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Possible Influences of Standardized Herbal Extract of Ashwagandha on the Pharmacokinetics and Toxicity of Ritonavir in Rats

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ABSTRACT

Objective: Herb-drug interactions of ashwagandha, an immunomodulatory agent along with ritonavir were studied in the context of their possible concomitant usage. This study investigates the possibility of pharmacokinetic interaction and also its effect on the toxicity effects between ritonavir and ashwagandha.
Methods: Single and multiple oral doses of ritonavir (30 mg/kg) and in combination with ashwagandha (300 mg/kg) in adult wistar rats were performed for one and nine days on two different groups (n = 6) respectively. PK parameters, CBP, SGPT, SGOT and serum creatinine levels of different groups were measured.
Results: Repeated oral administration of ashwagandha along with ritonavir caused the decrease in the plasma concentration of ritonavir 22.74 ± 1.61 (µg/h), increased the RBC number 4.98 ± 0.04 (m/cmm) and has significantly decreased the levels of SGOT and SGPT 24.66 ± 1.75 (U/L), 10.83 ± 1.47 (U/L) against ritonavir treated rats. The ritonavir plasma concentrations decreased significantly both in single and multiple doses groups of ashwagandha. Ashwagandha protected the deleterious effects caused by ritonavir on the blood cells. It has significantly decreased the SGOT and SGPT levels when compared to ritonavir treated rats. But, it did not decrease the serum creatinine levels.
Conclusion: Using ashwagandha along with ritonavir has no deleterious effects on the major organ systems. It also increases the absorption of ritonavir into the systemic circulation.

Keywords- Herb-drug interactions, Immunomodulation, Antiretroviral therapy.

INTRODUCTION

Several nutritional and dietary supplements available in markets are based on botanical products and are becoming increasingly popular as alternative medicines. Most herbal drugs are a complex mixture of chemical constituents. Often a complete characterization of the bioactive compounds from an herbal is unknown. Combination products composed of multiple natural products complicate matters further. Folkloric medicines which contain herbs are often taken in combination with therapeutic drugs in rural areas with the anticipation that it can raise the potential of pharmacokinetic and/or pharmacodynamic properties of drugs. This is a potential cause of herb-drug interactions. The combined use of herbs with drugs may mimic, increase or reduce the effects of either component, resulting in clinically important herb-drug interactions[1,2]. If it causes synergistic therapeutic effects it might lead to toxicities and even complicate the dosage regimen of long-term medications, while antagonistic interactions will result in decreased efficacy and therapeutic failure. The potential interaction of herbal medicines with drugs is a major safety concern, especially for drugs with narrow therapeutic indices (e.g. warfarin and digoxin) and may lead to adverse reactions that are sometimes life threatening[3,4].

Interactions between herbals and medications can be caused by either pharmacodynamic or pharmacokinetic mechanisms. Pharmacokinetic interactions occur when an herbal changes the absorption, distribution, metabolism, protein binding, or excretion of a drug that results in altered levels of the drug or its metabolites. Most of the current evidence of pharmacokinetic drug interactions involves metabolizing enzymes and drug transporters. Although drug interactions can involve enzymes such as glutathione-*S*-transferases and uridine diphosphoglucuronyl transfer eases (UGTs), most herbal-drug interactions are related to oxidative metabolism by the cytochrome P-450 system (CYP) or by the effect of a herbal on the efflux drug transporter *p*-glycoprotein[5].

The protease inhibitors such as indinavir, ritonavir, and neverapine are widely used in highly active antiretroviral therapy (HARRT). Many people use herbal remedies without knowing their effect when allopathic drugs are used concomitantly. Several protease inhibitors were studied for their interactions with natural health products[6-13]. Ashwagandha, commonly known as Indian ginseng, is the powder of dried roots of *Withania somnifera* Linn. (Solanaceae). It is found to possess immunomodulatory activities[14-17]. HIV patients on ritonavir therapy may consume ashwagandha for its beneficial effects. In this context, herb-drug interaction could arise and this study assesses the pharmacokinetic interactions of ritonavir with ashwagandha.

