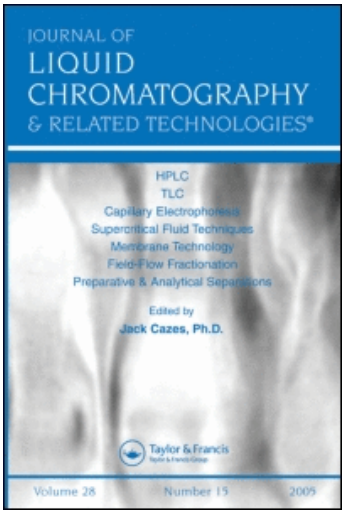

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SIMULTANEOUS DETERMINATION OF FIVE ANTIBACTERIALS IN SWINE MUSCLE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high performance liquid chromatographic method (HPLC) for the determination of olaquindox, morantel, furazolidone, nitrofurazone and carbadox residues in swine muscles was developed. The drugs were extracted from muscles with acetonitrile and cleaned up by alumina column. HPLC analysis was carried out on an Inertsil C8 column with a mobile phase of acetonitrile-water-acetic acid (3:97:1), and the drugs were detected at 340 nm. The average recoveries of all drugs added to muscles at 0.1 ppm level were more than 75% and the detection limit of each drug was 0.03 ppm in muscles.

INTRODUCTION

Furazolidone, nitrofurazone, carbadox, olaquindox, morantel and pyrantel (Fig.1) are often added to swine feeds to prevent and/or treat various infectious diseases to promote growth. Furazolidone and nitrofurazone

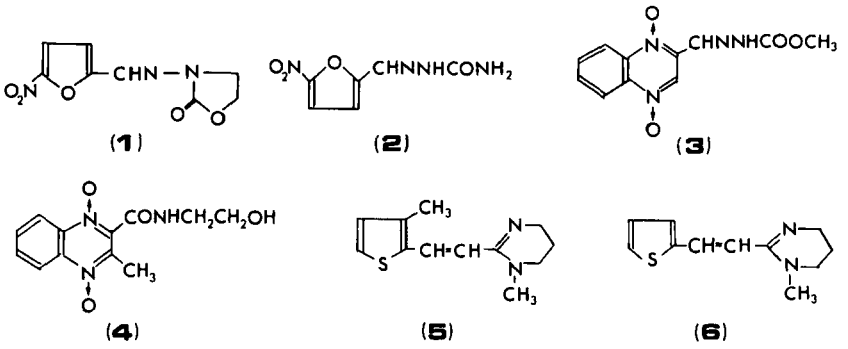

FIGURE 1. Chemical Structures of Five Drugs. (1) furazolidone, (2) nitrofurazone, (3) carbadox, (4) olaquindox, (5) morantel, (6) pyrantel,

are effective for treatment of necrotic enteritis in swine. Morantel is used for treatment of gastointestinal nematode infections and carbadox for prevention of swine dysentery and bacterial enteritis.

Residues of these drugs have been detected by spectrophotometry, gas chromatography and high performance liquid chromatography (HPLC). HPLC determination methods hitherto reported were for nitrofurazone (1-4), furazolidone (2,4,5-11), carbadox (3,13-15), olaquindox (16,17) and morantel (18,19). Among these methods, simultaneous determinations were carried out in combination with nitrofurazone and furazolidone (2,4), and with nitrofurazone and carbadox (3). Other analytical methods have been utilized for determination of each drug individually, and none of methods was simple enough to be employed routinely.

In the present study, an attempt was made successfully to simultaneously detect residues of olaquindox, morantel,

nitrofurazone, furazolidone and carbadox in swine muscle, in simple and rapid procedures, by HPLC.

EXPERIMENTAL

Caution : Sample and fortified extracts must be protected from direct sunlight or artificial light.

Reagents and chemicals

Drugs used in this study were olaquindox (Bayer Japan, Ltd., Tokyo, Japan), morantel succinate, pyrantel tartrate, carbadox (Pfizer-Sheiyaku Co., Ltd., Tokyo, Japan) and nitrofurazone, furazolidone (Sigma Chemical Company, St.Louis, MO, U.S.A.). Stock solutions were prepared by dissolving 10 mg of olaquindox, nitrofurazone, furazolidone and carbadox in 100 ml of N,N dimethyl formamide, 17.27 mg of morantel succinate and 18.72 mg of pyrantel tartrate in 100 ml of methanol at a final concentration of 100 μg base/ml.

The working standard solution was prepared at 1 μg /ml in methanol using 1 ml stock solution each. All other chemicals were analytical grade (Wako Pure Chemical Industry Ltd., Osaka, Japan). A chromatographic column, 300 mm x 15 mm i.d., was packed with 2 g alumina (Woelm N activity grade I , particle size, 50-200 μm, Woelm Pharma GmbH & Co., FRG) suspended in acetonitrile. The column was washed with 20 ml acetonitrile followed by 20 ml acetonitrile-water (95:5) solution.

