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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR THE *IN-VITRO* INTERACTION STUDY OF MEBENDAZOLE WITH ATORVASTATIN

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Keywords:

High-performance thin-layer chromatography, *In-vitro* interaction study, Mebendazole, Atorvastatin, Protein binding, Validation

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ABSTRACT: By the HPTLC method, a mobile phase system comprising of Ethyl acetate: Toluene: Methanol (5:4:1v/v/v) was selected, and the detection was carried out at 280 nm. The R_f values were found to be 0.65 ± 0.03 and 0.27 ± 0.03 for Mebendazole and Atorvastatin, respectively, which showed a good separation. The method was validated as per ICH guidelines. The percentage RSD values of repeatability, intraday, and interday were found to be less than 2 prove the precision of the method. The correlation coefficient value from the calibration graph was found to be 0.9949 for Mebendazole and 0.9979 for Atorvastatin. The percentage protein binding of Mebendazole and Atorvastatin was estimated by the equilibrium dialysis method. The in-vitro displacement interaction study was carried out using standard solutions of a mixture containing 1.5×10-4 M Mebendazole and Atorvastatin along with 1.5×10-4 M BSA. The percentage protein binding of individual Mebendazole and Atorvastatin after 6hr was found to be 86.14% and 91.18%, respectively. While they were evaluated in the presence of each other, the percentage protein binding was found as 84.4% for Mebendazole and 94.8% for Atorvastatin at the end of the study. The percentage protein binding of Mebendazole decreased by 1.74% and increased for Atorvastatin by 3.62%.

INTRODUCTION: Mebendazole (Methyl N-(6-benzoyl-1H-1, 3-benzodiazol-2-yl) carbamate) is a antihelminthic drug having molecular formula $C_{16}H_{13}N_3O_3$. Mebendazole causes degenerative alterations in the tegument and intestinal cells of the worm, which leads to diminished energy production, the parasite is immobilized and eventually dies[20]. Atorvastatin ((3R, 5R)-7-[2-(4-fluorophenyl) - 3 - phenyl - 4 - (phenyl-carbamoyl)-5-(propan - 2 - yl) - 1H - pyrrol - 1 - yl] - 3, 5-dihydroxyheptanoic acid) is a statin group of drug with molecular formula $C_{33}H_{35}FN_2O_5$. Atorvastatin is a competitive inhibitor of the enzyme HMG-CoA reductase.

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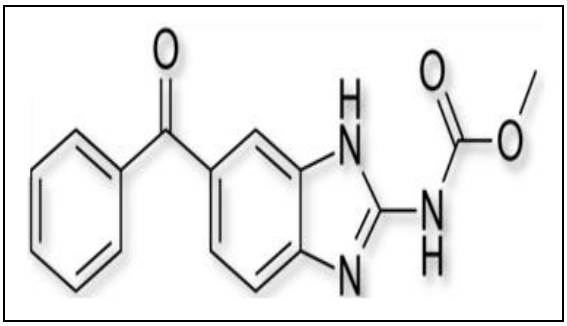
Atorvastatin also reduces VLDL-C, serum triglycerides (TG), and Intermediate Density Lipoproteins (IDL), as well as the number of apolipoprotein B (apo B) containing particles, but increases High-Density Lipoprotein Cholesterol [21]. Literature survey revealed that there is no reported HPTLC method for the *in-vitro* drug interaction of Mebendazole with Atorvastatin.

Hence, the aim and objective of the present work is the development and validation for the separation of Mebendazole and Atorvastatin and application of the developed HPTLC method for protein binding based *in-vitro* interaction of Mebendazole with Atorvastatin. Drug-drug interactions are very common in poly-drug prescriptions. Clinical case reports or studies are reported from time to time as evidence of interaction between drugs. Several studies explained such interaction at the molecular and enzyme level so that the relationship between clinical and experimental research shall be recognized.

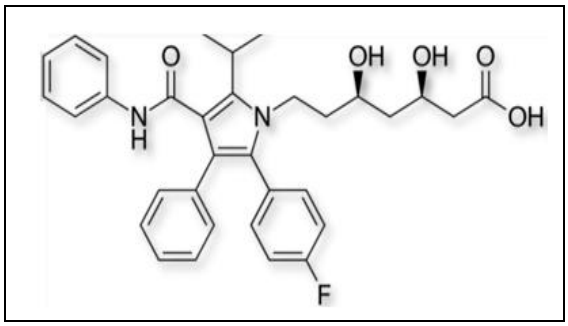
There are many approaches practiced for *in-vitro* drug interaction study such as metabolism-based, protein binding *etc*. Protein binding plays a major role in the bioavailability of the drugs.

