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Note

Rapid quantitation of ethopabate in chicken muscles using high-performance liquid chromatography with fluorimetric detection: purification from extractant by continuous liquid–liquid partition

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Ethopabate (4-acetamido-2-ethoxybenzoic acid methyl ester) is used as a coccidiostat in poultry feeds[1]. Published methods for its determination are based on gas–liquid (GLC)[2–5] and high-performance liquid chromatography (HPLC)[6] with spectrophotometric detection. These methods are tedious and time consuming, as ethopabate has to be derivatized in GLC methods and separated by column chromatography in the HPLC method.

This paper describes a conventional purification procedure involving droplet counter-current chromatography (DCCC)[7], and a sensitive HPLC determination with spectrofluorimetric detection. DCCC, a form of partition chromatography in which droplets of a mobile phase are passed through a column of stationary phase, has been used for the purification and separation of various samples. In this work, as ethopabate is fairly soluble in acetonitrile and hardly soluble in *n*-hexane, after the extraction of ethopabate from chicken muscles with acetonitrile, the extractant (used as the mobile phase) is continuously partitioned with *n*-hexane (stationary phase) and rapidly purified. This method has a limit of determination of 0.1 ng, compared with 1 ng for the ultraviolet absorption method.

EXPERIMENTAL

HPLC

A Shimadzu LC-3A HPLC instrument was used, equipped with an SPD-2A UV detector in combination with an RF 530 spectrofluorimeter and a column oven. The detector was connected to a Hitachi 065 1-mV recorder. Stainless-steel columns (50 × 4 mm I.D. as a guard column, followed by 250 × 4.6 mm I.D.) were packed with Zorbax ODS (5 μm), obtained from Shimadzu Seisakusho. Ethopabate was excited at 306 nm and the emission wavelength was 350 nm. UV monitoring of ethopabate was performed at 270 nm. The column oven was maintained at 50°C.

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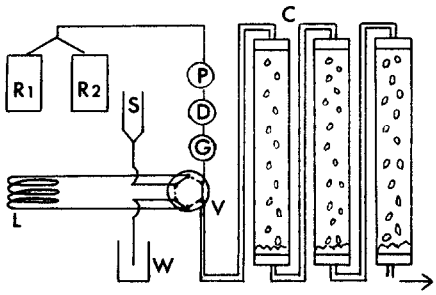

Fig. 1. Continuous liquid–liquid partition apparatus. R1, R2 = Solvent reservoir (acetonitrile, *n*-hexane); V = valve; D = damper; G = pressure gauge; C = PTFE tube; W = waste; L = sample loop; S = syringe; P = pump.

The mobile phase consisted of a mixture of acetonitrile, 0.01 *M* potassium dihydrogen orthophosphate and triethanolamine (40:59:1) acidified with orthophosphoric acid to pH 4.0. In all instances an injection volume of 10 μl, a flow-rate of 1.5 ml/min.

Continuous liquid–liquid partition

As illustrated in Fig. 1, the column system consisted of three PTFE tubes, each 220 × 9 mm I.D., which were joined in sequence top to bottom with 350 × 0.96 mm I.D. PTFE tubes. The total volume of stationary phase was about 48 ml and the volume of the injection loop was 25 ml. All experiments were carried out at room temperature. Before use, acetonitrile and *n*-hexane were allowed to equilibrate in a separating funnel, and then the upper phase was stored in the reservoir R2 and loaded into the PTFE columns as the stationary phase. The lower phase was stored in the reservoir R1 and sent off by pump as the mobile phase.

Reagents

Ethopabate was obtained from Dainippon Seiyaku and β-naphthol from Wako.

