Extractional spectrophotometric analysis of metronidazole, tinidazole, ornidazole and secnidazole bases through acid-dye complexation using bromothymol blue dye

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Abstract: An easy, precise and valid extractional-spectrophotometric technique is described for the assessment of metronidazole (MNZ), tinidazole (TNZ), ornidazole (ONZ) and secnidazole (SNZ) in pure state and in their pharmaceutical formulations. The technique includes first the reduction of above cited drugs using HCl and zinc powder, then the formation of intense yellow colored ion-association complex species (1:3 drug/dye) using bromothymol blue (BTB) in a buffered aqueous acidic medium at pH 3–3.50. The colored products are extracted into dichloromethane and quantitatively determined at 416–420 nm. The experimental operating factors influencing the ion-pairs development were studied and optimized to obtain the maximum color intensity. The Beer plots are obeyed in the concentration ranges 2.50–22.50, 2.50–30, 7.50–35 and 5–30 $\mu g ml^{-1}$ for MNZ, TNZ, ONZ and SNZ, respectively, with correlation coefficients not less than 0.9995. The proposed technique is recommended for the routine quality control analysis of the investigated drugs in commercial tablets with no observed interference from common pharmaceutical adjuvants. Results of such analysis were statistically validated and through recovery studies, showing excellent agreement with those achieved by the reported techniques.

Keywords*:* Metronidazole, tinidazole, ornidazole and secnidazole; Extractive spectrophotometry; Bromothymol blue; Pharmaceutical formulations.

INTRODUCTION

Nitroimidazoles have been introduced as one of the most recognizable anti-microbial agents, regarding to their remarkable potency and relatively low toxicity. The members of this group had extremely useful clinical activity against anaerobic pathogens that include both gram-negative and gram-positive bacteria, in addition to wide range of protozoans (Sweetman, 2009). Metronidazole, tinidazole , ornidazole and secnidazole are members of this group.

The significant value of such drugs promotes the introduction of many upto date reported techniques, which are developed for their determination either alone or in combination with other drugs within various pharmaceutical preparations and human biological fluids. Such techniques include, (Amer *et al*., 1989), polarography (El-Sayed 1997; Joshi *et al*., 1996; Sankar *et al*., 1989; Lichtig *et al*., 1996), voltammetry (Bartlett *et al*., 2005; Wang *et al*., 2006; Radi *et al*., 2000), Potentiometry (Visan *et al*., 1989), electrophoresis (Jin *et al*., 2000; Alnajjar *et al*., 2007; See *et al*., 2009), optical-fibre chemical sensor system (Liu *et al*., 1998), gas-chromatography (Liu *et al*., 2008; Bhatia *et al*., 1984), thin layer chromatography (Meshram *et al*., 2008), high performance thin layer chromatography (Gattavecchia *et al*., 1981; Vaidya *et al*., 2007; Ranjane *et al*., 2010), high and ultra performance liquid chromatography (Sagan *et al*., 2005; do Nascimento *et al*., 2005; Li *et al*., 2007; Ranjane *et al*., 2008; Singh *et al*., 2009; Silva *et al*., 2009; Maher *et al*., 2009; Liu *et al*., 2009), packed column supercritical fluid chromatography (Bhoir *et al*., 1997; Patel *et al*., 1998), chiral liquid chromatography (Wang *et al*., 2008), flow injection analysis (Mohamed *et al*., 1996; Lv *et al*., 2003), Chemiluminescence (David *et al*., 2000), spectrofluorimetry (Wang *et al*., 2004), proton nuclear magnetic resonance spectroscopy (Salem *et al*., 2006), spectrophotometry (Nagaraja *et al*., 2002; Saffaj *et al*., 2006; Mohamed *et al*., 2007; Mubeen *et al*., 2009) and official techniques (British Pharmacopoeia, 2007; The United States Pharmacopoeia, 2007).

Throughout the literature survey, ONZ and SNZ are not yet official in neither the British nor United States Pharmacopoeias, thus, there is a constant need for a simple, precise and economical analytical method that can be implied for the routine quality control analysis of such drugs. In addition, most of the reported spectro-photometric techniques suffer from drawbacks like showing low sensitivity as in (Mubeen *et al*., 2009) which could not show linearity below 16 $\mu g\ ml^{-1}$ for ONZ-Schiff base formation using Paradimethylaminobenzaldehyde (PDAB). Narrow range of determination (17.50 $\mu g\ ml^{-1}$) was also seen in (Nagaraja *et al*., 2002) using *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA).

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Furthermore, some methods involved either heating or cooling for the reaction to complete which by extension can lengthen the time needed for the analysis, other methods needed sophisticated precautions to be applied; such drawbacks were observed in (Mubeen *et al*., 2009) which required heating at 80 °C for 5 min or 37 °C for 15 min for the colored compound to develop, also, in (Nagaraja *et al*., 2002) and (Saffaj *et al*., 2006) both demanded cooling in ice nearly throughout the whole procedure. Finally, tedious pre-treatments and multi-reagents were required to be applied in either (Nagaraja *et al*., 2002) or (Saffaj *et al*., 2006) for first diazotization of the reduced drug forms prior their coupling with NEDA or □-naphtol, respectively, which in turn provoked the need for consequence removal of the excess interfering sodium nitrite (diazotizing agent) using the non-interfering 2% sulphamic acid solution.

