Micellar high performance liquid chromatographic determination of tinidazole in combination with ciprofloxacin or norfloxacin in bulk, pharmaceutical dosage forms and in spiked human plasma

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ARTICLE INFORMATION

ABSTRACT

A micellar liquid chromatographic method was developed for determination of tinidazole in bulk, dosage forms and human plasma using intersil cyano column and a mobile phase of 0.1 M sodium dodecyl sulphate, 20% 1-propanol, 0.3% triethylamine in 0.02 M orthophosphoric acid:water (60:40, *v:v*) (pH = 4). The U.V. detection was achieved at 311 nm. Various chromatographic parameters were investigated to select the optimum conditions for the separation, e.g. types of columns, pH of mobile phase, concentration of sodium dodecyl sulphate, 1-propanol, triethylamine, etc. The method was linear over the concentration range 40-200 µg/mL with regression coefficient 0.999. The result obtained by the proposed method was compared with that obtained by the reference HPLC technique. Furthermore, the proposed method was successfully applied as stability-indicating method for determination of tinidazole under different stressed conditions. The method showed good selectivity, repeatability, linearity and sensitivity according to the evaluation of the validation parameters.

1. Introduction

Tinidazole (TIN), is a 5-nitroimidazole compound with selective activity against anaerobic bacteria and protozoa. TIN has no activity against aerobic bacteria so, it must be combined with other antibacterial agents in the treatment of mixed infections involving aerobic and anaerobic bacteria. TIN has also been used successfully alone or in combination with other antimicrobial agents for prophylaxis in patients undergoing elective colonic and abdominal surgery, emergency appendectomy and gynecological surgery [1]. It is chemically described as 1-[2-(ethylsulphonyl) ethyl]-2-methyl-5-nitro-1*H*-imidazole [2] (M.W. = 247.3 g, pK_a = 0) (Figure 1). TIN bulk drug is official in British pharmacopeia [3], United State Pharmacopeia [4] and European Pharmacopeia [5].

Many methods for analysis of the cited compound have been reported, e.g. titrimetric method [3,5], spectrophotometric methods [6,7], flow injection analysis [8], spectrofluorimetric method [9,10], electrochemical methods [11,12] and chromatographic methods [13-16]. No micellar liquid chromatographic method for simultaneous determination of TIN is available in the literature

Micellar liquid chromatography (MLC) is a form of reversed phase liquid chromatography that uses an aqueous micellar solution as the mobile phase. This technique is used mainly to enhance retention and selectivity of various solutes that would otherwise be inseparable or poorly resolved.
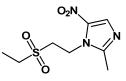

Figure 1. Structure of TIN, 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole.

One of the most advantageous is the ability to directly inject physiological fluids. Micelles have an ability to solubilize proteins which enables MLC to be useful in analyzing untreated biological fluids such as plasma, serum, and urine. MLC is a better choice than ion-exchange LC or ion-pairing LC for separation of charged molecules and mixtures of charged and neutral species. Another novel application of MLC involves the

separation and analysis of inorganic compounds, mostly simple ions. This is a relatively new area for MLC [17-19].

The aim of this work is to perform more accurate, reliable, fast and validated MLC method for determination of TIN alone or in combination with ciprofloxacin or norfloxacin in bulk, dosage form and spiked human plasma with a simple and rapid sample preparation especially for the routine analysis. Different chromatographic parameters were investigated to select the optimum conditions for the separation and applying as stability-indicating method for determination of TIN under different stress conditions. The result obtained by the proposed method was compared with that obtained by the HPLC technique [20]. The method was validated according to the international conference on harmonization guidelines (ICH) [21].

2. Experimental

2.1. Materials

TIN (99.5%) was kindly supplied by Multiapex Pharma Drug Company (Badr City, Egypt). Ciprofloxacin (CIP) (99.8%) was kindly supplied by Memphis Drug Company (Cairo, Egypt). Norfloxacin (NOR) (99.9%) was kindly supplied by Memphis Drug Company (Cairo, Egypt). The pharmaceutical products used were Ciprogyl tablets (under trial) labelled to contain 600 mg TIN/tab, 500 mg CIP /tab, were obtained from Research and Development Section in Memphis Company, Egypt. Conaz tablets (Batch 9160034) (Pharonia Pharmaceutical Company, Egypt) and Norfloxacin-TZ tablets (Batch 02321) (Sigma Pharmaceutical Company, Egypt) were purchased from the local pharmacy. They contain 600 mg TIN and 400 mg NOR/ tab. Plasma samples were obtained from Egyptian Research and Development Company and kept frozen until using after gentle thawing.

