1. INTRODUCTION

Amongst various illnesses to human beings, certain viral, bacterial and fungal infections are more common. Tendency of these microbes to develop new strains under any circumstances develops resistance with the available drugs; hence scientists have to make efforts to work on several molecules, novel entities to combat the illnesses, caused by them. For viral diseases, till date no antiviral candidate tested is able to completely inhibit replication of viruses, leading to envisage new class of antiviral agents with better pharmacokinetic-pharmacodynamic balance.

Effective antiviral therapy is the only hope for survival or alleviation of disease for tens of millions of individual worldwide suffering from chronic viral infections, including those caused by human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and others. In addition, there is a continuing threat of global outbreaks from influenza, severe acute respiratory syndrome (SARS), and other respiratory viruses. Despite considerable progress in the development of effective inhibitors that target specific aspects of the viral life cycles, therapeutic efficacy has been limited by the evolution of resistant virus. This problem not only results in the failure of therapy, but may limit the effectiveness of subsequent therapies. Moreover, attempts to counter drug resistance lead to complex, expensive, and toxic regimens. Antiviral drug resistance is therefore of paramount importance in dealing with growing epidemics caused by virus infections.

There are large number of drugs viz., valacyclovir, abacavir, famciclovir, darunavir, oseltamivir, ritonavir and lopinavir widely used in the treatment of selected viral infections solely or in combination with each other. Accurate and very precise bioanalytical methods are required to quantify these drugs in biological fluids e.g. saliva, plasma and aqueous humor to help medical community for fixing effective dose during treatment of viral ailments and to have better pharmacokinetic applications\(^1\),\(^2\).

In line to these expectations, over a decade there were many methods developed on antiviral drugs using HPLC-UV\(^{19,25-27,29}\), HPLC-fluorimetric\(^{16-18,28,30}\), GC and GC-MS\(^{20-22}\) instruments techniques. However, due to various limiting factors like low sensitivity, high plasma requirement for establishing methods, expensiveness, longer run times, thermal stability of molecules and difficulty in processing large samples using above said analytical techniques, it has have paved way for researchers to shift their focus to...
develop more sensitive and high throughput methods using HPLC coupled with MS and MS/MS\textsuperscript{31-34} which are used for clinical and pharmacokinetic applications.

The developed bio-analytical methods have to be sensitive enough to determine the biological sample concentration of the drug and/or its metabolite(s) at least for a period of about five elimination half-life’s after dosage of the drug. A bio-analytical method is a set of procedures involved in the collection, processing, storing, and analysis of an analyte(s)\textsuperscript{3} in biological matrix. Bio-analytical method analysis comprises of three phases namely a) Method development, b) Method validation and c) Method application (sample analysis).

During method development, finalizing a suitable analytical method for identification and quantification of drugs and their metabolites in various biological fluids is essential. The detection technique selected should be highly sensitive, precise and accurate for the estimation of drugs and their metabolites in the biological fluid. The sample processing method selected should have consistency in recovery, reproducibility and should deliver less or negligible interference with compounds of interest during instrumental analysis. Based on the nature of biological matrix and drug’s physico-chemical properties such as molecular structure, polarity, partition coefficient, solubility, dissociation constant etc., different extraction methodologies like protein precipitation extraction\textsuperscript{6-7}, liquid-liquid extraction\textsuperscript{8-11} and solid phase extraction\textsuperscript{12-15} are evaluated to extract neat form of the compounds of interest free from the endogenous substances like proteins, phospholipids, sugars, and salts present in the biological matrix. Most commonly endogenous substances such as phospholipids and proteins causes ion suppression\textsuperscript{4-5} during mass spectrometric analysis which leads to inaccurate results. Moreover, optimizing chromatographic conditions play a key role in analyzing the compounds of interest with high throughput, selectivity and sensitivity. Different chromatographic conditions like flow rate, column, column temperature, mobile phase combination and reconstitution solution need to be optimized. In case, any of the above parameters are not properly optimized, it may impact analysis resulting in poor sensitivity and inconsistent recoveries and leading to redevelopment of the method. Hence the method development process plays vital role in the bioanalysis.

