EFFECT OF GREEN TEA (*CAMELLIA SINENSIS*) ON THE PHARMACOKINETIC PROFILE OF CEPHALEXIN IN HEALTHY HUMAN VOLUNTEERS

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**BY**

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**AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

**JUNE, 2018**

# DECLARATION

I declare that the work in this Dissertation titled “ Effect of Green tea (*Camellia sinensis*) on the Pharmacokinetic profile of Cephalexin in Healthy Human Volunteers” has been carried out by me under the supervision of Dr. Aminu Musa and Prof. I.A Yakasai in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Mohammed Adamu AUWAL Name of student Signature Date

# CERTIFICATION

This Dissertation titled “EFFECT OF GREEN TEA (*Camellia sinensis*) ON THE PHARMACOKINETIC PROFILE OF CEPHALEXIN IN HEALTHY HUMAN

VOLUNTEERS” by MOHAMMED ADAMU AUWAL meets the regulations gorverning the award of the degree of Master of Science in Pharmaceutical Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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# ABSTRACT

Tea is a herb commonly consumed globally. Herbs are often taken concurrently with conventional medicines which results in increased potential of drug-herb interactions, and this may have important clinical significance based on an increasing number of reports of such interactions. This research work, determines the effects of green tea (*Camellia sinensis*) on the pharmacokinetic profile of cephalexin in healthy human volunteers. Quality control studies were conducted on a sample of cephalexin (500 mg) capsule to establish identity, weight uniformity, disintegration time, dissolution, and as well as assay using the methods of BP 1993 and BP 2013.A UV spectrophotometric method for determining cephalexin in plasma using water as a solvent at pH 4 was developed and validated based on international conference on harmonization (ICH) guidelines. For the *in vivo* study, six apparently healthy human volunteers were used throughout the study. The study involved four phases with one week washout period between the phases; phase I (administration of cephalexin alone), phase II (concurrent administration of cephalexin with aqueous extract of green tea), phase III (administration of cephalexin one hour after ingestion of aqueous extract of green tea) and phase IV (administration of cephalexin one hour before ingestion of aqueous extract of green tea). Blood samples (2 mL) were collected in a heparinized sample bottles from each of the six volunteers at 0, 0.5, 1, 2, 3, 4 and 6 hours. Plasma proteins were precipitated using 20 µL of 40 % perchloric acid, followed by centrifugation and buffering of the supernatant with 1 ml phosphate buffer pH 4, and subsequently volumes were adjusted to 5 ml with distilled water. Samples were analysed for cephalexin content using the developed UV method and pharmacokinetic parameters generated for each of these phases. The results of quality control studies revealed that the cephalexin 500 mg capsule passed the test. It was found to contain the labeled active

pharmaceutical ingredient and results for uniformity of weight, dissolution, and desintegration time were within the accepted range of 92.5 - 110 % , ˂ 7.5 %, ≥ 90 % and ˂ 15 minutes respectively. The calibration curve was found to be linear within the range of 5 - 150 µg/mL as its coefficient of determination (r2 = 0.9994) close to unity with a regression equation of y = 0.0045 - 0.0178. UV spectral analysis revealed 261 nm as the wavelength of maximum absorption of cephalexin in water at pH 4. Percentage recovery was found to be 84.5 - 85.5 % which is outside the accepted range of 98 - 102 %. The mean plasma concentration of cephalexin in phase I were found to be 13.50, 69.00, 58.60, 48.00, 42.60 and 12.40 (µg/mL) at time 0.5, 1, 2, 3, 4 and 6 (hours) respectively. The Cmax (µg/mL) for phases I, II, III and IV were found to be 69.00, 79.95, 68.00 and 87.07 (µg/mL) respectively at time 1 hour (Tmax) except for phase III

with Tmax of 2 hours. Their AUCs were found to be 235172, 302982, 354333 and 244449 µg/L\*h respectively. With the exception of Tmax of phase II and IV, Cmax of phase III, and also the clearance (Cl) of phase IV, all other pharmacokinetic parameters of phases II, III and IV determined were found to be significantly different (*p ˂ 0.05*) from those of phase I (control) . Therefore, it can be concluded that *Camellia sinensis* (green tea) aqueous extract was found to significantly (*p ˂ 0.05*) enhance the extent and the rate of absorption, decreased elimination rate of cephalexin as well as increase in its volume of distribution to the peripheral compartments.

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# ABBREVIATIONS

|  |  |
| --- | --- |
| ABUCUHSR | ahmadu bello university committee for use of human subjects in research |
| APA | american pharmacists association |
| API | active pharmaceutical ingredient |
| AUC | area under the curve |
| BP | british pharmacopoeia |
| BPCRS | british pharmacopoeia chemical reference standard |
| CV | coefficient of variation |
| Cmax | maximum plasma concentration |
| FTIR | fourier transform infra-red |
| GMP | good manufacturing practice |
| HSLC | high speed liquid chromatography |
| ICH | international conference on harmonization |
| IP | indian pharmacopoeia |
| Ka | absorption rate constant |
| Ke | elimination rate constant |
| LOD | limit of detection |

LQ limit of quantitation

MRT mean resident time

NAFDAC nigeria agency for food and drug administration control RSD relative standard deviation

SD standard deviation

SEM standard error of the meam

Tmax time to reach maximum plasma concentration TBAHS tetrabutylammonium hydrogen sulphate

USP united state pharmacopoeia

UV ultraviolet radiation

Vd volume of distribution

λmax maximum wavelength

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# CHAPTER ONE

# INTRODUCTION

## Pharmacokinetics

The term pharmacokinetics was first introduced by Dost (1953) in his book titled “Der blutspiegel”. Kinetics is that branch of knowledge which involves the change of one or more variables as a function of time. According to Gibaldi and Levy (1976) Pharmacokinetics is concerned with the study and characterization of the time course of drug absorption, distribution, metabolism, excretion and with the relationship of these processes to the intensity and time course of therapeutic and adverse effect of drugs. Wagner(1981) also defined Pharmacokinetics as the study of rate process associated with absorption, distribution, metabolism and excretion of drugs, and according to him the purpose of pharmacokinetics is to study the time course of drug and metabolites concentrations or amount in biological fluids, tissues and excreta, and also of pharmacological response, and to construct suitable models to interpret such data. Another more recent definition is the one put forward by Hedaya (2007) which defined pharmacokinetics as the study of kinetics of drug absorption, distribution, metabolism and elimination. It is important to study the rate of absorption of drug because faster drug absorption leads to faster onset of drug effect which is critical in treatment of acute condition and in emergency situations. Understanding the extent of drug absorption is also important because not all the total dose administered is the amount that is responsible for producing the drug effect but only the amount of drug absorbed and made available at site of action. Studying the drug distribution is necessary because the drug has to be taken to the site of action to elicit its effect. Also studying the rate of drug elimination is important so as to know the frequency of drug administration. Drugs that are

eliminated faster are administered more frequently so as to maintain an effective drug concentrations at all times during multiple drug administrations. Studying the organs responsible for drug elimination is also critical because patients with organ dysfunctions require dosage adjustments (Hedaya, 2007). Each pharmacokinetic process (absorption, distribution, metabolism and excretion) is associated with one or more parameters that are dependent on the drug, drug product and the patient (Hedaya, 2007).Pharmacokinetics is useful in selecting and adjusting drug dosage schedules and monitoring drug levels (therapeutic and toxic concentrations).Pharmacokinetic parameters include area under the curve (AUC), maximum plasma concentration (Cmax), time to attain maximum concentration (Tmax), absorption half- life(tα1/2), absorption rate constant (Kα), elimination half-life (tβ1/2), elimination rate constant (Kβ), Plasma clearance (Cl), volume of distribution (Vd) and lag time ( Tripathi, 2013).

## Absorption

This is the first stage, for orally administered drugs. Absorption is the movement of a drug from its site of administration into the central compartment (mostly blood) and the extent to which this occurs (Buxton, 2006). For solid dosage forms, absorption first requires dissolution of the tablet or capsule; this liberates the drug into the systemic circulation where it will be distributed to its sites of action, and clinicians are concerned primarily with bioavailability rather than absorption (Buxton, 2006). Drug absorption provides avenue for drug interactions, either negatively or positively. Negative interaction occurs when absorptionof a drug is greatly reduced by another drug i.e. Co-administration of iron preparation with tetracycline where the absorption of tetracycline is greatly reduced by iron. Positive interaction could occur by increasing the absorption of another drug. For example, anticholinesterases increase absorption of some drugs by increasing gastric emptying time (Katzung*et al*., 2004).

## Distribution

Following absorption or systemic administration into blood stream, a drug distributes into interstitial and intracellular fluids. This process reflects a number of physiological factors and the particular physicochemical properties of individual drug. Cardiac output, regional blood flow, capillary permeability, and tissue volume determine the rate of delivery and potential amount of drug distributed into tissues. Initially, liver, kidney, brain and other well-perfused organs receive most of the drug, whereas delivery to muscle, most viscera, skin and fat is slower. This second distribution phase may require minutes to several hours before the concentration of drug in tissue is in equilibrium with that in blood. It also involves a far larger fraction of body mass than does the initial phase and generally accounts for most of the extravascularly distributed drug. Therefore tissue distribution is determined by partitioning of drug between blood and a particular tissue and the more important determinant of that is the relative binding of drug to plasma proteins and tissue macromolecules (Buxton, 2006).

## Metabolism

Metabolism is the transformation of drug by chemical alteration to form metabolites. The metabolites are disposed of either in the urine or bile, this overall process is referred to as metabolism (Katzung*et al*., 2004). Metabolism of drugs and other xenobiotics into more hydrophilic metabolites is essential for their elimination from the body, as well as for the determination of their biological and pharmacological activity. In general, biotransformation reaction generates more polar, inactive metabolites that are readily excreted from the body. However, in some cases, metabolites with potent biological activity or toxic properties are generated. Drug metabolism or biotransformation reactions are classified as either phase I (Functionalization reactions) or phase II biosynthetic (Conjugation) reactions (Buxton, 2006).

The primary site of drug metabolism is liver; others are kidneys, intestines, lungs and plasma (Tripathi, 2013).

## Elimination

This is a general term referring to drug removal from the body by any mechanism (Katzung*et al*., 2004). Drugs are eliminated from the body either unchanged by the process of excretion or converted to metabolites. Excretory organs, the lung excluded, eliminate polar compounds more efficiently than substances with high lipid solubility. Lipid-soluble drugs are not readily eliminated until they are metabolized to more polar compounds. The kidney is the most important organ for excreting drugs and their metabolites. Drug substances excreted in the faeces are principally unabsorbed orally ingested drugs or drug metabolites excreted either in the bile or secreted directly into the intestinal tract and not reabsorbed (Buxton, 2006).

## Drug Interaction

A drug interaction is an interaction between a drug and some other substances, such as another drug or a certain type of food or herb, which prevents the drug from working correctly. An interaction can either increase or decrease the effectiveness and/or the side effects of a drug, or it can create a new side effect not previously seen before (APA, 2002).

## Classification of drug interaction

There are various categories of drug interactions which include drug-disease, drug-drug, drug- food, drug-herb and drug-environmental interactions (Linnarson, 1993).

