Research Article

A Comparative Study of Two Chromatographic Techniques for the Determination of Group of Imidazoles Simultaneously

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ABSTRACT

Reversed-phase high performance liquid chromatography (RP-HPLC) with UV detection and gas chromatography with flame ionization detection methods are described for the simultaneous determination of metronidazole and miconazole in vaginal ovules. In the former, a C_{18} column was used with a mobile phase of methanol–water (40:60 v/v) at a flow rate of 1.0 ml min^{-1} with detection at 220 nm, and sulfamethoxazole (SMX) used as an internal standard. Linear responses in the concentration ranges 10 -/80 and 5 -/40 µg ml^{-1} with detection limits 0.93 and 0.45 µg ml^{-1} were found for metronidazole (MNZ) and micanozole nitrate (MIC) respectively. Gas chromatography was performed on a capillary column (15 m × 0.32 mm i.d.) and nitrogen was used as a carrier gas at a flow rate of 1.5 ml min^{-1}. Linearity ranges were 200 - 1500 and 100 - 750 µg ml^{-1} for MNZ and MIC, with limit of detection values of 3 and 3.6 µg ml^{-1}, respectively, These method was found to be convenient and reproducible for the analysis of both active ingredients in ovule dosage forms, and could be extended to the determination of other imidazoles (ketoconazale, econazole and clotrimazole).

Keywords: Simultaneous determination, Metronidazole, Miconazole nitrate, High-performance liquid chromatography, Gas Chromatography, Flame Ionization Detector.

INTRODUCTION

Imidazoles are an aromatic heterocyclic alkaloids which contain 2 nitrogen atoms in the ring system (diazole) , substitution of imidazole nucleus with a variety of functional groups especially at N1 position has made it an important pharmacophore with a range of different pharmacological actions such as, microbicidal activity whether antifungal mediated by clotrimazole and miconazole or antiprotozoal, and antibacterial mediated by metronidazole. These two active ingredients are available as a mixture in formulated vaginal ovules to treatment a wide range of most commonly encountered causes of bacterial, fungal and trichomonal vaginal infections.
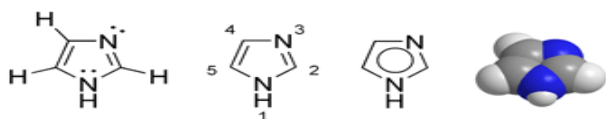
Figure 1: Structure of Imidazole

Due to the great importance of imidazoles in pharmacology and clinical medicine, efforts by analytical chemists have been made toward finding methods for determination of imidazoles through the use of versatile instrumental techniques like spectroscopy spectroscopy[1], polarography[2], packed columns supercritical fluid chromatography by using modified CO_2 with methanol as mobile phase[3], biamperometry[4] spectrophotometry[5], titrimetry[6], voltammetry [7], and LC–MS/MS[8].

Simultaneous determination of MNZ and MIC in pharmaceutical dosage forms have been reported firstly by Erk and Altun (2001) by utilizing ratio spectra derivative spectrophotometry as an quantitative multicomponent analytical technique and RP-HPLC as comparative method, Akay et al., 2002 modified a RP-HPLC method by adding an internal standard sulphamethoxazole (SMX) which is more relevant for use in samples requiring several and complicated extraction steps such as biological samples in order to minimize possible analytical errors associated with sample preparation and improve analytical performance of the method. Salem et al., (2006) developed a method for determination of MNZ, MIC and SMX using proton nuclear magnetic resonance and DMSO-d_6 as a solvent.

In 2009, Meshram et al., developed a new method exploiting high-performance thin-layer chromatographic (HPTLC) for the simultaneous determination of (MNZ) and (MIC) in a preparative gel by using toluene: chloroform: methanol (3.0:2.0:0.6 v/v) respectively on a thin layer chromatographic plates composed of silica gel. More recently, Ashour and Kattan (2010) have developed a gas chromatography method for the simultaneous determination of MNZ and MIC in tablets and ovules quantitatively by using flame ionization detector without the need for sample derivatization.

In this study optimized methods based on HPLC and GC have been compared for simultaneous determination of the active ingredients (MIC and MNZ) in vaginal ovules, and extended for the determination of other imidazole derivatives such as clotrimazole, econazole and ketoconazole, so the novelty in this study arise from its ability to determine a broad range of imidazoles under the same conditions.