On the light of these observations mentioned above, we introduce in this proposed communication, a modest trial to obtain an easy, valid, economic and precise extractional spectrophotometric technique for the determination of some 5-nitroimidazole drugs such as MNZ, TNZ, ONZ and SNZ in bulk state and in various commercial dosage forms available in the local markets. The concept of the introduced technique is the formation of dichloromethane extractable ion-association complexes between nitroimidazoles reduced forms and the laboratory commonly available acid-dye, BTB, in an acidic buffered medium. The different experimental conditions were thoroughly investigated and optimized, also the stiochiometric relationship was established. The introduced technique is easy, precise, reliable, economic and provides advantage over other reported spectrophotometric techniques.

MATERIALS AND METHODS

Chemicals and reagents

All materials and chemicals were of pharmaceutical or analytical class, utilized directly as received without any additional modification. The water used was constantly doubly glass distilled and de-ionized.

Absolute alcohol and HPLC grade methylene chloride (Sigma-Aldrich, USA), 0.10% (w/v) BTB (BDH, Pool, UK) was obtained through transferring the precise weighed quantity of 0.1gm to 1.50 ml of 0.10 M caustic soda, 20 ml of ethyl alcohol (96%) and sufficient water producing a 100 ml stock solution.

Britton-Robinson buffer solution of required pH was obtained through certain combination of boracic, phosphoric and ethylic acids with caustic soda (Britton *et al*., 1931).

MNZ (99.50% purity) and ONZ (99.68% purity) were gift samples from (Pharonia, Alexandria, Egypt), while TNZ (>99% purity) and SNZ (99.20% purity) were generously supplied by (MUP, Abo-sultan, Egypt) and (EPICO, 10th of Ramadan, Egypt), respectively. Reference solutions of 0.50 mg ml^{-1}, were obtained by dissolved each investigated drug in the minimal quantity of absolute methyl alcohol, then dilute to the volume with water. The stock solutions were stable for at least 72 h upon storage in a light protected cool (< 15°C) place.

Amrizole® tablets (Amriya, Alexandria, Egypt) and Flagyl® intravenous infusion (Amriya, Alexandria, Egypt) declared to comprise 500 mg MNZ per tablet and 500 mg MNZ per 100ml, respectively. Tibezole® tablets (Pharonia, Alexandria, Egypt) claimed to include 500 mg ONZ per tablet. Fasigyn® tablets (EPICO, 10th of Ramadan, Egypt) declared to comprise 500 mg SNZ per tablet. Finally, Protozole® tablets (MUP, Abo-sultan, Egypt) claimed to include 500 mg TNZ per tablet.

Instrumentation and equipments

A dual-ray Shimadzu (Tokyo, Japan) UV-1601 PC UV-vis spectrophotometer (10 mm optical path length matched quarts cell, 50 nm min^{-1} scan speed and 3nm fixed slit width) equipped with UVPC personal spectroscopy software version 3.70 (Shimadzu), applied for spectrophotometric determinations through a wavelength domain from 190 to 800 nm. A Cole Parmer pH-meter was equipped for pH adjustement. A side-bench WTB-Binder 1505 oven 300°C (Tuttlingen, Germany) used for controlling the temperature during investigating the temperatures influence on the stability of the ion-pair complexes. Hamilton automatic pipettes (50-200 and 200-1000 µl), glass micropipettes and burettes were used to deliver a wide range volumes.

Procedures

Preparation of standard solutions and calibration graphs

Ten milliliters of 1 N hydrochloric acid and 2 g of zinc dust were added to 50 ml of MNZ, TNZ, ONZ or SNZ (0.50 mg ml^{-1}) standard solutions in a 100 ml capacity Erlenmyer flask. With an occasional shaking, every 10 min, the flask was permitted to set aside for 30 min within ambient temperature (23±1°C). The reaction jumble was then filtered through a moisten Whatman Grade No.41 Quantitative filter paper (particle retention 20-25 µm) into a 100 ml capacity volumetric flask, the filter paper was rinsed twice with water and then the flask was filled to the volume with water.

An addition sequence of 5 ml Britton-Robinson buffer of pH 3 (MNZ) or pH 3.50 (TNZ, ONZ or SNZ), 1 ml of the reduced drug (0.25 mg ml^{-1}) and 5 ml of 0.10% (w/v) of BTB, were transferred to a 60 ml capacity separating funnel. After a standing time of 5 min, the intense yellow-colored ion-association complexes were extracted with 3 ml triplicate portions of dichloromethane, with a shaking time of 1 min per each portion. The organic layer

containing these colored complexes, was brought to be dried over anhydrous sodium sulfate then the filtrate was then collected in 10 ml capacity volumetric flask and filled to the volume with dichloromethane. The organic layer was analyzed at 416-420 nm for all drugs under investigation, against a reagent blank, obtained in similar pattern despite of the drugs addendum. Standard calibration curves were plotted between the absorbance values as a function of their corresponding concentrations.

Preparation and assay of tablet formulations

The total content of ten tablets of MNZ, TNZ, ONZ or SNZ were exactly weighed and grounded well to fine powder. The powder amounting to 100 mg of each drug was taken and solubilized in least amount of absolute methanol. After a 30 min of well shaking, the obtained mixtures were then filtered through a Whatman Grade No.41 Quantitative filter paper (particle retention 20-25 µm) and rinsed with water. Both the washings of drugs and the filtrate were gathered in 100 ml capacity volumetric flask and completed to the mark with water, then the comprehensive steps previously discussed was followed over the calibration concentration range of each drug.

Preparation and assay of intravenous infusion

Transfer an amount of the infusion containing 100 mg of MNZ into 100 ml capacity volumetric flask, 10 ml 0.10 M hydrochloric acid was added and the completed to mark with water. Then after, the comprehensive steps cited above were followed over the calibration range of MNZ.

