

Chromatographic methods for simultaneous determination of diiodohydroxyquinoline and Metronidazole in their binary mixture

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Abstract: Two chromatographic methods were developed for analysis of diiodohydroxyquinoline (DIHQ) and metronidazole (MTN). In the first method, diiodohydroxyquinoline and metronidazole were separated on TLC silica gel 60F254 plate using chloroform: acetone: glacial acetic acid (7.5: 2.5: 0.1, by volume) as mobile phase. The obtained bands were then scanned at 254 nm. The second method is a RP-HPLC method in which diiodohydroxyquinoline and metronidazole were separated on a reversed-phase C18 column using water : methanol (60 :40, V/V, PH=3.6)as mobile phase at a flow rate of 0.7 mL.min⁻¹ and UV detection at 220 nm. The mentioned methods were successfully used for determination of diiodohydroxyquinoline and metronidazole in pure form and in their pharmaceutical formulation.

Keywords: TLC-spectrodensitometry, RP-HPLC, diiodohydroxyquinoline and metronidazole.

INTRODUCTION

Metronidazole (MTN) is 2-methyl-5-nitroimidazole-1-ethanol (Merck, 2006). It is a 5-nitroimidazole derivative with activity against anaerobic bacteria and protozoa. MTN is an amoebicide at whole sites of infection with *Entamoeba histolytica*. The effectiveness of MTN against parasites in the bowel lumen is generally weak as a result of its rapid absorption so it should be used together with luminal amoebicide in the treatment of invasive amoebiasis (Martindale, 2006).

Diiodohydroxyquinoline (DIHQ) or iodoquinol (USP, 2009) is 5, 7-diiodoquinolin-8-ol (Merck, 2006) (1), is a halogenated hydroxyquinoline which can be used in the treatment of amoebiasis. DIHQ is poorly absorbed from the gastrointestinal tract and is amebicidal at the site of infection (Martindale, 2006).

This combination of two amoebicidal agents (DIHQ and MTN) is used for treatment of extra and intra-intestinal amoebiasis. The structural formulas of DIHQ and MTN are shown in fig. 1.

There are many reports for the determination of DIHQ and MTN either separately or in combination with other drugs including spectrophotometry (Nagaraja *et al.*, 2002; Erk *et al.*, 2001; Khashaba *et al.*, 1999; Shingbal *et al.*, 1980; Rizk *et al.*, 2000; Bhowal *et al.*, 1990; Saha *et al.*, 1988; Belal *et al.*, 1984; Windheuser *et al.*, 1967), electrochemistry (Lu *et al.*, 2004; Jin *et al.*, 2000), HPLC (Yuet *et al.*, 2002; Frasey *et al.*, 2004; Liu *et al.*, 2003; Shenet *et al.*, 2003; Storms and Stewart., 2002; Daeseleire *et al.*, 2000; Rizk *et al.*, 2002; Wojtowicz., 1984), HPTLC

(Argekaret *et al.*, 1996), GC (Wang *et al.*, 2001), atomic absorption spectrometry (Nejem *et al.*, 2008), iodometric titration (Soliman, 1975) and non aqueous titration (Kavarana, 1959; Paranjothy and Banerjee., 1973).

Few methods have been mentioned for analysis of DIHQ and MTN in binary mixture. The first method determined the two drugs spectrophotometrically using modified Vierordt equation (Chatterjee *et al.*, 1986). The second method used the bivariate calibration method for resolution of the binary mixture and compared results to those of derivative spectrophotometry (Lopez *et al.*, 1997). The third method determined DIHQ and MTN in pharmaceutical preparation by TLC (Parimoo *et al.*, 1992), in which a very complicated process was applied where the spots were detected by spraying 20% w/v methanolic solution of KOH and heating to 80°C till development of reddish pink color then the spots scraped and dissolved in 0.1 N HCl and finally the absorbances of supernatant were measured spectrophotometrically at 280 nm.

As noted, this method suffers a lot of drawbacks among which is the lack of precision, accuracy and specificity required in determination of drug combinations. Modern TLC densitometry offers a simple manner to quantify directly on TLC plate where the optical densities of the detached bands are measured quantitatively. The concentrations of compounds are detected by comparing to a standard curve from reference materials chromatographed simultaneously under the same conditions (Grinberg, 1990).

