IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
December 2021 Vol.:23, Issue:1

Development, Optimization and Validation of Rapid Chemometric Assisted RP-HPLC Method for The Simultaneous Estimation of One of The UTI Drug Combination in Bulk and In Tablet Dosage Form

IJPPR
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ISSN 2349-7203
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Submitted: 25 November 2021
Accepted: 30 November 2021
Published: 30 December 2021

Keywords: Multi-Criteria Decision Making Approach, Central composite design, Response Surface Methodology, RP-HPLC, Metronidazole, Norfloxacin.

ABSTRACT

Objective: This paper represents a simple, recently developed, optimized, and validated isocratic RP-HPLC design for the separation of two antibiotic drugs (Norfloxacin – NFX and Metronidazole - MNZ) in bulk and pharmaceutical formulation assisted with the quality by design and multi-criteria decision-making approach. **Methods:** The efficient chromatographic separation was achieved by utilizing the Intek Chromosal C_{18} Column (4.6 X 250mm id, 5µm molecule size) and PDA- UV- detection at 290nm. The purview of independent variables utilized for the designs was Acetonitrile concentration: 65-75%, pH: 3.8-4.2, and the flowrate: 0.8-1.2ml/min. **Results:** The optimized conditions for the assay were Acetonitrile: phosphate buffer *(*60:40*)*, the flow rate of 0.8ml/min, and the pH 3.8. For the evaluation of pharmaceutical formulation, a peak area ratio was utilized. The total chromatographic analysis run time per sample was approximately 5mins and the retention time for MNZ and NFX was 2.71mins and 3.55mins respectively. Then it was validated as per ICH guidelines under the optimized assay circumstance and applied for quantitative analysis of marketed tablets containing MNZ and NFX. **Conclusion:** The proposed method represents an efficient and easily accomplishable approach to resolving the problem of searching for the optimum RP-HPLC. The investigation also showed that the chromatographic technique coupled with chemometric tools provide useful information on separation and elution time, making this combined technique a powerful analytical tool. The validation study vindicated the determination of assay conditions by affirming that the assay was specific, accurate, linear, precise, rugged, and robust for the estimation of MNZ and NFX. Therefore, this method can be used for routine quality control analysis in the pharmaceutical environment.

INTRODUCTION

The bacterial infection occurred and affects the urinary tract is known as Urinary Tract Infection **(UTI)**[1]. Generally, UTI causing bacteria like *E. Coli* is typically entered to the bladder via the urethra, and also believed that the bacteria transmitted from bowel to urethra. Mainly females had a high risk of UTI due to their anatomy[2]. After the successful entry of bacteria into the bladder, it will be attached on its wall and forms a biofilm that resists the body's immune response[3].

Commonly UTI is classified into two types.

1. **Uncomplicated UTI** is Lower Urinary Tract Infection or Cystitis which is a Bladder infection. In many cases, the Uncomplicated UTI will resolve spontaneously without any treatment, but some patients seek treatment for their symptoms.

2. The Recurrent urinary infection with relapse or re-infection with a same or new organism, sometimes life-threatening due to the predisposing factors as well as multi-drug resistant bacteria is known as **Complicated UTI.** Any Male UTI is considered as Complicated[4,5].

UTI treatments are pointed towards the prevention of spreading the infection to the Kidney (upper parts) by inhibiting the bacterial enzymes like DNA gyrase and DNA topoisomerase which were required for bacterial DNA replication, transcription repair, and recombination.

