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Development of high-performance liquid chromatography for the determination of carbadox and olaquindox in animal feed

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High-performance liquid chromatography (HPLC) method with UV detection (373 nm) was developed for the determination of carbadox and olaquindox in animal feed. Among three different columns (LichroCART Purosphere RP-18, Waters Symmetry Shield RP-8 and Zorbax Phenyl SDB) that were studied, the Waters Symmetry Shield RP-8 column provided a slightly higher efficiency and lower peak tailing for both analytes. The optimal mobile phase conditions were established by varying the concentration of acetonitrile, pH and flow rate. Feed samples were extracted with methanol/water (50:50 v/v) for 60 min. Further solid-phase clean-up on a AccuBond Alumina N cartridge was performed to obtain an extract suitable for HPLC analysis. The calibration curves showed acceptable linearity in the concentration range from 0.1 to 50 mg/L with correlation coefficients (r^2) above 0.995. The limits of detection were 0.03 mg/L and 0.02 mg/L for carbadox and olaquindox, respectively. The mean recoveries of drugs from the feed samples spiked at 1 and 2 mg/kg were in the range of 95.3–97.2%. The applicability of this technique was demonstrated by the analysis of broiler chicken and porcine feeds.

Key words: high-performance liquid chromatography, carbadox, olaquindox, feeds

INTRODUCTION

Quinoxaline-1,4-dioxides are a group of synthetic antibacterial drugs often used as growth promoters as well as to prevent a number of diseases in animals [1]. They are administered orally or mixed with animal feed. Carbadox (methyl-3-(2-quinoalinylmethylene)carbazate-N^1,N^4-dioxide) and olaquindox (2-(*N*-2-hydroxyethylcarbamonyl)-3-methyl-quinoxaline-N^1,N^4-dioxide) are the best known members, but their use has been baned by the EU due to their carcinogenic, mutagenic and photoallergenic effects [2]. In order to control the compliance of the ban of carbadox and olquindox, a simple and effective analytical technique for monitoring of these drugs in animal feeds is of great significance.

High-performance liquid chromatography (HPLC) is one the most efficient techniques suitable for the determination of quinoxaline-1,4-dioxides [3–11]. Most of the published works, however, only deal with animal tissue matrices [4, 7, 9], require the derivatization of analytes [4, 7] or are devoted to the determination of carbadox [3, 5, 6].

To our knowledge, only a few reports on a conventional HPLC determination of carbadox and olaquindox in feeds have been reported to date [8, 10]. Nevertheless, these methods are not sensitive enough [8] or are very time-consuming [10] and, consequently, cannot be widely used for routine analysis.

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The aim of this research was to develop a simple and sensitive HPLC method for the determination of carbadox and olaquindox in feeds. For this purpose, HPLC separation and UV detection conditions were optimized and a simple sample preparation procedure including a liquid–liquid extraction and solid-phase extraction clean-up was established.

EXPERIMENTAL

HPLC analyses were performed using a Waters Alliance 2695 Separations Module (Milford MA, US) equipped with a quaternary solvent delivery system, an autosampler, and a column heater. HPLC separations were achieved using a LichroCART Purosphere RP-18 (250 mm × 4.6 mm, i.d., 5 µm particle size), Waters Symmetry Shield RP-8 (250 mm × 4.6 mm, i.d., 5 µm particle size) and Zorbax Phenyl SDB (150 mm × 4.6 mm, i.d., 5 µm particle size) columns, maintained at 20 °C with a mobile phase flow rate of 1.0 mL/min. The injection volume was 20 µL. Absorbance detection was performed at 373 nm using a Waters 2487 Absorbance Detector. AccuBond Alumina N (500 mg/5 mL) cartridges used for sample clean-up were obtained from Agilent (Milford, MA, USA).

Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Acetonitrile, methanol, acetic acid and ammonium acetate were HPLC grade and used as received (Merck, Darmstadt, Germany). Standards of carbadox and olaquindox were purchased from Sigma (St. Louis, MO,

USA). Chemical structures of the drugs used in this study are shown in Fig. 1. Stock solutions of carbadox and olaquindox at the concentration of 0.1 mg/mL were prepared in methanol and stored at 4 °C, protected from light. Working solutions used for spiking blank samples were obtained by appropriate dilution in the extraction solvent.

