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ANALYTICAL METHOD DEVELOPMENT FOR QUANTIFICATION OF TINIDAZOLE BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and rapid reversed-phase high performance liquid chromatography (HPLC) assay for determination of tinidazole level in human plasma was developed and validated. Hydrochlorothiazide *was used as an internal standard (IS). Human plasma (0.4 ml) were deproteinized with 20 µl perchloric acid, vortexed and centrifuged. The supernatant 100 µl was collected and injected into the HPLC system. Analysis was performed using Atlantis dC18 column with a mobile phase composed of 50 mM potassium phosphate, dibasic (pH=3.0) and acetonitrile (80:20, v:v). The eluent was monitored spectrophotometrically at 317 nm. No interference from blank plasma components or commonly used drugs was observed. The relationship of tinidazole concentration and peak area ratio of tinidazole / IS was linear ($R^2 \geq 0.9902$) in the range of 0.05 – 30 µg/ml, the intra and inter-day coefficient of variations were ≤8.9% and ≤8.2%, with a corresponding bias of ±8.2% and ±4.6%, respectively. Mean extraction recovery of tinidazole and the IS were 83% and 95%, respectively. The method was applied to assess the stability of tinidazole under various conditions encountered in the clinical laboratory. Tinidazole stability in processed samples (stored at room temperature for 24 hours or at -20°C 48 hours), unprocessed samples (stored for 24 hours at room temperature or, 8 weeks at -20°C), and after three freeze and thaw cycles was ≥88%, respectively.*

KEYWORDS: *Tinidazole, Hydrochlorothiazide, Human plasma, HPLC*

1. INTRODUCTION

Tinidazole, chemically known as 1-[2-(ethyl sulfonyl) ethyl]-2-methyl-5-nitroimidazole, utilized in the treatment of various bacterial infection that causes diarrhea and other intestinal issues [1-2]. Its oral bioavailability is about 99% with a peak plasma concentration of 10.1±0.6µg/ml within 1-2 hours after ingestion of 500 mg therapeutic dosage [3]. **Figure 1** depicts the chemical structures of tinidazole and the internal standard (IS) hydrochlorothiazide.

Several analytical methods have been reported for the determination of tinidazole concentration in pharmaceutical formulations and in various biological samples [4-18]. The techniques, which incorporates potentiometry [6], thin layer chromatography [7], polarography [8], spectrofluorometry [9] spectrophotometry [10], capillary electrophoresis [11], gas chromatography [12], high performance thin layer chromatography [13], high performance liquid chromatography (HPLC) [14-18] and HPLC coupled with tandem mass spectrometry [19]. Tinidazole level particularly in human serum/plasma were often determined by HPLC with UV detector [14-18]. Few reported HPLC assays utilized 95% chloroform as extraction solvent, whereas some were used metronidazole as internal standard in sample preparation [16-18]. Since chloroform and metronidazole both are carcinogenic in nature and an accordance to safety guidelines utilization of these chemicals ought to be maintained a strategic distance from or limit by substituting with reasonable nontoxic or less harmful chemical substances [20].

In the current study, we described a simple and rapid HPLC assay for determination tinidazole level in human plasma. We utilized low volume of perchloric acid for plasma protein precipitation and hydrochlorothiazide was used as internal standard in sample preparation. The method was fully validated and successfully applied to assess the stability of tinidazole under various laboratory conditions.

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2. MATERIAL AND METHODS

2.1 Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 system (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. We used a reversed-phase Atlantis dC18 (4.6 x 150 mm, 5 µm) column protected by guard column Symmetry C18 (3.9 x 20 mm, 5 µm). Data collected with a Pentium IV computer, using Empower Chromatography Software.

2.2 Chemical and reagents

All chemical reagents were of analytical-reagent grade except if expressed something else. Tinidazole and hydrochlorothiazide purchased from Sigma-Aldrich MO, USA. Methanol and acetonitrile, (both HPLC grade), perchloric acid (70%) and potassium phosphate (dibasic) were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

2.3 Chromatographic conditions

The mobile phase was composed of 0.05 M potassium phosphate (dibasic) (pH=3.0±0.1, adjusted with phosphoric acid) and acetonitrile (80:20, v:v) and was delivered at a flow rate of 1.0 ml/minute at ambient temperature with a run time of 10.0 minutes. A photodiode array detector set at 317 nm was used.

