



Research Article

DEVELOPMENT AND VALIDATION OF THE RP-HPLC METHOD FOR DETERMINATION OF BENZIMIDAZOLE CARBAMATES IN THE PRESENCE OF CYCLODEXTRINS

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ABSTRACT

Liquid chromatography is the universal method to determine drugs in biological matrices and pharmaceutical products. Different strategies have been developed in order to improve the aqueous solubility and bioavailability of benzimidazoles, such as the complexation with cyclodextrins. Although extensive studies on cyclodextrins have been performed, studies on the impact of cyclodextrins on the chromatographic process are scarce. The objective of this study was to develop and validate a suitable HPLC method for the quantification of benzimidazoles carbamates (albendazole and fenbendazole) in the presence of cyclodextrins (β -cyclodextrin and hydroxypropyl- β -cyclodextrin). The retention time of the benzimidazoles and their metabolites decreases in the presence of cyclodextrins. The formation of inclusion complexes between drugs and cyclodextrins can explain the change in retention times. Hydroxypropyl- β -cyclodextrin decreased the retention time of the solute in a more pronounced way than β CD. This phenomenon can be attributed to the greater hydrophilicity of Hydroxypropyl- β -cyclodextrin, which promotes the formation of more soluble complexes and, therefore, with less affinity for the stationary phase.

Key Words: Fenbendazole, Albendazole, Cyclodextrins, Reversed-Phase Chromatography

INTRODUCTION

Intestinal parasitoses represent one of the major public health problems, particularly in developing countries, where they have a great economic impact¹. Benzimidazole (BZD) and its derivatives are widely used in the treatment of intestinal helminths in animals and humans, with a large spectrum of activity and low toxicity². However, this class of drugs have low solubility and as a result, they have variable and incomplete bioavailability³⁻⁵.

Different strategies have been employed to increase the solubility of BZD, including the preparation of solid dispersions, the use of surfactants, liposomes and the development of inclusion complexes with cyclodextrins (CDs)^{3, 6-10}.

Although extensive studies on CDs have been performed, studies on the impact of CDs on the chromatographic process are scarce¹¹⁻¹³.

The objective of this study was to develop and validate a suitable HPLC method for the quantification of BZD carbamates (ABZ and FBZ) in the presence of CDs (β CD and HP β CD).

MATERIAL AND METHODS

Materials and Reagents

HPLC-grade acetonitrile was acquired from J.T. Backer Chemical Co. (Phillipsburg, NJ). Ultra purified water was prepared by using a Millipore Milli-Q system (Milford, CT, USA). Diammonium hydrogen orthophosphate, orthophosphoric

acid and the reference substances - fenbendazole (FBZ) and albendazole (ABZ) -, their sulphoxides – oxfendazole (FBZSO) and ricobendazole (ABZSO) -, and their sulphones - fenbendazole sulfone (FBZSO₂) and albendazole sulfone (ABZSO₂) - were acquired from Sigma-Aldrich (Milwaukee, WI, USA).

Instrumentation

The chromatographic analysis were performed on a Shimadzu HPLC LC-20AT system comprised of a SPD-20A UV detector, LC-20AB pump, DGU-20A degasser, SIL-20A/C autosampler, CTO-20AC column oven, CBM-20A controller and the LC-Solution as software (Shimadzu Co., Kyoto, Japan).

Chromatographic Conditions

The criteria used were based on the methodology described by Bull and Shume¹⁴. ABZ and FBZ were analyzed on a reversed-phase Kromazil RP 100 C18 column (5 μ m, 4.6x250 mm, Akzo Nobel). The mobile phase for these studies was acetonitrile-0.04M diammonium hydrogen orthophosphate (41:59, v/v). The pH of the mixture was adjusted to 7.5-7.6 drop wise with a 20% (m/v) orthophosphoric acid solution. The mixture was freshly prepared, filtered through a 0.45 μ m pore size cellulose acetate membrane filter and degassed under vacuum with the aid of an ultrasonic bath. An isocratic mobile phase elution was performed at a flow rate of 1.5 mL min⁻¹. The detection wavelength was 300 nm.

Validation of Chromatographic Method

The method was validated according to the ICH Harmonized Tripartite Guideline - Validation of Analytical Procedures: Text and Methodology Q2(R)¹⁵. The parameters evaluated were specificity, linearity, precision and accuracy, limits of detection and quantification, and robustness.

