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High Performance Liquid Chromatographic Determination of Ciprofloxacin Hydrochloride and Ornidazole in Human Plasma

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Abstract

A simple, rapid, sensitive selective chromatographic method has been developed for simultaneous determination of ciprofloxacin hydrochloride and ornidazole in human plasma by using internal standard. The method depends on reverse phase high performance liquid chromatography. The plasma sample was extracted using methanol: formic acid (5.5:0.5, v/v). A concentration range from 100-400 ng/ml for both drugs was used for calibration curve. The percent recoveries of ciprofloxacin and ornidazole were found to be 71.49-75.68 and 73.78-83.1 respectively. The mobile used consist of acetonitrile: methanol: water: triethylamine (40:20:40: 1% v/v/v) and flow rate 1 ml/min in isocratic mode. The separation was carried out by UV detector at wavelength 300 nm. The stability of ciprofloxacin hydrochloride 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 and by performing recoveries study for determination of ciprofloxacin hydrochloride and ornidazole in human plasma.

Keywords: Ciprofloxacin hydrochloride; Ornidazole; RP-HPLC; Human plasma; Liquid-liquid extraction

Introduction

Ciprofloxacin (Figure 1) [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid] 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-chloro-3-(2-methyl-5nitro-1H-imidazol-1-yl) propan-2-ol, used as an anti-infective agent. Use of ornidazole in combination with fluoroquinolone in the treatment of pelvic inflammatory disease and intra-abdominal 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 [3-8], spectrophotometry [9,10], HPLC [11-13], HPTLC [14], and spectrophotometric methods [15-17] have been reported for estimation of ornidazole alone as well as in combinations. RP-HPLC method was reported for estimation of Ciprofloxacin Hydrochloride and Ornidazole in Combined Pharmaceutical Dosage Form [18]. However no method was reported for simultaneous determination of ciprofloxacin hydrochloride and ornidazole in human plasma by RP-HPLC using liquid-liquid extraction. The proposed research work describes the simultaneous estimation of ciprofloxacin hydrochloride and ornidazole in human plasma by RP-HPLC 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 hydrochloride and ornidazole.

Materials and Methods

Instrumentation

A HPLC Knauer with Chromgate software version 3.1 having binary pumps Smartline 1000-1 and Smartline 1000-2. Detector Smartline UV-2600 was used. The analytical column employed was Eurosphere 100 C18 (250 mm × 4.6 mm × 5 µ) supplied by Knauer, Berlin, Germany. The working temperature was 28°C.

Chemicals

Ciprofloxacin hydrochloride, Ornidazole, tinidazole having 99.00%, 98.77%, 99.88% purity respectively were received from Aarti

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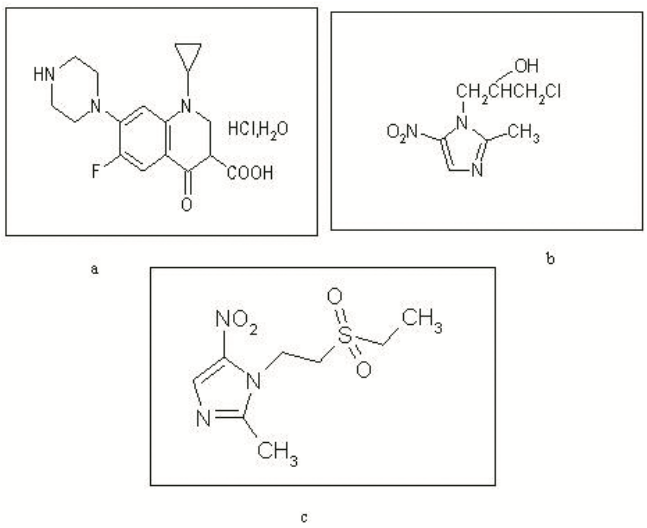

Figure 1: Structure of (a) ciprofloxacin hydrochloride, (b) ornidazole and (c) tinidazole (IS).