2.4 Preparation of standard and quality control samples

Stock solution of tinidazole and hydrochlorothiazide (1.0 mg/ml) were prepared in methanol. They were then diluted with blank human plasma and mobile phase to produce working solutions of 30 µg/ml and 100 µg/ml, respectively. Nine calibration standards in the range of 0.05 – 30 µg/ml and four quality control (QC) samples (0.05, 0.15, 15, and 27 µg/ml) were prepared in human plasma. Calibration standards and QC samples were vortexed for one minute and 0.4 ml aliquots were transferred in to 1.5 ml micro centrifuge polypropylene culture tubes and stored at –20 °C until used.

2.5 Sample preparation

Aliquots of 0.4 ml of calibration curves, or QC samples in culture tubes were allowed to equilibrate to room temperature. To each tube, 100 µl IS working solution was added and the mixture was vortexed for 10 seconds. 20 µl perchloric acid were added to the mixture and vortexed for one minute, and then centrifuged for 10 minutes at 11500 rpm at room temperature. The clear supernatant was transferred into an auto-sampler vail and 100 µl sample was injected into the HPLC system with a run time of 10 minutes.

2.6 Stability studies

Three of QC samples (0.05, 0.15, and 27 µg/ml) were used for stability studies. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20°C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed and stored at room temperature for 24 hours, or at –20°C for 48 hours before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20°C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20°C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

2.7 Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [21]. The validation parameter included specificity, linearity, accuracy, precision, recovery and stability.

3. RESULTS AND DISCUSSION

3.1 Optimization of chromatographic conditions

The mobile phase was composed of 0.05 M potassium phosphate (dibasic) (pH=3.0±0.1, adjusted with phosphoric acid) and acetonitrile (80:20, v:v) delivered at a flow rate of 1.0 ml/minute at ambient temperature, with a run time of 10 minutes. A photodiode array detector set at 317 nm was utilized. The retention times of IS and tinidazole were around 4.4 and 5.6 minutes, respectively.

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3.2 Specificity

Specificity is a measure of the ability of the analytical method to differentiate and quantify the analytes of interest in the presence of other components. No endogenous component from extracted human plasma co-eluted with hydrochlorothiazide or the IS. **Figure 2** depicts a representative chromatogram of drug free human plasma used in preparation of calibration standards and QC samples.

3.3 Linearity, Accuracy and Precision

Linearity of tinidazole assay was evaluated by analyzing nine curves of ten calibration standards over the range (0.05 - 30 µg/ml) prepared in human plasma. **Figure 3** represents an overlay of chromatograms of extracts of 0.4 ml human plasma spiked with the IS with or without nine concentrations of tinidazole. The peak area ratios were subjected to regression analysis. The suitability of the calibration curves was confirmed by back-calculating tinidazole concentration from the calibration curves (**Table 1**). All back-calculated concentrations were well within the acceptable limits. Precision and bias were also determined for four QC samples (0.05, 0.15, 15, and 27 µg/ml). The intra-day (n=10) and inter-day (n=20, over three consecutive days) precision was ≤8.9% and ≤ 8.2%, respectively. The intra-day and inter-day bias was in the range of -1.1 to 8.2% and of -3.7 to 4.6%, respectively. The results are summarized in **Table 2**.

3.4 Recovery

Tinidazole recovery was assessed by direct comparison of its peak area in plasma and in mobile phase samples, using five replicates for each of four QC samples (0.05, 0.15, 15, and 27 µg/ml). Similarly, the recovery of the IS was determined by comparing its peak area (at 10.0 µg/ml) in plasma and in mobile phase samples. The mean recovery of tinidazole and the IS was 83% and 95%, respectively.

3.5 Stability

As shown in **Table 3**, tinidazole stability in processed and unprocessed QC samples (0.05, 0.15, and 27 µg/ml) was investigated. Tinidazole was stable in processed samples for at least 24 hours at room temperature (≥ 96%) or 48 hours at -20 °C (≥ 93%). Tinidazole in unprocessed samples was stable for at least 24 hours at room temperature (≥ 88%), eight weeks at -20 °C (≥ 98%), or after three freeze-and thaw cycles (100%).

