Chapter - VI
Discussion
6. DISCUSSION:

In the present investigation, a UPLC-qToF-MS/MS method has been developed for the simultaneous determination of etoposide and PA-1. UPLC-qToF-MS/MS system is now considered a superior quantitative tool for providing better quality data in terms of increased detection limits, and chromatographic resolution with greater sensitivity.

The analytical method was validated to meet the acceptance criteria as per guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

The analytes were separated on a reverse phase C₁₈ column using methanol – water (72:28, v/v) mobile phase with a flow rate of 250μL/min. The QToF-MS was operated under multiple reaction monitoring (MRM) mode using electro-spray ionization (ESI) technique with positive ion polarity. The major product ions for etoposide and PA-1 were at m/z 185.1350 and 164.1581, respectively. The recovery of the analytes from mouse plasma was optimized using solid phase extraction (SPE) technique. The method was found to be specific as established by comparing blank plasma samples with those spiked with the analytes and was free from interference from endogenous components.

The total run time was 6 min and the elution of etoposide and PA-1 occurred at 1.24 min, and 2.84 min, respectively.

The CAL STD solutions were utilized for establishment of linearity and range (linear least-squares regression with a weighting index of 1/x). The calibration curves of etoposide as well as PA-1 were linear over the concentration range of 2 – 1000ng/mL (r², 0.9829), and 1 – 1000ng/mL (r², 0.9989), respectively.

The precision and accuracy parameters were ascertained in LLOQ, LQC, MQC, and HQC samples (5 replicates each in 3 sets) on the same day and on 3 consecutive days.
For etoposide intra-assay and inter-assay accuracy in terms of % bias was in between −7.65 to +6.26, and −7.83 to +5.99, respectively. For PA-1 intra-assay and inter-assay accuracy in terms of % bias was in between −7.01 to +9.10, and −7.36 to +6.71, respectively. The accuracy and precision of the method were within the acceptable limits of ±15%. The method was free from matrix effect.

The lower limit of quantitation for etoposide and PA-1 were 2.0 and 1.0ng/mL, respectively. Analytes were stable under various conditions (in autosampler, during freeze–thaw, at room temperature, and under deep-freeze conditions).

This method was used for a pharmacokinetic study to assess the effect of PA-1 on etoposide bioavailability.

When a drug exhibiting systemic effects is administered orally, its fate is usually described in the following way: the drug comes into contact with the contents of the GI system, is dissolved in intestinal juices, and brought into contact with intestinal epithelium. It is then absorbed through the gut wall and the enterocytes lining the gut wall (usually in the jejunum and ileum), and transported by the portal veins through the liver, before reaching the systemic circulation and hence different parts of the body. When the same drug is given by intravenous route, it enters the systemic circulation and is distributed throughout the body before reaching the liver for the first time.

The important difference between these two modes of administration lies in the pre-systemic fate of the drug. The time taken for an orally administered drug to reach the systemic circulation may cause a delay before a clinical effect is observed, and less than the total dose administered may reach the systemic circulation, with consequent differences in effect for the same dose. The extent of systemic availability is described with the pharmacokinetic term bioavailability (F). In the case of orally administered
drugs, \( F \) is theoretically determined in the following way: the drug is administered as a single dose i.v., and orally to determine area under concentration curve (AUC) from the time 0 to 'infinity'. Absolute bioavailability of the oral dose (\( F_{\text{oral}} \)) is: 

\[ F = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{i.v.}}} \]  

Pharmacokinetics is one important and preferred technique for the determination of bioavailability and obtaining other valuable information about the drug disposition. Small animals such as mice and rats are often used for PK studies. Assessment of bioavailability from plasma concentration-time data usually involves determining the maximum (peak) plasma drug concentrations (\( C_{\text{max}} \)) and the area under the plasma concentration – time curve (AUC). The intensity of the pharmacologic effect of a drug is related to the concentration of the drug at the receptor sites, located in the tissue cells, which are richly perfused with plasma. Therefore determination of drug concentration in plasma is employed to monitor if the dose is actually delivered to the site for the required therapeutic effect. The plasma drug concentration increases with the rate of absorption; therefore the most widely used general index of absorption is \( C_{\text{max}} \). AUC is another reliable measure of bioavailability. It represents the total amount of unchanged drug that reaches systemic circulation. Present investigation has revealed that PA-1 significantly enhanced both the extent and the absorption rate profile of etoposide in mice. The relative bioavailability (RB) as well as the absolute bioavailability (AB) of etoposide was enhanced by 2.32 fold in presence of PA-1. On the other hand etoposide did not influence the pharmacokinetic profile of PA-1. Further, this effect was not due to cytotoxicity. This was ascertained by measuring the release of tissue proteins from the intestinal mucosa, which was used as an index to assess tissue damage.

