THE USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC: A CASE STUDY OF GRISEOFULVIN AND CETIRIZINE HYDROCHLORIDE

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Faculty of Pharmacy and Pharmaceutical Sciences

by

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DECLARATION

This experimental work outlined in this thesis was carried out by the author at the Department of Pharmaceutical Chemistry, KNUST. This work has not been submitted for the award of any other degree.

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DEDICATION

I dedicate this work to my family.

ACKNOWLEDGEMENT

I give thanks to the almighty God, whose abundant grace, protection and love made this work possible.

Special thanks go to my parents and siblings for their prayers and support throughout this work.

I wish to express my appreciation to Prof. Kwakye, my supervisor for the guidance given me.

My sincere gratitude goes to Mr. Patrick Boamah, who provided the pure powder used in this research.

Lastly, I am grateful to lecturers and staff of the Department of Pharmaceutical Chemistry and my colleagues for their support.

God richly bless you.

ABSTRACT

A rapid and simple reverse-phase liquid chromatography method was developed and validated for the quantitative analysis of Griseofulvin and Cetirizine Hydrochloride using surrogate reference standards. Paracetamol, Ascorbic acid and Metronidazole were used as surrogate reference standards, for each of which a constant "k" was obtained.

The isocratic liquid chromatography (LC) analysis for Griseofulvin was performed on C18 Phenomenex column (250 x 4.6mm) using mobile phase composed of methanol and water in a ratio of 70: 30 (v/v) at a flow rate of 1 ml/min with a UV detector set at 254nm. The mean retention time in minutes for Griseofulvin, Paracetamol, Ascorbic acid and Metronidazole were 3.65 ± 0.06, 2.47 ± 0.03, 1.70 ± 0.02 and 2.65 ± 0.04 respectively. The constant K obtained for Paracetamol, Ascorbic acid and Metronidazole in relation to Griseofulvin were 0.3824 ± 0.0077, 0.3317 ± 0.0048 and 1.0902 ± 0.0067 in that order.

The isocratic LC analysis for Cetirizine hydrochloride was performed on C18 Phenomenex column (250 x 4.6mm) using mobile phase composed of methanol and sodium acetate buffer of pH 4.2 in a ratio of 60: 40 (v/v) at a flow rate of 1.5 ml/min with a UV detector set at 235nm. The mean retention times in minutes were 4.57 ± 0.06, 1.73 ± 0.05, 1.52 ± 0.04 and1.84 ± 0.04 for Cetirizine hydrochloride, Paracetamol, Ascorbic acid and Metronidazole respectively. The constant K obtained for Paracetamol, Ascorbic acid and Metronidazole in relation to Cetirizine hydrochloride were 0.3530 ± 0.0100, 0.3735 ± 0.0103, 0.4929 ± 0.0109 respectively.

The analytical methods were validated according to the International Conference on Harmonization (ICH) guidelines. The methods showed linearity over the working range. The coefficient of variation for repeatability and intermediate precision were less than 2%. The methods were applied to commercial preparations. Statistical comparison of the

outcome and that from standard methods revealed that the methods can be used for routine analysis of Griseofulvin and Cetirizine hydrochloride since they were comparable.

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LIST OF ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
GMP	Good Manufacturing Practice
WHO	World Health Organization
IUPAC	International Union of Pure and Applied Chemistry
ICH	International Conference on Harmonization
NPLC	Normal Phase Liquid Chromatography
RPLC	Reverse Phase Liquid Chromatography
Rf	Retardation Factor
SRS	Surrogate reference Standard
UV	Ultra Violet
AG	Griseofulvin manufactured by Ayrton Drugs Manufacturing Ltd
EG	Griseofulvin manufactured by Ernest Chemist Ltd
LG	Griseofulvin manufactured by Letap Pharmaceuticals Ltd
EC	Cetirizine hydrochloride manufactured by Ernest Chemist Gh. Ltd
HC	Cetirizine hydrochloride manufactured by Hovid
KC	Cetirizine hydrochloride manufactured by Kinapharma Ltd
SEM	Standard error of the mean
SD	Standard Deviation

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The quality, efficacy and safety of pharmaceutical products are a requirement for their release. Consumers receive effective drugs through the interplay between manufacturers and regulatory bodies. The quality of drug products has been ascertained in terms of their analytical characteristics. These include the physical, biological, biopharmaceutical as well as chemical processes aimed at defining the profile of the drugs in accordance with specifications laid down in the monographs of the official compendia to ensure safety and therapeutic efficacy (Levi *et al.*, 1964; World Health Organization, 2006).

Chemical analysis has evolved over time. It has advanced from classical methods to more sophisticated instrumental techniques through the exploration of scientist over the years. The classical methods measured obvious characteristics of analyte such as colour, odour, solubility in a series of solvents, boiling point among others with the addition of reagents that could yield these results mainly by precipitation, extraction and distillation. Gravimetric and volumetric measurements were also done for quantitative analysis (Skoog *et al.*, 2007). The modern instrumental techniques however measure the response of the chemical substance or analyte stimulated by its interaction with an external source of energy. Qualitative and quantitative analysis are based on comparison of parameters including chromatographic retention, absorbed or emitted radiation between the analyte and the standard e.t.c. The response given is the reflection of the physical or chemical characteristics of the analyte under study and can then be interpreted to obtain the desired information on analyte. The magnitude of this response is proportional to the amount of substance present. E.g. High Performance Liquid Chromatography (HPLC).

The HPLC is by far the most widely used analytical separation technique. This is due to the speed, reproducibility and sensitivity it offers. The technique is useful in the separation of compounds that vary widely in polarity in a single run. It is suitable for thermally fragile, non-volatile and even volatile compounds. It is also useful in the separation of compound of varying molecular weights like synthetic polymers (McMaster, 2007). HPLC has been instrumental to the pharmaceutical industry as most of its activities have derived immense benefit from the technique. It is used in developing viable synthetic routes for synthesizing active pharmaceutical ingredients, developing dosage forms and stability profiles for products. It is also used in the evaluation of metabolic and pharmacokinetic profiles of drugs in animal models and human clinical models. It is also applicable in quality control, in the assay, identification, dissolution and purity testing for the assessment of raw material, intermediate and finished pharmaceutical products against stipulated specifications.

The HPLC and other instrumental methods are indirect, so the detecting or measuring instrument requires calibration or standardization to establish the correlation between values indicated by the measuring instrument and the corresponding values obtained by standards under stated conditions (CITAC and Eurachem, 2002). Another form of calibration is the calibration of the analytical procedure which is to determine the relationship between the analytical response and the concentration or quantity of analyte. This is achieved in HPLC analysis by plotting data obtained from the response (peak area, peak height) against the concentration of analyte to produce a calibration curve. The unknown concentration of an analyte can then be obtained from the best-fit equation once its response signal is known. This however relies largely on the use of highly pure and well characterized chemical reference standards (Schiller, 1996; European co-operation for Accreditation, 2003)

Reference standards are well established and sufficiently homogenous physical specimens of drug substances, excipients, impurities, degradation products used by pharmaceutical and related industries to help ensure the identity, strength, quality and purity of medicines (drugs, biologics, and excipients). They are established through a collaborative testing process that involves at least three independent laboratories to standards and procedures enforceable by the appropriate institution ensuring their use is effective in demonstrating compliance with statutory requirements. Its uses include the assessment and calibration of measurement methods as well as assigning values to materials (Schiller, 1996; United States Pharmacopoeia, 2004).

Reference standards are unavailable in many countries to control the quality of the ever increasing pharmaceutical substances and preparations. Regulatory bodies are faced with the challenge of controlling the quality of imported drugs, as the best way to control imported drugs is to analyze representative samples in an official laboratory in the importing country (Olaniyi, 2000). Local manufacturers face the same difficulty as control of the quality of drugs is impeded by the lack of readily available reference standards. This is because procurement of these chemical reference standards is very expensive and time consuming as it has to be imported. These difficulties are manifested more in developing countries like Ghana.

Surrogate reference standards may be used in place of the reference material in analysis of drugs. Surrogate reference standards (SRSs) are drug substances that can be used to assay drug formulations in place of the pure reference standards. The surrogate standard should be:

- Readily available
- Highly pure

- Stable both on shelf and in solution
- Soluble in the solvent in which the drug is soluble
- Should not interfere with the analysis
- Should not react unfavourably or be converted to the analyte

A constant "K" of the surrogate reference standard in relation to the analyte under the HPLC conditions can be determined and used in the quantification of the analytes.

During HPLC method development, a series of factors are requisite to arrive at the optimum conditions. First of all, the goals of the method need to be spelt out. The questions of what type of analysis the method is to be developed, the number of samples to be analyzed at a time, whether it is necessary to resolve all sample components, level of accuracy and precision need to be answered prior to commencement of method development (Synder *et al.*, 1997). Again, the properties of the sample, level of detection and resolution required as well as how fast the analysis should be need to be considered. The chemical structure, pKa, molecular weight, stability and solubility of the sample are some properties of the sample that have to be known to guide in the selection of detector, column , mobile phase and to establish a sample preparation procedure (Wittrig, 2003). Once these factors have been settled on preliminary separations can be performed and then the conditions optimized based on the resolution desired.

As part of Good Manufacturing Practices (GMP) outlined by the World Health Organization (WHO) it is a requirement that every non - compendia analytical method or modified compendia method be validated and the results documented. As such, the alternative methods need to be carefully validated to ensure it yields its intended results. Also, significant tests should be done to ensure there is no significant difference between the outcome of the developed method and the standard method (Miller and Miller, 2005).

Method validation is the process to collect documented evidence to confirm that the analytical procedure performs according to its intended use. The validity of a method is established or demonstrated in a GMP compliance laboratory with samples and standards that are similar to unknown samples analyzed routinely according to a written down and predefined validation protocol (Jimidar *et al.*, 2007; Ravichandran *et al.*, 2010). Evidence on the specificity, linearity range, precision, accuracy, limit of detection, limit of quantitation and robustness has to be generated for regulatory submission or in - house application. Results from the method validation can be used to judge the quality, reliability and consistency of the analytical method. This shows that the method meets criteria such as ease of use, ability to be automated and to be controlled by computer systems, cost per analysis, sample throughput, turnaround time and environmental and safety requirements. In addition, validation ensures that the procedure has sufficient detail so that different analysts or laboratories following the same procedure obtain comparable results (Synder et al., 1997; Harvey, 2000).

This use of surrogate reference standards in quantitative HPLC has been undertaken in this institution over the last few years. Investigation has been conducted on Paracetamol, Indometacin, Prednisolone, Diclofenac sodium, Diazepam, Aspirin, Piroxicam, Mebendazole, Metronidazole and Chlorphenamine. This project seeks to investigate the use of surrogate reference standards in the analysis of Griseofulvin and Cetirizine hydrochloride.

1.2 AIM

To develop an alternative quantitative HPLC method for the analysis of Griseofulvin and Cetirizine Hydrochloride using Paracetamol, Ascorbic acid and Metronidazole as surrogate reference standards.

1.3 SPECIFIC OBJECTIVES

- Develop an HPLC method for the analysis of Griseofulvin and Cetirizine Hydrochloride using surrogate reference standards (SRS).
- Determine a constant K value for each SRS to be used in quantitative analysis
- Validate the method using parameters such as linearity, limit of detection and limit of quantification, precision.
- Determine the percentage content of different brands of commercial preparations of Griseofulvin and Cetirizine hydrochloride using the developed method
- Compare results obtained from the developed method to that from the standard method statistically.

1.4 HYPOTHESIS

The area under a peak in a chromatogram is proportional to the concentration of the analyte. That is,

$A \propto C \ldots\ldots\ldots\ldots eqn\ 1$

$Where\ A = Area$

$C = Concentration$

Introducing a constant Q, equation 1 becomes,

$A = QC$

$Q = \frac{A}{C} \ldots\ldots\ldots\ldots eqn\ 2$

In HPLC analysis, the constant is the same for similar compounds,

$\frac{Aa}{Ca} = \frac{As}{Cs} \ldots\ldots\ldots\ldots eqn\ 3$

For different compounds,

$\frac{Aa}{Ca} \neq \frac{As}{Cs}$

Introducing a constant K, equation 3 becomes,

$$\frac{Aa}{Ca} = K\frac{As}{Cs}$$

K is dependent on the nature of the surrogate compound in relation to the analyte.

Once K is known, Ca may be found from the relation

$$Ca = \frac{Aa \times Cs}{As \times K}$$

$Where\ Aa = Peak\ area\ of\ analyte$

$As = Peak\ area\ of\ Surrogate\ standard$

$Ca = Concentration\ of\ analyte$

$Cs = Concentration\ of\ Surrogate\ standard$

$$Thus, Percentage\ Content = \frac{Actual\ Concentration}{Nominal\ Concentration} \times 100\%$$

1.5 JUSTIFICATION

In HPLC analysis, reference standards of the drug being analyzed are required to prepare calibration curve from which the concentration of the unknown analyte can be found. However, these reference standards are very expensive to obtain making it difficult for industries as well as regulatory bodies to control these drugs as stated earlier. This results in industries not being able to meet their set target which might make them to release unwholesome drugs to the consumer which will rather complicate health condition of patients.

According to the British Pharmacopoeia Commission, with the agreement of the competent authority, alternative methods of analysis may be used for control purposes on condition that the method enables an unequivocal decision to be made as to whether

compliance with the standards of monographs would be realized if the official methods are used.

This makes apparent the need to develop alternative HPLC methods using surrogate reference standards, which are readily available and very affordable in our part of the world. This will enable regulatory bodies control drugs that are imported into the country and those manufactured in the country to ensure the safety of consumers.

Table 1-1 Cost of drug substances

Drug	Quantity	Price	Catalogue no
Griseofulvin	200mg	$204	1299007
Cetirizine hydrochloride	250mg	$204	1102929

Chapter 2

LITERATURE REVIEW

2.1 PROFILE OF DRUG SUBSTANCES

2.1.1 Cetirizine hydrochloride

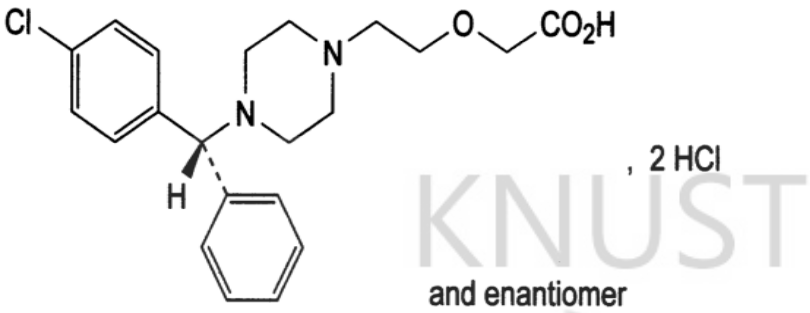

Figure 2-1 Chemical structure of Cetirizine hydrochloride

Cetirizine hydrochloride is a piperazine derivative with the chemical name (±) - [2 - [4-[(4-chlorophenyl) phenylmethyl] - 1 - piperazinyl] ethoxy] acetic acid, dihydrochloride. Cetirizine hydrochloride, a second-generation antihistamine, is a racemic selective H1 receptor inverse agonist (Rizk *et al.*, 2009).

It is a white to almost white crystalline powder with a melting point of 225°C. It is freely soluble in water, practically insoluble in acetone and dichloromethane (Galichet *et al.*, 2005)

Cetirizine is used to treat several allergy symptoms, including sneezing, itchy throat and watering of the eyes. Cetirizine hydrochloride is also used to treat hives (urticaria), hay fever, angioedema (Etolen, 2012).

2.1.2 Griseofulvin

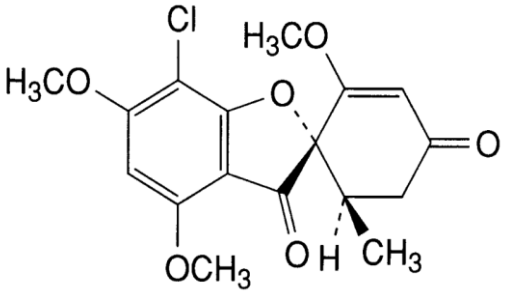

Figure 2-2 Chemical structure of Griseofulvin

Griseofulvin is an orally administered antifungal drug. It is a white to pale cream coloured powder with the melting point range of 217 ° to 224 ° (Galichet *et al.*, 2005).

It is practically insoluble in water, slightly soluble in methanol and ethanol and freely soluble in dimethylformamide and in tetrachloroethane (British Pharmacopoeia, 2007).

The antifungal substance derived from the mould *Penicillium griseofulvum*, is used to treat infections such as ringworm, athlete's foot, jock itch, and fungal infections of the scalp, fingernails and toenails (Martindale, 2005).

2.1.3 Paracetamol

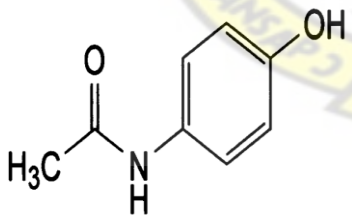

Figure 2-3 Chemical structure of Paracetamol

Paracetamol with IUPAC name *N* - (4 - Hydroxyphenyl) acetamide is a white crystalline powder in appearance. It melts in the range 168 ° to 172 °. It is slightly soluble in water and freely soluble in alcohol (Martindale, 2005).

Paracetamol also called acetaminophen has analgesic and antipyretic properties. Paracetamol is used to treat many conditions such as headache, muscle aches, arthritis, backache, toothaches, colds, and fevers (Ogbru, 2007).

2.1.4 Ascorbic acid

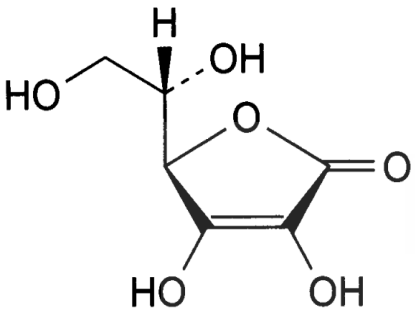

Figure 2-4 Chemical structure of Ascorbic acid

Ascorbic acid is a white to slightly yellow crystals or powder which gradually darkens on exposure to light. In the dry state, it is reasonably stable in air but oxidizes rapidly in solution. It is freely soluble in water and soluble in alcohol. It melts at about 190 ° with decomposition (Galichet *et al.*, 2005).

Ascorbic acid, a water-soluble vitamin, is essential for the synthesis of collagen and intercellular material. It is used in the treatment and prevention of deficiency. Ascorbic acid is also used as an antioxidant in pharmaceutical manufacturing and in the food industry (Olaniyi, 2005).

