ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 259–265

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

High performance liquid chromatographic assay of amprolium and ethopabate in chicken feed using solid-phase extraction[1]

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Received for review 27 July 1995; revised manuscript received 21 February 1996

Abstract

A method for the assay of mixtures of amprolium and ethopabate in chicken feed was developed utilizing reversed-phase high-performance liquid chromatography (HPLC) after sample clean-up of a methanolic extract by solid-phase extraction using CN cartridges. HPLC was done with benzocaine as internal standard on a C-8 column with methanol–water 40:60, containing octanesulfonic acid, triethylamine and acetic acid, as mobile phase. Eluate was monitored at 274 nm. Baseline separation was achieved with retention times of ≈ 7.5, 9.4, and 10.4 min, for amprolium, benzocaine, and ethopabate respectively. Feed constituents did not give peaks after 6.5 min. Peak area ratios were linear over 10–180 ng of amprolium, and 2–18 ng of ethopabate injected. Limits of quantitation at AUFS 0.05 were 0.5 and 0.3 ng respectively. Recovery studies from spiked feed ($n = 9$), covering $\pm 30\%$ of usual doses in feed, gave percent recoveries ($\pm$ SD) of $99.4 \pm 1.4\%$ for amprolium and $100.5 \pm 2.6\%$ for ethopabate. Applying the method to two different batches of commercial feed gave results which were comparable to those obtained by the AOAC spectrofluorometric methods.

Keywords: Amprolium; Chicken feed; Coccidiosis; Ethopabate; Ion-pair HPLC; Solid-phase extraction

1. Introduction

Chicken feed containing mixtures of 125 ppm of amprolium, 1-[(4-amino-2-propyl-5-pyrimidinyl)methyl]-2-methylpyridinium chloride hydrochloride, and 4 ppm of ethopabate, 4-acetamido-2-ethoxybenzoic acid methyl ester, is routinely fed to chickens as a starter feed to prevent coccidiosis. This disease, caused by several *Eimeria* species, can wipe out the entire poultry population of a farm in a matter of a few days. Studies

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PII S0731-7085(96)01829-8

undertaken in this laboratory to investigate the compliance with tolerance limits for residual drugs in chicken tissues as established in the Code of Federal Regulations (21 CFR Part 556) required the feeding of chicken feed containing these two coccidiostats to chickens. To assure that the drug contents in the feed were in conformity with the label claim, a simple, accurate assay method for these compounds in chicken feed was necessary.

Although amprolium and ethopabate are used as a mixture in feed, the literature does not reveal methods for the assay of these mixtures in feed. The AOAC official methods deal with feed containing amprolium or ethopabate only [1]. These methods are very laborious and time-consuming as they involve elaborate sample clean-up steps. After clean-up, amprolium was determined by timed color-forming derivatization steps and measurement by colorimetry or fluorometry. In the case of ethopabate, the compound was first hydrolyzed to the free amine, followed by derivatization into a colored product.

Chromatographic methods have also been applied to the assay of these compunds individually, after sample clean-up by (multiple) solvent extraction(s) or by conventional column chromatography. Amprolium in feed was assayed by high-performance liquid chromatography (HPLC) on specially prepared silica gel with detection at 270 nm [2]. Another HPLC method was reported for amprolium in chicken tissues using post-column fluorometric derivatization [3]. In feed mix, ethopabate was assayed by gas–liquid chromatography using a flame ionization detector [4]. Several HPLC methods were reported for ethopabate in feed or in tissues with UV detection [5] or by fluorometry utilizing its native fluorescence [6,7].

This paper reports a simple sample clean-up by solid-phase extraction (SPE) and an accurate HPLC assay method that allows the simultaneous assay of amprolium and ethopabate in chicken feed with a much shorter analysis time. In addition, it will also serve as the method for the quality control of commercial chicken feed containing mixtures of amprolium and ethopabate.