Inhibiting nucleic acid which leads to DNA disruption. These enzymes and nucleic acid were inhibited by certain antibiotics and their combinations like Quinolones (Nalidixic acid, Cinoxacin), FluoroQuinolones (Ciprofloxacin, Ofloxacin, NFXfloxacin, Levofloxacin etc.,), Nitroimidazoles (MNZnidazole, Tinidazole, Nimorazole, etc.,), etc.[6]

Norfloxacin (NFX) chemically known as 1-ethyl-6-fluro-4-oxo-7-piperazin-1-yl-1-quinolin-3-carboxylicacid. It is a whitish yellow crystalline powder and soluble in acetic acid, acetone, and chloroform, and slightly soluble in water. It is a fluro-quinoline derivative and broad-spectrum anti-bacterial agent against gram positive and negative organism[7]. Literature review discloses that remarkable analytical methods have been reported for the estimation of Norfloxacin and Tinidazole[8], Norfloxacin and Ornidazole[9], Norfloxacin, Nalidixic acid, Metronidazole and tinidazole[10]. Some data are also available for the estimation of Norfloxacin in biological samples[12] and by UV spectroscopy[13].

Metronidazole (MNZ) is chemically known as 2-(2-Methyl-5-nitro-1H imidazol-1-yl) ethanol. It is a pale yellow crystalline powder and soluble in water, ethanol, and methanol. It is a nitroimidazole derivative and effective against protozoa, anaerobic gram-positive, and negative bacteria. In 2019, it is one of the medicine present in the WORLD HEALTH ORGANISATION'S LIST OF ESSENTIAL MEDICINES[17]**.** In 2021, MNZnidazole was in the 119th place of most commonly prescribed medicine in the USA[18]. Recently Spectroscopic method[14] and Reverse-phase high-performance liquid chromatography (RP-HPLC)[11,15,16] have been reported for the simultaneous estimation of NFX and MNZ in pharmaceutical dosage forms and biological fluids were either expensive or tedious methods.

To the best of our vision, no advanced strategies are utilizing multiple criteria decision-making approach in high-performance liquid chromatography method in concurrent estimation of NFX and MNZ. So the synchronous assurance of these analytes becomes essential eventually. To develop the validated reverse-phase high-performance liquid chromatography for the simultaneous estimation of NFX and MNZ using Response surface methodology, the work proceeded. The consequence of studied factors was evaluated with the assistance of resolution, Fractional Factorial design, whereas the optimum chromatographic conditions were appraised by central composite design using the global mathematical optimization approach (Derringer's Desirability Constant). Finally, Linearity, Accuracy, Specificity, Precision, Ruggedness, and Robustness were tested for the proposed method using Experimental design following that the graphical interpretation of data performed by Response surface methodology[19].

MATERIALS AND METHODS:

APPARATUS

Chromatographic measurements were measured on the RP-HPLC Shimadzu Model. It prominence with High-Performance Liquid Chromatography consists of a PDA detector and used with LC empowered software. The system was controlled through the system controller and the Shimadzu Chromatographic software installed computer. Absorbance was recorded using a UV- visible spectrometer employing a quartz cell of 1.00cm of path length[20].

SOFTWARE

The Experimental Design, Data analysis, and Desirability functions were performed by using the Design Expert Trial Version 12.0 (Stat Ease Inc.., Minneapolis). The rest of the calculations were performed on Microsoft Excel 2007 (Microsoft USA).

CHEMICAL REAGENT:

Pure working standards of NFX and MNZ were gifted from Madras Pharmaceuticals, Chennai. Methanol (AR grade), Methanol (HPLC grade), Acetonitrile (HPLC grade), Water (HPLC grade), Potassium dihydrogen Phosphate (AR grade), Triethylamine (AR grade) were purchased from Modern Scientific and Chemicals. The pharmaceutical tablets were purchased from the local pharmacy.

STANDARD SOLUTIONS:

The standard stock solutions of MNZ and NFX (1mg/ml) were prepared with the mobile phase. The working stock solution was prepared by diluting the stock solution with mobile phase freshly during the analysis day. The calibration curve discloses the peak area ratios of MET and NFX were built up with the range of 10-50 µg/ml and 8-40 µg/ml and the prepared standard solution for the optimization procedure consists of MNZ and NFX at 10.0 µg/ml and 8.0 µg/ml respectively.

