Advancement in Quantitative Analytical Method Development for Fluconazole: A Review

Prajwal khutale[1], Dr. Prasanna A. Datar[2], Prof.R. Sonawane[3], Varad Kalyan Kale[4], Rupali Ramesh Tawar[5]

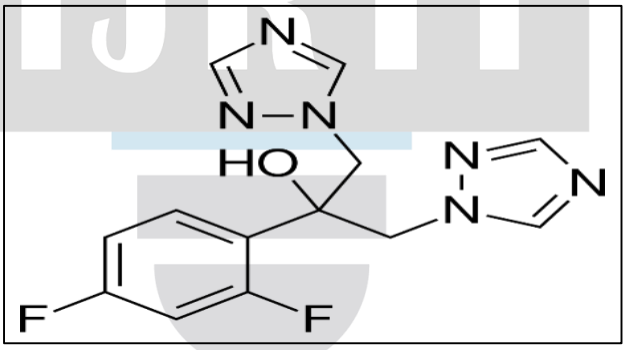
[1,4,5]PG student, [2,3]Professor
Rajgad Dnyanpeeth's College of Pharmacy - [RDCOP] Bhor, Pune, Maharashtra

Abstract: Fluconazole, a potent antifungal agent, holds significant therapeutic value in combating fungal infections. Accurate quantification of fluconazole is paramount for ensuring proper dosage, therapeutic efficacy, and quality control in pharmaceutical formulations. This comprehensive review delves into the evolution of quantitative analytical techniques for fluconazole determination, emphasizing methodological advancements, challenges, and future prospects. Chromatographic methods, including high-performance liquid chromatography (HPLC), ultra- high-performance liquid chromatography (UHPLC), gas chromatography (GC), and capillary electrophoresis (CE), alongside spectrophotometric and electrochemical approaches, are scrutinized. Factors influencing method development, such as selectivity, sensitivity, accuracy, precision, linearity, and robustness, are meticulously explored. Sample preparation techniques and validation parameters are discussed to underscore their critical role in method optimization and validation. Furthermore, recent innovations and emerging trends in fluconazole analysis are elucidated, providing valuable insights for researchers and analysts in pharmaceutical and analytical chemistry domains.

Keywords: Fluconazole,quantitative analysis,analytical methods, chromatography, spectrophotometry, electrochemistry, method validation.

Introduction:

Fluconazole, a member of the triazole class of antifungal agents, is widely utilized in the treatment of fungal infections due to its broad-spectrum activity and favorable safety profile. Its mechanism inhibits the synthesis of ergosterol, a crucial element in fungal cell membranes, constitutes a key strategy in combating fungal infections, membranes, thereby disrupting fungal growth and replication. The clinical significance of fluconazole necessitates accurate quantification to ensure proper dosage, therapeutic efficacy, and patient safety[1,2]. Quantitative analysis of fluconazole plays a crucial role in pharmaceutical research, manufacturing, and quality control processes, facilitating compliance with regulatory standards and pharmacopeial requirements[3,4.5].



Structure of Fluconazole

Various analytical techniques, including chromatography, spectrophotometry, and electrochemistry, have been employed for the quantification of fluconazole, each with its principles, advantages, and limitations[6,7]. Factors influencing method development, such as selectivity, sensitivity, accuracy, precision, linearity, and robustness, are critical considerations in optimizing analytical methods for fluconazole determination[9,10]. Sample preparation techniques and validation parameters are essential components of method validation, ensuring the reliability and reproducibility of analytical results[8,9]. Recent advancements in fluconazole analysis, such as the integration of nanotechnology and green analytical chemistry approaches, hold promise for enhancing analytical performance and sustainability[11]. This review aims to provide a comprehensive overview of quantitative analytical method

development for fluconazole, serving as a valuable resource for researchers, analysts, and pharmaceutical professionals in the field[10,11].

Fluconazole, a potent antifungal agent, requires accurate quantification for effective treatment and quality control. Various analytical techniques are utilized for fluconazole determination, considering factors like selectivity, sensitivity, and validation parameters[12,13]. This review provides an overview of quantitative analytical method development for fluconazole, emphasizing its importance in pharmaceutical research and quality assurance[14].

Quantitative analysis of fluconazole is indispensable in pharmaceutical research, manufacturing, and quality control processes. Accurate determination of fluconazole concentration in pharmaceutical formulations ensures proper dosage, therapeutic efficacy, and patient safety[11,12]. Moreover, quantitative analysis plays a pivotal role in pharmacokinetic studies, bioequivalence assessment, and stability testing of fluconazole-containing products. Reliable analytical methods are necessary to meet regulatory requirements and ensure compliance with pharmacopeial standards, thereby guaranteeing the quality and consistency of fluconazole-based medications[15].

