REPORT

Validated chromatographic and spectrophotometric methods for analysis of some amoebicide drugs in their combined pharmaceutical preparation

Eglal Adelhamid Abdelaleem and Nada Sayed Abdelwahab*
Analytical Chemistry Department, Faculty of Pharmacy, Benisuef University, Egypt

Abstract: This work is concerned with development and validation of chromatographic and spectrophotometric methods for analysis of Mebeverine HCl (MEH), Diloxanide furoate (DF) and Metronidazole (MET) in Dimetrol® tablets – spectrophotometric and RP-HPLC methods using UV detection. The developed spectrophotometric methods depend on determination of MEH and DF in the combined dosage form using the successive derivative ratio spectra method which depends on derivatization of the obtained ratio spectra in two steps using methanol as a solvent and measuring MEH at 226.4-232.2 nm (peak to peak) and DF at 260.6-264.8 nm (peak to peak). While MET concentrations were determined using first derivative (^{1}D) at λ = 327 nm using the same solvent. The chromatographic method depends on HPLC separation on ODS column and elution with a mobile phase consisting water: methanol: triethylamine (25: 75: 0.5, by volume, orthophosphoric acid to pH =4). Pumping the mobile phase at 0.7 ml min^{-1} with UV at 230 nm. Factors affecting the developed methods were studied and optimized, moreover, they have been validated as per ICH guideline and the results demonstrated that the suggested methods are reproducible, reliable and can be applied for routine use with short time of analysis. Statistical analysis of the two developed methods with each other using F and student's-t tests showed no significant difference.

Keywords: Mebeverine, diloxanide, metronidazole, successive derivative ratio spectrophotometric, RP-HPLC.

INTRODUCTION

Mebeverine Hydrochloride (MEH), is an official drug in British pharmacopoeia (British Pharmacopoeia, 2007) it is an antispasmodic drug that acts directly with direct on gastro intestinal tract used in conditions such as irritable bowel syndrome (Martindale, 2005). It is 3,4-dimethoxybenzoic acid 4-[ethyl(p-methoxy-alphamethylphenethyl)amino]butyl ester (The Merck Index, 2002). Diloxanide Furoate (DF), is an official drug in both British and United States pharmacopoeias (British Pharmacopoeia, 2007; USP, 2007), it is a frequently described antiamoebic drug (Al-Ghanam and Belal, 2001). It is chemically designated as N-dichloroacet-4-hydroxy-N-methyl anilide (Budavari, 2002). Metronidazole (MET), is an official drug stated in both British and United States pharmacopoeias (British Pharmacopoeia, 2007; USP, 2007), it is a derivative of nitroimidazole which has been used widely in protozoal diseases including trichromoniasis and giardiasis (Ghlivand and Torkashvand, 2011). It is designated as 1-(2-hydroxyethyl)-2-methyl-5-nitro imidazole (Budavari, 2002).

Several methods are used for determination of the proposed drugs singly or in combination with other drugs but no reports have been found for the determination of these drugs in their multi-component mixtures. British pharmacopoeia (British Pharmacopoeia, 2007) reported non aqueous titration methods for analysis of each of the suggested drugs in its dosage form; also USP (USP, 2007) determined each of DF and MET using the same technique. Recently MEH was determined by RP-HPLC (Zhang and Zhou, 2007; Haggag *et al.,* 2010; El masry *et al.,* 2011; Naguib and Abdelkawy, 2010), spectrophotometric (El didamony, 2008) and HPTLC Naguib and Abdelkawy, 2010) methods. On the other hand MET was determined in plasma (Quyang *et al.,* 2010; Marina *et al*., 2009; Fraselle *et al.,* 2007), blood (Faisal *et al.,* 2010), fish muscles (Maher *et al.,* 2008), procine liver (Xia *et al.,* 2007) and in different pharmaceutical preparations using different HPLC techniques (Tavakoli *et al.,* 2007; Ping *et al.,* 2007; Bassam *et al.,* 2008; El-Gindy *et al.,* 2010) The proposed drug has been also determined by spectrophotometric (El-Gindy *et al.,* 2010; Olajire Aremu and Offiony Edet; 2009) and voltametric methods (Ghlivand and Torkashvand, 2011). Both DF and MET have been determined in their combined dosage form by different methods including RP-HPLC (Adel and Diana, 2005; Al

*Corresponding author: nadasayed2003@yahoo.com

Shaalan, 2007) and spectrophotometric methods (Al Shaalan, 2007; El-Ghobashy and Abo-Talib, 2010; Bimalendu, 2003).

