A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF AZITHROMYCIN, FLUCONAZOLE AND ORNIDAZOLE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Objective: The objective of the study was to develop and validate a new rapid and more sensitive Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the simultaneous estimation of azithromycin, fluconazole and ornidazole in bulk and pharmaceutical dosage forms.

Methods: Separation was achieved with a cap cell pack C18 column (4.6 x 250 mm, 5 μ) with an isocratic mobile phase containing a mixture of acetonitrile and phosphate buffer pH 4.0 [adjusted with ortho-phosphoric acid] (50:50 % v/v) at the flow rate of 1 ml/min and detection was monitored at 210 nm.

Results: The retention time (Rt) of azithromycin, fluconazole and ornidazole were found to be 4.82±0.01, 5.25±0.01 and 6.33±0.01 min respectively. The precision was found with <1.5% of %RSD. The calibration curve was linear over the concentration ranging from 500-1000 µg/ml, whereas Limit of quantification limits (LOQ) was found to be 9.834, 2.667 and 7.980 µg/ml, respectively.

Conclusion: A simple isocratic liquid chromatographic method was developed and validated for simultaneous estimation of azithromycin, fluconazole and ornidazole in their formulations. Due to its simplicity, rapidness and specificity, it can be applied for routine quality control analysis of these drugs.

Keywords: Azithromycin, Fluconazole, Ornidazole, Method development, RP-HPLC

INTRODUCTION

Azithromycin is an antibiotic useful for the treatment of a number of bacterial infections, including middle ear infections, strep throat, bacterial infections, including middle ear infections, strep throat, pneumonia, traveler’s diarrhea and certain other infections. It is also used for sexually transmitted infections such as chlamydia and gonorrhea. It is chemically derived from erythromycin; it acts on bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. Thereby, inhibits peptidyl transferase activity and interferes with amino acid translocation during the process of translation.

Fluconazole is an anti-fungal medication which is being used in the number of fungal infections. It is primarily used in the treatment of candidiasis, blastomycosis, coccidiodymosis, cryptococcosis, histoplasmosis, dermatophytosis and pityriasis versicolor. It is a drug of choice in prevention of candidiasis during organ transplantation.

Ornidazole is a drug that cures some protozoan infections. It has been investigated for use in Crohn’s disease after bowel resection. It is used for the treatment of stomach, intestinal, urinary tract and genital infections. Formation of redox intermediate (an intracellular metabolite) is believed to be the key component responsible for killing microorganisms.

Combination of azithromycin, fluconazole and ornidazole were co-administered to treat a variety of skin infections such as eczema, fungal skin infections including ringworm etc. Combination therapy commonly administered in the trade name of AF-kit (azithromycin-1000 mg; ornidazole-750 mg and fluconazole-150 mg) (fig. 1). As these drugs are frequently co-administered in various infections, an effective, simple and specific analytical method is required for simultaneous estimation, thereby it will be helpful in the quantification of drugs in the formulation as well as in the biological matrices. An extensive literature survey suggests that development of various analytical techniques such as ultraviolet (UV)-visible spectroscopy [1-6], HPLC [6-24], HPTLC [25-28] and LCMS [29-32] for quantification of these drugs either in individual or combination with other drugs. Recently, Krishna et al. [21] reported an RP-HPLC method with the help of sodium dihydrogen ortho-phosphate (pH-5.2): acetonitrile in the ratio of 70:30 (%v/v) as mobile phase. Another study by Aranya et al. [24] reported a stability-indicating RP-HPLC method for simultaneous estimation of these drugs with the help of sodium dihydrogen ortho-phosphate (pH-5.2): acetonitrile in the ratio of 50:50 % v/v ratio as a mobile phase without using any IS. Moreover, the rapid elution with shorter runtime was observed at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min for azithromycin, fluconazole and ornidazole, respectively. In addition, the proposed method was validated as per ICH guidelines in terms of various parameters, and the results were found to be within acceptance criteria. In these studies, the method was found to be simple, rapid and more precise.

Fig. 1: Chemical structures of azithromycin, fluconazole and ornidazole
**Materials and Methods**

**Instrumentation**

Lab India UV-3000 double beam UV-Visible spectrophotometer was used to carry out absorption studies and the data were recorded by UV-Win software. Standard cuvettes of 10 mm path length were used for analysis. Cyber lab LC-100 HPLC system is accomplished with UV-detector, quantitative HPLC was performed on an isocratic mode using cap cell pack C18 (4.6 x 250 mm, 5μ) column with 20 μL injections of the sample loop. The output signal was monitored and integrated using Cyber lab LC-100 software. Lifecare ultra-sonic cleaner was used to sonicate the standard and sample solutions.