2.2. Reagents and chemicals

All Reagents were of analytical reagent grade and solvents were of HPLC grade. High purity water was obtained by Elga Labwater, Prima 7 (UK) and it was used throughout the study. Methanol, 1-propanol and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich (Germany). Ortho-phosphoric acid (85%), triethylamine (TEA) and sodium dodecyl sulphate (SDS, 99%) were obtained from Riedel-deHäen (Sleeze, Germany). Sodium hydroxide and hydrochloric acid (32%) were purchased from El-Nasr Company, Egypt. Hydrogen peroxide (30%) was obtained from Luna industrial group (6th of October City, Egypt).

2.3. Instrumentations

The MLC method was performed on Agilent1200 HPLC auto sampler system equipped with UV detector 1200 (Germany) and HPLC quaternary pump 1200 (Germany). Analytical data is stored in a computer equipped with Agilent software. Vortex used was VWR VV3 S540 International (West Charter, USA). Centrifuge used was centurion, (West Sussex, UK). The pH was measured with Jenway pH meter, 3510, (Essex, UK). The mobile phase was filtered through Charles Austen pumps Ltd filter, model-B100 SE (England, UK). Ultrasonic bath used was Falc., (Treviglio, Italy). The mobile phase and the injected solutions were filtered through 0.45 μm pore filters (Gelman, Germany).

2.4. Chromatographic conditions

MLC was performed on an intersil cyano column (5 μm particle size, 25 cm × 150 mm i.d.), (GL Sciences Inc., Japan) using micellar mobile phase consisting of 0.1 M SDS, 20% 1-propanol, 0.3% TEA in 0.02 M ortho-phosphoric acid: water (60:40, *v:v*) (pH = 4). The mobile phase was filtered and sonicated for 30 min before use. The flow rate was 1.0 mL/min and sample injection volumes were 20 μL at room temperature (25 °C). The UV detector was operated at 311 nm.

2.5. Standard solutions

Standard stock solutions of TIN / CIP combination (500 μg TIN and 416.7 μg CIP/mL) and TIN / NOR combination (500 μg TIN and 333.33 μg NOR/mL were prepared by dissolving 50 mg of TIN and 41.67 mg CIP or 33.33 mg NOR in 100 mL methanol then the solution was sonicated in an ultrasonic bath for 5 minutes. The solutions were found to be stable for 3 days at room temperature.

Working standard solutions (40-200 μg/mL) TIN combined with CIP or NOR were prepared by serial dilution of the standard solution with the mobile phase.

2.6. Preparation of pharmaceutical products

Take the contents of 20 tablets and determine the average weight of content per tablet. Accurate weight of the powder equivalent to 50 mg ITC was dissolved in 100 mL methanol then sonication for 15 min and filtered.

2.7. Plasma sample extraction

1 mL of human plasma was transferred into a centrifuge tube, spiked with 1 mL of TIN and deprotenized with 2 mL acetonitrile (ACN), then vortex. Final concentrations were 80, 100 and 120 μg TIN/mL, respectively. The solutions were centrifuged at 5000 rpm for 15 minutes. Then, the solutions were filtered through a disposable syringe filter (0.45 μm) before column injection.

2.8. Sample degradation

An appropriate amount of TIN powder at a final concentration of 500 μg/mL in 1 N alcoholic HCl was left for 24 hrs then the solution was filtered and 2 mL was taken into 10 mL volumetric flask and the volume was completed with the mobile phase and triplicate 20 μL injections were made. The same experiment was repeated but in 1 N alcoholic NaOH, 30% H_2O_2.

Each experiment was carried out in triplicate and all solutions were injected in triplicate. The nominal contents of each one were calculated using the constructed calibration graph or from the corresponding regression equation.

3. Results and discussion

The proposed MLC method represents a rapid and sensitive stability-indicating method for the separation and simulta-neous determination of TIN in combination with either CIP or NOR in bulk and commercial tablets. Moreover, it was extended to investigate the inherent stability of TIN under different stress conditions. By virtue of its high sensitivity, the proposed method was applied for the determination of TIN in human plasma with no need for tedious sample pre-treatment steps.