Therefore, the objective of this work is to develop sensitive, selective and reproducible methods for simultaneous determination of DIHQ and MTN.

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Chromatographic methods are well known for providing high selectivity and sensitivity when used for determination of pharmaceutical drugs. In this paper, two chromatographic methods, namely, TLC-spectro-densitometry and reversed phase high performance liquid chromatography (RP-HPLC) have been proposed for simultaneous determination of DIHQ and MTN that can be used for routine quality control analysis of these drugs either in bulk powder or in pharmaceutical formulation.

MATERIALS AND METHODS

Apparatus

- 1- UV lamp with short wavelength 254 nm (USA).
- 2- TLC scanner 3 densitometer (Camage, Muttentz, Switzerland).

The following requirements should be considered:

- Slit dimensions: 6.00x0.45, Micrometer
 - Scanning speed = 20 mm/s
 - Data resolution = 100 $\mu\text{m}/\text{step}$
- 3- Sample applicator for TLC Linomat IV with 100 μL syringe (Camage, Muttentz, Switzerland).
 - 4- TLC plates (20x20 cm) coated with silica gel 60 F 254 (Merck KgaA, Darmstad, Germany).
 - 5- Shimadzu Class-LC 10 AD Liquid Chromatograph equipped with Shimadzu SPD-10 A UV-VIS Detector (Shimadzu Corporation, Japan). Phenomenex C18 (25/cm x 4.6/mm i.d, 5/ μm particle size) column was used as a stationary phase for HPLC determinations (USA).
 - 6- Sonix TV ss-series ultrasonicator (USA).

Materials

Pure samples

DIHQ and MTN were kindly supplied by (CID) Co. Chemical Industries Development, Giza, Egypt. Their purities were found to be 100.01 ± 0.837 and 99.21 ± 1.602 , respectively, according to the spectrophotometric method of (CID) company.

Market samples

Paramibe compound[®] tablet (Batch No 114336W) labeled to contain 250/mg of DIHQ and 250/mg of MTN, is from (CID) Co. Chemical Industries Development, Giza, Egypt.

Reagents

All mentioned reagents and chemicals were of analytical grade and were used without extra purification

- 1- Chloroform, acetone, orthophosphoric acid and glacial acetic acid from (EL – NASR Pharmaceutical Chemicals Co., Abu - Zabaal, Cairo, Egypt).
- 2- Methanol HPLC grade (Sigma Aldrich, Germany).
- 3- Deionized water (SEDICO pharmaceutical Co., 6th October City, Egypt).

Preparation of standard solutions

- 1- Stock standard solution of DIHQ and MTN (0.5 mg.mL^{-1}): weigh accurately 0.05 gram of DIHQ and MTN into two separate 100-mL volumetric flasks, add 50 mL methanol to each flask, stir magnetically for 30 minutes to dissolve then complete the volume to the mark with methanol.
- 2- Working standard solutions (100 $\mu\text{g.mL}^{-1}$): transfer accurately 20 mL of DIHQ and MTN stock standard solutions respectively into two 100-mL volumetric flasks, then complete the volume to the mark with methanol.

Procedures

TLC-spectrodensitometric method

1. Linearity and construction of calibration curves:

Apply accurate aliquots equivalent to (0.2-1.8 $\mu\text{g.mL}^{-1}$) of DIHQ, (0.4-1.8 $\mu\text{g.mL}^{-1}$) of MTN from their corresponding working solutions (100 $\mu\text{g.mL}^{-1}$) to TLC plates (20x20cm) as band using the Camage TLC sampler. Leave a space of 1 cm between each band and 1.5 cm from the bottom edge of the plate. Develop the plate in a chromatographic tank previously saturated for one hour with the developing mobile phase consisting of chloroform: acetone: glacial acetic acid (7.5: 2.5:0.1, by volume) by ascending chromatography at room temperature (Parimoo *et al.*, 1992).

Detect the bands under UV-lamp and scan at 254 nm under the specified experimental conditions. Construct the calibration curves for each compound by plotting the peak area/100 versus the corresponding concentration and then compute the regression equations.

2. Analysis of laboratory prepared mixtures:

Prepare mixtures containing DIHQ and MTN in different ratios. Proceed as described in linearity and construction of calibration curves. Calculate the concentrations of the two compounds from their corresponding regression equations.