The fundamental parameters for LC-MS/MS method validation include selectivity, sensitivity, linearity, precision, accuracy, matrix effects, recovery, stability and dilution integrity\textsuperscript{23-24,35-36}. Measurement for each analyte in the biological matrix should be
validated. In addition, the stability of analyte in spiked samples should be determined. The successfully developed and validated method can be applied for pharmacokinetic, bioequivalence studies serving as useful tool in therapeutic drug monitoring.

In the current pharmaceutical environment, there is a prime need to determine antiviral agents in biological metrics solely as well as in their combined dosage forms, using LC-MS/MS methods to analyze antiviral agents which are more sensitive. Prompted by the present significance, present proposed work is aimed to achieve most selective, sensitive, rapid bioanalytical methods to analyze antiviral agents with the objective of minimizing various hurdles faced by researchers. These developed and validated methods could be used for clinical pharmacokinetic studies and therapeutic drug monitoring for its applicability.

Thus, objective of the present study was to develop and validate (ICH and US FDA guidelines)\textsuperscript{35-36}, simple, specific, sensitive and high throughput bioanalytical methods for estimation of antiviral agents like valacyclovir, abacavir, penciclovir, darunavir, oseltamivir, ritonavir and lopinavir.
1.1 DRUG PROFILE

1.1.1 Valacyclovir HCl

Valacyclovir hydrochloride is the salt of L-valyl ester of the antiviral drug acyclovir. The chemical name of valacyclovir hydrochloride is L-valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purine-9-yl) methoxy]ethylester, monohydrochloride.

Valaciclovir is a prodrug, an esterified version of Acyclovir, rapidly converted to acyclovir after oral administration via intestinal and hepatic first-pass metabolism.

Acyclovir is first converted to monophosphate derivative by the virus-specified thymidine kinase, and then to di and triphosphate compounds by host cell enzymes. The affinity of acyclovir for virus specific thymidine kinase is about 200 times greater than for the mammalian enzyme as a result acyclovir is selectively activated, and the active metabolite accumulates, only in infected cells. Acyclovir triphosphate inhibits viral DNA synthesis by two mechanisms: competition with deoxy GTP for the viral DNA polymerase, resulting in binding to the DNA template as an irreversible complex; and chain termination following incorporation into the viral DNA.

Unlike acyclovir, valacyclovir is a substrate for intestinal and renal peptide transporters. The relative oral bioavailability of acyclovir increases three- to fivefold approximately following valacyclovir administration.

Acyclovir distributes widely in body fluids, including vesicular fluid, aqueous humor, and cerebrospinal fluid. Compared with plasma, salivary concentrations are low, and vaginal secretion concentrations vary widely. Renal excretion of un metabolized acyclovir by glomerular filtration and tubular secretion is the principal route of elimination.

It is predominantly active against herpes simplex virus (HSV) types 1, 2 and to a lesser extent varicella-zoster virus (VZV). In immunocompetent persons, the clinical benefits of valacyclovir are greater in initial HSV infections than in recurrent ones, which typically are milder in severity. These drugs are particularly useful in immunocompromised patients because these individuals experience both more frequent and more severe HSV and VZV infections.

Approved uses of valacyclovir include treatment of first or recurrent genital herpes, suppression of frequently recurring genital herpes, and as a 1-day treatment for orolabial herpes. Once-daily dosing of valacyclovir (500mg) for chronic suppression in persons...
with recurrent genital herpes has been shown to markedly decrease the risk of sexual transmission. Valacyclovir has also been shown to be effective in preventing cytomegalovirus disease after organ transplantation when compared with placebo.

Valacyclovir is generally well tolerated, although nausea, vomiting, or rash occasionally occur. At high doses, confusion, hallucinations, and seizures have been reported. AIDS patient who received high-dosage valacyclovir chronically (ie, 8 g/d)\textsuperscript{41} had an increased incidence of gastrointestinal intolerance as well as thrombotic microangiopathies (thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome).

1.1.2 Abacavir

Abacavir is a synthetic carbocyclic nucleoside analogue. The chemical name of abacavir is \([(1S, \text{cis})-4-[2\text{-amino}-6-\text{cyclopropylamino}-9H\text{-purin}-9\text{-yl}]-2\text{-cyclopentene}-1\text{-methanol}] \text{(salt)} \text{ (2:1)}\textsuperscript{42}. Abacavir is the only approved antiretroviral that is active as a guanosine analog.