Specificity

The specificity of the method was evaluated for the presence of impurities (degradation products) and matrix components (CDs). ABZ and its degradation products were separately diluted in DMSO (1 mg mL⁻¹). Then 0.15 µL of each solution was transferred to a 1.5 mL amber vial and the volume was completed with the mobile phase. FBZ and its degradation products were evaluated following the same methodology. Standard solutions of the drugs - ABZ and FBZ - (100 µg mL⁻¹), and a placebo solution containing 10 mg mL⁻¹ of the CDs - βCD and HPβCD - were adequately prepared. Then, 1.5 mL of the standard solution (30 µg mL⁻¹) and 0.5 mL of the placebo solution (1 mg mL⁻¹) were transferred to a 5 mL volumetric flask and the volume was completed with the mobile phase. The solutions obtained this way were evaluated by liquid chromatography. The tests were performed in triplicate.

Linearity

The linearity of an analytical method was obtained through of external standard calibration and matrix superposition methods. For the external standard calibration method, a 100 µg mL⁻¹ standard solution of each drug - ABZ and FBZ - was prepared and appropriately diluted in the range of 2 to 100 µg mL⁻¹ corresponding to the application range from 0 to 120% of the expected value^{15,16}. For the matrix superposition method, a fixed volume of 1 mL of a stock solution of CDs 10 mg mL⁻¹ (βCD or HPβCD) was added to each of the prepared dilutions in the range of 2 to 100 µg mL⁻¹, such that the final concentration of CDs in each solution was 1 mg mL⁻¹.

Precision

The intermediate repetivity and precision of the test were calculated at three levels (2, 12 and 20 µg mL⁻¹) with three repetitions for ABZ and FBZ, resulting in 27 determinations for each drug, employing the following conditions: same analyst, different instruments and different days^{15,17}.

Accuracy

The accuracy was evaluated by adding known amounts of standard solution in the sample solution of binary complexes (drug:βCD or drug:HPβCD). The recovery experiments were performed in triplicate and data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added, and then multiplied by 100%. Accuracy values of between 85% and 115% were considered acceptable¹⁸. Binary complexes (drug:βCD or HPβCD) were prepared by lyophilization. Equimolar quantities of each drug - ABZ and FBZ - and CD - βCD and HPβCD -, were weighed and dispersed, separately, in purified water. The obtained dispersions were submitted to homogenization for 2 hours, cooled at -78.5 °C with solid carbon anhydride, and lyophilized in a Liotop mod. L101 lyophilizer for 120 hours.

Limit of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were determined based on standard deviation among responses and slopes of the regression equation of the calibration curve^{15,17}.

Robustness

The robustness was evaluated by varying the chromatographic column - Kromazil RP C18 and Kinetex[®] Evo C18, both with the same dimensions - 100 Å, 5 µm, 250x4.6mm -, the pH of the mobile phase (pH 7.5-7.6 and pH 8.32), the column temperature (ambient temperature and 40°C) and the flow speed (1.5 and 1.2 mL min⁻¹)^{15,17}.

Table 1: Mean values of the chromatographic parameters. The pH of the mobile phase was adjusted to 7.5-7.6 drop wise with a 20% (m/v) orthophosphoric acid solution

Drugs	t _r	W	N	k'	R	α
ABZ	9.104	0.6	3684	6.66	-	-
ABZ sulfone	2.936	0.2	3448	1.47	15.42	4.53
ABZ sulfoxide	2.096	0.2	1757	0.76	17.52	8.73
FBZ	14.226	0.6	8995	7.91	-	-
FBZ sulfone	4.385	0.2	7691	1.75	24.60	4.53
FBZ sulfoxide	2.766	0.3	3060	0.73	28.65	10.79

*t_r (retention time, expressed in minutes), w (peak width, expressed in centimeters), N (number of theoretical plates), k' (retention factor), R (resolution) and α (selectivity factor)

Table 2: Chromatographic parameters obtained for the drugs - ABZ and FBZ - employing different chromatographic columns

Parameters	ABZ		FBZ	
	Kromazil [®] RP	Kinetex [®] EVO	Kromazil [®] RP	Kinetex [®] EVO
Area	914511	908644	942889	938956
Peak height	75013 mAU	75214 mAU	49194 mAU	49462 mAU
Retention time, t _r	9.987 min.	10.263 min.	15.711 min.	16.107 min.
N ^a	9974.0169	10532.9169	10970.4676	11530.4644
HEPT ^b	0.0025	0.0024	0.0023	0.0022

^aN is the number of theoretical plates; ^bHEPT is the equivalent height of the theoretical plate

Table 3: Mean values of the chromatographic parameters obtained using the mobile phase without pH correction (pH 8.32)

Drugs	t_r	W	N	k'	R	α
ABZ	9.086	0.6	3669	6.83	-	-
ABZ sulfone	2.935	0.2	3446	1.53	15.38	4.47
ABZ sulfoxide	2.099	0.2	1762	0.81	17.47	8.45
FBZ	13.503	0.6	8104	7.87	-	-
FBZ sulfone	4.189	0.2	7019	1.75	23.29	4.49
FBZ sulfoxide	2.705	0.2	2027	0.77	27.00	10.13