Drugs Pvt. Ltd., Tarapur, Mumbai, Maharashtra, India. The HPLC grade acetonitrile, methanol, water, triethylamine were purchased from SD Fine Chem, Mumbai, India. Analytical formic acid was purchased from SD Fine Chem, Mumbai, India. Human plasma used for research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

Preparation of stock solutions and working standard solutions

Stock solutions 1000 µg/ml each of ciprofloxacin hydrochloride, ornidazole, tinidazole was prepared in methanol. The working standard solutions of 100 µg/ml of ciprofloxacin hydrochloride, ornidazole and tinidazole were prepared by further dilutions of stock solutions in mobile phase. Stock solution was stored at room temperature 28°C.

Preparation of plasma sample

In a 15 ml centrifuge tube 0, 1, 1.5, 2, 2.5, 3, 3.5, 40 µl of working stock solutions of ciprofloxacin hydrochloride and ornidazole were added to drug free plasma to provide calibration standards of 0 (no ciprofloxacin and ornidazole added) 100, 150, 200, 250, 300, 350, 400 ng/ml and 300 ng/ml of tinidazole (internal standard) was kept constant. The quality control (QC) samples were prepared in plasma for both drugs in concentration range 200, 300, 400 ng. Extractions of drugs were carried out by using methanol: formic acid (5.5 ml: 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 1 ml mobile phase. The analysis was carried on HPLC system.

Chromatographic condition

The mobile phase was selected as mixture of acetonitrile, methanol, water and triethylamine in the ratio of (40: 20: 40: 1%, v/v/v). The mobile phase was degassed prior to use under vacuum by filtration through Nylon 66 membrane of 47 mm size and 0.45 µm thicknesses with 20 µl injection. The detector was set at 300 nm. Flow rate was used 1 ml/min. An isocratic mode was used for the separation of the analyte.

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. Three replicate of three QC sample low, mid and high were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. The precision and recoveries of ciprofloxacin and ornidazole were calculated by comparison of the peak areas of low, mid, and high quality control sample (200, 300 and 400 ng/ml respectively) prepared in plasma (extracted) with unextracted ciprofloxacin hydrochloride and ornidazole in methanol by using peak areas of low, mid, high quality control sample (200, 300 and 400 ng/ml respectively) in similar manner. Stability experiments were undertaken to detect degradation of ciprofloxacin hydrochloride and ornidazole under certain condition. The stock solution stability of ciprofloxacin hydrochloride and ornidazole were examined at room temperature for 6 h. Freeze–thaw stability were determined at three QC concentrations (low, mid, high) 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 was assessed by comparing QC samples injected immediately after preparation with those left in auto sampler for 48 h.

Results and Discussion

Extraction procedure optimization

One of the most difficult parts during the method development was to achieve a low 200, mid 300 and high 400 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 are extracted. Different solvents were tried for the extraction of ciprofloxacin hydrochloride and ornidazole from human plasma. Five ml each of hexane and toluene were tried for the precipitation of 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 precipitation of protein 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 precipitation of protein and also the recovery which is reproducible and high as compare to other solvents. So methanol and formic acid (5.5 ml: 0.5 ml) was kept as final solvent for extraction of ciprofloxacin hydrochloride and ornidazole.

Optimization of chromatographic method for plasma study

For HPLC the method demonstrated good chromatographic specificity with no plasma interference at the retention times of ciprofloxacin hydrochloride, ornidazole and internal standard, representative chromatogram of plasma spiked with ciprofloxacin hydrochloride ornidazole and internal standard tinidazole. Ciprofloxacin hydrochloride, ornidazole, tinidazole well resolved with good symmetry and retention time of 2.13, 4.91, 6.86 min are shown in Figure 2. Initially plain solvent like acetonitrile, water, methanol were used but retention time for the all three drugs are above 10 min. Then methanol and water mixed in the ratio of (70:30 v/v) and vice-versa but no change was observed. Then addition of acetonitrile was done which leads to shorten the retention time and also shows good resolution between ciprofloxacin hydrochloride, ornidazole and tinidazole but the peak shape were not good. Then ratio of acetonitrile: methanol: water change to (40:20:40 v/v/v) by using this mobile phase resolution were increased for both the drugs and shows good separation in this mobile