MATERIALS AND METHODS

Ritonavir, zidovudine were kind gifts from Aurobindo Pharma, Hyderabad. Ashwagandha (of Himalaya Herbal Healthcare, Bangalore make) was purchased from a local ayurvedic medical shop in November 2010. A voucher specimen (ERR-WS-10) of the same is maintained in the herbarium of Vaagdevi College of Pharmacy, Warangal. Acetonitrile, methanol and water used during analytical experiments were of HPLC grade purchased from Finar Chemicals, Ahmedabad.

Animals

Experiments were performed on wistar rats procured from Mahaveera Enterprises (Hyderabad, T.S., India), weighing between 180-210 g. The animals were housed in colony cages (four per cage) under conditions of standard lighting,

temperature (22 ± 1°C) and humidity for at least one week before the beginning of experiment. They had free access to food and water. Approval of Institutional Animal Ethical Committee (IAEC Reg. No: 1047/ac/07/CPCSEA, dated 24-04-2007) was taken to perform the experiments.

Estimation of ritonavir by sensitive RP HPLC method

HPLC description

A Waters HPLC system is used in the study consisted of a pump (Model Waters 515 HPLC pump) operating at 1 mL/min, a syringe loading sample injector of 20 µL capacity, a C_{18} reverse phase column of 250 × 4.6 mm dimension and 5 µ particle size and a dual wavelength UV-Visible detector (UV-1800). The data analysis was done by Wufeng-chrom workstation (Shanghai Wufeng Scientific Instruments Co. Ltd Shangai).

Chromatographic conditions

The mobile phase consisted of acetonitrile: water (60: 40 % v/v) was pumped at a flow of 1 mL/min. The mobile phase was filtered through 0.4 µm membrane filter and degassed before use. The effluent was monitored at 240 nm. The total run time of the method was set at 15 min.

Preparation of calibration curve of ritonavir for *in vivo* samples

Stock solutions of 1000 µg/mL of ritonavir and zidovudine (internal standard) were prepared by dissolving in required quantity of acetonitrile. From the stock solution further dilutions of 0.1, 0.3, 0.5, 0.7, 1, 3, 5, 7 µg/mL of ritonavir were prepared by using acetonitrile. 100 µg/mL of zidovudine was prepared to be used as an internal standard. A standard graph was prepared by adding a known concentration of ritonavir to drug free plasma. Briefly, to each 100 µL of plasma sample, 100 µL of standard drug concentration and 100 µL internal standard (zidovudine) was added. Finally 100 µL acetonitrile was added and vortexed for 1 min and centrifuged at 10000 *g* for 3 min. The supernatant was transferred to clean, similarly labeled tube and was subsequently recentrifuge at 10000 *g* for 2 min and then evaporate the acetonitrile from these tubes and add the mobile phase. These samples were stored at -20°C until use. The resultant solution was injected into HPLC. Peak area ratios were calculated using the formula: Peak area ratio = Peak area of drug/Peak area of internal standard.

Construction of calibration curve

The calibration curve was constructed using peak area ratios of drug to internal standard vs nominal concentration. The slope of plot determined by the least square regression analysis was used to calculate the ritonavir concentrations in the unknown sample. A linear calibration curve in the range of 0.1 to 7 µg was established ($r^2 = 0.9993$). Retention times of zidovudine and ritonavir were 2.65 and 8.68 min, respectively.

Experimental design of pharmacokinetic and toxicological studies in normal healthy rats

Wistar rats of either sex were randomly distributed into four groups of six animals in each group; they are housed in well ventilated aluminum cages and maintained on uniform diet and temperature with 12 h light and dark cycle. Before experiment all animals fasted for Overnight and water *ad libitum*, water was withdrawn during experiment. After collection of initial blood samples, drugs were administered orally. The animals were divided into various groups in the following way:

Group I: Control (0.2 mL of 0.5% CMC sodium).

Group II: Receive ritonavir (30 mg/kg) - Single dose.

Group III: Receive ritonavir (30 mg/kg) + ashwagandha (300 mg/kg)- Single dose.
Group IV: Administer ashwagandha (300 mg/kg) for 9 days.
On 9th day administer (10 mg/kg) of ritonavir.

Blood (0.5 mL) for analysis was collected from orbital sinuses using heparinized capillaries into a micro centrifugation tubes contain anticoagulant at 1, 2, 4, 8 and 12 h after treatment. Plasma was analyzed separated by centrifugation and stored at -20°C until further analysis. These samples are used to analyze for pharmacokinetic and toxicological i.e SGOT-SGPT, Complete Blood Picture (CBP), Serum Creatinine Clearance test. Ritonavir levels were estimated by a sensitive RP-HPLC method.