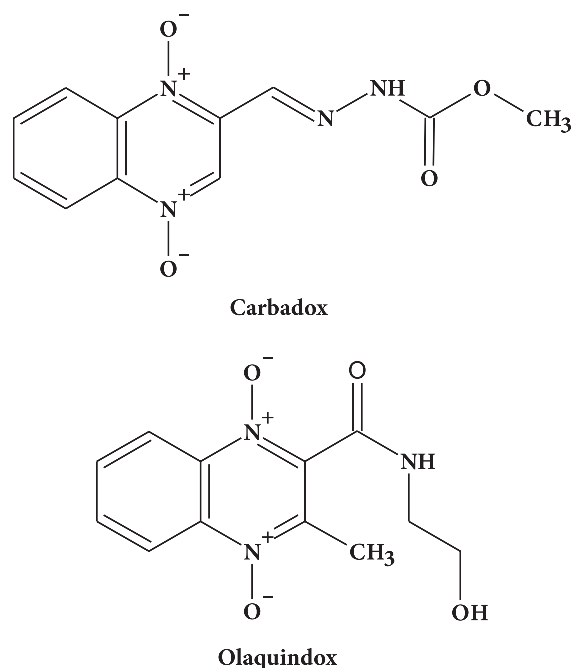

Fig. 1. Chemical structures of the drugs investigated

RESULTS AND DISCUSSION

Optimisation of separation and detection conditions

Since both analytes do not absorb in a visible range, in this work the UV region was used for detection. The detection conditions were optimized by measuring the UV absorption spectra of 0.05 mmol/L standard solutions in the range of 200–400 nm. The obtained results are demonstrated in Fig. 2. As can be seen, both analytes have relatively high absorbance in the range of 350–380 nm. Based on these results, a wavelength of 373 nm was selected for the further experiments.

Three commercially available columns representing different stationary phases (LichroCART Purosphere RP-18, Waters Symmetry Shield RP-8 and Zorbax Phenyl SDB) were compared in this study. The stationary phases have similar properties in terms of particle size, pore size and surface area, but their polarity slightly increases in the order RP-18 < RP-8 < Phenyl. The test mixture of the analytes was investigated under isocratic conditions at a flow-rate of 1 mL/min using a conventional reversed-phase HPLC mobile phase (10:90 v/v acetonitrile/water containing 25 mmol/L ammonium acetate). The results of the system suitability testing can be seen in Table 1. The retention of the analytes on the Phenyl column was greater than that on the 2.5-times longer RP-18 and RP-8 columns. These results clearly indicate that the Phenyl phase additionally exhibits π–π interaction with the analytes.

For the definition of the efficiency, two main parameters, namely the number of theoretical plates or height equivalent to a theoretical plate (H) were used. The latter was used in our case (tested columns differ in their length), because the theoretical plate number did not include the column length. Apparently,

Table 1. **Comparison of the performance data obtained for olaquindox and carbadox with different columns (n = 3)**

Analyte	Column	t_R, min	H, mm	A_s
Olaquindox	LichroCART Purosphere RP-18	5.1	0.16	1.3
	Waters Symmetry Shield RP-8	5.9	0.13	1.2
	Zorbax Phenyl SDB	6.8	0.15	1.3
Carbadox	LichroCART Purosphere RP-18	19.6	0.19	1.5
	Waters Symmetry Shield RP-8	20.4	0.16	1.3
	Zorbax Phenyl SDB	22.9	0.20	1.4