Apparatus

The HPLC system consisted of a Model 880-PU pump equipped with a Model 875-UV spectrometer, a Model 860-

CO column oven, (Japan Spectroscopic CO., Ltd., Tokyo, Japan) and a Model CR-6A Chromatopak integrator (Shimadzu sheisakusho, Kyoto, Japan). Inertsil C8 stainless steel columns, 150 mm x 4.6 mm i.d.(5 μm) and 10 mm x 4 mm i.d.(10 μm) (Gasukuro Kogyo, Inc., Tokyo, Japan), were used as analytical and guard column, respectively. The flow rate of mobile phase, acetonitrile-water-acetic acid (3:97:1), was 1.0ml/min. The column temperature was maintained at 45°C and the drugs were detected at 340 nm.

Sample preparation

10 g minced muscle was accurately weighed, homogenized for 3 min with 40 ml of acetonitrile, centrifuged for 10 min at 2,500 g and the supernatant was then filtered through cotton. The residue was homogenized with another 30 ml acetonitrile, the above procedure was repeated and the filtrate was collected in a separatory funnel. 30 ml n-hexane, which was saturated with acetonitrile before use, was added, shaken 5 min and allowed stand until two layers separated. The lower layer was transferred to the alumina column and the solvent was drained to ca. 5 mm above the alumina layer. 20 ml acetonitrile-water (95:5) was added to the column, and drugs were eluted at a flow rate of 5-6 ml/min. All eluates were collected and concentrated to ca. 2-3 ml under vacuum on a rotary evaporator at 65°C. The residual eluates were evaporated to dryness by blowing air with a bellows. The residue was dissolved in 1 ml

methanol, the solution was filtered through a filter membrane of 0.5 μm porosity and applied to the HPLC instrument.

RESULTS AND DISCUSSION

These drugs are quite sensitive to light, so all procedures have to be conducted quickly under subdued light to obtain good results.

As extraction solvents, ethyl acetate (1,4,9), acetone (8, 10), methylene chloride (6,15,18), chloroform (4), N,N dimethyl formamide (4,7), acetonitrile (12,13, 16), ethanol (3,14), water (2,17) and methanol (2,5,7, 11) have been previously used. In this experiment, however, acetonitrile was most appropriate as extraction solvent to obtain a clear extract and conduct the following procedures easily. The extract was partitioned with n-hexane to remove fat, which otherwise shortens the life span of HPLC column. In this partition procedure, small amounts of carbadox were transferred to the n-hexane layer. To inhibit the transfer of the drug into the n-hexane layer, the n-hexane was saturated with acetonitrile before use. To simplify the procedure and to obtain satisfactory recoveries of drugs, the extract was directly poured onto the alumina column without a concentration procedure.

A basic and a neutral alumina were examined as potential column supports. It was found that the recovery rate of morantel decreased when a basic alumina

was used as a support. A suitable amount of alumina was selected by testing 1-6 g neutral alumina. When 1-3 g were used, the recovery of each drug was almost satisfactory. However, when 4 g alumina or more were used, the recovery of drug, especially that of olaquindox decreased. To obtain a satisfactory chromatogram without extraneous peaks from the sample, at least 2 g of neutral alumina was necessary.

When acetonitrile was solely used as the column mobile phase, drugs were not eluted from the alumina. It was noticed that involvement of certain amounts of water was necessary in mobile phase. Comparative studies showed that 5% water in acetonitrile was the minimal requirement to elute drugs and was appropriate to retain interfering substances effectively. Increased polarity of the eluent did not improve the recovery of drugs.

At the evaporation stage, n-propyl alcohol was added to prevent bumping. Since it was found that recoveries of drugs decreased when the eluate was completely evaporated to dryness under vacuum on a rotary evaporator, the eluate was concentrated to 2-3 ml under vacuum on a rotary evaporator at first, then blown to dryness with air at 65°C. There was no difference between the recoveries of drugs when the eluate was blown by air or by nitrogen.