RESULTS

Reduction of the 5-nitro group placed on the imidazole ring possessed by the compounds under investigation, will increase the electron density around their tertiary heterocyclic amino group, thus easies its protonation at a suitable acidic pH, and association with the negatively charged dye, BTB. The yellow color of the ion-pairs formed are extractable in methylene chloride. Triple extraction is of a great necessity for a complete and quantitative recovery of all ion-association complexes into the organic layer. The extractive equilibria can be illustrated through the following equation:

$$\underset{\text{Colorless}}{MH^{+}_{(aq)}} + \underset{\text{Yellow}(\lambda_{max}\ 392\ nm)}{Y^{-}} \leftrightarrow MH^{+}Y^{-}_{(aq)} \leftrightarrow \underset{\text{Intense-yellow}\ (\lambda_{max}\ 416\text{-}420\ nm)}{MH^{+}Y^{-}_{(org)}}$$

Where MH^{+} is the protonated amino drugs and Y^{-} is the BTB anion form, the subscripted abbreviations *(org)* and *(aq)*, respectively stands to the organic and aqueous layers.

Spectral features

The spectral features of the dye, investigated drugs and drug-dye association complexes extracted in dichloromethane, all are illustrated in fig. 1. The complexes show absorbance maxima at 416, 418, 419 and 420 nm for SNZ, TNZ, MNZ and ONZ, respectively, against a reagent blank. BTB dye alone does not exhibit any interfering absorption around 420 nm under the same experimental conditions.

Optimal organic solvent scouting

A variety of water-immiscible extracting solvents were tested for optimal ion-associates recovery. Solvents like Toluene and benzene are not capable of satisfactory recoveries, while as carbon tetrachloride, ethylene chloride and dichloromethane proved useful for the quantitative recovery of those ion-association complexes. Dichloromethane was the most perfect solvent that yields the ultimate absorbance value and minimal blank readability. In addition, the high molar absorptivity values presented in table 2 for MNZ,TNZ, ONZ and SNZ-BTB ion-association complexes in dichloromethane, enforce

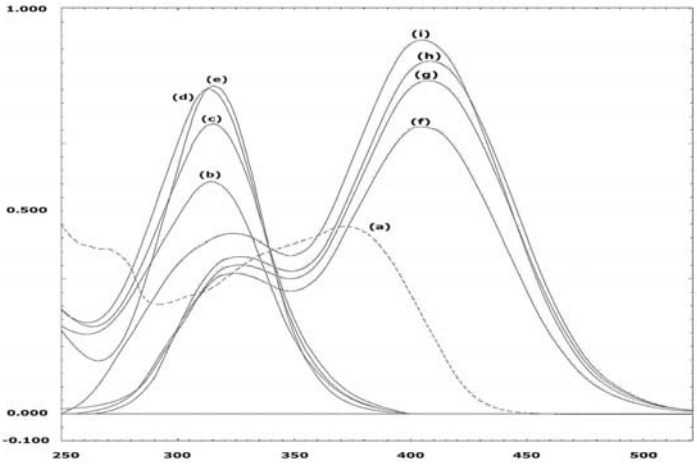


Fig. 1: Absorption spectrum of 25 µg ml^{-1} reduced nitroimidazoles and their-dye complexes extracted in 10 ml dichloromethane; (a) 0.10% (w/v) bromothymol blue dye, (b) metronidazole, (c) ornidazole (d) secnidazole, (e) tinidazole, (f) metronidazole-bromothymol blue complex, (g) ornidazole-bromothymol blue complex (h) secnidazole-bromothymol blue complex and (i) tinidazole-bromothymol blue complex.

the suitability of using dichloromethane as an extracting media, showing color stability for 72 h.

Influence of pH

The pH influence on the ion-pairs development was established, in the presence of 10, 12.50, 15 and 22.50 µg ml^{-1} of MNZ, TNZ, ONZ or SNZ, respectively, and through using different buffer solutions such as, Walpole (NaOAc-HCl), Clark and Lubs (KCl–HCl), (Potassium hydrogen phthalate-HCl), Sorensen (glycine-HCl), phosphate, Britton-Robinson and Mcilvaine buffer solutions, over the pH range 2-8. Britton-Robinson buffer solution shows the maximum absorbance values at pH 3 and 3.50 for MNZ and (TNZ, ONZ or SNZ), respectively, as indicated in fig. 2. As being observed beyond pH 4, sever absorption decline was a result of intensely colored blank.

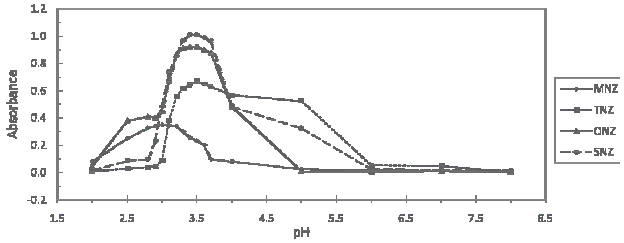


Fig. 2: Influence of pH on the acid-dye complex development of (10, 12.50, 15 and 22.50 µg ml^{-1}) of reduced metronidazole, tinidazole, orindazole and secnidazole, respectively, with BTB dye.

Influence of timing

The time influence on the ion-pairs development was carefully studied and presented in fig. 3. From these figures it is clear that, the total ion-pairs development needs 10 min for MNZ, TNZ and ONZ, while only 5 min for SNZ, before extraction with methylene chloride at ambient temperature (23±1°C). Also, a shaking time for 0.50-3 min reveals a maximum absorbance at 1 min shaking time.