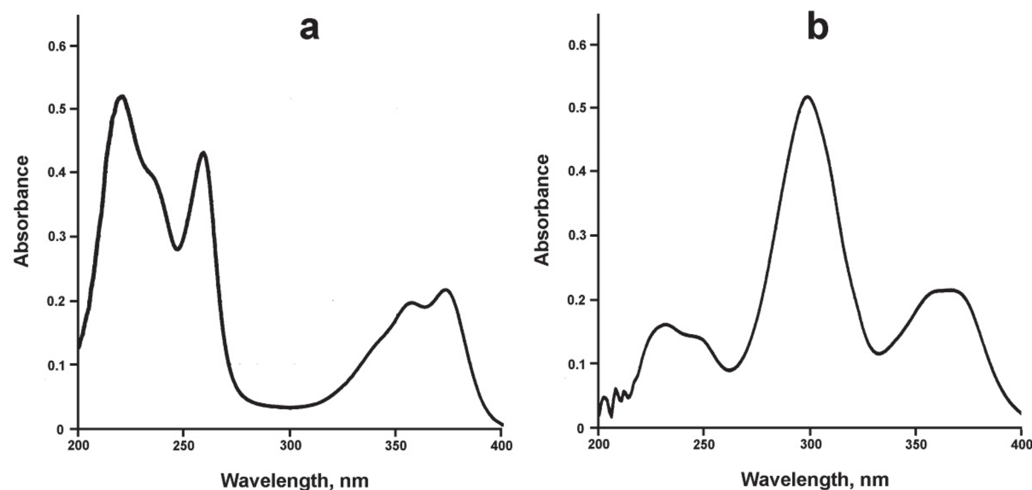

Fig. 2. Absorption spectra of olaquindox (a) and carbadox (b) standard solutions (0.05 mmol/L)

the efficiency of the Waters Symmetry Shield RP-8 column was slightly higher (lower H values) in comparison with the other two columns.

Peak asymmetry (A_s) is important for a precise peak integration and, thus, for quantitative information. However, a significant peak tailing was obtained for both compounds with all the three columns. Anyway, these values still meet the requirements for quantification, which recommend the A_s value to be less than 1.5. For both analytes the peak asymmetry was better in case of the Waters Symmetry Shield RP-8 column.

Peak resolution (R_s) describes the rate of compound separation. It was satisfactory (i. e. significantly higher than 1.5) for all the tested columns (results not shown). Based on the obtained results, the Waters Symmetry Shield RP-8 column was selected for the subsequent work.

Using an isocratic separation with mobile phase containing 10% acetonitrile the complete run time requires about 25 minutes. In order to accomplish the separation of carbadox and olaquindox in reasonable elution times, mobile phases containing various amounts of acetonitrile were evaluated (results not shown). As expected, an increased acetonitrile concentration greatly accelerated the elution of the analytes. However, at higher mobile phase elution strength, less hydrophobic olaquindox elutes in less than 4 minutes i. e. almost in the void volume of the column. Under these conditions, the quantification of the analyte is complicated due to the sample matrix interferences. The retention of a strongly retained compound in respect of less a retained one may be significantly reduced using a gradient elution mode. In order to elute olaquindox in about 5–6 minutes, 10% acetonitrile start concentration was necessary. On the other hand, acetonitrile content higher than 40% was required to elute carbadox in a reasonably short (less than 10 minutes) analysis time. These conditions lead to the adoption of the following gradient program: 0 min 10% CH_3CN, 5.0 min 20% CH_3CN, and 12.0 min 60% CH_3CN. Although the regeneration of the column following the gradient elution takes some time, the cycle time of the gradient elution is still shorter than that of the isocratic elution. Furthermore, the peak tailing was also reduced using this elution mode.

The influence of pH of the mobile phase on the retention behaviour of carbadox and olaquindox was studied in the pH range 3.5–7.0, and the results are shown in Fig. 3. As can be seen, the varying of pH has relatively little effect on the retention of olaquindox. In contrast, in the case of carbadox, its retention significantly increases when the pH of the mobile phase increases from 4.5 to 7.0. Unfortunately, since the protonation constants of the compounds studied are not available, an exact explanation of the obtained results is complicated. Most probably, when the pH of the mobile phase is over 4.5, the deprotonation of the analyte takes place. This deprotonation process results in reducing the polarity of carbadox and leads to much stronger hydrophobic interactions with the stationary phase. Based on these results, pH 4.0 was selected for further separations because it provided the shortest run time with acceptable resolution and peak efficiency.

Finally, the effect of the mobile phase flow-rate (from 1.0 to 2.0 mL/min) upon the efficiency was briefly studied. However, since no significant improvement in the peak efficiency was noticed for both compounds, the further work was carried out at 1 mL/min mobile phase flow-rate.