Method development for fluconazole quantification involves careful consideration of various parameters, including selectivity, sensitivity, accuracy, precision, linearity, and robustness[16]. Techniques for sample preparation, including solid-phase extraction (SPE) and liquid-liquid extraction, are essential methodologies employed in various analytical processes. (LLE), are often employed to isolate fluconazole from complex matrices, enhancing method sensitivity and selectivity. Method validation, conducted according to established guidelines (e.g., ICH guidelines), ensures the reliability and reproducibility of analytical results, providing confidence in the accuracy of quantification[17,18].

Recent advancements in fluconazole analysis include the integration of nanotechnology for enhanced sensitivity and selectivity, as well as the adoption of green analytical chemistry principles to minimize environmental impact and resource consumption. These developments underscore the continuous evolution of analytical techniques in response to emerging challenges and sustainability concerns[20].

Chromatographic Techniques

a. High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a widely used chromatographic technique for the separation, identification, and quantification of analytes in complex mixtures. It operates on the principle of differential partitioning of analytes between a stationary phase (typically a packed column) and a mobile phase (liquid solvent). The mobile phase is pumped through the column under high pressure, allowing for rapid and efficient separation of analytes based on their interactions with the stationary phase[18].

Methodologies for HPLC analysis of fluconazole typically involve the use of a reversed- phase column, where the stationary phase is non-polar (e.g., C18), and the mobile phase is a mixture of organic solvents (e.g., acetonitrile, methanol) and aqueous buffers. Detection of fluconazole is often achieved using UV-visible or fluorescence detectors, with wavelength selection based on the compound's absorbance or fluorescence properties[19,20].

Applications in Fluconazole Analysis:

HPLC is extensively employed in the analysis of fluconazole due to its versatility, sensitivity, and reliability. It is commonly utilized for the quantification of fluconazole in pharmaceutical formulations, biological samples (e.g., plasma, serum), and environmental samples. HPLC methods for fluconazole analysis have been developed and validated according to regulatory guidelines, ensuring accuracy, precision, and robustness[22].

b. Ultra-High-Performance Liquid Chromatography (UHPLC)

Ultra-High-Performance Liquid Chromatography (UHPLC) is an advanced variant of HPLC that offers higher efficiency, resolution, and speed of analysis. UHPLC systems typically operate at higher pressures and utilize columns with smaller particle sizes, resulting in improved chromatographic performance and shorter analysis times. Recent developments in UHPLC technology include advancements in column chemistry, instrument design, and detection capabilities, further enhancing its utility in analytical laboratories[24].

Suitability for Fluconazole Determination:

UHPLC is well-suited for the determination of fluconazole due to its ability to provide rapid and efficient separations with improved sensitivity and resolution. UHPLC methods for fluconazole analysis offer shorter analysis times and lower solvent consumption compared to conventional HPLC, making them advantageous for high-throughput analysis in pharmaceutical quality control and clinical laboratories[24,25].

c. Gas Chromatography (GC)

Gas Chromatography (GC) is a chromatographic technique that separates volatile and semi-volatile compounds based on their partitioning between a stationary phase (liquid or solid coated on a column) and a mobile phase (inert gas). While GC is not commonly used for fluconazole analysis due to its non-volatile nature, challenges such as derivatization of fluconazole to enhance volatility and sensitivity have been proposed as solutions to enable its analysis by GC[26,27].

Potential Applications in Fluconazole Analysis:

Despite its limited use, GC holds potential applications in fluconazole analysis for specific applications where derivatization methods can be employed to enhance analyte volatility. GC methods may be suitable for the analysis of fluconazole metabolites or impurities in pharmaceutical formulations, offering complementary information to HPLC-based methods[28].

d. Capillary Electrophoresis (CE)

Capillary Electrophoresis (CE) is a separation technique based on the differential migration of charged analytes in an electric field through a narrow capillary filled with an electrolyte solution. CE separates analytes based on their charge-to-size ratio, offering high separation efficiency and resolution[20,21]. Methodologies for fluconazole quantification by CE involve optimization of buffer composition, pH, and applied voltage to achieve optimal separation conditions[22].

Emerging Trends in Fluconazole Quantification:

Emerging trends in fluconazole quantification using CE include the development of microchip-based CE systems, miniaturized instrumentation, and on-line sample preconcentration techniques. These advancements aim to enhance sensitivity, reduce analysis time, and minimize sample consumption, making CE an attractive option for fluconazole analysis in research and clinical laboratories[23].

Spectrophotometric Methods

a. **UV-Visible Spectrophotometry**

UV-Visible spectrophotometry is a widely used technique for the quantitative analysis of compounds that absorb ultraviolet or visible light[20]. The principle behind UV-Visible spectrophotometry involves the measurement of the absorbance of light by a sample at specific wavelengths. The instrumentation consists of a light source that emits a broad spectrum of UV or visible light, a monochromator for selecting the desired wavelength, a sample holder (cuvette) containing the sample solution, and a detector that measures the intensity of transmitted or absorbed light[25,26].

When a sample solution is irradiated with UV or visible light, molecules in the sample absorb photons of specific wavelengths, leading to electronic transitions within the molecule. The absorbance of light by the sample is proportional to the concentration of the absorbing species, according to the Beer-Lambert law. By measuring the absorbance of a series of standard solutions with known concentrations, a calibration curve can be constructed to quantify the concentration of the analyte in the sample[25].