Estimation of SGOT-SGPT levels by modified IFCC-UV kinetic method

Enzyme reagent (1 mL) was added to 0.1 mL of plasma at room temperature. It was mixed well and absorption was measured against blank at 340 nm. The initial absorbance A0 was measured after exactly 1 min. A1, A2 and A3 were measured after every 30 s for 1 min 30 s.

The average change in absorbance per minute (Δ A/min) was calculated by the formula:

IU/L = ΔA/min X 1746 X tf
Temperature conversion factor (tf) =1.

Estimation of serum creatinine levels by modified Jaffe'S kinetic method

Enzyme reagent (1 mL) was added to 0.1 mL of plasma at room temperature. It was mixed well and absorption was measured against blank at 520 nm. The initial absorbance A1 for the Standard and Test were measured after exactly 30 s. Another absorbance A2 of the Standard and Test were measured exactly 60 s later. ΔA for both the Standard and Test were calculated.

For Standard ΔAS = A2S - A1S.
For Test ΔAT = A2T - A1T.
Calculate the Creatinine in mg/dl:
Creatinine in mg/dl = (Δ AT/ Δ AS) x 2.0

Data analysis

The data obtained was pooled for each group. Data were analyzed by one way ANOVA followed by Dunnett′s test for comparison of all other columns with control and at a significance level of $p < 0.05$. The data is presented as mean ± SD.

Estimation of complete blood picture (CBP): Automated blood count

The blood is well mixed (though not shaken) and placed on a rack in the analyzer (Cobus u 411). The cell counting component counts the numbers and types of different cells within the blood.

Blood (0.1 mL) is aspirated through narrow tubing which contains sensors that count the number of cells going through it, and can identify the type of cell by flow cytometry. The two main sensors used are light detectors, and electrical impedance. One way the instrument can tell what type of blood cell is present is by size. Other instruments measure different characteristics of the cells to categorize them.

Automated hematology analyzers also measure the amount of hemoglobin in the blood and within each red blood cell.

RESULTS AND DISCUSSION

Ritonavir is well absorbed from the gastrointestinal tract following oral dosing. Peak plasma concentrations are achieved with in 3-5 h. It is 98-99% bound to plasma proteins. It is extensively metabolized in liver, almost exclusively by the cytochrome P450 isoenzyme CYP3A4. Toxicity studies in animals identified major target organs as the liver, retina, thyroid gland and kidney. Hepatic changes involved hepatocellular, biliary and phagocytic elements and were

accompanied by increases in hepatic enzymes[18].

Pharmacokinetics of ritonavir after the oral administration of standardized herbal extract of ashwagandha

In the present study it is quite evident that single and multiple doses of ashwagandha decreased the plasma concentration of ritonavir. There is significant decrease in the AUC and increase in clearance. In the single dose studies the change in PK parameters could be because of the transient induction in intestinal P-gp and CYP 3A4. When ashwagandha was given in repeated doses (9 days) there could be induction of both the intestinal and hepatic CYP 3A4 subsequently increasing the metabolism and clearance of ritonavir, decreasing the overall AUC by more than 50% on repeated administration of ashwagandha (Table 1) showing a potential metabolic interaction. The decrease in plasma concentration of ritonavir and AUC may have direct impact on the pharmacodynamics in terms of the viral load.

Toxicity of ritonavir in the presence of standardized herbal extract of ashwagandha

In the present experiments blood profile, liver (SGPT, SGOT) and kidney (serum creatinine) function tests were studied to correlate the effect of the ashwagandha-ritonavir combination on the toxicity profiles. Ashwagandha have clearly shown the beneficial effect in the CBP, where there is significant improvement in blood cell count and lymphocytes in particular (Table 2). Studies clearly indicate the beneficial effect of ashwagandha on the deleterious effects caused by ritonavir on blood profile and most importantly could help the ailing immunity of the HIV patients on ritonavir therapy. Ashwagandha also improved the liver function compared to the elevated levels of SGPT/SGOT in ritonavir treated group (Table 3). The elevated levels could be because of the induction caused by the CYP that ultimately lead to toxicity which was controlled by ashwagandha. In contrary there is significant elevation in the serum creatinine levels in all the groups treated (Table 3).