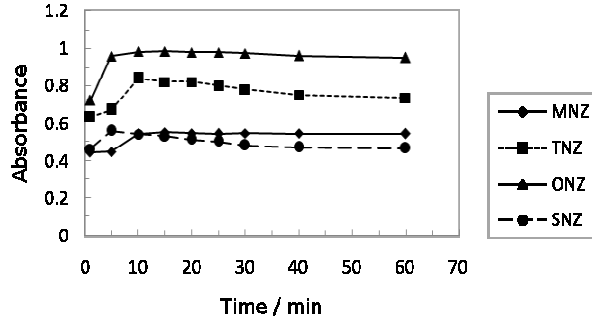


Fig. 3: Influence of standing time on the acid-dye complexes formation of (12.50, 12.50, 17.50 and 22.50 µg ml^{-1}) of reduced metronidazole, tinidazole, orindazole and secnidazole, respectively, with BTB dye.

Influence of aqueous to organic layers phase ratio and sequence of mixing

Triple extraction with 10 ml of dichloromethane (3 ml per each) and 1 min shaking time, produces much superior reproducible absorbance than 10 ml of dichloromethane at two times or one time for 1 min shaking. Also, the most favored addition order is buffer-drug-dye-dichloro-methane and the color remains stable for at least 72 h at this sequence.

Influence of temperature

The temperatures influence on the stability of the ion-pair complexes was studied within a temperature range 15-50°C. The complexes were placed in pre-heated side-bench oven, which is adjusted at a specific temperature, for 7 min prior each analyses. The Absorbance-temperature curves constructed at λ_{max} and represented in fig. 4 show a general increase in the absorbance as the temperature rises, due to the volatility of dichloromethane, which then reached its maxima at 35°C. The absorbance did not show significant change above this temperature. Therefore, it is concluded that the complexes were stable at high temperatures.

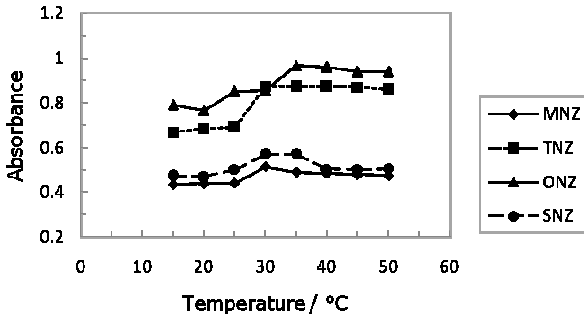


Fig. 4: Influence of temperature on full acid-dye complexes formation of (10, 12.50, 15 and 20 µg ml^{-1}) of reduced metronidazole, tinidazole, orindazole and secnidazole, respectively, with BTB dye.

Influence of dye and buffer concentration (v/v)

The buffer and counter-ion concentration influence on the development and extraction of the colored ion-pair complexes was investigated using constant MNZ, TNZ, ONZ and SNZ concentrations, while the volumes of BTB or buffer was varied from 1-9 ml of their stock solutions. Figs. 5 and 6 show that the absorbance of the recovered ion-pairs was raised by elevating the dye or buffer volumes till 5 ml of both, for MNZ, TNZ, ONZ and SNZ. Further increase in the dye or buffer concentration did not exhibit significant absorbance raise, rather than increasing the formation of an emulsion, which subsequently, prolongs the time needed for clear separation of the two phases. That is why 5 ml, of both dye and buffer stock solutions, is the most suitable volume to achieve reproducibility, reduced emulsion formation and minimized blank absorbance.

Pharmaceutical interference

The extent of potential interference of different excipients and tablet diluents within the assay was studied. Different pharmaceutical additives, that often escort the invest-tigated drugs in different pharmaceutical formulations, were studied and tabulated in table 1. Benzyl alcohol bacteriostatic preservative for the parental preparation and tablet fillers such as talc powder, starch, lactose, glucose, hydroxyl propyl cellulose and magnesium stearate were used. It was seen that the above additives at levels as high as 200-fold excess performed on laboratory prepared synthetic mixtures, posse no significant interference within the absorption spectra of drugs under investigation.

Stoichiometry of the reaction

The constitution of investigated ion-pair associations was settled by Job's technique of continuous variation using changing dye and drugs concentrations represented at fig. 7. In all drug cases the graphs reached peak value at 0.30 stoichiometric mole fraction, indicating the formation of [drug]:[BTB] ion-pairs at a stoichiometric ratios of 1:3, through an electrostatic attraction between the positively protonated reduced MNZ^{+3}, TNZ^{+3}, ONZ^{+3}, SNZ^{+3} and BTB^{-} anions as shown in the scheme 1.

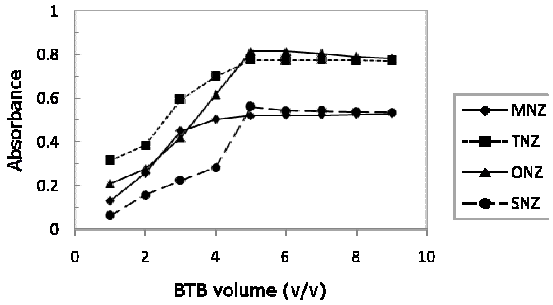


Fig. 5: Influence of dye concentration (v/v) on the acid-dye complexes formation of (15,12.50,15 and 20 µg ml^{-1}) of reduced metronidazole, tinidazole, orindazole and secnidazole, respectively.