Abacavir is converted inside cells to an active metabolite, carbovir 5-triphosphate, which is a potent inhibitor of the HIV-1 reverse transcriptase. It is initially monophosphorylated by adenosine phosphotransferase. The monophosphate is then converted to carbovir 3-monophosphate, which is then phosphorylated to the di and triphosphates by cellular kinases. Carbovir 5-triphosphate terminates the elongation of proviral DNA because it is incorporated by reverse transcriptase into nascent DNA but lacks a 3-hydroxyl group\textsuperscript{43}.

Abacavir oral bioavailability is greater than 80% regardless of food intake. Abacavir is eliminated by metabolism to the 5-carboxylic acid derivative catalyzed by alcohol dehydrogenase and by glucuronidation to the 5-glucuronide. These metabolites account for 30% and 36% of elimination, respectively\textsuperscript{44}. Abacavir is not a substrate or inhibitor of cytochrome P450 system (CYPs). Cerebrospinal fluid levels are approximately one third to those of plasma.

In initial monotherapy studies, abacavir reduced HIV plasma RNA concentrations up to 300 times more than with other antiretroviral nucleosides, and increased CD4+ lymphocyte counts by 80 to 200 cells/mm\textsuperscript{3}\textsuperscript{44}. Abacavir is effective in combination with other nucleoside analogs, non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors. Adding abacavir to zidovudine and lamivudine results in a substantially greater decrease in plasma HIV-1 RNA than seen with the two-drug regimen of zidovudine plus lamivudine in adults\textsuperscript{44} and children\textsuperscript{45}. Adding abacavir to
stable antiretroviral therapy can result in additional antiviral effect, but treatment-
experienced patients are much less likely to benefit from this drug if there are multiple
preexisting nucleoside resistance mutations.

The most important adverse effect of abacavir is a unique and potentially fatal
hypersensitivity reactions. Symptoms, which generally occur within the first 6 weeks of
therapy, include fever, malaise, nausea, vomiting, diarrhea, and anorexia. Respiratory
symptoms such as dyspnea, pharyngitis, and cough may also be present, and skin rash
occurs in about 50% of patients\(^{44}\).

This syndrome is characterized by fever, abdominal pain, and other gastrointestinal
complaints; a mild maculopapular rash; and malaise or fatigue. Respiratory complaints
(cough, pharyngitis, dyspnea), musculoskeletal complaints, headache, and paresthesias
are reported less commonly. Abacavir can never be restarted once discontinued for
hypersensitivity because reintroduction of the drug leads to rapid recurrence of severe
symptoms, accompanied by hypotension, a shock-like state, and possibly death. The
reported mortality rate of restarting abacavir in sensitive individuals is 4% \(^{44}\). Other
potential adverse events are rash, fever, nausea, vomiting, diarrhea, headache, dyspnea
and fatigue.

### 1.1.3 Famciclovir

*Famciclovir* is the diacetyl ester prodrug of 6-deoxy penciclovir, chemically known as 2-
\([2-(2-amino-9H-purin-9-yl)ethyl]-1,3-propanediol diacetate\(^{46}\).

After oral administration, famciclovir is rapidly converted by first-pass metabolism to
penciclovir. Penciclovir is active against HSV and VZV\(^{47}\). It is inhibitory also for HBV.
Penciclovir is an inhibitor of viral DNA synthesis. In virus infected cells, penciclovir is
phosphorylated initially by viral thymidine kinase. Penciclovir triphosphate serves as a
competitive inhibitor of viral DNA polymerase\(^{47}\). Unlike acyclovir, penciclovir does not
cause chain termination. Penciclovir triphosphate has lower affinity for the viral DNA
polymerase than acyclovir triphosphate, but it achieves higher intracellular
concentrations and has a more prolonged intracellular effect in experimental systems.