Distribution interaction studies between drugs involve interaction in the protein binding of two drugs.

Protein binding interactions are displacement reactions, which have been concerned as the mechanisms in many drug-drug interactions. The extent of drug binding to plasma proteins, determined by measuring the free active fraction, has a significant effect on the pharmacokinetics and pharmacodynamics of a drug.



MEBENDAZOLE



ATORVASTATIN

MATERIALS AND METHODS:

Instrumentation: Camag HPTLC system (with TLC Scanner-3, Win CATS software, and Linomat 5 as application device) was used for the method development and *in-vitro* interaction study.

Reference Substances, Reagents and Chemicals: Mebendazole and Atorvastatin were purchased from Sigma Aldrich, India. All the chemicals and solvents used were supplied by S.D. Fine Chemicals Ltd., Sigma Aldrich, and Merck India Ltd. Pre-coated silica gel 60F254 on aluminium sheets were procured from Merck, Germany.

Chromatographic Conditions: Pre-coated silica gel 60 F_{254} aluminum sheets (10 x10 cm) as the stationary phase, an analytical balance was used for weighing the compounds, plates were scanned using Camag win CATS software, bandwidth was 6 mm using Hamilton 100 µl syringe on precoated plates using automatic application device, Linear ascending development was carried out in twin trough glass chamber, and densitometric scanning was performed on Camag TLC scanner III in the absorption mode at 280 nm and operated by win CATS software.

Solution Preparation:

Preparation of Standard Stock Solution: About 2 mg of Mebendazole and 4mg of Atorvastatin were accurately weighed and transferred to 50 ml of volumetric flask and sonicated for 20 min to dissolve the sample.

Then the volume was made up with methanol to obtain a concentration of 40 µg/ml of Mebendazole and 80 µg/ml of Atorvastatin.

Preparation of Phosphate Buffer pH 7.4: The Phosphate buffer was prepared by taking 62.5 ml of 0.2 M potassium dihydrogen phosphate and added with 48.8 ml Sodium hydroxide. It was made up to 250 ml with distilled water.

Preparation of Bovine Serum Albumin Solution (1.5×10^{-4} M): The Bovine serum albumin (BSA) solution was employed for the estimation of protein binding of the drugs.

To prepare 1.5×10^{-4} M BSA solution, 0.48 g of BSA was dissolved in distilled water and made up to 50 ml.

Activation of Dialysis Membrane Tubes: The dialysis membrane tubes were cut into each 13 cm length, and its activation had been done by boiling the membrane for 2 h in 250 ml of distilled water at 70 ºC (±5 ºC). The boiled membranes were washed thoroughly with fresh distilled water and utilized for the study.

Preparation of Standard Solution of Mebendazole (1.5×10^{-4} M): A quantity of about 4 mg of Mebendazole was weighed and dissolved in sufficient volume of methanol with the aid of 15 min sonication and made up to 50 ml with the same to obtain 80 µg/ml.

Preparation of Standard Solution of Atorvastatin (1.5×10^{-4} m): A quantity of about 8 mg of Atorvastatin was weighed and dissolved in a sufficient volume of methanol. It was made upto 50 ml with methanol to obtain a concentration of 160 µg/ml.

Preparation of Standard Solution of Mixture (1.5×10^{-4} m): An accurately weighed quantity of about 4 mg Mebendazole and 8 mg of Atorvastatin was transferred to a 50 ml volumetric flask. It was dissolved and mixed thoroughly in methanol by sonication for 20 min and made up to 50 ml with methanol.

Validation of HPTLC Method: The method was validated as per ICH guidelines, and parameters like linearity, range, the limit of detection, the limit of quantification, precision, specificity, stability studies were performed.

Limit of Detection (LOD) And Limit of Quantification (LOQ): LOD & LOQ were calculated in terms of signal-to-noise ratio. The estimation was done using a set of five calibration curves used to determine method linearity.

$$LOD = 3\sigma / S \text{ and } LOQ = 10\sigma / S$$

Where, σ = the standard deviation of y-intercepts of regression lines, S= the slope of the calibration curve

Linearity and Range: The linearity of response was assessed by applying different volumes of stock solution *viz,* 2, 4, 6, 8, 10, 12 µl on TLC plate to obtain a linear concentration range of 0.04-0.24 µg/spot of Mebendazole and 0.08-0.48µg/spot of Atorvastatin. The spots were developed and evaluated densitometrically using the CAMAG HPTLC system. Peak areas were noted for each spot and plotted against their respective concentrations to get a linear graph.

Precision: The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Precision may be considered at three levels: repeatability, intermediate precision, and reproducibility.