Different parameters affecting the chromatographic performance of TIN were carefully studied in order to achieve the most suitable chromatographic system.

3.1. Choice of appropriate detection wavelength

The absorption spectrum of TIN in the mobile phase exhibits maximum absorption at 311 nm which allows also determination of CIP and NOR at this wavelength. So, 311 nm was selected as the optimum detection wavelength for detection and selective determination of TIN with a reasonable sensitivity in the presence of CIP or NOR.

Table 1. Performance data of the proposed method for determination of TIN/CIP and TIN/NOR in pure form.

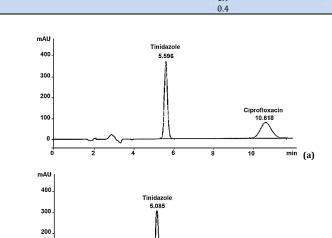| Parameter	Results	
	TIN/CIP	TIN/NOR
Concentration range (µg/mL)	40-200	40-200
LOD (µg/mL)	5.6	4.07
LOQ (µg/mL)	17.1	12.34
r	0.999	0.999
%RSD	1.9	1.2
% Error (% RSD/√n)	0.4	0.25

Figure 2. Representative chromatogram using the proposed method for simultaneous determination of TIN (100 µg/mL) in the presence of CIP (a), TIN (100 µg/mL) in the presence of NOR (b).

3.2. Choice of column

To investigate the chromatographic performance, different columns had been tried, these include reversed-phase C8, reversed-phase C18, two bonded phase cyano and bonded phase phenyl columns. Experimental trials revealed that the Intersil cyano column (5 µm particle size, 25 cm length, 150 mm i.d, GL Sciences Inc., Japan) was the most suitable one giving narrower symmetric peaks and highest number of theoretical plates within a reasonable analysis time.

3.3. Mobile phase composition

To achieve the best chromatographic conditions, the mobile phase composition was optimized to provide sufficient selectivity and sensitivity in a short separation time. The studied variables included; the pH of the mobile phase, flow rate, concentration of SDS, organic modifier and TEA.

3.3.1. pH of the mobile phase

In order to select the optimum pH value for the analysis of TIN, the pH of the mobile phase was studied over the range of 2.5-4.5. Mobile phases with pH values ranged from 3.5-4.0 provided suitable peak symmetry and better peak shape. Increasing the pH of the mobile phase more than 4.0 caused increase in the retention time of TIN. Finally, pH = 4.0 was selected as the optimum pH value for the mobile phase in this study yielding highest number of theoretical plates with good peak shape and lowest peak tailing.

3.3.2. Flow rate of the mobile phase

The effect of flow rate of the mobile phase on the retention of TIN was investigated over the range of 1.0-2.0 mL/min. Flow rate of 1.0 mL/min was chosen since it provides better peak

shape and better resolution within a reasonable time. After optimization of these variables, best peak shape, lowest peak tailing was achieved with well-defined peaks and good sensitivity within a reasonable analytical run time. Figure 2 represents a chromatogram indicating the good retention of TIN under the optimum chromatographic conditions.

3.4. Method validation

The validity of the proposed method was assessed by studying the following parameters in accordance to ICH guideline [21]: linearity, range, LOD, LOQ, accuracy, precision, selectivity, sample solution stability, mobile phase stability, system suitability and robustness.

3.4.1. Linearity and range

Under the optimum chromatographic conditions, a linear relationship was established by plotting average peak areas against TIN concentrations (µg/mL) as shown in Figure 3. The calibration graphs were found to be rectilinear over the concentration ranges of 40-200 (µg/ mL) TIN.

Statistical analysis of the data showed good correlation coefficients (*r*) and relative standard deviation as mentioned in Table 1.

$$y = 39.39x + 134.4 \ (r = 0.999) \text{ for TIN/CIP} \tag{1}$$

$$y = 41.24x + 3.599 \ (r = 0.999) \text{ for TIN/NOR} \tag{2}$$

Limit of detection (LOD) and limit of quantification (LOQ) for TIN were calculated according to ICH Q2 (R1) recommendation [21] and the results are shown in Table 1.

Table 2. Application of the proposed MLC method for the determination of TIN in combination with CIP.