MATERIALS AND METHODS

Instrumentation

The assays were performed with an HPLC system consisting of a Perkin Elmer series 200 model pumps and Perkin Elmer series 200 UV/Vis detector. The detector was set at 220 nm. Phenomenex Gemini C_{18} reverse phase column packed with 5 µm dimethyl octadecylsilyl bonded amorphous silica beads (250 mm × 4.6 mm) was used as the stationary phase, and a 7725 Rheodyne injector system with a 20-µl sample loop was used to deliver the sample to the column.

A Clarus 500 gas chromatograph (GC) equipped with a split/splitless injector and a flame ionization detector (FID) from Perkin Elmer Technologies together with a capillary column (15 m × 0.32 mm i.d., 0.25µm film thickness) from Alltech of the AT^{TM}_5ms type(5% phenyl)-95% methyl polysiloxane. Control valve for providing the switch and adjustment of air to FID and 1µL gas-tight syringe (SGE Analytical Science, Australia) were used.

Chemicals

Metronidazole (171.2 g mol^{-1}) and sulphamethoxazole (290.32 g mol^{-1}) were obtained from Fluka Analyticals, miconazole nitrate(479.1 g mol^{-1}), econazole nitrate (444.7 g mol^{-1}) , ketoconazole (531.44 g mol^{-1}) and clotrimazole (344.84 g mol^{-1}) were obtained from Sigma-Aldrich. Ultrapure deionized water and HPLC grade methanol were used for preparation of the mobile phase.

Pharmaceutical preparation

A commercial pharmaceutical preparation, Gyno-D® ovules (manufactured by Sandy Pharm. Ind., Syria), were assayed, lot no. : 23-148. Each ovule contained 500.0 mg metronidazole and 150.0 mg miconazole nitrate.

Standards stock solutions and calibration graphs

HPLC stock solutions of 1 mg ml^{-1} of MNZ and MIC were prepared by dissolving in methanol, then intermediate standard containing 100 µg.ml^{-1} of MNZ and 50 µg.ml^{-1} of MIC was prepared from the stock by dilution with the mobile phase. Five standard solutions were prepared from the intermediate solution with mobile phase by varying the concentration of MNZ in the range of 10-80 µg.ml^{-1} and MIC in the range of 5-40 µg.ml^{-1} each containing of sulfamethoxazole 0.25mg ml^{-1} were prepared. Calibration curve for HPLC analysis was obtained by plotting the average peak area ratio of the drugs to internal standard against the drugs concentration.

While in case of GC higher concentration of stock solution of MIC (4 mg mL^{-1}) and MNZ (8 mg mL^{-1}) were prepared in methanol. Five standard solutions for GC were prepared by dilution of the stock solution with the same solvent calibration graphs in the range of 200-1500 µg.ml^{-1} of MNZ and 100-750 µg.ml^{-1} were constructed, solutions of ketoconazole, clotrimazole and econazole nitrate were prepared with no respect for concentration as its determined qualitatively.

Chromatographic conditions

Separation of the analytEs by HPLC was carried out using reversed phase technique UV detection at 220 nm and sulfamethoxazole as an internal standard. A mixture of methanol //water (40:/60, v/v) was used as a mobile phase. One liter of the mobile phase was prepared, filtered with cellulose acetate filter with the aid of a vacuum and subsequently sonicated for 10 minutes before use. The mobile phase was delivered at a flow rate of 1.0 ml min^{-1}. Twenty micro liters of each solution was injected and chromatograms were recorded.

A 1 µl gas tight syringe was used to deliver a volume of 0.5 µl sample with a split ratio of 1:10. Both the detector and injector temperatures were set at 260°C. Nitrogen was used as carrier gas at a flow rate of 1.5 ml min^{-1}. A temperature gradient program in which the GC column temperature starting from 140°C for 3min, then raised to 180°C at a rate of 40°C min^{-1} and maintained for 2 min, finally raised with a rate of 20°C min^{-1} to a final temperature of 250°C was used.

Analysis of ovule dosage forms

The contents of five ovules were pooled and (give weight used here) exact of equivalent of the content of one ovule was weighed and transferred to a 100ml beaker. A 40 ml aliquot of methanol was added to the sample and mixture was placed on a hot plate. The sample was heated to 100° C with stirring at 500 rpm for 15min. The cold sample was filtered (Whatman1 filter paper) into a 100mL flask and the volume made up to mark with methanol. Subsequent dilutions were carried out with methanol.