## Consequences of drug interactions

Drug Interactions can result in one or more of the following outcomes: reduction in the desired effect of a drug, increase in adverse effects of a drug, unnecessary pain and suffering, increase in

the beneficial effect of a drug, decrease in the adverse effects of a drug and increased cost of treatment (APA, 2002).

Drug interactions may lead to an increase or decrease in the beneficial or the adverse effects of the given drugs(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011).When a drug interaction increases the benefit of the administered drugs without increasing side effects, both drugs may be combined to increase the control of the condition that is being treated(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011). For example, drugs that reduce blood pressure by different mechanisms may be combined because the blood pressure lowering effect achieved by both drugs may be better than with either drug alone(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011).The absorption of some drugs is increased by food. Therefore, these drugs are taken with food in order to increase their concentration in the body and, ultimately, theireffect(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011). Conversely, when a drug's absorption is reduced by food, the drug is taken on anempty stomach (*Bogenschutz and Bojrab*, *2003)*.

Drug interactions that are of greatest concern are those that reduce the desired effects or increase the adverse effects of the drugs(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011).

## Mechanism of drug interaction

Knowledge of the mechanism by which a given drug interaction occurs is often useful in practice, as the mechanism could influence both the time course and methods of evading the interaction (Linnarson, 1993).There are several mechanisms by which drugs interact withother drugs, food, and other substances (Baxter and Stockley, 2008).

An interaction can result when there is an alteration (whether increase or decrease) in one of the followings: absorption of a drug into the body, distribution of the drug within the body, metabolism and/or elimination of the drug from the body.

Most of the important drug interactions result from a change in the absorption, metabolism, or elimination of a drug. ((Baxter and Stockley, 2008).

* + - 1. *Change in absorption*

Most drugs are absorbed into the blood and then travelled to their sites of action. It is the most common drug interaction and is said to occur in the intestine. The mechanism of drug interaction due to absorption can be summarized as follows:

* + - * 1. an alteration in blood flow to the intestine;
        2. change in drug metabolism (breakdown) by the intestine;
        3. increased or decreased intestinal motility (movement);
        4. alterations in stomach acidity, and
        5. change in the bacteria that reside in the intestine.

Drug absorption can also be affected if the drug's ability to dissolve (solubility) is changed by another drug or if a substance (for example, food) binds to the drug and prevents its absorption (i.e. chelation) (Kushuba and Bertino, 2007).

* + - 1. *Change in drug distribution*

Change in drug interaction is said to occur when the concentration of a drug at the site of action is changed without necessarily altering its circulating concentration(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011).This becomes more alarming for drugs with intracellular

or central nervous system targets(Bogenschutz and Bojrab, 2003; Synder, *et al*., 2011). Examples of drugs that cause significant changes in the cell membrane transport of other drugs are:

1. verapamil which inhibits efflux transporters (e.g. P-glycoprotein) there by increasing the concentrations of substrates such as digoxin and cyclosporin.
2. probenecid by inhibiting anion transporters (e.g. OAT-1) causes increase in the concentrations of substrates such as methotrexate and penicillin.

Drug interactions involving transport are less well understood than drug interactions involving metabolism (Synder, *et al*., 2011).

## Herbal Medicines

Herbs and fruits have been used by man from prehistoric times. They are used for various reasons ranging from nutritional, therapeutic, to social reasons (include use as aphrodisiacs) (Odimgbe, 1998). Herbal medicines are becoming popular worldwide, despite their mechanisms of action being generally unknown, the lack of evidence of efficacy, and inadequate toxicological data. An estimated one third of adults in developed nations and more than 80% of the population in many developing countries use herbal medicines in the hope of promoting health and to manage common maladies such as colds, inflammation, heart disease, diabetes and central nervous system diseases (Zhou *et al.,* 2007).

There are more than 11,000 species of herbal plants that are in use medicinally to date, and of these about 500 species are commonly used in Asian and other countries. These herbs are often taken concurrently with conventional drugs, raising the potential of drug-herb interactions, which may have important clinical significance based on an increasing number of clinical reports of such interactions (Zhou *et al.,* 2007).

## Green tea

Tea is one of the herbs commonly consumed globally. It is the processed leaves of *Camellia sinensis* plant. *Camellia sinensis*is the source of black, green, oolong and white teas, it is an evergreen shrub indigenous to Southeast Asia. A difference in the method of processing of harvested leaves and buds of the plant is responsible for the varieties. White tea is made from very young tea leaves or buds; green tea is made from mature unfermented leaves; Oolong tea from partially fermented leaves; and black tea from fully fermented leaves (Reygaert,2014).

Studies have suggested that green tea may contribute to a reduction in the risk of cardiovascular diseases and some forms of cancer as well as promotion of oral health and other physiological functions such as anti-hypertensive effect, body weight control, ultraviolet protection, bone mineral density increase and neuro-protection power (Cabrera *et al*., 2006; Jazani *et al*., 2007).

## Statement of the Research Problem

Tea is a herb that is commonly consumed (Costa *et al*., 2002) and it has been estimated that, about 25% of all prescription drug users take tea concomitantly with conventional medications (Gurley *et al*., 2008). A good number of *in-vitro* studies have reported that green tea or its constituents enhance the antibacterial action of cephalosporins (Passat, 2012) and other groups of antibiotics (Lee *et al*., 2005; Jazani*et al*., 2007). However, there are other reports of green tea inhibiting the antibacterial action of cephalosporins (Passat, 2012) and other groups of antibiotics (Esimone*et al*., 2013; Ihekwereme*et al.*, 2015).

## Justification of the Research

There appears to be conflicting claims on the effect of green tea on the antibacterial activity of some cephalosporins and other antibiotics.However, there have been no reports on the pharmacokinetics interaction between green tea and cephalexin. Thus, the need to study the pharmacokinetic profile of cephalexin when co-administered with green tea, in order to explain the pharmacokinetic basis for some of the reported pharmacodynamic interactions.

## Aim and Objectives

## Aim

The aim of this study is to determine the effect of green tea on the pharmacokinetic profile of cephalexin in healthy human volunteers.

## Objectives

The objectives of this study are to;

1. carry out quality control assessment of both reference standard and cephalexin 500 mg capsule to be used in the study.
2. develop and validate a UV-Spectrophotometric method for the analysis of cephalexin in human plasma.
3. determine the pharmacokinetic profile of cephalexin when administered alone and when co-administered with green tea extract in healthy human volunteers.

## Hypothesis

## Null hypothesis

Green tea extract has no significant effect on the pharmacokinetics of cephalexin in healthy human volunteers.

## Alternate hypothesis

Green tea extract has significant effect on the pharmacokinetics of cephalexin in healthy human volunteers.

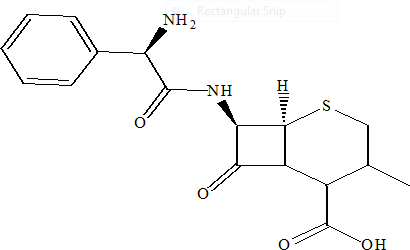
# CHAPTER TWO

# LITERATURE REVIEW

## Cephalexin

Cephalexin is a semi-synthetic first generation cephalosporin antibiotic. It is 7-(D-α-amino-α- phenylacetamido)-3-cephem-4-carboxylic acid and has a molecular formular C16H17N3O4S. It exist in two (2) salt forms i.e monohydrate and hydrochloride with a molar mass of 365.41 g/mol and 347.39 g/mol respectively. The compound is a zwitterion; i.e., the molecule contains both a basic and acidic group. The crystalline form of the drug is available as a monohydrate having a bitter taste.

Solubility in water is slow at room temperature; 1 or 2 mg/mL may be dissolved readily, but higher concentrations are obtained with increasing difficulty. It has a melting point of 326.8 ̊ C (NCCLS, 1997).



## Figure 2.1; Chemical structure of cephalexin

## Pharmacokinetics of cephalexin

Cephalexin is acid stable and may be given without regard to meals. It is rapidly absorbed after oral administration. Following doses of 250 mg, 500 mg, and 1 g, average peak serum level of approximately 9, 18 and 32 µg/mL respectively were obtained at 1 hour. Measurable levels were present 6 hours after administration. Cephalexin is excreted in the urine by glomerular filtration and tubular secretion (NCCLS, 1997). Binding to human serum proteins is low (15 %) and there is no measurable destruction or metabolism of cephalexin during its sojourn in the body fluids(Griffith, 1983). Seventy to 100 % of the dose is found unchanged in the urine 6-8 hr after each dose(Griffith, 1983). During this period, peak urine concentrations following the 250 mg, 500 mg, and 1 g doses were approximately 1000, 2000, and 5000 µg/mL respectively(NCCLS,

1997).

## Mechanism of action

Cephalexin is a beta-lactam antibiotic of the cephalosporin family. It is[bactericidal](https://en.wikipedia.org/wiki/Bactericidal) and acts by inhibiting synthesis of the [peptidoglycan](https://en.wikipedia.org/wiki/Peptidoglycan) layer of the bacterial cell wall. As cephalexin closely resembles d-alanyl-d-alanine, an amino acid ending on the peptidoglycan layer of the cell wall, it is able to irreversibly bind to the active site ofpeptidoglycan binding protein, which is essential for the synthesis of the cell wall. It is mostly active against[gram-positive cocci,](https://en.wikipedia.org/wiki/Gram-positive_cocci) and has moderate activity against somebacilli. However, some bacterial cells have the enzyme[β-lactamase,](https://en.wikipedia.org/wiki/Beta_lactamase) which hydrolyzes the beta-lactam ring, rendering the drug inactive. This contributes to antibacterial resistance towards cephalexin (Drawz and Bonomo, 2010).

## Uses and administration

Cephalexin is a first-generation oral cephalosporin antibiotic for the treatment of infections caused by susceptible Gram-positive and Gram-negative bacteria including infections of the respiratory and genito-urinary tracts, bones and skin. Doses are expressed in terms of equivalent amount of anhydrous cephalexin; 1.05 g of cephalexin monohydrate and 1.16 g of cephalexin hydrochloride are each equivalent to about 1 g of anhydrous cephalexin. It is given in doses ranging from 1 to 4 g daily in divided doses; 250 to 500 mg every 6 to 8 hours is typical. For skin and soft tissue infections, streptococcal pharyngitis, and uncomplicated urinary-tract infections a dose of 250 mg every 6 hours or 500 mg every 12 hours may be given. For the prophylaxis of recurrent urinary-tract infection, cephalexin may be given in a dose of 125 mg at night (Sweetman, 2009). Cephalexin does not treat [methicillin-resistant *Staphylococcus aureus*](https://en.wikipedia.org/wiki/MRSA)infections.

