Fluorimetric analysis of mebendazole and flubendazole with hydrogen peroxide

W. BAEYENS[1]*, F. ABDEL FATTAH[2] and P. DE MOERLOOSE[1]

[1] *Department of Pharmaceutical Chemistry and Drug Quality Control, Faculty of Pharmaceutical Sciences, State University of Ghent, Harelbekestraat 72, B-9000-Ghent, Belgium*
[2] *Pharmaceutical Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt*

Abstract: Analytically useful fluorescence was obtained from mebendazole and flubendazole after reaction with hydrogen peroxide in alkaline solution. The fluorescence produced ($\lambda_{ex} = 300$ nm; $\lambda_{em} = 360$ nm) is stable and concentrations of 0.1 µg ml^{-1} can be determined. The method has been applied to the analysis of some commercially available anthelmintic preparations.

Keywords: *Fluorescence spectroscopy; mebendazole; flubendazole; hydrogen peroxide derivatives; anthelmintics.*

Introduction

During a luminescence investigation of various pharmaceutically important imidazoles [1], when room temperature phosphorescence techniques [2] and fluorescence methods after alkaline hydrolysis [3] were shown to offer interesting possibilities for the analysis of some of these compounds, several general reagents described in the literature for fluorescence derivatization of organic compounds were tested. The drugs under investigation included the broad spectrum anthelmintics mebendazole and flubendazole (Scheme 1); levamisole (a potential drug for cancer and viral diseases); the antidepressant dexamisole; and tetramisole. Pure imidazole, benzophenone, benzimidazole and 2-aminobenzimidazole were used as reference compounds.

As the substituted imidazole drugs do not possess sufficiently conjugated systems to produce analytically useful fluorescence, it was hoped that reaction with fluorogenic reagents would produce more highly fluorescent derivatives with improved detection limits. This paper describes the analytical use of hydrogen peroxide for the fluorimetric analysis of mebendazole and flubendazole.
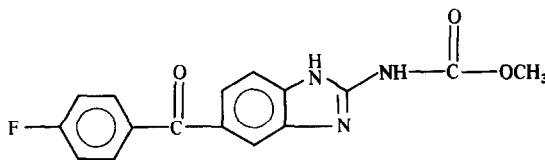
Experimental

Equipment

Luminescence spectra and measurements of fluorescence were made on an Aminco–Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.,

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*To whom correspondence should be addressed.
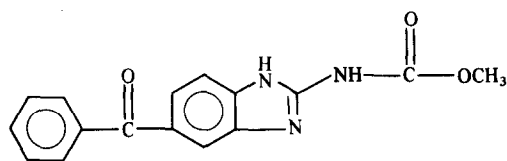
Scheme 1

Mebendazole

Flubendazole

USA), fitted with a Hanovia 150-W xenon arc lamp, grating excitation and emission monochromators, a photomultiplier (R446S) operated at 700–800V, and an X–Y recorder; the slits varied between 1 and 5 mm, the spectral bandpass being 5.5 nm mm^{-1} slit-width (Aminco off-axis ellipsoidal condensing system). Lamp stabilization was achieved by means of a magnetic arc stabilizer (Aminco); checks to ascertain that no fluctuations in lamp intensity occurred were carried out with polymer samples of fluorescence standards (e.g. anthracene, naphthalene, *p*-terphenyl, tetraphenyl-butadiene, rhodamine B) (ISA Belgium Groupe Instruments S.A., No. 16.156.139). No corrections were made for variations in source radiance, monochromator transmission or photomultiplier sensitivity with wavelength. The wavelength maxima reported are averages of at least five experimental values.

Ultraviolet absorption spectra were recorded on a Zeiss spectrophotometer, Model DMR 21, equipped with a hydrogen light source, 1-cm quartz cuvettes and a photomultiplier.

A Camag TL 29000 Universal UV lamp was used at 254 and 366 nm for viewing thin-layer chromatograms.