Famciclovir is well absorbed orally and converted rapidly to penciclovir by deacetylation
of the side chain and oxidation of the purine ring during and following absorption from
the intestine\(^{48}\). The bioavailability of penciclovir is 65% to 77% following oral
administration of famciclovir. Lower peak plasma concentrations of penciclovir but no
reduction in overall bioavailability occur in compensated chronic hepatic insufficiency. Penciclovir is excreted primarily in the urine.

Oral famciclovir is effective for the treatment of first and recurrent genital herpes, for chronic daily suppression of genital herpes, and for the treatment of acute zoster. Oral famciclovir (250mg three times a day for 7 to 10 days) is as effective as acyclovir in treating first-episode genital herpes. In patients with recurrent genital HSV, patient-initiated famciclovir treatment (125 or 250mg twice a day for 5 days) reduces healing time and symptoms by about one day. One-day usage of famciclovir (1000mg twice daily) significantly accelerates time to healing of recurrent genital herpes compared with placebo, by approximately 2 days. A single dose of 1500mg or two 750mg doses (bid) accelerates herpes labialis healing time.

Oral famciclovir is well tolerated but may be associated with headache, diarrhea, and nausea. Urticaria, rash, and hallucinations or confusional states (predominantly in the elderly) have been reported. No clinically important drug interactions have been identified with famciclovir or penciclovir.

1.1. 4 Oseltamivir Phosphate

Oseltamivir phosphate is chemically \([3R-(3\alpha,4\beta,5\alpha)]\)-ethyl 4-(acetylamino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate. Oseltamivir phosphate is an ethyl ester prodrug that lacks antiviral activity.

Oseltamivir cleaved by esterase’s in the gastrointestinal tract and liver to the active carboxylate. Oseltamivir carboxylate is a potent selective inhibitor of influenza A and B virus neuraminidases. Influenza neuraminidase cleaves terminal sialic acid residues and destroys the receptors recognized by viral hemagglutinin, which are present on the cell surface, in progeny virions, and in respiratory secretions. This enzymatic action is essential for release of virus from infected cells. Interaction of oseltamivir carboxylate with the neuraminidase causes a conformational change within the enzyme's active site and inhibits its activity. Inhibition of neuraminidase activity leads to viral aggregation at the cell surface and reduced virus spread within the respiratory tract.

Oral oseltamivir phosphate is absorbed rapidly and low blood levels of the phosphate are detectable, but exposure is only 3% to 5% of that of the metabolite. The bioavailability of the carboxylate is estimated to be approximately 80%. The carboxylate has a volume of distribution similar to extracellular water. Both the prodrug and active metabolite are
eliminated primarily unchanged through the kidney. Probenecid doubles the plasma half-life of the carboxylate, which indicates tubular secretion by the anionic pathway.

Oral oseltamivir is effective in the treatment and prevention of influenza A and B virus infections. Treatment of previously healthy adults (75mg twice daily for 5 days) or children aged 1 to 12 years (weight-adjusted dosing) with acute influenza reduces illness duration by about 1 to 2 days, speeds functional recovery, and reduces the risk of complications leading to antibiotic use by 40% to 50%. Treatment is associated with approximate halving of the risk of subsequent hospitalization in adults. When used for prophylaxis during the influenza season, oseltamivir (75mg once daily) is effective (approximately 70% to 90%) in reducing the likelihood of influenza illness in both unimmunized working adults and in immunized nursing home residents; short-term use (7 to 10 days) protects against influenza in household contacts. Early administration is crucial because replication of influenza virus peaks at 24-72 hours after the onset of illness.

Oral oseltamivir is associated with nausea, abdominal discomfort, and, less often, emesis, probably owing to local irritation. Gastrointestinal complaints usually are mild-to-moderate in intensity, typically resolve in 1 to 2 days despite continued dosing, and are preventable by administration with food. The frequency of such complaints is about 10% to 15% when oseltamivir is used for the treatment of influenza illness and less than 5% when used for prophylaxis. Increased frequencies of headache, fatigue, and diarrhea have been reported in a prophylaxis study in elderly adults.

1.1.5 Darunavir

Darunavir is chemically, [(1S,2R) - 3 - [[(4 - Aminophenyl)sulfonyl](2 methylpropyl)amino] - 2 - hydroxy - 1 - (phenylmethyl)propyl] - carbamic acid (3R,3aS,6aR)-hexahydropuro[2,3-b]furan-3-yl ester.