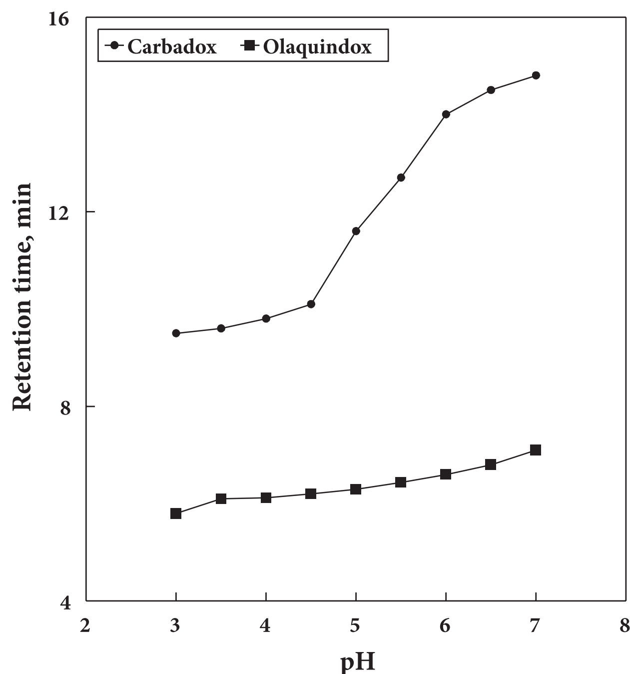

Fig. 3. Effect of mobile phase pH on the retention times of olaquindox and carbadox

The chromatogram obtained under optimum conditions for a standard solution is shown in Fig. 4.

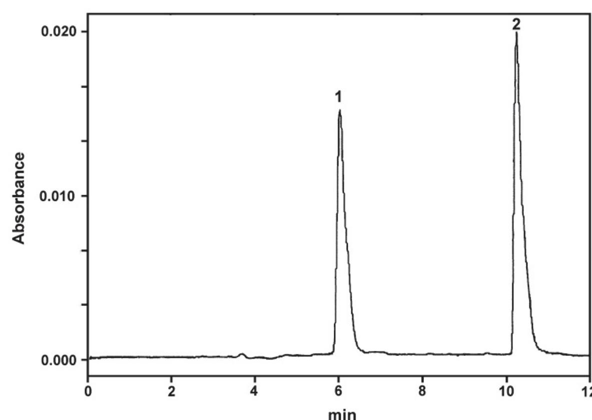

Fig. 4. Chromatogram of a standard solution (5 mg/L each analyte) under optimized conditions. Peaks: 1 – olaquindox; 2 – carbadox

Sample preparation

Common sample preparation strategies for veterinary drug residues in biological samples involve an extraction with an appropriate solvent (e. g., water, acetonitrile, methanol, ethyl acetate, etc.) followed by a sample clean-up procedure [5, 10–12]. Thus, preliminary studies were conducted to optimize solvents and extraction conditions. Based on the solubility properties of the analytes, the extraction efficiency from the spiked (at a 5 mg/kg fortification level) blank samples was studied using methanol/water mixtures at different volume ratios. The obtained results showed that 50:50 (v/v) methanol/water extractant provided acceptable (≥80%) absolute recoveries of both drugs from the spiked blanks.

Table 2. **Accuracy and precision data of the HPLC method for the determination of carbadox and olaquindox in spiked feed samples (n = 6)**

Parameter	Carbadox		Olaquindox	
	1.00 mg/kg	2.00 mg/kg	1.00 mg/kg	2.00 mg/kg
Found (mg/kg)	0.97	1.90	0.95	1.88
Mean recovery (%)	97.2	95.0	95.3	93.9
Precision (RSD%)	7.1	4.2	5.4	5.0

In the next step, the extraction of the drugs from the spiked blank samples was optimised by varying the extraction time from 20 to 80 min. However, extraction times greater than 60 minutes provided neither cleaner extracts nor higher analyte recoveries.

The complete procedure of sample extraction optimised for the analysis of carbadox and olaquindox residues was as follows: A 1 g of the feed sample and 10 mL of 50:50 (v/v) methanol/water solution were placed into a beaker and the mixture was agitated on a minishaker for 60 minutes. After centrifugation at 4000 rpm for 10 min, the methanolic/aqueous phase was ready for a clean-up procedure.

The extract from a biological sample contains many diverse compounds in addition to possible traces of the target analytes. In order to exclude these interfering substances, a number of clean-up methods have been developed for the analysis of veterinary drug residues. In most published methods for the determination of quinoxaline-1,4-dioxides in biological samples, at least one solid phase extraction (SPE) step with polar [10, 11] or reversed-phase [12] sorbent materials is included. In this study, polar AccuBond Alumina N (500 mg/5 mL) cartridges from Agilent were tested for the clean-up of the feed samples. Blank feed extracts spiked at 5 mg/kg were processed through all steps of the SPE in order to monitor the recoveries. Five milliliters of the extract was passed at 1–2 mL/min flow rate through the preconditioned cartridge. The first 0.5 mL of the effluent was directed to waste, and the next 2 mL fraction was collected, filtered and analysed. This SPE procedure yielded satisfactory high (≥90%) absolute recoveries for both analytes from the spiked blank extracts.