2.1.5 Metronidazole

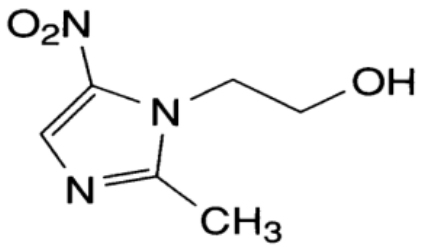

Figure 2-5 Chemical structure of Metronidazole

Metronidazole, a 5 - nitroimidazole derivative is an antibacterial. It is a white to yellowish crystalline powder in appearance with IUPAC name 2 - methyl - 5 - nitroimidazole - 1 - ethanol. Its molecular formula is $C_6H_9N_3O_2$. It has a molar mass of 171.15 and a melting range of 159 - 163 °C. Metronidazole is slightly soluble in water, acetone, and alcohol (Galichet *et al.*, 2005).

It is bactericidal against anaerobic bacteria. It also has some anti- protozoal activity (Martindale, 2005).

2.2 THEORY OF ANALYTICAL TECHNIQUES

2.2.1 Chromatography

The birth of chromatography is linked to Michael Tswett, a botanist who in the year 1903, isolated plant pigments on chalk columns (Ditz, 2005). It is the separation of components by the distribution between two or more immiscible phases. Chromatography is one of the most widely used techniques for the separation and purification of components. It has seen tremendous growth over the past four decades owing to its ease of operation, speed, relatively low cost and wide applicability. It can be used to separate mixtures of similar components such as proteins with great precision. Chromatography may be used to purify delicate compounds and even volatile substances. It is commonly used to isolate new compounds formed during chemical synthesis. In the pharmaceutical

industry, chromatography is used in the quality control during drug production to monitor the purity of drugs. Chromatography is also used in the qualitative and quantitative applications. Separation is brought about by the different affinity of components to the stationary phase. There is repeated adsorption and desorption as the components moves over the stationary phase and this determines the rate of separation (Gambhir, 2008). There are different types of chromatography based on the physical and chemical nature of the stationary phase, the purpose of the chromatographic experiment, the polarity of the stationary and mobile phases, the principle of separation among others. The different types of chromatography include:

- Thin layer chromatography
- Paper chromatography
- Column chromatography
- Gas chromatography
- Liquid chromatography
- Supercritical Fluid chromatography

While it may be possible to make use of more than one of the techniques in analysis, the choice of a particular chromatographic technique is dependent on the

- Availability and cost of equipment
- Ease and speed of the technique
- Chemical and physical characteristics of the compounds to be separated
- Complexity of sample
- Resolution required

2.2.1.1High Performance Liquid Chromatography

Advancement of research and the quest for further improvements in speed and efficiency of analysis led to the discovery and development of high performance liquid

chromatography (HPLC). HPLC is one mode of the chromatography systems which is widely used in the fields of clinical research, biochemical research, industrial quality control etc. As a result, HPLC has a high degree of versatility not found in other chromatographic systems. Its applications include detection, analysis, quantification and derivation of molecules from mixtures of biological, plant of medical importance by preparative HPLC (Dorsey and Stout, 2002; Shrivastava and Gupta, 2012).

This separation technique involves differential interaction of the sample between the stationary and mobile phases. HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase may be liquid or solid. These components are first dissolved in a suitable solvent, and then forced to flow through a chromatographic column containing stationary phase of small particle size under high pressure. The mixture separates into its components on the column. Retention is based on adsorption and also partition between the sample particles and the stationary phase depending on the mode of HPLC. The extent of interaction between the sample particles and the stationary phase affects resolution of sample components (Kar, 2005). The weaker the interaction with the mobile phase the longer the sample is retained on the stationary phase. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases.

Stationary phase

The stationary phase is the immobile packing material in the column. There are several types of matrices for support of the stationary phase. These include silica, polymers, alumina, and zirconia. Silica is the dominant support material used in HPLC columns (Kupeic *et al.*, 2005). This is because silica matrices are

- Robust
- Easily derivatized

- Do not tend to compress under pressure
- Chemically stable to most organic solvents and to low pH systems.

Porous polymer support materials as polyethers have been used mostly in ion exchange chromatography. Even though their performance has improved over the years, they still lag behind silica in terms of efficiency. Zirconia support materials are stable from pH 1 to 14 and at elevated temperatures. It can be derivatized for reversed - phase applications (Neue *et al.*, 2007).

The particles of the silica support come in a variety of sizes and this defines the quality, back-pressure and efficiency of the column. Particle sizes range from 2 - 5 µm for analytical columns and above 10 µm for preparative columns. Smaller particle size provides larger surface area resulting in a greater number of theoretical plates or increased separation efficiency.

Most support materials are porous thereby providing a larger surface area to maximize interaction between the sample and the stationary phase. Pore sizes range from 60 - 200 A°. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution (Dong, 2006).

Mobile phase

This is the liquid phase that moves the analyte across the stationary phase. The mobile phase interacts with the analyte and the stationary phase hence greatly influencing retention and separation. An ideal mobile phase or solvent should:

- Have the ability to interact with the stationary phase and the analyte to bring about the desired separation.
- Be highly pure
- Be relatively inexpensive
- Be compatible with the HPLC system and the detection system

- Have low UV cut - off if UV detector is used
- Have low viscosity
- Be safe for use i.e. low flammability and toxicity
- Highly inert (Liu and Vailaya, 2007)

Eluent polarity plays the major role in HPLC. Different proportions of different solvents are used to adjust the polarity of the mobile phase. Binary mixtures are most commonly used however ternary mixtures are also used. Methanol and acetonitrile are frequently used solvents in reversed phase chromatography and hexane is used in normal phase chromatography.

Mobile phase additives are used to control retention, enhance separation and peak shapes as well as reduce peak tailing. Buffers are added to control the pH of the mobile phase. They are most effective within ± 1.5 units of their pKa. Phosphate buffer is commonly commonly used with reversed phase HPLC. Acid modifiers such as Trifluoroacetic acid, phosphoric acid and acetic acid are commonly used to lower the pH of mobile phases to suppress ionization of weakly acidic analytes. Ion pairing reagents can be added to the mobile phase to provide retention for analytes (McPolin, 2009). Examples are heptanesulphonate and heptafluorobutyric acid. The mobile phase is propelled through the column by two types of elution:

a. Isocratic Elution

It is the simplest form of elution. It employs a mobile phase of constant composition throughout the analysis. It is achieved by either pumping the mixed mobile phase prepared prior to analysis through a single reservoir or by the delivery of a constant ratio of solvents by the binary pumps (Kupeic *et al.*, 2005). It is the preferred form of elution to maximize the loading capacity. The system as well as the column are equilibrated at all times and don't suffer from fast chemical changes.

b. Gradient Elution

The general problem of elution common with complex mixtures characterized by poor separation of components is reduced with this form of elution. It offers complete separation of the components with good peak resolution in reasonable time. It can be used in the separation of mixtures containing compounds of a wide range of polarities in shorter time without compromising resolution. It involves a continuous or steady alteration in mobile phase composition to increase the strength of the mobile phase**.** It enables the faster elution of strongly retained components while having the weakly retained components well resolved (Dorsey and Stout, 2002; Kazakevich, 2007).

Gradient elution separations generally provide faster and more efficient separations with improved limits of detection and less tailing for most compounds present in the sample even though chromatographers mostly avoid its use because of "ghost" peaks, baseline noise associated with it. Gradient elution requires more complex and expensive equipment and is difficult to maintain at a constant flow rate (Schellinger and Carr, 2006).

Separation mechanisms of HPLC

Liquid chromatography is further expanded according to the type of the interactions between the analyte and the stationary phase surface and according to the relative polarity of the stationary and mobile phases.

a. Adsorption Chromatography

It is the oldest of the chromatographic separation techniques. The two most common adsorbents are silica gel and alumina, with silica being by far the most popular. The separation is based on repeated adsorption and desorption between the analytes and the adsorbent. Adsorptive forces as hydrophobic, dipole-dipole, ionic interactions are

involved in this technique. The binding of the analyte to the stationary phase is proportional to the contact surface area around the analyte and the adsorbent (Hurtubise, 2003). The sample components are separated by the equilibration between the mobile phase and the stationary phase.

b. Partition chromatography

Partition chromatography, also known as liquid - liquid chromatography involves the distribution of solute between two immiscible solvents. The difference in partition coefficients of the solutes between the thin film of solvent on the stationary phase, which is usually the more polar solvent and the mobile phase accounts for the separation (Gambhir, 2008).

c. Ion-exchange chromatography

This technique is used almost exclusively with ionic or ionizable samples. The stationary phase consists of insoluble matrix to which charged groups have been covalently bound. Typical stationary phases are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to silica or polymeric materials. The mobile phase consists of aqueous buffers, where pH and ionic strength are used to control elution time. Separation in ion exchange chromatography is achieved by the reversible adsorption of ionic analytes to immobilized ion exchange sites or counter-ions of opposite charge on the ion exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces (Haddad and Jackson, 1990).

Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides, and other charged biomolecules. This is due to its widespread applicability, high resolving power, high capacity, simplicity and controllability of the method (Millner, 1999).

d. Size exclusion chromatography

Separation in size exclusion chromatography is on the basis of molecular size. The columns contain porous particles with precisely controlled pore sizes. The pore diameter defines the exclusion limit of the gel. Particles too large to enter the pores are excluded and have access to only the void volume and rapidly elute together in a single peak. Molecules that are smaller than the pore size enter the particles and their separation is determined by the pore size distribution within the pore volume. Among these, the larger ones are eluted earlier than the smaller particles. The flow rate of the sample entering the column is critical as it enables particles or molecules to diffuse in and out of the pores (Kazakevich and LoBrutto, 2007).

It is primarily for the analysis of large molecules such as polymers. It is the most widely used method for aggregation analysis of pharmaceutical proteins (Arakawa *et al.*, 2010).

e. Chiral chromatography

Chiral chromatography predominantly used in biomedical and pharmaceutical analysis involves the use of chemically bound chiral stationary phases. These include polysaccharide derivatives, cyclodextrins, pirkle type, proteins among others (Petterson and Persson, 1998). The stationary phase interacts with the analyte enantiomers to form short - lived diastereometric association. Intermolecular interactions including hydrogen bonding and ionic interactions occur between the analytes and the stationary phase material. It is a useful technique in the detection, separation and quantitation of optically active impurities in chiral active pharmaceutical ingredients (APIs) and drug products (Wang *et al.*, 2007).

Normal phase chromatography

Normal phase chromatography is a technique that employs the stationary phase material which is polar in nature in combination with a non - polar or moderately polar mobile phase to separate components of mixtures. There are a number of stationary phase materials or adsorbents but silica is the most common and provides very high selectivity for many applications. Non - polar solvents such as hexane and heptane are usually used (Meyer, 2006; Liu and Vailaya, 2007).

Retention of solutes is primarily a function of their relative polarity. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. Non - polar solvents like hexane with the addition of polar modifiers allow for control of retention of analytes to the stationary phase. Compounds that are not water soluble or that may decompose in water are better analyzed using normal phase chromatography. It is also useful for the separation of isomers and compounds that differ in the number or character of functional groups (Cooper, 2006).

Reversed phase chromatography

Reversed - phase chromatography also called bonded phase is widely used in pharmaceutical analysis. The packing material employed in this mode of chromatography is usually porous materials with hydrophobic surfaces. The separation mechanism depends on the hydrophobic binding interaction between the solute molecules and the immobilized hydrophobic ligands on the stationary phase. Majority of packing materials or adsorbents are chemically modified porous silica. Modern bonded phases are prepared by reacting the silanol groups on the surface of silica with an organochlorosilane (Ayim and Olaniyi, 2000). The mobile phase is usually a mixture of aqueous and organic solvents. Organic solvents most frequently used include methanol and acetonitrile. Here,

the more non - polar the material is, the longer it is retained. Reversed - phase chromatography is used for almost ninety percent of all chromatographic applications because

- A wide range of stationary phases are available to alter retention and selectivity
- Very simple mobile phases can be applied
- Columns are efficient and stable
- It allows precise control of type of organic solvent, pH and temperature
- It can be used to analyze both neutral and ionic compounds (Wittrig, 2003).

The hydrophobicity of an analyte can be influenced by pH. For this reason, buffers are used in most RPLC methods however covalently bound silane ligands of bonded-phase packing are hydrolyzed and the silica support is dissolved by aqueous mobile phases above pH 8. This often leads to premature column failure limiting the use of silica-based columns for applications requiring high pH (Kirkland *et al.*, 1995). Reverse phase chromatography has found both analytical and preparative applications in biochemical separation and purification.

Instrumentation

a. Pump

The high-pressure pumping system is an important part of the liquid chromatograph. It provides the high pressure required to propel the mobile phase and analytes through the densely packed column. Its performance directly affects the retention time and reproducibility. The flow is monitored by computer controlled devices. Most conventional pumping systems provide pressure up to 6000 psi. Ultrahigh Pressure Liquid Chromatography (UHPLC) introduced by MacNair et al. in order to take advantage of the

high efficiency potential of very small particles demonstrates high efficiency and high speed at pressures as high as 72 000 psi (Xiang *et al.*, 2003).

The pumping system must be able to provide constant and reproducible pressure up to 6000 psi, pulseless output and flow rates ranging from 0.1 to 10 ml/min. The reciprocating piston pumps, displacement type pumps and the pneumatic or the constant pressure pump are the types of pumps encountered (Jeffery et al., 1989; Ayim and Olaniyi, 2000).

b. Column

The column is an essential part of the High Performance Liquid Chromatograph. It holds the stationary bed which provides differential retention of components. It is usually a stainless steel tube filled with the packing material, often at ambient temperature. The stainless steel can be replaced with titanium or polyetheretherketone (PEEK) for more corrosion resistance in ion chromatography. Typical analytical columns are 50 - 250 mm long and 2.0 - 4.6 mm in diameter. Larger columns exist for preparative work. Shorter columns and smaller internal diameter analytical columns offer higher sensitivity, lower solvent usage and reduced analysis time (Dong, 2006). Columns are further categorized according to the different chromatographic modes of separation. Normal phase, reverse phase, size exclusion, ion exchange columns as well as specialized chiral columns may be used depending on the nature of the mixture to be separated. A typical column lifetime is about three to twenty four months.

Care of the column is key to obtaining reliable results in analysis and to the life time of the column. A guard column which is of the same nature as the main analytical column can be used to trap impurities or particles from the samples especially environmental samples where sample clean up may not be feasible. This is because impurities block

adsorption sites, change the selectivity of the column and cause peak splitting in the chromatogram. Buffers need to be thoroughly washed off by conditioning the column when they are used. Highly pure HPLC grade solvents can be used. Columns should be stored in the appropriate solvent after use.

c. Injector

Samples are injected into the HPLC via an injection port. The injection port of an HPLC consists of an injection valve and a sample loop. The sample dissolved in an appropriate solvent drawn into a syringe is injected into the loop via the injection valve. The rotation of the valve rotor closes the injection valve and opens the sample loop in order to inject the sample into the stream of the mobile phase. The loop volume ranges from 10 µl to over 500 µl (Beckett and Stenlake, 1988).

In more sophisticated liquid chromatography systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers. This is very useful in multiple analyses. It is always best to remove particles from the sample by filtering or by centrifuging to prevent blockage in the injection device (McPolin, 2009).

d. Detectors

The detector is the component of the HPLC instrument that emits response due to the eluting compound by monitoring properties inherent in them. Positioned immediately posterior to the column, it translates the changes in the chemical composition of the analyte at the column exit into an electrical signal. This signal is subsequently processed and recorded as a peak which gives information about the analyte (Dong, 2006). Detectors can be broadly classified as;

- Bulk property detectors

Bulk property detectors continuously monitor the difference in bulk property between the mobile phase containing the solute and the pure mobile phase. E.g. Refractive index, conductivity. Bulk property detectors have a finite signal in the absence of a solute. This results in two serious limitations of these detectors. First, the addition of a low concentration of solute will add only a small increment to what may already be a large background signal; as a result, these detectors generally have poor limits of detection and are generally not suitable for trace analysis. Second, as they also respond to the mobile phase, the signal changes with changes in mobile phase conditions. These detectors are therefore incompatible with gradient elution techniques (Scott, 1999).

- Solute property detectors

Solute property detectors respond to some unique chemical properties of compounds independent of the mobile phase. E.g. Redox behaviour, UV/Vis absorption, fluorescence. These detectors generally have high sensitivity and much lower limits of detection (Jeffery *et al.*, 1989).

- Desolvation detectors

This type of detector makes use of some property of the analyte when the mobile phase is removed. The mass spectrometer detector is an example of this type of detector (Ayim and Olaniyi, 2000).

Ultraviolet/Visible absorption Detector

It is based on the principle of absorption of UV or visible light by the solute according to Beer - Lambert law as it emerges from the column. The UV/Vis absorption detector is the most commonly used detector in pharmaceutical analysis as most pharmaceuticals have chromophores and therefore have UV absorbance. It is highly sensitive, reproducible and

easy to operate. It can detect as low as 1 ng of solute (Ayim and Olaniyi, 2000). The three types of UV detectors are:

- Fixed wavelength detector

A single UV lamp emits light at a specific wavelength. Even though other wavelengths are present the lamp emits light of very high intensity compared with that emitted at the same wavelength by broad spectrum emission lamps.

- Variable wavelength detector

It employs a lamp that emits light over a wide wavelength range. A monochromator can be used to select a particular wavelength for detection purposes.

- Diode array detector

The diode array detector is the most powerful UV detector. The xenon or deuterium lamp employed emits light over the UV spectrum range allowing the continuous monitoring of the entire spectrum. It enables the wavelength scan to be taken therefore samples whose maximum wavelengths are unknown can be analyzed (Scott, 1998).

Fluorescence Detector

A fluorescence detector monitors the emitted photon or fluorescent light of the solute after excitation by UV radiation. It is not versatile as it is limited to compounds that fluoresce. It is therefore applicable to many biological compounds however derivatisation schemes exist to add fluorophores to non-fluorescing compounds. It is selective and highly sensitive (pictograms to femtograms). It is useful in trace analysis in environmental and forensic analysis (Dorsey and Stout, 2002).

Refractive Index Detector

Refractive index detectors measure the change in refractive index of the mobile phase containing the solute and the mobile phase alone. It is useful especially for compounds that do not absorb UV light. It is usually used in the separation of carbohydrates.

Refractive index detectors are sensitive to temperature, pressure and dissolved gases. It is also sensitive to change in mobile phase conditions so are applicable only in isocratic separations. Refractive index detectors have very low sensitivity of about 0.1-10 µg (Dong, 2006).

Electrochemical Detector

The electrochemical detector measures the current generated with the oxidation and reduction of solutes as it emerges from the column. They include coulometric, polarographic, amperometric and potentiometric detectors. It offers high selectivity and sensitivity in picograms. They are incompatible with gradient elution. It is conveniently applicable with ketones, aldehydes, phenols etc. (Jeffery *et al.*, 1989).

Nuclear magnetic resonance absorption Detector

Nuclear magnetic resonance absorption detectors have grown rapidly due to improvement in instrumentation in recent years. NMR is used with liquid chromatography reduces analysis time. It allows for the structure elucidation of unknown compounds. This technique however offers very low sensitivity (Norwood *et al.*, 2007).