RP-HPLC method

1. Linearity and construction of calibration curves:

Transfer accurate aliquots equivalent to (50-1500) μg of DIHQ and (50-1000) μg of MTN from their corresponding working solutions (100 $\mu\text{g.mL}^{-1}$) into two separate sets of a series of 10-mL volumetric flasks. Complete the volume with methanol. Make triplicate 20 μL injections for each concentration. The separation was done on a C18 column using (water: Methanol 60: 40 v/v, pH adjusted to 3.6 with orthophosphoric acid) as a mobile phase. Record the chromatograms at ambient temperature maintaining the flow rate at 0.7mL.min^{-1} and detect the effluent at 220 nm. Construct the calibration curves for each compound by plotting the peak area/ 10^4 versus the corresponding concentration and then compute the regression equations.

2. Analysis of laboratory prepared mixtures:

Prepare mixtures containing DIHQ and MTN in different ratios. Proceed as described in linearity and construction of calibration curves. Calculate the concentrations of the two compounds from their corresponding regression equations.

Application of the proposed methods to pharmaceutical formulations

Thoroughly powder ten tablets of Paramibe compound[®] and mix, then weigh accurately an amount of the powder equivalent to 50/mg of DIHQ and 50/mg of MTN in 250-ml beaker. Add 70/mL of methanol; stir magnetically for 30 minutes then filter through filter paper into a 100-ml volumetric flask. Wash the beaker and the funnel then complete the volume with methanol to get a concentration of 0.5mg.mL⁻¹ for each drug. Appropriate dilutions were made to bring up a concentration of 50, 25 and 20µg.mL⁻¹ for each drug. The procedure of each method was followed and the concentrations of DIHQ and MTN were calculated from their corresponding regression equations.

RESULTS

Method development and optimization

TLC- spectrodensitometric method

The goal of this work is to develop a method that can be applied successfully for separation and quantification of the studied drugs.

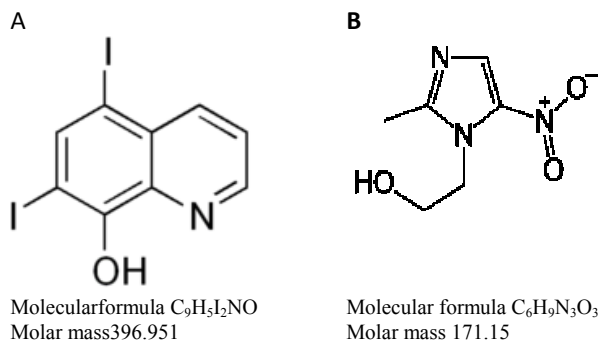


Fig. 1: chemical structure of DIHQ (A) and MTN (B)

Evaluation of the optimum parameters for best separation was carried out by investigating the effect of different variables. Different developing systems with different compositions and ratios were tried, but complete separation of DIHQ and MTN was achieved by using the reported developing mobile phase (31) consisting of chloroform: acetone: Glacial acetic acid (7.5:2.5:0.1, by volume). Also different scanning wavelengths were tried, but the best sensitivity obtained when 254 nm was used as scanning wavelength.

Different band dimensions were tried to give sharp and symmetrical peaks. The optimum band length chosen was 6 mm and the inter space between bands was 1 cm. The

slit dimensions of the scanning light beam should cover completely the band dimensions on the scanned track without any interference of adjacent bands. Different slight dimensions were tried where 6 mm x 0.45 mm provided the highest sensitivity the method is depend on the difference in R_f values of DIHQ (R_f = 0.67) and MTN (R_f = 0.26) as shown in fig. 2.

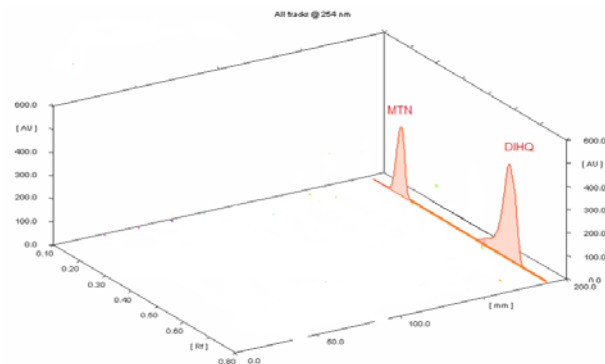


Fig. 2: 3D diagram showing an example of separated mixture of DIHQ and MTN in the spectrodensitometric method.

