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Original Article

New Analytical Method Development and Validation of Ciprofloxacin and Ornidazole in Human Plasma by High Performance Thin Layer Chromatography

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ABSTRACT

Objective: The objective of the method was to develop a simple, rapid, sensitive, selective and economic high performance thin layer chromatographic method for simultaneous determination of ciprofloxacin and ornidazole in human plasma by using tinidazole as an internal standard. **Method**: The plasma sample was extracted using methanol: formic acid (5.5:0.5 v/v) and known amount of extract was spotted on precoated silica gel 60 F_{254} plates using Camag Linomat V auto sampler. A concentration range from 100–700 ng/spot for both drugs was used for calibration curve. The percent recoveries of Ciprofloxacin and ornidazole were found to be 81.02 to 86.26 and 79.73 to 82.16 respectively. The mobile phase used consists of chloroform: methanol: triethylamine (9.0: 0.8: 0.4 v/v/v). Densitometric analysis was carried at wavelength 291 nm. **Result:** The R_f values for ciprofloxacin, ornidazole and tinidazole were found to be 0.18 ± 0.057, 0.49 ± 0.0.0057 and 0.75 ± 0.0054 respectively. The stability of ciprofloxacin and ornidazole in plasma were confirmed during three freeze-thaw cycles (-20°C), on bench during 12 h and post preparative stability study. The proposed method was validated statistically by performing recovery study for determination of ciprofloxacin and ornidazole in human plasma. **Conclusion**: The proposed method was found to be a simple, rapid, sensitive, selective and economic high performance thin layer chromatographic method for simultaneous determination of ciprofloxacin and ornidazole in human plasma In future this method can be used for clinical and pharmacokinetic studies.

Key words: Ciprofloxacin, Ornidazole, HPTLC, Human plasma, Liquid-liquid extraction.

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INTRODUCTION

Ciprofloxacin1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid (Figure 1) is broad spectrum fluoroquinolone antibacterial agent. It is effective in the treatment of a wide variety of infections including infections of bones and joints, particularly those caused by gram-negative pathogens. Gram-positive bacteria are generally susceptible or moderately susceptible. Ciprofloxacin only treats bacterial infections; it does not treat viral infections such as the common cold. For certain uses including acute sinusitis, lower respiratory tract infections and uncomplicated gonorrhea. Ciprofloxacin are not considered a first-line agent. Ciprofloxacin is one of the few broad spectrum antibacterial available in both intravenous and oral formulations. The primary mechanism of action of ciprofloxacin is inhibition of bacterial DNA gyrase.[1] Ornidazole, 1-(3 chloro-2-hydroxy propyl)-2 methyl-5-nitro imidazole, used as an anti-infective agent. Use of ornidazole in combination with fluroquinolone in the treatment of pelvic inflammatory disease and intraabdominal infection. It is an antimicrobial agent used in treatment of susceptible protozoal infections and anaerobic bacterial infection. It is prescribed to treat different health conditions due to anaerobic infections, amoebic liver abscess, amoebic dysentery, hepatic amoebias, The drug is available in both intravenous and oral formulations.The primary mechanism of action of ofloxacin appears to be the specific inhibition of DNA gyrase (topoisomerase II). This enzyme is responsible for the negative super coiling of the bacterial DNA and consequently for its topological configuration, governing functions such as RNA transcription, protein synthesis, DNA replication and repair functions.[2] The literature survey revealed that variety of analytical methods reported for estimation of Ciprofloxacin in human plasma and other biological fluids,[3–5] spectrophotometry,[6] fluorometry[7] and HPLC[8] methods have been reported for estimation of ornidazole alone as well as in combinations. UV spectrophotometry and RP-HPLC methods were reported for estimation of ciprofloxacin and ornidazole in Combined Pharmaceutical Dosage Form.[9,10] However no method has been reported for simultaneous determination of ciprofloxacin and ornidazole in human plasma by HPTLC using liquid-liquid extraction. The proposed research work describes the simultaneous estimation of ciprofloxacin and ornidazole in human plasma by HPTLC using tinidazole as an internal standard. A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of sample to give separate peaks in chromatogram to compensate for the losses of the compound of interest during sample pretreatment steps. It must have a completely resolved peak with no interferences; it must not be present in original sample. It must be stable, unreactive with sample components. In this method tinidazole is used an internal standard as it does not interfere with the peak area of Ciprofloxacin and ornidazole.