SAMPLE SOLUTIONS:

The marketed product of **GRAMOGYL (**contains MNZ 500mg and NFX 400mg) was selected and twenty tablets weighed accurately, the average mass per tablet was determined and finely powdered. Then transfer the fine powder equivalent to 50mg of MNZ and 40mg of NFX was transferred into 50 ml volumetric flask and 30 ml of mobile phase (consisting of 40:60 of Acetonitrile and Phosphate buffer and its pH was adjusted to 4.0 by triethylamine) was added then it was ultrasonicated for 20 mins and finally make up the volume (MNZ-1000µg/ml &NFX- 800 µg/ml) and filtered through the Whatman filter paper no 42. Reject the first portion of the filtrate and take 2.0 ml clear filtrate and diluted with 100ml of mobile phase (to obtain 20µg/ml for MNZ and 16µg/ml for NFX) and mix.

CHROMATOGRAPHIC SEPARATION

The chromatographic separation was carried out on C_{18}Intek Chromasol (4.6 x 250 mm, 5µm make ACE). The mobile phase consisted of 40 ml of ACN 60ml of Phosphate buffer and its pH was adjusted to 4.0 by using Triethylamine and the wavelength selected for the detection was 290nm. The sample injection volume was 20µl. The HPLC system was operated in an air-conditioned atmosphere (20°c ± 2°c) [21].

VALIDATION

The Validation studies were carried out by utilizing the optimized assay conditions from the standards of approval portrayed in ICH guidelines "Text on validation of Analytical Procedure" and Q2B Validation of Analytical Procedure: Methodology. The analytical parameters include accuracy, precision, linearity, detection limit and quantitation limit were evaluated. The calibration curves were tested using the one-way analysis of variance (ANOVA). The LOD and LOQ were standard deviations of response and slope of calibration curve of each drug. In addition to that, the robustness study for the proposed technique was evaluated by making a little modification in mobile phase concentration and flow rate[22,23].

RESULTS AND DISCUSSION:

Design of Optimization and Analysis:

To understand the sensitivity of the chromatographic factors on the separation of analytes and to optimize the resolution and analyzing time simultaneously, Chemometric protocols of Response surface design and Derringer's desirability function were successfully employed. The central composite design can be applied to optimize the separation and to assist the development of a better understanding of the interaction of several chromatographic factors (like Resolution, Void volume, Retention Factor, Separation Factor, Theoretical Plate, Peak Symmetry, and Asymmetry) on separation quality. In this work, the important chromatographic factors were selected and optimized by a central composite design experiment. The selection of factors for optimization was based on preliminary experiments and prior knowledge from the literature. Therefore, the key factors selected for the optimization process were Acetonitrile concentration (*A*), Buffer pH (*B*), and Flow rate (*C*). **Table 1** shows the levels of each factor studied for finding out the optimum values.

Table No. 1: Central composite arrangement and responses

S.NO	Run	Space type	Factor			Responses		
			A (%v/v)	B (pH)	C (ml/min)	k_1	$Rs_{1,2}$	tR_2
1	3	Centre	65	4	1	1.05	5.7	4.65
2	6	Centre	65	4	1	1.05	5.7	4.65
3	7	Centre	65	4	1	1.05	5.7	4.65
4	11	Centre	65	4	1	1.05	5.7	4.65
5	15	Centre	65	4	1	1.05	5.7	4.65
6	20	Centre	65	4	1	1.05	5.7	4.65
7	9	Axial	65	4	1.33	1.09	5.91	7.01
8	12	Axial	65	4.33	1	1	3.3	3.55
9	13	Axial	65	3.66	1	0.95	3.31	5.46
10	14	Axial	56.56	4	1	0.94	5.41	4.5
11	16	Axial	73.40	4	1	0.96	6.6	8.38
12	18	Axial	65	4	0.66	1	4.93	3.10
13	1	Factorial	60	4.2	0.8	1	6	6.26
14	2	Factorial	70	3.8	0.8	1.05	5.05	5.46
15	4	Factorial	60	3.8	0.8	1.11	4.36	5.31
16	5	Factorial	70	4.2	0.8	1.05	2.51	5.41
17	8	Factorial	60	3.8	1.2	1.07	4	3.66
18	10	Factorial	60	4.2	1.2	1	4	3.51
19	17	Factorial	70	3.8	1.2	1	5.88	4.30
20	19	Factorial	70	4.2	1.2	1	6.56	4.56