* t_r (retention time, expressed in minutes), w (peak width, expressed in centimeters), N (number of theoretical plates), k' (retention factor), R (resolution) and α (selectivity factor)

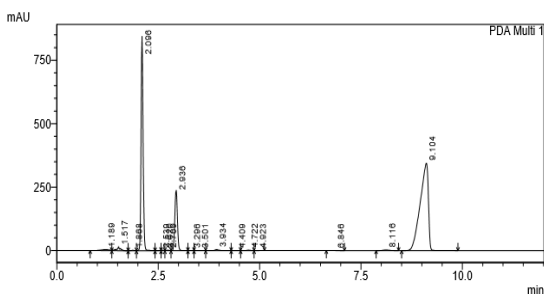


Figure 1: Chromatogram of ABZ and its degradation products - albendazole sulphoxide (ricobendazole) and albendazole sulfone at a concentration of 100 $\mu\text{g mL}^{-1}$

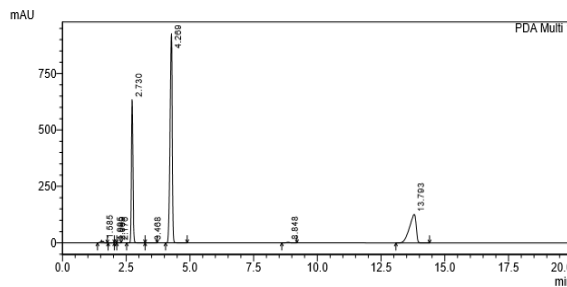


Figure 2: Chromatogram of FBZ and its degradation products - fenbendazole sulphoxide (oxfendazole) and fenbendazole sulfone at a concentration of 100 $\mu\text{g mL}^{-1}$

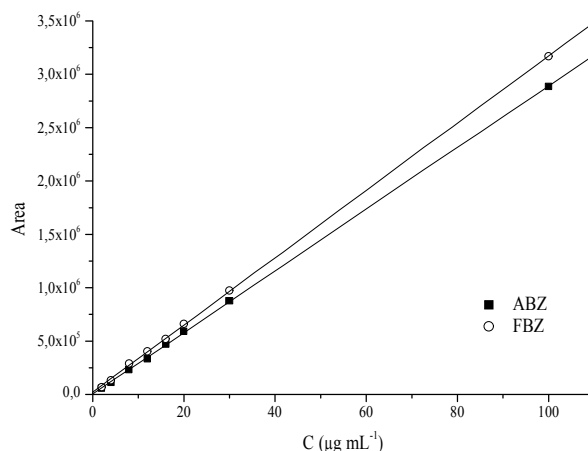


Figure 3: Mean analytical curve obtained for the drugs (ABZ and FBZ) with the external standard calibration method (n=3)

RESULTS AND DISCUSSION
Specificity

The specificity of the method was first assessed by evaluating the drugs - ABZ and FBZ - in the presence of their respective degradation products. The chromatographic conditions used enabled the separation between the drugs - ABZ and FBZ - and their degradation products (Figure 1 and 2).

The mean values of the chromatographic parameters obtained from the mean of three measurements for each drug - ABZ and FBZ -, and their respective degradation products, were calculated (Table 1).

The resolution (R) depends on 3 factors: the selectivity factor (α) - the extent of separation between two solutes ($\alpha > 1$); the

retention factor (k') - which describes the speed with which a compound migrates along the column ($1 < k' < 10$); and the number of theoretical plates (N), which should be as high as possible. Resolution values (R) equal to 1 indicate an almost complete separation of substances; resolution values (R) greater than 1.5 refer to the complete separation of substances; often, resolution values (R) between 4 and 5 are recommended so that the impurities can be separated.

The retention factor (k') showed that the drugs - ABZ and FBZ - had a lower migration speed along the column than their respective degradation products, which are more soluble and, therefore, have less affinity with the stationary phase.

The selectivity factor (α) was greater than 1, indicating an adequate separation of substances. The resolution (R) between the peaks of the degradation products and both original drugs was greater than 1.5, indicating the complete separation of substances.

The interference of the CDs (β CD and HP β CD) on the specificity of the method was studied through the comparison between the chromatograms of the substance of interest (ABZ and FBZ) and the chromatograms of the substance of interest added of matrix (β CD and HP β CD). The retention time of the benzimidazoles and their metabolites decreases in the presence of cyclodextrins. Hydroxypropyl- β -cyclodextrin decreased the retention time of the solute in a more pronounced way than β CD. This phenomenon was attributed to the greater hydrophilicity of β CD (RM β CD and HP β CD) derivatives, which promotes the formation of more soluble complexes and, therefore, with less affinity for the stationary phase.