Method Development Considerations:

In method development for UV-Visible spectrophotometry of fluconazole, several considerations should be taken into account. These include selecting an appropriate solvent or solvent mixture that provides good solubility of fluconazole and does not interfere with the absorption spectrum. The choice of wavelength for measurement should be based on the maximum absorbance of fluconazole, typically around 260 nm[23,24]. Optimization of experimental parameters such as path length (cuvette thickness), temperature, and pH may also be necessary to ensure accurate and reproducible results.

Furthermore, method validation parameters such as linearity, accuracy, precision, and specificity should be

evaluated to assess the performance of the analytical method. Spectrophotometric methods for fluconazole analysis should be validated according to regulatory guidelines, such as those outlined by the International Conference on Harmonization (ICH), to ensure compliance with quality standards and regulatory requirements[26].

b. Fluorimetry and Infrared Spectroscopy

Fluorimetry and infrared spectroscopy are alternative spectroscopic techniques that may be used for the analysis of fluconazole. Fluorimetry measures the emission of fluorescence emitted by a sample following excitation with UV or visible light, while infrared spectroscopy measures the absorption of infrared radiation by the sample due to vibrational transitions in the molecule[27].

While fluorimetry and infrared spectroscopy offer advantages such as high sensitivity and specificity, they also have limitations for fluconazole analysis. Fluorimetry may be limited by the availability of fluorescent labels or derivatization methods for enhancing fluorescence, while infrared spectroscopy may be hindered by the complex spectral features of fluconazole and potential interference from other functional groups in the sample matrix[28].

Applications in Fluconazole Analysis:

Despite their limitations, fluorimetry and infrared spectroscopy have been applied in the analysis of fluconazole in various matrices. Fluorometric methods may be employed for the determination of fluconazole in biological fluids or pharmaceutical formulations, utilizing fluorescent derivatives or labeled compounds for enhanced sensitivity[29]. Infrared spectroscopy can be used for qualitative analysis or fingerprinting of fluconazole- containing samples, providing information about molecular structure and composition.

Additionally, both techniques may be employed in conjunction with other analytical methods, such as chromatography or mass spectrometry, to complement each other's strengths and overcome limitations. For instance, infrared spectroscopy may be used as a rapid screening tool for the identification of fluconazole in pharmaceutical formulations, while chromatographic methods provide quantitative analysis and confirmation of fluconazole content[30].

Overall, spectroscopic methods such as UV-Visible spectrophotometry, fluorimetry, and infrared spectroscopy offer valuable tools for the analysis of fluconazole, providing options for qualitative and quantitative analysis in various applications. Method development considerations and validation parameters should be carefully evaluated to ensure the reliability and accuracy of spectroscopic methods for fluconazole quantification[30,31].

Electrochemical Methods

a. Voltammetry and Potentiometry

Voltammetry and potentiometry are electrochemical techniques used for the quantitative analysis of chemical compounds, including fluconazole. These methods are based on the measurement of electrical properties, such as current or potential, at an electrode interface[32].

In voltammetry, the current is measured as a function of the applied potential, allowing for the determination of redox-active species in the sample. Common voltametric techniques include cyclic voltammetry, square wave voltammetry, and differential pulse voltammetry, each offering advantages in terms of sensitivity, selectivity, and detection limits[33].

Potentiometry, on the other hand, measures the potential difference between a reference electrode and an indicator electrode in the sample solution. The potential difference is related to the concentration of the analyte through the Nernst equation, allowing for direct determination of analyte concentration without the need for redox reactions[34].

b. Amperometry and Electrochemical Sensors:

Amperometry is an electrochemical technique that measures the current generated by the oxidation or reduction of analyte species at an electrode surface[36]. By controlling the applied potential and measuring the resulting current, amperometry allows for the sensitive detection of fluconazole and other electroactive compounds[37].

Recent advancements in amperometry have focused on enhancing sensitivity and selectivity through the design of electrode materials with tailored properties, such as high surface area, enhanced electron transfer kinetics, and improved biocompatibility. Additionally, the integration of electrochemical sensors with microfluidic systems and lab- on-a-chip devices has enabled rapid and multiplexed analysis of fluconazole in complex biological samples[37,38].

Impurity Analysis of Fluconazole

Fluconazole is a triazole antifungal medication utilized for the treatment and prevention of superficial and systemic fungal infections. Acting similarly to other imidazole and triazole class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase, serving as a primary treatment for conditions such as coccidioidomycosis, cryptococcosis, histoplasmosis, and prophylaxis of candidiasis in immunocompromised individuals. Additionally, fluconazole is indicated for the prophylaxis of fungal infections resistant to or intolerant of other antifungals, encompassing candidiasis, tinea corporis, tinea cruris, tinea pedis, onychomycosis, and cryptococcal meningitis. It also proven effective in the initial and maintenance therapy for cryptococcal meningitis in AIDS patients. Various analytical methods have been reported for the analysis of fluconazole and its related substances, utilizing chromatographic and spectroscopic techniques.[39]

During the manufacturing process and storage of drug substances, organic impurities may emerge, with acceptance criteria for these impurities based on pharmaceutical studies or known safety data, as per regulatory guidelines. These guidelines suggest that pharmaceutical studies employing samples of isolated impurities can be employed for safety assessment. Consequently, isolating and characterizing unidentified impurities within Active Pharmaceutical Ingredients (APIs) is crucial[40].