## Adverse effects

The most common adverse effects of cephalexin, like other oral cephalosporins, are gastrointestinal (stomach area) disturbances and hypersensitivity reactions. Gastrointestinal disturbances include [nausea,](https://en.wikipedia.org/wiki/Nausea) vomiting and [diarrhea,](https://en.wikipedia.org/wiki/Diarrhea) diarrhea being most common. [Hypersensitivity reactions](https://en.wikipedia.org/wiki/Hypersensitivity_reaction) include skin rashes, [urticaria](https://en.wikipedia.org/wiki/Urticaria), [fever](https://en.wikipedia.org/wiki/Fever), and [anaphylaxis.](https://en.wikipedia.org/wiki/Anaphylaxis)[Pseudo](https://en.wikipedia.org/wiki/Pseudomembranous_colitis) [membranous colitis](https://en.wikipedia.org/wiki/Pseudomembranous_colitis) and[*Clostridium difficile*](https://en.wikipedia.org/wiki/Clostridium_difficile_colitis)have been reported with use of cephalexin (Haberfeld, 2009).

Signs and symptoms of an [allergic reaction](https://en.wikipedia.org/wiki/Allergic_reaction) include rash, itching, swelling, trouble breathing, or red, blistered, swollen, or peeling skin. Overall, cephalexin allergy occurs in less than 0.1% of patients, but it is seen in 1% to 10% of patients with a penicillin allergy (Haberfeld, 2009). Caution must be exercised when administering cephalosporin antibiotics to penicillin-sensitive

patients, because cross sensitivity with beta-lactam antibiotics has been documented in up to 10% of patients with a documented penicillin allergy (FDA, 2014).

## Interactions of cephalexin

Like other [β-lactam antibiotics,](https://en.wikipedia.org/wiki/%CE%92-lactam_antibiotic) renal excretion of cephalexin is delayed by [probenecid.](https://en.wikipedia.org/wiki/Probenecid) Alcohol consumption does not have a negative interaction with cephalexin, but reduces the rate at which it is absorbed (Barrio *et al.*, 1991). Cephalexin also interacts with[metformin,](https://en.wikipedia.org/wiki/Metformin) an [antidiabetic](https://en.wikipedia.org/wiki/Antidiabetic_drug) [drug](https://en.wikipedia.org/wiki/Antidiabetic_drug), and this can lead to higher concentrations of metformin in the body (Jayasagar *et al.*, 2002). There have been isolated reports of cephalexin decreasing the efficacy of oestrogen- containing oral contraceptives (Sweetman, 2009).

## Green Tea Constituents and their Interactions

Green tea is rich (35 % of dry weight) in catechins, a family of polyphenolic flavon-3-ols, and these are the putative bioactive agents (Williamson and Manach, 2005; Khan and Mukhtar, 2007; Yang *et al.*, 2009). The major catechin (about 60–70 %) in green tea is epigallocatechin gallate (EGCG), but there are also substantial amounts of epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) (Williamson and Manach, 2005; Khan and Mukhtar, 2007; Yang *et al.*, 2009). *In-vitro* studies have demonstrated the powerful antioxidant properties of catechins, particularly EGCG, and human studies have shown that the plasma total antioxidant capacity increases shortly after the ingestion of green tea (Benzie *et al*., 1999;Higdon & Frei, 2003; Williamson and Manach, 2005;Yang *et al.*, 2009). However, poor gastrointestinal absorption, rapid phase II metabolism and urinary excretion of conjugated catechins severely limits their bioavailability and their biological effects (Lee *et al*., 2002; Higdon & Frei, 2003; Williamson &

Manach, 2005; Del Rio *et al*., 2010). Including the absorption (assessed by their urinary excretion) of ring fission catechin metabolites produced from colonic bacteria increases

„catechin‟ bioavailability to about 40 % (ranging from < 3 to 100 %), but inter-individual differences in urinary concentrations of these metabolites are very large, and their physiological relevance is currently unknown(Stalmach *et al*., 2009).

OH OH



OH

OH

O

OH

O

O

OH

OH

C

OH OH

O

Epicatechin (EC)

Epicatechin gallate (ECG)

OH

O H

OH

OH



OH

OH

OH

O

OH

OH

HO

O

OH

OH

OH

Epigallocatechin (EGC)

O

OH

C

O

O

Epigallocatechin gallate (EGCG)

OH

OH

H

## Figure 2.2; Chemical structures of catechins present in green tea

## Pharmacodynamic interactions of green tea with cephalosporins

Passat (2012) conducted a study on the interaction of green tea and black tea with different groups of antibiotics including cephalosporins. He utilizes bacterial isolates (*E. coli*) collected from urine specimens of patients with urinary tract infection submitted to a diagnostic microbiology laboratory of a selected hospital (Al-Yarmook) during two months period. The results of this study shows thatgreen tea extract increased the diameter of inhibition zone (Synergetic effect) when combined with cefodizim and decreased the diameter of inhibition zone (Antagonistic effect) with cefipime.

To the best of my knowledge, There is no any report on the pharmacokinetic interactions of green tea with cephalexin.

## Pharmacodynamic interactions of green tea with other antibiotics

Passat (2012) in the above study also reported that green tea has synergistic activity with chloramphenicol, amoxicillin and azithromycin.

Synergetic effect has been reported with levofloxacin against anterohaemorrhagic *E. coli* (Tiwari

*et al.*, 2005).

However, Ihekwereme *et al.* (2015) reported an inhibitory action of green tea on the antimicrobial activity of ciprofloxacin using healthy adult volunteers who orally received green tea and ciprofloxacin simultaneously. A control group received ciprofloxacin alone. The ability of urine samples of volunteers to inhibit the growth of *E. coliin-vitro*was used to assess synergism. The result of this study revealed that tea reduced time to reach peak urinary excretion rate, and increased the urinary concentration of ciprofloxacin. In contrast, green tea not only failed to improve bacterial clearance of ciprofloxacin, but also antagonized the antibacterial activity at 1 hour and 5 hour. Antagonistic effect has also been reported with Ofloxacin (Esimone *et al.*, 2013) by reduction of bioavailability and increasing urinary concentration of this drug.

## Mechanism of interaction of green tea

Synergetic effect is achieved by catechin intercalation into phospholipid bilayers and it likely affects antibiotic resistance by perturbing the functions of key processes associated with the bacterial cytoplasmic membrane and make the micro-organisms more susceptible to the antimicrobial agents (Taylor *et al.*, 2005). While antagonistic effect may result from competing tea components with antimicrobial agent to bind to the micro-organism membrane, tea component may competitively inhibit the binding of antibiotic to the bacterial membrane and loss the activity against the bacteria in the presence of tea (Esimone *et al.*, 2007). The influence of competitive binding on the antimicrobial activity of these agents may depend on their structure, this may explain the differences in the antimicrobial activity of these agents in the presence of tea (Ikiqai *et al.*, 2005). In addition, the high element content of tea (potash, manganese, fluorine, aluminium and selenium) predisposes it to interaction with antimicrobial agents like tetracycline and fluoroquinolones (Kivisto *et al.*, 1989; Akerele and Okhamafe, 1991).

## Reported Methods of Cephalexin Analysis

Several analytical methods have been published for the quantification of cephalexin level in different matrixes, including spectrophotometry (UV, IR) (Panda *et al.*, 2013), High Performance Thin Layer Chromatography (HPTLC) (Jeswani *et al.*, 2009), Molecular Imprinted Solid Phase Extraction (Edward and Stanley, 2003), High Performance Liquid Chromatography (HPLC) (Lee and Lee, 1990; Anika *et al.,*1997; Hammami and Raja, 2014). Some of these methods suffered from a long processing time (Edward and Stanley, 2003), long chromatographic run time (Lee and Lee, 1990; Paul *et al.*, 1991; Edward and Stanley, 2003) or low recovery (Paul *et al.*, 1991).

## UV Spectrophotometry and its use in the analysis of cephalexin

UV-Visible spectrophotometry is one of the most frequently used techniques in pharmaceutical analysis. It involves measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution. Instrument which measure the ratio, or function of ratio, of the intensity of two beams of light in the UV-Visible region are called Ultraviolet-Visible spectrophotometers (Behera*et al.,* 2012).

Spectroscopy could be used in qualitative analysis (identification of analyte) and in quantitative analysis (to ascertain the quantity of analyte). Spectrophotometric technique is simple, rapid, moderately specific and applicable to small quantities of compounds (Behera*et al.,* 2012).

Quantitative analysis basically relates concentration of analyte and the intensity of light absorbed. The fundamental law that governs the quantitative spectrophotometric analysis is the Beer -Lambert law (Behera*et al.*, 2012).

Quantification of medicinal substance using UV spectrophotometer may be carried out by preparing solution in transparent solvent and measuring it‟s absorbance at suitable wavelength. The wavelength normally selected is wavelength of maximum absorption (λmax), where small error in setting the wavelength scale has little effect on measured absorbance.

Panda *et al.* (2013) developed two (2) UV spectroscopic methods for determination of cephalexin monohydrate in pharmaceutical dosage forms.

The methods were measured at 261 nm and 256-266 nm for the AUC method using a phosphate buffer (pH=5.5). The linearity was observed over a concentration range of 1.0– 120 μg/ml for the two UV spectroscopic methods (coefficient of determination, r2=0.999). The relative standard deviation values for the intra-day and inter-day precisions studies were < 2 % with accuracy of > 99 % for all the three developed methods. These developed methods were used successfully for the determination of cephalexin in dry syrup formulation.

Similarly, Khan *et al.* (2016) also developed and validated a new spectrophotometric method for determination of cephalexin monohydrate in pure form and pharmaceutical formulations. The method involves the addition of Ce IV to cephalexin in acidic medium, followed by the

determination of residual Ce IV by reacting with a fixed amount of methyl orange, and the absorbance was measured at 510 nm. Beer‟s law was obeyed in the concentration range of 0.6-20 µg/ml with a correlation coefficient of 0.992. The method was found to have good reproducibility with a relative standard deviation of 4.4 % (n = 9) and it was validated statistically by performing recovery studies and successfully applied for the determination of cephalexin in bulk powder and pharmaceutical preparations. Percentage recoveries were calculated and found to be within the range of 90.6 to 102.9 % for bulk powder and from 98.7 to

104.8 % for pharmaceutical preparations.

To the best of my literature search, there is no report of use of UV in the determination of cephalexin in the body fluids.

## HPLC: the modern method of cephalexin analysis

The application of high-pressure liquid chromatography (HPLC) to the analysis of antibiotics introduces a powerful tool for therapeutic drug monitoring as well as clinical research (Anhalt, 1980). The advantages of short turnaround time, method reliability, method sensitivity, and drug specificity justify the use of HPLC for various groups of antibiotics (Nilsson-ehle, 1977; Yoshikawa *et al*., 1980). The original reports of cephalosporin quantification by HPLC typically describe the analysis of a single cephalosporin in biological fluid (Wold and Turnipseed, 1977; Aziz *et al*., 1978; Nakagawa *et al*., 1978). Each of these methods presents a unique preparatory and chromatographic protocol for a single cephalosporin or its metabolite or both.

ProteinprecipitationandRP-HPLChavebeenusedformeasuringcephalexininplasmaand

serum sample.Methodsdiffermainlyinthechoiceofprecipitatingreagent,whichincludesmethanol (Nahata, 1981),methanolicsodiumacetate (Signs *et al*., 1984), 60 % Perchloric acid (Ticiano *et al*., 2009) andtrichloroaceticacid (Lecaillon *et al*., 1982).