4. CONCLUSIONS

The described HPLC assay is accurate, precise, and rapid. It requires only 0.4 ml plasma and utilizes a simple and convenient protein precipitation procedure. The assay was successfully applied to monitor stability of tinidazole under various conditions generally encountered in the clinical laboratories.

Conflict of Interest

None to declare

Table 1: Back-calculated tinidazole concentrations from ten calibration curves

Nominal Level (µg/ml)	Calculated level (µg/ml)		CV (%)*	Bias** (%)
	Mean	SD		
0.05	0.05	0.002	4.9	8.8
0.25	0.27	0.011	4.3	6.1
0.50	0.51	0.059	7.1	0.8
1.00	1.11	0.062	5.6	10.8
2.50	2.65	0.168	6.3	6.1
5.00	5.24	0.334	6.4	4.8
10.0	10.77	0.463	4.3	7.7
20.0	21.25	1.033	4.9	6.3
30.0	29.33	0.588	2.0	-2.2

*Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100. **Bias = measured level – nominal level divided by nominal level x 100.

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Table 2: Intra- and inter-day precision and bias of tinidazole assay

Nominal Level (µg/ml)	Measured level (µg/ml)		CV (%)*	Bias (%)
	Mean	SD		
		Intra-day (n=10)		
0.05	0.049	0.003	5.8	-1.1
0.15	0.153	0.014	8.9	2.2
15.0	15.022	0.707	4.7	0.1
27.0	24.798	2.121	8.6	8.2
		Inter-day (n=20)		
0.05	0.052	0.003	6.5	3.9
0.15	0.155	0.013	8.2	3.4
15.0	15.685	0.696	4.4	4.6
27.0	25.999	1.969	7.6	-3.7

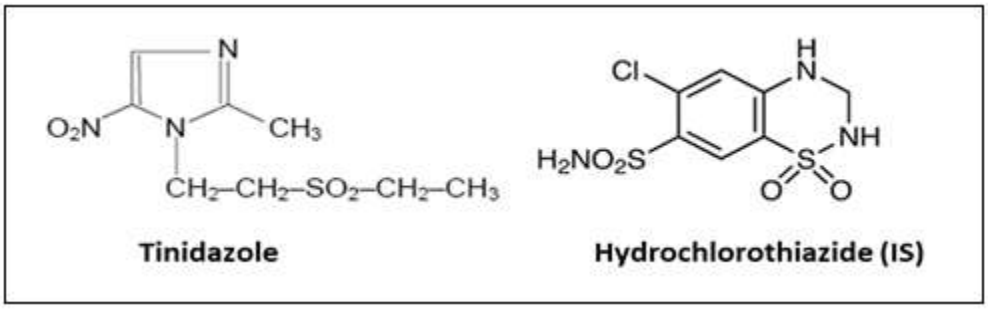
*CV, coefficient of variation (CV) = standard deviation (SD) divided by mean measured level x100.Bias = measured level - nominal level divided by nominal level x 100.

Table 3: Stability of tinidazole under various clinical laboratory conditions

Nominal level	Unprocessed		Processed		Freeze-Thaw Cycle		
(µg/ml)	24 hrs RT	8 wks -20 °C	24 hrs RT	48 hrs -20 °C	1	2	3
0.05	100	104	102	90	110	107	113
0.15	93	98	96	93	108	109	104
27.0	88	100	101	98	113	104	106

Data represent stability (%) calculated as mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after storing 24 hours at room temperature (24 hrs RT) or after freezing at –20ºC for 8 weeks (8 wks, –20ºC) or after 1 to 3 cycles of freezing at –20ºC and thawing at room temperature (freeze-thaw)., or were processed and then analyzed after storing for 24 hours at room temperature (24 hrs, RT) or 48 hours at –20ºC (48 hrs, -20ºC)

Figure 1: Chemical structures of tinidazole and hydrochlorothiazide (IS)



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Figure 2: Representative chromatogram of blank human plasma