From the previously mentioned literature review, it was shown that no published methods have been found for anlysis of the MEH, DF and MET in their ternary mixtures. So, this work has been aimed to development of a comparative study of recent, rapid, reproducible and sensitive methods. These methods include spectrophotometric and RP-HPLC methods for the determination of the previous drugs in their pure forms, synthetic mixtures and in their pharmaceutical preparation.

MATERIALS AND METHODS

Instruments

For spectrophotometric methods, a double beam UV-Visible spectrophotometer with quartz cell of 1 cm pathlength, model UV-1601 PC (SHIMADZU, Japan) connected to IBM compatible computer. UVPC personal spectroscopy software version 3.7 was used.

For HPLC method, HPLC instrument was equipped with a model series SCL-10 AVP controller, LC-10 ADVP pump, DGU-12 A Degasser and SPD-10 AVP UV-VIS detector (SHIMADZU, Japan), separation and quantitation were made on RP C18 column, 250 mm × 4.6 mm (i.d) (4.6 µm particle size). The detector was adjusted at 230 nm.

Samples

Pure samples

Standards MEH, DF and MET with claimed purity of 98.9, 100.5 and 100.4 % according to manufacturer certificate were kindly donated by EVA PHARMA for Pharmaceuticals and Medical Appliances S.A.E, Egypt) .

Pharmaceutical dosage form

Dimetrol® film coated tablets, labeled to contain 375 mg MET, 250 mg DF and 50 mg MEH per one tablet, Batch No.909537. (EVA PHARMA for Pharmaceuticals and Medical Appliances S.A.E, Egypt).

Reagents

All solvents and chemicals used through this work (ethanol , hydrochloric acid and sodium hydroxide) were of analytical grade and purchased from El- NASR Pharmaceutical Chemicals Co., Abu- Zabaal, Cairo, Egypt. Deionized water purchased from (SEDICO Pharmaceuticals Co., Cairo, Egypt). Acetonitrile, methanol, orthophosphoric acid and triethylamine were of HPLC grade (Sigma-Aldrich® Chemie GmbH, Germany).

Solutions

Stock standard solutions *of MEH, DF and MET* in the concentration of 1 mg ml^{-1} were prepared in methanol.

Working standard solutions *of MEH, DF and MET* in the concentration of 0.1 mg ml^{-1}, were prepared in methanol (for spectrophotometric methods) and in the used mobile phase (for HPLC method)**.**

Spectrophotometric methods

Spectral characteristics

Zero order absorption spectra of 10 µg ml^{-1} each of MEH, DF and MET were recorded from 200 to 360 nm using methanol as a solvent (fig. 1).

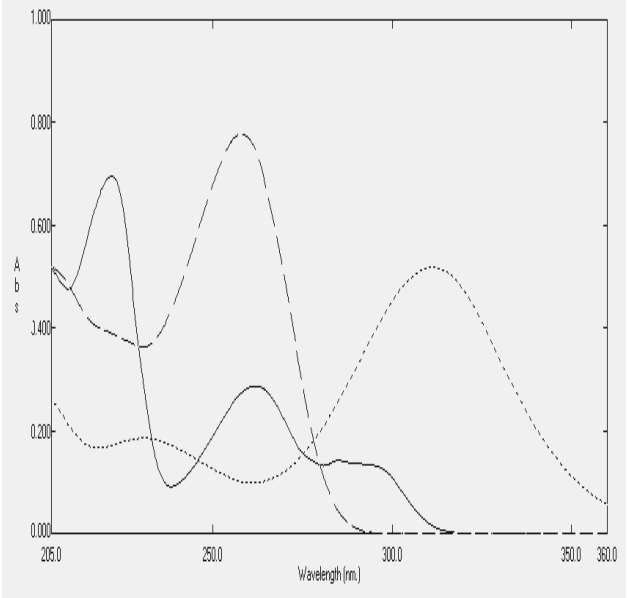


Fig. 1: Zero order absorption spectra of 10 µg ml^{-1} each of MEH (——), DF (------), MET (…….) using methanol as a solvent.

Method validation

The developed spectrophotometric methods were validated according to ICH guidelines (ICH, 2005) and USP requirements (USP, 2007).