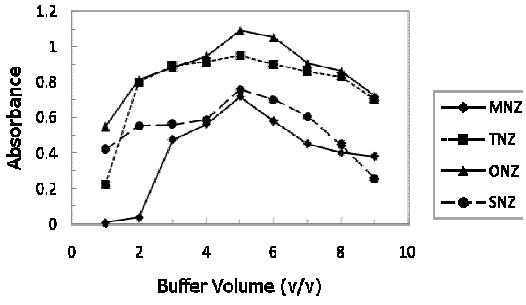
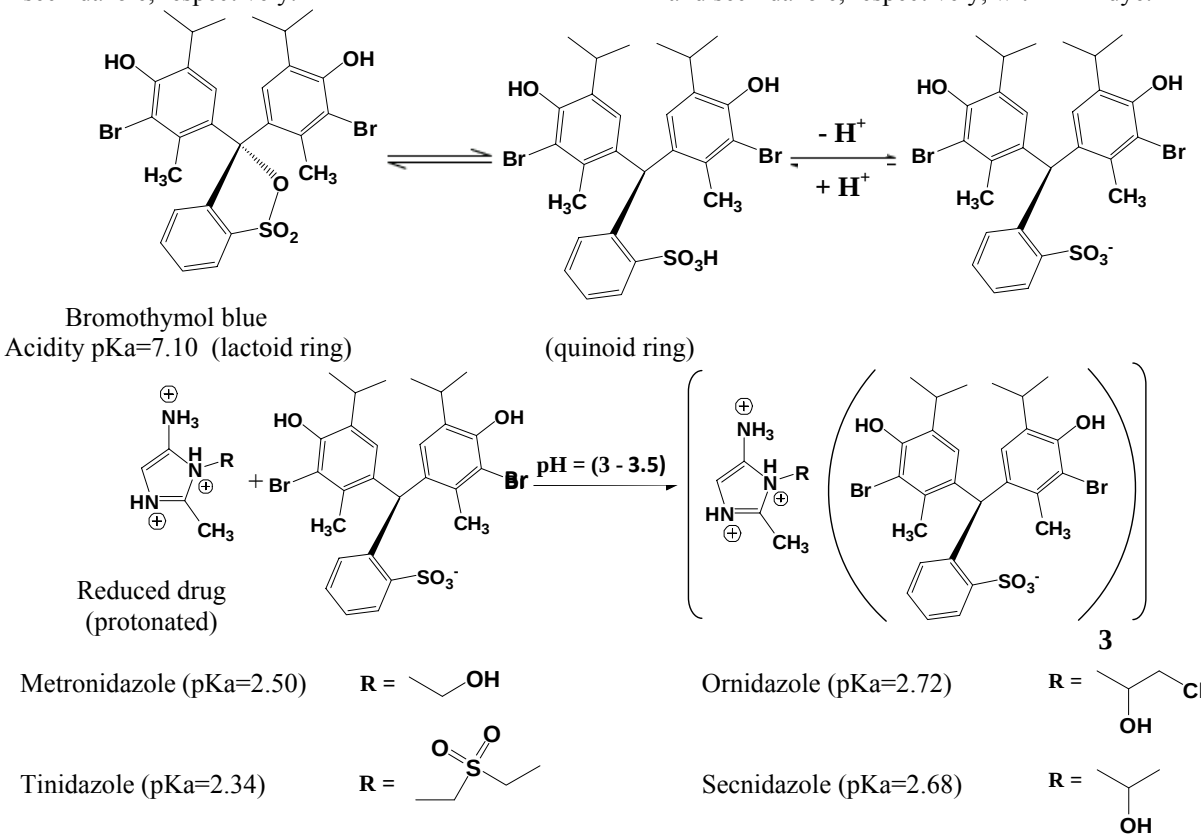


Fig. 6: Influence of buffer concentration (v/v) on the acid-dye complexes formation of (15, 12.50, 15 and 20 µg ml^{-1}) of reduced metronidazole, tinidazole, orindazole and secnidazole, respectively, with BTB dye.



Scheme 1. Suggested mechanism for the formation of reduced MNZ, TNZ, SNZ and ONZ – BTB (1:3) ion-pair complexes.

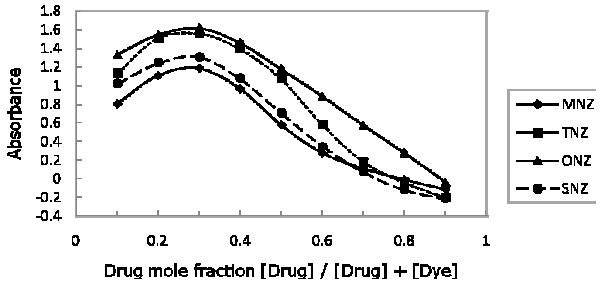


Fig. 7: Continuous variation plots of reduced metronidazole, tinidazole, orindazole and secnidazole – bromothymol blue complex systems in dichloromethane (1×10^{-3} M).