The ranges of each factor used were: MeCN concentration (60–70%), Phosphate buffer pH (3.8–4.2), and Flow rate (0.8–1.2 mL/min). As response variables, the capacity factor of MNZ($k1$), the resolution between two pairs MNZ and NFX ($Rs_{1,2}$), and the retention times of NFX (tR_2), were chosen. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Central composite design with quadratic equation was represented as

$$Y = \beta_0 + \beta 1X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta 11X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y is the response to be modeled, β is the regression coefficients and X_1, X_2, and X_3 represent factors A, B, and C respectively. Statistical parameters obtained from ANOVA for the reduced models were given in table 6. The insignificant terms ($p>0.05$) were eliminated from the model through a backward elimination process to obtain a simple and realistic model. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected.[24]

Table No. 2: Reduced response models and statistical parameters obtained from ANOVA for CCD

Responses	Regression model	Adjusted R^2	Model p value	C.V (%)	Adequate Precision
k_1	+1.05=0.0034*A-0.0070*B+0.0008*C+0.022*AB+0.0075*AC+0.0050*BC-0.0251*A^2-0.0163*B^2+0.0085*C^2	0.9481	<0.0001	4.33	7.25
$Rs_{1,2}$	+5.70+0.2249*A-0.0591*B-0.2462*C-0.3662*AB-0.1788*AC-	0.9524	<0.0001	7.33	12.26
tR_2	0.8863*BC+0.1087*A^2-0.8459*B^2-0.0981*C^2 +4.95+0.5503*A-0.16138*B+0.0677*C	0.9676	<0.0001	8.57	23.46

The adjusted R^2values were well within the acceptable limits of $R^2 \geq 0.80$[25], which revealed that the experimental data showed a good fit with second-order polynomial equations. For all the reduced models, *p*-value of < 0.05 was obtained, implying these models were significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio. A ratio greater than 4 is desirable.[26] The ratio was found to be in the range of 4.33 – 8.57 which indicated an adequate signal and therefore the model was significant for the separation process. The coefficient of variation (C.V) is a measure of reproducibility of the model and as a general rule, a model can be considered reasonably reproducible if it is less than 10%.

In table 2 the interaction with the largest absolute coefficients among the fitted model was AB (+0.022) of the k_1 model. The positive interaction between A and B was statistically significant (< 0.0001) for k_1. The study revealed that changing the fraction of MeCN from

low to high resulted in a rapid decline in the retention time of MNZ both at the low and high levels of buffer pH. Further at a low level of factor *A*, an increase in the buffer pH resulted in a marginal decrease in the retention time. This might be due to reduced silanol effects as a result of the higher buffer pH used. Therefore, when the MeCN concentration was set at its lowest level, the buffer pH had to be at its highest level to shorten the run time. The existence of such interactions emphasized the necessity to carry out active multifactor experiments for the optimization of chromatographic separation. To gain a better understanding of the results, the predicted models were presented in the form of perturbation plots 1and 3D response surface plots 2.

