Chapter — III

Review of Literature
3. REVIEW OF LITERATURE:

3.1 Pharmacokinetics

Pharmacokinetics (PK) is the branch of pharmacology that considers what the body does to a drug, as distinguished from pharmacodynamics (PD), which is the study of what a drug does to the body. PK essentially describes the fate of a drug after administration to a living being, and involves several features like the extent and rate of absorption, distribution, metabolism and excretion referred to as the ADME. PK helps in understanding important aspects of drug selection, and monitoring for appropriate therapeutic or toxic effects for an individual.

Absorption is the movement of a drug into the bloodstream and is of primary importance since the drug must be absorbed before any medicinal effects can take place. A drug's PK profile may be altered by adjusting factors that affect absorption. Stomach is the first place where in its aqueous environment an orally administered drug will dissolve. The rate of dissolution is a key target for controlling the duration of a drug's effect. Absorption is also influenced by gut motility, food intake and presence of other drugs. After oral administration, drug absorption occurs predominantly within the small intestine: the villous structures of the absorptive cells enormously increase the surface area available for the absorption.

Distribution describes the reversible transfer of drug from one space to another within the body and is dependent on certain physiological and physicochemical factors. Physicochemical factors are partition coefficient, degree of ionization and molecular size of the drug. Physiological factors are the permeability between tissues, blood flow, perfusion rate of tissue and plasma protein binding. A drug begins to be broken down as soon as it enters the body.
Metabolism is the irreversible transformation of a drug. The majority of small-molecule drug metabolism is carried out in the liver by redox enzymes, termed cytochrome P450 (CYP) enzymes (biotransformation). Biotransformation is thus the process which terminates the drug action and facilitates its excretion.

Excretion refers to the elimination of drug from the body. Unless excretion is complete, accumulation of foreign substances can adversely affect normal metabolism.

Absorption, biotransformation and elimination are first-order, i.e., rate depending upon the concentration of the drug. When body's capacity to process the drug limits the rate, saturated, zero-order kinetics is observed.

Overall disposition of a drug is assessed by measuring its concentrations in the blood plasma at given time intervals following administration. From plasma concentration-time curve, information can be obtained for (i) peak plasma concentration ($C_{\text{max}}$), (ii) time between the administration and the achievement of the peak concentration ($T_{\text{max}}$), and (iii) total amount absorbed from the area under the curve (AUC) (Fig. 2A).

![Fig. 2A: Plasma conc. of a drug versus time curve after single oral dosing, showing (i) peak height to plasma conc. ($C_{\text{max}}$), (ii) rate of absorption (the time between the administration and achievement of the peak height conc. ($T_{\text{max}}$), and (iii) total amount absorbed (measured as the area under the plasma conc.-time curve), AUC (area under curve). (Source: Csaky, 1984 [27])](image-url)
The constants $C_{\text{max}}$, $T_{\text{max}}$ and AUC are the kinetic determinants of oral bioavailability of a drug. Variations in $C_{\text{max}}$, $T_{\text{max}}$ and AUC affect the quantitative action of the drug. Three hypothetical examples are depicted below (Fig. 2B - C) for the same drug in which the rate of absorption varied while the total amount absorbed (AUC) remained unchanged.

![Drug concentration vs. time graph](image)

Fig. 2B: In this example the rate of absorption varied while the total amount absorbed (AUC) was unchanged. A. Therapeutic level not attained; B. Therapeutic level attained; C. Toxic level attained (Source: Csaky, 1984 [27]).

Three examples as depicted below show that while the rate remained the same, but the fraction of the amount absorbed varied.

![Drug concentration vs. time graph](image)

Fig. 2C: In this example the rate of absorption remains same but the extent of absorption varied. A. Therapeutic levels not attained; B. Therapeutic level attained and C. Toxic level attained (Source: Csaky, 1984 [27]).
These examples show that for a rapid action drug or for drugs with a narrow safety margin, the rate of absorption is important, while for long acting and with repeated dosing of a drug, the amount absorbed remains important [27].

The area under curve (AUC) corresponds to the integral of the plasma concentration versus an interval of definite time. In practice, \( \text{AUC} = \int ([C] \times Dt), \) where \([C]\) is measured concentration and \(Dt\) interval of time between two measurements. The precision of the AUC grows with the number of measurements of concentration taken. The AUC is expressed in mass \((\text{ng/ \mu g} \times \text{liter}^{-1} \times \text{time (min/ hour)})\). One of its uses is to allow the measurement of the bioavailability of a drug.

3.2 Pharmacokinetic constants

**Volume of distribution** \((V_d)\) and **Clearance** \((C_l)\):

Drug dosage regimens are determined by two basic parameters; (i) volume of distribution \((V_d)\), which determines the amount of drug required to achieve a target concentration and (ii) clearance \((C_l)\), which determines the dosage rate to maintain an average steady state concentration.