Increased plasma concentrations of etoposide may be due to an increased absorption or a decreased elimination. In PA-1 treated group volume of distribution (\( V_D \)) of
etoposide decreased with a concomitant decrease in its clearance ($C_L$). $V_D$ is the hypothetical volume, in which the drug would have been distributed by supposing that its concentration is homogeneous, i.e. the average tissue concentration is identical to that of the plasma. It is expressed as $V_D = \text{dose}/C_0$ (initial concentration). Variability in $V_D$ reflects the proportion of the administered dose that remains in the plasma. The total $C_L$ depends on the constant of elimination and thus on $t_{1/2}$ and on $V_D$. It is related to the volume in which the drug is dissolved ($V_D$) and the rate at which it goes out (related to $t_{1/2}$ or the elimination constant, $k_{el}$). Therefore clearance is defined as the product of $V_D \times k_{el}$. These changes in $V_D$ and $C_L$ along with a longer $t_{1/2}$ in PA-1 treated group compared to untreated group, suggested that an increase in the overall rate of drug elimination is slowed down during the terminal phase, which could be a potential contributing factor in the observed enhancement of etoposide bioavailability.

In an earlier study i.v. pharmacokinetics of etoposide is described, where it was shown that etoposide disappears from the systemic circulation according to a capacity-limited elimination process [140].

The present results are in accordance with several reported studies where many compounds which were assessed for their role in increasing oral bioavailability of etoposide also caused an enhanced AUC accompanied by a reduction in the $V_D$ and $C_L$ of etoposide. Substances like amiodarone, cyclosporine A (Cy A) and SDZ PSC 833, verapamil, ketoconazole etc.) Which were assessed to restore the sensitivity of multidrug resistant tumor cells to etoposide showed modification of etoposide pharmacokinetics with respect to AUC and the clearance of the drug [2]. In microdialysis infusion studies performed in rat, CyA was found to decreased non-renal as well as systemic clearance of etoposide, leading to higher etoposide concentration in CNS [141,142].
In another study infusion of CyA in rats treated with etoposide caused a decrease in the plasma clearance, and an increase in plasma and tissue concentrations of etoposide [143]. In rats pretreated with a CyA analogue PSC 833, bioavailability and blood levels of etoposide were significantly increased, and the disappearance rate of etoposide from the intravascular compartment was considerably slowed down [144]. In a Phase I trial study pharmacokinetic effects of CyA have resulted in a concentration dependent decrease in renal and non-renal clearance etoposide while increasing its systemic exposure [145-147]. In children with acute myeloid leukemia CyA increased etoposide AUC with a decrease in clearance [148]. In pediatric patients of solid tumors given a combination of high-dose CyA and etoposide, the etoposide AUC was found to increase [149].

Verapamil significantly increased AUC of co-dosed etoposide in rats, and significantly reduced its total body clearance, while the absolute bioavailability (F) of etoposide increased by 1.38 to 1.47 fold [150].

In patients of advanced cancer who received ketoconazole along with an escalating dose of oral etoposide, the etoposide oral AUC was increased. Ketoconazole reduced the apparent clearance of oral etoposide [151]. A synergistic suppression of prostate cancer clonogenic cell growth by ketoconazole in the presence of etoposide has also been demonstrated [152].

The possibility of increasing the activity of etoposide by combining this drug with indomethacin has been explored. Non-toxic concentrations of indomethacin were found to enhance the sensitivity to etoposide in cultured Lewis lung carcinoma cells [153].