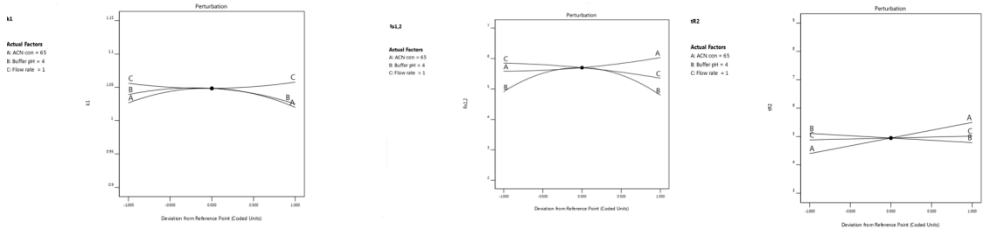


Fig. 1(a) Capacity factor **Fig. 1(b) Resolution** **Fig. 1(c) Retention time**

Figure No. 1: Perturbation plots for Responses

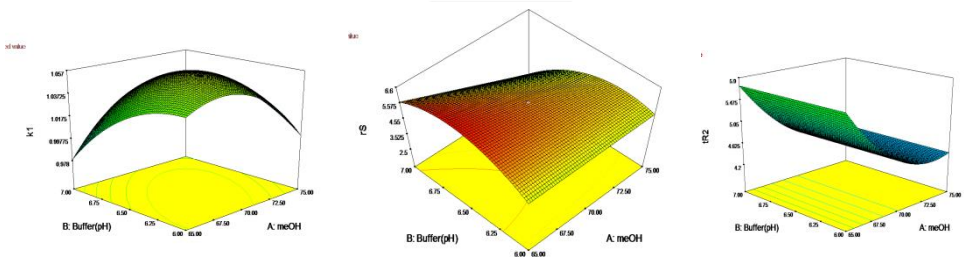


Fig. 2(a) Capacity factor **Fig. 2(b) Resolution** **Fig.2(c)Retention Time**

Figure No. 2: Response surface plots for Responses

Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. The perturbation plot provided silhouette views of the response surface plots where it showed how the response changes as each factor moved from a chosen reference point, with all factors held constant at the reference value. The steepest slope or curvature indicated the sensitiveness of the response to a specific factor. Figure 2c showed that MeCN concentration (factor A) had the most important effect on retention time (tR_2) following factor C. The rest of the factors had a significant effect on k_1 and $Rs_{1,2}$. Figure 3a showed that k_1 values increased as the level of

flow rate increased and that k_1 values decreased as the level of MeCN concentration increased. The value of the resolution ($Rs_{1,2}$) increased with increasing levels of A and B. Analysis of the perturbation plots and response plots of optimization models revealed that factors A, B, and C had a significant effect on the separation of the analytes.

GLOBAL OPTIMIZATION

Derringer's desirability function was employed for the global optimization of three responses and to select different optimal conditions for the analysis of formulation in the present study. The identified criteria for the optimization were resolution between the peaks, capacity factor, and elution time. Derringer's desirability function, D, is defined as the geometric mean, weighted or otherwise of the individual desirability functions. The expression that defines Derringer's desirability function is:

$$D = [d_1^{p2} \times d_2^{p2} \times d_3^{p2} \times \ldots\ldots\ldots \times d_n^{pn}]^{1/n}$$

where pi is the weight of the response, n is the number of responses and di is the individual desirability function of each response. Desirability function (D) can take values from 0 to 1. Weights can range from 0.1 to 10. Weights lower than 1 give less importance to the goal, whereas weights greater than 1 give more importance to the goal. The criteria for the optimization of each response were shown in table 3.

Table No. 3: Criteria for the Optimization of the Individual Responses

Response	Lower limit	Upper limit	Criteria/Goal
k_1	0.94	1.11	Maximize
$Rs_{1,2}$	2.51	6.6	Maximize
tR_2	3.51	8.38	Minimize

In criteria, the responses tR_2 was minimized in order to shorten the analysis time and $Rs_{1,2}$ was maximized to separate the MNZ and NFX. In order to separate the first eluting peak (MNZ) from the solvent front, k_1 was maximized. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function was presented in figure 3.