\(V_d\) is used to quantify the distribution of a drug between plasma and the rest of the body after oral or parenteral dosing. It is defined as the theoretical volume in which the total amount of drug would need to be uniformly distributed to produce the desired blood concentration of a drug. Drugs that are water soluble or highly bound to plasma proteins have a high plasma concentration relative to the dose, hence small \(V_d\). In contrast drugs that are lipid soluble or bind extensively to tissues are present in plasma in low concentration, and therefore have large \(V_d\). \(V_d\), multiplied by the known effective concentration of a drug in plasma \((C_p)\) determines the **loading dose**.
A drug starts to be eliminated as soon as it is absorbed. Clearance (Cl) defines how a dose is to be given, at a rate that balances its clearance rate. It is not an indicator of how much drug is being removed, but only represents the theoretical volume of blood; serum or plasma completely cleared of drug per unit of time, and has units of volume/time (i.e., L/hr or ml/min). It is also related to the weight of the patient (L/hr/kg). Total clearance is the fraction of the Vd, which is completely purified per unit of time. For intravenous route, the clearance, Clp = Dose/AUC. When referring to the function of the kidney, Cl is considered to be the amount of liquid filtered out of the blood that gets processed by the kidneys or the amount of blood cleared per time. Cl of a substance is sometimes expressed as the inverse of the time constant that describes its removal rate from the body divided by its Vd (or total body water). In steady-state, it is defined as the mass generation rate of a substance (which equals the mass removal rate) divided by its concentration in the blood. For substances that exhibit substantial plasma protein binding, Cl is generally defined as the total concentration (free + protein-bound) and not the free concentration.

The biological half-life or elimination half life of a substance is the time it takes for the blood plasma concentration of a substance to half its steady-state. When half-life varies with the concentration of the drug, it is proportional to the initial concentration of the drug Ao and inversely proportional to the zero-order rate constant k0 where:

$$t_{1/2} = \frac{0.5A_0}{k_0}$$

This process is usually a logarithmic process - that is, a constant proportion of the agent is eliminated per unit time. Thus the fall in plasma concentration after the administration of a single dose is described by the following equation:
\[ C' = C_0 e^{-kt} \]

Where \( C'_t \) is concentration after time \( t \); \( C_0 \) is the initial concentration (\( t=0 \)); \( k \) is the elimination rate constant.

The relationship between the elimination rate constant and half-life is given by the following equation:

\[ k = \frac{\ln 2}{t_{1/2}} \]

Half-life is determined by clearance (\( C_L \)) and volume of distribution (\( V_D \)) and the relationship is described by the following equation:

\[ t_{1/2} = \frac{\ln 2 \cdot V_D}{C_L} \]

The knowledge of the half-life is useful for the determination of the frequency of administration of a drug (the number of intakes per day) for obtaining the desired plasma concentration. Generally, the half-life of a particular drug is independent of the dose administered. In certain exceptional cases, it varies with the dose: it can increase or decrease according to, for example, the saturation of a mechanism (elimination, catabolism, binding to plasma proteins etc [28-30].

### 3.3 Bioavailability

Bioavailability is one of the principal pharmacokinetic properties of drugs, which is used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation, and is available at the site of action. By definition, when a drug is administered intravenously, its bioavailability is 100%. However, when a drug is administered via other routes (such as oral), its bioavailability decreases (due to
incomplete absorption or first-pass metabolism). **Absolute Bioavailability (AB)** describes the availability of the active drug in systemic circulation after parenteral administration. In order to determine $AB$ of a drug, a pharmacokinetic study must be done to obtain a plasma drug concentration vs. time plot for the drug after both intravenous (i.v.), and non-intravenous administration. **Relative Bioavailability (RB)** measures the bioavailability of a certain drug when compared with another formulation of the same drug, usually an established standard.

The absolute bioavailability ($AB$) ($F$) is the dose-corrected $AUC_{\text{non-i.v.}}$ divided by $AUC_{\text{i.v.}}$. $F$ for a drug administered by the oral route is:

$$F = \frac{[AUC]_{\text{po}} \times \text{dose}_{\text{IV}}}{[AUC]_{\text{IV}} \times \text{dose}_{\text{po}}}$$

Therefore, a drug given by the i.v. route will have an $AB$ of 1 ($F=1$), while the absolute bioavailability of a drug, when administered by an extra vascular route, is usually less than one (i.e. $F<1$) [28-30].

### 3.4 Factors affecting oral drug bioavailability

Various physiological factors reduce the availability of drugs prior to their entry into the systemic circulation. Whether a drug is taken with or without food will also affect absorption, other drugs taken concurrently may alter absorption and first-pass metabolism; intestinal motility alters the dissolution of the drug and may affect the degree of chemical degradation of the drug by intestinal microflora.

Other factors may include, physical properties of the drug (hydrophobicity, pKa, solubility), drug formulation (excipients used, manufacturing methods, immediate - modified - delayed - extended - sustained release etc.), gastric emptying rate, circadian
differences, interactions with other drugs, individual variation in metabolic differences (age, drugs metabolized more slowly in fetal, neonatal, and geriatric populations), phenotypic differences, diet and gender. Disease states affecting liver, GIT or kidney metabolism will also have an effect.

Each of these factors may vary from patient to patient (inter-individual variation), and indeed in the same patient over time (intra-individual variation). In clinical trials inter-individual variation is a critical measurement used to assess the bioavailability differences from patient to patient in order to ensure predictable dosing [31].

