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Trace analysis of endectocides in milk by high performance liquid chromatography with fluorescence detection

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ABSTRACT

An analytical method has been developed for the simultaneous determination of the following endectocide drugs in milk: ivermectin, abamectin, doramectin, moxidectin, eprinomectin, emamectin and nemadectin. Samples were extracted with acetonitrile, purified with solid-phase extraction on a reversed phase C_8, derivatised with *N*-methylimidazole, trifluoroacetic anhydride and acetic acid to a stable fluorescent derivative, and were further analysed by gradient high performance liquid chromatography (HPLC) on an endcapped reversed phase Supelcosil LC-8-DB. The derivatisation step was mathematically optimised and the method was validated according to the requirements of Commission Decision 2002/657/EC, using fortified raw bovine milk. Mean recovery was between 78 and 98%. The repeatability (CV_r) and within-laboratory reproducibility (CV_W) ranged from 4.6 to 13.4% and from 6.6 to 14.5%, respectively. Decision limits (CCα) for analytes with MRL values, namely eprinomectin and moxidectin, were determined to be 24.8 and 50.6 μg kg^{-1}, respectively. CCα values for unauthorised endectocides ranged from 0.1 to 0.2 μg kg^{-1}. Due to high acceptability regarding the required criteria and applicability to ovine and caprine milk, giving similar results, this multi-analyte method has been successfully implemented in pharmacokinetic research studies as well as statutory residue monitoring in Slovenia.

1. Introduction

Macrocyclic lactone endectocides are derivatives of the natural fermentation products of soil-dwelling microorganisms from the genus *Streptomyces*, which are in very low doses extremely effective against (endo-) internal and (ecto-) external parasites and are probably the most widely used anti-parasitic drugs. These 16-member macrocyclic compounds are structurally broadly divided into avermectins: ivermectin (IVM), abamectin (ABM), doramectin (DOR), eprinomectin (EPR), emamectin (EMA) and selamectin; and milbemycins: milbemycin oxime, moxidectin (MOX) and nemadectin (NEM), which in brief differ in the presence of the sugar moiety, but share a common mode of pharmacological activity related to a unique tri-partite pharmacophore [1]. Due to their high lipophilicity [2], endectocides are substantially excreted also in milk, with residues reaching up to 5% of the dose administered [3,4]. For reasons of consumer safety, their use in animals from which milk is produced for human consumption is mostly unauthorised in the European Union (EU). The exceptions are EPR and MOX, for which maximum residue limits (MRLs) for the parent drug of 20 μg kg^{-1} in bovine and 40 μg kg^{-1} in bovine and ovine milk, respectively, have been established [5,6] and a zero withdrawal period set for milk.

A comprehensive overview of methodologies for determination of endectocide residues in biological matrices, including milk, was published by Danaher et al. [7]. High-performance liquid chromatography with fluorescence detection (HPLC-FLD) of endectocides evidently predominates over liquid chromatography with mass or tandem mass spectrometric (MS or MS/MS) detection. This is due to higher sensitivity, better reproducibility and lower cost of HPLC-FLD. A disadvantage of HPLC-FLD is the requirement of pre-column derivatisation to convert non-fluorescing parent compounds into their fluorescent derivatives, which are problematic from the stability point of view as they are known to be degradable to some extent with time [8–10]. EPR is particularly problematic, because the formation of its fluorescent derivative after reaction with trifluoroacetic anhydride (TFAA) and *N*-methylimidazole (NMIM) is slow, incomplete and quite unstable [11,12]. In the past, this analytical problem had been overcome by either purely qualitative determination [11], "on-line" pre-column derivatisation [13,14] or a photochemical post-column reactor [15]. Later it was found that the stability of the EPR fluorescent derivative

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could be enhanced by longer incubation time (90 min), higher temperature (65 °C) [16] and additionally by 100% acetic acid in the derivatised extract [17]. The reaction mechanism of the derivatisation of endectocides from an organic chemistry point of view was recently described and optimised by Berendsen et al. [18].

Using the method described below, residues of all relevant endectocides that can be officially or illegally used in food-producing animals, including EPR (component B_{1a}), MOX, EMA (component B_{1a}), NEM, ABM (component B_{1a}), DOR and IVM (component H_2B_{1a}) residues in milk were simultaneously determined. In Slovenia, residues of endectocides have been followed during state monitoring of veterinary drug residues in food of animal origin since 1994, when analysis of IVM as the first such substance was introduced [19,20]. Since then, endectocide analysis has developed according to progressive addition of specific substances for research and monitoring purposes [21–24].

2. Experimental

2.1. Standards, reagents and labware

Reference standards of EPR, EMA, ABM and DOR were purchased from Dr Ehrenstorfer (Augsburg, Germany), and of IVM from Calbiochem (San Diego, CA, USA). MOX (Cyanamid, Princeton, NJ, USA) and NEM (BASF, Ludwigshafen, Germany) standards were obtained through the EU Community Reference Laboratory for Residues (BVL, Berlin, Germany). Standard solutions were prepared in previously silanised dark glassware using acetonitrile (MeCN) and were kept in a refrigerator. Stock solutions were individually prepared at a concentration of 100 μg mL^{-1} (B_{1a} or H_2B_{1a} equivalents).