Linearity of the suggested successive derivative of ratio spectra method for analysis of MEH and DF were tested by recording the spectra of different pure standard solutions of each in the concentration range of 2-25 and 1-25 µg ml^{-1} for MEH and DF, respectively in methanol and then storing the recorded spectra. For MEH, the stored spectra were divided by the standard spectrum of 20 µg ml^{-1} MET and first derivative of the produced ratio spectra were then obtained. Then these vectors (first derivative of the first ratio spectra) were divided by (d/dλ) (DF/MET) corresponding to the first derivative of the ratio spectra of 20 µg ml^{-1} of each to obtain the second ratio spectra and first derivative of these ratio spectra were then obtained using $\Delta\lambda = 4$. Similarly, the recorded spectra of DF were divided by the spectrum of 20 µg ml^{-1} MET and the first derivative of these vectors were then divided by (d/dλ) (MEH/MET) corresponding to the first derivative of the ratio spectra of 20 µg ml^{-1} of each and the second ratio spectra were obtained. The first derivative of these

vectors were then obtained with $\Delta\lambda = 4$. Metronidazole linearity were tested by recording the absorption spectra of pure MET in methanol (1-28 μg ml^{-1}) then getting the first derivative (^1D) of these spectra using $\Delta\lambda = 4$. The calibration graphs were obtained by plotting the amplitudes from 226.6 nm to 232.4 nm (peak to peak), 260.6 nm to 264.8 nm (peak to peak) and at 327 nm for MEH, DF and MET, respectively, versus the corresponding concentrations.

Specificity of the suggested spectrophotometric methods were ascertained by analyzing different synthetic mixtures prepared from different ratios of MEH, DF and MET and following the procedure under linearity to obtain the concentration of each drug.

RP-HPLC method

Chromatographic conditions

Chromatographic analysis was performed in isocratic mode with water: methanol: triethylamine (25: 25: 0.5, by volume pH = 4 with phosphoric acid) as a mobile phase delivered at 0.7 ml min^{-1}, injection volume 20 μl and scanning at 230 nm at room temperature. The run time was 10 min and the total peak area was used to quantify each of the studied drugs.

Method validation

Linearity of the developed RP-HPLC method was evaluated by preparing standard solutions of each drug at different concentration levels ranging from 2-25 μg ml^{-1} for MEH and DF and from 4-25 μg ml^{-1} for MET. Injections in triplicates were made for each concentration then the calibration curves were obtained by plotting the area under the curve against the corresponding concentration of each drug. Specificity of the method can be defined as absence of any interference at retention times of peaks of interest and it was evaluated by observing the chromatograms of the ternary mixture and that of Dimetrol® tablets. Robustness of the method was demonstrated by verifying system suitability parameters by making small deliberate changes in the conditions of the chromatographic separation, e.g. change in flow rate by ± 0.05 ml min^{-1}, change in pH by ± 0.2 units, change in the organic composition of the mobile phase by ± 1% and change in triethylamine by ± 0.05 %, then calculating the resolution among the studied drugs. System suitability test (SST) parameters were tested during method development and optimization as well as through the validation procedure. SST parameters include resolution (Rs), tailing, capacity (k'), selectivity factors (α) and column efficiency (number of theoretical plates, (N).

Accuracy (recovery study) of the suggested spectrophotometric and RP-HPLC methods was calculated as the percentage recoveries of blind pure drugs. It was further assessed by application of standard addition technique at different levels (80, 100 and 120%), by addition of known amounts of pure drugs to known concentrations of Dimetrol® tablets and then analyzing the prepared mixtures. *Precision* was studied with respect to repeatability and intermediate precision. Repeatability was calculated by determination of different three concentrations of pure drugs (5, 10 and 15 μg ml^{-1}) in triplicates on the same day in the same equipment. To determine the intermediate precision, the experiment was repeated on the same concentration seven times on four consecutive days.

Application to pharmaceutical dosage form

Twenty coated tablets of Dimetrol® were separately weighed. Accurately weighted portion equivalent to 150 mg MET, 100 mg DF and 20 mg MEH were separately transferred into 100-ml volumetric flask and 75 ml methanol was then added. The prepared solution was sonicated for 30 minutes, the volume was completed to the mark with the same solvent and the prepared solution was then filtered. Appropriate dilution of this solution was made to prepare the working solution (0.1 mg ml^{-1}) and then the proposed methods were followed.