3.5 Biochemical regulators of drug bioavailability

P-glycoprotein (P-gp)

The membranes around absorptive epithelial cells in the intestinal mucosa are lipid bilayers containing proteins (e.g. receptors and carrier molecules). A variety of transporter proteins located at the apical membrane of the enterocytes control the fate of drugs by influencing ADME. Among these P-gp is the most widely studied, and probably the most important efflux pump in controlling the disposition of drugs [3, 32, 33].

P-gp, is a 170 kDa transmembrane protein that belongs to the superfamily of ATP-binding cassette (ABC) transporters which is predominantly present in apical membrane of the enterocytes and also in canalicular membranes of the liver, proximal tubules of the kidney and endothelial cells of the blood-brain barrier. It acts as an energy-dependent drug efflux pump that lowers intracellular drug concentrations [34]. Drugs that are substrates for P-gp bind to the transporter and are transported back to the apical side of the intestinal mucosal cells via an ATP-dependent process that greatly reduces
their overall permeability and oral bioavailability. Overall P-gp plays a major physiological role as a barrier for entry of xenobiotics as well as a mechanism to eliminate xenobiotics from systemic circulation. Structurally diverse compounds have been identified as substrates for P-gp: anticancer drugs are the most notable examples [35].

The over expression of P-gp is a significant factor in chemotherapy failure due to the ability of this pump to limit the cell accumulation of anti-neoplastic drugs [35-39].

The use of P-gp-expressing cell lines, the generation of P-gp knockout mice as well as studies in animals and humans has contributed to an understanding on the role of induction and inhibition of this protein as an important underlying mechanisms for low drug bioavailability in humans [40].

The high transport capacity, broad substrate specificity and wide expression of P-gp makes it a very significant factor governing the pharmacokinetic profile of drugs, and has now become an important target of intense investigation: P-gp transport screening play a key role in the drug discovery process. The modulation of P-gp activity has been seen as a useful strategy for increasing the penetration and retention of anticancer drugs in resistant tumor cells. Selective inhibitors of P-gp activity has been shown to increase the oral bioavailability of P-gp substrate anticancer drugs including etoposide [25], which is pumped out from the intestinal epithelium into the lumen by P-gp [41-44].

The cytochrome P450 monooxygenase (CYP) system

CYP superfamily of hemoprotein enzymes found on the membrane of endoplasmic reticulum are the principle enzymes involved in the biotransformation of ~75% of drugs and other foreign compounds (Phase I first pass metabolism). CYPs are also known as
mixed function oxidases or mono-oxygenases, as metabolism of a substrate by a CYP consumes one molecule of molecular oxygen and produces an oxidized substrate and another molecule of oxygen appears in water as a byproduct.

\[
RH + NADPH + H^+ + O_2 = R-OH + NADP^+ + H_2O
\]

RH denotes parent drug and R-OH is oxidized product.

CYPs catalyze variety of reactions including hydroxylation, N-dealkylation, O-dealkylation, S-oxidation, and epoxidation. CYPs are also called polysubstrate mono-oxygenases, as one isoenzyme can have multiple substrates [45].

CYP super family is sub-divided into families and subfamilies that are classified solely on the basis of amino acid sequence homology. In humans 21 families, 20 subfamilies and 57 genes have been described. However, only three main P-450 gene families, CYP1, CYP2, and CYP3 isoforms currently are thought to be responsible for approximately 70% drug metabolism.

CYP1A

CYP1 family comprises 3 members, namely 1A1, 1A2, and 1B1, with CYP1A2 being one of the major CYPs in human liver that metabolizes about 15% of clinical drugs, chemicals, environmental toxins, and several endogenous compounds, suggesting its potential role in some physiologic processes in addition to xenobiotic metabolism. CYP1A2 is subject to induction and inhibition by a number of compounds. Phenacetin, caffeine, and theophylline have been frequently used as model substrates for evaluating the activity of CYP1A2 \textit{in vivo}. For \textit{in vitro} studies, O-deethylation of substrates like phenacetin, 7-ethoxycoumarin, and 7-ethoxyresorufin are commonly used probes for determining CYP1A2 activity [46].
CYP 2B1

The CYP2B subfamily is a major CYP isoform expressed constitutively and inducibly [47] in both liver and small intestine of rodents. It metabolizes compounds like pentoxyresorufin, testosterone, and androstenedione and some drugs. CYP2B is induced by diverse compounds, such as phenobarbital, and some plant products, picrotoxin, pinenes camphor and limonene. It can be measured as 7-benzyloxyresorufin O-dealkylase, 7-pcetylresorufin O-depentylase, benzyloxyresorufin O-dealkylase [48, 49].

CYP3A

CYP3A4 family accounts for 30% of total hepatic content and 70% of gut wall content. There are four isoforms expressed in humans, 3A4, 3A5 and 3A43 (in liver and gut), and 3A7 in fetal liver. CYP3A4 is predominantly expressed in human liver, and non-hepatic tissues, most notably the intestine, and is central to the metabolism of a wide array of chemicals, dietary constituents and fully or partially of over 50% of clinically used drugs. Different CYP3A4 substrates have been used experimentally as probes of this enzyme, which are structurally diverse and exhibit a wide range of sizes, and affinities, such as erythromycin, nifedipine, midazolam, diazepam, steroids, terfenadine, and cyclosporine [50-52].