Conditional stability constants (K_f) of the Ion-Pair complexes

The conditional stability constants (K_f) of the ion-pair associates for the reduced MNZ,TNZ, ONZ and SNZ under the experimental conditions described above, were obtained from the continuous variation data using the cited equation (Britton, 1952):

$$K_f = \frac{A/A_m}{[1 - A/A_m]^{n+2} C_M (n)^n}$$

Where *A* and *Am* are the observed maximum absorbance and the absorbance value when all the drug present is associated, respectively. C_M is the molar concentration of drug at the maximum absorbance and *n* is the stoichiometric constant with which the dye complexes with drug. Also the free energy changes *(ΔG)* were calculated using the cited equation (Elshanawane *et al*., 2006):

$$\Delta G = -2.303RT\text{Log}K_f$$

Where, *R* is the universal gas constant (8.31 J), *T* is the absolute temperature (273+25°C), K_f is conditional stability constants. The *log* K_f values for reduced

Table 1: Effect of pharmaceutical excipients on the analysis of metronidazole, tinidazole, orindazole and secnidazole

Excipients	Excipient added	% Recovery of the drug ± % R.S.D. [a]			
		Metronidazole	Tinidazole	Ornidazole	Secnidazole
Talc powder	40 mg	98.80 ± 0.85	99.03 ± 0.66	98.33 ± 0.59	98.42 ±0.97
Starch	40 mg	101.20 ± 0.65	101.31 ± 0.65	100.42 ± 0.97	100.73 ± 0.98
Lactose	40 mg	100.30 ± 0.70	99.82 ± 0.66	99.80 ± 0.99	100.16 ± 0.89
Glucose	40 mg	98.91 ± 0.93	100.47 ± 0.53	100.21 ± 1.01	100.14 ± 0.60
Magnesium octadecanoate	40 mg	102.42 ± 0.52	101.54 ± 0.95	102.19 ± 0.88	102.66 ± 0.76
Hydroxyl propyl cellulose	40 mg	99.80 ± 1.01	99.79 ± 0.94	99.46 ± 0.80	98.82 ± 0.86
Benzyl alcohol	40 ml	100.43 ± 1.10	–	–	–
Mixture[b]		103.62 ± 0.86	97.61 ± 1.33	101.11 ± 0.79	100.21 ± 1.38
% R.S.D.		1.45	1.36	1.08	1.39

20 µg ml^{-1} of each drug is taken.
[a] Average of five determinations.
[b] 5 mg of each of the excipients (not including benzyl alcohol) in the above columns.

Table 2: Analytical parameters and regression characteristic for the microanalysis of metronidazole, tinidazole, orindazole and secnidazole, using the proposed technique

Parameter	Drug			
	Metronidazole	Tinidazole	Ornidazole	Secnidazole
λ max (nm)	419	418	420	416
Beer's law concentration limit (µg ml^{-1})	2.50-22.50	2.50-30	7.50-35	5-30
Molar absorpitivity *(ε)* (L mol^{-1} cm^{-1})	4.34×10^3	$8.50\ x\ 10^3$	1.13×10^4	7.76×10^3
Sandell's sensitivity (g cm^{-2})	3.11×10^{-5}	4.17×10^{-5}	5.52×10^{-5}	4.60×10^{-5}
Slope (b)	3.15×10^{-2}	4.56×10^{-2}	5.14×10^{-2}	4.66×10^{-2}
S.D. of slope	5.09×10^{-4}	7.13×10^{-4}	4.15×10^{-4}	6.60×10^{-4}
% R.S.D. of slope	4.36×10^{-3}	4.28×10^{-3}	5.20×10^{-3}	3.77×10^{-3}
Confidence limit of the slope at $P \leq 0.05$	3.11×10^{-2} – 3.19×10^{-2}	4.60×10^{-2} – 4.72×10^{-2}	5.08×10^{-2} – 5.20×10^{-2}	4.61×10^{-2} – 4.71×10^{-2}
Intercept (a)	-3.20×10^{-3}	-5.66×10^{-2}	3.82×10^{-2}	-1.50×10^{-3}
S.D. of intercept	9.65×10^{-3}	1.35×10^{-2}	7.29×10^{-3}	1.32×10^{-2}
% R.S.D. of intercept	6.03×10^{-2}	8.12×10^{-2}	9.13×10^{-2}	7.54×10^{-2}
Confidence limit of the intercept at $P \leq 0.05$	(-2.40×10^{-3}) – 8.80×10^{-3}	4.58×10^{-2} – 6.74×10^{-2}	2.72×10^{-2} – 4.92×10^{-2}	(-9.10×10^{-3}) – 1.21×10^{-2}
Correlation coefficient (r)(n = 6)	0.9995	0.9995	0.9995	0.9996
LOD (µg ml−1)	5.33×10^{-2}	5.16×10^{-2}	5.01×10^{-2}	4.67×10^{-2}
LOQ (µg ml−1)	1.61×10^{-1}	1.56×10^{-1}	1.52×10^{-1}	1.42×10^{-1}

MNZ,TNZ, ONZ and SNZ ion-associates are 6.36 ± 0.32, 6.59 ± 0.50, 6.97 ± 0.29 and 6.69 ± 0.35, respectively. While, the *ΔG* values are -36268, -37583, -39782 and -38165, respectively; thus, indicating a spontaneous and stabile reaction nature.

DISCUSSION

Linearity and Beer's law obedience

Guided by the optimum operational conditions previously illustrated, standard calibration graphs for MNZ, TNZ, ONZ and SNZ were constructed by plotting the absorbance, against concentration. The linearity limits, regression equations, Sandell's sensitivity values (*S*) and the molar absorpitivity (ε) for each drug were presented in table 2. The curves were linear showing a Beer's law obeyence over the concentration domains 2.50-22.50, 2.50-30, 7.50-35 and 5-30 μgml^{-1} for MNZ, TNZ, ONZ and SNZ, respectively. The correlation coefficient, slope and intercept are described by the regression analysis (Miller, 1991), $Y = a + b\,x$ (where Y is the 10 mm layer absorbance, b and a are the slope and the intercept, respectively, while, x is the drug measured solution concentration of in μg ml^{-1}) developed by the linear least-squares treatment. Regression analysis reveals a satisfactory correlation (r^2 = 0.9990-0.9992). The high Sandell's sensitivity and molar absorpitivity values ranging from 3.11×10^{-5} to 5.52×10^{-5} μg cm^{-2} and from 4.34×10^{3} to 1.13×10^{4} L mol^{-1} cm^{-1}, respectively, refer to the towering specificity and selectivity of the introduced