In fact oral BA of a drug happens to be a function of (i) the fraction of dose (Fa) absorbed from the GIT, (ii) the intestinal availability (Fg), that correspond to the
fraction of dose not metabolized in the intestinal wall during the absorption process, and (iii) the fraction of dose ($Fh$) not metabolized in the liver before reaching the systemic circulation. Therefore, the values of $(1-Fg)$ and $(1-Fh)$ mean the first-pass metabolism of orally administered drugs in GI tract and liver respectively [154].

![Diagram of drug absorption and metabolism](image)

Though oral administration is the most popular route for drug administration, being convenient, and non-invasive, but the GIT mucosa represents a major physical and biochemical barrier to the systemic availability of orally ingested pharmacologically active molecules. The pharmacological behavior of a drug is generally related to its concentration at the sites of action, which is turn is related to the amount administered (dose) and to the drug’s absorption, distribution, metabolism, and/or excretion (ADME). Pharmacokinetic drug interactions can occur via inhibition or induction of intracellular metabolic enzymes or specific membrane transport systems [155, 156]. In this context, the application of P-gp/CYP 3A4 inhibitors (such as CyA, verapamil, ketoconazole as exemplified above), have overwhelmingly suggested that the oral bioavailability of etoposide remains contingent on circumventing pharmacokinetic limitations arising out of a functional interplay of these proteins.

Accordingly the role of PA-1 was further examined on some major biochemical determinants of poor oral bioavailability of etoposide which are now understood in terms of
(A) Reduced absorption/rapid efflux from the GIT

(B) Excessive first pass metabolism, and

(C) Extensive plasma protein binding, which is also considered an important contributor to the broad and variable first-pass extraction [157].

In subsequent studies a quantitative assessment of these cellular events was carried out in order to understand the mode of action of PA-1.

**Role of PA-1 in the modulation of etoposide absorption/ P-gp mediated efflux from GIT**

It is now established that etoposide oral bioavailability is limited primarily by P-gp. Etoposide is a substrate of P-gp which overwhelmingly regulates its overall disposition in defining the intestinal permeability of etoposide, and there is substantial excretion of etoposide via the intestinal mucosa. Evidence for the above comes from experiments from various in vitro and animal-derived models, where specific P-gp inhibitors have been shown to modify the absorption/efflux of etoposide, such as, in glioma cell lines, brush border membrane vesicles, Caco-2, other cancer cell lines, and several other tissues like olfactory and respiratory mucosae, and etoposide resistant-cancer cell lines [41,158-165].
A P-gp modulator trifluoperazine was found to inhibit etoposide transport out of the extracellular fluid as supported by the increase in the blood brain ratio of etoposide at steady state in a rat model [142]. In a multidrug etoposide-resistant small cell lung cancer line VPR-2, cyclosporine and verapamil, both P-gp inhibitors, enhanced accumulation of etoposide in the cells [166]. A similar finding was also reported in luminal epithelial cells [143] and in canalicular membrane preparations in presence of cyclosporine [141]. Verapamil also enhanced etoposide transport and kinetics in rabbit intestinal tissue, where etoposide was shown to be a substrate for multiple efflux transporters [167]. In a recent study N-octyl-O-sulfate chitosan was shown to potentially inhibit P-gp to enhance uptake of etoposide in Caco-2 cells [117]. When studied in rat intestinal tissues etoposide efflux was inhibited by LTC$_4$ (specific inhibitor of MRP1) and quinidine [159]. Using isolated mouse ileum it was also revealed that handling of etoposide by intestine is a complex process due to involvement of multiple transporters other than P-gp [41].

After oral ingestion, drug absorption is the first step for expression of pharmacological effect, and has many difficulties and unknown factors. It is important to overcome the absorption barrier. Several methods are utilized to evaluate drug absorption potential. These methods include insilco models, artificial membranes as absorption models, Using chamber, Caco-2 monolayers, in situ intestinal perfusion and in vivo absorption studies. Although all the above tissues are used as potential screening models, the everted gut sac and intestinal loop are considered more advanced models in the sense that they incorporate alterations due to intestinal metabolism [168], and the presence of absorptive epithelial layer where P-gp is prominently expressed [169]. In addition an in vitro model using artificial lipid membrane such as parallel artificial membrane permeability assay (PAMPA) is also widely used. Typically, a
combination of these models is used routinely in assessing intestinal permeability. A tiered approach is often used, which involves high throughput (but less predictive) models for primary screening followed by low throughput (but more predictive) models for secondary screening and mechanistic studies [170].