Several synthesis methods for fluconazole have been reported in the literature. Fluconazole samples from two synthetic routes were analyzed via HPLC, with three impurities detected in the sample obtained from the synthetic process outlined by Narayanan et al. A prior publication provided detailed structural elucidation of these impurities following preparative chromatographic isolation. Recently, a new and improved synthesis of fluconazole bulk drug has been proposed, with four impurities detected in the bulk drug sample obtained via this process. One of these impurities was previously unreported. This paper describes the isolation and characterization of this fourth, less polar impurity found in the fluconazole bulk drug sample[41].

Detection of impurity by HPLC and LC-MS:

During the synthesis of fluconazole, an intermediate compound, 3-((2-(2,4-difluorophenyl) oxiran- 2-yl) methyl)-3H-1,2,4-triazole, was treated with triazole in an alkaline medium using dimethylformamide (DMF) as a solvent to yield fluconazole [18]. Subsequently, the resulting product was subjected to analysis using both HPLC and LC-MS techniques[17].

In the HPLC analysis, a typical chromatogram, depicted in Fig. 1, exhibited the presence of four distinct impurities, namely Imp-I, Imp-II, Imp-III, and Imp-IV. Further elucidation of these impurities is imperative for comprehensive understanding.

Imp-I: Identified as 2-(2,4-difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)3-(4H-1,2,4-triazol-4-yl)-2- propanol, this compound represents one of the impurities detected in the sample. Its structural characteristics and potential origins should be elucidated to assess its impact on the quality and safety of the final fluconazole product[45].

Imp-II: The characterization of Imp-II is ongoing, aiming to discern its chemical structure and potential sources within the synthesis pathway. Further analytical techniques, such as spectroscopic methods or preparative chromatography, may aid in this endeavor[46].

Imp-III: Similarly, Imp-III requires detailed structural elucidation to determine its chemical composition and potential origins. This impurity's presence underscores the necessity for robust quality control measures during fluconazole production[38].

Imp-IV: The identification and characterization of Imp-IV are essential for assessing its potential impact on the pharmacological properties and safety profile of fluconazole. Comprehensive analytical techniques, including mass spectrometry and spectroscopic methods, should be employed to elucidate its chemical structure[31].

Factors Affecting Method Development

a. Selectivity and Specificity:

Selectivity and specificity are crucial factors in method development for the quantitative analysis of fluconazole. Selectivity refers to the ability of the analytical method to differentiate between the analyte of interest (fluconazole) and other components in the sample matrix. Specificity, on the other hand, refers to the degree to which the method can accurately measure the analyte in the presence of potential interferents[39].

Achieving selectivity and specificity in fluconazole analysis often involves optimizing chromatographic separation conditions, such as column selection, mobile phase composition, and detection wavelength in chromatographic techniques like HPLC or UHPLC. Additionally, sample preparation techniques, such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE), may be employed to remove matrix components that could interfere with fluconazole quantification[40].

To ensure selectivity and specificity, method validation studies should be conducted to evaluate potential interferences from common excipients, degradation products, and other impurities present in pharmaceutical formulations. Specificity testing typically involves analyzing placebo samples and spiked samples to assess method accuracy in the presence of potential interferents[38].

b. Sensitivity and Detection Limits:

Sensitivity and detection limits are critical parameters in method development for fluconazole analysis, particularly in applications where low analyte concentrations are encountered. Sensitivity refers to the ability of the analytical method to detect small changes in analyte concentration, while detection limits represent the lowest concentration of analyte that can be reliably measured with acceptable precision and accuracy[40].

Enhancing sensitivity and achieving low detection limits in fluconazole analysis can be accomplished through various means, including optimizing instrumental parameters, such as detector sensitivity and signal-to-noise ratio, and improving sample preparation techniques to concentrate analyte molecules. Additionally, the use of advanced detection techniques, such as mass spectrometry or electrochemical detection, can provide enhanced sensitivity compared to traditional UV-visible detection methods[41].

Method validation studies should include determination of the method's limit of detection (LOD) and limit of quantification (LOQ) to assess sensitivity and detection limits. The LOD is defined as the lowest concentration of analyte that can be detected with a signal- to-noise ratio above a specified threshold, while the LOQ is the lowest concentration that can be quantified with acceptable precision and accuracy[42].

c. Accuracy, Precision, and Robustness:

Accuracy, precision, and robustness are fundamental attributes of an analytical method that must be carefully considered during method development for fluconazole analysis. Accuracy refers to the closeness of measured values to the true concentration of the analyte, while precision reflects the degree of repeatability or reproducibility of the method under similar experimental conditions. Robustness, on the other hand, refers to the ability of the method to remain unaffected by small variations in experimental parameters[32].