Materials

All imidazole drugs studied were generously given by Janssen Pharmaceutica, Beerse, Belgium and were used without further purification. The pharmaceutical preparations were also obtained from this manufacturer. Imidazole (analytical grade, Merck) was used without further purification; 2-aminobenzimidazole and benzophenone (Merck) were used after recrystallization from water or ethanol, respectively. All reagents were of analytical grade (Merck, UCB and J.T. Baker).

The purity of water (de-ionized or bi-distilled) and of the organic solvents used was controlled by luminescence scanning at highest instrumental sensitivity settings. A series of buffer solutions (pH 1–13) [1] were prepared for studying the pH-dependence of fluorescence.

Procedures for fluorescence measurements

Aqueous or ethanolic stock solutions of all imidazole derivatives were prepared in concentrations varying from 0.05 to 1 mg ml^{-1}, depending on their solubilities. Further dilutions were performed using 1 M ethanolic ammonium hydroxide solution, 1 M

ethanolic hydrochloric acid solution or ethanol. For those compounds soluble in water 1 M aqueous sodium hydroxide, 1 M aqueous hydrochloric acid or water were used. Spectra of neutral, acidic and alkaline solutions (1, 10 and 50 μg ml^{-1}) of all compounds were recorded.

The pH-dependence of the native fluorescence of mebendazole and flubendazole was examined by mixing equal volumes of the ethanolic solution (10 μg ml^{-1}) with buffer in the range pH 1–13. The excitation and emission spectra of the solutions and of the corresponding blank solutions were recorded directly.

Dimethylsulfoxide (DMSO) and ethanol were compared for their suitability as solvents for fluorescence measurements of mebendazole and flubendazole. DMSO was found to give the better results. The fluorescence intensities of each compound were measured as a function of concentration, the linear range determined and the detection limits calculated.

Optimization of analytical conditions

For the optimization of the various parameters, stock solutions of mebendazole and flubendazole were prepared in 1 M sodium hydroxide solution (1 mg ml^{-1}) by heating in a boiling water-bath. These solutions were diluted (1+99) using bi-distilled water, prior to the application of the proposed analytical procedure.

Effect of pH. One milliliter of mebendazole or flubendazole solution in 0.01 M NaOH (10 μg ml^{-1}) was transferred to a test-tube and mixed with 4 ml buffer solution ranging from pH 1 to 13. This was followed by the addition of 1 ml aqueous reagent solution (0.3% v/v H_2O_2) and subsequent mixing. The tubes were then stoppered and heated over a boiling water-bath for 15–20 min, cooled to room temperature and scanned for excitation and emission maxima. Blank solutions were prepared simultaneously and scanned at the wavelength maxima of the fluorophore in order to compensate for background fluorescence.

Effect of H_2O_2-concentration. One milliliter of mebendazole or flubendazole solution in 0.01 M sodium hydroxide (10 μg ml^{-1}) was transferred to a test-tube and mixed with 4 ml of the required buffer solution. This was followed by the addition of 1 ml aqueous hydrogen peroxide solution, varying in concentration from 0.003 to 30% (v/v). The tubes were stoppered, re-mixed and heated over a boiling water-bath for 15–20 min. After cooling to room temperature, the solutions were scanned for excitation and emission maxima and readings were taken at the specific maxima of each compound. Appropriate blank solutions were run simultaneously, starting with 1 ml 0.01 M sodium hydroxide solution.

Optimum analytical procedure and concentration curves. The optimum analytical conditions (pH, sodium hydroxide concentration, H_2O_2 concentration, heating time and wavelength maxima) were used for the quantitative analysis of mebendazole and flubendazole. To obtain the spectra and the relevant calibration curves, stock solutions could not be prepared directly in pH 9.5 buffer because of the low solubility of mebendazole and flubendazole; as a result, solutions were prepared in 1 M sodium hydroxide. However, mebendazole dissolves rapidly in pH 12.6 buffer, so that it can be used as a starting solution. In all cases, however, a final check on the pH of the reaction mixture was carried out.