RP-HPLC method

A validated isocratic RP-HPLC method with UV detection was developed for simultaneous determination of DIHQ and MTN. To optimize the mentioned RP-HPLC method, all of the experimental conditions were tested. The stationary phase used was selected to be the reversed-phase over the normal phase separation due to the defects of the normal phase, e.g. hydration of silica with water that can cause tailing of peak. C18 column is preferred for mixture separation than C8 column, as it gives higher resolution.

Mobile phase systems of different compositions and ratios were tried e.g. (water : methanol) using different acids (H₃PO₄ and CH₃COOH) at different pH values. Complete separation of DIHQ and MTN without interference within a suitable time was achieved using water: methanol (60:40, v/v pH adjusted to 3.6 with H₃PO₄ acid). In addition, the effect of pH was studied by using acids of different pK_a values (H₃PO₄ and CH₃COOH). It was found that pH = 3.6 with H₃PO₄ acid was suitable for proper resolution and peak shape. Different wavelengths were studied for detection of the effluent; it was found that detection at 220 nm gave maximum sensitivity for both compounds.

Also different flow rates were tested to provide good separation within acceptable run time where 0.7 mL.min⁻¹ was the best flow rate. Finally, an effective separation was done by using C18 (25cm×4.6mm i.d. 5µm particle size) column as a stationary phase and using water: methanol (60:40, v/v pH adjusted to 3.6 with H₃PO₄ acid) as a mobile phase, maintaining the flow rate at 0.7 mL min⁻¹ with UV detection at 220 nm. The retention times

for DIHQ and MTN were 3.9 and 5.8 min respectively, fig. 3.

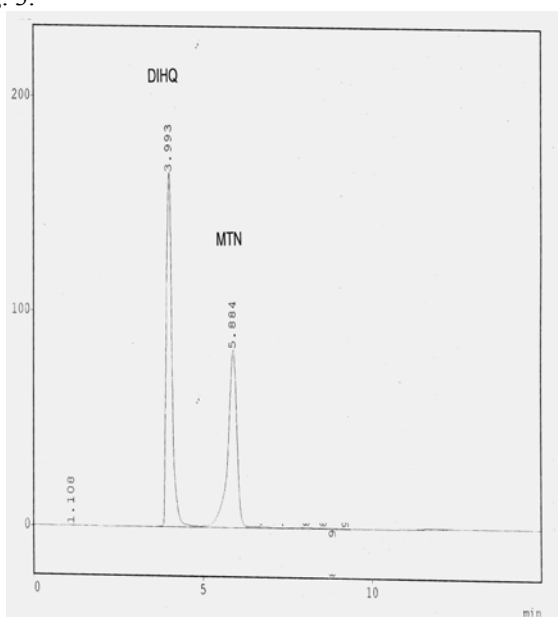


Fig. 3: HPLC chromatogram of DIHQ and MTN using methanol: water (40:60, v/v) PH adjusted to 3.6 with H₃PO₄ acid as a mobile phase with UV detection at 220 nm at a flow rate of 0.7/mL.

Method validation

Method validation was performed according to ICH guidelines (ICH, 2005). Linearity of the described methods was evaluated and it was evident in the concentration range of 0.2-1.8 μg.band⁻¹ for DIHQ and 0.4-1.8/μg.band⁻¹ for MTN in case of TLC spectrodensitometric method and 5-150 μg.mL⁻¹ for DIHQ and 5-100/μg.mL⁻¹ for MTN in case of RP-HPLC method, good linearity was evident by the high value of the correlation coefficient and the low intercept value, (table 1).

The regression equations were calculated and found to be:
- In case of TLC-densitometric method

$$Y_1 = 2.5443C_1 - 0.0258 \quad r_1 = 0.9991$$

$$Y_2 = 1.1695C_2 + 0.1827 \quad r_2 = 0.9986$$

Where Y₁ and Y₂ are the peak area /100, C₁ and C₂ are DIHQ and MTN concentrations in μg. band⁻¹ respectively and r₁ and r₂ are the correlation coefficients.