Darunavir is a human immunodeficiency virus (HIV) protease inhibitor which prevents HIV replication by cleaving HIV encoded Gag-Pol polyproteins in infected cells, reducing viral maturation, load and increasing CD4+T lymphocytes.

Darunavir is primarily metabolized by CYP3A4. Darunavir is used as an adjunct therapy with low dose ritonavir, which inhibits cytochrome P450 3A4, which increases the bioavailability and half life of darunavir. The absolute oral bioavailability of a single 600
mg dose of darunavir alone and after co-administration with 100 mg ritonavir twice daily was 37% and 82% \(^{61}\), respectively.

A mass balance study in healthy volunteers showed that after single dose administration of 400 mg \(^{14}\)C-darunavir, co-administered with 100 mg ritonavir, approximately 79.5% and 13.9% of the administered dose of \(^{14}\)C-darunavir was recovered in the feces and urine, respectively. Unchanged darunavir accounted for approximately 41.2% and 7.7% of the administered dose in feces and urine\(^{61}\), respectively.

Co-administration of darunavir / ritonavir is contraindicated with drugs that are highly dependent on CYP3A4 for clearance and for which elevated plasma concentrations are associated with serious and / or life-threatening events (narrow therapeutic index)\(^{61}\).

1.1.6 Lopinavir and Ritonavir

Lopinavir is chemically designated as \([1S-[1R^*,(R^*), 3R^*, 4R^*]]-N-[4-[(2,6-dimethylphenoxy)acetyl]amino]-3-hydroxy-5-phenyl-1(phenylmethyl)pentyl]tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H)-pyrimidineacetamide. Lopinavir is structurally similar to ritonavir\(^{62}\).

Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12 tetra azatridecan -13-oic acid, 5-thiazolylmethy]ester, \([5S-(5R^*,8R^*,10R^*,11R^*)]\)\(^{62}\).

Both lopinavir and ritonavir are peptidomimetic HIV protease inhibitors, more active against HIV-1 than HIV-2. Lopinavir is available only in coformulation with low doses of ritonavir, which is used to inhibit CYP3A4 metabolism\(^{63}\) and increase concentrations of lopinavir.

Lopinavir and ritonavir are selectively toxic by potently inhibiting the HIV-encoded protease but not host-encoded aspartyl proteases. Both are absorbed rapidly after oral administration. A moderate- to high-fat meal increases oral bioavailability of both drugs up to 50 and 13% respectively\(^{62}\). Although the capsules contain lopinavir–ritonavir in a fixed 4:1 ratio, the observed plasma concentration ratio for these two drugs following oral administration is nearly 20:1, reflecting the sensitivity of lopinavir to the inhibitory effect of ritonavir on CYP3A4. Approximately 90% of total drug in plasma is the parent compound lopinavir, and less than 3% of a dose is eliminated unchanged in the urine.

Ritonavir is metabolized primarily by CYP3A4 and to a lesser extent by CYP2D6.
Ritonavir and its metabolites are mainly eliminated in feces (86% of parent drug and metabolites), with only 3% of the drug eliminated unchanged in the urine\textsuperscript{62}.

The most common adverse events reported with lopinavir-ritonavir coformulation have been gastrointestinal, including loose stools, diarrhea, nausea, and vomiting. These are less frequent and less severe than those reported with the 600-mg twice-daily standard dose of ritonavir. The most common laboratory abnormalities include elevated total cholesterol and triglycerides. Because the same adverse effects occur with ritonavir, it is unclear whether the side effects are due to ritonavir, lopinavir, or both \textsuperscript{63-64}.

Because lopinavir metabolism is highly dependent on CYP3A4, concomitant administration of agents that induce CYP3A4, such as rifampin, may lower plasma lopinavir concentrations considerably. St. John's wort is a known inducer of CYP3A4, leading to lower concentrations of lopinavir and possible loss of antiviral effectiveness. Coadministration of other antiretrovirals that can induce CYP3A4, including amprenavir, nevirapine or efavirenz which may require higher dose of lopinavir \textsuperscript{63}.
REFERENCES