Concentrations varying from 0.01 to 50 µg ml^{-1} of drug were examined. From the analytical curves obtained the linear response ranges were established and detection limits calculated.

Application to pharmaceutical preparations

Standard solution. Standard solutions were prepared by dissolving mebendazole or flubendazole (1 mg ml^{-1}) in 1 M sodium hydroxide solution by heating and stirring in a boiling water-bath and diluting with pH 9.5 buffer to a final concentration of 1–10 µg ml^{-1}.

Blank solutions. Reagent blank solutions were prepared in the same media as the samples and subjected to the same analytical procedures.

Excitation and emission maxima. Excitation and emission maxima of 300 and 360 nm, respectively, were used for measuring fluorescence intensities of samples, standard and blank solutions.

Determination of mebendazole in Vermox® tablets. Ten tablets, weighed accurately, were reduced to a homogeneous fine powder in a mortar and an amount corresponding to 50 mg mebendazole was transferred into a 100-ml volumetric flask using 1 M sodium hydroxide solution. The suspension obtained was stirred and heated over a boiling water-bath for about 30 min. After cooling, the suspension was made up to 100 ml with 1 M NaOH and centrifuged for 10 min at 3000 rpm. Finally, the supernatant was diluted to give a drug concentration of 1–10 µg ml^{-1} using pH 9.5 buffer solution. An aliquot of 5 ml of the prepared solution was mixed with 1 ml aqueous reagent solution (0.3% v/v H$_2$O$_2$) in a test-tube. The tubes were stoppered, heated in a boiling water-bath for 20 min, cooled and the fluorescence measured at the specific excitation and emission maxima.

Determination of mebendazole in Vermox® suspension. The average weight of 1.00 ml of the suspension was determined and a weight corresponding to 20 mg mebendazole was transferred to a 100-ml volumetric flask; the procedure was then completed as under Vermox® tablets.

Determination of flubendazole in Flumoxal® tablets and Flumoxal® suspension. The procedures described for Vermox® tablets and for Vermox® suspension were applied. Buffer pH 9.5 was used for the final dilutions; a longer heating period (40 min) had to be used for the flubendazole–H$_2$O$_2$ reaction mixture.

Stability of the fluorophore

In order to determine the stability of the fluorophore in the analytical procedures, solutions of mebendazole and of flubendazole (10 µg ml^{-1}) in the selected medium were measured at 1-h intervals after preparation and then daily at the appropriate wavelengths of excitation and emission, the solutions being kept at room temperature and protected from light. Two reference standards in a polymer matrix were measured simultaneously: naphthalene (λ_{ex} = 287 nm, λ_{em} = 338 nm) and tetraphenylbutadiene (λ_{ex} = 355 nm, λ_{em} = 425 nm). Blank solutions were prepared to correct the results.

In addition, the influence of oxygen exclusion from samples (10 µg ml^{-1}) was tested

by passing nitrogen gas through the prepared reaction mixtures and any changes in fluorescence intensities were calculated. The results were compared with those obtained from another series of solutions prepared in the same way, where oxygen gas was used instead of nitrogen.

Results and Discussion

Hydrogen peroxide has been widely used as an analytical reagent for the fluorimetric analysis of several organic compounds [4].

Jensen and Pflaum [5] have investigated the reaction of some antihistamines with hydrogen peroxide and studied the fluorescence characteristics of the products. These compounds included pyridine, picoline, pyrimidine and imidazole derivatives.

Preliminary experiments were carried out on the imidazole drugs under investigation, starting with acid (50% v/v acetic acid) or alkaline (0.1N NaOH) solutions (10, 20 and 50 μg ml^{-1}). Aqueous hydrogen peroxide solution (3%; v/v) was added and the solutions were heated in a boiling water-bath for 30 min, cooled, and scanned to determine the excitation and emission maxima. Five compounds produced positive results in alkaline medium, the excitation maxima being around 300 nm, the emission maxima being in the 400 nm region. These compounds included mebendazole, flubendazole, tetramisole, dexamisole and levamisole. Imidazole itself produced no fluorescent products, while 2-aminobenzimidazole yielded relatively intense signals in alkaline medium (λ_{ex} = 325 nm, λ_{em} = 450 nm).

