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Spectrofluorimetric analysis of ethopabate in veterinary formulations with application to residue determination in chicken muscles and liver

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ABSTRACT: Ethopabate is a veterinary drug used in the prophylaxis and treatment of coccidiosis in chickens. The presence of drug residues in edible tissues can be dangerous to human consumers. It may cause direct toxic effects, allergic reactions and increased bacterial resistance. A highly sensitive, simple and rapid spectrofluorimetric method was developed for the determination of ethopabate in its veterinary formulations. The proposed method is based on measuring the native fluorescence of ethopabate in water at 364 nm after excitation at 270 nm. The fluorescence–concentration plot was rectilinear over the range of 2–100 ng/mL, with a limit of detection of 2.9 ng/g and a limit of quantification of 9.8 ng/g for ethopabate. The method was successfully applied to the analysis of ethopabate in its commercial veterinary formulations and the results were in good agreement with those obtained with the reference method. The method was extended to the determination of ethopabate residues in chicken muscles and liver, and the results were satisfactory. The recoveries obtained were in the 108.36–113.42% range. No organic solvents are used in the procedure, so it can be considered a type of 'green' chemistry.

Keywords: ethopabate; spectrofluorimetry; veterinary formulations; chicken muscles; liver

Introduction

Ethopabate (ETH), methyl—4-acetamido-2-ethoxybenzoate (1) (Fig. 1), is a coccidiostat frequently used in the prophylaxis and treatment of coccidiosis and leukocytozoonosis in chickens (2). It has a synergistic effect with some anticoccidial drugs (3). Edible tissues containing veterinary drug residues can pose risks to human health, including direct toxic effects, allergic reactions and increased bacterial resistance (4). Coccidiosis is caused by an obligate intracellular protozoan parasite of the genus *Eimeria*. Coccidiosis is considered to be one of the most significant diseases of poultry and costs the world's commercial chicken producers at least US$1.5 billion annually (5). The symptoms of coccidiosis in poultry may be one or more of the following: bloody diarrhea, high mortality, reduction in feed and water intake, emaciation and loss of egg production (6). A wide range of drugs is available for the prevention and treatment of coccidiosis. Withdrawal times have been established for these substances, but residues are frequently found in products derived from poultry eggs and meat (7). The use of ETH as a feed additive is banned in many countries, and the US Code of Federal Regulations (CFR) has established a maximum residue limit (MRL) of 0.5 ppm in chicken muscles and 1.5 ppm in chicken liver (8).

Reviewing the literature revealed that there was only one method for the determination of ETH in veterinary preparations using thin-layer chromatography (TLC) and ion-pair high-performance liquid chromatography (HPLC) with UV detection (9). To determine ETH residues in food, liquid chromatographic techniques are most commonly employed. ETH was determined in poultry liver using UV detection after solid-phase extraction (SPE) for sample cleanup (10). It was also determined in chicken muscles using fluorimetric detection after SPE (11) or liquid extraction for sample purification (12). ETH was determined in chicken muscles, chicken liver, eggs and baby food by micellar liquid chromatography with fluorescence detection after aqueous sodium dodecyl sulfate (SDS) extraction (13). However, these methods suffer from some limitations such as column clogging, involving complex procedures, need personal expertise and require expensive equipment.

Eliminating the need for chromatography and simplifying the extraction and cleanup would provide the simple and more rapid methods required by the food industry to screen tissue samples for veterinary drug residue violations (14). Spectrofluorimetric techniques are highly sensitive, selective, easily operated and economical and therefore have been used to estimate drug residues in tissues (14) and milk (15,16).

The aim of this study was to exploit the native fluorescence of ETH to develop a rapid, simple, sensitive and selective spectrofluorimetric method for determining ETH in veterinary formulations in addition to the determination of ETH residues in chicken muscles and liver.

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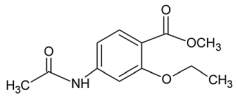

Figure 1. Structural formula of ethopabate.