Sample preparation

A 20-g amount of chicken muscles was homogenized with 50 ml of acetonitrile and centrifuged at 2000 *g* for 10 min. The supernatant was filtered through cotton-wool. Extraction and centrifugation were repeated and the filtrate was diluted to 100 ml with acetonitrile. A 20-ml volume of this solution was loaded into the sample loop of the liquid–liquid partition column, then the mobile phase was pumped at 7 ml/min through the sample chamber and passed through a narrow tube into the top of the first PTFE tube. From the last column, 30 ml of eluate was collected and evaporated to dryness at 40°C. The residue was dissolved in 1 ml of acetonitrile containing β-naphthol as an internal standard and filtered with a 0.45 nm membrane filter. A 10-μl volume of the filtrate was injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

As shown in Fig. 2, when ethopabate was detected using a fluorescence detec-

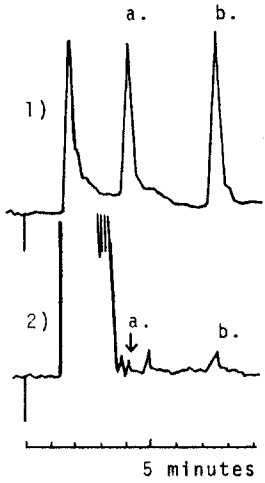

Fig. 2. Typical chromatogram obtained from chicken muscles under the conditions given in the text. (a) Ethopabate, 1 ng; (b) β-naphthol, 2 ng. Detector: (1) spectrofluorimeter, excitation at 306 nm and emission at 350 nm; (2) UV, 270 nm.

tor, there were no interfering peaks on the chromatogram. On the other hand, when detected by ultraviolet absorption, there were many interfering peaks. Also, the sensitivity of the former method was about ten times greater than that of the latter.

The optimal HPLC operating conditions was established by varying the mobile phase composition, column temperature and flow-rate. The retention time of the internal standard (β-naphthol) was longer than that of ethopabate, so triethanolamine was added to the mobile phase and the temperature of the column oven was set at 50°C. The flow-rate was set at 1.5 ml/min. By adjusting the pH of the mobile phase to below 5 with orthophosphoric acid, constant and well defined chromatograms of ethopabate and β-naphthol could be obtained.

A calibration graph was constructed for the range 0.05–2 ng of ethopabate

TABLE I

RECOVERY OF ETHOPABATE ADDED TO CHICKEN MUSCLES

Ethopabate added (μg)	*Recovery (%)*	*Average (%)*	*Standard deviation (%)*	*Coefficient of variation (%)*
0.2	84.2	90.2	3.1	3.44
	84.9			
	91.3			
	94.2			
	96.4			
1.0	95.3	96.7	0.085	0.09
	93.0			
	97.5			
	101.0			
	96.9			

with a constant amount of 2 ng of β-naphthol present. The ratio of the peak height of ethopabate to that of the internal standard was plotted against the concentration of ethopabate, and was found to be linear.

In the continuous liquid–liquid partition procedure, to remove as much interfering material from the extract as possible, the effect of the number of PTFE tubes (1-6) and the flow-rate of the mobile phase (3, 5, 7 and 10 ml/min) were studied. After evaporation of the extract, the weights of the residues were compared. It was found to be necessary to connect at least three PTFE tubes in order to purify the extract effectively. With one or two tubes, even if the *n*-hexane in the stationary phase was replaced for each sample, the extract was not sufficiently purified. The flow-rate of the mobile phase had no effect on the purification.

More than 95% of the ethopabate was recovered in the first 20 ml of eluate, so 30 ml were collected.

A recovery test was carried out using three connected PTFE tubes and a flow-rate of the mobile phase of 7 ml/min. Ethopabate was added to chicken muscles at levels of 0.05 and 0.25 ppm. As shown in Table I, recoveries of 90.2% (0.05 ppm level) and 96.7% (0.25 ppm level) were obtained, with coefficients of variation of 3.44% and 0.09%, respectively.

In conclusion, the method presented is simple, rapid and sensitive, appears to have satisfactory precision and accuracy and can be used easily in a regulatory laboratory.

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