MATERIALS AND METHODS

Instrumentation

HPTLC Camag with precoated silica gel plate 60 F_{254} (20×10 cm) 250 µm thickness (E.Merck, Darmstadt, Germany) was used as stationary phase. Sample applications were done using Camag 100 µl syringe and Camag Linomat V applicator. The samples were sprayed in the form of narrow bands of 8 mm length at a constant rate 2 µl/s. Linear ascending development was carried out in 20×10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was

performed by using Camag TLC scanner III supporting win CATS software (V 1.4.2.8121 Camag). Evaluations of chromatograms were done by using peak areas.

Chemicals

Ciprofloxacin, Ornidazole and tinidazole were received from Aarati drugs pvt ltd Tarapur, Mumbai, Maharashtra, India. The HPLC grade chloroform and methanol were purchased from Fisher scientific India. Analytical Reagent grade triethylamine and formic acid were purchased from S.D. Fine Chem., Mumbai, India. Human plasma used for research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

Preparation of stock solution and working standard solutions

Stock solutions 1000 µg/ml each of ciprofloxacin, ornidazole and tinidazole were prepared in methanol. The working standard solutions of 100 µg/ml of ciprofloxacin, ornidazole, tinidazole were prepared by further dilutions of stock solutions in methanol.

Preparation of plasma sample

To 1ml of fresh human plasma in separate 15 ml centrifuge tube 0,1,2,3,4,5,6,7 µl of working stock solutions of Ciprofloxacin and ornidazole were added to drug free plasma to provide calibration standards of 0 (no ciprofloxacin and ornidazole added) 100, 200, 300, 400, 500, 600, 700 ng/ml and 400 ng of tinidazole (internal standard) was kept constant. The quality control (QC) samples were prepared low, mid and high in plasma for both drugs in concentration range 200, 400, 500 ng /spot. Extractions of drugs were carried out by using methanol: formic acid (5.5: 0.5 ml) by vigorous vortex using remi mixer for 1.5 min and centrifuged at 8000 rpm at 10 min. The organic phase recovered and evaporated to dryness on hot plate. The residual mass reconstituted with 1ml methanol. The analysis was carried on HPTLC.

Chromatographic condition

The mobile phase was selected as mixture of chloroform, methanol and triethylamine in the ratio of (9.0: 0.8: 0.4, v/v/v) for the development of plates. Time for chamber saturation was optimized to 14 min. The length of chromatographic development was 70 mm. The densitometric scanning was performed at wavelength 291 nm.

Method validation

The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery and stability. The validation of the method was based on FDA guidelines and on standard Bioanalytical Method validation recommendation. The selectivity of method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure (Figure 2). Five replicate of three QC sample low 200, mid 400 and high 500 ng/ spot were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. Precision and recoveries of ciprofloxacin and ornidazole were calculated by comparison of the peak areas of low, mid, and high quality control sample (200, 400 and 500 ng/ spot respectively) prepared in plasma (extracted) with unextracted ciprofloxacin and ornidazole in methanol and formic acid by using peak areas of low, mid and high quality control sample (200, 400 and 500 ng/spot respectively) in similar manner. Stability experiments were undertaken to detect degradation of Ciprofloxacin and ornidazole under certain condition. The stock solution stability of ciprofloxacin and ornidazole were examined at room temperature for 6 h. Freeze–thaw stability were determined at three QC concentrations (low 200, mid 400 and high 500 ng/ spot) after freezing (−20°C) and thawing for three cycles and compared with nominal value. Bench-top stability of both drugs was assessed for low mid and high QC samples by comparing with nominal value which stored at room temperature for 12 h. The effect of storage within the auto-sampler

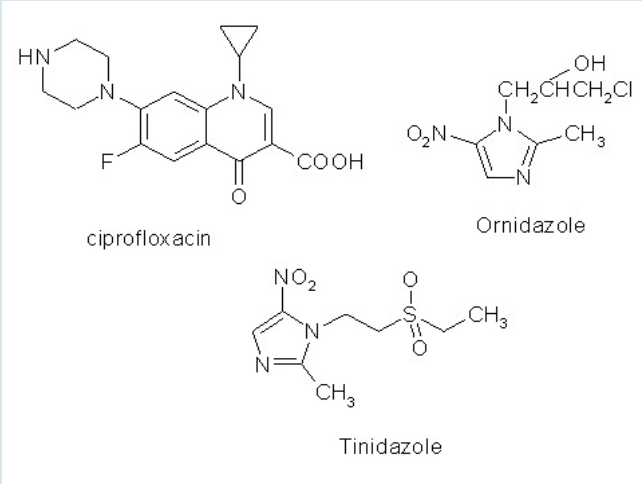

Figure 1: Structure of (a) ciprofloxacin, (b) ornidazole and(c) tinidazole (IS) IS=Internal Standard.