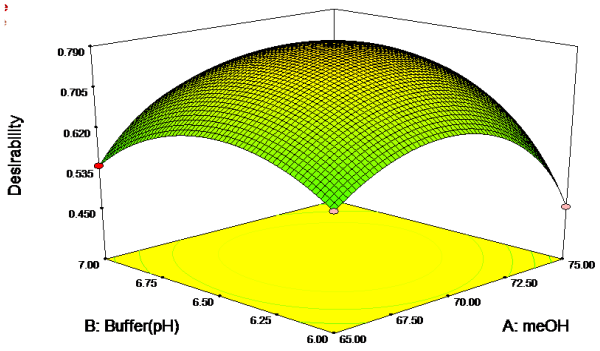


Figure No. 3: Graphical representation of overall desirability function

From the figure 3 it could be concluded that there was a set of coordinates producing high desirability value (D = 0.898) were MeCN concentration of 60.0%, buffer pH of 3.8 and flow rate of 0.8 ml/min. The optimized assay conditions were MeCN: phosphate buffer (60.0:40.0%v/v) (pH 3.8) as mobile phase at a flow rate of 0.8 ml/min. and UV detection at 290 nm. The predicted response values corresponding to the later value of D were k_1 = 1.04, $Rs_{1,2}$ = 3.70 and tR_2 = 3.55min. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram was shown in figure 4. The observed difference between the predicted and experimental responses were found to be in good agreement, within a difference of 4.0% was shown in table 4**.**

Table No. 4: Comparison of experimental and predictive values of different objective functions under optimal conditions

Optimum conditions	Methanol (%v/v)	Buffer (pH)	Flow rate (ml/min)	K_1	$Rs_{1,2}$	tR_2
Predictive	60.0	3.80	1.0	1.04	3.70	3.55
Experimental	60.0	3.80	1.0	1.09	3.77	3.51
Average error				4.80	1.89	1.12
Desirability Value (D)=0.898						

ASSAY METHOD VALIDATION:

The final step of the study was to check the method validation for Accuracy, Precision, Linearity Ruggedness and Robustness. System suitability test provides the added assurance

that on a specific occasion the method is giving, accurate and precise results as shown in table 5.

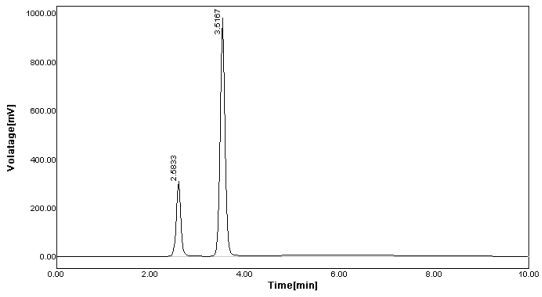
Table No. 5: System suitability parameter

Parameters	MNZnidazole	NFXfloxacin
Capacity factor (K')	1.0	1.05
Retention time (Rt) in min ± %RSD	2.71±0.52	3.55±0.75
Theoretical plates (N)	3006.7	3930.9
Resolution (Rs)	3.33 minutes	

The optimized assay method was specific in relation to the placebo used in this study because there was no excipients peak co-eluted with the analytes. No interferences were observed as shown in figure 4. The method was also selective because there were no interferences observed from any of the excipients in the tablet formulation tested. An excellent linearity was set up at five levels in the range of 10-50µg/ml for MNZ and 8-40 µg/ml for NFX with the R^2 value of 0.9997 for MNZ and 0.9995 for NFX. The Slope and the Intercept of the calibration curve were 60883X + 12994 MNZ and 56409X + 25643 for NFX. The LOD & LOQ for MNZ and NFX were 0.0204 µg/ml, 0.0416 µg/ml and 0.620 µg/ml, 0.1262 µg/ml respectively. Accuracy was performed by spike recovery method and the results were found to be 99.6%, 100.3%, and 100.8% for MNZ and 101.5%, 99.3% and 101.3% for NFX and these results were within the acceptance range of 100 ± 2% which reflect that there was no interference of excipients. For the precision study, the % RSD was well within the acceptance range of ≤ 2. The Robustness study discloses that that small changes in the did not alter the resolution, retention time, and retention factors.