The fluorescence of mebendazole and of flubendazole in hydrogen peroxide mixtures is greatly enhanced at alkaline pH; practically no fluorescence could be detected at acidic pH values (Fig. 1). Optimum fluorescence intensities were observed around pH 9.5 for the two compounds. Important wavelength shifts were observed around pH 7 (Fig. 2). The analogous behaviour of mebendazole and flubendazole towards hydrogen peroxide indicates a more or less similar fluorophore formation.

For the tetramisole–H$_2$O$_2$ reaction, maximum fluorescence intensity is observed at around pH 7. However, irreproducible results were obtained at this pH, probably due to
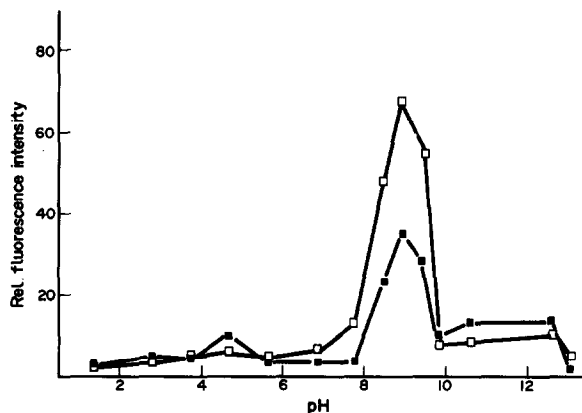
Figure 1
Influence of pH on the fluorescence observed for equal concentrations of mebendazole (□) and flubendazole (■) with H$_2$O$_2$.
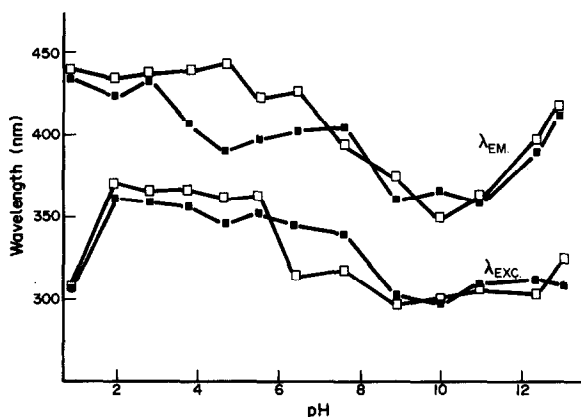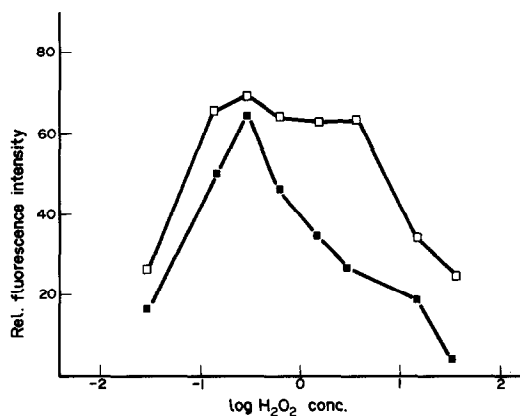
Figure 2
Excitation and emission wavelengths as a function of pH for mebendazole (□) and flubendazole (■) H_2O_2 derivatives.

instability of the fluorophore. Various attempts were made to stabilize the reaction products by the addition of a reducing agent such as formaldehyde, by exclusion of oxygen (passing nitrogen gas into the solution), by the addition of some metal ions or by the use of other buffers. All these attempts were unsuccessful for tetramisole.

Various concentrations of H_2O_2 ranging from 0.003 to 30% v/v were tested at pH 9.5 in order to increase the sensitivity of the proposed analytical method, as illustrated in Fig. 3. The highest fluorescence signals were observed using 0.3% v/v hydrogen peroxide. Concentrations higher than 1% v/v H_2O_2 solution lead to fluorescence quenching, while no fluorescent products could be detected with H_2O_2 concentrations lower than 0.015% v/v.