**Chemicals and reagents**

Standard drugs of azithromycin, fluconazole and ornidazole were obtained as gift samples from Cipla Laboratory, Malapur and Aurobindo Pharmaceuticals, Hyderabad. Market formulation (AF-kit, Madras Pharmaceuticals, Chennai) was procured from the local market. HPLC grade acetonitrile and methanol were obtained from Merck Life Sciences, Mumbai, India. Analytical grade solvents and other chemicals were acquired from SD Fine Chemicals, Mumbai, India. Water was obtained from millipore with milli Q system, filtered through 0.45 μ nylon membrane for the HPLC experiments.

**Preparation of phosphate buffer**

Phosphate buffer was prepared by dissolving 13.6 g of potassium dihydrogen ortho-phosphate in 1000 ml of water (HPLC grade) and pH was adjusted to 4.8 with ortho-phosphoric acid and solution was filtered through 0.45 μ millipore nylon filter.

**Preparation of the mobile phase**

Acetonitrile: phosphate buffer (pH-4.8) in the ratio of 50:50 % v/v was prepared and it was filtered through 0.45 μ millipore nylon filter. The solution was degassed with ultrasonic cleaner for 15 min. The resultant solution was used as the mobile phase.

**Chromatographic conditions**

The method was developed by using a cap cell pack C18 column (4.6 x 250 mm, 5μ) with an isocratic mobile phase which consists a mixture of acetonitrile and potassium dihydrogen ortho-phosphate buffer (pH-4.8 adjusted with ortho-phosphoric acid) in 50:50 % v/v ratio. The mobile phase was filtered through 0.45 μ millipore nylon filter under vacuum filtration. Flow rate of the mobile phase was adjusted to 1 ml/min. The eluted compounds were detected at the wavelength of 210 nm. The sample injection volume was 20 μL.

**Preparation of a standard mixture of azithromycin, fluconazole and ornidazole**

Transfer 100 mg of azithromycin, 15 mg of fluconazole and 75 mg of ornidazole into a 100 ml of volumetric flask and add 70 ml of diluent. Then it was sonicated for 15 min and the volume made up to 100 ml with diluent. The resulted solution (20 μL) was injected into the HPLC system by employing optimized chromatographic conditions.

**Preparation of sample mixture of azithromycin, fluconazole and ornidazole**

Twenty tablets of each of azithromycin, fluconazole and ornidazole (AF-Kit) were weighed and the average weight of each tablet was determined individually. The tablets were crushed into a fine powder, accurately weighed tablet powder equivalent to 100 mg (0.1121 g) of azithromycin, 15 mg (0.239 g) of fluconazole and 75 mg (0.169 g) of ornidazole and transferred into a clean 100 ml volumetric flask. Add 70 ml of diluents, and then sonicated for 15 min to dissolve, made up to the volume with diluents. The resulted solution was filtered through 0.45μ membrane filter and then above resulted solution 20 μL was injected into the HPLC system.

**Selection of detection wavelength**

The standard stock solution of azithromycin, fluconazole and ornidazole in the concentration of 10 μg/ml was prepared and each solution was scanned in UV range (200-400 nm) in 10 mm path length against the solvent blank. The overlain spectrum of three drugs was recorded against solvent blank.

**Method validation**

Method validation was performed using standard and sample solutions of analytes as per ICH guidelines for proposed method [33-35]. The following validation parameters performed such as specificity, linearity, precision, accuracy, robustness, LOD and LOQ etc.

**Results**

The purpose of the present study was to develop a rapid and sensitive RP-HPLC method for the simultaneous estimation of azithromycin, fluconazole and ornidazole in their dosage form using cap cell pack C18 analytical column with UV detection.

**Selection of detection wavelength**

The study of the absorption spectrum for three drugs revealed that well-defined λmax. The iso-absorptive point was found at 210 nm for three drugs at the given concentration. Obtained wavelength maxima and iso-absorptive point for the three drugs were used for the simultaneous estimation by using RP-HPLC method. The overlain spectrum of azithromycin, fluconazole and ornidazole was shown in fig. 2.