Parameter	Proposed method			Comparison method [20]
	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found *	% Found*
	40	39.20	98.0	99.2
	60	61.4	102.3	101.5
	80	80.7	100.9	102.1
	100	98.0	98.0	98.5
	120	122.5	102.1	103.8
	200	199.9	100.0	101.3
x±SD	100.2±1.9			101.1±1.9
t	0.8 (1.8) **			
F	1.0 (5.1) **			

* Each result is the average of three separate determinations.
** Values between parentheses are the tabulated t and F values at p = 0.05 [22].

Table 3. Application of the proposed MLC method for the determination of TIN in combination with NOR.

Parameter	Proposed method			Comparison method [20]
	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found *	% Found
	40	40.7	101.8	100.5
	60	60.8	101.3	100.9
	80	79.7	99.6	101.2
	100	100.3	100.3	99.4
	120	118.3	98.6	97.9
	140	142.2	101.6	100.5
	200	200.2	100.1	99.2
x±SD	100.5±1.2			99.9±1.2
t	0.8 (1.8) **			
F	1.0 (3.8) **			

* Each result is the average of three separate determinations.
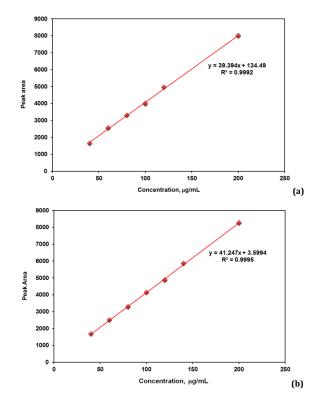** Values between parentheses are the tabulated t and F values at p = 0.05 [22].

Figure 3. Calibration curve for simultaneous determination of TIN in pure form using the proposed MLC method in presence of (A) CIP in pure form, (B) NOR in pure form.

3.4.2. Accuracy

To prove the accuracy of the proposed method, the results of the assay of TIN in pure form by the proposed MLC method were compared with those obtained using a comparison HPLC method [20]. Statistical comparison of the results obtained by the proposed MLC method with those obtained by the comparison method using *t*-test and *F*-test revealed no significant differences between the performance of the two methods regarding the accuracy and precision, respectively. The results are shown in Table 2 and 3.

The comparison HPLC method involved the use of a mobile phase consisting of 0.05 M SDS: 6% propan-1-ol (47:3, *v:v*) and µbondapack cyano column at 320 nm [20].

Table 4. Precision data of the proposed method for the determination of TIN in dosage forms.

	% Found±SD	%RSD	%Error
Intra-day precision			
Ciprogyl tab	108.0±1.0	0.9	0.3
Conaz tab	94.3±0.6	0.6	0.2
Norfloxacin-TZ tab	104.4±0.7	0.7	0.2
Inter-day precision			
Ciprogyl tab	107.0±2.4	2.2	0.5
Conaz tab	95.7±2.2	2.3	0.5
Norfloxacin-TZ tab	102.2±2.6	2.5	0.6

Table 5. System suitability test parameters for the developed MLC method for TIN.

Parameter	TIN/CIP	TIN/NOR
No of theoretical plates, N	7145	4859
Capacity Factor, K'	0.84	0.84
Selectivity factor, α	1.95	1.92
Resolution factor, R_s	8.2	5.51

Table 6. Application of the proposed and comparison method to determination of TIN in tablets.

Pharmaceutical preparation	% Found *, mean±SD	
	Proposed	Reference [20]
Ciprogyl tab	105.8±1.6	105.2±1.7
t **	0.5	-
F **	1.8	-
Conaz tab	97.3±1.7	97.8±1.7
t **	0.6	-
F **	1.1	-
Norfloxacin-TZ tab	101.4±1.3	101.3±1.5
t **	0.4	-
F **	2.1	-

* Each result is the average of four separate determinations and each one repeated three times.
** The tabulated *t* = 1.9 and F = 9.3 at *n* = 12 and *p* = 0.05 [22].

3.4.3. Precision

Intra-day precision of the proposed method was achieved by six replicate determinations of 100 μg/mL TIN in the three dosage forms on three successive times in the same day. Inter-day precision was performed as intra-day but on three successive days. Small values of %error and %RSD revealed the precision of the proposed method. The results are illustrated in Table 4.

3.4.4. Selectivity

The specificity of the proposed MLC method was established by its ability to determine TIN in commercial tablets without interference from other drugs or common tablet excipients. Neither the combined drugs nor the common tablet excipients show any interfering peaks at the retention times of the drug, which proved the homogeneity and purity of the peak. Furthermore, to evaluate the specificity of the method to determine TIN in human plasma, blank plasma was diluted with the micellar mobile phase and injected under the recommended chromatographic conditions. No endogenous interference was observed at the retention time of TIN, proving the specificity of the method.