Table 3: The intra-day and inter-day precision for the microanalysis of metronidazole, tinidazole, orindazole and secnidazole, using the proposed technique (n = 3)

Drug	Added (μg ml^{-1})	Intra-day				Inter-day			
		Found [a] (μg ml^{-1})	Recovery (%)	S.D. [a]	R.S.D.[a] (%)	Found [a] (μg ml^{-1})	Recovery (%)	S.D. [a]	R.S.D.[a] (%)
Metronidazole	5	5.09	101.76	3.20×10^{-2}	3.15	5.06	101.17	1.51×10^{-2}	1.46
	12.50	12.30	98.39	1.92×10^{-2}	1.88	12.26	98.07	1.64×10^{-2}	1.66
	20	19.87	99.33	6.01×10^{-3}	0.62	19.87	99.36	1.10×10^{-3}	0.13
Tinidazole	5	4.92	98.37	1.50×10^{-2}	1.51	4.99	99.73	1.44×10^{-2}	1.39
	15	15.11	100.75	3.01×10^{-3}	0.34	14.99	99.95	7.33×10^{-3}	0.70
	25	24.83	99.32	1.42×10^{-2}	1.44	24.75	99.01	3.24×10^{-3}	0.27
Orindazole	7.50	7.41	98.81	3.10×10^{-3}	0.34	7.37	98.32	2.01×10^{-2}	2.01
	12.50	12.75	102.00	2.02×10^{-3}	0.23	12.72	101.74	2.94×10^{-2}	2.90
	22.50	22.40	99.54	1.33×10^{-2}	1.33	22.19	98.62	9.42×10^{-3}	0.96
Secnidazole	7.50	7.44	99.26	3.00×10^{-2}	2.97	7.42	98.99	1.74×10^{-2}	1.70
	12.50	12.63	101.07	9.14×10^{-3}	0.92	12.52	100.16	3.13×10^{-2}	3.14
	27.50	22.04	97.95	6.05×10^{-3}	0.64	21.93	97.47	4.44×10^{-3}	0.46

[a]Means and percentage relative standard deviations (% R.S.D.) for five replicates performed on four constitutive days.

Table 4: The accuracy data for the microanalysis of metronidazole, tinidazole, orindazole and secnidazole, using the proposed technique (n = 5)

Dosage form	Amount taken (μg ml^{-1})	Amount added (μg ml^{-1})	Total amount found[a] (μg ml^{-1})	Recovery ± S.D. [a]	R.S.D. [a] (%)	Er [a] (%)
Amrizole® tablet	5	5	11.89	$100.02 \pm 1.6\,x\,10^{-2}$	1.60	0.02
		12.50	21.46	$100.03 \pm 3.32\,x\,10^{-2}$	3.26	0.03
		20	28.98	$100.14 \pm 1.04\,x\,10^{-2}$	0.97	0.14
Flagyl® infusion Solution	5	5	11.66	$97.27 \pm 2.71\,x\,10^{-2}$	2.77	-2.73
		12.50	21.12	$98.34 \pm 1.44\,x\,10^{-2}$	1.46	-1.66
		20	28.61	$97.89 \pm 6.05\,x\,10^{-3}$	0.57	-2.11
Protozole® tablet	5	5	23.29	$96.90 \pm 9.08\,x\,10^{-3}$	0.94	-3.10
		12.50	30.94	$99.99 \pm 8.15\,x\,10^{-3}$	0.83	-0.01
		25	42.40	$97.10 \pm 1.73\,x\,10^{-2}$	1.78	-2.90
Tibezole® tablet	5	7.50	13.77	$97.66 \pm 8.82\,x\,10^{-3}$	0.85	-2.34
		15	20.93	$101.10 \pm 1.01\,x\,10^{-2}$	0.98	1.10
		27.50	33.73	$99.76 \pm 1.11\,x\,10^{-2}$	1.10	-0.24
Fasigyn® tablet	5	7.50	4.93	$98.64 \pm 7.00\,x\,10^{-3}$	0.66	-1.36
		15	12.58	$100.63 \pm 1.42\,x\,10^{-2}$	1.38	0.63
		27.50	26.58	$96.64 \pm 7.04\,x\,10^{-3}$	0.69	-3.36

[a]Means and percentage relative standard deviations (% R.S.D.) for five replicates.

Table 5: Microanalysis of metronidazole, tinidazole, orindazole and secnidazole in pharmaceutical formulations using the proposed and reported techniques