![Fig. 2: Overlain spectrum of azithromycin, fluconazole and ornidazole](image)

**Method optimization**

Optimization of chromatographic conditions for isocratic RP-HPLC detection, parameters such as mobile phase composition, pH (4.8-6.3) and flow rate (1 ml/min±0.2) were varied for system suitability studies. The variation in the mobile phase led to considerable changes in the chromatographic parameters like asymmetric factor, capacity factor and retention time (Rt). Varying in pH showed that optimized conditions were reached at pH 4.8, producing well resolved and sharp peaks for three drugs. Henceforth, in the present method, the acetonitrile and phosphate buffer (50:50 % v/v) (pH-4.8) was used as a mobile phase with 1.0 ml/min flow rate as optimal conditions. The detection of peaks achieved at 210 nm for the three analyte drugs. For quantitative determination of azithromycin, fluconazole and ornidazole in formulations, initially mixed standard solutions in appropriate concentrations were injected into the column and Rt's of azithromycin, fluconazole and ornidazole was found to be 4.82±0.01, 5.25±0.01 and 6.33±0.01 min, respectively (fig. 5).

**Method validation**

Method validation was performed as per ICH guidelines for simultaneous determination of the azithromycin, fluconazole and ornidazole. The results of parameters such as specificity, linearity, precision, accuracy, robustness, detection limit, quantification limit were described in the following sections.

**Specificity**

No interference of additives peak was found in the chromatogram for three drugs in tablet formulation, which indicates the proposed method is specific. Blank determination also performed where no appearance or interference of peaks observed.
Linearity

Standard solutions at six different concentration levels ranging from 500-1000 µg/ml for azithromycin, 75-150 µg/ml for fluconazole and 375-750 µg/ml for ornidazole were prepared and analyzed in order to demonstrate the linearity relationships. For linearity study, the standard solutions were prepared as per label claim amount in the tablets. The regression curve was obtained by plotting the peak area v/s concentration of each analyte (fig. 4-6). It was obtained by least-squares method [15]. The correlation coefficient ($r^2$) values were found to 0.999 for all the drugs. Detailed Linearity data were summarized in table 1.

![Fig. 3: Standard chromatogram of azithromycin, fluconazole and ornidazole in acetonitrile and phosphate buffer pH-4.8 (50:50%v/v)](image)

![Fig. 4: Calibration curve of azithromycin (1000-500 µg/ml)](image)

![Fig. 5: Calibration curve of fluconazole (75-150 µg/ml)](image)
Table 1: Linearity data of azithromycin, fluconazole, ornidazole

| Analyte    | Concentration (µg/ml) | Peak area (mV) | Linear regression equation |
|------------|-----------------------|----------------|---------------------------|
| Azithromycin | 500                   | 212776         |                           |
|            | 600                   | 262627         | $y = 439.5x + 1549$       |
|            | 700                   | 312991.6       | $r^2 = 0.999$             |
|            | 800                   | 353358.8       |                           |
|            | 900                   | 395606.7       |                           |
|            | 1000                  | 438629         |                           |
|            | 75                    | 197526.9       |                           |
| Fluconazole | 90                    | 239585.4       | $y = 2643x + 1247$        |
|            | 105                   | 282508.2       | $r^2 = 0.999$             |
|            | 120                   | 315641.9       |                           |
|            | 135                   | 357006.2       |                           |
|            | 150                   | 438629         |                           |
|            | 375                   | 288696         |                           |
| Ornidazole | 450                   | 346460.7       | $y = 772.2x + 1448$       |
|            | 525                   | 409934.6       | $r^2 = 0.999$             |
|            | 600                   | 464002.2       |                           |
|            | 625                   | 490068.5       |                           |
|            | 750                   | 570405.9       |                           |
Accuracy
Standard addition experiments were employed for accuracy studies in which the percent (%) recovery was determined. Accuracy of the method was evaluated in triplicate at three concentration levels, i.e. 50 %, 100 % and 150 % of target concentration and the percentages of recoveries were calculated. Resulted chromatograms at these levels were shown in fig. 7-9. Percentage recoveries of three drugs found in between 98.3-101.2 %, which indicates the results obtained were within the potency range (table 2-4).