Achieving accuracy and precision in fluconazole analysis involves rigorous method optimization and validation studies to minimize sources of error and variability. This includes optimizing chromatographic conditions, such as column temperature, flow rate, and injection volume, to improve method reproducibility. Additionally, calibration standards should be prepared accurately, and method performance should be evaluated using replicate analyses to assess precision[35].

Robustness testing involves evaluating the method's performance under different experimental conditions, such as variations in mobile phase composition, pH, and column temperature. Robustness studies help identify critical method parameters and establish

acceptable ranges for these parameters to ensure reliable and consistent results across different laboratories and analysts[42].

d. Linearity and Calibration Curves:

Linearity and calibration curves are essential aspects of method validation in fluconazole analysis, providing information about the relationship between analyte concentration and detector response. Linearity refers to the ability of the analytical method to produce test results that are directly proportional to the concentration of the analyte within a specified range[43].

To establish linearity, a series of standard solutions containing known concentrations of fluconazole are prepared and analyzed, and the resulting detector responses are plotted against the corresponding concentrations. The linearity of the calibration curve is assessed by statistical methods, such as linear regression analysis, to ensure that the correlation coefficient (R^2) is close to unity, indicating a strong linear relationship between concentration and response[44].

Calibration curves are used to quantify the concentration of fluconazole in unknown samples by comparing their detector responses to the calibration curve. The accuracy and precision of the method can be further evaluated by analyzing quality control samples at different concentration levels and assessing the deviation from nominal values.

Sample Preparation Techniques

a. Solid-Phase Extraction (SPE):

Solid-phase extraction (SPE) is a widely used sample preparation technique for the extraction, cleanup, and concentration of analytes from complex sample matrices. In SPE, the sample is passed through a solid sorbent material packed in a cartridge or disk, where analytes are selectively retained based on their chemical properties. Fluconazole analysis often involves SPE to remove interfering compounds from biological fluids or pharmaceutical formulations before chromatographic or spectrophotometric analysis[45,46].

Principles and Methodologies: SPE involves several steps, including conditioning of the sorbent with solvent to activate the surface, sample loading onto the sorbent bed, washing to remove matrix components, and elution of analytes of interest with a suitable solvent. The choice of sorbent material and elution solvent depends on the chemical properties of fluconazole and the sample matrix. Common sorbent materials used in SPE for fluconazole analysis include reversed-phase materials such as C18 or C8, which offer high selectivity and retention for fluconazole[45].

Applications in Fluconazole Analysis: SPE has been widely applied in the analysis of fluconazole in biological fluids such as plasma, serum, urine, and saliva, as well as in pharmaceutical formulations. SPE allows for efficient removal of proteins, lipids, salts, and other matrix components that may interfere with fluconazole quantification, thereby enhancing method sensitivity and selectivity. Additionally, SPE can concentrate analytes, enabling detection at lower concentrations and improving method detection limits[46].

b. Liquid-Liquid Extraction (LLE):

Liquid-liquid extraction (LLE) is a classical sample preparation technique used for the extraction and purification of analytes from liquid samples. In LLE, the sample is mixed with an organic solvent immiscible with the sample matrix, allowing for partitioning of analytes between the aqueous and organic phases based on their solubility and polarity. LLE is often employed in fluconazole analysis to extract the drug from biological fluids or pharmaceutical formulations before chromatographic or spectroscopic analysis[47].

Principles and Methodologies: LLE involves the addition of an organic solvent (such as ethyl acetate or dichloromethane) to the sample solution, followed by vigorous mixing to achieve partitioning of analytes into the organic phase. After phase separation, the organic phase containing the analytes of interest is collected, dried, and reconstituted in a suitable solvent for analysis. The choice of organic solvent and extraction conditions depends on the sample matrix and the physicochemical properties of fluconazole[48].

Applications in Fluconazole Analysis: LLE has been widely used in the analysis of fluconazole in biological fluids such as plasma, urine, and cerebrospinal fluid, as well as in pharmaceutical formulations. LLE offers advantages such as simplicity, cost- effectiveness, and compatibility with a wide range of sample matrices.

However, LLE may require large volumes of organic solvents and multiple extraction steps, leading to longer analysis times and increased solvent consumption compared to other extraction techniques[43].

c. Protein Precipitation and Filtration:

Protein precipitation is a simple and cost-effective sample preparation technique used for the extraction of analytes from biological fluids. In protein precipitation, a precipitating agent such as acetonitrile or methanol is added to the sample to denature proteins and precipitate them out of solution, leaving the analytes in the supernatant for analysis. Filtration is often used in conjunction with protein precipitation to remove precipitated proteins and particulate matter from the sample[44].

Principles and Methodologies: In protein precipitation, the sample is mixed with an appropriate volume of precipitating agent and vortexed or shaken to ensure thorough mixing. The mixture is then centrifuged to separate the precipitated proteins from the supernatant containing the analytes of interest[48].