2. Experimental

2.1. Apparatus

The liquid chromatograph consisted of a Beckman Model 110B Solvent Delivery Module equipped with a 20 μl injection valve (Beckman Instruments, Fullerton, CA), a Model 783 programmable absorbance detector (Kratos Analytical–Perkin-Elmer Corp., Norwalk, CT), a Model 4270 electronic integrator (Varian Instruments, Walnut Creek, CA), and a 12-port Visiprep Solid Phase Extraction Vacuum Manifold (Supelco, Inc., Bellefonte, PA).

2.2. Reagents and materials

The following reagents and materials were used: amprolium hydrochloride (Sigma Chemical Co., St. Louis, MO), ethopabate (courtesy Merck & Co., Inc., Rahway, NJ), benzocaine, methanol (Fisher, Fairlawn, NJ), solid-phase extraction cartridges (LC-CN, Supelco, Inc.), commercial egg-laying feed mixture (Layeena®, Ralston Purina, St. Louis, MO). All other chemicals were reagent-grade and were used as received.

2.3. HPLC conditions

A 15 cm × 4.6 mm i.d. LC-8-DB 5 μm column (Supleco, Inc.), was used at ambient temperature. The mobile phase consisted of methanol:water 40:60 v/v, containing 4.6 mM sodium 1-octanesulfonate, 0.5% triethylamine, and 0.5% glacial acetic acid, and was pumped at 1 ml min^{-1}. The mobile phase was filtered through a 0.45 μm Nylon-66 filter (Rainin Instrument Co. Inc., Woburn, MA) and vacuum-degassed before use. The eluate was monitored at 274 nm (0.08 AUFS). The attenuation of the integrator was programmed to run at 2 during the first 8.5 min of the chromatographic run and at 0.5 thereafter.

2.4. Internal standard solution

About 10 mg of benzocaine was accurately weighed, tranferred into a 10 ml volumetric flask, dissolved, and diluted to volume with methanol.

Exactly 0.5 ml of this solution was pipetted into a 50 ml volumetric flask and diluted to volume with methanol.

2.5. Standard solutions preparations

2.5.1. Amprolium

About 25 mg of amprolium was weighed accurately and transferred to a 100 ml volumetric flask, dissolved and diluted to volume with methanol. Exactly 5.0 ml of this solution was pipetted into a 50 ml volumetric flask and diluted to volume with methanol to give a concentration of about 0.025 mg ml^{-1}.

2.5.2. Ethopabate

About 16 mg of ethopabate was weighed accurately and transferred to a 100 ml volumetric flask, dissolved and diluted to volume with methanol. Exactly 1.0 ml of this solution was pipetted into a 100 ml volumetric flask and diluted to volume with methanol to give a concentration of about 0.0016 mg ml^{-1}.

2.5.3. Standard working solution

Volumes of 2.0 ml of the diluted amprolium standard solution and 1.0 ml of the diluted ethopabate standard solution were pipetted into a 10 ml volumetric flask. After the addition of 0.5 ml of internal standard solution, the solution was diluted to volume with methanol.

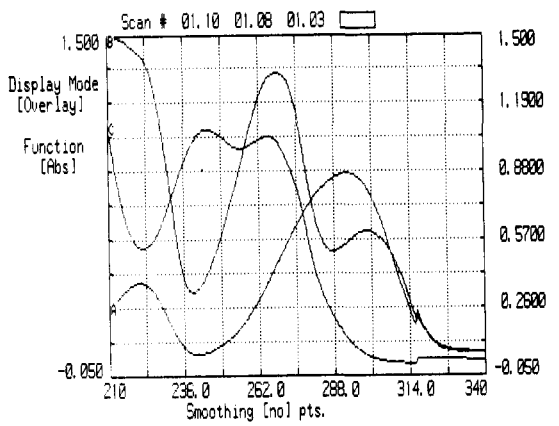

Fig. 1. UV absorption spectra of amprolium (C), ethopabate (B) and benzocaine (A) in methanol.

2.6. Sample solution preparation

About 10 g of commercial chicken feed, containing a mixture of 0.0125% amprolium and 0.0004% ethopabate, was accurately weighed into a 125 ml Erlenmeyer flask and suspended in 25.0 ml of methanol. The feed was sonicated in an ultrasonic bath for about 5 min, and subsequently set aside for 30 min with occasional shaking. The supernatant was filtered through a dry, fluted filter paper, discarding the first few milliliters of the filtrate.