Mass Spectrometer detector

This type of detector is based on the ionization of compound being separated. This technique offers highly efficient separation, high sensitivity with reduced analysis time (Ogundaini *et al.*, 2000a).

2.2.1.2Thin layer Chromatography

Thin - layer chromatography (TLC) is a fast, inexpensive and versatile technique which is widely used for the purpose of identification since outcome of quantitative analysis is generally not of high precision and accuracy (Jeffery *et al.*, 1989). In thin - layer chromatography, a mobile phase moves by capillary action across the stationary phase,

usually finely ground silica, alumina, cellulose or kieselguhr particles mixed with a binding agent such as calcium sulphate and a fluorescent indicator coated on a glass slide, plastic sheet or aluminium foil (Ayim and Olaniyi, 2000)

By the manipulation the mobile phase, organic compounds can be separated. The molecules are distributed by partition between the mobile and stationary phase. Different compounds depending on their interactions with the stationary and mobile phase will adhere to the stationary phase more or less than the other compounds allowing efficient separation. Visualization techniques commonly used are UV light of wavelength 254 nm or an iodine chamber. Spraying with a solution of sulphuric acid is commonly used for organic compounds (Christian, 2004). The main uses of TLC are:

- qualitatively determine the number of components in a mixture
- to determine the purity of a compound
- determine appropriate conditions for running column chromatography
- analyze the fractions obtained from column chromatography (Magdum *et al.*, 2008).

The chromatographic measurement of a substance in TLC is the retardation factor which is expressed as

$$Rf = \frac{Distance\ travelled\ by\ substance\ from\ origin}{Distance\ travelled\ by\ solvent\ from\ origin}$$

The Rf value is expressed either as a fraction or as a percentage. In modern times, TLC has seen advancement in instrumentation. This includes High Performance Thin Layer Chromatography (HPTLC) and TLC - MS which helps achieve increased chromatographic efficiency and quite useful in quantitative work (Ayim and Olaniyi, 2000).

2.2.2 UV/Visible Spectroscopy

Ultraviolet - visible spectrophotometry involves the measurement of the absorption of electromagnetic radiation of wavelengths between 200 and 800 nm by molecules which have electrons or atoms possessing unshared electron pairs in solution. It is one of the most frequently employed in pharmaceutical analysis as a wide variety of pharmaceutical substances absorb radiation in the ultraviolet (190 - 380 nm) and visible (380 - 800 nm) regions of the electromagnetic spectrum (Carey, 1996; Bloch, 2006). UV / VIS spectrometry has limited applications in qualitative analysis however it is probably the most useful tool available for quantitative determinations in diverse areas due its versatility, accuracy and sensitivity. It can be used for direct determination of a large number of organic, inorganic and biochemical species accurately at fairly low concentrations, 10^{-4} to 10^{-5} or even lower. In addition to these, the convenience of conducting the determination and its reasonable selectivity make it a method of choice for quantitative determinations (Miyawa and Schulman, 2002).

When electromagnetic radiation is passed through an absorbing medium, some light is absorbed and the rest is transmitted. The intensity of the transmitted light is measured and is found to depend on the thickness of the absorbing medium and the concentration. This dependence forms the basis of spectrometric determinations. The transmitted radiation has information about the nature and the amount of the absorbing species and is given in terms of two fundamental laws. Lambert's law, which expresses the relationship between the light absorption of the sample and the thickness of the absorbing medium or the optical path length;

$$Absorbance = \log_{10}(Io/I) = kl$$

The other is Beer's law, which expresses the relationship between the light absorption of the sample and its concentration.

$$Absorbance = \log_{10}(Io/I) = kc$$

A combination of the two laws gives Beer - Lambert's law given as

$$Absorbance = abc$$

$Where\ Io = Incident\ light$

$I = Transmitted\ light$

$k = constant\ of\ proportionality$

$l, b = Path\ length\ in\ cm$

$c = Concentration$

$a = Specific\ absorbance$

The combined Beer - Lambert law is the basis of all analytical absorption and holds when the incident light is monochromatic, solutions are dilute and stray radiations are excluded. Some of its uses in pharmaceutical analysis include tablet dissolution, analysis of multicomponent mixtures as well as the analysis of raw materials, intermediate and finished products (Ogungbamila and Olaniyi, 1991; Ogundaini *et al.*, 2000b).

2.2.3 Titrimetry

Titrimetry is a frequently used technique in quantitative analysis and pharmaceutical assays. This is because it is a generally simple and inexpensive procedure. It involves the measure of titrant (a solution of accurately known concentration) that is required to bring about a complete chemical reaction with an analyte. The completion of titration is accompanied by some physical or chemical transitions in the analyte (Gupta *et al.*, 2011). This can be identified visually or by instrumental techniques.

- Visual detection

This involves the use of indicators which sharply define the end point by a change in physical properties as the formation of colour or precipitate. These indicators usually are

composed of highly conjugated organic constituents that give rise to the colour over a pH range. Methyl orange, phenol red and phenolphthalein are some commonly used indicators in acid alkali titrations (Ogungbamila and Olaniyi, 1991).

- Potentiometric titration

It involves the measure of electric potential across an indicator electrode and a reference electrode. It makes use of potential as a function of titrant volume, where the actual change in concentration as the titrant is added is measured. The end point is indicated by the largest break in potential. It is very accurate compared to volumetric titrations because of the high sensitivity of the pH meter as compared to visual indicators. The reaction taking place dictates the indicator electrode to be used. The commonly used indicator electrode for redox reactions is the platinum wire. The glass electrode or any other pH responsive electrode is used for neutralization reactions while in precipitation reactions, an indicator that readily comes into equilibrium with one of the ions present is used (Beckett and Stenlake, 1988).

The precision, accuracy and reproducibility of a titrimetric method depends on accurate weighing, preparation of solutions, tolerance of glassware and the ability to accurately locate the end point of a reaction (Olaniyi, 2000). There are different chemical reactions in titrimetric analysis.

Reactions in titrimetric analysis

a. Neutralization titration

This form of titration involves the transfer of protons (Bronsted - Lowry) or electron - pairs (Lewis) from one of the reacting species to the other in solution. The focus here is the changes in H^+ ion concentration. The end point is detected by a sudden change in pH. In order for the titration reaction to go to completion, a strong acid or a strong base is the

usual choice for a titrant with leveling effect in aqueous medium in mind (Gupta *et al.*, 2011).

b. Non - aqueous titration

Non - aqueous titration involves the use of a solvent other than water as the medium for titration. This is done to enhance the acidity or basicity of the compound being determined and hence improve the detection of the end point. This titrimetric method is the method of choice when:

- The sample is insoluble in water.
- The sample and/or titrant reacts with water in an undesirable way.
- The analyte is very weakly acidic or basic.
- Selectivity is enhanced particularly in analysis of analytes with similar dissociation constants (Ayim and Olaniyi, 2000).

A common titrant for non - aqueous acid - base titrations is $HClO_4$ in glacial acetic acid. Perchloric acid dissolved in acetic acid has greater tendency to donate protons as acetic acid functions as a base combining with the proton donated by perchloric acid to form onium ion which is a strong acid. Similarly, a weak base in acetic acid enhances its basicity. The titration of a weak base in acetic acid with acetous perchloric acid helps bring the reaction to a sharp end point with acetic acid exerting a leveling effect. KOH in methanol may be used in the titration of weakly acidic drugs (Svehla, 1979; Chtaurvedi, 2009).

c. Redox titration

Redox titrations are based on the simultaneous oxidation and reduction reaction between analyte and titrant. For the reaction to undergo completion with a sharp end point there

must be a sufficiently large difference between the oxidizing and reducing capabilities of these agents.

$$Ox + Red \leftrightarrow Red' + Ox'$$

The end point is detected commonly by the use of a redox indicator, by potentiometry or by self-indication (Ayim and Olaniyi, 2000; Gupta *et al.*, 2011). A redox indicator produces a sudden change in the electrode potential in the vicinity of the equivalence point during a redox titration. E.g. Ferroin. This is possible when the indicator itself is redox active. Common oxidizing and reducing agents are $KMnO_4$ and sodium thiosulphate respectively (Jeffery *et al.*, 1989; Kar, 2005).

Chapter 3

MATERIALS AND METHODS

3.1 INSTRUMENTS AND GLASSWARE

- Adam-analytical weighing balance
- Eutech instruments pH meter
- Stuart Melting point apparatus SMP10
- Clifton Sonicator
- PG Instruments T90 + UV/Vis Spectrophotometer
- HPLC Chromatograph
    - Kontron instrument HPLC pump 422
    - Perkin Elmer 785A UV/Vis Detector
    - Powerchrome 280 software integrator
- Conical flasks
- Burette
- Measuring beakers (25 ml)
- Volumetric flasks (1000 ml, 250 ml, 100 ml, 50 ml, 25 ml)
- Transfer pipettes (25 ml, 20 ml, 10 ml)
- Graduated pipettes (1 ml, 5 ml, 10 ml)
- Test tubes
- No. 1 Whatman filter papers
- Glass funnel
- Melting point capillary tubes
- Pre-coated TLC plates (Gf 254, 0.25 mm Merck W.)

3.2 MATERIALS AND REAGENTS

- Sodium hydroxide (BDH)
- Hydrochloric acid (36 %, BDH)
- Sulphuric acid (98 %, BDH)
- Sulphamic acid (BDH)
- Perchloric acid (70 %, Aldrich)
- Glacial acetic acid (BDH)
- Acetic anhydride (BDH)
- Potassium hydrogen phthalate (BDH)
- Mercuric acetate
- Crystal violet
- Iodine
- Potassium iodide
- Starch solution
- Sodium thiosulphate (BDH)
- Sodium acetate (BDH)
- Ethyl acetate (BDH)
- Dichloromethane
- Toluene
- Ammonia
- Methanol
- Ethanol
- Acetonitrile (BDH)
- Potassium dichromate
- Nitric acid

- Sodium nitrite

Table 3-1 Profile of pure samples

Drug	Source	Batch number	Date of Manufacture	Date of Expiry	Assay
Cetirizine hydrochloride	Ernest Chemist Gh. Ltd	CDH/002/10	June, 2010	May, 2013	99.80 ± 0.29%
Griseofulvin	Ayrton Drugs	110438	April, 2011	April, 2015	100.04±0.44%
Paracetamol	Ayrton Drugs	1104125	April, 2011	April, 2015	99.80±0.70%
Ascorbic Acid	Amponsah Effah Ltd	0911847	June, 2004	June, 2012	99.78±0.31%
Metronidazole	Ayrton Drugs	20110340	March, 2011	March, 2015	100.14±0.92%

Table 3-2 Brands of Griseofulvin

Tablet	Manufacturer	Strength	Batch number	Date of Manufacture	Date of expiry
Griseofulvin (AG)	Ayrton Drugs Ltd	125mg	FN014	June, 2012	May, 2015
Griseofulvin (EG)	Ernest Chemist Gh. Ltd	125mg	0204L	April. 2011	April, 2015
Griseofulvin (LG)	Letap Pharmaceuticals Ltd	125mg	1270112	Feb. 2012	Feb, 2014

Table 3-3 Brands of Cetirizine hydrochloride

Tablet	Manufacturer	Strength	Batch number	Date of Manufacture	Date of expiry
Cetirizine hydrochloride (EC)	Ernest Chemist Gh. Ltd	10mg	0109L	Sept. 2011	Sept. 2014
Cetirizine hydrochloride (HC)	Hovid	10mg	BA11432	Nov. 2011	Nov. 2015
Cetirizine hydrochloride (KC)	Kinapharma Ltd	10mg	12006	Feb. 2012	Feb. 2016

3.3 MELTING POINT DETERMINATION

A capillary tube was filled with the pure sample of each drug. The capillary tube was then put into the melting point determination apparatus and the melting points determined.

3.4 IDENTIFICATION TESTS

3.4.1 Cetirizine hydrochloride

20 mg of the pure sample was dissolved in 50 ml of 1.03 %w/v of HCl and diluted to 100 ml with the same acid. 10 ml of this solution was then diluted to 100 ml. The resulting solution was scanned in the UV spectral range 210 nm to 350 nm. The specific absorbance was then calculated from the absorbance obtained (British Pharmacopoeia, 2007).

3.4.2 Griseofulvin

5 mg of pure Griseofulvin powder was weighed and dissolved in 1 ml concentrated sulphuric acid. 5 mg potassium dichromate was then added and colour change was observed (British Pharmacopoeia, 2007).

3.4.3 Paracetamol

About 0.1 g of the powder was diluted to 100 ml with methanol. 0.5 ml of 1.03 %w/v of HCl was added to 1 ml of this solution and diluted to 100 ml with methanol. The absorbance of the resulting solution was read at 249 nm and the specific absorbance calculated (British Pharmacopoeia, 2007).

3.4.4 Ascorbic Acid

1.0 g of pure ascorbic acid was dissolved in 20 ml of carbon dioxide free water. 0.2 ml of 2 M nitric acid and 0.2 ml of 1.7 %w/v of silver nitrate were added to 1ml of this solution and the colour change was observed (British Pharmacopoeia, 2007).

3.4.5 Metronidazole

About 40.0 mg of pure metronidazole powder was dissolved in 0.1M hydrochloric acid and diluted to 100.0 ml with the same acid. 5.0 ml of the solution was diluted to 100.0 ml with 0.1M hydrochloric acid. The resulting solution was examined between 230 nm and 350 nm and the specific absorbance at the maximum was then determined (British Pharmacopoeia, 2007).

3.5 SOLUBILITY TESTS

An amount of 0.05 g of the pure samples was dissolved in 5 ml of each of water, methanol and observations recorded.

3.6 THIN LAYER CHROMATOGRAPHY ANALYSIS OF PURE SAMPLES AND TABLETS

3.6.1 Griseofulvin

10 mg of pure was Griseofulvin dissolved in methanol and made to 10 ml with the same solvent. A quantity of the powdered Griseofulvin tablets containing 10 mg of Griseofulvin was also dissolved in methanol to 10 ml. About 20 µl portions of the test solution and the standard solution were spotted on a pre - coated TLC plate and allowed to dry. The chromatogram was developed in a chamber containing a mobile phase made of toluene: ethyl acetate: glacial acetic acid (6: 4: 0.5 v/v/v).The plate was removed, air dried and examined under UV light at 254 nm.

The Rf values of both the standard and sample were compared.

3.6.2 Cetirizine hydrochloride

10 mg of pure Cetirizine hydrochloride was dissolved to 10 ml with distilled water. A quantity of the powdered Cetirizine hydrochloride tablets containing 10 mg of Cetirizine hydrochloride was also dissolved with distilled water to 10 ml. About 20 µl portions of the test solution and the standard solution were spotted separately on a pre - coated TLC

plate and allowed to dry. The chromatogram was developed with ammonia: methanol: methylene chloride (1: 10: 90 v/v/v) as mobile phase. The plate was removed when the solvent had moved over the measured distance from the point of application, air dried and then examined under UV light at 254 nm.

The Rf values of both the standard and sample were compared.

3.7 STANDARDIZATION OF SOLUTIONS

3.7.1 Standardization of 0.1 M Perchloric acid

0.5 g of potassium hydrogen phthalate was weighed and dissolved in 25 ml glacial acetic acid. This was titrated with 0.1M Perchloric acid using crystal violet as indicator. This was triplicated.

3.7.2 Standardization of 0.05 M Iodine

25 ml of the 0.05 M iodine was pipetted into a conical flask. It was then titrated with the 0.1 M Sodium thiosulphate.

3.7.3 Standardization of 0.1 M Sodium Nitrite

About 30 ml of water was added to 25 ml of 0.02 M $KMnO_4$. 4 ml of concentrated sulphuric acid was added to the mixture with swirling. The solution was titrated with 0.1 M sodium nitrite.

3.8 ASSAY OF PURE SAMPLES

3.8.1 Assay of Griseofulvin

80 mg of pure Griseofulvin was dissolved in ethanol to 200 ml. 2 ml of this solution was then diluted to 100 ml with ethanol. The absorbance of this solution was taken at 291 nm. The content of $C_{17}H_{17}ClO_6$ was calculated with specific absorbance as 686 (British Pharmacopoeia, 2007).

3.8.2 Assay of Cetirizine hydrochloride

0.100 g of the pure powder was dissolved in 25 ml glacial acetic acid. 6 ml of mercury (II) acetate in glacial acetic acid and drops of crystal violet indicator were then added. The solution was titrated with 0.1 M perchloric acid. A blank titration was carried out.

1 ml of 0.1 M perchloric acid is equivalent to 23.09 mg of $C_{21}H_{27}Cl_3N_2O_3$.

3.8.3 Assay of Paracetamol

About 0.25 g of Paracetamol powder was weighed. 10 ml of 70 g/l hydrochloric acid was added and the mixture boiled under reflux for an hour. The condenser was washed with 30 ml of water and 1 g of potassium bromide was then added. The solution was titrated with 0.1 M sodium nitrite.

1 ml of 0.1M sodium nitrite is equivalent to 15.12 mg of $C_8H_9NO_2$ (International Pharmacopoeia, 2006).

3.8.4 Assay of Metronidazole

0.150 g of metronidazole powder was dissolved in 50 ml of glacial acetic acid. This solution was titrated with 0.1 M perchloric acid with crystal violet as the indicator.

1 ml of 0.1 M perchloric acid is equivalent to 17.12 mg of $C_6H_9N_3O_3$ (British Pharmacopoeia, 2007).

3.8.5 Assay of Ascorbic Acid

0.150 g of the pure powder was dissolved in a mixture of 10 ml of 1 M sulphuric acid and 80 ml of carbon dioxide free water. 1ml of starch solution was then added to the mixture and titrated with 0.05 M iodine.

1 ml of 0.05 M iodine is equivalent to 8.81 mg of $C_6H_8O_6$ (British Pharmacopoeia, 2007).

3.9 UV SCAN OF THE PURE SAMPLES

About 10 mg of pure samples was weighed and dissolved in 10 ml of water and methanol. A solution of concentration 0.001 %w/v was prepared from this solution by serial dilution. The resulting solution was then scanned over the wavelength range of 200 - 400 nm.

3.10 PREPARATION OF BUFFER

2.1 g of sodium acetate was dissolved in 500 ml of distilled water. The pH was adjusted to 4.2 with 2.5 ml of acetic acid.

3.11 HPLC METHOD DEVELOPMENT

Different proportions of the solvents making up the mobile phase were tried at different flow rates to investigate their effect on resolution and the shape of peaks. The following conditions gave sharp and well resolved peaks.