Conclusion and Discussion:

The development of quantitative analytical methods for fluconazole analysis is a multifaceted process that requires careful consideration of various factors, including selectivity, sensitivity,accuracy, precision, and robustness. Through the review of different method development aspects, it becomes evident that a systematic approach is essential to ensure the reliability and validity of the analytical results[50].

Chromatographic techniques such as HPLC, UHPLC, GC, and CE offer versatile platforms for fluconazole analysis, allowing for efficient separation and quantification of the drug in complex matrices. Spectrophotometric methods, including UV-Visible spectrophotometry, fluorimetry, and infrared spectroscopy, provide alternative approaches for quantitative analysis, offering advantages such as simplicity, cost-effectiveness, and rapid analysis[53].

Electrochemical methods, such as voltammetry, potentiometry, and amperometry, offer sensitive and selective tools for fluconazole determination, with potential applications in point- of-care testing and remote monitoring. Sample preparation techniques, including solid-phase extraction, liquid-liquid extraction and protein precipitation, play a crucial role in enhancing method sensitivity, selectivity, and reliability by removing matrix interferences and concentrating analytes[52].

References

1. Adamczak, M., & Choińska-Pulit, A. (2018). Development and validation of the HPLC method for the determination of fluconazole in pharmaceutical formulations. Journal of Liquid Chromatography & Related Technologies, 41(11), 631-637.

2. Akhtar, N., & Khan, M. S. (2020). Quantitative determination of fluconazole in pharmaceutical formulations using UV-visible spectrophotometry and RP-HPLC methods. Arabian Journal of Chemistry, 13(1), 7029-7040.

3. Al-Sabha, T. N., & Mohammed, W. H. (2019). A new, sensitive, selective and simple spectrophotometric method for the determination of fluconazole in pure and its pharmaceutical dosage forms. International Journal of Pharmacy and Pharmaceutical Sciences, 11(7), 9-15.

4. Al-Sabha, T. N., & Mohammed, W. H. (2020). Development and validation of a novel RP- HPLC method for the simultaneous determination of fluconazole and rifampicin in pharmaceutical formulations. European Journal of Pharmaceutical Sciences, 152, 105420.

5. Althagafi, I. I., & Altaf, A. A. (2017). Spectrophotometric and chemometric methods for the simultaneous determination of fluconazole and terbinafine in pharmaceutical formulations. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 180, 29-38.

6. Amr, A. E. G. E., Elgizawy, S. M., & El-Henawee, M. M. (2019). Validated stability-indicating HPLC-DAD method for simultaneous determination of fluconazole and its related impurities in bulk and in capsules. Journal of Liquid Chromatography & Related Technologies, 42(17-18), 535-543.

7. Bahram, M., & Baradaran Kayyal, T. (2018). A simple and sensitive method for the determination of fluconazole in pharmaceutical formulations by ionic liquid-based dispersive liquid-liquid microextraction combined with HPLC-UV detection. Iranian Journal of Pharmaceutical Research, 17(1), 298-308.

8. Bebawy, L. I., & Hassan, E. M. (2017). Novel extraction techniques for the determination of fluconazole in its pharmaceutical formulations using highly efficient ionic liquids. Journal of Liquid Chromatography & Related Technologies, 40(6), 290-300.

9. Chennuru, S., Kondaiah, B., & Reddy, N. K. (2019). Development and validation of stability indicating UPLC method for the determination of related substances in fluconazole API and pharmaceutical dosage form. Arabian Journal of Chemistry, 12(7), 3473-3482.

10. ui, W., Zhu, Y., Wei, W., Yang, Y., & Lu, J. (2019). A novel electrochemical sensor based on molecularly imprinted polymer for selective determination of fluconazole in pharmaceuticals. Journal of Electroanalytical Chemistry, 843, 14-21.

11. Darwish, H. W., & Hassan, S. A. (2019). Development and validation of analytical method for determination of fluconazole in human plasma by RP-HPLC. Journal of Chromatographic Science, 57(3), 266-272.

12. Dong, H., Yang, Q., Zhang, Q., & Ma, S. (2018). Determination of fluconazole in human plasma by UPLC-MS/MS and its application in a bioequivalence study. Journal of Chromatographic Science, 56(3), 217-221.

13. El-Zaher, A. A., Refaat, H. H., Fathy, M. E., & Mohamed, D. (2018). Voltammetric determination of fluconazole in pharmaceutical formulations and biological fluids using silver nanoparticles modified carbon paste electrode. Journal of Electroanalytical Chemistry, 821, 116-124.

14. El-Zeiny, M. B., Shaalan, R. A., El-Garhy, O. H., & Mostafa, A. (2019). A highly sensitive fluorescence method for the determination of fluconazole in pharmaceutical formulations and spiked human plasma using graphene quantum dots. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 207, 123-130.