2.7. SPE

The LC-CN solid phase extraction cartridges were conditioned by passing methanol followed by distilled water. Exactly 2.0 ml of the above sample filtrate was passed through the conditioned SPE cartridge and the excess fluid eluting out of the cartridge was collected in a 10 ml volumetric flask. The remaining retained analytes were eluted by passing 1.5 N HCl through the cartridge and collected in the same 10 ml volumetric flask. The sample solution in the flask was neutralized with ammonium hydroxide solution

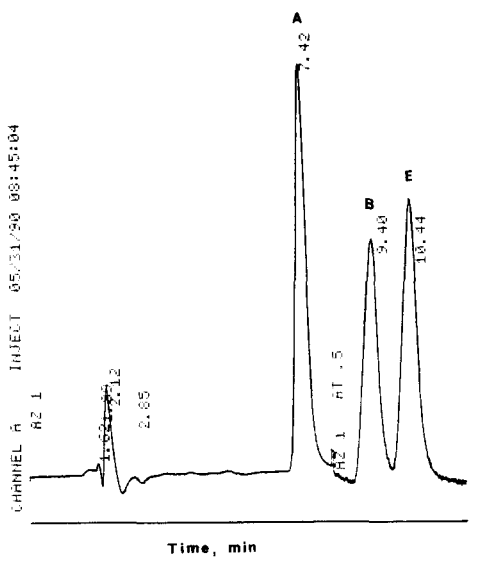

Fig. 2. Liquid chromatogram of methanolic standard solution run under conditions described in the text: A, amprolium; B, benzocaine; E, ethopabate.

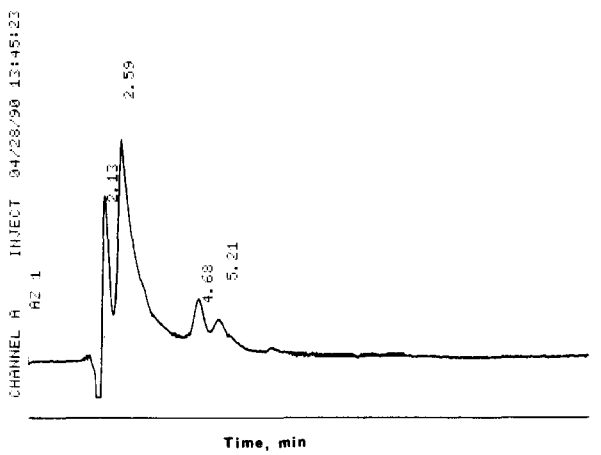

Fig. 3. Liquid chromatogram of chicken feed placebo run under conditions described in the text.

and diluted to volume with methanol. The solution was then transferred to a centrifuge tube and centrifuged for 5 min. A volume of 5.0 ml of the supernatant was pipetted into a 10 ml volumetric flask. After the addition of 0.5 ml of internal standard solution, the solution was diluted to volume with methanol.

2.8. Chromatographic procedure

Using the 20 μl sample valve, the sample solution and the standard solution were injected separately into the HPLC instrument under the operating conditions described above. Quantitation was based on relating the compound:internal standard peak area ratio of the sample to that of the standard.

3. Results and discussion

It was virtually impossible to find an internal standard that was related to both amprolium and ethopabate because these two compounds differ significantly in terms of chemical structure. Attempts to find a chemical entity that could serve as an internal standard and be added to the feed mix sample prior to sample preparation was not sucessful. However, trials with a number of compounds indicated that benzocaine can be used as an internal standard for correcting variations, if any, during the chromatographic run. Although sample preparation was conducted in the absence of an internal standard, the efficiency of this step was verified by recovery studies as well as by comparisons of the results with the official AOAC method as further described later.

The UV absorption spectra of amprolium, ethopabate and benzocaine in methanol were scanned for the selection of the HPLC UV detector wavelength. Although their λ_{max} values will be shifted in the presence of mobile phase, the spectral shift will be minimal. Fig. 1 is an overlay spectrum of the UV absorption spectra of the three compounds in methanol. From this spectrum, a compromise wavelength of detection of 274 nm was selected because this wavelength is close to the λ_{max} value of amprolium and ethopabate and is in a region where benzocaine still shows appreciable absorption.