- In case of RP-HPLC method

$$Y_1 = 0.8861C_1 + 25.724 \quad r_1 = 0.9997$$

$$Y_2 = 4.9775C_2 - 12.539 \quad r_2 = 0.9992$$

Where Y₁ and Y₂ are the peak area /10⁴, C₁ and C₂ are DIHQ and MTN concentrations in μg.mL⁻¹ respectively and r₁ and r₂ are the correlation coefficients. Precision of the proposed TLC-spectrodensitometric and RP-HPLC methods were evident as shown in table 1.

Accuracy of the proposed methods was checked by applying the mentioned methods for analysis of different blind samples of DIHQ and MTN. The concentrations were calculated from the corresponding regression equations. The results obtained as shown in table 2.

Accuracy of the methods was assured by applying the standard addition technique on paramibe compound[®] tablets where good recoveries were obtained revealing no interference from excipients and good accuracy of the proposed methods. According to ICH guidelines, for chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labeled. Specificity of the proposed methods is evident from the TLC-spectrodensitometric and RP-HPLC chromatograms in figs. 2 and 3, respectively.

According to the ICH recommendations, the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits, LOD=3.3S.D./Slope, LOQ=10S.D./Slope (table 1).

Robustness of the proposed methods was evaluated in the

Table 1: Results of assay validation parameters of the proposed methods for the determination of DIHQ and MTN in their mixture

| Parameters | TLC-spectrodensitometric method | | RP-HPLC method | |
|-----------------------------|----------------------------------|---------------------------------|-----------------------------|------------------------------|
| | DIHQ | MTN | DIHQ | MTN |
| Range | 0.2-1.8 (μg.band ⁻¹) | 0.4-1.8(μg.band ⁻¹) | 5-150(μg.mL ⁻¹) | 5-100 (μg.mL ⁻¹) |
| Slope | 2.5443 | 1.1695 | 0.8861 | 4.9775 |
| Intercept | -0.0258 | 0.1827 | 25.724 | -12.539 |
| Correlation coefficient (r) | 0.9991 | 0.9986 | 0.9997 | 0.9992 |
| Accuracy (mean ± SD) | 100.21±1.537 | 100.14±1.807 | 99.94±0.773 | 99.89±0.909 |
| (RSD%) ^{a*} | 1.264 | 1.201 | 1.464 | 1.311 |
| (RSD%) ^{b*} | 1.622 | 1.324 | 1.619 | 1.256 |
| LOD | 1.99(μg.band ⁻¹) | 0.51(μg.band ⁻¹) | 0.85(μg.mL ⁻¹) | 0.61(μg.mL ⁻¹) |
| LOQ | 6.04(μg.band ⁻¹) | 1.55(μg.band ⁻¹) | 2.56(μg.mL ⁻¹) | 1.86(μg.mL ⁻¹) |

a and b the intra-day and inter-day relative standard deviations of the average of concentrations 0.4 , 0.6,1.0 μg.band⁻¹ for each in case of TLC-spectrodensitometric method and 20.00 , 40,00 and 60.00 μg.mL⁻¹ for each drug in case of RP-HPLC method

Table 2: Results of accuracy for determination of pure DIHQ and MTN by the proposed spectrodensitometric and RP-HPLC methods

| TLC-spectrodensitometric method | | | | | | RP-HPLC method | | | | | |
|-----------------------------------|------|---|------|-------------|-------------|-----------------------------------|--------|---|--------|-------------|-------------|
| Taken($\mu\text{g. band}^{-1}$) | | Found ^a ($\mu\text{g. band}^{-1}$) | | Recovery% | | Taken($\mu\text{g. band}^{-1}$) | | Found ^a ($\mu\text{g. band}^{-1}$) | | Recovery% | |
| DIHQ | MTN | DIHQ | MTN | DIHQ | MTN | DIHQ | MTN | DIHQ | MTN | DIHQ | MTN |
| 0.40 | 0.40 | 0.40 | 0.41 | 100.00 | 102.50 | 10.00 | 5.00 | 9.93 | 4.91 | 99.30 | 98.20 |
| 0.60 | 0.60 | 0.59 | 0.59 | 98.33 | 98.33 | 15.00 | 10.00 | 14.98 | 10.07 | 99.87 | 100.70 |
| 0.80 | 0.80 | 0.82 | 0.81 | 102.33 | 101.25 | 20.00 | 20.00 | 20.09 | 19.92 | 100.45 | 99.60 |
| 1.00 | 1.00 | 0.99 | 0.99 | 99.00 | 99.00 | 50.00 | 40.00 | 49.92 | 40.15 | 100.16 | 100.38 |
| 1.20 | 1.20 | 1.20 | 1.18 | 100.00 | 98.33 | 100.00 | 80.00 | 98.87 | 80.32 | 98.87 | 100.40 |
| 1.40 | 1.40 | 1.42 | 1.42 | 101.43 | 101.43 | 150.00 | 100.00 | 151.50 | 100.08 | 101.00 | 100.08 |
| | | | | 100.21 | 100.14 | | | | | 99.94 | 99.89 |
| | | | | ± 1.537 | ± 1.807 | | | | | ± 0.773 | ± 0.909 |