Chromatographic Conditions for Cetirizine hydrochloride and SRSs

Column:	ODS C18 Phenomenex 250 x 4.6 mm
Mobile phase:	Methanol: Sodium acetate buffer pH 4.2 (60: 40 v/v)
Flow rate:	1.5 ml/min
Wavelength of detection:	235 nm
Mode of elution:	Isocratic

Chromatographic Conditions for Griseofulvin and SRSs

Column:	ODS C18 Phenomenex 250 x 4.6 mm
Mobile phase:	Methanol: Water (70: 30 v/v)
Flow rate:	1.0 ml/min
Wavelength of detection:	254 nm

Mode of elution: Isocratic

3.12 VALIDATION OF THE DEVELOPED METHODS

3.12.1 Linearity

A stock solution of 0.01 % w/v of drugs and surrogate reference standards were prepared and serially diluted to different concentrations. They were filtered and injected. The concentrations were plotted against the peak areas obtained.

3.12.2 Repeatability

A mixture of the Griseofulvin (0.006 %w/v) and surrogate reference standards (0.003 %w/v) was prepared, filtered and injected. This was repeated six times. The content was calculated from the peak areas obtained. This was repeated for Cetirizine hydrochloride and the surrogate reference standards.

3.12.3 Intermediate Precision

Intermediate precision was carried out by inter – day precision study. This was done on three different days. 0.006 %w/v of Griseofulvin tablets was mixed with 0.003 % w/v of the surrogate reference standards. The mixture was filtered and injected. The percentage contents were calculated from the peak areas obtained. This was done for Cetirizine hydrochloride as well.

3.12.4 Stability of solutions

About 5 mg of the powder was weighed and dissolved to 10 ml with methanol. The solution was filtered and injected at an interval of 10 minutes for a period of one hour.

3.12.5 Robustness

The analysis was carried out varying one parameter at a time while keeping the others constant to investigate the robustness of the method. The parameters varied were wavelength and flow rate.

3.13 DETERMINATION OF THE "K" VALUE OF THE SURROGATE REFERENCE STANDARDS

Different concentrations chosen from the linear portion of the calibration curve of the drugs and surrogate reference standards were prepared. The mixtures of drug and surrogate were filtered and injected. It was repeated five times and the results computed. The k value of each surrogate in relation to the analyte was then calculated.

3.14 UNIFORMITY OF WEIGHT TEST

Twenty different tablets each of the brands of the tablets were weighed individually. The average weight was found and the percentage deviation calculated.

3.15 ASSAY OF TABLETS WITH THE STANDARD METHOD

3.15.1 Assay of Griseofulvin

0.125 mg of Griseofulvin pure powder was dissolved in methanol. A quantity of powdered Griseofulvin tablets containing 62 mg Griseofulvin was also dissolved in methanol and diluted to 50 ml with the mobile phase. The solutions were filtered and injected at 254 nm. The mobile phase used was water: acetonitrile: tetrahydrofuran (60: 35: 5 v/v/v) at flow rate of 1 ml/min (United States Pharmacopoeia, 2004).

3.15.2 Assay of Cetirizine hydrochloride

5 mg of Cetirizine hydrochloride pure powder was dissolved in the mobile phase. A quantity of the powdered tablet containing 20 mg Cetirizine hydrochloride was also dissolved in the mobile phase. 1 ml of this solution was further diluted to 100 ml with the

mobile phase. The standard and test solutions were filtered and injected. The wavelength of detection was 230 nm. The mobile phase used was dilute sulphuric acid: water: acetonitrile (0.4: 6.6: 93 v/v/v) at flow rate of 1 ml/min

3.16 ASSAY OF TABLETS USING THE K VALUES

3.16.1 Assay of Griseofulvin tablets using the k values

An amount of powdered tablets containing 10 mg of Griseofulvin was weighed, dissolved in methanol to 10 ml and filtered. A 0.006 %w/v solution was prepared from this stock and added to 0.004 %w/v of the surrogate reference standard. The mixture was filtered, injected and the results recorded.

3.16.2 Assay of Cetirizine hydrochloride tablets using the k values

An amount of powdered tablets containing 10 mg of Cetirizine hydrochloride was weighed, dissolved in methanol to 10 ml and filtered. A solution containing 0.008 %w/v of Cetirizine hydrochloride was prepared by serial dilution and added to 0.005 %w/v of the surrogate reference standard. The mixture was then filtered, injected and the results recorded.

Chapter 4

RESULTS AND CALCULATIONS

4.1 MELTING POINT DETERMINATION

Table 4-1 Melting points of pure drug substances

Drug	Melting point		Literature value
	1st Det.	2nd Det.	
Griseofulvin	217-223 °C	216-224 °C	217-224 °C
Cetirizine Hydrochloride	222 °C	224 °C	225 °C
Paracetamol	169-171 °C	169-172 °C	168-172 °C
Ascorbic acid	190-192 °C	189-192 °C	190-192 °C
Metronidazole	159-163 °C	158-163 °C	159-163 °C

4.2 IDENTIFICATION TESTS

Table 4-2 Identification tests of Cetirizine hydrochloride, Paracetamol and Metronidazole by the BP method. Results represent mean ± SEM of triplicate determinations.

Drug	Observation	Literature value (BP)	Inference
Cetirizine hydrochloride	371.9 ± 8.2	359 - 381	Present
Paracetamol	903.4 ± 2.6	860 - 980	Present
Metronidazole	377.7 ± 2.9	365 - 395	Present

Table 4-3 Colour tests of Griseofulvin and Ascorbic acid by the BP method .

Drug	Observation	Literature value (BP)	Inference
Griseofulvin	A wine colour was observed	Wine colouration	Present
Ascorbic acid	A grey precipitate was formed	Grey precipitate	Present

4.3 THIN LAYER CHROMATOGRAPHY ANALYSIS

$$Rf = \frac{Distance\ travelled\ by\ substance\ from\ origin}{Distance\ travelled\ by\ solvent\ from\ origin}$$

$$= \frac{3.5}{4.2}$$

$$= 0.833$$

Table 4-4 Comparison of Rf values of brands of Cetirizine hydrochloride with that of pure Cetirizine hydrochloride

Analyte	Manufacturer	Rf value of tablet	Rf value of pure sample
EC	Ernest Chemist Gh. Ltd	0.833	0.833
HC	Hovid	0.833	0.833
KC	Kinapharma Ltd	0.829	0.829

Table 4-5 Comparison of Rf values of brands of Griseofulvin with that of pure Griseofulvin

Analyte	Manufacturer	Rf value of tablet	Rf value of pure sample
AG	Ayrton Drugs	0.814	0.814
EG	Ernest Chemist Gh. Ltd	0.800	0.800
LG	Letap Pharmaceuticals	0.800	0.800

4.4 PERCENTAGE PURITY OF PURE SAMPLES

$Weight\ of\ Griseofulvin = 0.0399g$

$Absorbance\ at\ 291nm = 0.547$

$Concentration\ of\ solution\ A = 0.0399\%w/v$

$Specific\ absorbance = 686$

$$From\ C_1V_1 = C_2V_2$$

$$0.0399 \times 1 = C_2 \times 50$$

$$C_2 = \frac{0.0399}{50}$$

$$C_2 = 0.000798\%w/v$$

$$From\ Beer's\ Law, A = abc$$

$$c = \frac{A}{ab}$$

$$c = \frac{0.547}{686}$$

$$c = 0.000797\%w/v$$

$$\%\ Purity = \frac{Actual\ Concentration}{Nominal\ Concentration} \times 100$$

$$= \frac{0.000797}{0.000798} \times 100$$

$$= 99.90\%$$

Table 4-6 Percentage purity of pure drug substances. Data represents mean ± SD of triplicate determinations.

Drug	Percentage purity	Literature value (BP)
Cetirizine Hydrochloride	99.8 ± 0.29	99 - 100.5 %
Griseofulvin	100.04 ± 0.17	97 - 102 %
Paracetamol	99.80 ± 0.28	98.5 - 101 %
Ascorbic acid	99.78 ± 0.31	99 - 100.5 %
Metronidazole	100.14 ± 0.37	99 - 101 %

4.5 HPLC METHOD DEVELOPMENT

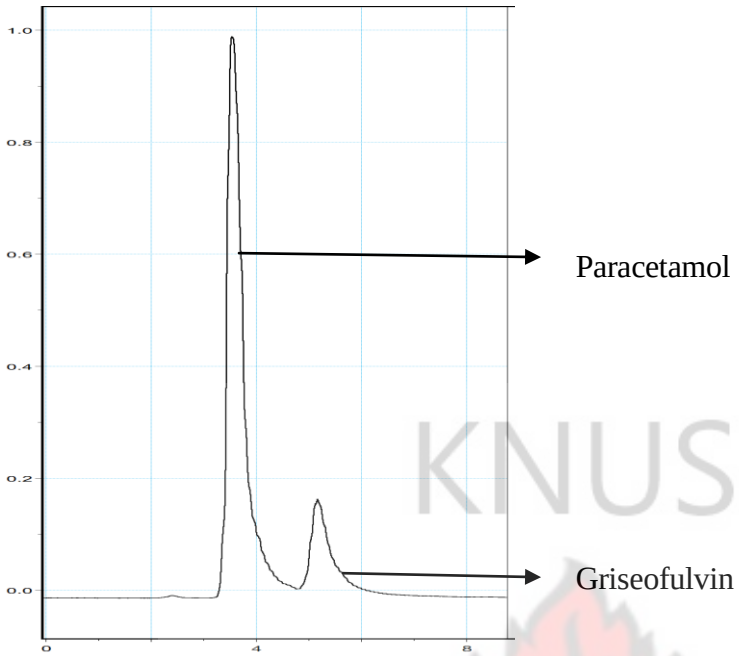

Figure 4-1 Chromatogram of Griseofulvin and Paracetamol

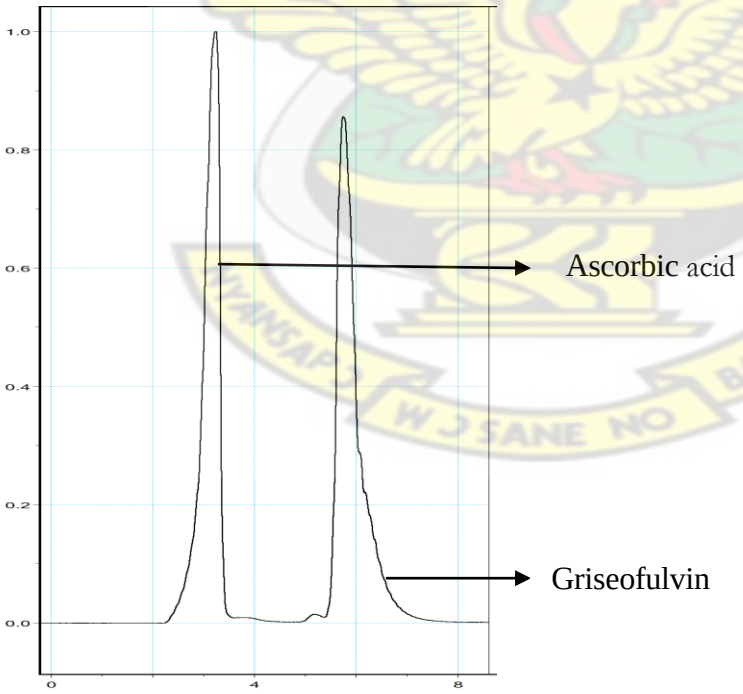

Figure 4-2 Chromatogram of Griseofulvin and Ascorbic acid

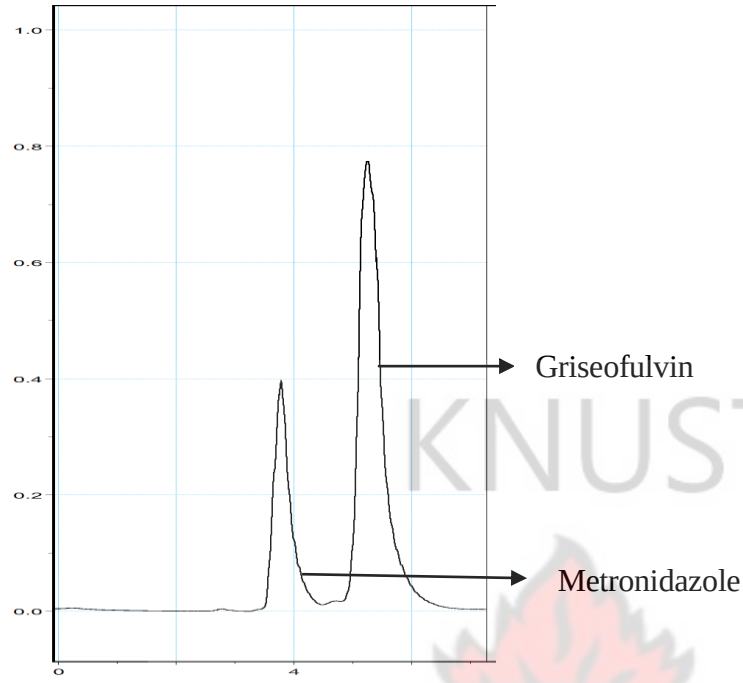

Figure 4-3 Chromatogram of Griseofulvin and Metronidazole

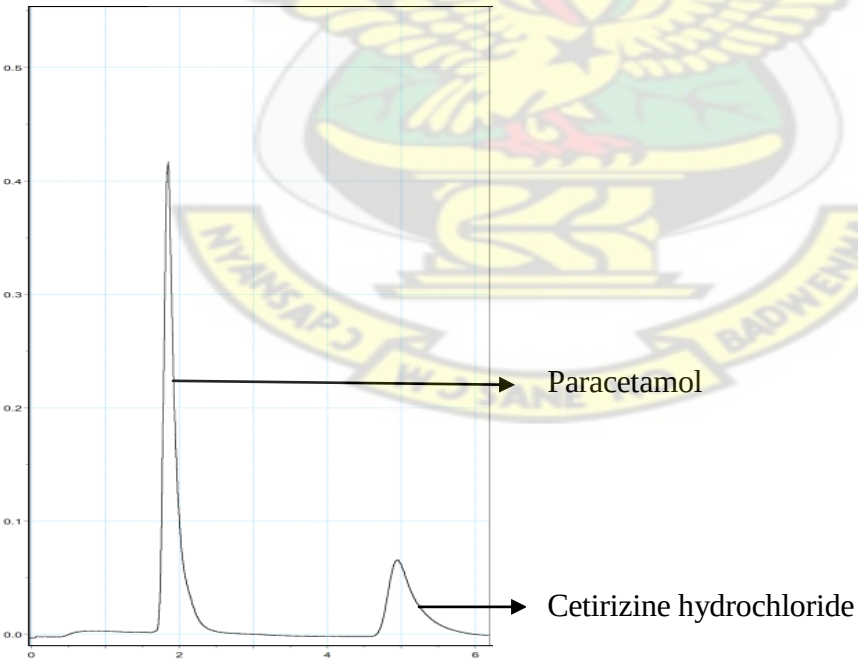

Figure 4-4 Chromatogram of Cetirizine hydrochloride and Paracetamol

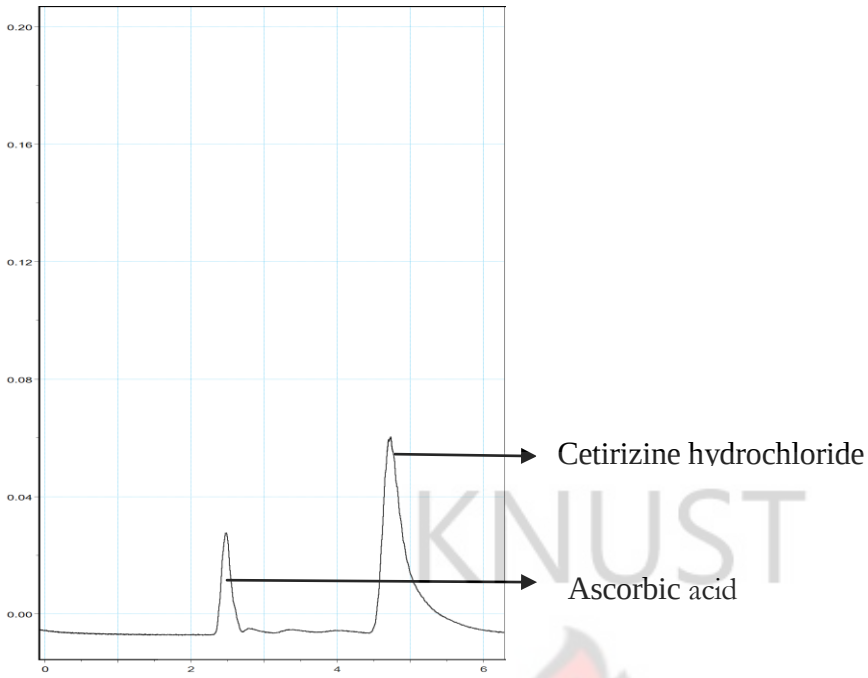

Figure 4-5 Chromatogram of Cetirizine hydrochloride and Ascorbic acid

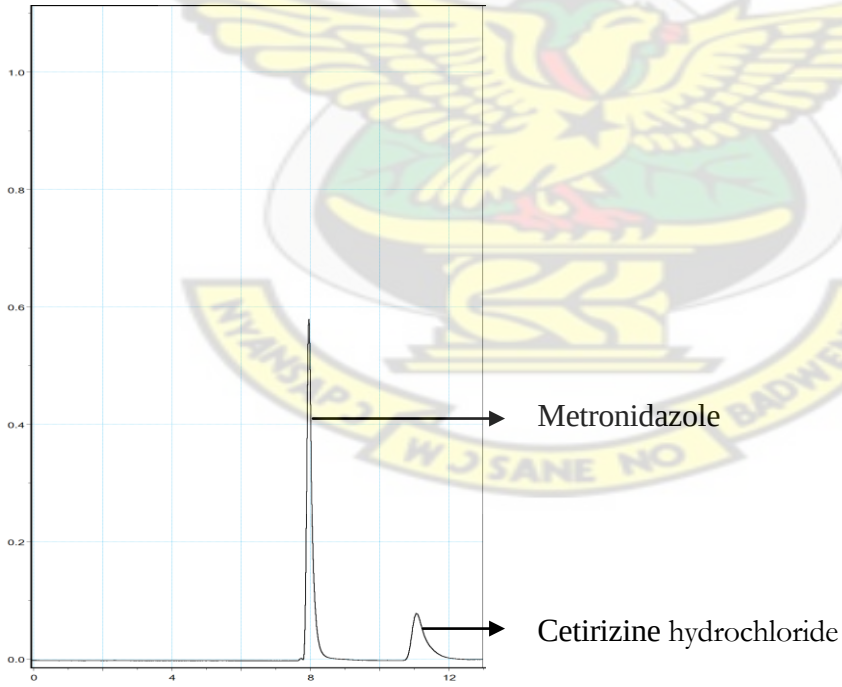

Figure 4-6 Chromatogram of Cetirizine hydrochloride and Metronidazole

Table 4-7 Mean retention times of Griseofulvin and Cetirizine hydrochloride in the presence of the SRSs. Data represents the mean ± SD of six determinations.