In the first place it would be worthwhile to elaborate diverse features of the two intestinal models. The everted gut sac happens to be a 'leaky' epithelia, and a simple and efficient two-compartment screen to assess kinetics of drug transport, across the epithelial layer. Gut sac is considered more indicative of in vivo intestinal transport [27,125,171], and there a linear [126].

The model of in situ intestinal perfusion is another widely accepted technique for estimating carrier-mediated absorptive as well as secretory processes of the intestine and has been shown to be a precise preclinical permeability models to predict in vivo oral absorption in humans [172,173]. In the in situ intestinal perfusion system, an intestinal segment is exposed in an anesthetized rat and drug solution is perfused through the lumen in a single-pass or re-circulating fashion. Drug permeability is derived from the rate of disappearance of drug from the perfusate.

The everted intestine and in situ intestinal perfusion techniques have been potentially indentified to address the problem of poor membrane permeation as a cause of low oral bioavailability. The in vitro and in situ methods are now commonly used for studying problems and possible solutions. With these methods an investigator can better control and individually examine the physiological and biopharmaceutical variables affecting drug bioavailability [174].

These two models represent potential site for drug metabolism contributing substantially to the first pass metabolism, due to the presence of P-gp and CYP 3A4 which is highly expressed in duodenum and jejunum, second only to liver, and the data
originating from these two models (everted gut sac and in situ perfusion) represent integrative aspects of first-pass metabolism [175,176].

Using these intestinal models P-gp has been implicated to regulate etoposide permeability, cellular and tissue distribution and pharmacokinetics to such an extent that P-gp mediated efflux activity remains inversely correlated to the extent of intestinal absorption of etoposide and its bioavailability [67,177-179].

The present investigation have shown that PA-1 enhanced the permeation characteristics of the intestine indicated by a significant increase in the transport rate of etoposide as well as its apparent permeability coefficient (Papp) in gut sac model, thus suggesting that the observed enhancement of etoposide bioavailability could possibly be via enhancing the absorptive transport and/ or inhibiting P-gp mediated etoposide efflux. In collateral experiment verapamil as an inhibitor of P-gp/ CYP3A4 was used as standard reference, also enhanced the etoposide transport in this model. Verapamil is a conventional mammalian P-gp inhibitor [180].

Our observations were in accordance with earlier studies carried out in this model, where inhibition of P-gp has been shown to increase the absorption of etoposide, and reduce its secretory permeability across intestine with considerable bioavailability implications. Everted gut sac has been used for studying quantitative transport of anticancer drugs including etoposide, and potential P-gp modifiers [181]. In earlier studies it has been shown that etoposide is absorbed from luminal site to the serosal site in the jejunum and ileum sacs, while it was secreted by means of P-gp after its absorption across the intestinal epithelium [182]. The mucosal-to-serosal transfer of etoposide begins after some lag period, and has a significant active, saturable and concentration-dependent secretory transport component in intestinal mucosa, and in vitro permeability values appear to be consistent with the clinical results in humans.
Similarly a very poor absorption of etoposide has been demonstrated in in situ absorption studies carried out in rat [184]. The transport/absorption of etoposide was significantly increased from the luminal to serosal site in the jejunum by 2-4 folds in presence of hydroxyzine and quinidine, both P-gp inhibitors. In this model a P-gp monoclonal antibody (C219) increased the absorption of etoposide in rat gut sac [182,183,185]. Furthermore, when the effect of diet on etoposide absorption was studied using a rat everted gut sac model, quercetin a plant derived flavonoid with P-gp inhibitory activity was observed to enhance the etoposide absorption in the jejunum and ileum [186]. Conversely pre-treating rats with P-gp inducers (like sodium arsenite, sodium butyrate) decreased etoposide absorption in both jejunum and ileum [183].