Analytical performance

In order to evaluate the practical applicability of the proposed technique, several important analytical performance characteristics were measured under optimized conditions.

The linearity of the method was tested by preparing calibration curves for both analytes with ten points. The tested concentration range was from 0.1 to 50 mg/L and each concentration level was injected for five times. The calibration curves were linear in the tested concentrations range with acceptable correlation coefficients (r^2) of more than 0.995. The limits of the detection defined as the concentration, which produced a signal equal to three times the background noise level, were 0.03 mg/L and 0.02 mg/L for carbadox and olaquindox, respectively.

The accuracy and precision of the method were determined by spiking blank feed samples with analytes at two concentration levels (1 and 2 mg/kg) and six samples per concentration level. The accuracy was expressed in terms of recovery rates and precision as relative standard deviation (RSD). These results are summarized in Table 2. According to the EU guidelines for approval of a method for drug residue analysis, the average recovery of quantitative methods at analyte concentrations higher than 1 mg/kg should be 80 to 110%, and the RSD should not exceed 16%. The results presented in Table 2 indicate that both analytes meet these criteria.

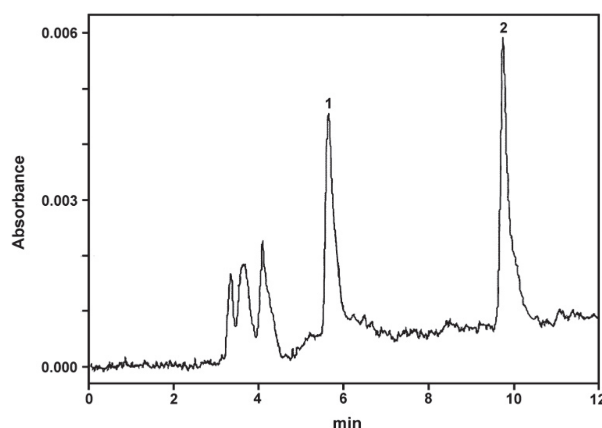

Fig. 5. Chromatogram of a porcine feed sample spiked at 3 mg/kg of olaquindox (1) and carbadox (2)

Finally, the HPLC method was applied to broiler chicken feed and porcine feed samples collected in Lithuania during 2006 year. Fig. 5 shows a typical chromatogram of the porcine feed sample spiked at 3 mg/kg of carbadox and olaquindox. Among the 25 samples analysed, none were found positive for the drugs studied.

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KARBADOKSO IR OLAKVINDOKSO NUSTATYMAS PAŠARUOSE EFEKTYVIOSIOS SKYSČIŲ CHROMATOGRAFIJOS METODU

Santrauka

Optimizuotas efektyviosios skysčių chromatografijos metodas karbadoksui ir olakvindoksui pašaruose nustatyti. Palyginus tris kolonėles (LichroCART Purosphere RP-18, Waters Symmetry Shield RP-8 ir Zorbax Phenyl SDB) nustatyta, kad truputį geresniu efektyvumu bei smailių simetriškumu analitėms pasižymi Waters Symmetry Shield RP-8 kolonėlė. Ištirta acetonitrilo koncentracijos judrioje fazėje, pH ir tėkmės greičio įtaka karbadokso ir olakvindokso atskyrimui. Vaistai iš pašarų mėginių ekstrahuojami vandens / metanolio (50 : 50 v/v) mišiniu, ekstraktas papildomai išvalomas kietafazės ekstrakcijos būdu leidžiant jį per polinį AccuBond Alumina N adsorbentą. Abiems junginiams išmatuotos kalibracinės kreivės tiesinės, esant 0,1–50 mg/kg koncentracijai ($r^2 \geq 0{,}995$). Aptikimo ribos – 0,03 mg/L karbadoksui ir 0,02 mg/L olakvindoksui. Vidutinės standartinių priedų (1 ir 2 mg/kg) pašarų matricoje išgavos siekia 95,3–97,2%. Metodas pritaikytas viščiukų lesalo ir paršelių pašaro analizei.