3.6 First Pass effect

The first-pass effect (also known as first-pass metabolism or pre-systemic metabolism) is a phenomenon whereby the concentration of a drug is greatly reduced before it reaches the systemic circulation. It has been widely believed that the liver is the major site of first-pass metabolism because of its size and its high content of drug -
metabolizing enzymes, but the amount of an orally administered drug that reaches the systemic circulation can be reduced by both intestinal and hepatic metabolism.

Earlier poor oral drug bioavailability was generally believed either due to physico-chemical properties, poor permeability through the intestinal membrane, or significant first-pass hepatic metabolism. Two decades ago the importance of transporter-enzyme interplay and its effects on drug bioavailability and hepatic disposition were first recognized due to interactive biochemical processes in the intestine and/or the liver.

The first pass effect (metabolism and efflux) in the small intestine has received a great deal of attention, where the synergistic alliance between efflux and metabolism by P-gp and CYP enzymes is overwhelmingly implicated in controlling the bioavailability of drugs [53-57], thus making GIT mucosa a major physical and biochemical barrier to the systemic availability of orally ingested, pharmacologically active molecules.

Significant overlap in function and expression exists between P-gp and the drug metabolizing enzyme CYP3A4. CYP3A is the most abundantly expressed CYPs in the human liver and intestine and contributes to the metabolism of a wide spectrum of pharmaceutical agents. Although differences have been noted between these proteins, CYP3A4 and P-gp have been found to display overlapping tissue expression patterns in liver, intestine, and kidney. It is suggested not only by their joint presence at sites of drug absorption (and elimination) i.e., within the digestive tract and liver, but also by the extensive overlap in their substrate specificities. They are also co-inducible in response to many drugs. These two proteins also share many substrates, inducers and inhibitors including etoposide [36, 58]. The function of P-gp may allow CYP3A4 to have repeated and prolonged access to its substrate molecules. (Fig. 3) depicts these various factors responsible for poor bioavailability of drugs.
P-gp and CYP3A functionally interact in three ways: [i] a portion of the xenobiotic molecules extruded from inside the enterocytes into the intestinal lumen by the P-gp can be reabsorbed into the enterocytes and is thus exposed again to metabolism by CYP3A4, [ii] P-gp keeps intracellular drug concentrations within the linear range of the metabolizing capacity of CYP3A, and [iii] P-gp prevents further interaction of CYP3A4-generated metabolites with the enzyme (which might result in product inhibition) by transporting drug metabolites formed in the mucosa back into the gut lumen thus extruding them out of the enterocytes. In comparison with the gut mucosa, in hepatocytes the spatial sequence of CYP3A and P-gp is reversed, resulting in different effects when the activity of one or both are changed [59].

It is noteworthy that drugs that are substrates of both proteins often have a low oral bioavailability, and simultaneous inhibition of P-gp/CYP3A4 has been found to increase the bioavailability of P-gp/ CYP3A substrates [60]. Evidence for these have come from studies on the bioavailability of several drugs using P-gp/ CYP3A inhibitors, and in P-gp knockout animals. Human clinical studies have provided further evidence of this functional interaction between these two proteins [53-57, 61, 62]. The effectiveness of this system is optimized through dynamic regulation of transporter and enzyme
expression; tissues have a remarkable capacity to regulate the amount of protein both at transcriptional and post-transcriptional levels in order to maintain homeostasis [63].

In 2005, Wu and Benet have proposed a Biopharmaceutical Drug Disposition Classification System (BDDCS), a modification of the FDA's Biopharmaceutics Classification System (BCS), as a tool for predicting the relevance of transporters and enzymes in drug disposition [64].

CYP3A and P-gp are both regulated by nuclear receptors such as the pregnane X receptor (PXR) [65]. There is significant genetic variability of CYP3A, P-gp and PXR and their expression, activity is dependent on co-administered drugs, herbs, food, age, hormonal status and disease. There exists a sex-related variation in the levels of expression and activities of drug transporters and metabolizing enzymes [66].

The interaction between drug-metabolizing enzymes and active transporters are now an emerging concept in pharmacokinetics and widely implicated in the disposition of etoposide [67].

3.7 Bio-analytical aspects

Earlier several HPLC procedures for the analysis of etoposide and its metabolites have been developed (cf. Appendices section). In the present investigation an analytical procedure to determine etoposide concentrations in aqueous and plasma matrix was developed using UPLC- Qtof-MS/MS.

UPLC- Qtof-MS/MS:
The ultra performance liquid chromatography (UPLC) connected to a time-of-flight (ToF) mass spectrometer is commonly referred to as UPLC/Q-Tof. A UPLC QTof system used in the present investigation is shown in (Fig. 4).
UPLC is a relatively new technique in liquid chromatography, designed in a special way to decrease solvent consumption, with shortening analysis time up to nine times comparing to the conventional system. The separation on UPLC is performed under very high pressures with no negative influence on analytical column or other components of chromatographic system. The use of smaller particles provides enhanced chromatographic speed, resolution, and sensitivity.

