



Analysis of 76 veterinary pharmaceuticals from 13 classes including aminoglycosides in bovine muscle by hydrophilic interaction liquid chromatography–tandem mass spectrometry



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ABSTRACT

A multiresidue/multiclass method for the simultaneous determination of 76 veterinary drugs and pharmaceuticals in bovine muscle tissue has been developed and validated according to the requirements of European Commission Decision 2002/657/EC. The analytes belong in 13 different classes, including aminoglycoside antibiotics, whose different physicochemical properties (extremely polar character) render their simultaneous determination with other veterinary drugs quite problematic. The method combines a two-step extraction procedure (extraction with acetonitrile followed by an acidic aqueous buffer extraction) with hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) determination, allowing confirmation and quantification in a single chromatographic run. Further cleanup with solid phase extraction was performed using polymeric SPE cartridges. A thorough ionization study of aminoglycosides was performed in order to increase their sensitivity and significant differences in the abundance of the precursor ions of the analytes were revealed, depending on the composition of the mobile phase tested. Further gradient elution optimization and injection solvent optimization were performed for all target analytes. The method was validated according to the European Commission Decision 2002/657. Quantitative analysis was performed by means of standard addition calibration. Recoveries varied from 37.4% (bromhexine) to 106% (kanamycin) in the lowest validation level and 82% of the compounds showed recovery >70%. Detection capability (CC β) varied from 2.4 (salinomycin) to 1302 (apramycin) $\mu\text{g kg}^{-1}$.

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1. Introduction

During the last decades, a large number of veterinary drugs have been used in order to improve animal health and prevent stress-induced animal death but also as growth promoters for intensive animal production [1]. Antibiotics and anthelmintics are the most commonly used classes of drugs along with coccidiostats which are commonly used to prevent and treat coccidiosis in poultry [2]. Their improper use, non-respect of withdrawal periods, and cross-contamination can lead to the presence of residues of veterinary drugs in food of animal origin. The possible adverse effects on public health include allergic reactions in hypersensitive or sensitized individuals, and the development of resistant strains of bacteria following the ingestion of sub-therapeutic doses of antimicrobials [3].

Different legal frameworks ensure that residues are kept in check through the establishment of Maximum Residue Limits (MRLs) and the control of veterinary drug residues in samples taken from food-producing animals. The use of veterinary drugs was regulated through EU Council Regulation 2377/90/EC [4], which has been repealed by Council Regulation 470/2009/EC [5] and describes the procedure for establishing MRLs for veterinary medicinal products in foodstuffs of animal origin. Commission Regulation (EC) No 124/2009 establishes Maximum Levels (MLs) for the presence of coccidiostats or histomonostats in food [6] and most recently, Commission Regulation 37/2010/EC [7] sets a list of pharmacologically active substances and their MRLs in foodstuffs of animal origin. To ensure that testing is carried out according to high analytical standards guidelines in Commission Decision 2002/657/EC specifies the rules for the analytical methods to be used for testing food samples of animal origin for the presence of residues and contaminants [8].

Moreover, general pharmaceuticals for human medication are considered as widespread emerging pollutants with potential to enter the food chain [9]. Several studies have pointed out that

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excreted human pharmaceuticals or drugs of abuse end up in the environment, can be detected in surface water and drinking water and eventually enter the food chain [9,10]. This occurs through insufficient treatment of wastewater in wastewater treatment plants [11,12].

To fulfill the regulatory requirements the development of sensitive, selective and reliable analytical methods is necessary. Mass spectrometric techniques and especially LC–QqQ–MS (utilizing selected reaction monitoring (SRM) mode, i.e. two transitions, to provide the required degree of confidence), have been the starting point for the development of such methods [13–15]. The interest in multi-analyte methods for the simultaneous determination of many substances among different antibiotic classes has continuously increased within the last few years. However, multiclass methods for veterinary drugs face a number of analytical challenges that need to be overcome [16]. Previous studies of multi-residue methods often contained only some different classes of drugs and/or test only a few compounds within the families of antibiotics [2,17–23].

Multi-residue methods are mainly based on reversed phase (RP) separation due to its efficient separation of analytes within broad range of polarity. However, highly polar compounds undergo early elution on traditional RP stationary phases, leading to lower sensitivity of the mass spectrometric detection. The analysis of highly hydrophilic and polar compounds by HILIC coupled to MS has been demonstrated as a valuable complementary approach to RPLC [24].

The concept of HILIC is as follows: a polar stationary phase retains polar analytes that are eluted by a mixture of organic solvent (usually ACN) and water [25]. The main advantages of HILIC comprise: (a) satisfactory retention of polar compounds, (b) increased MS sensitivity due to mobile phase's high organic content which tends to improve desolvation and increase ionization efficiency, (c) better suited for larger volume injection of extracts with high organic content and (d) higher flow rates possible because of the high organic content (lower viscosity) of typical mobile phases [25–27].