DISCUUSSION

Spectrophotometric methods

Several spectrophotometric determinations have been applied for resolving mixtures of components with overlapping spectra. When these methods were compared with each other, derivative spectrophotomrtry is found to be more selective, reliable, and sensitive than normal spectrophotomrtry (Dinc and Onur, 1998). Salins *et al* (Salinas *et al.,* 1990) developed the derivative ratio spectrophotometric method for analysis of binary mixtures. On the other hand, Berzas Nevado *et al* (Berzas Nevado *et al., 1992)* developed derivative ratio spectra zero crossing method for ternary mixtures resolution, Dinc et al (Salinas *et al.,* 1990; Dinc, 1999; Dinc *et al.,* 2002) suggested resolution of ternary mixtures using double divisor ratio spectra derivative method. Recently Afkami and Bahram (Afkami and Bahram, 2005) developed a new method for determination of ternary mixtures which depends on the successive derivative of ratio spectra in two successive steps. In this work, this method has been successfully applied for determination of MEH and DF in their combined mixtures with MET, while measuring MET in the same mixtures using first derivative (^1D) spectrophotometric method. The suggested methods have been optimized and applied for determination of the studied drugs in their combined dosage form. The theoretical background of the developed successive derivative of ratio spectra method has been illustrated by Afkhami and Bahram (Afkami and Bahram, 2005).

Method development and optimization

The main step in the development of an analytical method is to improve the conditions and parameters which should

be followed in the development and validation. Different solvents were studied (methanol, ethanol, acetonitrile, 0.1 N NaOH, 0.1 N HCl and water), the criteria employed were the sensitivity of the method and availability of the solvent. From a solvent effect studies and spectral behaviors of MEH, DF, and MET, methanol was selected as a solvent for the suggested spectrophotometric methods. The divisor concentration effect on the method selectivity and analytical parameters such as correlation coefficient, intercept and slope of the calibration equations was tested. Different concentrations of MEH, DF, and MET were tested (5, 10, 15 and 20 µg ml^{-1}, each). It was found that the divisor concentration had a great effect on the method selectivity, 20 µg ml^{-1}, each of MEH, DF, and MET were used as divisors. The amount of Δλ had no considerable effect on the suggested first derivative and successive derivative of ratio spectra methods (for both the steps), Δλ = 4 was used.

For determination of MEH, the absorption spectra of different concentrations were divided by the spectrum of 20 µg ml^{-1} of MET to obtain the first ratio spectra and the first derivative of this ratio spectra were obtained. Then these vectors (first derivatives of the ratio spectra) were divided by (d/dλ) (DF/MET) corresponding to the derivative of the ratio of the spectra of 20 µg ml^{-1} of each and therefore second ratio spectra were obtained. The first derivatives of these vectors were obtained from which MEH was determined by measuring the total amplitudes from 226.6 nm to 232.4 nm (peak to peak) (fig. 2).

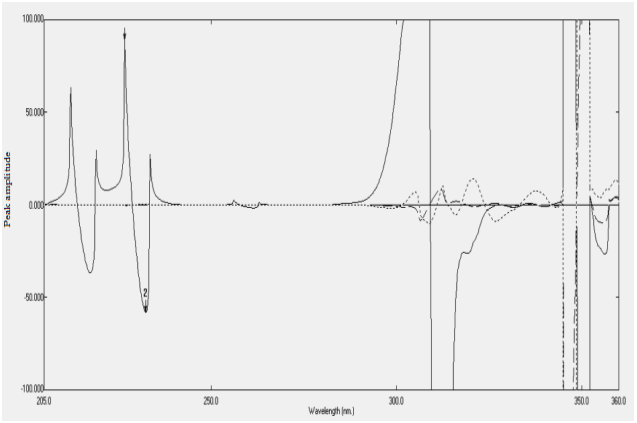


Fig. 2: First derivative of second ratio spectra of 10 µg ml^{-1} each of MEH (——), DF (------), MET (…….) using 20 µg ml^{-1} each of MET and DF as divisors and methanol as a solvent.

By the same way, the concentrations of DF were determined by measuring the total peak amplitudes of the first derivative of the second ratio spectra from 260.6 nm to 264.8 nm (peak to peak, fig. 3, using the spectra of 20 µg ml^{-1} each of MET and MEH to obtain the first and second ratio spectra.