The UPLC/Q-ToF system is a novel system in many aspects as it uses very low particle size column packing which provides excellent chromatographic separation to generate high peak capacities in short times. Equipped with an electrospray (ES) source, the system has a quadrupole (Q) unit, which is connected to a collision cell followed by a time of flight (ToF) tube. The quadrupole is the most widely used analyzer due to its ease of use, mass range covered, good linearity for quantitative work, resolution and quality of mass spectra. In the quadrupole, selected compounds are allowed for transmission, while others are vented into the atmosphere.
The transmitted compounds enter the collision cell, where they are fragmented and the generated fragments (product ions) enter the ToF tube, where they are detected with accurate mass (accuracy of typically 5 p.p.m or better) between the found and the theoretical molecular weight of the compound in the ToF tube.

Sample preparation: The common methods employed in sample preparation are weighing, dilution, filtration, evaporation, pH adjustment, vortexing, internal standards addition, centrifugation, liquid-liquid extraction and solid-phase extraction.

Liquid-liquid extraction (LLE): LLE partitions a sample between two immiscible phases to separate analytes from interfering matrix. Out of the two phases, one is usually aqueous and the other is organic. Analytes extracted into the organic phase are easily recovered by evaporation of the solvent, while analytes extracted into the aqueous phase can be injected directly onto a chromatography column.

Solid phase extraction (SPE): SPE is the latest method for sample preparation, compared to classical liquid-liquid extraction. The advantages of SPE are the low solvent consumption, the enormous time saving and the potential for automation. SPE offers a multitude of adsorbents for polar, hydrophobic and/or ionic interactions, while liquid-liquid extraction is limited to partition equilibriums in the liquid phase. The main objectives of SPE are to prepare selective and specific sample by removing interfering matrix components.

Enrichment of samples can increase the detection sensitivity by 100 to 5000 times. The analytes can be either absorbed on the SPE packing material or directly flow through while the interfering substances are retained. Primary goal of SPE is selective extraction of components of interest from a complex sample or much larger sample volumes prior to actual analysis. The main steps of the SPE procedure are (a)
conditioning of the adsorbent, (b) Sample application (adsorption), (c) washing of the adsorbent, and (d) elution.

3.8 Validation of Analytical method

Analytical method validation is a critical component. In order to minimize variation, assay of samples are carried out on a single day, relating to standard curve of each day. Quality control (QC) samples are of known concentration (low, medium and high), prepared by spiking drug-free biological fluid with drug, and are run each day. The QC samples help in determining intra-day accuracy and precision of the analytical method. The standard solutions are of concentrations different from QC samples and from lowest to highest that could be anticipated for a particular study. The regression line to fit the linear (straight line) standard curve is based on least squares analysis.

Sensitivity: The limit of detection (LOD) is the lowest concentration of drug that will yield an assay response significantly different from that of a sample blank, whereas the limit of quantitation (LOQ) (sensitivity) is the lowest concentration of drug that can be determined with acceptable precision and under the given experimental conditions.

Accuracy: Accuracy is how close a measured value is to the actual (true) value. ‘Accuracy’ is thus a measure of the degree to which a mean (m) obtained from a series of experimental measurements agrees with the value m, which is accepted as the true or correct value for the quantity measured. Absolute accuracy of a mean is defines m- m, and of an individual measurement, xI -m. Relative accuracy of a mean is calculated by (m - m)/m, and percent accuracy by 100 x (m - m)/ m. Generally from replicate measurements, more reproducible data is obtained and the mean is considered to be the best estimate of the true value (μ).
Precision: Precision is how close the measured values are to each other. ‘Precision’ (σ) describes the reproducibility of measurements within a set. It is used to show scatter or dispersion between numeric values in a set of measurements that have been determined under the same analytical parameters: small value of σ indicates higher precision than a large value of σ. Generally ‘standard deviation’ (s) is a more reliable expression of precision and is widely used for determining statistical significance. The standard deviation is the square root of the variance. The variance is defined as the average of the squared differences from the mean.

Relative standard deviation: For comparison of variations (e.g. precision) it is often more convenient to use the relative standard deviation (RSD). RSD is expressed more usually as a percentage and is then called coefficient of variation (CV). It is equal to 100 × [(standard deviation of array X)/(average of array X)] [68].

Matrix Effect: The mechanism and the origin of the matrix effect is not fully understood, but it may originate from the competition between an analyte and the co-eluting, matrix components reacting with primary ions formed in the UPLC-MS/MS interface. The current regulatory requirements include the need for the assessment and elimination of the matrix effect in the bio-analytical methods. Determination of the matrix effect allows the assessment of the reliability and selectivity of an existing UPLC-MS/MS method. Qualitatively, experiments confirming the presence of matrix effect in biological matrixes in comparison with the MS/MS response in neat solvents or in mobile phases have been proposed.
3.9 Etoposide

IUPAC Systematic Name; 4'-Demethyl epipodophyllotoxin 9- (4,6-O-(R)- ethylidene-β-D-glucopyranoside) (C_{29}H_{32}O_{13}; molecular mass: 588.57).

Etoposide is a semi-synthetic derivative of podophyllotoxin and was first synthesized in 1963. Podophyllotoxin is isolated from the dried roots and rhizomes of species of the genus Podophyllum. The first clinical trial of etoposide was reported in 1971. The drug was approved for use in the USA in 1983.