A possible involvement of P-gp in mode of action of PA-1 was further clarified using Rho - 123 in gut sac model. P-gp is an effective efflux transporter of Rho - 123 [187], and is therefore recognized as a valuable probe substrate for P-gp-mediated efflux from the intestinal tissue into the lumen [34]. It has also been used earlier to determine Pgp-mediated efflux of etoposide in this model [127,183,185]. Rho - 123 is a fluorescent dye which has an ester moiety in its structure and is metabolized by intestinal esterase, and some of its metabolites (like rho-110, a deacetylated product) also have fluorescent intensity [126]. A significant relationship between Rho - 123 clearance ratios and P-gp expression support its useful role as an index for the P-gp function in vivo [188].

The results of the present investigation have revealed a significant potential of PA-1 to inhibit Rho - 123 efflux in rat gut sac, and therefore P-gp activity.

In intestinal epithelium P-gp modulates drug metabolism by increasing the exposure of a drug to intracellular CYP 3A4 through repeated cycles of drug absorption and efflux [59]. Therefore a model of single pass in situ perfusion in rat was used to further clarify the role of PA-1.
The results showed that PA-1 considerably reduced the intestinal exsorption rate, exsorption clearance and the total plasma clearance of etoposide. This is also supported by earlier studies in which it was shown that when etoposide was infused intraluminally simultaneously with quinidine a P-gp inhibitor, the rate of etoposide absorption was increased, while the intestinal clearance was decreased resulting in higher serum concentration of the drug [182]. An in situ single pass perfusion technique has earlier been used to assess the exsorption of etoposide; quinidine decreased the efflux of etoposide [183]. Quinidine also inhibited etoposide efflux completely in the ileal and colonic segments of rat intestine [160]. These effects were suggested to be due to inhibition of etoposide transport by P-glycoprotein present in the gut.

From the present investigation it could thus be inferred that PA-1 is a potential P-gp-inhibiting agent, which might increase the etoposide absorption while decreasing its efflux from the blood into the intestinal lumen, thereby increasing the oral bioavailability of etoposide.

Effect of PA-1 on of P-gp dependent ATPase

The involvement of P-gp in the mode of action of PA-1 was further evaluated in a subsequent P-gp dependent ATPase assay, since etoposide efflux happens to be an ATP dependent process [159,167,189,190]. P-gp is an unusual ATP-driven transporter, in that it has a low affinity for ATP and exhibits a high level of constitutive or basal ATPase activity. This protein has 12 transmembrane domains contained in two homologous halves and two ATP-binding cassette domains in each half that catalyze ATP hydrolysis. One characteristic of this protein is that it couples binding and hydrolysis of ATP at the two nucleotide binding domains to drug export by
transmembrane domains. Therefore ATP hydrolysis happens to be an inherent property of P-gp. P-gp-dependent ATPase assay is a widely used in vitro method to investigate the affinity of a substance for P-gp, since one interesting property of this enzyme is its vulnerability to stimulation or inhibition by a variety of chemotherapeutic agents, natural products and other xenobiotics, suggesting that there exists some type of communication, or coupling, between drug-binding sites, which are believed to reside within transporter domains of the membrane. The transport activity of P-gp is related to the amount of inorganic phosphate (Pi) that is liberated upon hydrolysis of ATP so that this assay is able to distinguish a substrate from an inhibitor [129].

P-gp ATPase is sensitive to vanadate, a phosphate analogue which inhibits its hydrolytic activity by forming a complex with MgADP at a catalytic site that resembles the pentacovalent phosphorus of chemical transition state. With the use of three ionic pump inhibitors, ouabain (an inhibitor of Na+/K+-ATPase), sodium azide (an inhibitor of Fo-F1 ATPase) and EGTA (an inhibitor of Ca++-ATPase), the remaining ATPase could therefore be attributed to P-gp [129].

In the present investigation rat jejunal membrane preparations were incubated with ATP, MgCl₂ and vanadate in the presence and absence of PA-1. The reaction is allowed to proceed through ATP hydrolysis to products ADP and Pi. The results showed that PA-1 inhibited the vanadate-sensitive ATP hydrolysis in rat jejunal membrane preparations, suggesting its P-gp inhibitory profile.