Forced Degradation studies were also performed to evaluate the validity of the method. The results obtained indicated that the proposed MLC method is selective and able to determine TIN in presence of its degradation products.

3.4.5. Sample solution stability and mobile phase stability

Evaluation of the stability of TIN in its three dosage forms solution was achieved by quantification of TIN on three successive days and comparison to freshly prepared dosage forms solution. Similarly, the stability of the mobile phase was checked. No significant changes were observed, proved that the sample solution and mobile phase used during the assay were stable up to 3 days.

3.4.6. System suitability test (SST)

Evaluation of SST parameters was performed during the development and optimization of the method. The test was performed by injecting the standard sample in triplicate and the parameters were calculated as reported by USP [4]. SST parameters include capacity factor (k'), selectivity factor (α), resolution factor (R_s), column efficiency (Number of theoretical plates, N). The final SST parameters under the optimum chromatographic conditions are illustrated in Table 5.

3.4.7. Robustness

To assess the robustness of the proposed MLC method, chromatographic conditions were deliberately altered. The tested variables included; concentration of 1-propanol (20%±0.5, *v:v*), strength of ortho-phosphoric acid (0.02±0.005 M), concentration of TEA (0.3±0.01%). The efficiency of the separation of TIN was not affected by all the varied chromatographic conditions indicating the reliability of the proposed method.

3.5. Applications

3.5.1. Application of the proposed method to the determination of TIN in tablets

The developed MLC method was applied successfully for the assay of TIN in commercial tablets either in combination with NOR or CIP. No interferences were encountered from common tablet excipients.

The results obtained by the developed MLC method were statistically compared with those of the comparison HPLC method [20] using *t*-test and F-test. In both tests, the calculated values did not exceed the theoretical values at the 95% confidence level, which indicated that there were no significant differences between the developed and comparison methods regarding accuracy and precision, respectively. The results are shown in Table 6.

Table 7. Assay results for the determination of TIN in spiked human plasma using the proposed method.

Parameter	Conc. added (µg/mL)	Conc. found (µg/mL)	% Recovery *
Spiked plasma	80	80.9	101.1
	100	105.2	105.2
	120	121.1	103.9
Mean%±SD	103.4±2.1		
%R.S.D	2.0		
% Error	0.7		

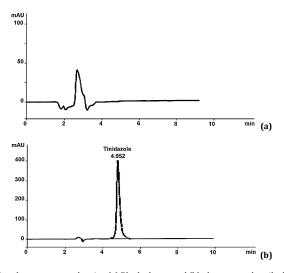* Each result is the average of three separate determinations.

Figure 4. Representative chromatograms showing (a) Blank plasma and (b) plasma sample spiked with 120 µg/mL TIN.

3.5.2. Application of the proposed method to the determination of TIN in spiked human plasma

MLC allows biological samples to be analysed without prior tedious pre-treatment for plasma protein precipitation or elimination of other interfering substances, thus considerably reducing the cost and analysis time. The proposed MLC method was successfully applied to the determination of TIN in spiked human plasma.

The proposed method showed satisfactory results for determination of TIN in spiked human plasma. The assay results using the proposed method are summarized in Table 7. Figure 4 shows representative chromatograms for blank and spiked plasma samples.

3.5.3. Results of stress testing studies

The proposed method was applied to assess the inherent stability of TIN under different conditions like: alkaline, acidic, oxidative and photolytic conditions. The studied compound is found to be highly labile to alkaline hydrolysis, the solution upon addition of NaOH, acquires a yellow colour [23]. Where the acidic degradation using 1 N HCl for 24 hrs resulted in about 2%. Exposure of TIN to oxidative degradation by 30% H_2O_2 for 24 hrs, about 62.5% degradation of TIN was observed.

4. Conclusions

The proposed MLC method represents a new rapid and sensitive stability-indicating assay for the separation and simultaneous determination of TIN in bulk and pharmaceutical dosage form containing TIN/CIP or TIN/NOR. The proposed method was applied for the determination of TIN in human plasma with no need for tedious sample pre-treatment steps. In addition, the proposed method was extended to investigate the inherent stability of TIN under different stress conditions.

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