Etoposide is one of the most active and useful anti-neoplastic agents, and is now used routinely in first line combination chemotherapy of testicular cancer, small-cell lung cancer, non-Hodgkin's lymphoma, and poorly differentiated carcinoma of unknown primary site. In addition, substantial anti-neoplastic activity is well documented in acute leukemia, non-small-cell lung cancer, ovarian cancer, Hodgkin's disease, and several childhood malignancies, acute non-lymphocytic leukemia, and Kaposi's sarcoma associated with acquire immunodeficiency syndrome [31].

Etoposide is a chiral drug and only the trans-isomer is pharmacologically active. Etoposide increases topoisomerase II-mediated DNA breakage primarily by inhibiting the ability of the enzyme to re-ligate cleaved nucleic acid molecules. The formation of a topoisomerase – drug – DNA ternary complex appears to be critical for nucleic acid cleavage. The drug enters the complex through a direct interaction with the ATP-bound enzyme monomer in such a way that each drug molecule stabilizes only a single-stranded break. Thus, depending on the molar ratio between etoposide and topoisomerase II either single stranded or double stranded DNA breaks are generated. [69]. An ATM (ataxia telangiectasia mutated) -dependent activation of AMPK (AMP-activated protein kinase), and activated p53 pathway and caspase are suggested to play a role in etoposide-induced DNA damage response [70, 71].
The chemistry, pharmacology, pharmacokinetics, clinical efficacy, adverse effects, and pharmacodynamics of etoposide have been extensively reviewed earlier [4, 5, 72-76]. The following review of documented literature is based on original articles and reviews search, which were identified using a PubMed search strategy (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) covering the time period up to 2010.

Etoposide displays narrow therapeutic index and erratic pharmacokinetics, and dose individualization has to be achieved for overcoming the inter-patient variability, so as to maintain proper drug exposure within a therapeutic range. Clinical pharmacokinetic studies have revealed substantial inter-individual variability regarding AUC values and steady-state concentrations of etoposide, and also variability in clearance and systemic exposure. There is evidence that the bioavailability is dose-dependent, with decreasing absorption of doses probably due to a concentration-dependent reduction in the solubility of etoposide in the stomach and small intestine. A daily oral administration of etoposide shows low bioavailability and large inter-, and intra-individual variability [4-9, 77-79].

Despite its extensive use, the main pharmacokinetic determinants of this drug are still not completely defined in order to optimize the pharmacotherapeutic parameters of etoposide, including dose, therapeutic schedule, and route of administration. Numerous studies have been reported on the pharmacokinetics of etoposide in animals and humans (cf. Appendices section).

There were no significant race-related or age and gender differences in the pharmacokinetic parameters of etoposide. The half-life for etoposide absorption is 0.4 hours, with peak concentrations occurring between 0.5 - 4 hours. The terminal half-life is approximately 5 to 10 hours in those investigations that used HPLC. Approximately
6% to 25% of unchanged drug is recovered in the urine and most of excretion occurred in the first 12 hours. The pharmacokinetics of i.v. administered etoposide in children was found similar to that in adult, with a total plasma clearance in the range of 15 - 40 mL/ min/ m², a distribution volume of 5 - 17 L/ m² and an elimination half-life of 3 - 8 hr. In most studies, a bi-exponential elimination is described, with a distribution half-life of about 1 hr. Studies with high doses of etoposide have shown tri-exponential elimination, with a terminal half-time of 18 hr or longer, possibly reflecting release of etoposide from tissues. With standard doses of 100 mg/ m² delivered over 1 - 2 hr, the peak concentrations are 10 - 20 µg/ mL [74].

In a population PK-PD study of oral etoposide in patients with solid tumors, exposure to free etoposide during prolonged oral treatment was found to be highly variable and is the main determinant of pharmacodynamic effects. The population PK model based on Creatinine clearance is poorly predictive of exposure. Therapeutic drug monitoring suggested for dose individualization or to study the relationship between exposure and antitumor effect [9]. In humans etoposide clearance was found reduced in small cell lung cancer patients with elevated serum Creatinine [80].

In a pharmacokinetics study of etoposide in children treated for newly diagnosed acute myeloid leukemia, no significant correlation was found between etoposide pharmacokinetics and the remission rate or the relapse rate [81]. The overall bioavailability was 60.6 ± 22.4% (ranged from 17.6% to 91.2%) in childhood acute lymphoblastic leukemia patients [82].

In a recent study, the oral bioavailability and pharmacokinetic disposition of etoposide after an i.v. formulation administered orally to tumor-bearing dogs was characterized. Oral administration of etoposide resulted in significantly lower values for
the maximum plasma concentration and the area under the plasma etoposide concentration-versus-time curve, compared with results for i.v. administration. Oral bioavailability of etoposide was low and highly variable among dogs [83].

The efficacy of etoposide is schedule-dependent (The total systemic exposure under oral etoposide treatment varies considerably between individuals) [8]. Saturable absorption of etoposide was reported for doses greater than 200 mg/day, whereas lower doses were associated with increased bioavailability, although they were characterized by high inter- and intrapatient variability. Various pharmacodynamic models have been proposed to guide etoposide oral dosage [84].