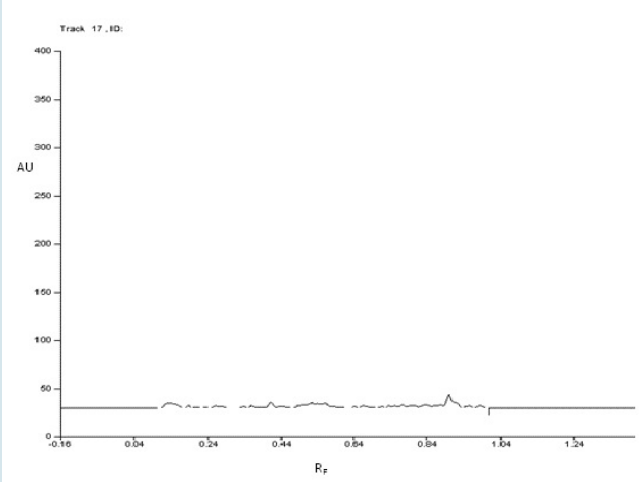

Figure 2: Densitogram of blank plasma showing no interference of drugs.

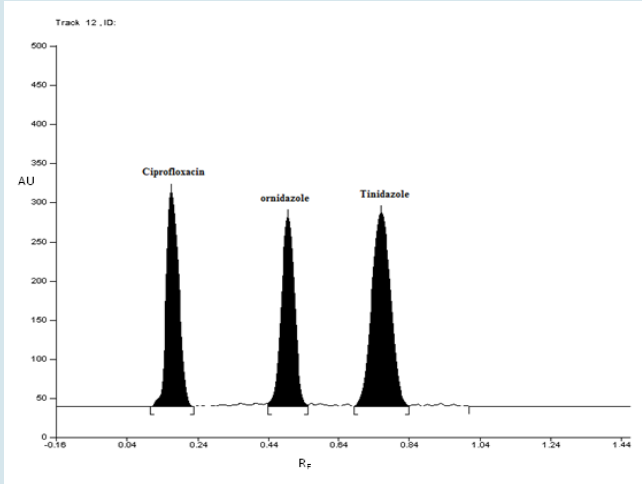

Figure 3: Densitogram of ciprofloxacin 100 ng/spot (Retention factor = 0.18 ± 0.0057), ornidazole 100 ng/spot (Retention factor =0.49 ± 0.0057), tinidazole (IS) 100 ng/spot (Retention factor 0.75 ± 0.0054).

Table 1: Calibration parameters of Ciprofloxacin hydrochloride and Ornidazole in human Plasma by HPTLC

Parameter	Ciprofloxacin hydrochloride	Ornidazole
Beer's law range (ng/ml)	100-700	100-700
Regression Equation (y = mx + c)		
Slope (m)	0.003	0.001
Intercept (c)	0.085	0.028
Correlation coefficient (r2)	0.9910	0.9960
Limit of detection(LOD g/ml)	16.35	80.13
Limit of quantitation(LOQ µg/ml)	49.55	242.81

Table 2: Result of recovery of ciprofloxacin hydrochloride and ornidazole in human plasma by HPTLC

Concentration (ng/spot)	Ciprofloxacin hydrochloride		Ornidazole	
	% Recovery*	% RSD	% Recovery*	% RSD
200	86.26	1.77	82.16	1.21
400	81.02	2.98	79.73	1.77
500	83.13	3.48	81.31	2.08
One Way Analysis of Varience(ANOVA)	P=0.226 <(theoretical p (3,0.05)= 3.18)			

* Average of 3 determinations, %RSD=percent relative standard deviation.

Table 3: Result of precision of ciprofloxacin hydrochloride and ornidazole in human plasma by HPTLC

Precision	Concentration (ng/spot)	Ciprofloxacin hydrochloride			Ornidazole		
		SD*	%RSD	% RE	SD*	%RSD	% RE
Intra-day	200	0.023	2.56	1.03	0.013	2.15	0.60
	400	0.013	1.09	0.60	0.09	1.01	1.77
	500	0.014	1.95	0.79	0.022	2.21	2.13
Inter-day	200	0.043	4.80	1.93	0.021	2.14	1.86
	400	0.023	2.33	1.03	0.024	2.51	1.08
	500	0.035	3.64	1.58	0.022	2.22	2.71

SD*= standard deviation, a verage of 5 determinations, %RSD = percent relative standard deviation, % RE=percent relative error

Table 4: Stability study of ciprofloxacin hydrochloride and ornidazole in human plasma by HPTLC