Experimental

Apparatus

The fluorescence spectra and measurements were recorded using a Perkin–Elmer UK model LS 45 luminescence spectrometer, equipped with a 150 W xenon arc lamp; the excitation and emission wavelengths were 270 and 364 nm, respectively. The slit width for both monochromators was set at 10 nm, and the photomultiplier voltage was set to auto. A 1 cm quartz cell was used. A Consort NV P901 pH meter calibrated with standard buffers was used for pH measurements. A TDL-60B centrifuge (Anke, Taiwan), a BHA-180 T sonicator (Abbotta Corp, USA) and a Tissue Master-125 homogenizer with a 7 mm stainless steel generator probe (Omni International, USA) were used for sample preparation.

Reagents and materials

All chemicals used were of high purity, analytical grade, and distilled water was used throughout the study. ETH, analytical standard (100% purity, Vetranal®) was purchased from Sigma-Aldrich, Germany, and was used as received. Amprobate® powder (batch # 409067), labeled as containing 250 g of amprolium HCl and 16 g of ETH/1000 g, was produced by Memphis Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt, and was purchased from local veterinary centers. Extra-Cox® powder (batch # 11777), labeled as containing 26.5 g of amprolium HCl and 1.6 g of ETH/100 g, was produced by Medizen Pharmaceutical Industries for Panax Pharmaceuticals, Cairo, Elobour City, Egypt, and was purchased from local veterinary centers. Methanol, propanol, acetonitrile and acetone were obtained from Sigma-Aldrich (Germany). Acetate buffer solutions (0.2 M), covering a pH range of 3.6–5.6, and borate buffer solutions (0.2 M), covering the pH range of 6.5–9.5 were from BDH, UK. SDS (95%), cetrimide (CTAB, 99%; Winlab, UK), carboxymethyl cellulose (CMC; El Nasr Chem.Co., Cairo, Egypt) and Tween-80 (Adwic Co., Egypt) were used, a 0.5% aqueous solution of each was prepared. Hydrochloric acid (10 M) and sodium hydroxide were obtained from El Nasr Chem. Co., Cairo, Egypt. Chicken muscle and liver samples were purchased from the local market. Syringe filters (Minisart RC25) of pore size 0.45 μm were purchased from Sartorius-Stedim (Goettingen, Germany).

Standard stock and working solutions

A stock standard solution of 100.0 μg/mL of ETH was prepared in distilled water. A working standard solution of 10.0 μg/mL was prepared by further dilution with distilled water as appropriate.

Procedure for calibration graph

Accurately measured aliquots of the working solution were transferred into a series of 10.0 mL volumetric flasks and completed to the volume with distilled water so that the final concentration was in the range 2–100 ng/mL. The fluorescence intensity was measured at 364 nm after excitation at 270 nm. The relative fluorescence intensity was plotted against the final concentration of the drug. Alternatively, the corresponding regression equation was derived.

Procedure for veterinary formulations

An accurately weighed quantity of each of powdered Amprobate® and Extra-Cox® equivalent to 10 mg of ETH was transferred into a 100.0 mL volumetric flask and sonicated with 80.0 mL of methanol for 30 min. The volume was completed to the mark with methanol and filtered. The solution was further diluted with water to obtain solution containing 10.0 μg/mL of ETH. The procedure described under 'Construction of the calibration graph' was followed using water to prepare the working solution. The nominal powder content was calculated using the calibration graph or the corresponding regression equation.

Samples preparation

Samples of chicken muscle and liver (2.5 g each) were accurately weighed and spiked with aliquots of ETH solution. The spiked samples were mixed with 25 mL of extracting solvent (methanol/water 80: 20 v/v). The chicken muscles and liver samples were homogenized at 5000 rpm for 5 min, then the homogenate was sonicated for 30 min and then centrifuged at 3000 rpm for 5 min. The supernatant of all samples was filtered through 0.45 μm syringe filters. The filtrate was diluted with water to obtain solution containing 10.0 μg/mL of ETH. The procedure described under 'Construction of calibration graph' was followed. The ETH content was calculated using the calibration graph or the corresponding regression equation.

Results and discussion

ETH was found to exhibit an intense native fluorescence in aqueous solution at 364 nm emission after excitation of 270 nm (Fig. 2). This property allows us to develop a new spectrofluorimetric method for the determination of ETH in its veterinary formulations, either alone or in the presence of co-formulated drugs like amprolium, without interference. It also permitted the determination of ETH residues in chicken muscles and liver. The proposed method offers high sensitivity because concentrations of ETH low as 0.49 ng/mL could be detected accurately. Different experimental parameters were studied.