Both standards and samples contain 0.25 mg.ml^{-1} SMX as internal standard.
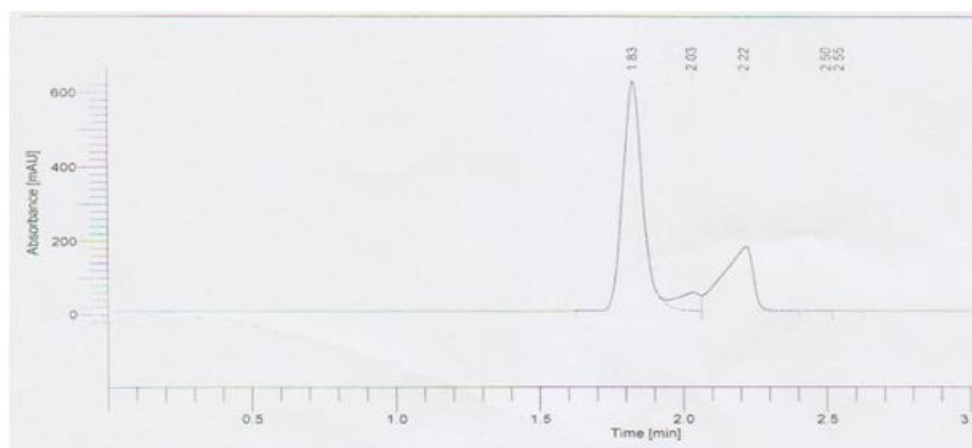
RESULTS

HPLC method

Both MIC and MNZ were found to be more soluble in methanol than other solvents, and as a result it was chosen as the solvent in both chromatographic separation methods.

In the HPLC method, a binary mixture of methanol and water (40:60v/v) was used as the mobile phase as it gave good resolution and sensitivity with minimum tailing for both MNZ and MIC in agreement with the of Akay et al.

Absorbance measurement at 220 nm gave good detector response and flow rate of 1.0 ml min^{-1} was optimized as it provided good peaks separation, with very short analysis time, MIC (t_r: 1.84 min), SMX (t_r: 3.24 min) and MNZ (t_r: 4.05 min) Figure 2. Parameters such as resolution, sensitivity and peak tailing were investigated in order to test system suitability.
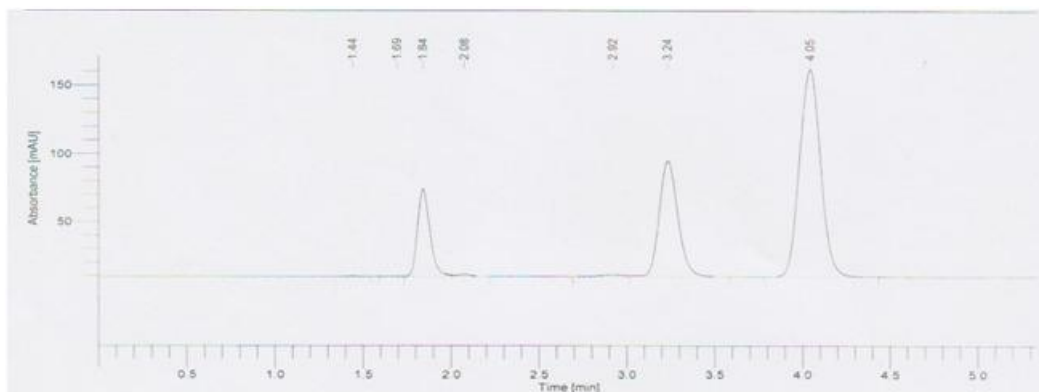
Figure 2: The chromatogram of a solution of miconazole nitrate (MIC) (1.84 min) and metronidazole (MNZ) (4.05 min) with Sulfamethoxazole (SMX) (3.24 min) (25 µg ml^{-1}).