Stability parameters	Concentration (ng/spot)	Ciprofloxacin hydrochloride		Ornidazole	
		SD (n=5)	CV (%)	SD (n=5)	CV (%)
Short term 6h	Low 200	0.0057	0.89	0.023	8.94
	Mid 400	0.040	2.32	0.011	0.90
	High 500	0.049	2.03	0.011	0.68
Freeze-thaw	Low 200	0.0094	1.26	0.01	1.44
	Mid 400	0.031	3.76	0.0086	1.12
	High 500	0.010	1.47	0.011	1.38
Bench top 12 h	Low 200	0.014	2.42	0.039	6.34
	Mid 400	0.032	3.93	0.023	3.21
	High 500	0.0043	0.54	0.071	9.72
Post-preparative	Low 200	0.017	3.58	0.0037	1.08
	Mid 400	0.046	5.97	0.0095	1.094
	High 500	0.01	1.46	0.0065	0.80

was assessed by comparing QC samples injected immediately after preparation with those left in auto-sampler for 48 h.

RESULT AND DISCUSSION

Extraction procedure optimization

Most difficult parts during the method development was to achieve low 200, mid 400 and high 500 ng/ml in triplicate and reproducible recovery from the solvent which is used for extraction of the drug and also difficult task to select such single extracting solvent from which both the drugs were extracted. Different solvents were tried for the extraction of ciprofloxacin and ornidazole from human plasma. First 5 ml each of hexane and toluene were tried for the extraction of the drug from plasma but the recovery was very less. Ethyl acetate and chloroform were also tried up to 5.0 ml. It gave 50 to 55% of recovery because of less for extraction of ciprofloxacin and ornidazole from plasma. At the last methanol was tried and 70 to 80% of recovery was obtained. It was found that the addition of formic acid (0.5 ml) increases the extraction of ciprofloxacin and ornidazole from plasma and also the recovery which is reproducible and high as compare to other solvents. So methanol and formic acid (5.5:0.5 v/v) was kept as final solvent for extraction of ciprofloxacin and ornidazole.

Optimization of chromatographic method

Initially plane solvents like methanol, ethyl acetate, chloroform were tried. The spots were developed with chloroform and methanol but no proper resolution observed between ornidazole and tinidazole and ciprofloxacin also shows the tailing. Then chloroform and methanol in the ratio of (8:1v/v) was tried but again there was no proper resolution obtained. Then proportion of chloroform was increased by 1 ml showing good resolution but tinidazole show greater R_f value lastly by decreasing the concentration of methanol from 1ml to 0.8 ml and addition of 0.4 ml triethylamine, good resolution with symmetrical peaks of ciprofloxacin ornidazole and tinidazole were obtained. Finally mobile phase used consisted of chloroform: methanol: triethylamine (9: 0.8: 0.4 v/v/v) which gave good resolution of peaks for ciprofloxacin, ornidazole and tinidazole. The R_f values for ciprofloxacin, ornidazole and tinidazole were found to be 0.18 ± 0.0057, 0.49 ± 0.0057, and 0.75 ± 0.0054 respectively. Well defined spots were obtained by prewashing the plate using methanol followed by activation at 120°C for 20 min. Chamber was saturated with mobile phase for 14 min at room temperature (Figure 3).

Calibration curves

The seven point calibration curve was constructed for each of Ciprofloxacin and ornidazole 100, 200, 300, 400, 500, 600, 700 ng/ml and 400 ng of tinidazole (internal standard) were constructed by plotting the peak response ratio of ciprofloxacin to IS versus concentration of Ciprofloxacin and ornidazole to IS versus concentration of ornidazole in plasma. Correlation coefficients are 0.9910 and 0.9960 for ciprofloxacin and ornidazole respectively. Linearity was found over the range 100–700 ng/spot. The lower limit of quantification is lowest concentration in the calibration curve. The ciprofloxacin and ornidazole can be determined at LLOQ 100 ng/spot (Table 1).

Recovery

Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of same concentration. Recovery data was determined in triplicate at three concentrations (low, mid and high) as recommended by FDA guidelines.[11] Recovery was calculated with comparison of areas obtained with standard drug spiked with plasma before extraction (unextracted) at room temperature and area of slandered drug with spiked plasma after extraction (extracted). The recovery at three concentrations 200, 400, 500 ng/spot was found to be 86.26, 81.02, 83.13% for Ciprofloxacin and 82.16, 79.73, 81.31% for ornidazole. Average recoveries obtained in each instance were compared with One Way Analysis of Varience (ANOVA) TEST. As observed P=0.226 value is less than theoretical P(3,0.05)=3.18 value, it is concluded that the recoveries obtained were in agreement for drug means no significant difference will obtained. The results are shown in (Table 2).