Intra-Day Precision: Intra-day precision was studied by carrying out the analysis of the standard drug of two different concentrations three times on the same day, and % RSD was calculated.

Inter-Day Precision: Inter-day precision was studied by carrying out the analysis of the standard drug of two different concentrations for three different days and %RSD was calculated.

Repeatability:

Repeatability of Sample Application: Repeatability of sample application was carried by spotting 6 times 10 µl of drug solution on a pre-coated TLC plate followed by the development of plate and % RSD was calculated.

Repeatability of Sample Measurement: Repeatability of sample application was determined by spotting 10 µl of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and % RSD was calculated.

Specificity: Specificity is the ability to assess the analyte in the presence of components that are expected to be present. The peak purity of Mebendazole and Atorvastatin was assessed by comparing its respective spectra at three different levels, that is, peak start (S), peak apex (M), and peak-end(E) positions of the spot.

Stability Studies: When the developed chromatographic plate is exposed to atmosphere, the analytes are likely to decompose. It is necessary to study the stability of the drug on a plate.

It was studied by scanning the plate at a different time interval, and peak areas were compared with the peak area of the freshly scanned plate. The % change in the peak areas was calculated.

RESULTS AND DISCUSSION:

Method Development and Optimization: The selection of solvent is done based on the drug solubility and stability. Accordingly, methanol was selected as the solvent.

Different mobile phase compositions were tried for the development and validation of Mebendazole and Atorvastatin in combination with the HPTLC method. Among these trial mobile phase systems, Ethyl acetate: Toluene: Methanol (5:4:1 v/v/v) was

selected as the compact and dense spots were obtained with good separation. A wavelength of 280 nm was fixed for scanning the plates since the response was good at this wavelength. A standard densitogram obtained is given in **Fig. 1.**

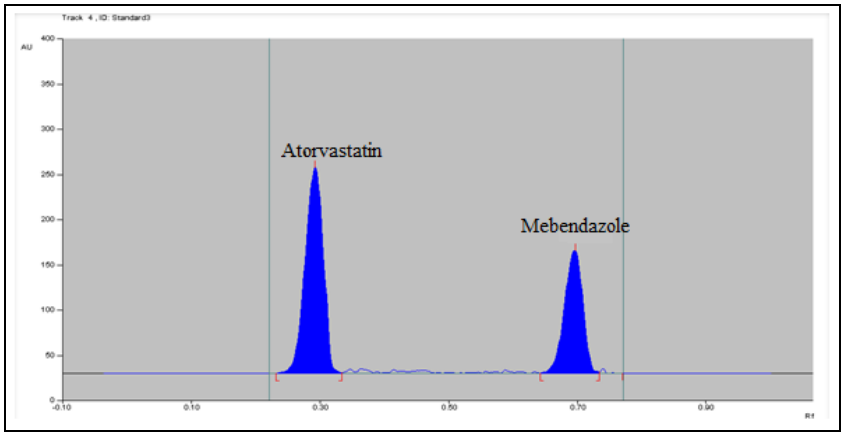


FIG. 1: DENSITOGRAM OF MEBENDAZOLE 0.12 µg/SPOT AND ATORVASTATIN 0.24 µg/SPOT

Validation:

Linearity and Range: The linear regression data showed a good linear relationship over a concentration range of 0.04-0.24 µg/spot of Mebendazole and 0.08-0.48 µg/spot of Atorvastatin. The linear regression equation found for Mebendazole and Atorvastatin is presented in **Table 1**. The peak areas of Mebendazole and Atorvastatin were noted with R_f value 0.65 ± 0.03 and 0.27 ± 0.03, respectively. The calibration graph obtained for Mebendazole and Atorvastatin is shown in **Fig. 2** & **3**. Overlain densitogram is given in **Fig. 4.**