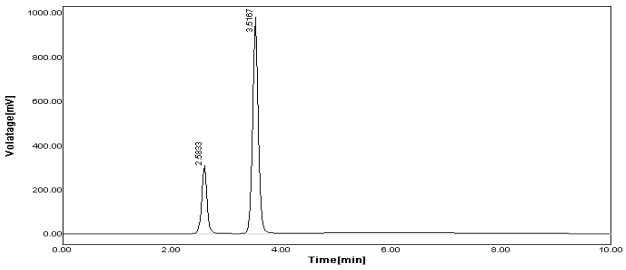
APPLICATION OF METHOD

Finally, the commercially marketed tablet containing 500 mg of MNZ and 400 mg of NFX was assayed by the proposed RP-HPLC assay method, and their chromatograms were presented in fig 5.



Standard Chromatograms for Analytes

Figure No. 4



Sample Chromatograms (Gramogyl tablet)

Figure No. 5

The procured results were 99.7% and 99.3%. Excellent conformity was found between the assay results and the label claim of the product and the % RSD for the tablet is < 2, indicating the Precision of the assay method.

CONCLUSION

An efficient and easily accomplishable approach to resolving the problem of searching for the optimum RP-HPLC[27]. The experimental design and response surface technique procedure provide a better insight into the sensitivity of chromatographic factors and their interaction effects on the attributes of separation.[28] This investigation also showed that the chromatographic technique coupled with chemometric tools provide useful information of separation and elution time, making this combined technique a powerful analytical tool for the simultaneous estimation of MNZ and NFX[27]. The validation study vindicated the determination of assay conditions by affirming that the assay was specific, accurate, linear, precise, rugged, and robust for the estimation of MNZ and NFX. Moreover, the previously reported method addresses only separation of both drugs with a traditional approach with a longer run time of more than 10 mins (MNZ: 7.5mins and NFX: 9.9min)[16] while our proposed method can quantify MNZ and NFX in a run time of <5(MNZ: 2.71min and NFX: 3.55min) min using a novel approach, multivariate statistic techniques. Therefore, this method can be used for routine quality control analysis in a pharmaceutical environment.

REFERENCES

1. Centre for Disease Control & Prevention (2015) Urinary Tract Infection (https://www.cdc.gov/antibiotic-use/community/for-patients/common illnesses/uti.html).
2. Flores-Mireles AL, Walker JN Caparon M and Hultgren SJ (2015).Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews. Microbiology. 13 (5): 269–284.