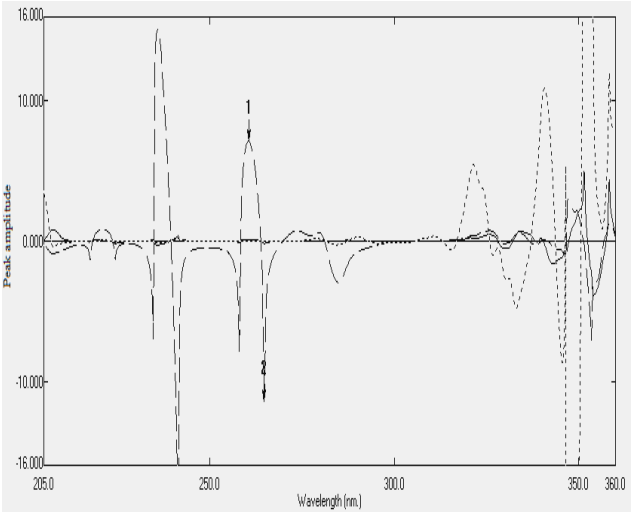


Fig. 3: First derivative of second ratio spectra of 10 µg ml^{-1} each of MEH (——), DF (------), MET (…….) using 20 µg ml^{-1} each of MET and MEH as divisors and methanol as a solvent.

For selective determination of MET, zero order absorption spectra at 311 nm were tested, unfortunately bad results (regarding selectivity) were obtained. Metronidazole was successfully determined by measuring the (^{1}D) amplitudes at 327 nm as shown in fig. 4.

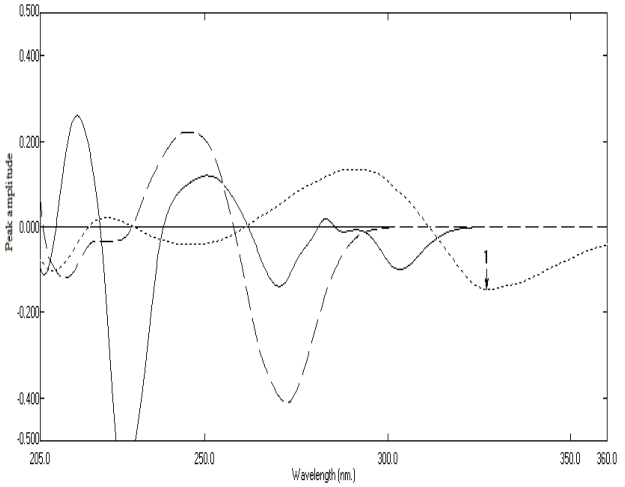


Fig. 4: First derivative spectra of 10 µg ml^{-1} each of MEH (——), DF (------), MET (…….) using methanol as a solvent.

RP-HPLC method

A RP-HPLC method was optimized and validated for determination of MEH, DF and MET in their ternary mixtures without physical separation. The developed method has high sensitivity, selectivity, reproducibility and short analysis time.

Method development and optimization

Combination between polarities of the analytes, stationary phase and mobile phase was necessary to develop a LC

method to get a good separation in a suitable analysis time (El masry *et al.,* 2011). The parameters affecting the chromatographic separation had been studied and optimized:-

a- The stationary phase

The stationary phase has a very important role that leads to the best separation. Different stationary phases were tried (C_{18}, C_8 and CN columns) using mobile phase of acetonitrle : water (70: 30, v/v) using C_{18} column gave the most acceptable peak shape for the studied drugs.

b- The mobile phase

The mobile phase composition was based on providing good baseline, adequate separation and sharp peaks in a suitable time of analysis so the effect of changing the pH of the mobile phase and the organic modifier were studied.

- Effect of the organic modifier

Initially mobile phases of water: acetonitrile in different ratios was tested. It was observed that using acetonitrile as an organic modifier gave bad baseline with broad peaks and hence bad resolution among the separated peaks. The next step is the replacement of acetonitrile with methanol which is reported to influence the retention characters of basic solutes (like MEH, DF and MET) (Sykora *et al.,* 1997). Different ratios of methanol were tested (50-80%) where in all ratios, good base line was observed but with poor resolution between peaks of MEH and MET. The ratio of 75% methanol was chosen to reduce the analysis time.

- Effect of triethylamine

From the molecular structures of the studied drugs (The Merck Index, 2003), all the studied drugs contain amino groups which interact with silanol groups of the stationary phase (McNair and Polite, 2007) leading to peak broadening and tailing. To suppress this interaction, silanol blockers (e.g. triethyl amine) should be added to the mobile phase (SYkora *et al.,* 1997). Different ratios of triethyl amine (0.1-0.6%) were tested where acceptable peaks with minimum tailing were obtained upon using 0.5% triethyl amine but without improving the resolution between MEH and MET.