Figure 3: The chromatogram of a solution of Ketoconazole (1.83 min) and Econazole nitrate (2.03 min) with Clotrimazole (2.22 min)

Table 1: System suitability results of the investigated HPLC and GC methods

Method	Resolution	Selectivity	Efficiency	Tailing factor
HPLC	11	2.2	4178 for MNZ 2407 for MIC	1.1 for MNZ 1.3 for MIC
GC	27	6.6	1264 for MNZ 37791 for MIC	1.1 for MNZ 1.3 for MIC

Table 2: Determination of recovery, and relative standard deviation (RSD %) of laboratory prepared mixtures of metronidazol and miconazole nitrate by RP-HPLC

Nominal concentration µg ml^{-1}	Assayed concentration (µg ml^{-1}) Mean ± S.D.	RSD%	Recovery
Metronidazole (MNZ)			
10	9.3 ± 0.13	1.42	93
20	20.5 ± 0.4	2.00	102.7
40	40.95 ± 0.82	2.16	102.4
60	63.73 ± 0.99	1.56	106.2
80	76.8 ± 1.65	2.15	96
Miconazole (MIC)			
5	4.92 ± 0.04	0.90	98.5
10	10.69 ± 0.32	3.02	106.9
20	20.98 ± 0.59	2.82	104.9
30	32.25 ± 0.73	2.26	107.5
40	37.7 ± 0.93	2.47	94.3

The acceptance limits were Capacity factor, ќ >2, Peak resolution, R (>2) between MNZ and MIC, Number of theoretical plate, N (> 2000) for MNZ and MIC, USP, tailing factors, T (< 2). Resolution and selectivity factors for this system were found to be 11 and 2.2, respectively. Tailing factors were obtained as 1.1 for MNZ and 1.3 for MIC (Table 2). Qualitative analysis of other imidazoles (clotrimazole, ketoconazole and econazole nitrate) in this study under the same conditions also gave short analysis times: ketoconazole (t_r: 1.83 min), econazole (t_r: 2.03 min) and clotrimazole (t_r: 2.22 min) but not highly resolved (figure 3).

According to results shown in Table 1, the developed method satisfies the acceptance criteria mentioned above within acceptable limits

Linearity

Linearity was obtained for MNZ in the concentration range of 10 – 80 µg ml^{-1} and MIC in the concentration range 5 – 40 µg ml^{-1} .The regression coefficients were 0.9946 for MNZ and 0.9904 for MIC, indicating good linearity (Figure 4) the calibration curves could be represented by the linear regression equations:

$Y_{MIC} = 0.0204X + 0.008$

$Y_{MNZ} = 0.0421X + 0.035$
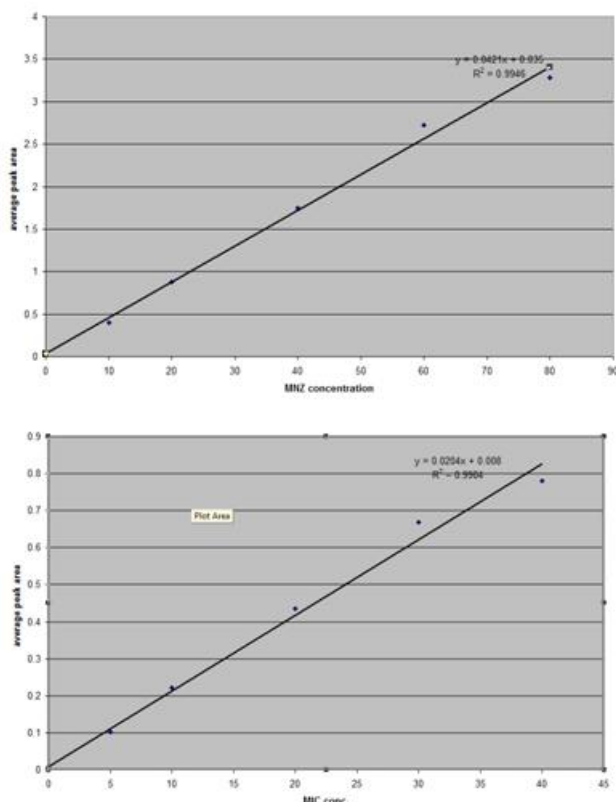
Figure 4: Linear regression curves of MIC and MNZ in HPLC

Limits of detection and determination

The limits of detection and determination represent indicators for method sensitivity were calculated as defined by IUPAC ,LOD (k=3) = $k \times$ y /m and LOQ (k=10) =

$k \times$ y /m criterions, respectively, where y , is the standard deviation of the peak area ratios (three injections) of the sample and m is the slope of the corresponding calibration curve. In the present study, LOD were found to be 0.93 and 0.45 µg/ml and the LOQ values for MIC and MNZ were 3.1 and 1.5 µg/ml, respectively.