About 94% of a dose of etoposide is bound to protein in adult cancer patients with normal hepatic function and 97.5% in children. The hematological toxicity of etoposide correlated better with the AUC for free compound than with that for total etoposide. Less etoposide penetrates into other fluid spaces, almost certainly because of its extensive protein binding. Even at high concentrations, etoposide is not found in appreciable concentrations in the cerebrospinal fluid, indicating that it does not cross the blood-brain barrier into the central nervous system, and is therefore useless in the treatment of tumors within the brain. Inter-animal variation in the unbound clearance of etoposide was observed. On an average, only 20% of the dose was excreted unchanged in the urine after 24hr. This is in contrast to human studies that demonstrate that renal elimination (up to as much as 50%) plays a major role in the excretion of etoposide. Non-renal elimination fate of the etoposide dose is poorly understood, and is responsible for 80% of etoposide clearance, which is even greater than that reported in humans [6, 7].
Etoposide is degraded via complex metabolic pathways. Hepatic glucuronidation of etoposide is a major metabolic elimination pathway. Etoposide glucuronides have been shown to be formed in isolated perfused rat liver models, and also by human liver microsomes, possibly catalyzed by UDP-glucuronyltransferase 1A1. Approximately 50% (range, 20% - 81%) of an etoposide dose is recovered in the urine as parent drug or glucuronide conjugate, being major urinary metabolite of etoposide in humans [85-87]. The catechol metabolite resulting from conventional multiple-day dosing of etoposide has also been reported in patients and may be associated with a higher risk for secondary malignancies and genotoxicity [10]. The metabolism of etoposide to its catechol metabolite is more in pediatric patients receiving multiple-day bolus etoposide infusions, associated with risk of leukemia as a treatment complication [88].

Oxidative activation of etoposide by cytochrome P450 monooxygenases, MPO, prostaglandin synthetase, and tyrosinase are reported to contribute to its cytotoxicity [89]. Etoposide can also be metabolized to DNA-inactivating catechol, ortho-quinone and semi-quinone free radical derivatives which may contribute to its cytotoxicity [88, 90].

**Strategies for enhancement of etoposide oral bioavailability**

To improve the pharmaceutical characteristics of etoposide, etoposide phosphate has been developed as a more water-soluble pro-drug, etoposide phosphate, for clinical use. Once this drug enters the systemic circulation, the phosphate is rapidly and completely cleaved by circulating phosphatases to the active drug etoposide. Early clinical trials showed that equimolar doses of etoposide and etoposide phosphate resulted in equivalent concentrations of etoposide in plasma. Etoposide phosphate can be rapidly infused in modest fluid volumes at dosages with minimal acute side-effects. In patients with established solid tumors the bioequivalence of etoposide phosphate, to etoposide
has been demonstrated. Conversion of prodrug to etoposide during incubation with gastric juice was negligible. Variable conversion of etoposide phosphate to etoposide would be expected within the intestinal lumen after oral administration. This could have important pharmacokinetic consequences. The toxicities of EP were virtually identical to those seen with etoposide [91].

Several documented pharmaceutical and pharmacological approaches are reviewed below.

**Pharmaceutical approaches**

Variability in absorption of etoposide is related to its instability in gastric or intestinal solutions [92]. It has been suggested that etoposide absorption was dissolution rate limited, and the low equilibrium aqueous solubility, slow intrinsic dissolution rate, and chemical instability at pH 1.3 could account for the low oral bioavailability [93, 94].

In order to overcome incomplete and variable bioavailability of etoposide due to its concentration and pH-dependent stability in gastric and intestinal fluids, the use of agents that may influence etoposide stability and, thereby, bioavailability, was investigated in a number of clinical studies. Drugs that influence the rate of gastric emptying, while modulating the time of drug absorption, did not significantly alter the etoposide AUC or bioavailability [8].

Attempts to enhance the aqueous solubility and dissolution rate of etoposide were made by preparing various polymorphs of etoposide by crystallizing it from organic solvents [95]. A microcrystal suspension in oil formulation of etoposide has been used in rats via intra-peritoneal bolus injection, but was found to deliver significantly less etoposide than the aqueous etoposide solution [96]. The in vivo release and tissue distribution of etoposide delivered sub-conjunctivally by a bio-erodible drug-
polyanhydride controlled release device on the ocular surface have been found to be sufficient to reduce fibroblast proliferation studied in rabbits [97]. A microdialysis technique for the assessment of the local drug delivery system using microfibrous collagen as a drug carrier has been demonstrated for etoposide [98]. The effect of cremophor and Tween 80 were also examined. Both these agents increased AUC and decreased the total and biliary clearance of etoposide in rat [99].

Encapsulation of etoposide was achieved in unilamellar cationic liposomes which showed increased antitumor efficacy and reduction in the adverse effects associated with drug, was found to alter the pharmacokinetics of etoposide, administered to mice (increased AUC of the free drug) [100]. A solid formulation was designed resulting in the enhancement and modification of the etoposide solubilization process considered equivalent, in vitro, to the current marketed product [101].

A polymeric solubilizer, poly (N-vinylpyrrolidone)-block-poly (D, L-lactide) has been reported to produce self-assembly in water to yield polymeric micelles that efficiently solubilize etoposide [102]. Etoposide was also incorporated in an injectable parenteral emulsion, using soybean oil and phosphatidylcholine as emulsifier and its pharmacokinetics and tissue distribution studied in mice. This formulation showed high AUC, MRT (mean residence time), and lower clearance, and higher concentrations in liver, spleen, and lung [103].