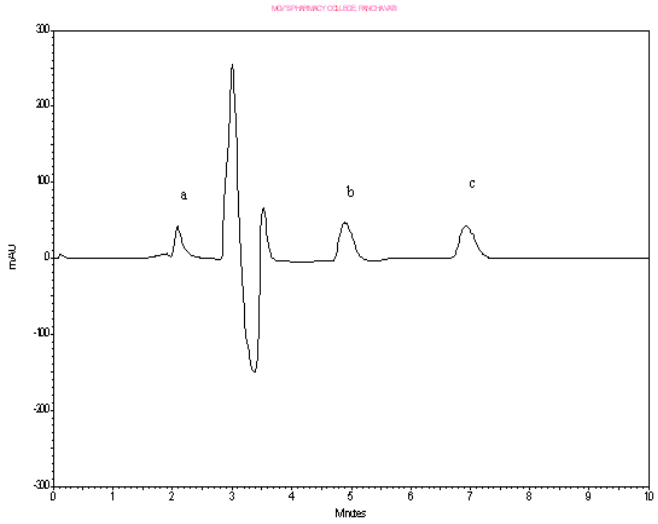

Figure 2: HPLC chromatogram of ciprofloxacin hydrochloride 200 ng/ml (Retention Time=2.13), ornidazole 200 ng/ml (Retention Time=4.91), tinidazole (Internal standard) 300 ng/ml.

phase composition addition of 1% triethylamine leads to good shape of peaks for all three drugs. So ratio of acetonitrile: methanol: water: triethylamine (40:20:40: 1% v/v/v/v) was selected as final mobile phase. For validation purpose flow rate of 1 ml/min, wavelength of detection 300 nm and working area temperature 28°C were used.

Calibration curves

The calibration curve was constructed for each of ciprofloxacin hydrochloride and ornidazole 100, 150, 200, 250, 300, 350 and 400 ng/ml in triplicate by plotting the peak response ratio of ciprofloxacin hydrochloride to IS versus concentration of ciprofloxacin hydrochloride and ornidazole to IS versus concentration of ornidazole in plasma. Correlation coefficients were found to be 0.9995 and 0.9993 for ciprofloxacin hydrochloride and ornidazole respectively. Linearity's were found over the range 100-400 ng/ml. The lower limit of quantification was defined as lowest concentration in the calibration curve. The ciprofloxacin hydrochloride and ornidazole can be determined at LLOQ 100 ng/ml (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 200, mid 300 and high 400 ng/ml) as recommended by FDA guidelines [19]. Recovery was calculated with comparison of areas obtained with standard drug spiked with plasma before extraction (unextracted) at room temperature and area obtained of slandered drug with spiked plasma after extraction (extracted). The recovery at three concentrations 200, 300, 400 ng/ml was found to be 71.49, 77.23, 75.68% for ciprofloxacin hydrochloride and 73.78, 71.15, 83.1% for ornidazole, 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 300 and high 400 ng/ml) in three replicate. The inter-day and intra-day precision and accuracy for the ciprofloxacin hydrochloride and ornidazole evaluated by assaying the QC samples (low, mid, high) n=3 in % RSD. In this assay the intra-run precisions were found in the range of 0.5 to 8% for ciprofloxacin hydrochloride and 0.69 to 4.07% for ornidazole, and the inter-run precision were 4.50 to 5.38% and 1.96 to 2.8.09% respectively for ciprofloxacin hydrochloride and ornidazole. The accuracy was within 0.60 to 6.40% and 4.00 to 12.66% for ciprofloxacin hydrochloride and ornidazole respectively. The above values were within acceptable range. It shows that the method is accurate and precise. The result of inter-day, intra-day precision and accuracy for ciprofloxacin hydrochloride and ornidazole are shown in Table 3.