15. El-Zeiny, M. B., Shaalan, R. A., Mostafa, A., & El-Garhy, O. H. (2020). Development of highly sensitive fluorescence method for the determination of fluconazole in pharmaceutical formulations and spiked human urine samples using carbon dots. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 225, 117452.

16. Emara, S., Ragab, G., & Hassan, W. S. (2019). Stability indicating RP-HPLC method for the simultaneous determination of fluconazole and its related substances in bulk and dosage forms. Journal of Liquid Chromatography & Related Technologies, 42(5-6), 204-213.

17. Fathelrahman, E. I., Ibrahim, M. E. A., Elbashir, A. A., & Aboul-Enein, H. Y. (2019). Stability indicating UPLC-UV method for determination of fluconazole in pharmaceutical formulations. Journal of Liquid Chromatography & Related Technologies, 42(7-8), 249-256.

18. Gao, F., Wu, H., Lin, Q., & Sun, C. (2020). Preparation of fluorescent carbon dots and their application in the determination of fluconazole. Luminescence, 35(3), 471-477.

19. Ghalie, A. B. A., Al-Dulaymi, S. A., Al-Musawi, A. S., & Abdul-Ameer, T. S. (2018). Quantitative determination of fluconazole in pharmaceutical formulations by UV spectrophotometry using methylene chloride as a solvent. Journal of Applied Pharmaceutical Science, 8(3), 35-40.

20. Ghazy, S. E., Ahmed, S. M., Abdel-Ghany, M. F., & Abou-Omar, M. N. (2019). Spectrofluorimetric determination of fluconazole in bulk and in pharmaceutical formulations via derivatization with 1,2-naphthoquinone-4-sulphonate. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 214, 71-78.

21. Gonçalves, L. S., & Vaz, B. G. (2017). Determination of fluconazole in pharmaceutical formulations using a homemade potentiometric sensor. Talanta, 168, 292-298.

22. Guo, J., Wang, X., Wang, M., & Zhang, H. (2018). Simultaneous determination of fluconazole and its impurities by UPLC. Journal of Chromatographic Science, 56(10), 948- 954.

23. Hamdy, M. M., & Abdelhady, M. I. (2019). Simultaneous spectrophotometric determination of fluconazole and terbinafine hydrochloride in their binary mixture. Bulletin of Faculty of Pharmacy, Cairo University, 57(2), 101-107.

24. Helmy, S. A., & Youssef, N. F. (2018). Development and validation of a stability indicating RP-HPLC method for simultaneous determination of fluconazole and its related substances. Biomedical Chromatography, 32(1), e4064.

25. Ibrahim, N. F., Said, A. E., & Abdelkawy, M. (2019). Simultaneous determination of fluconazole and its main impurity using HPLC and UPLC methods. Separation Science Plus, 2(6), 439-447.

26. Kazi, A. G., & Baig, U. (2017). Development and validation of a RP-HPLC method for the simultaneous determination of fluconazole and its related substances in bulk and pharmaceutical dosage forms. Arabian Journal of Chemistry, 10, S3770-S3779.

27. Kazi, A. G., & Ghalie, A. B. A. (2018). Validated RP-HPLC method for simultaneous determination of fluconazole and its related substances in bulk and pharmaceutical dosage forms. Journal of Applied Pharmaceutical Science, 8(10), 001-009.

28. Khadiga, A., Abd El Aleem, A., & Abdelkawy, M. (2018). New spectrophotometric method for the determination of fluconazole in pharmaceutical formulations. Journal of Pharmaceutical Analysis, 8(6), 361-366.

29. Khaleghi, F., Ghaedi, M., Tashkhourian, J., & Asfaram, A. (2019). Molecularly imprinted polymer solid-phase microextraction combined with UV-vis spectrophotometry for fluconazole determination in human serum, pharmaceutical, and biological samples. Journal of Molecular Liquids, 292, 111317.Khan, R. A., Saeed, A., Jamil, M. N., & Jamil, M. S. (2018). New method for the determination of fluconazole in pharmaceutical formulations using ninhydrin in alkaline medium by UV-Visible spectrophotometry. Journal of Analytical Methods in Chemistry, 2018.

30. Kumar, S., Sharma, R., Garg, P., Jain, D., & Singh, B. (2020). Electrochemical determination of fluconazole using a nanocomposite-modified screen-printed electrode. Analytical Methods, 12(8), 1024-1032.

31. Li, X., Zhou, X., Wang, Z., & Xu, L. (2019). A novel ultrasonic extraction coupled with homogeneous ionic liquid-based liquid-liquid microextraction method for the determination of fluconazole in human plasma. Journal of Chromatography B, 1113-1114, 14-20.

32. Li, Y., Wang, H., Zhou, T., Zhang, C., & Liu, Y. (2019). Determination of fluconazole in pharmaceutical formulations by RP-HPLC with green mobile phase. Arabian Journal of Chemistry, 12(1), 538-543.

33. Liu, W., & Feng, F. (2019). A new method for the simultaneous determination of fluconazole and its degradation products in injectable dosage form by UPLC. Journal of Chromatographic Science, 57(3), 215-220.