Nakagawa *et al.* (1978) developed a rapid and accurate high speed liquid chromatographic method for the determination of cephalexin in human plasma and urine. The method involves micro-pore filtration of urine specimens and methanol extraction of plasma samples followed by HSLC separation on a bonded reverse phase column utilizing a mobile phase of methanol-water containing acetic acid. The quantitative UV response at 254 nm covered a wide range of cephalexin concentrations down to 0.5 µg/ml, and no metabolite peaks were detected. The time course of plasma level and urinary excretion were determined until 6 hours after oral administration of cephalexin capsules to healthy volunteers.

Also, a high-pressure liquid chromatographic method was described by Steven *et al.* (1984) fortheanalysisofawiderangeofcephalosporin congeners. CephalosporinswereresolvedonaC- 18reverse-phasecolumn,utilizinga mobile phase of variouspercentagesof0.01 Msodium acetate, acetonitrile, and methanol mixtures. Eachantibioticdemonstratedexcellentlinearitythroughoutthetherapeuticrange. Percentage recoveriesof93to101 % were reported with detection limitsrangingfrom0.2to1.0µg/ml and a retention time of 4to6minforthese drugs.

Similarly, Hammami and Raja (2014) optimized and validated a simple, precise, and rapid reversed phase high performance liquid chromatographic (HPLC) method for determination cephalexin in human plasma and applied it in clinical settings. The mobile phase consisted of a mixture of equal volumes of 0.01 M cetyltrimethyl ammonium bromide and 0.01 M dipotassium hydrogen phosphate (pH 6.5, adjusted with phosphoric acid), acetonitrile, and triethylamine (60 : 40 : 0.001, v : v : v) using Waters 2998 photodiode array detector set at 260 nm to detect the analyte.

Finally, a more recent work was that of Panda *et al.* (2013) where he developed an ultra fast liquid chromatographic method for determination of cephalexin using an isocratic separation technique on an Enable C18G column (250 mm x 4.6 mm in diameter with a particle size of 5 µm) and using methanol: 0.01 M TBAHS solution (50:50, v/v) as the mobile phase at a flow rate of 1.0 ml/min at an ambient temperature.

# CHAPTER THREE

# MATERIALS AND METHODS

## Materials

## Drugs

Brand of cephalexin capsule(CelexinR500 mg) Cephalexin standard powder (Sigma Aldrich Germany) Green Tea (*Camellia sinensis*) bags

## Glass wares

250 mL Extraction bottles (Pyrex England) 100 mL measuring cylinder (Pyrex England)

100 mL and 250 mL conical flasks (Pyrex England) 25 mL and 50 mL beakers (Pyrex England)

10 mL test tube (Pyrex England)

15 mL centrifuge tubes (Pyrex England)

5 mLHeparinized sample bottles (Skytec Medical)

5, 10, 25, 50, and 100 mL volumetric flasks (Pyrex England) Funnel

Automatic Pipette Pump

Pipette (Pyrex England) Filter paper

## Equipment

Electronic weighing balance (Metlers Pr 63 Switzerland) Disintegration apparatus (Erweka G.M.B.H Germany) Centrifuge machine (Gallen Kamp, Philip Harries, Ltd England) Gallen Kamp Hot Air Oven (Philip Harries, Ltd England) Thermometer (Mc Donald Scientific International, England)

Dissolution machine (DA-6D, Veego Scientific devices Mumbai, India) Stop watch (Smith England clock system)

UV Spectrophotometer (Biomate 6)

Infra-red spectrophotometer (Agilent Technology) pH Meter (Milwaukee, Hungary)

## Chemicals and reagents

All Reagents were of analytical grades Distilled water (BPH Chemical, England) Methanol (BPH Chemical, England)

* 1. N HCl (BPH Chemical, England) 0.1N NaOH (BPH Chemical, England) Chloroform (BPH Chemical, England) Phenolphthalein indicator

40 % Perchloric acid

## Methods

## Sample collection

* + - 1. *Cephalexin capsule* (*500 mg*)

A brand of Cephalexin capsule (500 mg) was purchased from a registered pharmacy in Zaria, Kaduna state. Standard cephalexin powder was obtained from Sigma- Aldrich Germany with Product Number C4895 and Batch Number 066M4755V.

* + - 1. *Green tea*

Green tea was purchased from a reputable herbal shop inside Ahmadu University Zaria (Samaru main campus). Informations on the pack such as manufacturing date, expiry date, batch number and NAFDAC Registration number were noted.

* + - 1. *Preparation of the green tea extract*

The green tea extract was prepared by adding one tea bag to a cup containing freshly boiled water (added to the 150 ml mark), agitating the tea bag gently for 3 minutes. Finally the tea bag was discarded (Fung *et al*., 2013).

* + - 1. *Collection and preparation of blank plasma*

Fresh blood from a cubital vein was collected in a heparinized sample bottles (5 ml) from a volunteer after undergoing an overnight fasting. It was then centrifuged at 2000 rpm for 10 minutes and the supernatant layer (plasma) decanted and finally stored in a refrigerator at -4 ̊ C.

## Quality control of cephalexin standard powder

* + - 1. *Identification of cephalexin standard powder*

The standard powder of cephalexin was identified using Fourier transform infra-red (FTIR) spectroscopy and melting point.

*Fourier transformed infra-red spectroscopy (FTIR):*

Standard cephalexin powder was identified by means of FTIR spectrophotometry. The FTIR spectrum obtained was compared with the cephalexin referenceFTIR spectrum (BP, 2013).

*Melting point determination:*

Melting point was determined by adding a pinch of the standard powder inside asmall capillary tube and tapped on a hard surface to a height of about 3 mm. The capillary tube was then placed inside the melting point machine. Melting process was observed via the magnifying lens. The melting point obtained was compared with that of official value (BP, 2013).

## Quality control of cephalexin capsule

* + - 1. *Inspection of NAFDAC label requirement*

The address of manufacturer, batch number, manufacturing date and expiry date were examined and recorded.

* + - 1. *Identification of cephalexin capsule*

The cephalexin capsule was identified using Fourier transform infra-red (FTIR) spectroscopy and color of precipitate formed when treated with 0.1 ml of a 1 % w/v solution of copper (II) sulphate and 0.1 ml of 2 M sodium hydroxide as described below:

*Fourier transform infra-red spectroscopy (FTIR):*

Twenty(20) cephalexin capsules (500 mg) were emptied into porcelain mortar and pestle.

A pinch from the powdered drug was mixed with potassium bromide powder. The mixture was grounded into a fine powder using mortar and pestle. The fine powder was then placed inside a cylindrical holder, pressed together with ahelp of a hydraulic pressing machine. Afterwards, the pellet or disc in the holder, was placed into the instrument and spectrum acquired. Finally, FTIR spectrum obtained was compared with FTIR spectrum of cephalexin standard powder (BP, 2013).

*Color of precipitate formed:*

This test was done by shaking a quantity of the contents of the capsules containing a quantity equivalent to 0.5 g of anhydrous cephalexin with 1 mL of water and 1.4 mL of 1 M hydrochloric acid. The mixture was filtered and the filtrate washed with 1 mL of water. A precipitate was formed after slow addition of a saturated solution of sodium acetate to the filtrate. Methanol (5 ml) was added to the precipitate, then filtered and washed with two 1-mL quantities of methanol.

The residue obtained was compared with that of test C conducted by mixing 20 mg of cephalexin with 0.25 mL of a 1 % v/v solution of glacial acetic acid and adding 0.1 mL of a 1 % w/v solution of copper (II) sulphate and 0.1 mL of 2 M sodium hydroxide (BP, 1993).

* + - 1. *Assay of cephalexin capsule*

Assay of cephalexin capsule was carried out according to the method specified in B.P (1993). Briefly, 100 mL of water was added to the equivalent of 0.25 g of anhydrous cephalexin and then stirred for 30 minutes. Sufficient amount of water was then added to produce 250 mL solution and then filtered. 10 mL of the filtrate was transferred to a Stoppered flask; 5 mL of 1 M sodium hydroxide was then added and allowed to stand for 20 minutes. 20 mL of a freshly prepared buffer solution containing 5.44 % w/v of sodium acetate and 2.40 % w/v glacial acetic acid was added. 5 mL of 1 M hydrochloric acid and 25 mL of 0.01 M iodine were added and allowed to stand for 20 minutes while protected from light. Excess of iodine was then titrated with 0.02 M sodium thiosulphate using starch mucilage as indicator. To a further 10 mL of the solution, 20 mL of the buffer solution and 25 mL of 0.01 M iodine were added and allowed to stand for 20 minutes, and then titrated with 0.02 M sodium thiosulphate using starch mucilage added towards the end of the titration as indicator. The difference between the two titrations represents the amount of iodine equivalent to cephalexin present. The content of C16H17N3O4S was calculated from the difference obtained by carrying out the assay at the same time using 0.25 g of cephalexin *BPCRS*in place of the contents of the capsules and from the declared content of C16H17N3O4S in cephalexin *BPCRS.*

* + - 1. *Uniformity of weight test*

Twenty cephalexin capsules were weighed individually, then the contents of the shells wereemptied and later the shell reweighed with the aid of an analytical balance. The difference between the capsules with the content and the shell alone was recorded as the weight of each capsule. The average weights of the capsules as well as their percentage mean deviation from the mean weight were calculated (BP, 2009).

* + - 1. *Dissolution test*

The dissolution test was carried out using Erwekadissolution apparatus Germany (basket type). The dissolution medium was 900 mL of 0.1N HClwhich was maintained at 37 ± 0.5 °C. The basket was rotated at a speed of 100 rpm for 30 minutes. Afterwards, 10 mL of the sample was withdrawn and filtered. The absorbance was measured using a UV spectrophotometer at 262 nm (USP, 29)

* + - 1. *Disintegration test*

Six (6) cephalexin capsules were randomly picked and placed in the6 basket units of Erweka disintegration machine,containing 0.6 % v/v solution of HCl in place of water, maintained at 37

± 10 oC.The time taken for all the capsule particles in each unit to pass through the mesh was recorded. Average disintegration time and percentage mean deviation was determined.The BP specification for disintegration is that at the end of specified time, all the capsules should completely break up and pass through the sieve within 15 minutes (BP, 1993).

## Analytical method

* + - 1. *Preparation of stock solution*

Stock solution of cephalexin was prepared by dissolving 0.15 g (150 mg) of accurately weighed cephalexin standard powder in 20 mL distilled water contained in 100mL volumetric flask and making up to volume with the same solvent to obtain 150μg/mL stock solution.

* + - 1. *Determination of wavelength of maximum absorption*

One millilitre (1 mL) of stock solution was withdrawn and transferred into a 10 mL volumetric flask and made up to volume with distilled water to make 15 µg/mL solution. This was then scanned through a wavelength range (200-400nm) to obtain the wavelength of maximum absorption.