3. Salvatore S, Cattoni E, Siesto G, Serati M, Sorice P and Torella M (2011). "Urinary tract infections in women". European Journal of Obstetrics, Gynecology, and Reproductive Biology. 156 (2): 131–136.
4. Bradley J and Susan J (2003) Affect regulation and the development of Psychopathology, New York, Guliford Press.
5. Shingo Y and Yamamoto (2016) Prevention and treatment for the complicated UTI infection. Urological Science. 27 (4): 186-189.
6. Colgan R and Williams M (2011). "Diagnosis and treatment of acute uncomplicated cystitis". American Family Physician. 84 (7): 771–776.
7. www. drug bank.com.
8. Mayank Pant, Khemchand Dadare, N. C. Khatri, Application of UV spectrometric methods for Simultaneous estimation of Norfloxacin and Tinidazole bulk and in tablet dosage form Pharma Chem. 2012(4) : 1041-1046.
9. S. B. Wankhede, A. Prakash, B. B. Kumari, S. S. Chitlange (2011), Simultaneous spectrometric estimation of Norfloxacin and ornidazole in tablet dosage form Indian J Pharm Sci. 73 (3): 300 – 330.
10. R. K. Maheshwari, S. C. Chaturvedi and N. K. Jain (2006), A Novel spectrometric estimation of some poorly soluble drug using a hydrotropic solubilizing agent. Indian J Pharm Sci. 68 (2): 195-198.
11. Bikshal Babu Kasimala, Venkadeshwara Rao Anna and Useni Reddy Mallu (2014) Reverse Phase liquid chromatographic method for simultaneous estimation of antibiotic drugs: Metronidazole, nalidixic acid, tinidazole and norfloxacin. Der Pharmacia Lettre. 6(4): 411-419.
12. Zhenping Liu, Mingliang Jin, JiepingCao, Jaun Wang and Xin Wang (2018). High sensitive electrochemical sensor for the determination of Norfloxacin and its metabolism using MWCNT – CPE / pRGO- ANSA/Au. Sensors and Actuators B: Chemical. 257, 1065-1075.
13 Kiran B Alphae, Raymond M. Lawrence and Amit Kasabe (2020) New validated UV Spectrometric Method for the estimation of Norfloxacin and tinidazole in bulk and tablet dosage form. IJPPR. Human. 19(1): 192-198.
14. S. Harshini, M. Akiful Haque, V. Rojakumara, M. Pravalika, and S. Prasanna kumara (2014) Simultaneous estimation of Norfloxacin and metronidazole in bulk and pharmaceutical dosage form by using UV spectrometric method. IJMN. 1(1): 27-32.
15. S. Basak, Sanchitha Mandal and Moitreyee Chattopadhyay (2010) Simultaneous spectrometric assay for estimation of Norfloxacin and metronidazole tablets. Asian J Chem. 1(8) 5971-5975.
16. Minnal R Ghante, Harpreet K. Pannu, Amruta Loni and Tejashree Shivsharan (2012) Development and validation of RP-HPLC method for simultaneous estimation of Metronidazole and Norfloxacin in bulk and tablet dosage. Int J Pharm & Pharm Sci. 4: 241-245.
17. World Health Organization (2019). World Health Organization model list of essential medicines: 21st list 2019. Geneva: World Health Organization. hdl:10665/325771. WHO/MVP/EMP/IAU/2019.06. License: CC BY-NC-SA 3.0 IGO.
18. https://clincalc.com/DrugStats/Top300Drugs.aspx.
19. Mahmoud M. Elkhoudary, Randa A. Abdel Salam and Ghada M. Hadad (2016) Development and Optimization of HPLC Analysis of Metronidazole, Diloxanide, Spiramycin and Cliquinol in Pharmaceutical Dosage Forms Using Experimental Design.Journal of Chromatographic Science. 54(10): 1701–1712.
20. Shimadzu instruction manual.
21. Erdal Dinc, Adbil Ozdemir, Hali Aksoy, Ozgur Ustundag Chemometric Determination of Naproxen Sodium and Pseudoephedrine Hydrochloride in Tablets by HPLC. Chemical and Pharmaceutical Bulletin 2006 volume 54 Issue 4 pages 415-421.
22. International Conference on Harmonization guidance for Industry. (1994) Q2A Text on validation of Analytical methods. Switzerland, IFPMIA, 1-4.
23. International Conference on Harmonization guidance for Industry. (1996) Q2B Text on validation of Analytical methods. Switzerland, IFPMIA,1-8.
24. Parajo JC, Alonso JL, Lage MA and Vazquez D.(1992) Empirical modeling of eucalyptus wood processing, Bioprocess Engineering.8 : 129-136.
25. Lundstedt T, Seifert E, Abramo L, Thelin B, Nystrom Å, Pettersen J, Bergman R. (1998) Experimental Design and optimization. Chemom. Intell. Lab.Syst. 42: 3−40.
26. Beg Q, Sahai V and Gupta R. (2003) Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor, Process Biochemistry,39(2): 203-209.

27. P. Giriraj and T. Sivakumar (2014) Development and Validation of Rapid chemometric assisted RP-HPLC with PDA detection method for the simultaneous estimation of Pyridoxine HCl and Doxylamine Succinate in Bulk and Pharmaceutical Dosage form, Chromatography Research International, 2014 Article ID 827895.
28. Balamurugan Krishnan and Kirtimaya Mishra (2020) Qbd based Development & Validation of RP-HPLC method for the simultaneous estimation of Sitagliptin and metformin Int. J. Pharm. Investigation, 10(4):512-518.