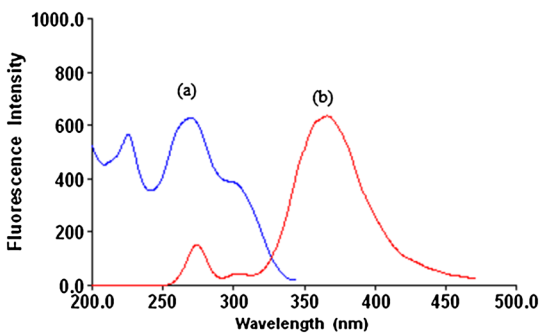

Figure 2. (a) Excitation and (b) emission spectra of 100 ng/mL ETH in water.

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Effect of pH

The influence of pH on the native fluorescence of ETH was studied by adding 2 mL of acetate buffer (pH 3.6–5.6), borate buffer (pH 6.5–9.5), 0.1 M NaOH and 0.1 M HCl. It was found that use of any of these buffers either does not affect the fluorescence intensity of ETH or even decreases it. Therefore, for simplicity, no buffer was used throughout this study.

Effect of different organized media

The fluorescence properties of ETH in various organized media were studied, using anionic surfactant (SDS), cationic surfactant (CTAB), non-ionic surfactant (Tween-80) and different macromolecules, CTAB, methyl cellulose and HP-β-CD, where 1 mL of each surfactant (0.5% w/v) was added to an aqueous solution of the drug (final concentration 0.1 μg/mL). Tween-80 caused a very slight increase in the relative fluorescence intensity (RFI), whereas SDS, CTAB, methyl cellulose and HP-β-CD caused a decrease in the RFI of the drug (Fig. 3). It is obvious from these results that the presence of surfactants has no significant effect or may decrease the fluorescence intensity. Therefore, no surfactant was used throughout this study.

Effect of diluting solvent

Dilution with different solvents including water, methanol, acetone, acetonitrile, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) was employed (Fig. 4). Water only gave the highest relative fluorescence intensity for ETH compared with the other solvents. Thus, water was chosen as the diluting solvent throughout the study.

Effect of time

The fluorescence intensity of ETH was measured at different times. It was found that the fluorescence intensity developed immediately and was not affected by time over a period of 24 h.

Effect of temperature

Another factor that affects fluorescence intensity is temperature. The effect of temperature was studied over the range 40–100 °C

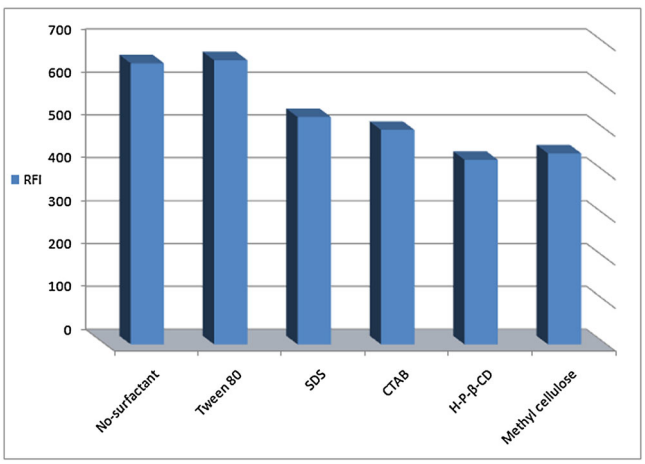

Figure 3. Effect of the type of organized media (1 mL of a 0.5% solution of each) on the RFI of ETH (100 ng/mL).

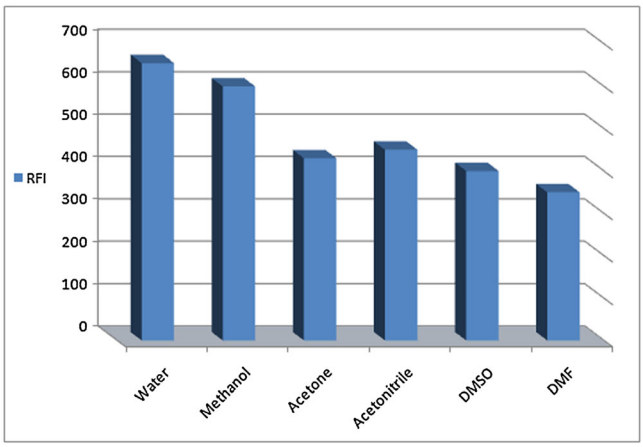

Figure 4. Effect of diluting solvent on the RFI of ETH (100 ng/mL).

in a thermostatically controlled water bath. It was found that increasing the temperature resulted in a decrease in the RFI. This effect can be explained by higher internal conversion as the temperature increases, facilitating non-radiative deactivation of the excited singlet state (17). Therefore, all the experiments were carried out at room temperature.