*Average of three determinations

Table 4: Statistical analysis of parameters required for system suitability testing of the proposed method.

| Parameters | For TLC-densitometric method | | | Parameters | For RP-HPLC method | | |
|--------------------------------|------------------------------|------|------------------|--|--------------------|--------|--|
| | Obtained value | | Reference value | | Obtained value | | Reference value |
| | DIHQ | MTN | | | DIHQ | MTN | |
| Resolution (R_s) | 1.82 | | > 1.5 | Resolution (R_s) | 2.50 | | > 1.5 |
| Capacity factor(K') | 0.49 | 2.85 | 0- 10 acceptable | Capacity factor(K') | 2.55 | 4.36 | 1- 10 acceptable |
| Symmetry factor | 1.25 | 1.0 | ≈ 1 | Tailing factor (T) | 1.33 | 1.40 | $< 1.5-2$ |
| Selectivity factor(γ) | 5.82 | | > 1 | Selectivity factor(γ) | 1.71 | | > 1 |
| | | | | Number of Theoretical plate(N) | 676.00 | 556.96 | Increases with increases efficiency |
| | | | | HETP (cm.plate ⁻¹) Height equivalent to theoretical plate | 0.037 | 0.045 | The smaller the value, the higher the efficiency |

Table 5: Statistical analysis of the results obtained by proposed methods and reference method for the determination of DIHQ and MTN

| Parameter | Spectrodensitometric method | | RP-HPLC method | | Reference method | |
|--------------------------------------|-----------------------------|--------|----------------|-------|------------------|-------|
| | DIHT | MTN | DIHT | MTN | DIHT | MTN |
| Mean% | 100.21 | 100.14 | 99.94 | 99.80 | 100.01 | 99.21 |
| SD | 1.537 | 1.807 | 0.773 | 0.909 | 0.837 | 1.602 |
| N | 6 | 6 | 6 | 6 | 6 | 6 |
| Student's t-test (2.23) ^b | 0.265 | 0.943 | 0.151 | 0.785 | - | - |
| F-value (5.05) ^b | 3.372 | 1.272 | 1.172 | 3.106 | - | - |

a manufactured method personal communications .

b the values between parenthesis are the theoretical values for t and F at P=0.05.

development phase by making small changes in the composition of mobile phase and detection wavelength. The low value of %RSD shows that the methods are robust and that deliberate small changes in the studied factors did not lead to a significant change in retention values, area or symmetry of the peaks.

System suitability tests are depend on the idea that the equipment, electronics, analytical operations and samples

constitute an integral system that should be considered as whole. System suitability is used to evaluate method performance before or during the analysis of the drugs. System suitability was checked by calculating the capacity factor (K'), tailing factor (T), column efficiency (N), the selectivity factor (γ) and resolution (R_s), where the system was found to be adequate as shown in table 4.

DISCUSSION

The mentioned methods are effective for providing sensitive, accurate and reproducible quantitative analysis for simultaneous determination of DIHQ and MTN in bulk powder and in pharmaceutical preparation, without interference from excipients. TLC-spectrodensitometric method has the advantages of that many samples can be developed simultaneously using a small volume of mobile phase and provides high sensitivity and selectivity. RP-HPLC method provides rapid, short analysis time, simple and selective method.

CONCLUSION

The suggested methods provide selective, accurate and sensitive analytical procedures for the determination of DIHQ and MTN. They are suitable for routine analysis and quality control of DIHQ and MTN in its pharmaceutical formulation.

Statistical analysis was performed by comparing the results of the mentioned methods with those of manufacturer method. No significant difference was observed regarding accuracy and precision, as shown in table (5).

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