![Chromatogram of 150% standard addition (level-3)](image)

**Table 2: Accuracy data of azithromycin (n = 3)**

| S. No. | Spiked level | Sample area | Sample height | % Recovery | % mean recovery±SD |
|--------|--------------|-------------|---------------|------------|-------------------|
| 1      | 50%          | 228316      | 2446          | 101.4      | 101.2±0.288       |
| 2      | 100%         | 438962      | 49343         | 99.2       | 99.3±0.472        |
| 3      | 150%         | 668649      | 74832         | 98.2       | 98.3±0.264        |

**Table 3: Accuracy data of fluconazole (n= 3)**

| S. No. | Spiked level | Sample area | Sample height | % Recovery | % mean recovery±SD |
|--------|--------------|-------------|---------------|------------|-------------------|
| 1      | 50%          | 205368      | 21689         | 101.0      | 100±0.0577        |
| 2      | 100%         | 395978      | 43189         | 98.5       | 99.8±1.209        |
| 3      | 150%         | 603246      | 64783         | 99.1       | 99±0.0577         |

**Table 4: Accuracy data of ornidazole (n = 3)**

| S. No. | Spiked level | Sample area | Sample height | % Recovery | % mean recovery±SD |
|--------|--------------|-------------|---------------|------------|-------------------|
| 1      | 50%          | 295162      | 35021         | 100.8      | 100.1±0.578       |
| 2      | 100%         | 579209      | 70861         | 101.1      | 100.5±0.871       |
| 3      | 150%         | 869543      | 105856        | 99.7       | 99.5±0.378        |

**Precision**
Precision of the method was determined by injecting the standard and sample solutions of azithromycin, fluconazole and ornidazole separately for six times and measured % RSD with the help of peak area for all six injections. System precision was established by injecting six replicate injections of standard solutions into the chromatographic system by maintaining the optimized conditions. Method precision was established by injecting six replicate injections of sample solution into the chromatographic system by...
maintaining the optimized conditions. In both cases, the % RSD found to be <2%, which indicate the proposed method was more precise. The precision data were summarized in table 5 and 6.

Robustness

Robustness experiments were carried by altering chromatographic conditions such as flow rate and detection wavelength to demonstrate any deliberate changes in the proposed method. The changes were made in the chromatographic conditions, viz. change in flow rate by ±0.2 ml/min and change in the wavelength ±5 °C. The % RSD was found <2% for three drugs even slight change of flow rate as well as detection wavelength (table 7, 8 and 9). As there were no significant variations in elution time for all the three drugs, indicate the method was robust.

Table 5: System precision data of azithromycin, fluconazole and ornidazole (n=3)

| S. No | Injection | Azithromycin | Fluconazole | Ornidazole |
|-------|-----------|--------------|-------------|------------|
|       | Rt (min)  | Peak area    |             |            |
| 1     | 4.81      | 438629       | 38393       | 560485     |
| 2     | 4.82      | 438732       | 383871      | 564479     |
| 3     | 4.81      | 429781       | 383878      | 566369     |
| 4     | 4.82      | 429658       | 386958      | 568349     |
| 5     | 4.81      | 429268       | 386215      | 566332     |
| 6     | 4.82      | 429658       | 386115      | 568911     |
| mean±SD | 4.83   | 432621±3268.5 | 38507±232.5 | 56517±1639.1 |
| % RSD |          | 0.75         | 0.06        | 0.28       |

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 6: Method precision data of azithromycin, fluconazole and ornidazole (n=3)

| S. No | Azithromycin | Fluconazole | Ornidazole |
|-------|--------------|-------------|------------|
|       | Rt (min)     | Peak area   |             |            |
| Injection-1 | 4.81   | 438654      |             |            |
| Injection-2 | 4.81   | 438962      |             |            |
| Injection-3 | 4.82   | 446756      |             |            |
| Injection-4 | 4.82   | 448756      |             |            |
| Injection-5 | 4.81   | 448851      |             |            |
| Injection-6 | 4.81   | 446231      |             |            |
| mean±SD | 4.82   | 444701.0±3031.6 |             |            |
| % RSD |          | 0.68         | 0.02        | 0.16       |

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 7: Robustness data of azithromycin

| Factor   | Level | Mean±SD of area | %RSD |
|----------|-------|-----------------|------|
| Flow rate (ml/min) | 0.8   | 427494.5±1579.11 | 0.36 |
| Wavelength (nm) | 205   | 554261±84.852    | 0.01 |
|           | 210   | 435941.7±3800.6  | 0.87 |
|           | 215   | 544333±1323.13   | 0.16 |

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 8: Robustness data of fluconazole

| Factor   | Level | Mean±SD of area | %RSD |
|----------|-------|-----------------|------|
| Flow rate (ml/min) | 0.8   | 385962.5±744.58  | 0.19 |
| Wavelength (nm) | 205   | 484532±5124.91   | 0.26 |
|           | 210   | 383603±58.6      | 0.01 |
|           | 215   | 485995±500.63    | 0.10 |