Validation of the method

The validity of the method was checked by testing linearity, LOD, LOQ, accuracy, repeatability, precision and specificity according to ICH recommendations (18).

Linearity and range. The linearity of the assay method was assayed by analyzing eight sets for the drug ($n = 8$ for the standard calibration plot), the fluorescence intensity vs concentration plot was linear over a concentration range of 2–100 ng/mL. Linear regression analysis of the data gave the following equation:

$$\mathrm{RFI} = 35.79 + 6.19C (r = 0.9999)$$

Where RFI is the relative fluorescence intensity and C is the final concentration of ETH in ng/mL. Statistical analysis of the data gave small values for the standard deviation of the residual ($S_{y/x}$), the standard deviation of the intercept (S_a), the standard deviation of the slope (S_b) and the percentage relative error (% Er), as shown in Table 1.

Limit of detection and limit of quantification. LOD was determined by evaluating the smallest concentration that can be detected and was found to be 2.9 ng/g (1.22×10^{-9} M). LOQ was determined by establishing the smallest concentration that can be measured, below which the calibration graph is non-linear, it was found to be 9.8 ng/g according to ICH Q2R1 recommendations (18):

$$\mathrm{LOQ} = 10S_a/b$$

$$\mathrm{LOD} = 3.3S_a/b$$

where: S_a is the standard deviation of the intercept of the calibration curve and b is the slope of the calibration curve.

Accuracy and precision. Intraday precision was evaluated by determining three concentrations of the drug in pure forms on three successive occasions in the same day. Interday precision

Table 1. Analytical performance data for the spectrofluorimetric determination of ethopabate

Parameter	Results
Wavelength ($\lambda_{ex}/\lambda_{em}$) (nm)	270/364
Linearity and range (ng/mL)	2–100
Limit of detection (ng/mL)	0.29
Limit of quantification (ng/mL)	0.98
Intercept (*a*)	37.44
Slope (*b*)	6.19
Correlation coefficient (*r*)	0.9999
SD of residuals ($S_{y/x}$)	2.47
SD of intercept (S_a)	0.61
SD of slope (S_b)	0.03
% RSD	1.98
% Err	0.70

Where: $S_{y/x}$ standard deviation of the residuals, S_b standard deviation of the slope,
S_a standard deviation of the intercept, $\%\ Error = \%\ RSD/\sqrt{n}$

Table 3. Assay results for the determination of ethopabate in pure form by the proposed and reference methods

Parameter	Method	
	Proposed	Reference
% Recovery	98.56	98.29
	104.67	101.71
	98.9	99.43
	99.34	
	101.61	
	99.29	
	100.75	
	100.18	
Mean ($\overline{X}$)	100.41	99.81
± SD	1.99	1.74
Students *t*-value[a]	0.65	
Variance ratio *F*-value[a]	1.31	

[a]Tabulated *t*- and *F*-values at $P = 0.05$ are: 2.262 and 4.74, respectively.

was also evaluated through replicate analysis of three concentrations over a period of three successive days. The results of intraday and interday precision are summarized in Table 2. The relative standard deviations were found to be very small, indicating reasonable repeatability and intermediate precision of the proposed method.

The results of the proposed method were favorably compared with those obtained using the reference method (19). Statistical analysis of the results obtained using the proposed and reference methods showed no significant difference in the performance of the two methods using Student's *t*-test and variance ratio *F*-test (Table 3). The proposed procedure offers additional advantages over the reference procedure in that the proposed is more sensitive with good accuracy and precision. The reference method depends on the reversed phase liquid chromatographic determination of ETH in poultry feeds, with UV detection using a C_{18} column and a mobile phase composed of acetonitrile/water (30: 70 v/v) at a flow rate of 1.4 mL/min. Detection was carried out at 280 nm using SPE for sample cleanup (19).