Precision and accuracy

Precision of the method was determined by repeatability (intra-day) and intermediate precision (inter- day) and accuracy for set of quality control (QC) samples (low 200, mid 400 and high 500 ng/spot) in five replicate. The inter-day and intra-day precision and accuracy for the Ciprofloxacin and ornidazole evaluated by assaying the QC samples (low, mid and high) n=5 in %RSD. In this assay the intra-run precisions were found in the range of 1.086 to 2.56% for Ciprofloxacin and 1.006 to 2.21% for ornidazole and inter-run precision were 2.33 to 4.80% and 2.14 to 2.51% respectively for Ciprofloxacin and ornidazole. The accuracy was found to be 0.6 to 1.03% and 0.6 to 2.13% for ciprofloxacin and ornidazole respectively. The above values were obtained within acceptable range; it shows that the method is accurate and precise. The results are shown in (Table 3).

Sensitivity and selectivity

Selectivity should be assessed to show that the intended analytes were measured and their quntitation was not affected by presence of biological matrix. There was no significant interference observed and no changes in R_f of ciprofloxacin and ornidazole, the method is selective. Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision which is lower than 20%.[12] The accuracy and precision at lower limit of quantitation LLOQ was analyzed by using five replicates (n=5) of the sample at the LLOQ concentration. The accuracy is determined by % relative error at this LLOQ concentration. The lower limit of quantitation which could be detected and were found to be 100 ng/spot with % relative error=3.3 and % relative standard deviation=8.35 for Ciprofloxacin % relative error=1.22 and % relative standard deviation=2.75 for ornidazole which is within acceptable limit.

Stability

In stock solution stability three concentration levels low 200, mid 400, high 500 ng/ml and 400 ng/ml of tinidazole as internal standard were used. QC samples were thawed and left at room temperature for 6 h. Comparison of the results for QC samples (extracted) with freshly prepared stock solutions (unextracted) showed that there was no significant difference between response of freshly prepared solutions and sample of ciprofloxacin and ornidazole after 6 h. Freeze-thaw stability was determined after three freezes-thaw cycles for three replicate of low, mid and high QC samples. The samples were stored at -20°C for 24 h. Then thaw at room temperature. No significant difference between freeze-thaw sample and freshly prepared sample was observed. The result of stability study shows that no significant degradation occurred at ambient temperature for 12 h for bench-top stability. And also for the post-preparative stability studies for 48 h after comparing with freshly prepared sample. Standard stock solutions of Ciprofloxacin, ornidazole and internal standard tinidazole were stable for 12 days at 4°C. Results of stability are shown in (Table 4).

CONCLUSION

The proposed method was found to be a simple, rapid, sensitive, selective and economic high performance thin layer chromatographic method for

simultaneous determination of ciprofloxacin and ornidazole in human plasma by using tinidazole as an internal standard. The method was successfully applied to simultaneous determination of ciprofloxacin and ornidazole in human plasma without any interference from the additives. Sensitivity of the method is suitable for handling various plasma levels of the drug. In future this method can be used for clinical and pharmacokinetic studies.

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CONFLICT OF INTEREST

No conflict of interest.

ABBREVIATIONS USED

HPTLC: High Performance Thin Layer Chromatography; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **UV:** Ultra violet; **CV:** Coefficient of Variance; **SD:** Standard Deviation; **RSD:** Relative standard deviation; **Rf:** Retardation factor, **RE:** Relative error.

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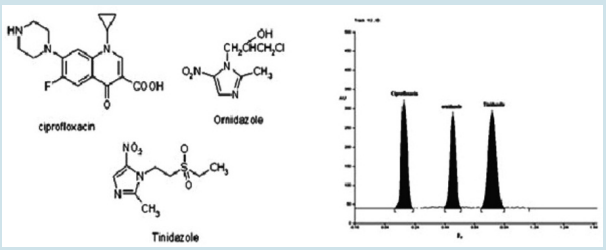
PICTORIAL ABSTRACT



SUMMARY

- HPTLC Method was Developed and Validated for Ciprofloxacin and Ornidazole in Human Plasma by high performance thin layer chromatography.
- The analysis was done using aluminum plates pre-coated with silica gel 60F254 as stationary phase and methanol: formic acid (5.5:0.5 v/v) as mobile phase.
- Developed method was applied for estimation of drugs in Human Plasma.
- The proposed method was validated statistically by performing recovery study.

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