Analyte	Mean retention time
Griseofulvin	3.65 ± 0.06
Cetirizine Hydrochloride	4.57 ± 0.06

Table 4-8 Mean retention times of SRSs in the presence of Griseofulvin. Data represents the mean ± SD of six determinations.

Analyte	Mean retention time
Paracetamol	2.47 ± 0.03
Ascorbic acid	1.70 ± 0.02
Metronidazole	2.65 ± 0.04

Table 4-9 Mean retention times of SRSs in the presence of Cetirizine hydrochloride. Data shows the mean ± SD of six determinations.

Analyte	Mean retention time
Paracetamol	1.73 ± 0.05
Ascorbic acid	1.52 ± 0.03
Metronidazole	1.84 ± 0.04

4.6 VALIDATION OF DEVELOPED METHODS

4.6.1 Linearity

Table 4-10 Regression characteristics of developed method for Griseofulvin with SRSs

Drug	Slope	Intercept	Coefficient of correlation (R^2)
Griseofulvin	915.03	0.7991	0.9960
Paracetamol	1224.2	0.8014	0.9970
Ascorbic acid	570.95	0.0731	0.9949
Metronidazole	1532.3	0.6519	0.9980

Table 4-11 Regression characteristics of developed method for Cetirizine hydrochloride with SRSs

Drug	Slope	Intercept	Coefficient of correlation (R^2)
Cetirizine Hydrochloride	182.5	0.2804	0.9964
Paracetamol	936.79	0.4357	0.9961
Ascorbic acid	163.26	0.2543	0.9951
Metronidazole	1477.9	2.4379	0.9951

4.6.2 Limit of Detection and Quantitation

Table 4-12 Calculation of residual standard deviation

Concentration (x)	Peak area (y)	y_{model}	$y-y_{model}$	$(y-y_{model})^2$
0.001	1.89	1.71	0.176	0.03093
0.002	2.57	2.63	-0.059	0.00350
0.004	4.42	4.46	-0.039	0.00154
0.006	6.19	6.29	-0.099	0.00986
0.007	6.98	7.20	-0.224	0.05031
0.008	8.07	8.12	-0.049	0.00243
0.009	9.33	9.03	0.296	0.08740

$$Limit\ of\ Detection = {}^{3.3\sigma}/_{s}$$

$$Limit\ of\ Quantitation = {}^{10\sigma}/_{s}$$

$$Where\ \sigma = Residual\ standard\ deviation$$

$$s = slope$$

$$Equation\ of\ line = y = 915.03x + 0.7991$$

$$\text{Residual Standard Deviation} = \frac{\sqrt{\sum(y - ymodel)^2}}{n-1}$$

$$= \frac{\sqrt{0.18597}}{6}$$

$$= 0.17605$$

$$Limit\ of\ Detection = \frac{3.3 \times 0.17605}{915.03}$$

$$= 0.000634$$

$$Limit\ of\ Quantitation = \frac{10 \times 0.17605}{915.03}$$

$$= 0.00192$$

Table 4-13 LOD and LOQ of Griseofulvin and SRSs

Drug	LOD	LOQ
Griseofulvin	0.00063	0.0019
Paracetamol	0.00051	0.0016
Ascorbic acid	0.00072	0.0022
Metronidazole	0.00052	0.0016

Table 4-14 LOD and LOQ of Cetirizine hydrochloride and SRSs

Drug	LOD	LOQ
Cetirizine Hydrochloride	0.00093	0.0028
Paracetamol	0.00050	0.0015
Ascorbic acid	0.00053	0.0016
Metronidazole	0.00050	0.0015

4.6.3 Stability profile of drug substances

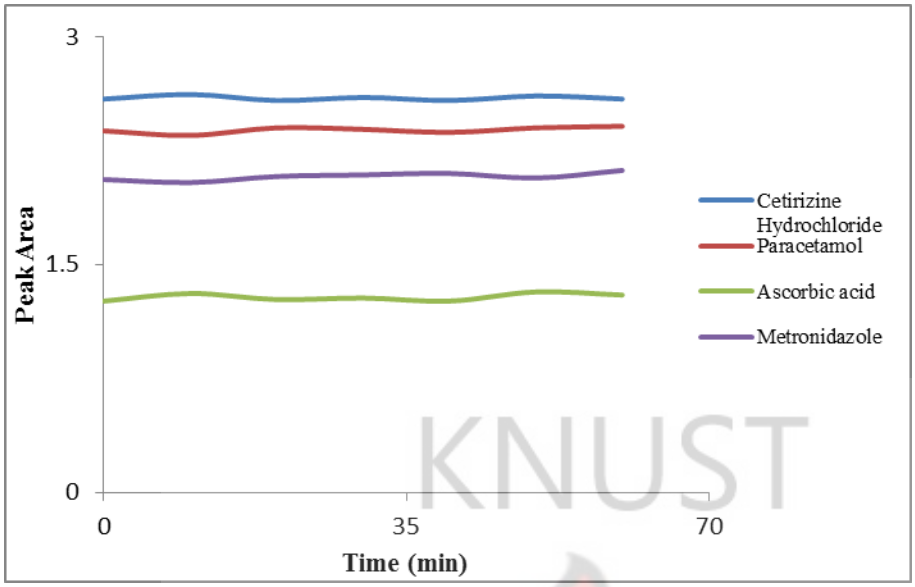

Figure 4-7 Peak area versus time in minutes showing the stability profile of Griseofulvin and SRSs in solution over a period of an hour.

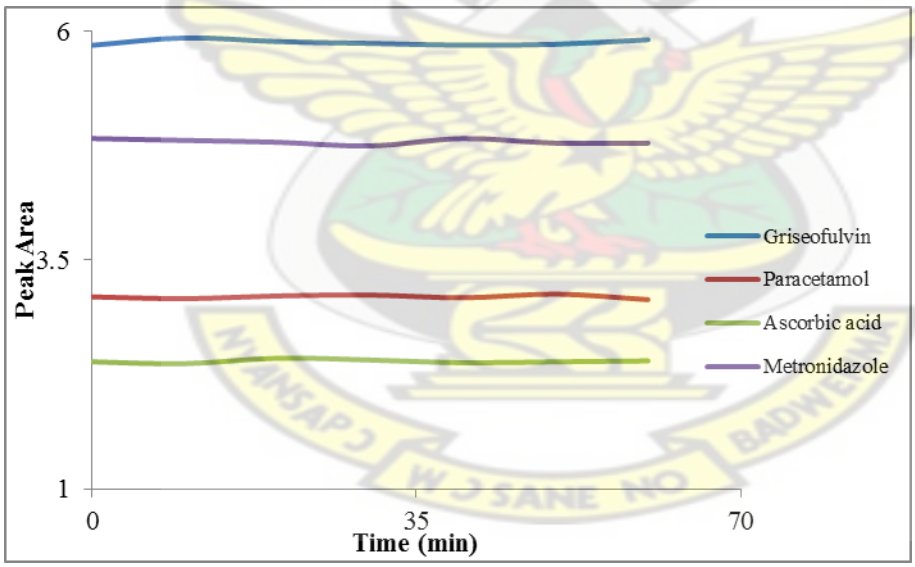

Figure 4-8 Peak area versus time in minutes showing the stability profile of Cetirizine hydrochloride and SRSs in solution over a period of an hour

4.6.4 Repeatability

Table 4-15 Repeatability of method for Griseofulvin. Data represents mean ± SD of six replicate measurements.

SRS	Mean % content	Coefficient of variation
Paracetamol	99.56 ± 0.93	0.9351
Ascorbic acid	99.88 ± 1.07	1.0701
Metronidazole	100.48 ± 0.53	0.5328

Table 4-16 Repeatability of method for Cetirizine hydrochloride. Data indicates mean ± SD of six replicate measurements.

SRS	Mean % content	Coefficient of variation
Paracetamol	99.53 ± 0.54	0.5484
Ascorbic acid	100.40 ± 1.20	1.1973
Metronidazole	100.07 ± 0.92	0.9238

4.6.5 Intermediate precision

Table 4-17 Inter - day precision of method for Griseofulvin. Data indicates mean ± SD of six replicate determinations .

	Surrogate Reference Std.	Mean % content	Coefficient of variation
Day 1	Paracetamol	99.85 ± 0.50	0.5054
	Ascorbic acid	99.98 ± 0.98	0.9754
	Metronidazole	100.71 ± 0.75	0.7500
Day 2	Paracetamol	100.62 ± 0.41	0.4088
	Ascorbic acid	99.72 ± 1.01	1.0142
	Metronidazole	100.11 ± 1.12	1.1167
Day 3	Paracetamol	100.56 ± 0.76	0.7642
	Ascorbic acid	99.73 ± 1.05	1.0570
	Metronidazole	100.40 ± 0.99	0.9850

Table 4-18 Inter - day precision of method for Cetirizine hydrochloride. Data indicates the mean ± SD of six replicate determinations.

	Surrogate Reference Std.	Mean % content	Coefficient of variation
Day 1	Paracetamol	100.06 ± 0.69	0.6901
	Ascorbic acid	99.61 ± 0.85	0.8551
	Metronidazole	99.93 ± 1.21	1.2087
Day 2	Paracetamol	100.19 ±0.38	0.3883
	Ascorbic acid	100.38 ±1.29	1.2863
	Metronidazole	99.47 ± 0.77	0.7795
Day 3	Paracetamol	99.90 ± 1.06	1.0570
	Ascorbic acid	99.46 ± 1.14	1.1435
	Metronidazole	99.50 ± 1.32	1.3228

4.7 CALCULATION OF K VALUES

$$k = \frac{Aa \times Cs}{As \times Ca}$$

Where $Aa = Peak\ area\ of\ analyte$

$As = Peak\ area\ of\ Surrogate\ standard$

$Ca = Concentration\ of\ analyte$

$Cs = Concentration\ of\ Surrogate\ standard$

$$k = \frac{4.91 \times 0.004}{8.59 \times 0.006}$$

$$= \frac{0.01964}{0.05154}$$

$$= 0.38106$$

Table 4-19 Mean K value of SRSs in relation to Griseofulvin. Data represents mean ± SD of six replicate determinations.

Surrogate Reference Standard	K value
Paracetamol	0.3824 ± 0.0073
Ascorbic acid	0.3317 ± 0.0046
Metronidazole	1.0902 ± 0.0063

Table 4-20 Mean K value of SRSs in relation to Cetirizine hydrochloride. Data represents mean ± SD of six determinations.

Surrogate Reference Standard	K value
Paracetamol	0.3530 ± 0.0092
Ascorbic acid	0.3735 ± 0.0100
Metronidazole	0.4929 ± 0.0104

4.8 UNIFORMITY OF WEIGHT TEST

Table 4-21 Uniformity of weight test conducted on various brands of Griseofulvin and Cetirizine hydrochloride

Drug	Brand	Inference
Cetirizine Hydrochloride	EC	Passed
	HC	Passed
	KC	Passed
Griseofulvin	AG	Passed
	EG	Passed
	LG	Passed

4.9 CALCULATION OF PERCENTAGE CONTENT WITH K VALUE

$$Average\ weight\ of\ tablets = 0.1995g$$

$$125mg = 0.1995g$$

$$4mg = \frac{4 \times 0.1995}{125}$$

$$= \frac{0.798}{125}$$

$$= 0.0064$$

$$Area\ of\ analyte = 15.78$$

$$Area\ of\ standard = 9.11$$

$$k\ value\ of\ Metronidazole = 1.09$$

$$Concentration\ of\ standard = 0.004$$

$$Concentration\ of\ analyte = \frac{Aa \times Cs}{k \times As}$$

$$= \frac{15.78 \times 0.004}{1.09 \times 9.11}$$

$$= \frac{0.0631}{9.9299}$$

$$= 0.00635$$

$$Percentage\ content = \frac{Actual\ Concentration}{Nominal\ Concentration} \times 100$$

$$= \frac{0.00635}{0.0064}$$

$$= 99.32\%$$

Table 4-22 Mean percentage content of brands of Griseofulvin calculated with the K value. Data represents mean ± SD of six determinations.

Surrogate reference standard	Percentage content		
	AG	EG	LG
Paracetamol	99.90 ± 0.86	100.07 ± 0.44	99.87 ± 0.68
Ascorbic acid	99.74 ± 1.03	99.54 ± 1.13	100.12 ± 1.06
Metronidazole	100.23 ± 0.63	99.89 ± 1.07	100.34 ± 0.94

Table 4-23 Mean percentage content of brands of Cetirizine hydrochloride calculated with the K value. Data represents mean ± SD of six determinations.

Surrogate reference standard	Percentage content		
	EC	HC	KC
Paracetamol	100.16 ± 1.22	100.05 ± 0.46	100.54 ± 0.81
Ascorbic acid	99.78 ± 0.62	100.11 ± 0.52	100.44 ± 1.23
Metronidazole	99.63 ± 0.90	99.71 ± 0.49	99.99 ± 1.16

Table 4-24 Mean percentage content obtained from the assay of brands of Griseofulvin using the standard USP method. Results represent mean ± SD of six measurements.

Brand	Mean percentage content
AG	100.67 ± 1.11
EG	100.12 ± 1.28
LG	100.57 ± 1.34

Table 4-25 Mean percentage content obtained from the assay of brands of Cetirizine hydrochloride using the standard BP method. Results represent mean ± SD of six measurements.

Brand	Mean percentage content
EC	100.96 ± 1.25
HC	100.68 ± 1.08
KC	100.84 ± 1.02

4.10 COMPARISON OF DEVELOPED METHOD TO THE STANDARD METHODS

Table 4-26 Comparison of standard USP method to developed method for Griseofulvin. Data was analysed using the paired t - test at 95 % confidence level.

Surrogate reference standard	Mean of developed method	Mean of standard method	t_{stat}	t_{crit}
Paracetamol	99.99	100.13	0.23	2.78
Ascorbic acid	100.46	100.13	0.85	2.78
Metronidazole	100.37	100.13	0.49	2.78

Table 4-27 Comparison of standard BP method to developed method for Cetirizine HCL. Data was analysed using the paired t - test at 95 % confidence level.

Surrogate reference standard	Mean of developed method	Mean of standard method	t_{stat}	t_{crit}
Paracetamol	100.27	100.55	0.39	2.78
Ascorbic acid	100.12	100.55	0.62	2.78
Metronidazole	100.31	100.55	0.26	2.78

Chapter 5

DISCUSSIONS

5.1 MELTING POINT DETERMINATION

The melting point of pure samples used in the analysis was determined to confirm their identity. It was also done to measure their purity as pure compound melt over a narrow range and the presence of impurities cause a depression of the melting point and the widening on the melting range (Ogungbamila and Olaniyi, 1991).

5.1.1 Cetirizine hydrochloride

The melting point obtained for the pure samples of Cetirizine hydrochloride in the first and second determinations were 222 °C and 224 °C respectively. This agrees with 225 °C stated by the Clark's analysis of drug and poisons indicating the sample contained cetirizine hydrochloride

5.1.2 Griseofulvin

The melting range obtained for the pure samples of Griseofulvin in the first and second determinations were 217 - 223 °C and 216 - 224 °C respectively. This agrees with the range 217 - 224 °C stated by the British Pharmacopoeia indicating the sample contained Griseofulvin.

5.1.3 Paracetamol

169 - 171 °C and 169 - 172 °C were obtained in the first and second determinations respectively. The results are within the range 168 - 172 °C specified by the British Pharmacopoeia. The sample therefore contained Paracetamol and is free of impurities.

5.1.4 Ascorbic acid

The melting range obtained were 190 - 192 °C and 189 - 192 °C in the first and second determinations respectively. This agrees with the range 190 - 192 °C stated by the British Pharmacopoeia indicating the sample contained Ascorbic acid and is free of impurities.

5.1.5 Metronidazole

159 - 163 °C and 158 - 163 °C were obtained in the first and second determinations respectively. The results are within the range 159 - 163 °C specified by the British Pharmacopoeia. The sample therefore contained Metronidazole.

5.2 IDENTIFICATION TESTS

Identification tests are very important tests designed to confirm or prove that the compound or sample contains the active ingredient it is said to contain. The identity of pure compounds used as reference standards as well as formulated products needs to be verified unambiguously to ensure they contain the active ingredient they are purported to. Various chemical tests can be performed to ascertain the identity of a compound. They include TLC analysis, visual chemical tests (colour test), UV/Vis analysis, Infrared absorption analysis and NMR analysis.

5.2.1 Griseofulvin

Griseofulvin was dissolved in sulphuric acid to provide an acidic medium for oxidation with potassium dichromate. The reaction gave rise to a wine colouration. This indicates the powder contained Griseofulvin.

5.2.2 Cetirizine Hydrochloride

Cetirizine hydrochloride was identified by UV/Visible spectroscopy. The sample dissolved in hydrochloric acid and scanned over 210 - 350 nm gave an absorption maximum at 232 nm. This was close to 231 nm specified by the British Pharmacopoeia. The specific absorbance calculated was 371.9 ± 8.2. This fell within the range 359 to 381 specified by the British Pharmacopoeia indicating the sample contained cetirizine hydrochloride.

5.2.3 Paracetamol

The phenolic OH of paracetamol remained undissociated on the addition of HCl. This is because the phenolic OH is protonated making electrons not available for conjugation with the ring. There is a shift to a shorter wavelength therefore absorbance was read at 249 nm. The calculated specific absorbance was 903.4 ± 2.6. This falls within 860 - 980 specified by the British Pharmacopoeia indicating the sample contained Paracetamol.

5.2.4 Ascorbic acid

Dilute nitric acid was added to ascorbic acid dissolved in water to create an acidic environment to enable the reaction to proceed. Ascorbic acid was then oxidized on the addition of silver nitrate forming a grey coloured solution. This indicates the presence of Ascorbic acid.

5.2.5 Metronidazole

The pure Metronidazole powder and scanned over the spectral range 230 - 350 gave absorption maximum and minimum at 277 nm and 242 nm respectively. This is similar to 277 nm and 240 nm stated by the British Pharmacopoeia. Specific absorbance calculated at the absorption maximum was 377.7 ± 2.9. This occurs within the specified British Pharmacopoeia range of 365 - 395. This shows the presence of Metronidazole.

5.3 TLC ANALYSIS OF PURE SAMPLES

The TLC analysis of the pure analytes revealed one principal spot indicating the samples were suitable for use in the analysis since they contained no impurity.

5.3.1 Cetirizine Hydrochloride

TLC analysis conducted on all brands of Cetirizine Hydrochloride revealed spots with similar Rf values as the pure sample. This was done to determine if the tablets contained the active ingredient. The three brands of Cetirizine Hydrochloride EC, HC and KC had Rf values of 0.833, 0.833 and 0.829 respectively as the pure sample. This indicates all brands contained Cetirizine Hydrochloride.

5.3.2 Griseofulvin

TLC analysis conducted on the brands AG, EG and LG of Griseofulvin revealed one spot with Rf values of 0.814, 0.800 and 0.800 respectively as the pure sample meaning they contained the active ingredient they were purported to.