Drug	Name of the dosage form	[Drug] taken (μg ml^{-1})	Recovery % ± % R.S.D. [a]		*t*-test	*F*-value
			Proposed	Reported [b]		
Metronidazole	Amrizole® tablet	5	99.33 ± 0.95	100.20 ± 1.51	0.69	0.36
		12.50	102.08 ± 0.70	99.84± 0.85	1.66	3.03
		20	99.23 ± 0.65	98.18 ± 0.38	1.69	1.17
	Flagyl® infusion Solution	5	98.60 ± 0.88	101.07 ± 0.98	1.55	2.21
		12.50	98.85 ± 0.71	98.72 ± 0.49	0.26	1.16
		20	98.73 ± 0.54	102.65 ± 0.93	1.70	1.33
Tinidazole	Protozole® tablet	5	98.93 ± 0.65	102.07 ± 0.30	1.70	1.01
		12.50	101.28 ± 0.70	98.51 ± 1.11	1.63	3.28
		25	100.68 ± 1.00	96.61 ± 0.91	1.73	3.15
Ornidazole	Tibezole® tablet	7.50	102.58 ± 1.52	98.22 ± 0.88	1.67	2.88
		15	98.29 ± 0.39	100.98 ± 1.62	1.54	1.02
		27.50	98.47 ± 0.55	101.01 ± 1.51	1.52	2.11
Secnidazole	Fasigyn® tablet	5	97.87 ± 1.48	98.53 ± 2.05	0.47	2.93
		12.50	98.59 ± 0.53	98.56 ± 0.64	0.14	0.40
		27.50	98.84 ± 0.87	100.88 ± 1.70	1.39	3.15

[a]Average of five determinations.
[b]Reported techniques (Saffaj *et al*., 2006), Nagaraja *et al*., 2002, Mubeen *et al*., 2009 and Saffaj *et al*., 2006) for metronidazole (tablet), metronidazole (infusion), tinidazole, ornidazole and secnidazole, respectively.
Tabulated *t*-value at 95% confidence limit = 2.13 and degree of freedom = 4. Tabulated *F*-value at 95% confidence limit = 3.24 and degrees of freedom = 3 and 16.

technique. This is also supported by the calculated values of detection limit (LOD), which is the analyte lowest concentration, that gives the measurable reading, and limit of quantitation (LOQ), which is the analyte lowest concentration, which gives a reading that can be precisely mounted. Both can be obtained using the following equations (Miller *et al*., 1993), $LOD = (3.3 \times Standard\ deviation)\ slope^{-1}$ and $LOQ = (10 \times Standard\ deviation)\ slope^{-1}$, that were found to be ranging 1.09 x 10^{-1} - 2.02 × 10^{-1} μg ml^{-1} and 3.29 × 10^{-1} - 6.12 x 10^{-1} μg ml^{-1} for LOD and LOQ, respectively.

Accuracy and precision

The accuracy and precision of the proposed technique were examined through recovery analyses. Both precision represented as percentage relative standard deviation (% R.S.D.) while accuracy introduced as percentage relative error (% Er), were valued and stated in tables 3 and 4. Accuracy was applied through standard addition technique and calculated using the following equation (Suslu, *et al*. 2002), $\%\ Er = [(found - added)/added] \times 100$. The low values of both the inter and intra-day % R.S.D. and the % Er., indicate that the introduced technique is highly repeatable and reproducible for the microanalysis of MNZ, TNZ, ONZ and SNZ, via ion-pair formation using the sulphonphthalein dye (BTB).

Ruggedness and robustness

The technique ruggedness was evaluated through comparing the within- and intra-day precision values that has been executed in the exact analytical lab by two different analysts. The % R.S.D. values did not override 3.70%, ensuring that the technique is rugged. Also, robustness was determined by making slight deliberate changes in the operation parameters, such as pH (±0.2-0.3), dye volume, shaking and standing time during extraction process, room temperature, and wavelength of detection (±1 nm). No marked alterations in the absorbance intensity were noticed, and regarding pH robustness, % R.S.D. values ranging from 1.27%-1.84%. Thus, indicating the robustness of the developed technique.

Analysis of the pharmaceutical formulations

The validity and the applicability of the proposed colorimetric technique was valued through its utilization in the determination of MNZ in both tablets and intravenous infusions while TNZ, ONZ and SNZ in tablets manufactured by Egyptian companies. The recovery values were obtained referencing the calibration graphs, and satisfactory results were obtained, as presented in table 5. There was no drugs spectrum maxima alteration as a result to the co-existence of other ingredients within the dosage forms. The data obtained were judged and statistically compared using Student's *t*-test and the one-way analysis of variance (ANOVA test) with that calculated by the reported techniques. At $p < 0.05$, both the *t*-test values and the variance ratio *F*-values obtained, did not override the theoretical tabulated values. Therefore, there is no important variation between the introduced and the reported techniques, indicating an excellent agreement and a high similarity between the techniques accuracy and precision. In addition the

introduced technique possesses less time consumption, more color stability and wider range of determination

CONCLUSION

The introduced technique is easy, precise, inexpensive, fast and practically applicable in the microanalysis of MNZ, TNZ, ONZ and SNZ in bulk state and commercial pharmaceutical formulations with no interaction from commonly used additives nor excipients. The proposed technique offers preferential advantages over most of the reported techniques. This technique is sufficiently precise to allow analysis down to 2.50, 7.50 and 5 μg ml^{-1} for MNZ or TNZ, ONZ and SNZ, respectively, showing relatively wide applicable range of determination (20-27.50 μg ml^{-1}). Neither cooling, heating nor complicated pre-treatments were of any need, where the maximum ion-pair complex colors were developed at once only after the buffer and dye reagent were added, thus, allowing all the analyses to be done only within 8-10 min after the solutions and reduced drug forms were prepared. There is also, no involvement of any critical and hazard experimental conditions, expensive reagents nor sophisticated instruments (as in HPLC and gas chromatography techniques), which an ordinary analytical laboratory cannot afford. In addition, this proposed paper thoroughly investigates and optimizes every factor that can affect the formation of such ion-pair complexes, also the stiochiometric relationship was established. The introduced technique can be recommended for the routine quality control assessment of MNZ, TNZ, ONZ and SNZ in pure state and in formulations, with speed at low expenses without losing the accuracy.

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