Table 2. Accuracy and precision data for the determination of ethopabate using the proposed method

Parameter	Ethopabate concentration (ng/mL)		
	20.0	50.0	100.0
Intraday[a]			
% Recovery	100.32	99.54	100.72
	99.61	99.73	100.51
	99.83	100.19	100.89
Mean ($\overline{X}$)	99.92	99.82	100.71
± SD	0.36	0.33	0.19
% Err	0.21	0.19	0.11
Interday[b]			
% Recovery	101.00	100.62	99.62
	100.87	100.13	99.84
	100.65	99.71	100.17
Mean ($\overline{X}$)	100.84	100.15	99.88
± SD	0.18	0.45	0.28
% Err	0.10	0.26	0.16

Each result is the average of three separate determinations.
[a]Three measurements taken on one day
[b]Measurements taken on three consecutive days

Selectivity. The selectivity of the method was investigated by observing any interference encountered from common excipients or co-formulated drugs such as amprolium in different formulations. It was shown that these compounds did not interfere with the results of the proposed method.

Applications

Veterinary formulations. The proposed method was applied to the determination of ETH in its veterinary formulations Amprobate® and Extra-Cox®. It is a powder intended to be dissolved in the drinking water of chickens. Because ETH and powder excipients are soluble in water and ETH is soluble in methanol, methanol was used to extract the drug. The specificity of the method was investigated by observing no interference from common excipients. This was proved by good recovery values obtained during determination of ETH in Amprobate® and Extra-Cox® powder formulations (Table 4).

Food samples. The applicability of the developed procedure for the determination of ETH was tested by analyzing ETH in spiked chicken muscle and liver. All samples were bought at a local supermarket. Table 5 shows the results of the ETH analysis determined in all samples after homogenization with extracting solution, sonication, centrifugation and filtration. Samples were spiked at the following ETH concentrations: 20, 50 and 100 ppb. The data obtained (Table 5) show satisfactory recoveries for ETH in all samples. These results reveal how a surveillance program for ETH residues can be performed under the proposed conditions.

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Table 4. Assay results for the determination of ethopabate in its different veterinary formulations by the proposed and reference methods

Parameter	Proposed method			Reference method
	Amount taken	Amount found	% found	% found
Amprobate® (16 g ETH/kg)	20.00	19.96	99.81	100.30
	50.00	50.06	100.13	99.75
	100.00	99.89	99.89	100.24
Mean			99.94	100.09
± SD			0.17	0.30
t-test		0.77		
F-test		3.28		
Extra-Cox® (16 g ETH/kg)	20.00	20.04	100.20	99.82
	50.00	50.04	100.09	99.93
	100.00	99.75	99.75	100.15
Mean			100.01	99.97
± SD			0.23	0.17
t-test		0.28		
F-test		1.95		

[a]Tabulated *t*- and *F*-values at $P = 0.05$ are: 2.776 and 19.00, respectively.

Table 5. Assay of ethopabate in food samples using the proposed and reference methods

Method	Chicken muscles		Chicken liver	
	Proposed	Reference	Proposed	Reference
% Recovery	112.92	108.34	110.32	108.24
	109.35	115.15	112.78	109.77
	115.26	116.78	108.94	107.08
Mean $(\overline{X})$	112.51	113.42	110.68	108.36
± SD	2.98	4.48	1.94	1.35
Students *t*-test[a]	0.29		1.69	
Variance ratio *F*-value[a]	2.26		2.08	

[a]Tabulated *t*- and *F*-values at $P = 0.05$ are: 2.776 and 19.00, respectively.

Conclusion

A simple, sensitive, rapid and economic spectrofluorimetric method was developed for the determination of ethopabate in its raw state and in veterinary formulations. The simplicity of the method allows the successful determination of the studied drug in its formulations either alone or in the presence of co-formulated drugs without prior separation and does not require the elaborate treatment associated with chromatographic methods. Moreover, the method was extended to the determination of ETH residues in chicken muscle and liver. The proposed method is useful for food quality testing and control to determine the ETH content in chicken muscles and liver samples. Validation according to ICH regulations provides satisfactory results in terms of sensitivity, linearity, accuracy and recoveries and at the ppb level. The proposed method is a non-pollutant methodology, because no organic solvents are used in the procedure, and it can therefore be considered as a type of 'green' chemistry.

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