### Assay precision and recovery

To establish reliability of the method, 5 standards with different concentrations were assayed (n=3) (Table 2), the obtained data indicated considerable level of reproducibility for the method during one analytical run.

The recoveries of these two active ingredients in pharmaceutical dosage form are obtained from spiked samples. In this study the investigated sample was supposed to contain 40 µg/ml of MNZ and 12 µg/ml of MIC and it was found that recoveries were 94.9% and 92.9% for MNZ and MIC respectively; the relative standard deviations were found to be 0.84% for MNZ and 0.26% for MIC, indicating very good repeatability of the proposed method.

GC method

The chromatographic separations were performed on an AT^{TM}_5ms capillary column which gave a good separation of both active ingredients. Sharp and symmetrical peaks were achieved by programming the oven temperature at 140°C for 3min, then programmed to 180°C at a rate 40°C min^{-1} and maintained for 2 min, finally was raised with a rate of 20°C min^{-1} to a final temperature of 250°C. The retention times of MNZ and MIZ were about 1.57 and 9.72min, respectively (Fig. 5). A flow rate value of 1.5 ml min^{-1} was chosen for the optimum separation of the compounds, as at this value the analyte peaks were well defined and resolved.
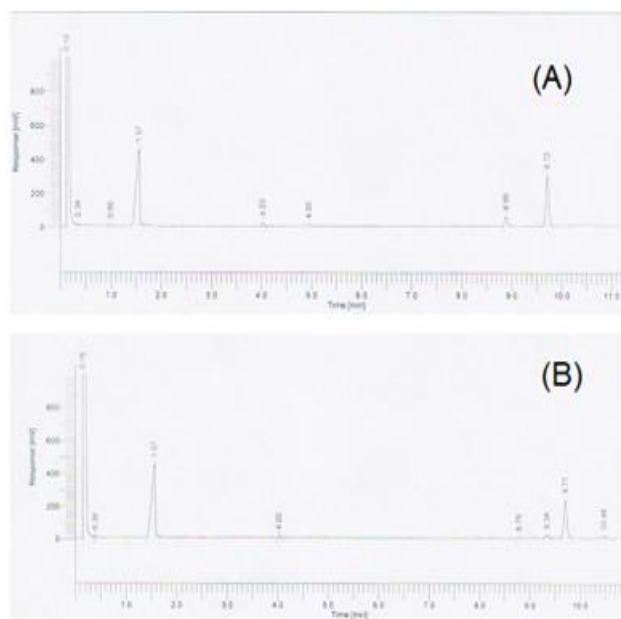

Figure 5 Gas chromatograms from (A) mixture of standard solution of MNZ and MIC and (B) sample solution prepared from the ovules.

The same parameters of resolution, sensitivity and peak tailing have been investigated to test system suitability through selection of representative chromatogram of freshly prepared standard solutions of MNZ and MIC.

Resolution and selectivity factors for this system were found 27 and 6.6 and Tailing factors 1.1 for MNZ and 1.3 for MIC respectively.

Qualitative analysis of other imidazoles (clotrimazole, ketoconazole and econazole) in this study under the same conditions has also gave short analysis time: clotrimazole (t_r: 9.00 min), econazole (t_r: 9.29 min) as shown in (Fig. 6) with a resolution of 2.9
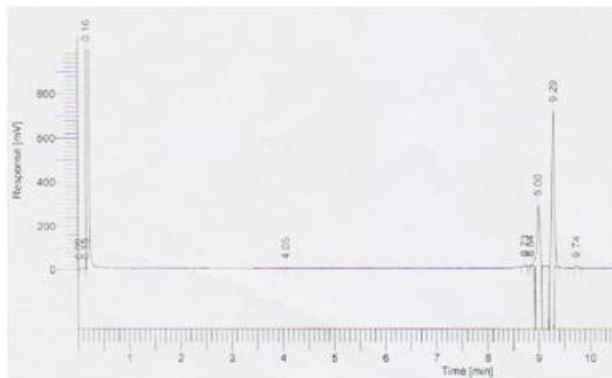
Figure 6 Typical gas chromatogram of clotrimazole (t_r : 9.00 min) and econazole (t_r :9.29 min) at flow rate 1.5 ml min^{-1}.