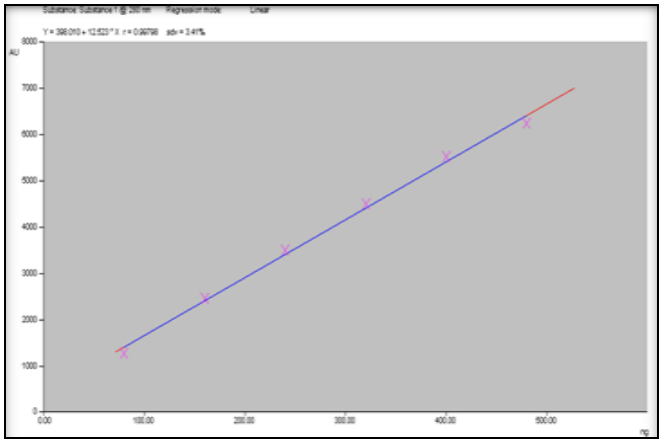


FIG. 2: CALIBRATION GRAPH OF MEBENDAZOLE

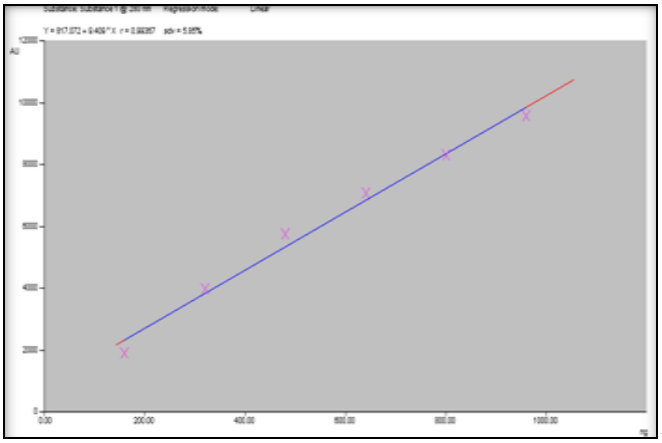


FIG. 3: CALIBRATION GRAPH OF ATORVASTATIN

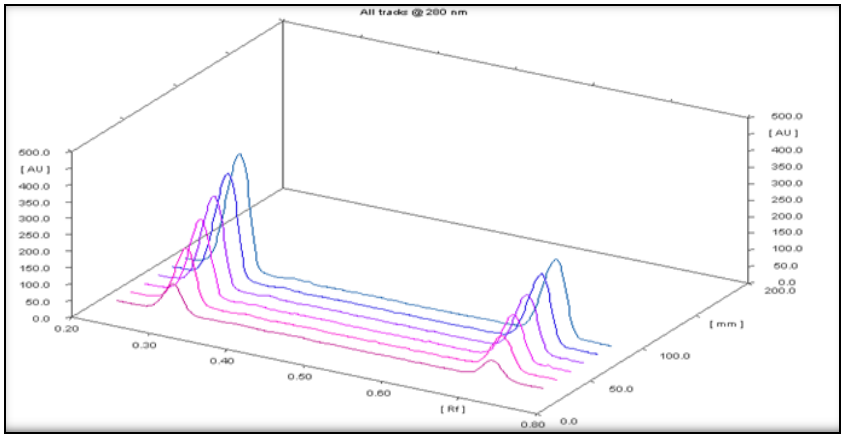


FIG. 4: OVERLAIN DENSITOGRAMS OF MEBENDAZOLE AND ATORVASTATIN

Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ of the mixture were found to be 0.01µg/spot and 0.04µg/spot for Mebendazole; 0.02 µg/spot and 0.08µg/spot for Atorvastatin respectively shown in **Table 1.**

TABLE 1: VALIDATION PARAMETERS FOR MEBENDAZOLE AND ATORVASTATIN

Parameters	Mebendazole	Atorvastatin
Linearity range	0.05 - 0.5µg/spot	0.08-0.48µg/spot
Regression equation	y = 8.160x +179.906	y = 9.118 x+294.28
Correlation coefficient	0.9978	0.9977
LOD	0.03µg/spot	0.05µg/spot
LOQ	0.045µg/spot	0.075µg/spot

Precision: Intraday, interday, repetability of the sample application and measurement was carried out, and the % RSD values were found to be less than 2 shows the precision of the method. The % RSD obtained is given in **Table 2.**

TABLE 2: PRECISION DATA

PRECISION								
Application Volume (µL)	Interday Precision		Intraday Precision		Repeatability of Sample Application		Repeatability of Measurement	
	MBZ	ATV	MBZ	ATV	MBZ	ATV	MBZ	ATV
2	0.16	0.19	0.21	0.48	-	-	-	-
8	0.06	0.10	0.06	0.11	0.06	1.62	0.04	0.62
12	0.05	0.06	0.04	0.08	-	-	-	-

Specificity: The peak purity of both Mebendazole and Atorvastatin was assessed by comparing their respective spectra at peak start, peak apex, and peak end positions of the spot. The good correlation among the spectra acquired at start (s), apex (m), and end (e) indicates the high purity of both drugs. It can be concluded that no impurities or degradation products migrated with the peaks obtained from standard solutions of the drugs.

Stability Studies: In the developed plate, Mebendazole was found to be stable for 15 min, and Levamisole was stable for 2 h.