Effect of PA-1 on passive transport of etoposide

Though active absorption and exorption remains important factors in limiting drug bioavailability, but the issue of passive drug permeability also merits consideration, since absorption of majority of xenobiotics is largely facilitated by passive diffusion. P-
gp can act both as an active secretion system and as an absorption barrier during the transport of drugs from enterocytes into the intestinal lumen [34]. In addition, P-gp efflux from the cell also competes with passive trans-membrane drug influx. Consequently the inhibition of secretary transport (from the enterocyte back into the intestinal lumen) may increase permeation in the absorptive direction. In other words the efflux runs countercurrent to the absorptive transport or diffusion of drugs, thereby restricting the extent of oral absorption [191].

To clarify whether PA-1 affects non-carrier mediated drug absorption in the intestine, we used PAMPA. PAMPA is an excellent alternative to cellular models for ADME, and is a frequently used as an in vitro non-cell based model to assess intestinal passive permeability screen [192]. In this assay rate of permeation across the membrane barrier has been shown to correlate well with the extent of drug absorption in humans [170].

PAMPA enjoys wide popularity in pharmaceutical industry, which stems from its potential for high throughput, and cost effectiveness. The acronym PAMPA, for 'parallel artificial membrane permeability assay', was coined by Manfred Kansy and coworkers at Hoffmann-La Roche in 1998. PAMPA is a non-cell based assay designed to predict passive transcellular permeability of drugs. The assay was carried out in a 96-well multi-screen permeability plate, to measure the ability of a compound to diffuse from a donor to an acceptor compartment separated by a hexadecane liquid layer on a polycarbonate membrane support. PAMPA experiments are performed in a "sandwich-like" apparatus: two microtitre plates are joined to form a'sandwich; one is referred to as the "donor" plate and the other as the "acceptor" plate. Donor compartment carries a hydrophobic filter material coated with a mixture of lecithin/phospholipids dissolved in an inert organic solvent creating an artificial lipid membrane barrier that mimics the
intestinal epithelium. Permeability is determined by observing the disappearance of compound from the donor and the appearance in the acceptor. The compartment that contains drug at the start of the experiment is referred to as the donor. The precise fit between the two sandwich plates enables the compound to transfer between compartments, passing through the filter barrier. Earlier using this model Hwang et al., [135] have determined percentage transport of etoposide across the lipid bilayer (%T) to be 0.63%, and the calculated anticipated fraction absorbed in humans (Fa) to be 41%.

Results of the present investigation however showed that PA-1 did not modify passive transport pattern of etoposide in this assay.

Role of PA-1 in the modulation of CYP mediated first-pass metabolism of etoposide

Desired therapeutic plasma levels of orally administered drugs are dependent not only on adequate intestinal absorption, but also on the extent of first pass effect. This pre-systemic metabolism can principally take place anywhere before the drug reaches the systemic circulation. Although drug metabolism can occur in the intestine, the liver has been considered the primary site of all clinically significant pre-systemic metabolism. Drugs that most efficiently cleared from the blood by the liver (high clearance drugs) have been shown to have low oral bioavailability as a result of extensive metabolism during the first pass ('first-pass metabolism') through the liver on the way from GI tract to the systemic circulation [139].

Etoposide is known to undergo biotransformation predominantly via O-demethylation in rat and humans, mediated mainly by CYP3A4 and to a minor extent by CYP1A2 and 2E1, and were inhibited by CYP3A4 inhibitors, ketoconazole,
troleandomycin, verapamil and cyclosporine [193-196]. CYP 3A4 expression has been detected in a high percentage of tumors such as soft tissue carcinomas [132].

Earlier O-demethylation of etoposide to a 3', 4'-dihydroxy derivative has also been demonstrated in isolated mouse liver microsomes [196-199]. Etoposide metabolism has also been studied in vitro in human liver microsomes, where Michaelis-Menten one-enzyme kinetic behavior was observed [194,196].

In the present investigation the effect of PA-1 on CYP modulatory role was assessed in liver microsomes, since the microsomes which are abundantly rich in CYP enzymes are difficult to prepare from the intestine compared to liver, primarily for the reason that intestinal microsomes are exposed to intestinal proteases during their isolation. With the result their typical marker activities remain highly variable and unstable and it is very difficult to validate the drug oxidation by intestinal microsomes [176].