- Effect of mobile phase pH

To improve the resolution between MEH and MET, different mobile phase pH values were tested (3-8 pH value). It was noticed that the retention of DF and MET were almost unchanged with pH variation while MEH showed dramatic changes in retention with pH variation affecting its resolution from DF and MET. It was highly retained on the stationary phase on using basic pH value while it was rapidly eluted on using strong acidic pH value. pH = 4 gave the best resolution among the eluted drugs with acceptable retention times.

c-The mobile phase flow rate and the detection wavelength

The mobile phase was delivered at different flow rates (0.5, 0.7, 1 and 1.5 ml min^{-1}) where optimum separation with reasonable analysis time was obtained with a flow rate of 0.7 ml min^{-1}. The optimum absorption wavelength for detection of the studied drugs was chosen especially with regard to absorption spectrum of MEH (of the lowest ratio in the combined dosage form). Wavelengths of 220, 230 and 254 nm were tried, the highest detector response was obtained at 220 nm but with low signal to noise ratio. Therefore the final detection wavelength was 230 nm at which acceptable detector response for all the proposed drugs with acceptable noise to signal ratio were obtained.

After method optimization, the chromatographic separation was performed on ODS C_{18} column with a mobile phase consisting of water: methanol: triethyl amine (75: 25: 0.5, by volume pH = 4 with orthophosphoric acid) delivered at 0.7 ml $min^{-1}$and detection of the separated peaks at 230 nm. The obtained chromatogram is shown in fig. 5.

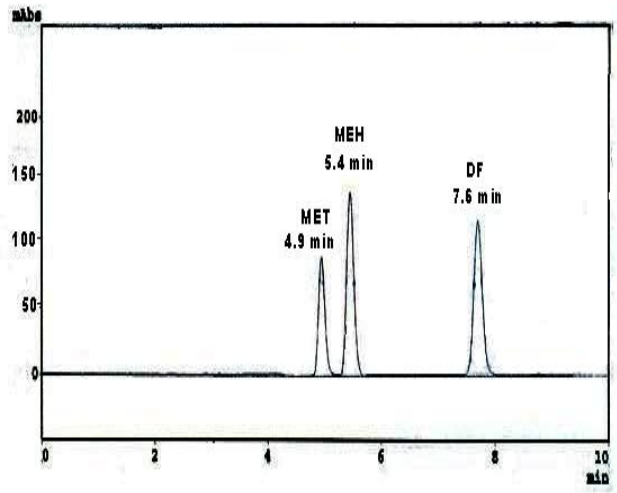


Fig. 5: HPLC chromatogram of MET, MEH and DF using methanol : water : triethylamine (75: 25; 0.5%, by volume pH=4 with phosphoric acid) as a mobile phase.

RESULTS

The developed spectrophotometric and RP-HPLC methods have been successfully applied for determination of the ternary mixture in Dimetrol® tablets, table 1. Moreover, the results obtained from the suggested spectrophotometric methods for analysis of the three studied drugs in their combined dosage form were statistically compared to those obtained from RP-HPLC one (using F and student's t-test) with no significant difference among the proposed methods, table 1, regarding both accuracy and precision.

Methods validation

Linearity

Beer's Lamber's law was obeyed in the concentration ranges of 2-25, 1-25 and 1-28 µg ml^{-1} for MEH, DF and MET, respectively (for spectrophotometric methods) and in the range of 2-25 for both MEH and DF and 4-25 µg ml^{-1} for MET (for RP-HPLC method). The evaluation parameters like correlation coefficients, intercept and slope were calculated and presented in table 2.

Accuracy and precision

The good percentage recoveries and the acceptable RSD% indicating that the proposed methods are accurate and precise table 2.

Specificity

-Spectrophotometric methods: when the suggested spectrophotometric methods were applied for analysis of number of laboratory prepared mixtures containing MEH, DF and MET in different ratios, good percentage recoveries and low SD values were produced, table 1, confirming the high specificity of the developed methods.

-RP-HPLC method: the specificity of the method was confirmed by good separation of the three proposed drugs. Furthermore, matrix components, e.g. excipients do not interfere with the three components as shown in fig. 6.