5.4 ASSAY OF PURE SAMPLES

The percentage purity of a drug is necessary to determine the reputation or potency of the drug. This was done to guide in the weight of solute to take during the preparation of solutions

5.4.1 Griseofulvin

Griseofulvin was assayed by UV/Vis Spectroscopy. This is because it contains chromophores. It also has methoxy groups (auxochromes) which enhance or increases its wavelength of absorption. It was dissolved in ethanol and absorbance read at 291 nm. The percentage purity obtained was 100.04 ± 0.17. The standard of the British Pharmacopoeia was met as this fell within 97 - 102 % specified.

5.4.2 Cetirizine hydrochloride

Cetirizine hydrochloride was assayed by non-aqueous titration. Mercuric acetate was used to prevent interference by halogen acid which will be formed in its absence. The percentage purity obtained was 99.80 ± 0.29. This agrees with 99.0 - 100.5 stated by the Btitish Pharmacopoeia.

5.4.3 Paracetamol

The assay of paracetamol was by diazotization with sodium nitrite in the presence of hydrochloric acid. The percentage purity stated by the International Pharmacopoeia is 98.50 - 101.0 and that from the calculation was 99.80 ± 0.28. This implies the standards of the International Pharmacopoiea were met.

5.4.4 Ascorbic acid

Ascorbic acid was assayed by iodimetry. The ascorbic acid was dissolved in water and acidified to enhance the oxidizing activity of the iodine. The enediol group was readily oxidized to dehydroascorbic acid and the iodine reduced to hydrogen iodide simultaneously. Starch was added to visually detect the endpoint due to its tendency to give a deep blue colour with excess iodine when all the ascorbic acid is oxidized. The percentage purity stated by the British Pharmacopoeia is 99 - 100.5 and that from the calculation was 99.78 ± 0.31.This implies the standards of the BP were met.

5.4.5 Metronidazole

Metronidazole was assayed by non-aqueous titration due to its weak basicity. It was dissolved in glacial acetic acid to enhance its basicity. It was titrated against acetous perchloric acid which behaves a strong acid thereby bringing the reaction to a sharp endpoint. The calculated percentage purity was 100.14 ± 0.37. This falls within the

range 99 - 101 required by the BP implying the sample met the British Pharmacopoeias' standard of purity.

5.5 UNIFORMITY OF WEIGHT TEST

5.5.1 Cetirizine hydrochloride

According to the British Pharmacopoeia, for tablets with average weight 80 mg or less, not more than two tablets should deviate from the average weight by more than 10 %. Based on this, it can be inferred that all batches of the three brands of Cetirizine Hydrochloride passed the uniformity of weight test. This is because none of the brands of Cetirizine Hydrochloride deviated by more than 10 % .The highest percentage deviation for EC, HC and KC were 2.84, 3.63 and 4.75 respectively.

5.5.2 Griseofulvin

For uncoated and film - coated tablets with average weight more than 80 mg but less than 250 mg, not more than two tablets should deviate from the average weight by more than 7.5 % (British Pharmacopoeia, 2007). The highest percentage deviation for AG, EG and LG were 3.52, 2.15 and 4.02 respectively. The brands of Griseofulvin passed the uniformity of weight test.

5.6 HPLC METHOD DEVELOPMENT

The goal was to develop a simple, fast and cost effective method. The C18 column was used because very simple mobile phase can be applied to alter retention and selectivity. The UV detector was used because of the level of detection it offers and again because it is the most commonly used mode of detection. A number of trials were conducted to determine the optimum conditions under which the analytes together with each surrogate reference standards would elute with the speed and the resolution desired. Methanol was used as it is relatively low - priced, has low UV cut - off, has low

viscosity and is water miscible. The analytes were slightly or freely soluble in methanol and water which constituted the mobile phases.

5.6.1 Griseofulvin

Different proportions of methanol and water were investigated. The optimum mobile phase composition was (70: 30 v/v) methanol: water because the peaks were sharp and free from tailing. The wavelength of detection used was 254 nm as analytes and surrogates reference standards absorb significantly at this wavelength. Flow rates between 0.8 and 1.2 ml/min were studied. The flow rate of 1.0 ml/min gave a reasonable separation time. The mean retention time for Griseofulvin, Paracetamol, Ascorbic acid and Metronidazole were 3.65 ± 0.06, 2.47 ± 0.03, 1.70 ± 0.02 and 2.65 ± 0.04 respectively.

5.6.2 Cetirizine Hydrochloride

A proportion of (60: 40 v/v) methanol: sodium acetate buffer pH 4.2 gave sharp and well resolved peaks. The wavelength of detection was 235 nm. The flow rate of 1.5 ml/min was used because it gave a reasonable separation time. The mean retention time for Cetirizine Hydrochloride, Paracetamol, Ascorbic acid and Metronidazole was 4.57 ± 0.06, 1.73 ± 0.05,1.52 ± 0.03 and 1.84 ± 0.04 respectively.

5.7 VALIDATION OF ANALYTICAL METHOD

As stated earlier, validation studies are an essential part of GMP outlined by the World Health Organization. The developed methods were therefore evaluated and tested to demonstrate their suitability for their intended purpose.

5.7.1 Stability

Stability of the analytes in solution was studied to evaluate the duration of sample handling. This was done to ascertain the duration under which the sample is stable in

solution. From the graph, the peak area of the analyte and standards remained quite stable within the time of study. It can be inferred that the analyte and surrogate reference standards were stable within the analytical run time.

5.7.2 Linearity

Linearity of an analytical method is the ability of the method to produce test results or response that is directly proportional to the concentration of analytes in the sample over the range of analyte concentration of interest. Linearity of the method for Griseofulvin and its surrogate reference standards was investigated over the working range of 0.001 - 0.01 %w/v. From the plot of response against concentration, the coefficient of correlation for Griseofulvin, Paracetamol, Ascorbic acid and Metronidazole were 0.9960, 0.9970, 0.9949 and 0.9980 respectively. The linearity of the method for Cetirizine Hydrochloride was evaluated over the working range 0.002 - 0.02 %w/v and 0.001 - 0.009 %w/v for the surrogate reference standards. The coefficients of correlation were 0.9964, 0.9961, 0.9951 and 0.9951 for Cetirizine hydrochloride, Paracetamol, Ascorbic acid and Metronidazole respectively. The linear regression data indicates the response is linear or is proportional to concentration of analyte over the range studied.

5.7.3 Limit of Detection (LOD)

The minimum concentration of analyte that can confidently be detected in a sample in the midst of baseline noise for the method for Griseofulvin and its surrogate reference standards were 0.0063, 0.00051, 0.00072 and 0.00052 for Griseofulvin, Paracetamol, Ascorbic acid and Metronidazole respectively. The LOD for Cetirizine hydrochloride, Paracetamol, Ascorbic acid and Metronidazole were 0.00093, 0.00050, 0.00053 and

0.00050. This was calculated from the residual standard deviation of the regression lines.

5.7.4 Limit of Quantitation (LOQ)

The LOQ calculated for the method for Griseofulvin and SRSs were 0.0019, 0.0016, 0.0022 and 0.0016 for Griseofulvin, Paracetamol, Ascorbic acid and Metronidazole respectively as recorded in Table 4 - 13. The LOQ for the method developed for Cetirizine hydrochloride and surrogates were 0.0028, 0.0015, 0.0016 and 0.0015 for Cetirizine hydrochloride, Paracetamol, Ascorbic acid and Metronidazole respectively as recorded in Table 4 - 14. This shows the minimum amount of analyte that can confidently be analyzed with acceptable or defined precision and accuracy.

5.7.5 Precision

Precision was studied under repeatability and intermediate precision as defined by the ICH Guidelines on the Validation of Analytical Procedures.

5.7.5.1Repeatability

Repeatability was performed to measure the closeness of the analytical results. Six replicate measurements were taken and expressed as coefficient of variation and recorded in Table 4 -15 and 4 - 16 for Griseofulvin and Cetirizine hydrochloride respectively. The coefficient of variation of the analysis of Griseofulvin with Paracetamol, Ascorbic acid and Metronidazole were 0.9351, 1.0701, and 0.5328 % respectively.

The coefficient of variation calculated for Cetirizine hydrochloride with Paracetamol, Ascorbic acid and Metronidazole were 0.5484, 1.1973 and 0.9238 in that order. The coefficient of variation fell below 2 % specified by the United States Pharmacopoeia. This is an indication that the methods were precise.

5.7.5.2Intermediate precision

This was performed to establish the effect of random variations on the precision of analytical results. Days were varied for this evaluation. Data obtained from the evaluation for the method for Griseofulvin and Cetirizine hydrochloride are summarized in Table 4 - 17 and 4 - 18 respectively. The coefficients of variation calculated for the analysis of Griseofulvin on day 1 were 0.5054, 0.9754, 0.7500 with Paracetamol, Ascorbic acid and Metronidazole as SRSs. The coefficients of variation recorded on the second day were 0.4088, 1.0142 and 1.1167 and that for the third day were 0.7642, 1.0570 and 0.9850.

The coefficients of variation calculated from the analysis of Cetirizine hydrochloride with Paracetamol, Ascorbic acid and Metronidazole as SRSs were 0.6901, 0.8551 and 1.2087 respectively on the first day. The second day had coefficient of variation of 0.3883, 1.2863 and 0.7795 and the third day had 1.0570, 1.1435 and 1.3228 respectively. The coefficient of variation fell below 2 % specified by the United States Pharmacopoeia indicating the methods were precise.

5.7.6 Robustness

The evaluation of robustness of developed methods was done by analyzing samples under the analytical conditions and by the variation some of the conditions. Flow rate and wavelength were varied. The data obtained from the assay are reported in APT - 9 and 10 for the method for Griseofulvin and Cetirizine hydrochloride respectively. The results were subjected to ANOVA test to see if there is a significant difference between results obtained under the varied conditions as well as the variation in results with the surrogate reference standard used. There was no significant in the mean assay results as the calculated F was lower than the critical value of F under the varied

conditions. There was also no significant difference in results with the surrogate standard used. The results from the statistical analysis are indicated in APT - 31 to 34.

5.8 CALCULATION OF K VALUE

K value is dependent on the absorption of the surrogate reference standard in relation to the analyte at the wavelength of detection. This in turn is dependent on the chromophores and auxochromes present in the chemical structure of the surrogate reference standard which influence the absorption at the wavelength of study. The concentration and peak area of analyte and surrogate reference standards injected were used in the calculation of the k value using the relation stated earlier. The k value for Paracetamol, Ascorbic acid and Metronidazole in relation to Griseofulvin were 0.3824 ± 0.0073, 0.3317 ± 0.0046 and 1.0902 ± 0.0063 respectively. The k value for Paracetamol, Ascorbic acid and Metronidazole in relation to Cetirizine Hydrochloride were 0.3530 ± 0.0092, 0.3735 ± 0.0100 and 0.4929 ± 0.0104 in that order.

The concentration was varied to determine its effect on the k value. Results obtained were analyzed by ANOVA test. The comparison revealed that there is no significant difference in the k value with change in concentration as the calculated F values were lesser than the critical F values of 3.89 as indicated in APT -11 to APT - 16.

5.9 DETERMINATION OF PERCENTAGE CONTENT WITH THE K VALUES

Three brands each of Griseofulvin and Cetirizine Hydrochloride were analyzed with their respective developed method. Percentage contents obtained fell within 95 - 105 % and 90 - 110 % stipulated by the BP and USP respectively. This shows that the results from the developed method are comparable to those obtained from the standard methods. The mean percentage content for AG, EG and LG with Paracetamol as surrogate reference standard were 99.90 ± 0.86, 100.07 ± 0.44 and 99.87 ± 0.68. The mean percentage content for AG, EG and LG with Ascorbic acid were 99.74 ± 1.03,

99.54 ± 1.13 and 100.12 ± 1.06. The mean percentage content for AG, EG and LG with Metronidazole were 100.23 ± 0.63, 99.89 ± 1.07 and 100.34 ± 0.94.

The mean percentage content for EC, HC and KC with Paracetamol as surrogate reference standard were 100.16 ± 1.22, 100.05 ± 0.46 and 100.54 ± 0.81. The mean percentage contents for EC, HC and KC with Ascorbic acid as surrogate reference standard were 99.78 ± 0.62, 100.11 ± 0.52 and 100.44 ± 1.23. The mean percentage content for EC, HC and KC with Metronidazole were 99.63 ± 0.90, 99.71 ± 0.49and 99.99 ± 1.16.

5.10 DETERMINATION OF PERCENTAGE CONTENT WITH STANDARD METHODS

The three brands of Griseofulvin were analyzed using the method specified by the USP. The results obtained for AG, EG and LG were 100.67 ± 1.11, 100.12 ± 1.28 and 100.57 ± 1.34. The results fell within the range stipulated range 90 - 110 % of the USP. The brands of Cetirizine hydrochloride EC, HC and KC were analyzed with the method specified in the BP. The percentage contents obtained were 100.96 ± 1.25, 100.68 ± 1.08 and 100.84 ± 1.02. They all fell within 95 - 105 % stipulated by the BP. The brands of both Griseofulvin and Cetirizine hydrochloride passed by the standards of the USP and BP respectively.

5.11 COMPARISON OF THE STANDARD METHOD TO THE DEVELOPED METHOD

The developed methods and the standard methods were compared statistically to determine if there is a significant difference between the outcomes of both methods. The results were subjected to the paired t test. The results are summarized in Table 4 - 26 and 4 - 27. The calculated t value obtained using Paracetamol, Ascorbic acid and Metronidazole as surrogate reference standards in the analysis of Griseofulvin compared to the standard USP method were 0.23, 0.85 and 0.49 respectively. They fell below the critical t value 2.78 implying there is no significant difference between

outcome of the standard USP method and the developed method for Griseofulvin at 95 % confidence level.

The calculated t value obtained from the comparison of the standard BP method to that developed for Cetirizine Hydrochloride using Paracetamol, Ascorbic acid and Metronidazole as surrogate reference standard were 0.39, 0.62 and 0.26 respectively. They were lesser than the critical t value at five degrees of freedom 2.78 meaning there is no significant difference between the outcomes of the developed method and the standard BP method at 95 % confidence level.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

The possibility of the use of surrogate reference standards in the analysis of Griseofulvin and Cetirizine hydrochloride was investigated. The identity and purity of pure samples were examined before use in analysis. The results revealed that the samples contained the various active ingredients. TLC analysis conducted on the pure samples revealed one spot implying the samples were free of impurities and therefore suitable for use. TLC analysis conducted on the formulations showed they contained the active ingredients.

A simple, fast and economical HPLC method was developed for both Griseofulvin and Cetirizine hydrochloride using Paracetamol, Ascorbic acid and Metronidazole as surrogate reference standards. The optimum condition found for Griseofulvin and the surrogate reference standards was methanol: water (70: 30 v/v) at a wavelength 254 nm and a flow rate of 1 ml/min. The condition for Cetirizine hydrochloride and the surrogate reference standards was methanol: sodium acetate buffer pH 4.2 (60: 40 v/v) at a wavelength of 235 nm and a flow rate of 1.5 ml/min.

The developed methods were validated under linearity, LOD, LOQ, repeatability, intermediate precision and robustness and the results analyzed statistically.

Three brands each of marketed formulations of both Griseofulvin and Cetirizine hydrochloride were analyzed with the developed methods using the calculated K values of each surrogate reference standard. The brands Griseofulvin and Cetirizine Hydrochloride were analyzed with the methods stipulated by the USP and BP respectively. Uniformity of weight test conducted prior to the assay revealed the

tablets passed the uniformity of weight test by the standards of the British Pharmacopoeia.

The outcomes of both determinations were analyzed statistically to compare the methods. There was no significant difference between the standard method and the developed methods. The developed method can therefore be adopted for routine analysis of the compounds.

6.2 RECOMMENDATION

Further investigations should be conducted on the use of surrogate reference standards in the assay of other pharmaceutical preparations to confirm the validity of this method for routine analysis.

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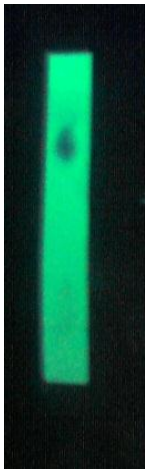
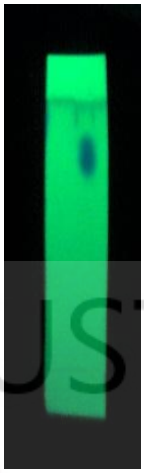
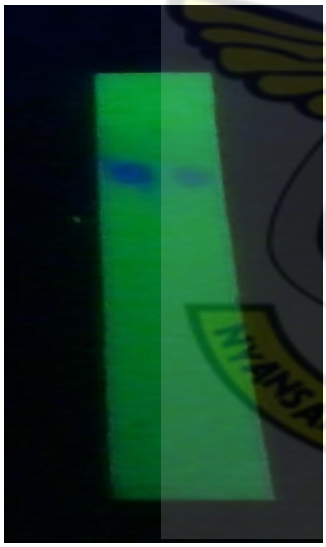
APPENDIX



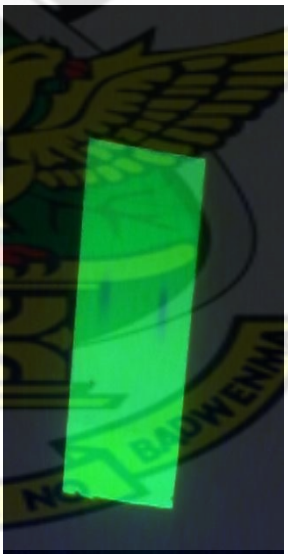
APF 1 - TLC chromatogram of pure Cetirizine hydrochloride



APF 2 - TLC chromatogram of pure Griseofulvin



APF 3-TLC chromatogram of pure Cetirizine hydrochloride and tablet (EC)



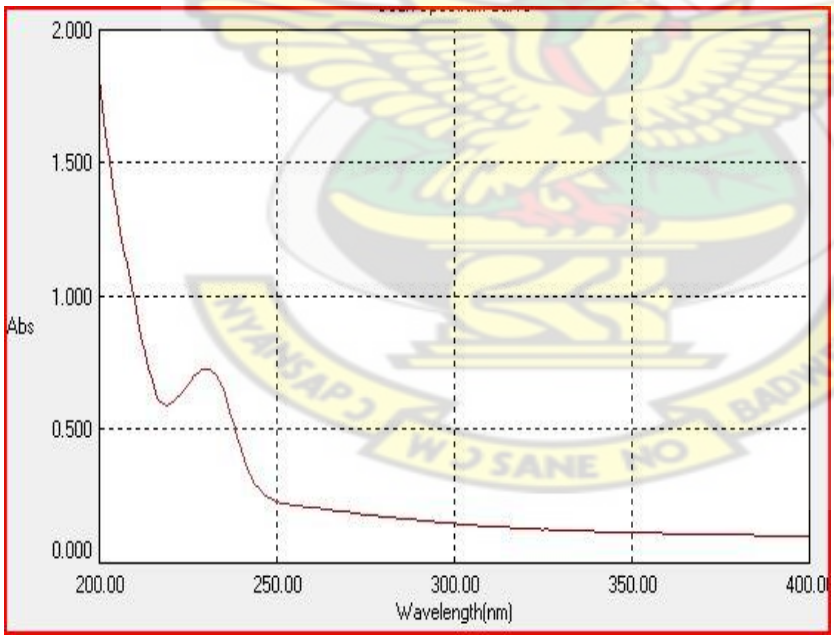
APF 4 - TLC chromatogram of pure Griseofulvin and tablet (AG)

