCATS software, version 1.4.5.2027 (CAMAG).

2.5. HPTLC-mass spectrometry

Zones of interest were located under UV light illumination at 254 nm and 366 nm for non-derivatized and derivatized plates, respectively, and marked with a soft pencil. Through the TLC-MS interface (CAMAG) equipped with an oval shaped elution head, analytes on the plates were extracted with eluent provided by a quaternary 1100 HPLC pump (Agilent) at the rate of 0.2 mL/min for 60 s. For non-derivatized plates, the eluent consisted of acetonitrile/20 mM ammonium formate buffer (73, v/v), and for derivatized plates of methanol/20 mM ammonium formate buffer (7/3, v/v). The mass spectrometric evaluation of zone extracts was simultaneously performed with a G1956B MSD single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface (Agilent, Waldbronn, Germany), operated by ChemStation B.02.01 SR2 software. Full scan MS data acquisition was carried out in both positive and negative mode with following