Several applications of HILIC in the analysis of veterinary drugs and pharmaceuticals in food products have been reported the last decade [28–42]. A brief review of employed columns, mobile phases and type of samples is presented in Table 1. As it can be noticed, high attention has been paid in the determination of aminoglycoside antibiotics with hydrophilic interaction chromatography [31,34–37,39–42]. Their determination in food and environmental matrices was recently reviewed [43]. Aminoglycosides (AGs) are very polar compounds containing many amino and hydroxyl groups in their structures. These groups make them difficult to separate using conventional RPLC without ion pairing. The use of HILIC MS provides the advantage of direct determination (no derivatization or ion-pairing reagents) and higher sensitivity comparing to ion pairing RPLC [25,43]. However, the simultaneous determination of AGs and other drugs in a single method is very difficult due to their rather different physicochemical properties. The simultaneous separation and determination of AGs and other veterinary drugs with HILIC–MS/MS has only been reported once so far, by Chiaoan et al. [35].

Despite the advantages on the detection techniques, when dealing with real complex samples (e.g. foods, edible tissues from animals), an appropriate sample preparation is required in order to decrease interferences and avoid possible matrix effects.

Solvent extraction with aqueous, low-pH extraction solvents has been mainly applied for the extraction of AGs from various matrices [31,35–37,39–42,44]. AGs' extraction from food samples shows some difficulties mostly due to the fact that these compounds tend to bind to proteins and to form complexes with metal ions. As a result, the use of strong acids such as trichloroacetic acid (TCA) and chelating agents such as ethylenediaminetetraacetic acid

(EDTA) is recommended [39]. In several multi-analyte methods ACN or a mixture of ACN and alcohol, ethanol or methanol (MeOH), is often used as the extraction solvent [17,18,20,22,23]. Also, extraction with a mixture of ACN and aqueous buffer has been tested and gave satisfactory results in some cases [17,19].

Many of these studies, use SPE as a cleanup and preconcentration step [17,18,31,36] while others employ a dSPE cleanup following a modified QuEChERS approach [17,20,22]. For aminoglycosides SPE cartridges which are commonly used are weak cation exchange cartridges [31,40–42,44]. Lately, new polymeric sorbents that include novel functional groups in the polymeric structure resulting in a hydrophilic–hydrophobic balance material have been used for aminoglycosides analysis, (HLB cartridges) [36,39]. HLB cartridges are also widely used in multi-residue methods for the extraction and clean up of several veterinary drugs since they ensure higher loading capacity, smaller volume of the elution solvent and no silanol activities, as typically observed when using silica based materials [45].

The purpose of this study involves the development of a sensitive and reliable multiclass/multi-analyte method for the quantitative determination of 76 veterinary drugs belonging in 13 different classes, including AGs, in bovine tissue. As AGs present the lowest sensitivity among all target analytes, a thorough ionization study was performed under HILIC conditions, along with an optimization of several chromatographic parameters, aiming to maximize AGs sensitivity.

The method was validated according to the European Commission 2002/657/EC [8] and was applied to real bovine muscle samples.

To the best of our knowledge, this is the first study reported using HILIC–MS/MS that quantifies such a great number of compounds belonging in more than 10 different classes, simultaneously, including AGs. Strongly retained AGs like apramycin and neomycin are also included which have never been previously included in a multi-residue method. Additionally, the ionization study of AGs as well as the optimization of the gradient program and injection solvent constitution provides novel and significant information for HILIC's applicability in multiresidue analysis.

2. Materials and methods

2.1. Chemicals and reagents

All the analytes studied are presented in Table S1. All veterinary drug and pharmaceutical standards were of high purity grade (>90%) and the majority of them was purchased from Sigma-Aldrich (Steinheim, Germany). Nine isotopically labeled standards, nigericin, amikacin and flubendazole were used as internal standards (IS). Amikacin and flubendazole are not licensed for use in cattle and so it is extremely unlikely to detect residues in bovine muscle tissue. Arprinocid, salinomycin, semduramicin, manduramycin, narasin, albendazole sulfone, flunixin, meloxicam, nigericin, flunixin d₃ and meloxicam d₃ were donated by the Veterinary Drug Residues Laboratory of the State General Laboratory of Cyprus. Sulfadiazine d₄, sulfadimidine d₄ and sulfadimethoxine d₄ were obtained from Toronto Research Chemicals (Toronto, Canada).

ACN and MeOH LC–MS grade were purchased from Merck (Darmstadt, Germany). Formic acid 99% and ammonium formate (>90% purity) were purchased from Fluka Buchs, Switzerland. EDTA, sodium hydroxide monohydrate (NaOH) and sodium chloride (NaCl) were purchased from Panreac (Barcelona, Spain). TCA and ammonium acetate from Fisher Scientific (Loughborough, UK). Distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Regenerated cellulose syringe filters (15 mm diameter, 0.22 µm pore size) were provided

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