Derivatisation reagents (NMIM, TFAA, glacial acetic acid, 100% anhydrous) and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany) and were of p.a. purity; MeCN and methanol (MeOH) were supplied by J.T. Baker (Phillipsburg, NJ, USA) and were of Ultra Gradient HPLC Grade. Silanizing reagent Sylon CT was obtained from Supelco (Bellefonte, PA, USA).

Polypropylene centrifuge tubes of 50 and 15 mL were from Sarstedt (Nümbrecht, Germany) and polypropylene round flasks of 50 mL were from Brand (Wertheim, Germany). Silanized vials, dark glass, 2 mL were from Agilent (Palo Alto, CA, USA).

2.2. Apparatus

An ultrasonic bath type Sonis 4 was from Iskra (Šentjernej, Slovenia), vibromixers of type EV-100 and 314 EVT were from Tehtnica (Železniki, Slovenia), the centrifuge of type Minifuge 3 S-R and the ventilation oven of type Kendro T 6030 were from Heraeus (Osterode, Germany), the vacuum manifold used for solid-phase extraction (SPE) was Visiprep from Supelco (Bellefonte, PA, USA), while nitrogen evaporators were of type No. 111 from Organomation Associates, Inc. (Berlin, MA, USA) and Liebisch 2366 (Bielefeld, Germany).

An HPLC system Agilent 1100 (Agilent, Palo Alto, CA, USA) was used, consisting of quaternary pump G1311A, vacuum degasser G1322A, automatic injector G1329A with temperature controlled sample tray G1330A, column thermostat G1316A, fluorescence detector G1321A and integration software ChemStation G2170AA and G2180AA.

2.3. Extraction/clean-up and derivatisation

Milk samples (5 g) were extracted with 20 mL of MeCN, by manual shaking (5 min), sonicating (15 min) and manual shaking again (5 min). After centrifuging at 3290 ***g*** (10 min), 50 μL of TEA was added to 15 mL of MeCN extract, which was further diluted to 50 mL with distilled water (H_2O) and applied to a C_8 SPE cartridge, 6 mL/500 mg (J.T. Baker, Phillipsburg, NJ, USA), being pre-conditioned with MeCN (5 mL) and MeCN:H_2O:TEA (30:70:0.1, v/v/v, 5 mL). The SPE cartridges were washed with MeCN: H_2O:TEA (50:50:0.1, v/v/v, 5 mL) and elution was done under gravity with MeCN (5 mL) [25].

Sample eluates and standard solutions for calibration were evaporated to dryness under nitrogen at 50 °C and allowed to cool before addition of NMIM:MeCN (1:1, v/v, 100 μL) and TFAA:MeCN (1:2, v/v, 150 μL) [26,27]. After dilution with MeCN (630 μL), acetic acid (120 μL) was added and the mixture was vortexed (1 min). An aliquot was transferred to an HPLC vial and incubated in an oven (65 °C, 50 min), cooled in a desiccator (4 °C, 3 min) and equilibrated at room temperature for at least 12 min prior to injection onto the HPLC system. During temperature equilibration before HPLC analysis, the sample derivatives were centrifuged at 3290 ***g*** for 10 min.

2.4. HPLC analysis

A binary gradient separation was achieved on a stainless steel analytical column (150 × 4.6 mm), packed with Supelcosil LC-8-DB 3 μm particles (Supelco, Bellefonte, PA, USA) maintained at 27 °C. Guard column (20 × 4 mm) was packed with the same material (5 μm). The mobile phase was pumped at a flow rate of 1.0 mL min^{-1} and was composed of MeCN:MeOH:H_2O in the ratios 425:425:150 (v/v/v) and 460:460:80 (v/v/v), respectively. The gradient profile was as follows (1) 0–15 min, 100% A; (2) 15–16 min, 100% A → 100% B; (3) 16–22 min, 100% B; (4) 22–23 min, 100% B → 100% A; (5) 23–27 min, 100% A. The total run time per single injection was 27 min. An injection volume of 20 μL was used. Fluorescence of the derivatives was detected at excitation and emission wavelengths of 364 and 470 nm, respectively. Calculations were performed according to the external standard method using a standard calibration curve, constructed by plotting the peak area as a function of analyte concentration.

2.5. Validation of the analytical procedure

Validation was performed using fortified raw milk samples determined to be free of endectocide residues. The method was validated for raw bovine milk, although its applicability to sheep and goat milk has also been tested.

The procedure was validated in accordance with Commission Decision 2002/657/EC [28], as a quantitative confirmatory method, with EPR and MOX considered as substances with a permitted limit in milk, and other endectocide substances as not allowed in milk. The following validation parameters were evaluated: specificity, linearity, recovery, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), stability and ruggedness.

2.5.1. Specificity

The ability of the method to chromatographically separate the endectocides studied was evaluated for standards (mixture with individual concentration of 25 μg L^{-1}) as well as for milk (fortified samples with individual standard addition of 2–40 μg kg^{-1} for a particular substance). Twenty negative bovine and four ovine milk samples were analysed under within-laboratory reproducibility conditions and the eventual background in the area of endectocide retention times was evaluated to detect the presence of possible interference.

2.5.2. Linearity

Linearity was determined for standards by the least squares method to calculate regression and correlation parameters between chromatographic peak areas and standard concentrations