Linearity

Under the optimum conditions for GC-FID, the calibration curves showed a linear range in the concentration range of 200 – 1500 µg ml^{-1} and 100 – 750 µg ml^{-1} for MNZ and MIC, respectively (Table 4). The regression curve was calculated by the excel program and the correlation coefficients were 0.9762 for MNZ and 0.9855 for MIC, indicating relatively good linearity. The calibration curves could be represented by the linear regression equations:

$Y_{MIC} = 7112.2X + 7158$

$Y_{MNZ} = 6505.9X + 1173$

Limits of detection and determination

The minimum level at which the investigated compounds can be reliably detected (limit of detection, LOD) and quantified (limit of quantitation, LOQ) were determined experimentally. LOD was expressed as the concentration of drug that generated a response to three times of the signal to-noise (S/N) ratio, and LOQ was 10 times of the S/N ratio. The LOD of MNZ and MIC attained as defined by IUPAC, LOD (k=3) $= k \times Y/m$ (where m is the slope of the calibration curve and Y is the standard deviation of the intercept), was found to be 3.0 and 3.6 µg ml^{-1}, respectively. The LOQ was also attained according to the IUPAC definition, LOQ (k=10) $= k \times Y/m$, and was found to be 10 and 12 µg ml^{-1}, respectively.

Assay precision and recovery

The precision and accuracy of the method were evaluated by analysis of standard solutions of the mixture (n = 4). The standard deviation, relative standard deviation, and recovery of different amounts tested were determined from the calibration curve. As shown in Table 3, the relative standard deviation (R.S.D.) was about 5%.

The proposed method was applied for analysis of ovule samples. The results obtained by the proposed method are in satisfactory agreement. The analytical results are summarized in Table 4.

Table 3: Accuracy and precision for the determination of miconazole nitrate (MIC) and metronidazole (MNZ) by gas chromatography

Nominal concentration µg ml^{-1}	Assayed concentration (µg ml^{-1}) Mean ± S.D.	RSD%	Recovery
Metronidazole			
200	230 ± 10.3	4.47	115
500	427 ± 16.4	3.85	85
750	604 ± 34	4.55	80.5
1000	977 ± 40.8	4.17	97.7
1500	1608 ± 73	4.53	107.2
Miconazole			
100	125 ± 4.1	3.2	125
250	257.6 ± 7.1	2.84	103
375	375 ± 12	3.26	98.6
500	553 ± 22.3	4.03	110
750	710 ± 23.8	3.18	94.6

Table 4: Analysis of miconazole nitrate and metronidazole in pharmaceutical dosage forms

R.S.D %	Recovery	Found (mg)	Label claim (mg)	Gyno- D Preparation
2.27	83	414.5	500	Metronidazole
3.36	97	145.5	150	Miconazole

CONCLUSION

The results obtained in this study with respect to the determination of MNZ and MIC by HPLC has shown that it has great deal of agreement with work of Akay et al. (2002) in terms of convenience and reproducibility for analysis of these drugs in ovule dosage forms. However, in this study a shorter analysis time (</5 min) compared to /6 min obtained by Akay et al. The proposed HPLC method was validated by evaluation of the LOD, LOQ values, relative standard deviation (RSD %), recovery, resolution, selectivity, tailing and capacity factors for this technique.

Tailing factors of both methods are almost the same (1.1 for MNZ and 1.3 for MIC) whereas resolution and selectivity is much better than the reported HPLC method by a factor of 5 and 2 respectively. The proposed method is also found to have nearly the same precision with that

of reported method since the RSD values of standard solutions were found to be 2.16 and 3.02% for MNZ and MIC, respectively with nearly the same good linearity (> 0.99) for both drugs, also in terms of LOD and LOQ the proposed method were higher than the reported method. This method has proved to have broader range of applicability than reported method of Akay in terms of qualitative determination of other imidazoles (ketoconazole, econazole and clotrimazole) within short analysis time (< 3 min) and this gives greater scope for determination of wide range of imidazoles simultaneously.

Determination of MNZ and MIC by GC method has shown some points of agreement with works of Ashour and Kattan (2009) especially in terms of sensitivity and specificity for analysis of these drugs in ovule dosage forms in shorter analysis time (</10 min) than that obtained in the work of Ashour and Kattan (13 min.). Assay parameters used in this study improved resolution and selectivity. Limits of detection and quantitation (LOD and LOQ) in both methods are almost the same (3.1 µg/ml for MNZ and 3.6 µg/ml for MIC) whereas the precision and accuracy obtained by this method was less than that obtained by the reported method and the expected reason for this imprecision is might be due to the absence of internal standard which was not used in this method.

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