In the chromatographic separation process, the difference in magnitude of the physical and chemical forms that act between the solutes and the two phases - mobile and stationary - determine the separation of individual solutes. The elementary forces that act on the molecules are London dispersion forces or Van der Waals forces, induced dipole interactions, hydrogen bonds, dielectric interactions and electrostatic and coulombian interactions. The variables that affect these intermolecular forms will influence the degree of separation obtained by the passage of the solutes in the chromatographic column¹⁹.

Feng et al.¹³ studied the behavior of the retention time of isoflavone in the presence of CDs using the k_1/k_0 ratio. The k_1/k_0 values for β CD and derived β CD followed the order: RM β CD > HP β CD > β CD, which suggests that the derived CDs diminish the retention time of the solute in a more pronounced way than β CD. The k_1/k_0 ratio varies depending on the type and concentration of the CD.

Linearity

The external standard calibration method is performed when no systematic error from the matrix is suspected, while the matrix superposition method compensates for the effect of the matrix^{16,17,20}. A linear correlation was obtained for the analytical curves with the external standard calibration method and matrix superposition method for both drugs - ABZ and FBZ -, with a correlation coefficient (r) exceeding 0.99. The analysis of the residue showed normal distribution of points around the line indicating that the model is adequate (Figure 3).

Precision

The relative standard deviations were lower than 5%, suggesting that the proposed method is precise^{15,17}.

Accuracy

The mean recovery percentages obtained for 3 determinations were 98.43% for ABZ: β CD, 99.42% for ABZ:HP β CD, 98.22% for FBZ: β CD, and 99.31% for FBZ:HP β CD.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The values for the limit of detection (LOD) obtained for the drugs ABZ and FBZ were 0.4855 and 2.4575 $\mu\text{g mL}^{-1}$, respectively. The values for the limit of quantification (LOQ) obtained for the drugs ABZ and FBZ were 1.6183 and 8.1918 $\mu\text{g mL}^{-1}$, respectively.

Robustness

The efficiency of a column, i.e. the column's capacity of producing narrow and sharp peaks, is expressed as the number of theoretical plates (N) or the equivalent height of the theoretical plate (HETP). The number of theoretical plates (N) indicates the efficiency of the column: $N = 16 \left(\frac{t}{w}\right)^2$, where t is the retention time and w is the peak width at the base. The equivalent height of the theoretical plate (HEPT) is calculated by the ratio between the column length (L), expressed in centimeters, and the number of theoretical plates (N). The efficiency of the column and the equivalent height of the theoretical plate (HEPT) correlate according to: excellent (< 0.06), good (0.06 to 0.10), acceptable (0.10 to 0.15), and bad (0.30). The change of the chromatographic column, from Kromazil[®] R C18 to Kinetex[®] EVO C18, had no impact on the chromatographic parameters and the sensitivity of the method. The greatest number of theoretical plates was observed for the column Kinetex[®] EVO C18, suggesting greater efficiency, but the values of the equivalent height of the theoretical plate (HEPT) indicated that both columns had excellent efficiency (Table 2).

BZDs have a imidazolic ring containing nitrogen atoms of an acid and basic nature. Under specific conditions, the molecule can be protonated ($\text{p}K_a \cong 5-6$) or deprotonated ($\text{p}K_a \cong 12$). ABZ is found in its neutral form in the pH range 7.7-11.2, while FBZ is found in its neutral form between pH 7.1-9.8²¹. The influence of the pH of the mobile phase on the retention time and peak definition of the drugs was evaluated. The mobile phase had a pH of 8.32 when the correction with o-phosphoric acid 20% was not performed. Through a comparison of Tables 1 and 3, one can see that with a pH of 8.32, the selectivity factor (α) and resolution (R) values suffered no significant alteration; a small reduction in retention time (t) of both drugs was observed.

The column temperature was varied from ambient temperature to 40°C to assess the impact on the chromatographic parameters. A small reduction in retention times of both drugs - ABZ and FBZ - was observed at the high temperature.

The deformation of the peaks is associated to the flow of the mobile phase due to longitudinal diffusion of molecules and mass transfer resistance, and, as such, a high flow can lead to a broadening of the chromatographic band. An increase in the retention time was observed for both drugs when the flow speed was reduced from 1.5 mL min^{-1} to 1.2 mL min^{-1} .

CONCLUSION

This work reported the development and validation of a RP-HPLC method for the simultaneous determination of benzimidazoles and their metabolites - ABZ or FBZ - in the presence of CDs.

CDs dramatically reduce the retention time of benzimidazoles and their metabolites in the chromatographic process. The formation of inclusion complexes between drugs and CDs can be explain the change in retention times.

The proposed method proved to be specific, precise and accurate for the determination of the benzimidazoles and their metabolites - ABZ or FBZ - in the presence or absence of CDs, since it was able to analyze the substances unequivocally.

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