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 9: Robustness data of ornidazole

| Factor   | Level | Mean±SD of area | %RSD |
|----------|-------|-----------------|------|
| Flow rate (ml/min) | 0.8   | 567256±2265.5   | 0.39 |
| Wavelength (nm) | 205   | 625212.5±566.39 | 0.09 |
|           | 210   | 563655±2268.7   | 0.04 |
|           | 215   | 625656.5±371.23 | 0.05 |

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation
Limits of detection and quantification (LOD and LOQ)

The sensitivity of the method was measured by calculating the LOD and LOQ. LOD and LOQ were assessed as per ICH guidelines, i.e., at signals to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations (fig. 10 and 11). The linear regression equation was plotted for concentration v/s area of the peak. The standard deviation of y-intercepts and slope were considered for determination of sensitivity ranges for LOD and LOQ (table 10 and 11).

Table 10: LOD data of azithromycin, fluconazole and ornidazole (n = 3)

| S. No. | Drug       | Peak area | LOD (µg/ml) |
|--------|------------|-----------|-------------|
| 1      | Azithromycin | 2157      | 5.810       |
| 2      | Fluconazole | 9950      | 1.790       |
| 3      | Ornidazole  | 57739.2   | 4.924       |

n = number of determinations.

Table 11: LOQ Data of azithromycin, fluconazole and ornidazole (n = 3)

| S. No. | Drug       | Peak area | LOQ (µg/ml) |
|--------|------------|-----------|-------------|
| 1      | Azithromycin | 3878      | 9.834       |
| 2      | Fluconazole | 14887     | 2.667       |
| 3      | Ornidazole  | 96232.1   | 7.980       |

n = number of determinations.

DISCUSSION

The present study describes the development and validation of a RP-HPLC method for simultaneous estimation of azithromycin, fluconazole and ornidazole in bulk drug and in their tablet dosage form. The separation was achieved on cell pack C18 column (4.6 × 250 mm, 5µ) with UV detection. The method involves with acetonitrile and phosphate buffer pH-4.8 (50:50 % v/v) as mobile phase. The flow rate was monitored at 1.0 ml/min with an injection volume 20 µl. The separation was achieved by UV-detection wavelength at 210 nm. The peaks were eluted at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min for azithromycin, fluconazole and ornidazole, respectively which was found with a short time of elution and well resolved to that of the reported method [22].

The decrease in buffer content (aqueous content) as well as adjusting pH at 4.8 of the mobile phase system resulted in rapid elution of fluconazole (RT-5.25 min). The system suitability parameters such as theoretical plates and tailing factor were found to be 8310, 1.0 (azithromycin), 8288, 1.8 (fluconazole) and 11846,
1.5 (ornidazole). Linearity was found over the concentration range of 500-1000 µg/ml for azithromycin, 75-1500 µg/ml for fluconazole and 375-750 µg/ml for ornidazole with a correlation coefficient (r) of 0.999 for three drugs. The method found to be more accurate with % recoveries of 100-102 %, 99-100 % and 98-102 % for azithromycin, fluconazole and ornidazole, respectively. LOD values were 5.810 µg/ml (azithromycin), 1.790 µg/ml (fluconazole) and 4.924 µg/ml (ornidazole). LOQ values were 9.834 µg/ml (azithromycin), 2.667 µg/ml (fluconazole) and 7.980 µg/ml (ornidazole). The present liquid chromatographic method was found that it is as more appropriate for simultaneous estimation of such multi-component (three-drug combinations) pharmaceutical dosage forms.

CONCLUSION
A simple, accurate and precise RP-HPLC method has been developed for the estimation of azithromycin, fluconazole and ornidazole in tablet dosage form using UV-detector. A RP-cell pack C18 column (4.6 × 250 mm, 5 µ) with a mobile phase consisting of acetonitrile and phosphate buffer (pH-4.8) (50:50 % v/v) at 1 ml/min flow rate was used and the effluents were monitored at 210 nm. The results obtained by the proposed method were found as highly resolved, rapid with shorter runtime over previous reported methods. The peaks were eluted at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min, respectively. The percentage recovery was found to be 100-102 %, 99-100 % and 98-102 % with accepted limits of % RSD (<2%) for three drugs, respectively. The present RP-HPLC method developed was well suitable for routine analysis of these drugs in their pharmaceutical formulation and it can also be applicable to biological samples.

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AUTHORS CONTRIBUTIONS
All authors contributed equally to this manuscript

CONFLICTS OF INTERESTS
The authors declare no conflict of interest. It has not meant to publish elsewhere. And it has not meant simultaneously presented for publication elsewhere. All authors have decided to the submission to the journal.

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