SOLUBILITY TESTS

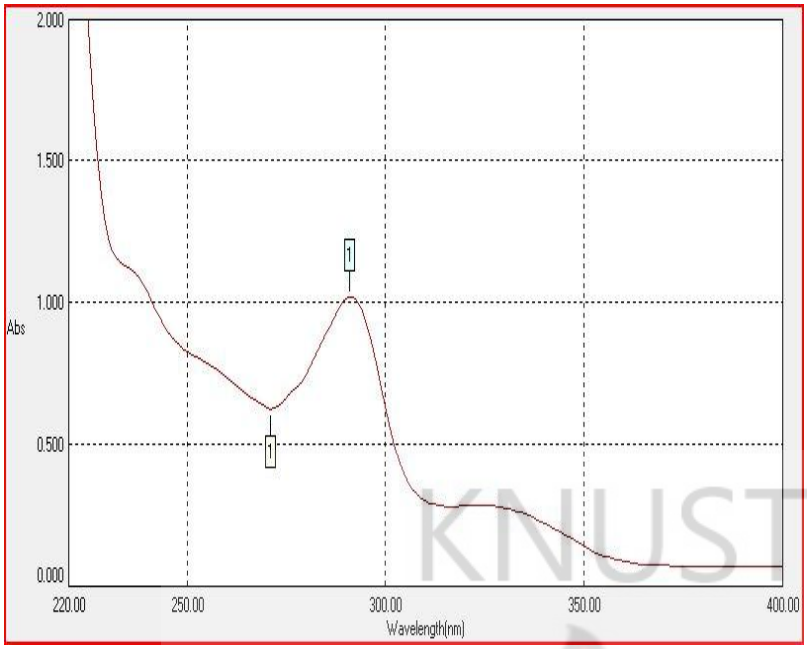
Drug	Water		Methanol	
	Observation	Literature	Observation	Literature
Griseofulvin	Practically insoluble	Practically insoluble	Slightly soluble	Slightly soluble
Cetirizine Hydrochloride	Freely soluble	Freely soluble	Soluble	Soluble
Paracetamol	Sparingly soluble	Sparingly soluble	Freely soluble	Freely soluble
Ascorbic acid	Freely soluble	Freely soluble	Soluble	Soluble
Metronidazole	Slightly soluble	Slightly soluble	Slightly soluble	Slightly soluble

APT 1 - Solubility of drug substances in methanol and water

UV SPECTRUM OF ANALYTES

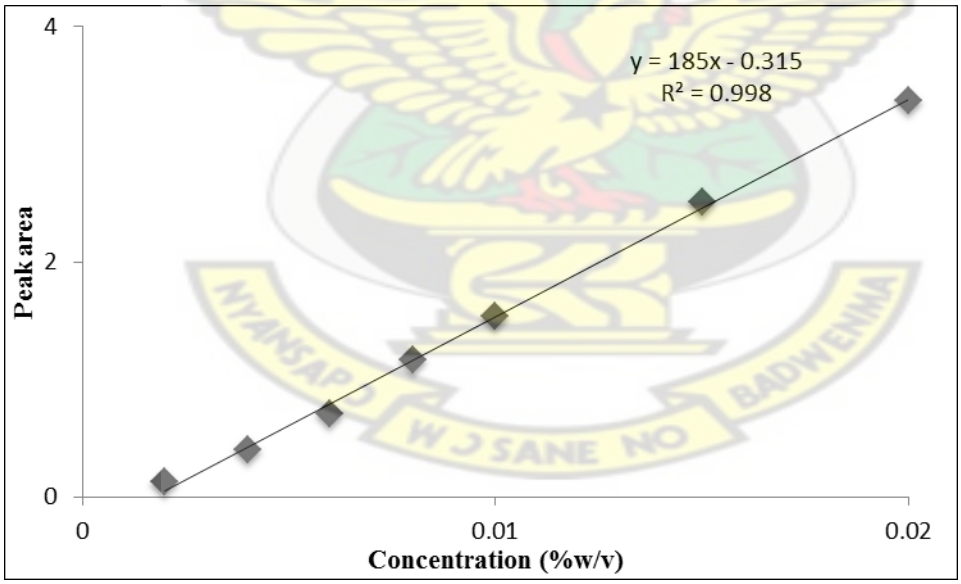


APF 5 - UV spectrum of Cetirizine hydrochloride

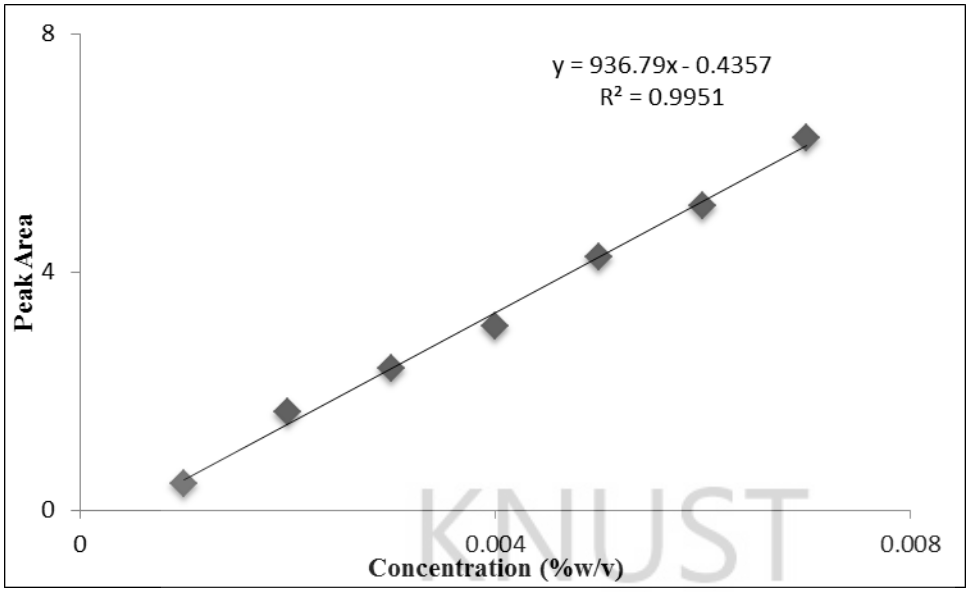


APF 6 - UV spectrum of Griseofulvin

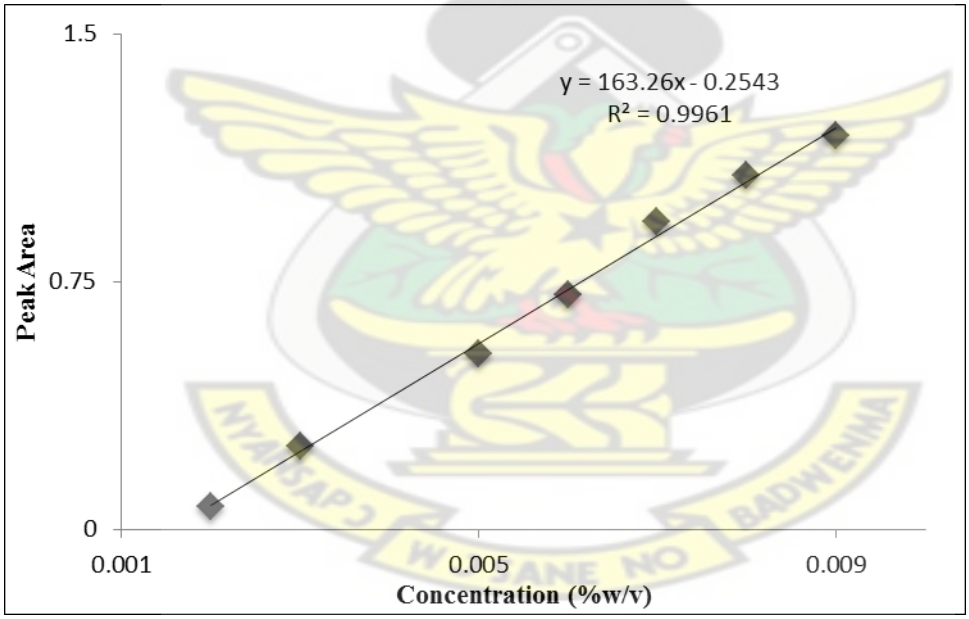
CALIBRATION CURVES OF CETIRIZINE HYDROCHLORIDE AND SURROGATE SURROGATE STANDARDS



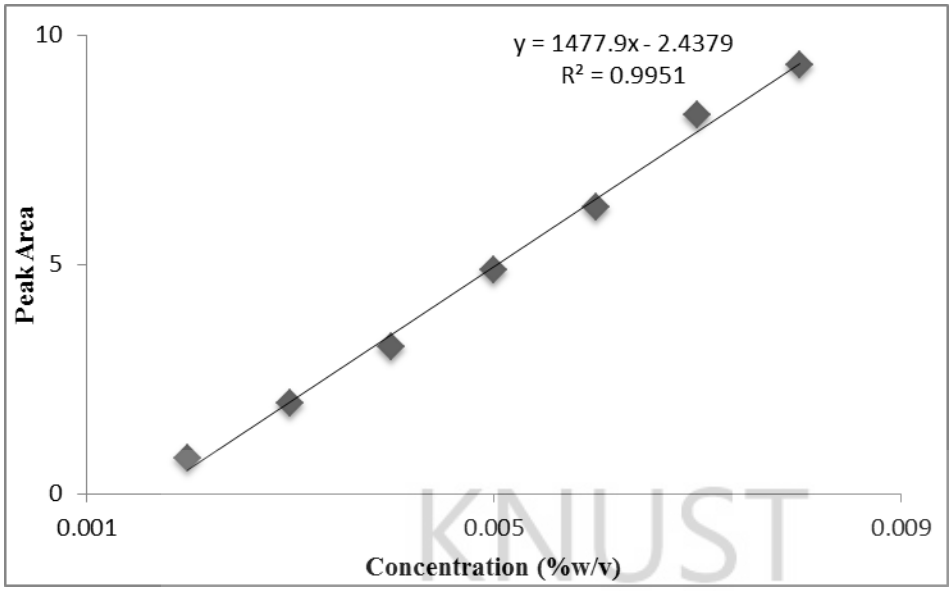
APF 7 - Calibration curve of Cetirizine Dihydrochloride



APF 8 - Calibration curve of Paracetamol

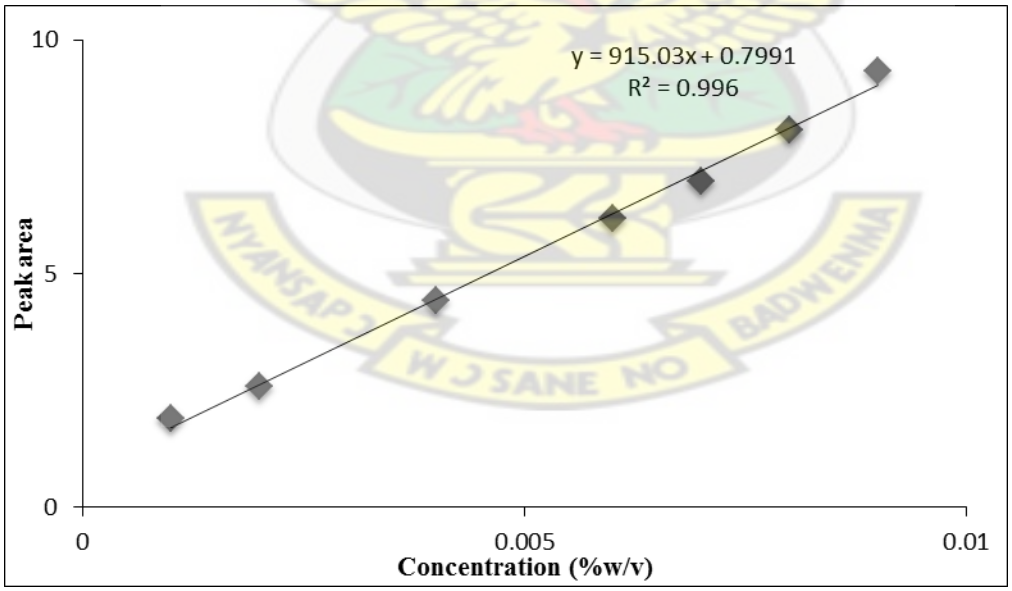


APF 9 - Calibration curve of Ascorbic acid

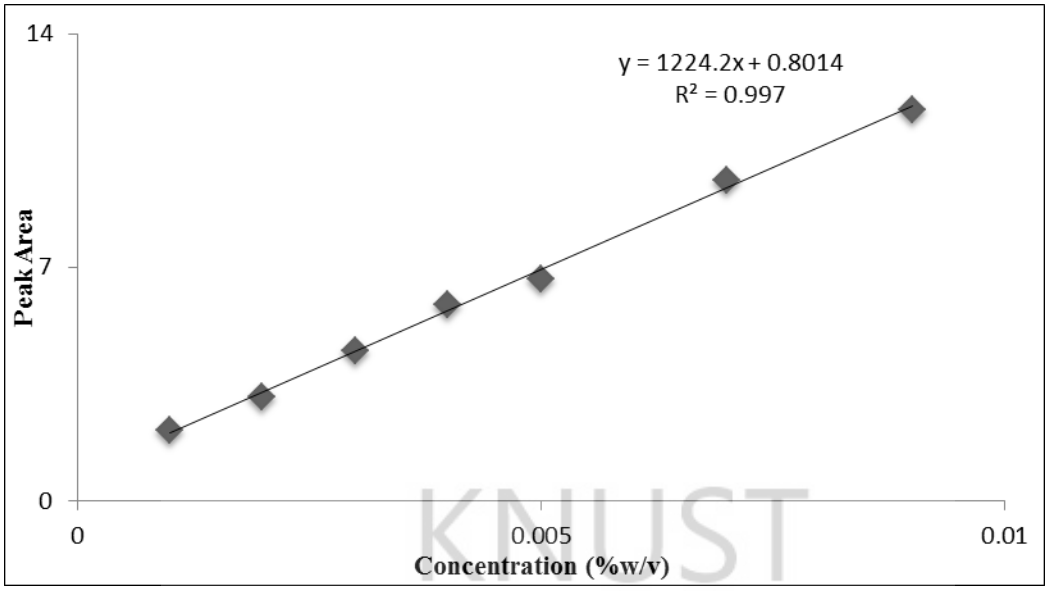


APF 10 - Calibration curve of metronidazole

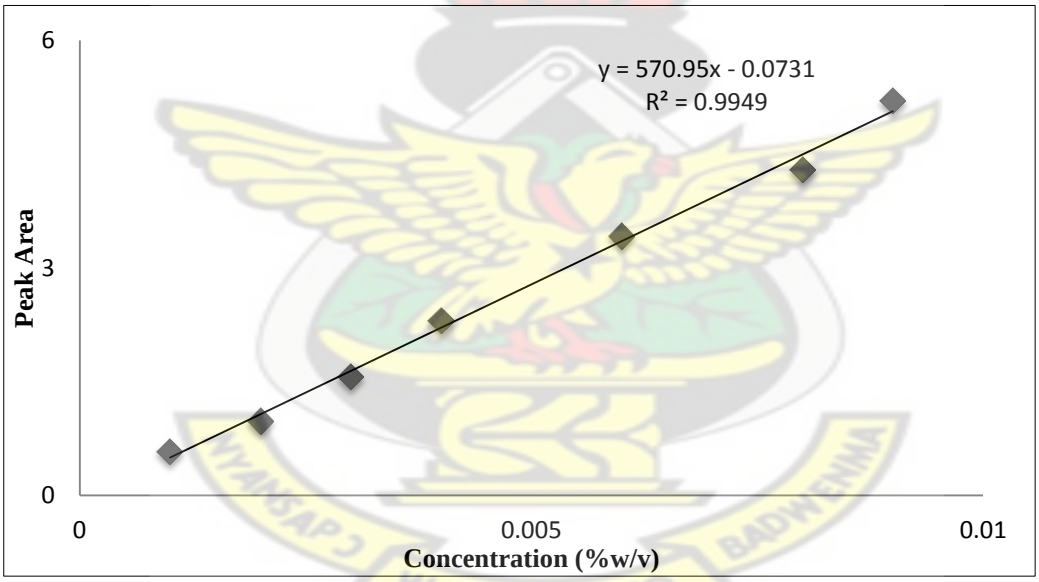
CALIBRATION CURVES OF CETIRIZINE HYDROCHLORIDE AND SURROGATE SURROGATE STANDARDS



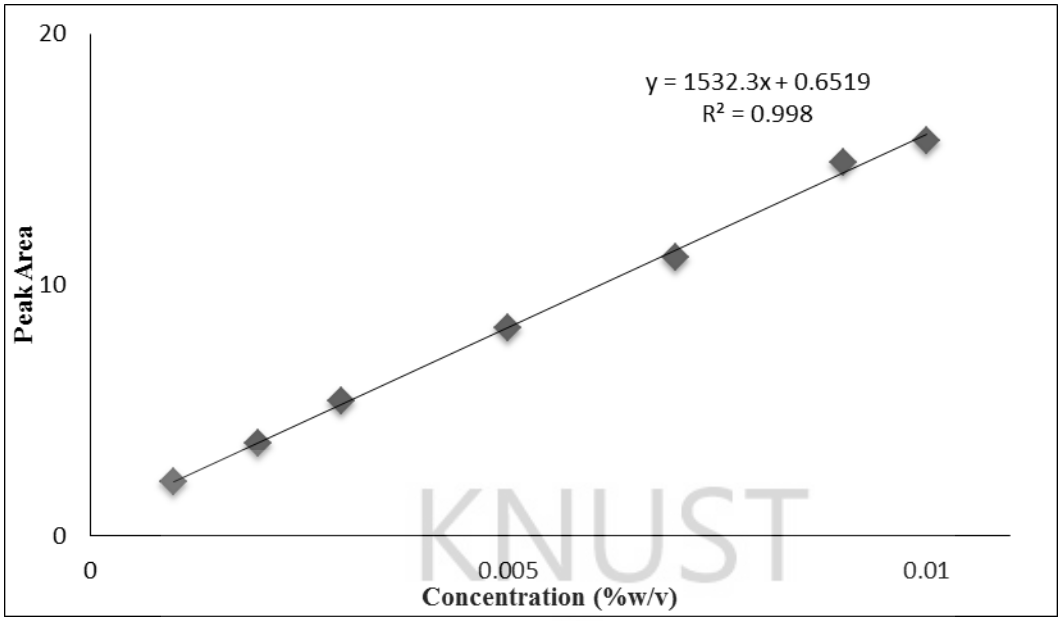
APF 11 - Calibration curve of Griseofulvin