* + - 1. *Determination of pH of maximum absorption*

To five test-tubes (labeled 1, 2, 3, 4 and 5) each containing 2 mL of a 150 μg/mL solution

cephalexin, 1 mL of buffered solution pH 3, 4, 5, 6 and 7 was respectively added and the volume made up to 5 mLwith distilled water to obtain 60 μg/mL and then mixed vigorously. The absorbance of each solution was measured at 261 nm so as to obtain the pH of maximum absorption.

* + - 1. *Extraction procedure for cephalexin in plasma*

Method of Ticiano *et al.* (2009) was adopted and modified as follows; 1 mL plasma samples contained in an extraction centrifuge tubes were mixed with 20 µL of 40 % perchloric acid for one minute. The precipitate formed was then centrifuged at 3000rpm for 10minutes. The supernatants were transferred into 5 mL volumetric flask with aid of Pasteur pipette and buffered with 1 mL of 2 M phosphate buffer (pH 4). The content of the flask was made up to the mark with distilled water,and vortex mixed afterwards absorbance was measured at 261 nm.

* + - 1. *Preparation of calibration curve*

Ten point calibration curves was constructed by spiking 1 mL blank plasma contains in ten labeled test tubes with 0.17, 0.3, 0.5, 0.67, 1.3, 2.67, 3.3, 4, 4.67 and 5 mL stock solution of cephalexin, 20 µL of a precipitating agent (40 % perchloric acid) was added and vortex mixed for one (1) minute. The mixture was then centrifuge at 3000 rpm for 10 minutes. The supernatant was transferred into 5 mL volumetric flask, buffered with 1 mL of phosphate buffer (pH 4) solution and made up to volume with distilled water. Their absorbances were recorded at 261 nm. The absorbances obtained were plotted against their corresponding concentrations with aid of Microsoft excel 2007.

## Validation of the developed analytical method

The developed method was validated for its linearity, precision, accuracy or percentage recovery, limit of detection (LOD), and limit of quantification (LOQ) according to International Conference on Harmonization (ICH) guideline 1995.

* + - 1. *Linearity*

The linearity of this developed method was established by least square with the aid Microsoft Excel 2007.

* + - 1. *Precision*

This was done by measuring the absorbance of a solution of fixed concentration of cephalexin (40 μg/mL) using the proposed method. It consists of Within-day and between day precision. Expressed as RSD ≤ 2%

*Within-day precision*:

15, 20 and 40 μg/mL solutions of cephalexin were used for the within-day precision. Their absorbances were measured hourly at 261 nm for up to three (3) times in order to get nine (9) determinations. Their means, standard deviations and relative standard deviations (RSD %) were then calculated.

*Between-day precision*:

This consists of measuring the absorbance of the 40 μg/mL solution in triplicate for three consecutive days in order to get nine (9) determinations. The mean, standard deviation and relative standard deviation (RSD %) were calculated.

* + - 1. *Percentage recovery / Accuracy*

Accuracy of the analytical method was determined by recovery study where 18, 20 and 22 (μg/mL) of standard drug were added respectively to three different test tubes each containing

0.7 mL of stock solution, while a fourth test tube, also containing 0.7 mL of the stock solution was left unspiked. The absorbance was then measured and the percentage recovery was calculated as follow:

% Recovery = 𝑂𝑏𝑠𝑒𝑟𝑣𝑒𝑑𝑐𝑜𝑛𝑐𝑒𝑛𝑡𝑟𝑎𝑡𝑖𝑜𝑛

𝐸𝑥𝑝𝑒𝑐𝑡𝑒𝑑 𝑐𝑜𝑛𝑐𝑒𝑛𝑡𝑟𝑎𝑡𝑖𝑜𝑛

× 100 − − − − − − − − − − − −1

* + - 1. *Limit of Detection*

The limit of detection (LOD) may be expressed as:

LOD = 3.3∗σ − − − − − − − − − − − − − − − − − − − − − − − 2

S

Where σ = the standard deviation of the response, S = the slope of the calibration graph

* + - 1. *Limit of Quantification*

The quantificationlimit (QL) may be expressed as:

QL =

10 . σ

S − − − − − − − − − − − − − − − − − − − − − − − 3

Where σ = the standard deviation of the response, S = the slope of the calibration graph

## *In-vivo* interaction studies of cephalexin and green tea aqueous extract

* + - 1. *Study protocol*

Six healthy adult male volunteers (age 28 ±5years, weight 60 ±5kg) were used for the study. The human volunteers were healthy, non-smoking, non-alcoholic and free from liver and kidney diseases. Individuals who are hypersensitive to cephalexin or penicillin allergy were excluded. Ethical approval was sought for from Ahmadu Bello University Committee for the Use of Human Subjects in Research (ABUCUHSR) (Appendix XIX). Also, consent was obtained from each subject participating in this study after adequate explanation of the aims, methods, objectives, and potential hazards of the study.All volunteers were instructed to abstain from drugs or any herbal medicines two weeks before the commencement of the study.

* + - 1. *Study design*

This study was designed into four (4) phases in which the drugs were administered to the volunteers after an overnight fasting.

* + - 1. *Drug administration and collection of blood samples*

*Phase I:*After administration of cephalexin 500 mg capsule with a glass of water, blood samples (2 mL) were collected into a heparinized sample bottles from each of the six healthy human volunteers at time 0, 0.5, 1, 2, 3, 4 and 6 hours. Plasma samples were extracted, buffered andstored at – 4oC before analysis.

*Phase II (Concurrent administration):*Following a concurrent administration of cephalexin (500 mg) with 200 mL of green teaaqueous extract,blood samples (2 mL) were collected into a heparinized sample bottles from each of the six healthy human volunteers at time 0, 0.5, 1, 2, 3, 4 and 6 hours. Plasma samples were extracted, buffered and stored at – 4oC before analysis.

*Phase III (Delayed administration of cephalexin):*Following ingestion of 200 mL of green tea aqueous extract with administration of cephalexin 500 mg capsule an hour later,blood samples (2 mL) were collected from each of the six healthy human volunteers at time 0, 0.5, 1, 2, 3, 4 and 6 hours.Plasma samples were extracted, bufferedand stored at – 4oC before analysis.

*Phase IV (Post administration of cephalexin):*Following administration of cephalexin 500 mg capsule with ingestion of 200 mL of green tea aqueous extract an hour later,blood samples (2 mL) were collected into a heparinized sample bottles from each of the six healthy human volunteers at time 0, 0.5, 1, 2, 3, 4 and 6 hours. Plasma samples were extracted, buffered and stored at –4oC before analysis.

Cephalexin was extracted from all the plasma samples and the concentration therein determined using the method developed.

## Data analysis

The absorbances were converted to concentration using the calibration graph. Pharmacokinetic parameters were generated using Kinetica 5.0 software. Results were expressed as mean ± S.D and statistical analysis was conducted using ANOVAat 95 % confidence interval.

# CHAPTER FOUR

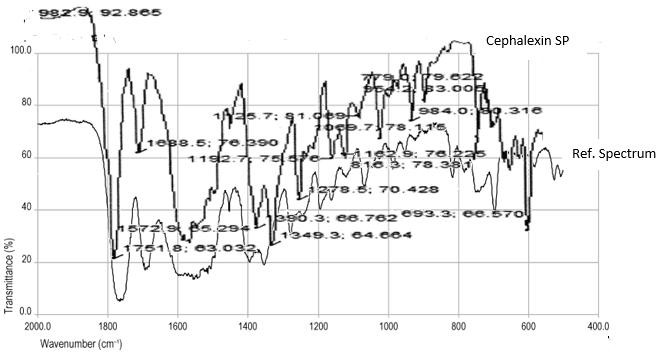
# RESULT

## Quality Control of Cephalexin Standard Powder

The test conducted on cephalexin standard powder showed conformity with official requirement (BP, 2013).

## Identification of cephalexin standard powder

The FTIR spectrum of the cephalexin standard powder was found to be superimposable with that of the reference and this comprised of only their fingerprint regions (Figure 4.1) as shown below;



## Figure 4.1: Superimposed FTIR spectra of cephalexin standard powder and reference

The powder was further confirmed to be cephalexin by the result of melting test and it was found to be 324 – 326 ̊ C which is within the official range.

## Quality Control of Cephalexin Capsule

## Physical evaluation of cephalexin capsule sample

The labeled information of cephalexin capsulesused in this study is presented in the Table 4.1.

## Table 4.1 Labeled information on thecapsule sample

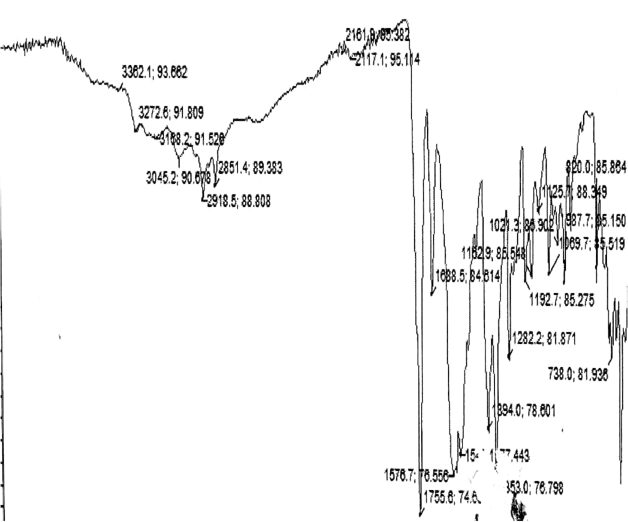
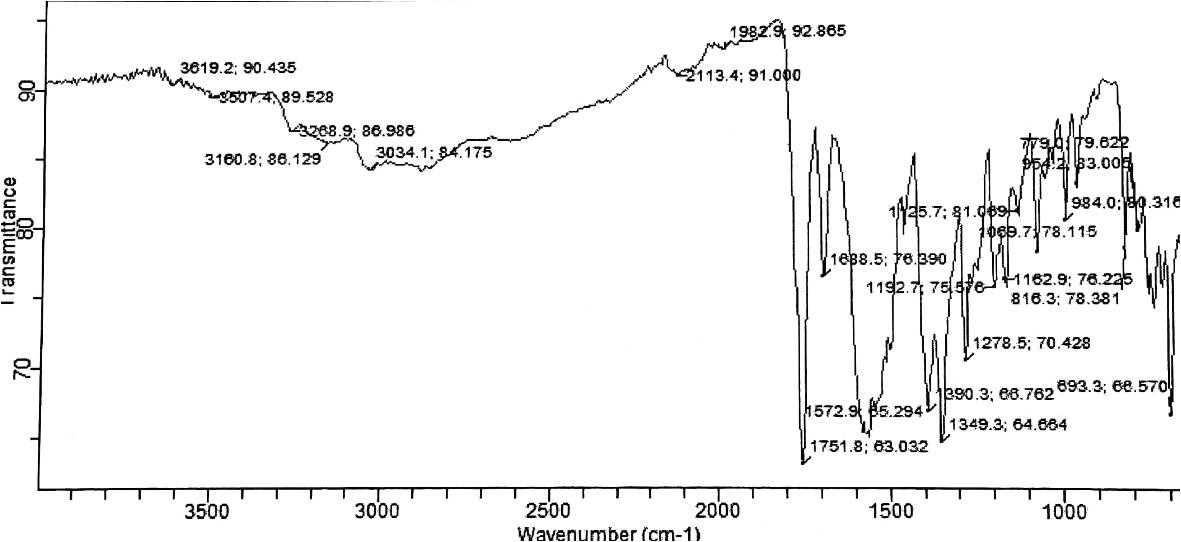
Generic name NAFDAC No.Mfd Expd Batch No Country of

Origin

Cephalexin 500 mg 04-3000 12/14 12/17 BE12473 Malaysia

## Identification of cephalexin capsule

The FTIR spectrum of the cephalexin capsule was found to be superimposable with FTIR spectrum of standard cephalexin powder (Figure 4.2) as shown below;



## Figure 4.2: Superimposed spectra of cephalexin capsule and standard cephalexin powder

Thecolour of the solution of cephalexin capsule produced during the colour test was found to be olive-green which conformed to the official result (BP, 1993).