Encapsulation of etoposide in lipid nanospheres (LN) improved the anticancer activity and a further inclusion of polyethylene glycol-distearoyl phosphatidyl ethanolamine increased the circulation time and stability of LN [104]. Etoposide oleate associated to a cholesterol-rich micro emulsion (LDE) was reported to be taken up by malignant cells overexpressing low-density lipoprotein receptors. Drug was found to be
retained in the microemulsion particles after its intravenous administration until its removal from the circulation and internalization by the cells. In addition, LDE-etoposide oleate has the ability to concentrate in malignant ovarian tissues [105].

A vasopermeability-enhancing peptide (37-amino-acid linear sequence of IL-2) given i.v. 2 hours before the injection of suboptimal doses of etoposide in mice bearing established solid tumors substantially improved the efficacy of anticancer drug by virtue of its ability to increase the uptake of drugs in solid tumors selectively [106]. One study showed the capability of tripalmitin nanoparticles in enhancing the tumor uptake of etoposide, in Dalton’s lymphoma tumor bearing mice [107].

Etoposide encapsulated parenteral emulsion has been prepared using soybean oil, egg lecithin and cholesterol. Etoposide encapsulated long circulating parenteral emulsion was also prepared using PEG 2000 as a stealth agent, which showed improved pharmacokinetic parameters with 5.5 times higher AUC than etoposide conventional formulation in rats and mice. Its improved activity was due to enhanced permeability and retention effect [108]. Etoposide delivery through polysorbate 20 micelles (EPM) has been attempted. Subcutaneous injection of EPM resulted in significantly higher tumor uptake and prolonged tumor retention, and relatively high brain concentrations compared to etoposide, in Dalton’s lymphoma in mice [109].

Preparations like etoposide-loaded tripalmitin (ETP) nanoparticles, etoposide-loaded glycerol monostearate, and etoposide-loaded glycerol distearate have also been to show greater and prolonged apoptotic induction properties [110]. Lipid nanocapsules loaded with etoposide showed sustained release over a period of several days in a cell culture with 4-fold higher efficiency [111]. PLGA and PCL nanoparticles as drug carriers for etoposide in enhancing the bioavailability and reducing the etoposide-associated
toxicity were evaluated in mice and rabbits [112]. Etoposide solubilized in poly (ethylene glycol)-block-poly (d, l lactic acid) micelles were shown to deliver clinically relevant doses of the drug [113].

A liposomal etoposide showed biphasic pharmacokinetic profiles with 60% increase in AUC with a 35% decrease in clearance, resulting in a 70% increase in the MRT of etoposide. The uptake of etoposide from this form of etoposide was higher in macrophage-phagocyte endowed tissues [114]. In a recent study an etoposide loaded biodegradable nanoparticles for sustained release were prepared [115]. A freeze-drying technique has also been used to produce a submicron etoposide-phospholipids emulsion which showed significantly improved liposolubility of the drug [116]. A formulation comprising of N-octyl-O-sulfate chitosan showed higher intestinal absorption of etoposide in rat jejunum and ileum, and higher uptake in Caco-2 cell [117].

Many patents on various formulations of etoposide with oils, phospholipids and other emulsifiers/ surfactants/ excipients/ adjuvants have been filed to provide high bioavailability of etoposide [118].

From above studies it becomes apparent that a common pharmaceutical approach has been to conferring water solubility to drugs by using variety of amphiphilic, solubilizing agents. However, many of the chemical substances themselves are associated with a number of pharmacokinetic and pharmaceutical concerns.

Pharmacological approaches

Diverse natural compounds from medicinal plants have been evaluated in order to explore enhancement of bioavailability of many clinically important drugs. In this context application of P-gp / CYP 3A4 dual role inhibitors in improving per oral drug delivery have gained special interest. Therefore the possibility of improving the
bioavailability of etoposide by combining this anti-cancer agent with several pharmacologically active substances specially P-gp/ CYP3A inhibitors have been explored in recent times.

Orally administered morin (an inhibitor of CYP isozyme and P-gp) significantly increased the AUC, \( C_{\text{max}} \) and the absolute bioavailability of orally administered etoposide [119].

In rats kaempferol was found to enhance AUC, and peak concentration (\( C_{\text{max}} \)), and also the absolute bioavailability of orally administered etoposide, and decreased total body clearance (\( C_{\text{L}} \)) via inhibition of CYP 3A4 and P-gp [120].

Quercetin, a P-gp/ CYP3A inhibitor altered the pharmacokinetic parameters of orally administered etoposide in rat. It significantly increased AUC and absolute bioavailability of orally administered etoposide and decreased its \( C_{\text{L}} \) mainly due to inhibition of P-gp-mediated efflux and CYP3A4-catalyzed metabolism in the intestine [121].

In a recent study potentiating effect of wogonin, a flavone in the roots of *Scutellaria baicalensis*, was observed to potentiate the anticancer action of etoposide due to P-gp inhibition and accumulation of this agent in etoposide-induced apoptosis in tumor cells [122,123].

The foregoing account amply suggests that development of newer strategies to enhance the bioavailability of oral etoposide is being investigated intensely by researchers worldwide.