The microsomal erythromycin N-demethylase activity was determined as a marker for CYP 3A4. Microsomal 7-ethoxyresorufin O-deethylation (EROD) was determined as a marker for CYP1A2. Microsomal 7-methoxycoumarin O-demethylation (7-MOCD) was determined as a marker for CYP2B1. The most widely employed assays for O-deethylase activity involves measurement of the fluorescent product resorufin after the metabolic conversion of the substrate ethoxyresorufin. O-demethylase activity involves measurement of the fluorescent product 7-hydroxycoumarin after the metabolic conversion of the substrate 7-methoxycoumarin. Measurement of formaldehyde by NASH reagent as a result of metabolic conversion of the substrate erythromycin is generally employed for the assay of N-demethylase activity.

In the present investigation a significant CYP inhibitory profile of PA-1 was revealed by its ability to inhibit two major NADPH-assisted reactions i.e., (i) O-dealkylation represented by O-deethylation and O-demethylation, and (ii) N-demethylation of
etoposide, which could significantly prevent the CYP mediated elimination of etoposide.

**Role of PA-1 on etoposide plasma protein binding**

Extensive plasma protein binding is also considered an important contributor to the broad and variable first-pass extraction [157]. The unbound fraction of a drug in plasma is increased when it is displaced by some compound at the plasma protein binding sites. Subsequent alterations in plasma concentration profiles can be caused by changes in both $C_L$ and $V_d$ of the drug. The effect on the steady-state concentration ($C_{ss}$) and AUC is also influenced from the change in $C_L$. Therefore the effect of protein binding replacement depends on the magnitude of $C_L$ and the route of administration. On the other hand the protein binding replacement has little effect on the $C_{ss}$ and AUC for unbound drugs after oral administration, which are parameters directly related to the pharmacological and adverse effects, irrespective of the magnitude of $C_L$. The alteration of $V_d$ caused by protein binding replacement also has an effect on the blood drug concentration. In the case of drugs with a relatively large $V_d$, $V_d$ increases in parallel with unbound fraction. Although this leads to a transient reduction in total blood concentration caused by the redistribution of the drug into tissues, the unbound concentration is not affected. However, in the case of drugs with a small $V_d$, which depend on unbound fraction to a lesser extent, the total blood concentration is not affected so much by the change in unbound fraction, but the unbound concentration is greatly altered [200]. More than 95% of etoposide is bound to plasma protein in vivo [194].

The results of the present investigation have shown that PA-1 did not modify the etoposide plasma protein binding.
PA-1 a dual inhibitor of P-gp and CYP

In recent times first-pass effect in the small intestine has received a great deal of attention concerning the absorption and/or exsorption of xenobiotics following oral administration. It is now understood that the pharmacokinetic interference between anticancer drugs and P-gp inhibitors is due primarily to competition for drug metabolizing enzymes [201]. Reduction of the barrier function via modulation of these proteins could affect the pharmacokinetics of the drug, causing elevation of plasma drug concentrations [202]. PA-1 has been found to be a dual inhibitor of CYP3A4/ P-gp; CYP 3A4 remains localized in the small intestine at the columnar epithelial cells lining the intestinal lumen, and P-gp is found on the apical surface (that is, luminal entrance site) of these cells, serve as major determinants of bioavailability of orally administered drugs, as both these proteins are functionally linked, and act synergistically in the small intestine to limit oral drug bioavailability [53-57, 61].

The intestinal permeability and consequently overall bioavailability enhancing profile of PA-1 was not due to cytotoxicity. This was ascertained by measuring the release of tissue proteins from the intestinal mucosa, which was used as an index to assess tissue damage. PA-1 was also found to devoid of any acute or repeated dosing (sub-acute) toxicity. Taken together present results have shown that PA-1 is endowed with a prominent dual inhibitory activity against both the proteins, P-gp and CYP 3A4, which may thus explain its role as a bioavailability enhancer for oral etoposide, acting by counteracting the metabolic barriers presented by intestinal efflux and liver oxidative biotransformation of absorbed etoposide after its oral administration.