## Assay of cephalexin capsule

The percentage content of cephalexin in the capsule used was found to be 103.33 % which is within the accepted limit.

## Uniformity of weight and dissolution test

The results of the uniformity of weight and dissolution test carried out on the cephalexin capsule are in conformity with the official (BP, 2013; USP, 29) specifications (Table 4.2).

## Table 4.2Results for uniformity of weight and dissolution test

|  |  |  |  |
| --- | --- | --- | --- |
| Test | Result obtained (%) | Official limit (%) | Remark |
| Uniformity of weight | ≤ 2.6 | < 7.5 | passed |
| Dissolution | 93 | ≥ 90 | passed |

* + 1. **Disintegration time test of cephalexin capsule**

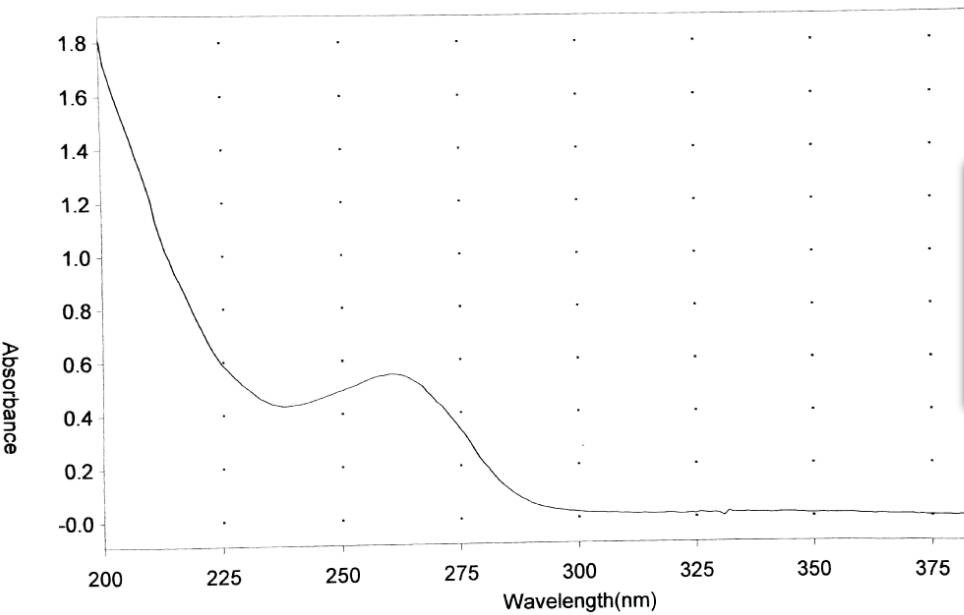
All the six capsules chosen for this test were found to disintegrate within the officially specified time (Table 4.3).

|  |  |
| --- | --- |
| **Table 4.3 Disintegration time** |  |
| Capsule | Disintegration time (min) |
| 1 | 2.90 |
| 2 | 3.21 |
| 3 | 2.95 |
| 4 | 3.12 |
| 5 | 3.07 |
| 6 | 3.26 |
| Mean | 3.59 |

## Results of Analytical Method

* + 1. **Wavelength of maximum absorption**

The wavelength of maximum absorption of cephalexin solution (15µg/mL in water) was found to be 261 nm (Figure 4.3).



## Figure 4.3: Spectrum of a 15 µg/mL solution of cephalexin in water.

## Determination of pH of maximum absorption

The pH of maximum absorption of cephalexin in water at 261 nm was found to be 4 (Table 4.4).

## Table 4.4: Effect of pH on absorption of cephalexin at wavelength of maximum absorption

|  |  |  |
| --- | --- | --- |
| **pH** |  | **Absorbance** |
| 3 | 0.666 |  |
| 4 | 0.678 |  |
| 5 | 0.644 |  |
| 6 | 0.648 |  |
| 7 |  | 0.639 |

## Calibration curve

The calibration curve of cephalexin in plasma (pH 4) at 261 nm was found to be linear in the concentration range of 5 – 150 µg/mL (Figure 4.4)



200

150

100

**CONCENTRATION ( µg/ml )**

50

0

0

0.1

0.2

Series1

Linear (Series1)

0.3

0.4

0.5

y = 0.004x - 0.017

R² = 0.999

0.6

0.7

**ABSORBANCE**

**Figure 4.4: Calibration curve of cephalexin in plasma (pH 4) at 261 nm**

## Validation of the Analytical Method

## Precision of the analytical method

The intra-day and inter-day precisions were found to be < 2 % as indicated by their percentage RSD (Table 4.5 and 4.6).

**Table 4.5 Within-day precision using 15, 20 and 40 µg/ml cephalexin solution**

|  |  |  |
| --- | --- | --- |
| **Concentration** | **Mean ± SD** | **RSD (%)** |
| 15 | 0.050 ± 0.0008 | 1.6 |
| 20 | 0.073 ± 0.002 | 2.7 |
| 40 | 0.152 ± 0.001 | 0.65 |

**Table 4.6 Between-day precision using 40 µg/ml cephalexin solution**

|  |  |  |
| --- | --- | --- |
| **Days** | **Mean ± SD** | **RSD (%)** |
| 1 | 0.157 ± 0.0012 | 0.74 |
| 2 | 0.154 ± 0.0005 | 0.37 |
| 3 | 0.153 ± 0.0016 | 1.06 |

* + 1. **Accuracy and percentage recovery of the analytical method**

The percentage recovery was found to be less than the acceptable limit of 98-102% (Table 4.7)

## Table 4.7 Percentage recovery of standard cephalexin powder spiked in blank plasma

|  |  |  |  |
| --- | --- | --- | --- |
| **S/No** | **Amount added (ug/mL)** | **Amount found (ug/mL)** | **Percentage recovery** |
|  | **(n=3)** | **(n=3)** | **( %)** |
| 1 | 18 | 34.6 | 84 |
| 2 | 20 | 36.6 | 85.5 |
| 3 | 22 | 38.18 | 84.9 |

* + 1. **Limit of quantification (LOQ) and limit of detection (LOD) of the developed method**

The results obtained for thelimit of detection and limit of quantification are shown in Table 4.8.

## Table 4.8 LOQ and LOD of the developed method

S/No Parameters Result obtained

1 Limit of detection 183.33 ng/mL

2 Limit of quantification 555.56ng/mL

* 1. **Results of *In-vivo* Studies**

The *in-vivo* studies of cephalexin in the various phases are shown in the Tables 4.9 and 4.10. Also in Figures 4.5, 4.6 and 4.7.

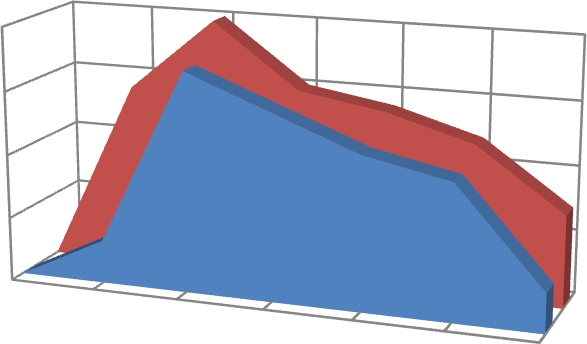
## Table 4.9 Mean plasma concentration of cephalexin (ug/mL) in all the four phases

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Time** | **phase I** | **phase II** | **phase III** | **phase IV** |
| 0 | nd | nd | nd | nd |
| 0.5 | 13.50 | 57.10 | 21.00 | 30.20 |
| 1 | 69.00 | 79.95 | 26.00 | 87.07 |
| 2 | 58.60 | 60.18 | 68.00 | 61.95 |
| 3 | 48.20 | 55.73 | 58.00 | 46.84 |
| 4 | 42.60 | 47.95 | 52.40 | 39.95 |
| 6 | 12.40 | 28.84 | 24.00 | 6.30 |

nd= not detected

**Mean Conc. (µg/ml)**

**Figure 4.5: Mean plasma concentration of cephalexin alone and cephalexin with green tea when taking concurrently.**



80

60

40

PHASE I (Cephalexin alone)

20

0

PHASE II (Cephalexin+Green

tea concurrently)

1

2

3

4

5

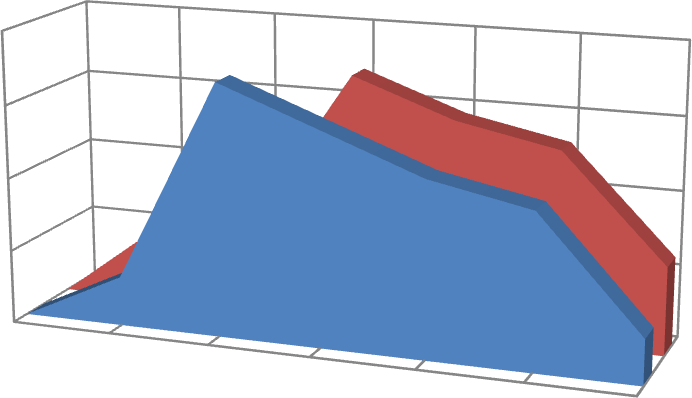
6

7

**Time (hours)**

**Mean Conc. (μg/ml)**

**Figure 4.6: Mean plasma concentration of cephalexin alone and cephalexin one hour after ingestion of green tea**



80

60

40

PHASE I (Cephalexin alone)

20

PHASE III (Green tea+Cephalexin

one hour later)

0

0

0.5

1

2

3

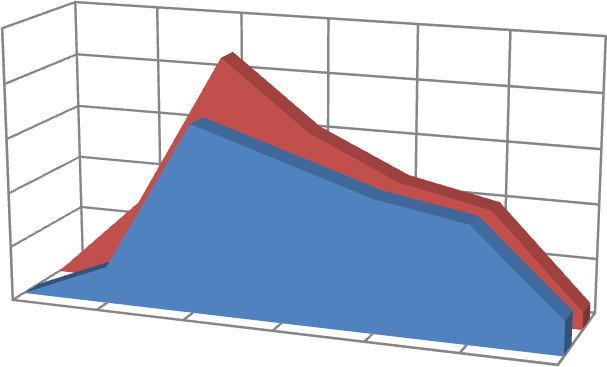
4

6

**TIME (Hours)**

**Mean Conc. (µg/ml)**

**Figure 4.7: Mean plasma concentration of cephalexin alone and green tea one hour after administration of cephalexin**



100

80

60

PHASE I (Cephalexin alone)