Under the proposed experimental conditions, amprolium, ethopabate and benzocaine eluted as peaks well separated from one another (Fig. 2) with typical tailing factors at 5% of peak height of 1.3 for amprolium, and 1.1 for benzocaine and amprolium. The benzocaine (internal standard) peak eluted between amprolium and ethopabate. The approximate retention times were 7.5 min for amprolium, 10.4 min for ethopabate and 9.4 min

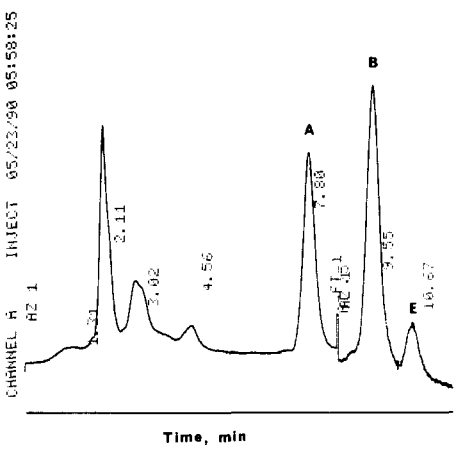

Fig. 4. Liquid chromatogram of the SPE eluate from spiked chicken feed placebo run under conditions described in the text: A, amprolium; B, benzocaine; E, ethopabate.

Table 1
Recovery data from spiked chicken feed palcebo

Sample	Amount added[a] (ppm)		Amount found[b] (ppm)		Recovery (%)	
	Amp.	Eth.	Amp.	Eth.	Amp.	Eth.
AOAC method	125.0	4.0	126.4	3.92	101.1	98.0
HPLC method						
A1	87.5	2.8	88.6	2.97	101.3	106.1
A2	87.5	2.8	86.5	2.75	98.9	98.2
A3	87.5	2.8	86.4	2.81	98.7	100.4
B1	125.0	4.0	124.5	4.01	99.6	100.3
B2	125.0	4.0	124.0	4.04	99.2	101.0
B3	125.0	4.0	121.5	3.91	97.2	97.8
C1	162.5	5.2	162.5	5.25	100.0	101.0
C2	162.5	5.2	165.0	5.27	101.5	101.3
C3	162.5	5.2	159.7	5.12	98.3	98.5
				Overall recovery:	99.4	100.5
				SD	1.38	2.48

[a] Covering ± 30% of commercial potency; Amp. = amprolium, Eth. = ethopabate.
[b] Average of duplicate runs.

for benzocaine, corresponding to retention factors, k', of 2.5, 3.9, and 3.4 respectively. The resolution between amprolium and internal standard was 3.1 and between ethopabate and internal standard it was 1.4. Determination of the column efficiency, expressed in terms of the height equivalent to a theoretical plate (HETP), gave HETP values of 0.05, 0.05 and 0.06 mm plate^{-1} for amprolium, ethopabate and benzocaine respectively.

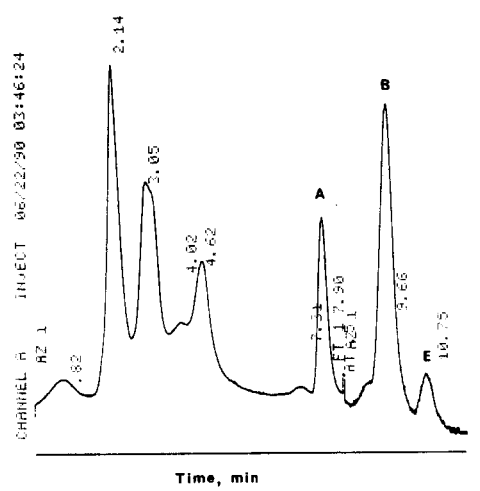

Fig. 5. Liquid chromatogram of the SPE eluate from commercial starter feed run under conditions described in the text: A, amprolium; B, benzocaine; E, ethopabate.