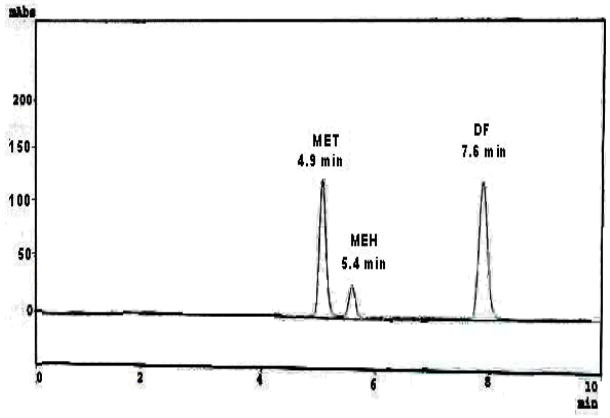


Fig. 6: HPLC chromatogram of Dimetrol® tablets contains 22.5 µg ml^{-1} of MET, 3 µg ml^{-1} MEH and 15 µg ml^{-1} DF using methanol: water : triethylamine (75: 25; 0.5%, by volume pH=4 with phosphoric acid) as a mobile phase.

Table 1: Determination of the studied drugs in the laboratory prepared mixtures (L.P.) and pharmaceutical preparation by the proposed methods and statistical comparison with the developed RP-HPLC method.

Parameters	Spectrophotometric methods			RP-HPLC method		
	MEH	DF	MET	MEH	DF	MET
L.P. Mixtures [a]	99.48 ± 1.201	100.45 ± 2.30	100.67 ± 2.038			
Dimetrol® tablets [b] (B.No.909537)	101.20 ± 1.218	100.75 ± 0.730	100.59 ± 1.102	99.84 ±0.994	100.88 ± 0.841	99.21 ±1.094
Standard addition [a]	98.38 ± 1.415	99.36 ± 2.041	99.28 ± 1.042	100.38 ± 1.997	99.05 ± 1.434	99.49 ± 1.664
Degree of freedom F-test	9 (5.192) [c] 1.500	10 (5.053) [c] 1.326	10 (5.053) [c] 1.014			
Degree of freedom Student's – t test	9 (2.262) [c] 2.036	10 (2.228) [c] 0.282	10 (2.228) [c] 2.174			

a: Average of 3 determinations. b: Average of 5 for MEH and 6 determinations for DF and MET .
c: The values in the parenthesis are the corresponding theoretical values at p= 0.05.

Table 2: Regression and analytical parameters of the proposed methods for determination of Mebeverine HCl, Diloxanide Furoate and Metronidazole.

Parameters	Spectrophotometric methods			HPLC method		
	MEH	DF	MET	MEH	DF	MET
Calibration range	2-25µg ml^{-1}	1-25µg ml^{-1}	1-28µg ml^{-1}	2-25µg ml^{-1}	2-25µg ml^{-1}	4-25µg ml^{-1}
Slope	1.2803	1.7870	0.0147	1.0129	0.9066	0.6014
Intercept	-0.3924	0.3040	0.0006	-0.2466	0.5907	0.0896
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9996	0.9998	0.9999
Accuracy	99.92	99.67	100.41	99.82	99.67	100.10
Precision						
Repeatability	0.799	0.643	1.002	0.945	1.302	0.859
Intermediate precision	1.040	1.250	0.798	1.750	0.640	1.950

Robustness of RP-HPLC method

The method was demonstrated to be robust over an acceptable working range of its HPLC co-operational conditions except the change in the mobile phase pH. It was found that the retention time of MEH affected by any variation in the mobile phase pH which affected the resolution among the three studied drugs, hence it was concluded that the method is sensitive to mobile phase pH which should be carefully controlled.

System suitability test for RP-HPLC method

The system suitability test confirms that the analytical procedure is valid as well as ensures the resolution between different peaks of interest. All critical parameters tested met the accepted criteria (table 3).