Protein Binding Study of Mebendazole and Atorvastatin: About 2.5 ml of 1.5×10^{-4} M Mebendazole solution and 1.5×10^{-4} M BSA solution were taken inside the previously activated dialysis membrane tubes and sealed. The tubes were immersed in 30 ml of phosphate buffer taken in measuring cylinders. The samples were taken in replicates and all the measuring cylinders were placed in a mechanical shaker at 40 rpm for sufficient period of time to complete dialysis. The samples withdrawn from the buffer compartment at regular intervals were analyzed using the newly developed HPTLC technique. Similarly, protein binding studies were also conducted for Atorvastatin also.

Assessment of Protein Binding and Displacement Interaction Study: The *in-vitro* displacement interaction study was carried out for Mebendazole in the presence of its interacting drug Atorvastatin. About 2.5 ml of a standard solution of a mixture containing 1.5×10^{-4} M Mebendazole and 1.5×10^{-4} M Atorvastatin along with 1.5×10^{-4} M BSA was added to the previously activated dialysis membrane tubes and sealed. The tubes were immersed in 30 ml of phosphate buffer taken in measuring cylinders. The samples were prepared in replicates, and all the measuring cylinders were placed in a mechanical shaker at 40 rpm for a sufficient period of time to complete dialysis. The samples withdrawn from the buffer compartment at regular intervals were analyzed using the newly developed HPTLC technique. The results obtained are given in **Table 3.**

TABLE 3: RESULTS OF *IN-VITRO* INTERACTION STUDY OF MEBENDAZOLE AND ATORVASTATIN

Concentration (µg/ml)		Concentration of Unbound Drug (µg/ml)		% Protein Binding		% Displacement	
MBZ	ATV	MBZ	ATV	MBZ	ATV	MBZ	ATV
80	160	12.3	8.94	84.42	94.8	15.58	5.2

The chromatograms were recorded, and the concentration of unbound drug was determined by calculating peak area ratios with the standards. The percentage of protein binding (F) was calculated as follows:

$$F = (B - A) / B \times 100$$

Where, A = Concentration of free drug in buffer compartment. B = Concentration of total drug in buffer compartment.

The percentage of protein binding (F) was calculated, and the difference in the percentage protein binding of Mebendazole was calculated to find the percentage displacement of Mebendazole due to interaction with Atorvastatin.

The densitogram of Mebendazole and Atorvastatin admixture after equilibrium dialysis study for 6 h is given in **Fig. 5.**

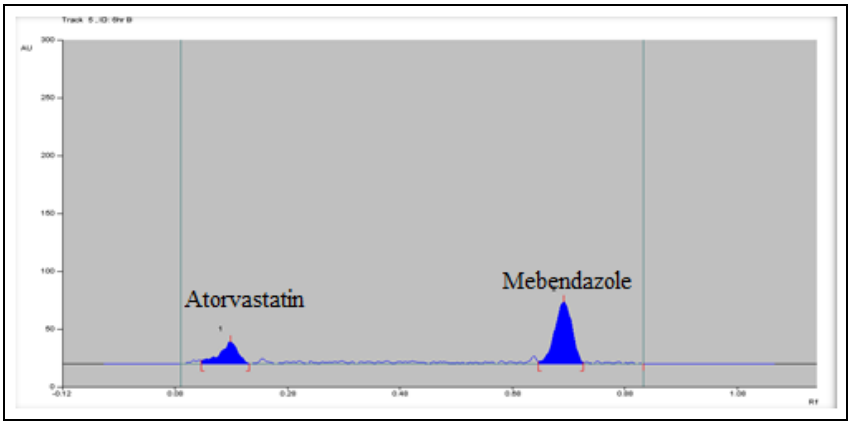


FIG. 5: DENSITOGRAM OF MEBENDAZOLE AND ATORVASTATIN ADMIXTURE AFTER EQUILIBRIUM DIALYSIS (6 h)

CONCLUSION: So far, no *in-vitro* interaction methods were reported for the interaction of Mebendazole with Atorvastatin. Hence, this method was successfully employed for protein binding based *in-vitro* evaluation study of the same. From the protein binding study, a significant displacement was observed for Mebendazole and Atorvastatin. Hence their concomitant use should be avoided since it may lead to anaphylaxis and other severe hypersensitivity reactions. The immediate medical intervention of drug discontinuance is required if anaphylaxis of hypersensitivity reaction occurs. However, further *in-vitro* studies in animals and humans are requested for clinical decision making, choosing the appropriate treatment requirement, and eliminating drug-related problems. HPTLC methods developed were validated as per ICH guidelines and found to be specific, accurate, and precise. The newly developed HPTLC method might be an alternative step forward compared to the analytical methods available

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CONFLICTS OF INTEREST: Nil

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