The fluorescence emission intensity was measured after heating over a boiling water-bath for various periods. The reaction mixtures were prepared using pH 9.5 buffer solution and 0.3% v/v H_2O_2 solution. The maximum fluorescence intensity occurred

Figure 3
Influence of H_2O_2 concentration on the fluorescence intensity of equal concentrations of mebendazole (□) and flubendazole (■).

after 20–25 min heating for mebendazole and after 40–45 min in the case of flubendazole (Fig. 4). The longer heating period for flubendazole can be attributed to the presence of a fluorine atom which may hinder the reaction to some extent. Moreover, for mebendazole the fluorescence intensity is ca 2·2 times higher than for flubendazole. Heating periods longer than 45 min result in a marked decrease in fluorescence for both compounds.
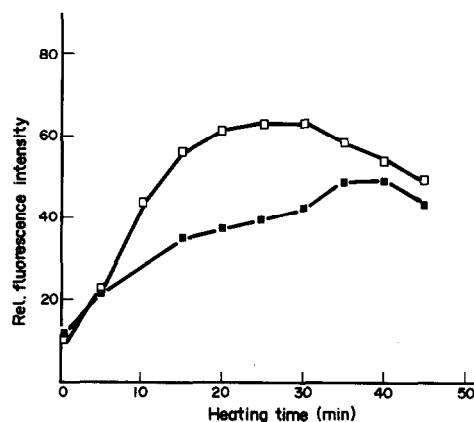
Figure 4
Influence of heating time upon the reaction of H_2O_2 with equal concentrations of mebendazole (□) and flubendazole (■).

A fall in fluorescence intensity of about 50% was observed both for mebendazole and for flubendazole reaction mixtures within 24–48 h. Oxygen exclusion from the samples did not influence the fluorescence readings.

Pure imidazole gave no fluorescence emission when treated with hydrogen peroxide under analogous conditions; 2-aminobenzimidazole, however, yielded intense signals in these circumstances (λ_{ex} = 325 nm; λ_{em} = 450 nm). It is assumed that hydrogen peroxide reacts with the alkaline hydrolysis products of mebendazole and flubendazole.

Concentration curves for the mebendazole and flubendazole hydrogen peroxide derivatives were established under the optimum analytical conditions described. Linear response of fluorescence with concentration of the analyte was observed for mebendazole and for flubendazole up to 6 and 8 μg ml^{-1} respectively, higher concentrations leading to negative curvature. Solute–solute interactions, self-absorption or dimer formation may be the origin of the fluorescence quenching effects observed. The regression data for each compound under the specified conditions were as follows: mebendazole–concentration range 0.01–6 μg ml^{-1}–y = 0.457x + 4.11 (n = 6; SE in intercept, 0.108; r = 0.9956); flubendazole–concentration range 0.01–8 μg ml^{-1}–y = 0.593x + 5.33 (n = 6; SE in intercept, 0.111; r = 0.9971). Concentrations at 0.1 μg ml^{-1} can be determined for both molecules using the proposed method. Table 1 illustrates the results obtained with some commercial anthelmintic preparations.

Attempts to isolate and characterize the fluorescent species using preparative thin-layer chromatography and liquid chromatography have not been successful. It seems that the fluorescent species is difficult to extract from alkaline, neutralized or acidified reaction mixtures using various organic solvents. In most of the cases the fluorophores remained in the aqueous phase, indicating their strong polar properties.

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Table 1
Fluorimetric determination of mebendazole and flubendazole in some
pharmaceutical preparations using the H_2O_2 reaction

	Average recovery (%)	Relative standard deviation (%) ($n \geqslant 6$)
Mebendazole		
Vermox® tablets	99.30	2.16
Vermox® suspension	101.75	3.01
Flubendazole		
Flumoxal® tablets	99.00	1.05
Flumoxal® suspension	103.9	0.90

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