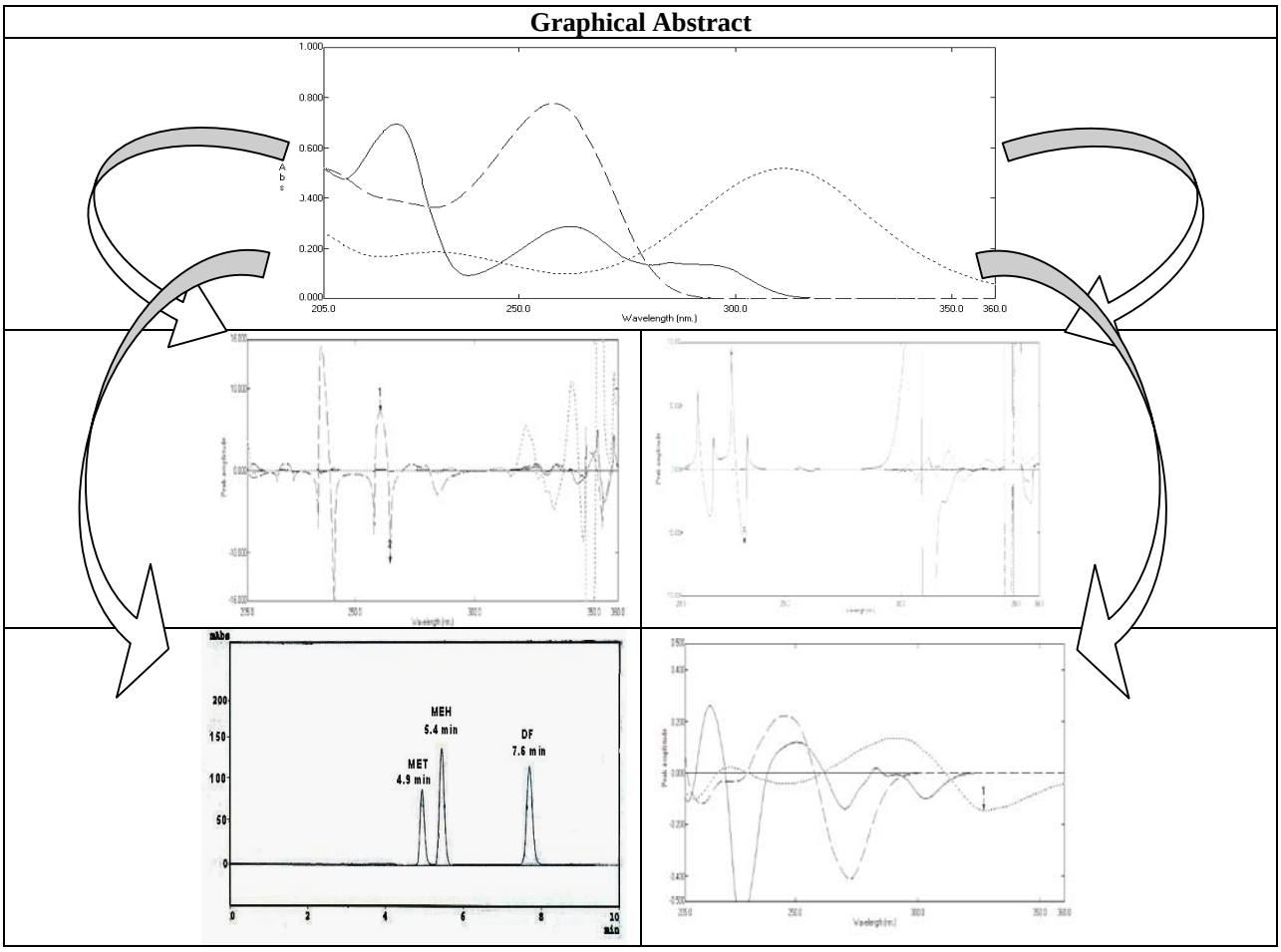
CONCLUSION

The developed methods represent fast analytical procedures for determination of MEH, DF and MET in

Table 3: Statistical analysis of parameters required for system suitability testing of RP- HPLC method.

Parameters	Obtained value			Reference value
	MET	MEH	DF	
Resolution (Rs)	1.54	5.86	> 1.5	
Relative retention (α)	1.1	1.41	> 1	
Tailing factor (T)	1.0	1.0	1.09	T = 1, for a typical symmetrical peak
Capacity factor (k')	3.9	4.4	6.6	1-10 acceptable
Number of theoretical plates (n)	4266	3809	5776	Increase with the efficiency of the separation
HETP	5.86×10^{-3}	6.56×10^{-3}	4.32×10^{-3}	The smaller the value the higher the column efficiency

HETP = height equivalent to theoretical plates (cm/plate)



their combined dosage form. The preparation of the sample is simple and the time of analysis is short. The proposed spectrophotometric methods are simple, sensitive, economic and easy to understand and apply. On the other hand the developed RP-HPLC method is more specific than the developed spectrophotometric methods but it needs high cost equipment and materials. Standard addition can be applied easily in the suggested methods and matrix effects can be removed. Therefore, these methods can be used for resolution the studied mixture in complex samples with unknown matrices. All the obtained results confirmed the applicability, accuracy and precision of these methods.

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