The relationship between the compound:internal standard peak area ratio and amount of compound injected was established. Linearity was obtained between 10–180 ng of amprolium and 2–18 ng of ethopabate injected. Typical regression equations were: $A = 32.3C - 0.08$ for amprolium; and $A = 79.8C - 0.04$ for ethopabate, where A = compound:internal standard peak area ratio, and C = amount (μg) of compound injected. The correlation coefficients, r, were 0.999 for both amprolium and ethopabate. The limit of quantitation (LOQ) was determined by a modified method of Glaser et al. [8] at 0.05 AUFS. The modification involved assaying samples ($n = 7$) at three different concentration levels near the preliminary estimated quantitation limit and determining the standard deviation (SD) of each level. The Y intercept (S_0) of the SD vs. concentration plot was multiplied by 10. The

Table 2
Recovery data from commercial starter feed[a]

Sample	Amount found (ppm)[b]				Recovery (%)			
	HPLC		AOAC		HPLC		AOAC	
	Amp.	Eth.	Amp.	Eth.	Amp.	Eth.	Amp.	Eth.
AT1	111.3	4.00	114.8	3.74	89.0	100.0	91.8	93.5
AT2	112.4	3.99			89.9	99.8		
AB	107.8	3.76			86.2	94.0		
BT1	107.5	4.12	116.5	3.87	86.0	103.0	93.2	96.7
BT2	101.9	3.96			81.5	99.0		
BB	113.1	4.00			90.5	100.0		

[a] Containing 125 ppm amprolium and 4 ppm ethopabate; Amp. = amprolium, Eth. = ethopabate.
[b] Average of duplicate runs.

value of "10 S_0" was used to determine the LOQ following the compendial method [9]. This method gave LOQ values of 0.5 and 0.3 ng of injected amprolium and ethopabate respectively, at the 95% confidence level.

Recovery studies were performed on chicken feed placebo spiked with amprolium and ethopabate, covering the range between −30% and +30% of the regular concentration levels of the two compounds in feed. A typical chromatogram of a chicken feed placebo, that was subjected to the SPC cleanup, is shown in Fig. 3. The chromatogram shows that the SPE cleanup procedure was sufficiently effective since no feed components peaks are observed beyond 6.5 min. Fig. 4 is the liquid chromatogram of chicken feed placebo spiked with amprolium and ethopabate. The sharp spike, indicated by "FT" on the chromatogram, was the result of the programmed change in electronic integrator attenuation from 2 to 0.5. This change in integrator attenuation was necessary to show all peaks on the chart due to the much lower concentration of ethopabate in feed mix as compared to the amprolium concentration. Overall percent recoveries ($\pm$ SD, $n = 9$) for amprolium and ethopabate were $99.4 \pm 1.38\%$ and $100.5 \pm 2.48\%$ respectively (Table 1). For comparison purposes a chicken feed placebo spiked with the usual levels of amprolium and ethopabate was assayed by the AOAC method [1]. The percent recovery ($n = 2$) was 101.1% and 98.0% for amprolium and ethopabate respectively. Comparison of the 125 and 4 ppm results showed that the results obtained by the proposed HPLC method are statistically ($p = 0.05$) not different from those obtained by the AOAC method.

The method was applied to commercial starter feed. Samples from two different batches, A and B, of commercial starter feed were assayed. AT1, AT2, BT1 and BT2 were sampled from the top of the bag of each respective batch while AB and BB were sampled from the bottom of the bag of each respective batch. No conclusion can be drawn from the data as to whether shifting of drug components had occurred in the bags. One sample from each batch of commercial feed (AT1 and BT1) were also assayed according to the method of the AOAC [1]. Table 2 shows the recovery data obtained from these two methods and Fig. 5 shows a typical liquid chromatogram of the SPE eluate from a commercial starter feed.

Acknowledgements

Thanks are due to the National Institutes of Health for financial support. This study is part of NIH grant No. R15 DK40148-01 entitled *Coccidiostat Residues Determination in Chickens and Eggs*. The generosity of Merck & Co., Rahway, NJ is acknowledged for supplying the ethopabate raw material used in this investigation. This work is part of the M.S. thesis of P.R.

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