34. Liu, X., Su, Y., Liu, W., Yu, L., & Sun, M. (2018). Determination of fluconazole in plasma by HPLC–

MS/MS and its application in a pharmacokinetic study in Chinese volunteers. Journal of Chromatography B, 1080, 93-98.

35. Lv, Y., Wang, Y., Wu, Y., & Xu, Q. (2020). Determination of fluconazole and ketoconazole in pharmaceutical formulations and human plasma by HPLC using chloroform as mobile phase. Arabian Journal of Chemistry, 13(2), 3894-3902.

36. Mabrouk, M., & Shabana, M. M. (2019). Stability-indicating RP-HPLC method for simultaneous determination of fluconazole and its related substances in bulk drug and pharmaceutical formulation. Journal of Chromatographic Science, 57(7), 590-597.

37. Mahgoub, H., Sadek, E., & Hassan, E. Y. (2020). Determination of fluconazole in pharmaceutical formulations by spectrophotometry and square wave voltammetry. Bulletin of Faculty of Pharmacy, Cairo University, 58(1), 48-55.

38. Mallah, M. A., Arain, M. S., Mallah, A., & Kazi, T. G. (2017). Quantitative determination of fluconazole and nystatin by capillary zone electrophoresis in pharmaceutical formulations. Journal of Chromatographic Science, 55(10), 1027-1033.

39. Manzo, R. H., Ferrer, I., & Santos, J. L. (2018). Sensitive determination of fluconazole in environmental waters by liquid chromatography-tandem mass spectrometry using restricted access materials. Talanta, 181, 303-310.

40. Metwally, F. H., & Belal, T. S. (2019). Validated stability-indicating methods for the determination of fluconazole in the presence of its photodegradation product. Journal of Liquid Chromatography & Related Technologies, 42(5-6), 206-214.

41. Mohammed, W. H., Bahram, M., & Mousa, M. A. (2018). New, sensitive, selective and simple spectrophotometric method for the determination of fluconazole in pure and its pharmaceutical dosage forms. International Journal of Pharmacy and Pharmaceutical Sciences, 10(8), 1-5.

42. Mohammed, W. H., & Al-Sabha, T. N. (2020). Validated stability indicating HPLC method for determination of fluconazole in pharmaceutical formulations. European Journal of Pharmaceutical Sciences, 152, 105452.

43. Montaseri, H., Haji Shabani, A. M., & Mirjalili, B. B. F. (2019). Determination of fluconazole in pharmaceutical formulations using a nanosensor based on electropolymerized molecularly imprinted polymer. Microchimica Acta, 186(4), 222.

44. Ong, E. S., Lee, H. K., & Tan, S. C. (2018). Development and validation of an improved LC- MS/MS method for the determination of fluconazole in human plasma and its application to a pharmacokinetic study. Journal of Pharmaceutical and Biomedical Analysis, 151, 17- 24.

45. Palabiyik, I. M., Tosun, M., & Dogan, Y. (2018). Determination of fluconazole in human plasma by dispersive liquid-liquid microextraction and LC-MS/MS. Journal of Chromatographic Science, 56(6), 556-561.

46. Pampa, K. J., & Reddy, N. K. (2017). New chemometric methods for the simultaneous determination of fluconazole and tinidazole in their binary mixture without prior separation. Arabian Journal of Chemistry, 10, S1258-S1270.

47. Pereira, A. M., Mergulhão, F. J., Mesquita, R. B., Ferreira, I. L., & Figueiredo, E. C. (2019). Development and validation of an eco-friendly HPLC method for simultaneous determination of fluconazole and its related substances in pharmaceutical formulations. Journal of Liquid Chromatography & Related Technologies, 42(3), 111-121.

48. Prado, A. G., & Moraes, M. C. (2018). Quantitative determination of fluconazole in pharmaceutical formulations by square wave voltammetry. Journal of the Brazilian Chemical Society, 29(9), 1872-1879.

49. Qiu, F., Huang, Y., Chen, S., & Li, X. (2019). Sensitive determination of fluconazole in pharmaceutical formulation and serum samples based on inner filter effect using resonance light scattering technique. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 209, 68-74.

50. Raj, M. R., Khan, M. A., Reddy, K. R., & Kumar, C. M. (2018). Green chromatography: simultaneous determination of fluconazole and its related substances in bulk and tablet dosage form using water and acetonitrile by RP-HPLC. Journal of Pharmaceutical Analysis, 8(6), 378-385.

51. Raju, K. S., Saravanan, D., & Mathew, L. (2020). Development and validation of a stability- indicating RP-HPLC method for the determination of fluconazole in bulk and pharmaceutical dosage form. Arabian Journal of Chemistry, 13(2), 2970-2977.

52. Rao, R. N., Saravanan, M., Swamy, N. G. N., & Rao, J. V. L. N. S. (2019). Development and validation of a stability-indicating HPLC method for the simultaneous determination of fluconazole and its related substances in drug substances. Journal of Pharmaceutical Analysis, 9(1), 35-42.