APF 12 - Calibration curve of Paracetamol



APF 13 - Calibration curve of Ascorbic acid



APF 14 - Calibration curve of Metronidazole

STANDARDIZATION OF SOLUTIONS

Standardization of $HClO_4$

APT - 2 Titre values for the standardization of $HClO_4$

	First determintion	Second determination	Third determination
Final titre	30.20	30.30	30.30
Initial titre	0.00	0.00	0.00
Titre value	30.20	30.30	30.30

Standardization of I_2

APT - 3 Titre values for the standardization of I_2

	First determintion	Second determination	Third determination
Final titre	23.70	47.20	23.60
Initial titre	0.00	23.70	0.00
Titre value	23.70	23.50	23.60

Average titre= $\frac{23.70+23.50+23.60}{3}$

$= \frac{70.8}{3}$

$= 23.60$

APT - 4 Titre values for the standardization of $NaNO_2$

	First determintion	Second determination	Third determination
Final titre	12.60	30.00	42.40
Initial titre	0.00	12.60	30.00
Titre value	12.60	12.40	12.40

Average titre= $\frac{12.60 +12.40 +12.40}{3}$

$= \frac{37.40}{3}$

$= 12.47$

Repeatability

APT - 5 Data obtained from repeatability studies of method for Griseofulvin

	Percentage Content					
SRS	1	2	3	4	5	6
Paracetamol	99.74	99.03	98.89	98.54	101.06	99.56
Ascorbic acid	98.88	99.89	101.13	99.48	101.18	99.89
Metronidazole	100.65	101.07	100.84	99.87	100.67	99.76

APT - 6 Data obtained from the repeatability studies of method for Cetirizine hydrochloride

	Percentage Content					
SRS	1	2	3	4	5	6
Paracetamol	99.45	98.78	99.78	100.16	99.99	00.03
Ascorbic acid	101.20	100.64	99.33	101.28	101.80	98.75
Metronidazole	98.63	99.82	100.85	100.63	101.02	99.51

Intermediate precision

APT - 7 Data obtained from the inter - day precision of developed method for Griseofulvin

	SRS	Percentage content					
		1	2	3	4	5	6
Day 1	Paracetamol	99.59	100.28	98.99	100.32	99.8	100.09
	Ascorbic acid	99.25	100.19	100.50	98.83	99.59	101.54
	Metronidazole	99.37	101.59	101.13	100.48	100.94	100.73
Day 2	Paracetamol	100.66	100.12	100.96	100.84	101.02	100.10
	Ascorbic acid	98.26	98.70	100.03	100.59	100.72	100.02
	Metronidazole	98.70	100.42	101.05	98.70	100.75	101.50
Day 3	Paracetamol	99.81	101.54	100.30	100.34	99.89	101.49
	Ascorbic acid	98.80	101.54	99.12	100.12	98.82	99.98
	Metronidazole	100.91	98.56	99.98	100.84	101.02	101.10

APT - 8 Data obtained from the inter - day precision of developed method for Cetirizine hydrochloride

SRS		Percentage content					
		1	2	3	4	5	6
Day 1	Paracetamol	99.25	100.31	101.08	99.61	100.54	99.62
	Ascorbic acid	98.59	99.72	100.69	99.52	100.39	98.72
	Metronidazole	98.83	101.08	101.22	100.73	99.15	98.57
Day 2	Paracetamol	100.65	100.55	100.18	99.67	99.82	100.28
	Ascorbic acid	100.41	102.1	98.25	99.79	100.84	100.91
	Metronidazole	98.65	99.34	100.54	98.87	99.12	100.30
Day 3	Paracetamol	100.02	100.43	101.05	98.27	100.60	99.00
	Ascorbic acid	97.76	100.37	100.01	99.90	98.29	100.40
	Metronidazole	98.55	99.67	100.07	98.56	101.78	98.35

APT - 9 Data obtained from the evaluation of robustness of developed method for Griseofulvin

Parameter	Variation	Percentage content		
		Paracetamol as SRS	Ascorbic acid as SRS	Metronidazole as SRS
Wavelength	252	98.68	100.82	101.55
	254	99.39	102.40	100.04
	255	100.42	98.84	101.33
Flow rate	1.0	98.92	101.50	101.03
	1.1	101.92	100.72	102.32
	1.2	101.16	100.81	100.78

APT - 10 Data obtained from the evaluation of robustness of developed method for Cetirizine hydrochloride

Parameter	Variation	Percentage content		
		Paracetamol as SRS	Ascorbic acid as SRS	Metronidazole as SRS
Wavelength	233	98.91	101.35	100.27
	235	102.02	99.77	98.48
	236	101.02	100.15	101.21
Flow rate	1.3	99.88	101.37	100.13
	1.4	98.82	100.09	100.50
	1.5	100.17	100.78	99.60

APT - 11 K value of Paracetamol in relation to Griseofulvin

Concentration of analyte	Concentration of SRS	K value					
		1	2	3	4	5	f_{stat}
0.006	0.004	0.3811	0.3811	0.3758	0.3965	0.3825	
0.004	0.003	0.3759	0.3922	0.3994	0.3802	0.3773	0.05
0.002	0.001	0.3803	0.3936	0.3791	0.3838	0.3863	

Data analyzed with ANOVA

APT - 12 K value of Ascorbic acid in relation to Griseofulvin

Concentration of analyte	Concentration of SRS	K value					
		1	2	3	4	5	f_{stat}
0.006	0.004	0.3293	0.3270	0.3367	0.3363	0.3265	
0.004	0.003	0.3297	0.3287	0.3417	0.3343	0.3196	0.58
0.002	0.002	0.3177	0.3326	0.3276	0.3350	0.3216	

Data analysed with ANOVA

APT - 13 K value of Metronidazole in relation to Griseofulvin

Concentration of analyte	Concentration of SRS	K value 1	2	3	4	5	f_{stat}
0.006	0.004	1.0907	1.0889	1.0881	1.0917	1.1001	
0.004	0.002	1.0891	1.0911	1.0863	1.0957	1.0815	0.91
0.002	0.001	1.0875	1.0890	1.0916	1.0873	1.0883	

Data analysed with ANOVA

APT - 14 K value of Paracetamol in relation to Cetirizine hydrochloride

Concentration of analyte	Concentration of SRS	K value 1	2	3	4	5	f_{stat}
0.008	0.004	0.3468	0.3418	0.3589	0.3495	0.3536	
0.006	0.003	0.3628	0.3489	0.3567	0.3624	0.3412	0.71
0.005	0.002	0.3533	0.3677	0.3456	0.3487	0.3690	

Data analysed with ANOVA

APT - 15 K value of Ascorbic acid in relation to Cetirizine hydrochloride

Concentration of analyte	Concentration of SRS	K value 1	2	3	4	5	f_{stat}
0.008	0.005	0.3737	0.3590	0.3654	0.3862	0.3776	
0.006	0.003	0.3579	0.3736	0.3735	0.3841	0.3580	0.75
0.005	0.001	0.3688	0.3653	0.3854	0.3789	0.3901	

Data analysed with ANOVA

APT - 16 K value of Metronidazole in relation to Cetirizine hydrochloride

Concentration of analyte	Concentration of SRS	K value 1	2	3	4	5	f_{stat}
0.008	0.004	0.4778	0.5034	0.4955	0.4976	0.4835	
0.006	0.003	0.4935	0.4989	0.4855	0.5079	0.4938	0.71
0.005	0.002	0.5027	0.4795	0.4837	0.4815	0.4958	

Data analysed with ANOVA

APT - 17 Uniformity of weight test of AG

No	Weight/g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.1864	0.0019	1.0298
2	0.1846	0.0004	0.0542
3	0.1796	-0.0049	2.6558
4	0.1879	0.0034	1.8428
5	0.1806	-0.0039	2.1138
6	0.1848	0.0003	0.1626
7	0.1799	-0.0046	2.4932
8	0.1903	0.0058	3.1436
9	0.1910	0.0065	3.5230
10	0.1857	0.0012	0.6504
11	0.1880	0.0035	1.8970
12	0.1809	-0.0036	1.9512
13	0.1889	0.0044	2.3848
14	0.1820	-0.0025	1.3550
15	0.1828	-0.0017	0.9214
16	0.1857	0.0012	0.6504
17	0.1836	-0.0009	0.4878
18	0.1796	-0.0049	2.6558
19	0.1820	-0.0025	1.3550
20	0.1825	-0.0020	1.0840

Weight of twenty tablets= 3.6891g

Average weight = 0.1845g

APT - 18 Uniformity of weight test of EG

No	Weight /g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.1995	0.0000	0.0000
2	0.1999	0.0004	0.2005
3	0.1998	0.0003	0.1504
4	0.2013	0.0018	0.9023
5	0.2000	0.0005	0.2506
6	0.2034	0.0039	1.9549
7	0.1952	-0.0043	2.1554
8	0.2002	0.0007	0.3509
9	0.2013	0.0018	0.9023
10	0.1990	-0.0005	0.2506
11	0.1982	-0.0013	0.6516
12	0.1968	-0.0027	1.3534
13	0.1963	-0.0032	1.6040
14	0.1977	-0.0018	0.9023
15	0.1998	0.0003	0.1504
16	0.1985	-0.0010	0.5013
17	0.2005	0.0010	0.5013
18	0.2014	0.0019	0.9524
19	0.2011	0.0016	0.8020
20	0.1992	-0.0003	0.1504

Weight of twenty tablets= 3.9890g

Average weight = 0.1995g

APT - 19 Uniformity of weight test of LG

No	Weight /g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.1945	-0.0018	0.9170
2	0.2031	0.0068	3.4641
3	0.1934	-0.0029	1.4773
4	0.1948	-0.0015	0.7641
5	0.2042	0.0079	4.0245
6	0.2039	0.0076	3.8716
7	0.1917	-0.0046	2.3434
8	0.1930	-0.0033	1.6811
9	0.1973	0.0010	0.5094
10	0.1987	0.0024	1.2226
11	0.1970	0.0007	0.3566
12	0.1920	-0.0043	2.1905
13	0.2041	0.0078	3.9735
14	0.2005	0.0042	2.1396
15	0.2038	0.0075	3.8207
16	0.1979	0.0016	0.8151
17	0.1915	-0.0048	2.4452
18	0.1918	-0.0045	2.2924
19	0.2003	0.0040	2.0377
20	0.1929	-0.0034	1.7320

Weight of twenty tablets= 3.9268g

Average weight = 0.1963g

APT - 20 Uniformity of weight test of EC

No	Weight /g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.1889	0.0022	1.18
2	0.1833	-0.0034	1.82
3	0.1858	-0.0009	0.48
4	0.1873	0.0006	0.32
5	0.1894	0.0027	1.45
6	0.1867	0.0000	0.00
7	0.1887	0.0020	1.07
8	0.1871	0.0004	0.21
9	0.1877	0.0010	0.54
10	0.1874	0.0007	0.37
11	0.1851	-0.0016	0.86
12	0.1853	-0.0014	0.75
13	0.1872	0.0005	0.27
14	0.1835	-0.0032	1.71
15	0.1862	-0.0005	0.27
16	0.1896	0.0029	1.55
17	0.1885	0.0018	0.96
18	0.1821	-0.0046	2.46
19	0.1814	-0.0053	2.84
20	0.1912	0.0045	2.41

Weight of twenty tablets= 3.7331g

Average weight = 0.1867g

APT - 21 Uniformity of weight test of HC

No	Weight /g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.0867	-0.0015	1.70
2	0.0885	0.0003	0.34
3	0.0893	0.0011	1.24
4	0.0886	0.0004	0.45
5	0.0872	-0.0010	1.13
6	0.0914	0.0032	3.63
7	0.0859	-0.0023	2.61
8	0.0902	0.0020	2.27
9	0.0855	-0.0027	3.06
10	0.0873	-0.0009	1.02
11	0.0863	-0.0019	2.15
12	0.0874	-0.0008	0.91
13	0.0901	0.0019	2.15
14	0.0878	-0.0004	0.45
15	0.0888	0.0006	0.68
16	0.0854	-0.0028	3.17
17	0.0866	-0.0016	1.81
18	0.0882	0.0000	0.00
19	0.0892	0.0010	1.13
20	0.0875	-0.0007	0.79

Weight of twenty tablets= 1.7644g

Average weight = 0.0882g

APT - 22 Uniformity of weight test of KC

No	Weight /g (x)	Deviation (x-t)	% Deviation (x-t/t) × 100
1	0.1910	-0.0006	-0.3132
2	0.1917	0.0001	0.0522
3	0.1885	-0.0031	1.6180
4	0.1872	-0.0044	2.2965
5	0.1935	0.0019	0.9916
6	0.1947	0.0031	1.6180
7	0.2007	0.0091	4.7495
8	0.1951	0.0035	1.8267
9	0.1897	-0.0019	0.9916
10	0.1935	0.0019	0.9916
11	0.1963	0.0047	2.4530
12	0.1866	-0.0050	2.6096
13	0.1959	0.0043	2.2443
14	0.1829	-0.0087	4.5407
15	0.1871	-0.0045	2.3486
16	0.1912	-0.0004	0.2088
17	0.1950	0.0034	1.7745
18	0.1988	0.0072	3.7578
19	0.1860	-0.0056	2.9228
20	0.1997	0.0081	4.2276

Weight of twenty tablets= 3.8317g

Average weight = 0.1916g

APT - 23 Percentage contents of brands of Griseofulvin with Paracetamol as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EG	99.59	100.28	99.35	100.32	100.09	99.80
LG	99.44	99.35	100.21	99.25	99.90	101.05
AG	99.49	100.18	100.59	101.06	99.38	98.72

APT - 24 Percentage contents of brands of Griseofulvin with Ascorbic acid as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EG	100.45	99.51	99.33	98.54	98.20	101.19
LG	99.25	100.99	100.50	98.83	99.59	101.54
AG	99.57	99.62	99.05	101.35	98.41	100.45

APT - 25 Percentage contents of brands of Griseofulvin with Metronidazole as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EG	99.17	100.50	100.65	101.10	98.24	99.69
LG	99.79	101.01	100.28	100.72	98.87	101.47
AG	99.37	100.59	101.13	100.48	100.04	99.75

APT - 26 Percentage contents of brands of Cetirizine hydrochloride with Paracetamol as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EC	100.68	99.03	98.38	100.54	99.21	100.31
HC	99.78	100.08	100.02	99.66	99.85	100.95
KC	101.12	100.41	100.36	101.28	99.07	101.01

APT - 27 Percentage contents of brands of Cetirizine hydrochloride with Ascorbic acid as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EC	100.32	99.45	98.79	100.42	100.07	99.61
HC	100.07	100.58	100.62	100.41	99.66	99.32
KC	98.33	99.66	100.68	101.25	101.58	101.13

APT - 28 Percentage contents of brands of Cetirizine hydrochloride with Metronidazole as SRS

Brand	Percentage Content					
	1	2	3	4	5	6
EC	98.84	98.57	100.09	99.72	99.58	101.08
HC	100.24	100.10	99.12	99.22	99.51	100.07
KC	99.15	100.73	101.22	98.22	99.76	100.87

APT - 29 Data obtained from the comparison of the standard USP method to the developed method for Griseofulvin

Standard method	Paracetamol as SRS	Ascorbic acid as SRS	Metronidazole as SRS
99.57	100.59	100.65	100.99
99.62	101.06	98.88	100.50
100.28	99.38	100.59	98.73
99.71	98.72	100.21	99.59
100.45	99.44	100.50	101.44

APT - 30 Data obtained from the comparison of the standard BP method to the developed method for Cetirizine hydrochloride

Standard method	Paracetamol as SRS	Ascorbic acid as SRS	Metronidazole as SRS
101.13	98.48	98.84	99.51
100.40	99.76	102.08	100.11
100.36	100.55	100.42	99.29
101.28	101.21	99.72	101.12
99.57	101.35	99.53	102.14

APT - 31 Effect of wavelength and surrogate reference standard on the outcome of developed method for Griseofulvin. Data was analysed using MS Excel 2010.

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
252 nm	3	301.05	100.35	2.2249
254 nm	3	301.83	100.61	2.5087
255 nm	3	300.59	100.1967	1.587433
Paracetamol	3	298.49	99.49667	0.765433
Ascorbic acid	3	302.06	100.6867	3.181733
Metronidazole	3	302.92	100.9733	0.665433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Wavelength	0.261956	2	0.130978	0.058451	0.944015	6.944272
SRS	3.678822	2	1.839411	0.820868	0.502683	6.944272
Error	8.963244	4	2.240811			
Total	12.90402	8				

APT - 32 Effect of flow rate and surrogate reference standard on the outcome of developed method for Griseofulvin. Data was analysed using MS Excel 2010.

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
1.0 ml/min	3	301.45	100.4833	1.888233
1.1 ml/min	3	304.95	101.65	0.6907
1.2 ml/min	3	302.75	100.9167	0.044633
Paracetamol	3	301.99	100.6633	2.420033
Ascorbic acid	3	303.03	101.01	0.1821
Metronidazole	3	304.13	101.3767	0.683033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Flow rate	2.086667	2	1.043333	0.930786	0.465685	6.944272
SRS	0.763467	2	0.381733	0.340555	0.730167	6.944272
Error	4.483667	4	1.120917			
Total	7.3338	8				

APT - 33 Effect of flow rate and surrogate reference standard on the outcome of developed method for Cetirizine hydrochloride. Data was analysed using MS Excel 2010.

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
1.5 ml/min	3	302.38	100.7933	0.640033
1.4 ml/min	3	299.41	99.80333	0.767233
1.3 ml/min	3	300.55	100.1833	0.348233
Paracetamol	3	298.87	99.62333	0.505033
Ascorbic acid	3	302.24	100.7467	0.410433
Metronidazole	3	301.23	100.41	0.5913

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Flow rate	1.4966	2	0.7483	1.973192	0.253385	6.944272
SRS	1.994067	2	0.997033	2.629076	0.186669	6.944272
Error	1.516933	4	0.379233			
Total	5.0076	8				

APT - 34 Effect of wavelength and surrogate reference standard on the outcome of developed method for Cetirizine hydrochloride. Data was analysed using MS Excel 2010.

Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
233 nm	3	300.53	100.1767	1.494933
235 nm	3	300.27	100.09	3.2097
236 nm	3	302.38	100.7933	0.319433
Paracetamol	3	301.95	100.65	2.5207
Ascorbic acid	3	301.27	100.4233	0.680133
Metronidazole	3	299.96	99.98667	1.923433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Wavelength	0.882467	2	0.441233	0.188439	0.835201	6.944272
SRS	0.682067	2	0.341033	0.145646	0.868848	6.944272
Error	9.366067	4	2.341517			
Total	10.9306	8				