40

20

0

PHASE IV (Cephalexin+Green tea

one hour later)

0

0.5

1

2

3

4

6

**Time (Hours)**

**Table 4.10 Mean values of pharmacokinetic parameters for all the phases**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **PK parameter** | **Phase I** | **Phase II** | **Phase III** | **Phase IV** |
| C max (µg/mL) | 69.00 | 79.95\* | 68 | 87.07\* |
| T max (hour) | 1 | 1 | 2\* | 1 |
| T1/2 (hour) 1.456 | 3.087\* | 2.251\* | 0.983\* |  |
| T lag (hour) | 0 | 0 | 0 | 0 |
| MRT (hour) | 3.294 | 5.063\* | 4.605\* | 2.627\* |
| Ke (/hour) | 0.476 | 0.224\* | 0.307\* | 0.705\* |
| Vd (liters) | 3.990 | 5.138\* | 4.833\* | 2.787\* |
| AUC (μg/L\*h) | 235172 | 302982\* | 354333\* | 244449\* |
| Cl (L/h) | 1.9 | 1.156\* | 1.488\* | 1.965 |

**\*** Significant difference atp< 0.05

# CHAPTER FIVE

# DISCUSSION

## Quality Control of Cephalexin Standard Powder and Capsule

Based on the results of tests that were carried out on the standard powder, it was identified as cephalexin standard powder as its IR spectrum was superimposable with that of the reference spectrum (Figure 4.1) and had a melting point within the official range of 324 ̊ C to 326 ̊ C (BP 2013)

The sample of cephalexin capsule used in this study was found to be of good appearance, shape, color, packaging,content and has NAFDAC registration number and it is within the shelf live (Table 4.1) at the time of this study. The sample was identified as cephalexin capsule as its IR spectrum is in conformity with that of standard cephalexin powder (Figure 4.2) and also produced an olive-green colour which is in conformity with the BP (1993). Furthermore, it passed the assay as its percentage content was within 92.5-110 % (BP, 2013). Uniformity of weight serves as anindicator to good manufacturing practices (GMP) as well as amount of the active pharmaceutical ingredient (API) contained in the formulation(Ngwuluka*et al*., 2009). The capsule had a percentage weight deviation from the mean weight of ≤ 2.6 % (Table 4.2). Therefore it complies with the compendial specifications for uniformity of weight which stated that for capsules weighing greater than 300 mg, the percentage weight deviation should be equal to or less than 7.5 % (BP, 2013). The BP (1993) stated that all capsules should disintegrate within ˂15 min, and hence sample capsule had passed this test as all the six capsules disintegrated in less than 15 minutes (Table 4.3).

For a drug to elicit its pharmacological activity it has to release appreciable amount of its active pharmaceutical ingredient (API) into the biological media and this is determined by dissolution studies. The official specification is that the amount of drug released (dissolution) should not be less than 90% of the stated amount at 30 min (BP, 2013).Based on the findings of this study, thecapsule sample released 93 % of its active pharmaceutical ingredient (API) which complies with the compendial specifications for dissolution (Table 4.2). Therefore, the quality control results of this capsule sample shows that it had no negative impact on the outcomes of the *in- vivo* studies conducted on the healthy human volunteers since it was of good quality.

## Analytical Method Development

Cephalexin contains a benzene chromophore in its structure which absorbed UV light and therefore enabling it to be analyzed using UV spectroscopy. The UV spectrophotometric method developed was simple, selective and precise for analysis of cephalexin in biological fluids (plasma). Cephalexin was found to have a wavelength of maximum absorbance (λmax) of 261 nm (Fig. 4.1) and absorbed maximally at pH 4 (Table 4.8). This is in accordance with the reports of Panda *et al.* (2013) where cephalexin was measured at 261 nm and 256 – 266 nm for the two UV methods developed respectively.

The developed method was found to be linear within the range of 5 -150 µg/mL as its correlation determination (r2) was found to be 0.9994 (Fig. 4.2) which is also similar to the reports of Panda *et al* (2013) with a linear coefficient of determination (r2 = 0.999) over a concentration range of

1.0 – 120 µg/mL of cephalexin.

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The method was found to be precise as indicated by the average RSD values for both intra-day (1.65 %) and inter-

day precision (0.71 %) which is less than 2% as specified by the ICH (1995). The percentage recovery of the developed method was found to be 84 - 85.5 % which is outside the accepted limit of 98 – 102 % (ICH, 1995). This agrees with the reports of Joy *et al.* (1987) who reported a recovery of 82.3 % for cephalexin in plasma. Similarly, Ticiano *et al.*(2009) reported a percentage recovery of 82.0 ± 1.6% (*n* = 3) for cephalexin after precipitation of plasma with 40

% perchloric acid followed by buffering with sodium acetate 2.5 M (pH 4.5) post-centrifugation. The buffering step had the inconvenience of diluting the sample and decreasing the accuracy and sensibility of the method (Ticiano *et al*., 2009). Thus, it can be seen that cephalexin generally has a relatively low recovery from plasma.

**5.3*In-vivo*Studies**

The result of this study revealed that cephalexin when administered alone (phase I) to the six (6) healthy adult volunteers had a mean plasma concentrations as shown in Table 4.9 and it differs from the reports of Nakagawa *et al.* (1978) mean cephalexin plasma concentration 8.79 ± 3.42,

21.29 ± 5.54, 6.46 ± 1.12, 2.47 ± 0.49, 1.17 ± 0.24 and 0.41 ± 0.16 µg/mL at time 0.5, 1, 2, 3, 4

and 6 hours respectively. Extended bioavailability for cephalexin in the gastrointestinal tract (G.I.T) was highly observed when cephalexin was administered concurrently with the green tea aqueous extract in phase II (Figure 4.5) as compared with the other co-administration phases (Figure 4.6 and 4.7 respectively), and this implies concurrent administration of cephalexin with green tea aqueous extract provides maximum therapeutic outcome. The maximum plasma concentrations (Cmax) of phases I, II, III and IV, and their time to reach maximum plasma concentration (Tmax)(Table 4.10) varies with the reports of Spyker *et al*. (1978) who reported a Cmax of 27.6 ± 6.4 μg/ml and a Tmax of 1.35 ± 0.83 hours. With the exception of Tmax of phases II and IV, Cmax of phase III, and also the clearance (Cl) of phase IV, all other

pharmacokinetic parameters of phases II, III and IV were found to be significantly different (*p<0.05*) from those of phase I (control).

Discrepancies in the results as compared with other studies could be attributed to the genetic make-up, diet or geographic location or in one way to the buffering step used in the extraction of cephalexin from the plasma samples of these individual subjects.

Drug bioavailability is determined by Cmax and AUC0-∞. *Camelliasinensis* (Green tea) aqueous extract was found to significantly (*p<0.05*) enhance the extent of absorption of cephalexin and also increases its distribution to peripheral compartment except for phase IV where it decreases (table 4.10). Furthermore, green tea extract was observed to significantly (*p < 0.05*) decreases the elimination rate of cephalexin except for phase IV (administration of cephalexin one hour after ingestion of green tea) where it increases the elimination rate and subsequently decreases the mean residence time (table 4.10), and this can be further proven by the observed increase in the mean residence time (MRT) for cephalexin when given together with green tea in phase II and III of this study (Table 4.10). Clearance of this drug from the plasma has also been reduced significantly *(p ˂ 0.05*) in all the phases exception in phase IV. These factors would make the drug level high in the system and could result in to toxicity of this antibacterial agent especially when repeated doses were given in chronic conditions.

# CHAPTER SIX

# SUMMARY AND CONCLUSION

## Summary

In this study, quality control studies were conducted on both the cephalexin standard powder and the cephalexin sample capsules used. A simple, selective and preciseUV spectrophotometric method for the analysis of cephalexin from plasma was developed and validated according to ICH guidelines. For the *in vivo* interaction study between cephalexin and green tea, six healthy human volunteers were used in the four phases of the study; phase I (administration of cephalexin alone), phase II (administration of cephalexin with green tea aqueous extract concurrently), phase III (administration of cephalexin one hour after ingestion of green tea aqueous extract) and phase IV (ingestion of green tea aqueous extract one hour after administration of cephalexin).

The results of quality control studies showed that both the standard powder and the cephalexin sample capsules contained the labeled amount of the APIs.

The pharmacokinetic parameters of phases II, III and IV were found to be significantly different (*p<0.05*) from those of phase I except for Tmax of phase II and IV, Cmax of phase III and Cl of phase IV. *Camellia sinensis*(Green tea) aqueousextract was found to significantly (*p<0.05*) enhance the extent of absorption of cephalexin as evidenced from the increased Cmax and AUC in the co-administration phases of this study. Furthermore, the extract was observed to significantly (*p*<*0.05*) decreases the elimination rate of phase II and III. Significant (*p ˂ 0.05*) increase in elimination rate of phase IV and volume of distribution of cephalexin to the peripheral compartments in all the co-administration phases was also observed.

## Conclusion

The cephalexin capsule sample used in this study is of good quality and it has shown no any negative influence on the outcome of the *in-vivo*interaction aspect of this study. A simple, precise and affordable UV spectrophotometric method for determination of cephalexin in plasma was developed and validated according to ICH guidelines (1995), and that green tea (*Camellia sinensis*) has been found to significantly *(p˂0.05*) enhance the extent of absorption, decreases the elimination rate, and increases the half-life (T1/2) of cephalexin in phase IV. This made the drug more available in the plasma for systemic circulation.

## Recommendations

The following recommendations are of utmost importance:

* This study should be replicated using highly selective and sensitive equipment such as HPLC for the analysis of cephalexin in human plasma.
* Pharmacodynamic interactions studiesshould be carried out between green tea and cephalexin, and this might provide solutions to the effect of increased bioavailability observed with cephalexin when co-administered with green tea in humans as well as possible toxicity it might cause.