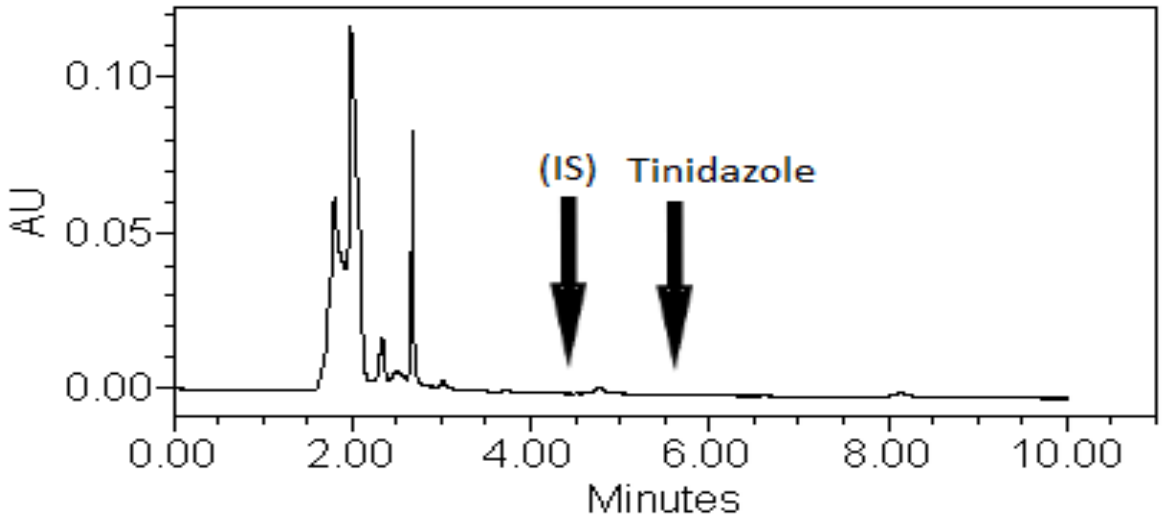
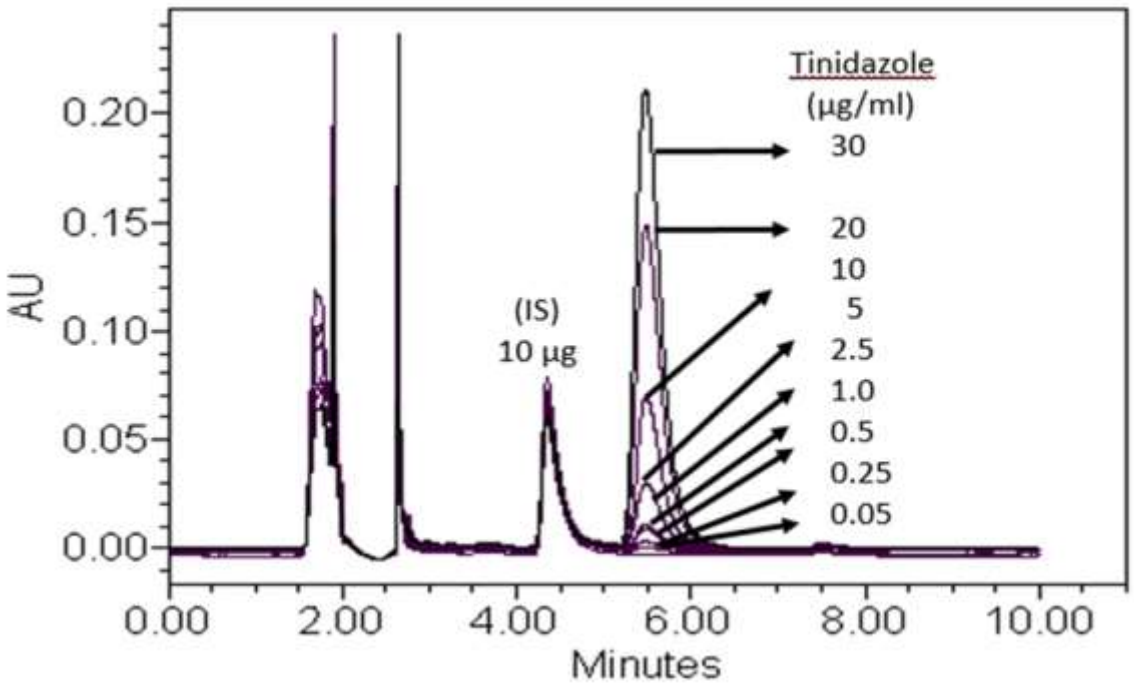


Figure 3: Overlay chromatograms of extracts from 0.4 ml human plasma spiked with the internal standard (IS) alone or with tinidazole at one of nine concentrations



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