Nucleosil C18 on 150 mm and 250 mm columns, and an Inertsil C8 on 150 mm column were compared for their

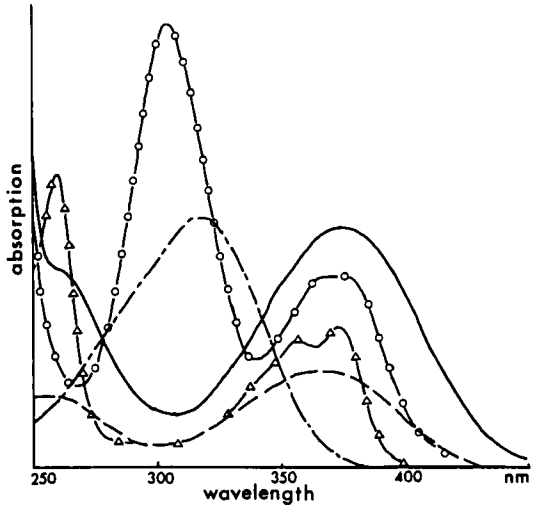

FIGURE 2. Absorption Spectra of Five Drugs. Concentration of each drug was 5 μg/ml in HPLC mobile phase. -Δ-:olaquindox, ——:nitrofurazone, -·- :morantel - -:furazolidone, -O-:carbadox,

efficiency. When the Nucleosil C18 columns were used, olaquindox was not retained to the column and a tailing phenomenon was observed in the morantel peak. Moreover, in the 150 mm column, nitrofurazone and carbadox were not separated. By using the Inertsil C18 column, olaquindox was retained on the column and a sharp peak for morantel was obtained. As for the mobile phase, an acidic condition was necessary to retain morantel on the column. With acetonitrile-water-acetic acid (3:97:1) as the mobile phase, five drugs were readily separated. The absorption spectra of five drugs were observed as shown in Figure 2. Detection wavelength was set at 340

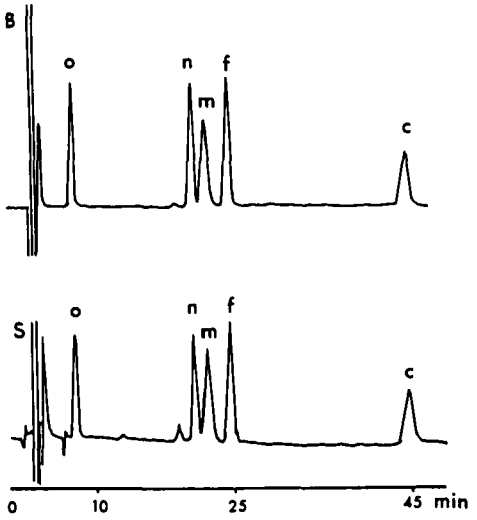

FIGURE 3. Chromatograms of Standards and Muscle Extracts. B::standards, S:swine muscle fortified with five drugs. o:olaquindox, n:nitrofrazone, f:furazolidone, m:morantel, c:carbadox, (20 ng each)

TABLE 1.

Recovery Study of Five Drugs from Fortified Swine Muscles

Added (ppm)		Olaquindox	Morantel	Nitro-furazon	Furazolidone	Carbadox
0.05	Rec.,(%)	65.9	85.7	82.1	80.0	84.3
	SD	0.032	0.061	0.015	0.017	0.029
	C.V.,(%)	9.70	14.2	3.65	4.25	7.00
1.0	Rec.,(%)	75.9	89.1	87.8	82.8	81.0
	SD	0.031	0.063	0.048	0.041	0.061
	C.V.,(%)	4.08	7.07	5.46	4.98	7.53
2.0	Rec.,(%)	71.9	88.5	88.5	84.3	90.9
	SD	0.063	0.085	0.073	0.051	0.103
	C.V.,(%)	4.37	4.78	4.12	3.02	5.66

Each value is the average of five examinations.

nm to compromise the different optimal wavelengths of the drugs. The chromatograms of standards and muscle extracts are shown in Figure 3. Other drugs, which are often administered to swines, such as sulfonamides, tyrosine and lincomycin, did not interfere with the detection of the target drugs, since the optimal wavelengths of these non-target drugs were towards shorter wavelengths. Neither nitrofuran nor nifursol interfered with the detection of drugs under these HPLC conditions.

A recovery test was carried out by adding 0.5, 1 and 2 ml of the working standard solutions to muscles and analyzed as described above. The results of recovery are shown in Table 1.

The calibration curve for each drug was as follows;

$X = 5.2651Y + 0.0624$, $r = 0.9967$ in olaquindox,

$X = 3.8519Y + 0.0642$, $r = 0.9943$ in nitrofurazone,

$X = 3.2599Y + 0.0836$, $r = 0.9977$ in morantel,

$X = 2.9554Y + 0.0122$, $r = 0.9988$ in furazolidone,

$X = 4.2653Y + 0.0694$, $r = 0.9988$ in carbadox,

where Y = peak area of drug, X = concentration (μg/ml) of drug and r = correlation coefficient, respectively.

The calibration curve was linear over the range of 3-40 ng in each drug and the detection limit, (5-fold noise level), was 0.03 ppm in each drug.

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