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# APPENDICES APPENDIX I

Uniformity of Weight Raw Data (g) n=20

|  |
| --- |
| S/NO. Weight in grams |
| 1 0.654  2 0.645  3 0.606  4 0.649  5 0.633  6 0.653  7 0.646  8 0.634  9 0.648  10 0.648  11 0.653  12 0.666  13 0.686  14 0.693  15 0.656  16 0.630  17 0.692  18 0.682  19 0.646  20 0.675 |

# APPENDIX II

Disintegration Time Test Raw Data (Minutes) n=4

|  |  |
| --- | --- |
| Capsule | Disintegration time (min) |
| 1 | 2.90 |
| 2 | 3.21 |
| 3 | 2.95 |
| 4 | 3.12 |
| 5 | 3.07 |
| 6 | 3.26 |
| Mean | 3.59 |

# APPENDIX III

Dissolution test raw data ( 45 minutes)

|  |
| --- |
| Generic % release (t = 45) Remark |
| Cephalexin capsule 93 passed |

# APPENDIX IV

Assay of cephalexin capsule raw data

|  |
| --- |
| Brand Strength (g) E. Weight (g) Final volume (mL) Percentage content |
| Celexin 0.5 0.28 16 103.33 |

# APPENDIX V

Preparation of 0.1 N HCl

1.7 ml of the conc. HCl solution was measured and made up to 200 ml with distilled water to make 200 ml of 0.1 N HCl solution as follows:

C1 = 12 N, V1 = ?, C2 = 0.1 N and V2 = 200 ml C1 V1 = C2 V2, 12 x V1 = 0.1 x 200

V1 = 1.7 ml

# APPENDIX VI

Preparation of 0.1 N NaOH

16 g of NaOH was weighed and then dissolved into 200 ml of distilled water contained in 500 ml volumetric flask to make 200 ml of 2 M NaOH solution as follows:

V = 200 ml (0.2 ml), Conc. = 2 M

Mol = 0.2 x 2, Mol = 0.4

Mass = Mol x Molar mass, Mass = 0.4 x 40 Mass = 16 g

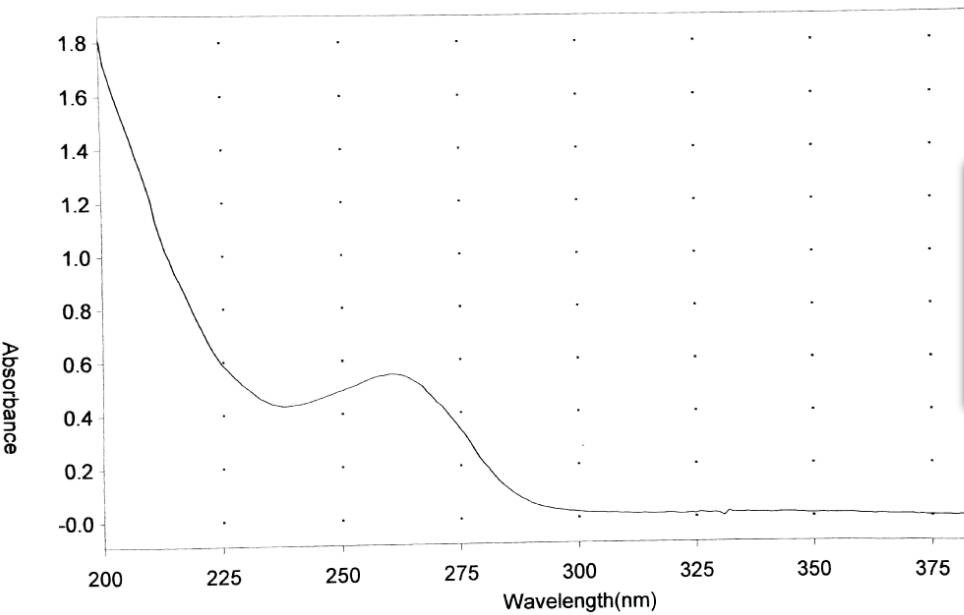
# APPENDIX VII

Preparation of pH solution data

pH solution of 4 and 5 were prepared by dissolving each of the pH 4 and pH 5 buffer tablets into 100 mL distilled water. pH 3, 6 and 7 were made by adjusting the previous pHs with either 0.1N HCl or 0.1N NaOH as the case may be.

# APPENDIX VIII

Spectrum of 15 µg/ml solution of Cephalexin monohydrate standard powder in water at 261 nm



# APPENDIX IX

Precisions Raw Data

Within-day precision using 15, 20 and 40 µg/ml cephalexin solution

|  |  |
| --- | --- |
| **Concentration** | **Absorbance (s)** |
| 15 | 0.051, 0.051, 0.050 |
| 20 | 0.075, 0.073, 0.070 |
| 40 | 0.150, 0.152, 0.152 |

|  |  |
| --- | --- |
| Between-day precision using 40 µg/ml cephalexin solution |  |
| **Days** | **Absorbance** |
| 1 | 0.157, 0.157, 0.159 |
| 2 | 0.154, 0.155, 0.154 |
| 3 | 0.153, 0.155, 0.151 |

# APPENDIX X

Accuracy/ recovery studies of standard cephalexin powder spiked in blank plasma

|  |  |  |  |
| --- | --- | --- | --- |
| **S/No** | **Amt added (ug/mL) (n=3)** | **Amt expected (µg/mL)** | **Amt found (µg/mL) (n=3)** |
| 1 | 18 | 36 | 34.6 |
| 2 | 20 | 38 | 36.6 |
| 3 | 22 | 40 | 38.18 |

# APPENDIX XI

Sample conversion of absorbances of plasma samples containing cephalexin into concentrations (mean of phase I)

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Time (hrs.) | Absorbance | Concentration (µg/mL) |
| 1 | 0 | nd | 0.00 |
| 2 | 0.5 | 0.043 | 13.5 |
| 3 | 1 | 0.292 | 69 |
| 4 | 2 | 0.246 | 58.6 |
| 5 | 3 | 0.199 | 48.2 |
| 6 | 4 | 0.174 | 42.6 |
| 7 | 6 | 0.038 | 12.4 |

nd = Not detected

# APPENDIX XII

Sample conversion of absorbances of plasma samples containing cephalexin into concentrations (mean of phase II)

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Time (hrs.) | Absorbance | Concentration (µg/mL) |
| 1 | 0 | nd | 0.00 |
| 2 | 0.5 | 0.239 | 57.1 |
| 3 | 1 | 0.342 | 79.95 |
| 4 | 2 | 0.253 | 60.18 |
| 5 | 3 | 0.233 | 55.73 |
| 6 | 4 | 0.198 | 47.95 |
| 7 | 6 | 0.112 | 28.84 |

nd = Not detected

# APPENDIX XIII

Sample conversion of absorbances of plasma samples containing cephalexin into concentrations (mean of phase III)

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Time (hrs.) | Absorbance | Concentration (µg/mL) |
| 1 | 0 | nd | 0.00 |
| 2 | 0.5 | 0.077 | 21.00 |
| 3 | 1 | 0.097 | 26.00 |
| 4 | 2 | 0.288 | 68.00 |
| 5 | 3 | 0.244 | 58.00 |
| 6 | 4 | 0.218 | 52.40 |
| 7 | 6 | 0.089 | 24.0 |

nd = Not detected

# APPENDIX XIV

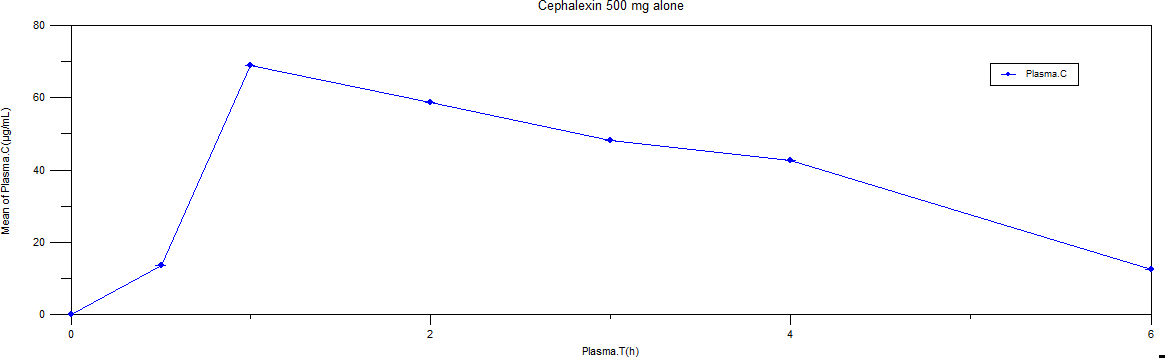
Sample conversion of absorbances of plasma samples containing cephalexin into concentrations (mean of phase IV)

Sample Time (hrs.) Absorbance Concentration (µg/mL)

|  |  |  |  |
| --- | --- | --- | --- |
| 1 | 0 | nd | 0.00 |
| 2 | 0.5 | 0.118 | 30.20 |
| 3 | 1 | 0.374 | 87.07 |
| 4 | 2 | 0.261 | 69.95 |
| 5 | 3 | 0.194 | 46.84 |
| 6 | 4 | 0.162 | 39.95 |
| 7 | 6 | 0.011 | 6.30 |
| nd=Not detected |  |  |  |

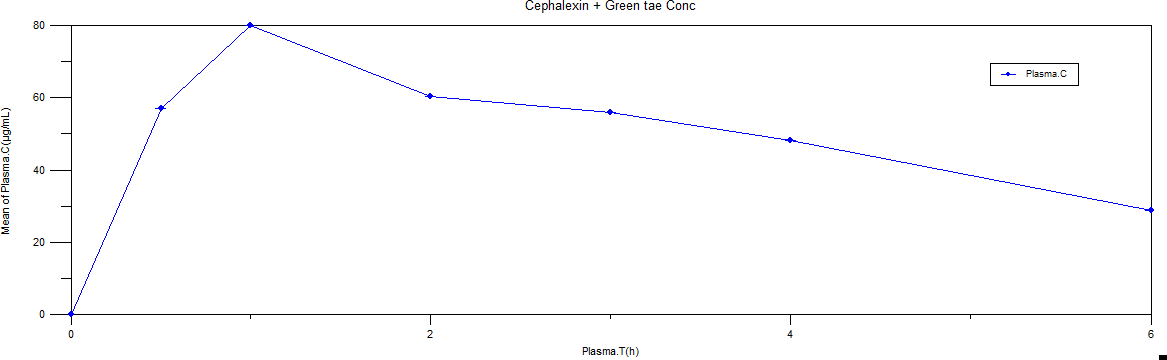
# APPENDIX XV

Concentration-Time curve of Phase I



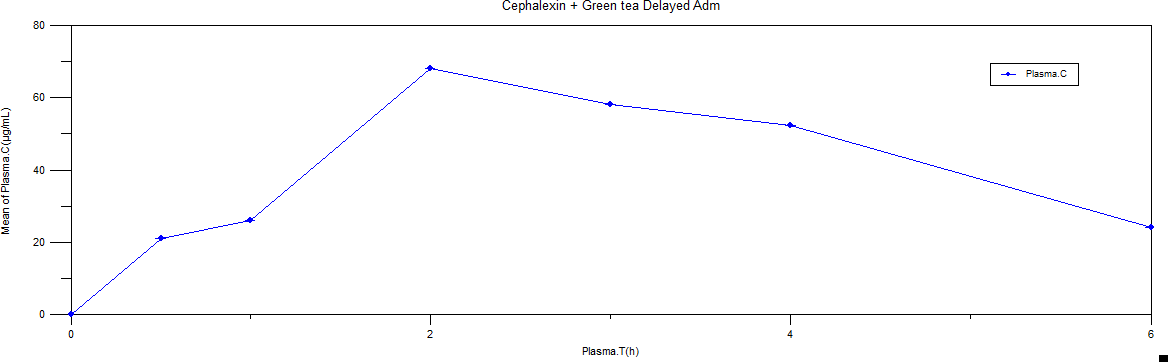
# APPENDIX XVI

Concentration-Time curve of Phase II



# APPENDIX XVII

Concentration-Time curve of Phase III



# APPENDIX XVIII

Concentration-Time curve of Phase IV