Sensitivity and selectivity

Selectivity should be assessed to show that the intended analytes are measured and that their quantitation is not affected by presence of biological matrix. There was no significant interference observed and no changes in retention time of ciprofloxacin and ornidazole which shows 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% [20]. The accuracy and precision at lower limit of quantitation (LLOQ) analyzed by using five replicates (n=5) of the sample (100 ng/ml) at the LLOQ concentration. The accuracy is determined by %RE at this LLOQ concentration. The lower limit of quantitation which could be detected and were found to be % Relative Error=11 and %Relative Standard Deviation=6.54 for ciprofloxacin hydrochloride and % Relative error=10.80 and % Relative Standard Deviation=1.75 for ornidazole which is within acceptable limit.

Stability

From stock solution for stability three concentration levels low 200, mid 300, high 400 ng/ml and 300 ng/ml of tinidazole as internal standard

Parameter	Ciprofloxacin hydrochloride	Ornidazole
Beer's law range (ng/ml)	100-400	100-400
Regression Equation (y=mx+c)		
Slope (m)	0.038	0.024
Intercept (c)	0.051	0.00714
Correlation coefficient (r^2)	0.9995	0.9993
Limit of detection (LOD g/ml)	0.501	2.75
Limit of quantitation (LOQ µg/ml)	1.50	8.33

Table 1: Calibration parameters of Ciprofloxacin hydrochloride and Ornidazole in human plasma by HPLC.

Concentration (ng/ml)	Ciprofloxacin hydrochloride		Ornidazole	
	% Recovery (n=3)	% RSD	% Recovery (n=3)	% RSD
200	71.49	1.03	73.78	1.69
300	77.23	1.60	71.78	4.85
400	75.68	0.35	83.10	3.91

Table 2: Result of recovery of ciprofloxacin hydrochloride and ornidazole in human plasma. % RSD: Relative standard deviation.

Precision	Concentration (ng/ml)	Ciprofloxacin hydrochloride			Ornidazole		
		SD (n=3)	% RSD	% RE	SD (n=3)	% RSD	% RE
Intra-day	200	0.080	8.00	6.40	0.043	4.07	5.00
	300	0.025	2.50	1.03	0.011	0.73	12.66
	400	0.005	0.50	3.50	0.015	0.69	5.00
Inter-day	200	0.034	5.24	0.60	0.085	8.09	7.30
	300	0.036	5.38	2.13	0.045	3.01	4.00
	400	0.037	4.50	3.50	0.049	1.96	3.32

Table 3: Precision of ciprofloxacin hydrochloride and ornidazole in human plasma. SD: Standard deviation; % RSD: Relative standard deviation; % RE: Relative error.

Stability parameters	Concentration (ng/ml)	Ciprofloxacin hydrochloride		Ornidazole	
		SD (n=3)	CV (%)	SD (n=3)	CV (%)
Short term 6 h	Low 200	0.08	1.40	0.17	1.20
	Mid 300	0.23	1.31	0.38	1.29
	High 400	0.58	1.39	0.99	1.33
Freeze-thaw	Low 200	0.70	1.54	0.25	1.55
	Mid 300	0.96	1.62	0.15	1.52
	High 400	0.10	1.44	0.28	1.36
Bench top 12 h	Low 200	0.11	1.53	0.60	1.39
	Mid 300	0.49	1.509	0.41	1.38
	High 400	0.79	1.46	1.21	1.45
Post-preparative	Low 200	0.43	3.03	0.13	1.46
	Mid 300	0.35	2.05	0.52	1.022
	High 400	0.77	1.45	0.08	1.080

Table 4: Stability study of ciprofloxacin hydrochloride and ornidazole in human plasma. SD: Standard deviation (n=3); CV (%): Coefficient of variance.

were used. QC samples were thawed and left at room temperature for 6 h. Comparison of the results for QC samples with freshly prepared stock solutions showed that there was no significant difference between response of freshly prepared solutions and sample of ciprofloxacin hydrochloride 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 hydrochloride, ornidazole and internal standard tinidazole were stable for 12 days at 4°C. Results of stability are shown in Table 4.

Conclusion

The method was successfully applied to simultaneous determination of ciprofloxacin and ornidazole in human plasma without any interference from the additives and endogenous substances. It is a simple, selective and sensitive method requiring inexpensive reagents. 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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