Ashwagandha is a very popular Indian traditional medicine and is used as a general tonic. It has been found to possess potent immunomodulatory actions. It is often sold on OTC even by druggists and chemists as a health supplement. HIV patients are immuno-compromised and because ashwagandha is a general tonic with immunomodulatory effects, it is possible that both are concomitantly used by such patients. Therefore, there is a chance for herb-drug interaction in HIV patients with the use of ashwagandha and ritonavir.

The above studies revealed that plasma concentrations of ritonavir decreased in the presence of ashwagandha below the MEC. The ritonavir plasma concentrations decreased significantly both in single and multiple doses groups of ashwagandha. There are significant changes in the PK parameters. In single dose groups of ashwagandha - ritonavir AUC decreased with increase in clearance. Induction of CYP3A4 by repeated administration of ashwagandha could be the major reason for the decrease in the AUC. Ashwagandha protected the deleterious effects caused by ritonavir on the blood cells and liver, it could not protect those effects on kidney.

CONCLUSION

The studies clearly indicate a potential interaction (metabolic) between ashwagandha and ritonavir. The interaction could lead to sub therapeutic levels of ritonavir and obvious increase in the viral load and failure of the therapy. Hence the present study recommends that while such herb – drug combinations are used, dosage

adjustments are to be strongly advocated and the above interaction in HIV patients has to be still envisaged further in a controlled clinical set-up.

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Declaration of interest statement

All the authors have no conflict of interest.

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Table 1. Comparison of pharmacokinetic parameters of ritonavir (30 mg/kg) and also following pretreatment with ashwagandha (300 mg/kg) by oral administration in healthy rats (n = 6)

Parameter	Ritonavir	Ritonavir + Ashwagandha (1st day)	Ritonavir + Ashwagandha (9th day)
AUC (µg/h)	52.67 ± 3.37	34.74 ± 1.68***	22.74 ± 1.61***
AUMC (µg/ml/h)	61.08 ± 2.16	55.71 ± 1.92***	60.5 ± 1.57
CL_F (L/h)	83.12 ± 2.42	124.84 ± 4.42***	163.81 ± 5.64***
CL_F (L/h/kg)	576.49 ± 19.68	853.25 ± 15.78***	1071.39 ± 28.72***
T_{max} (h)	1.06 ± 0.03	1.06 ± 0.03	1.04 ± 0.028
C_{max} (µg/mL)	12.45 ± 0.74	10.59 ± 0.38***	9.32 ± 0.49***
MRT (h)	5.47 ± 0.33	1.60 ± 0.10**	1.20 ± 0.09***

**p value < 0.01, ***p value <0.0001,*p value<0.05.
Compared with Ritonavir treated group.

Table 2. Complete blood picture (CBP) (mean ± SD) changes after oral administration of ritonavir and combination with ashwagandha in healthy rats (n = 6)

Cells	Control	Ritonavir	Ritonavir + Ashwagandha (1st day)	Ritonavir + Ashwagandha (9th day)
Haemoglobin (g)	14.3 ± 0.21	12.9 ± 0.16**	13.55 ± 0.327***	14.21 ± 0.40
R.B.C (m/cmm)	4.98 ± 0.04	4.03 ± 0.103**	4.2 ± 0.33***	4.98 ± 0.04
Total count (TC) (m/cmm)	4008.33 ± 66.45	3616.66 ± 354.49*	3823.33 ± 2.065	4166.66 ± 163.29
Neutrophils (%)	42.33 ± 0.81	35 ± 1.78***	36.66 ± 2.065***	41.33 ± 2.06
Lymphocytes (%)	53.5 ± 1.37	47.33 ± 1.36*	43 ± 1.41*	55 ± 1.41
Monocytes (%)	2.5 ± 0.54	1.83±0.75*	2.33±0.81	2.66 ± 0.51
Eosinophils (%)	1 ± 0.63	1 ± 0.63	1 ± 0.63	1.33 ± 0.51

***p< 0.001, **p<0.01,*p<0.05 Compared to Control.

Table 3. Mean ± SD changes in SGOT, SGPT and serum creatinine levels after oral administration of ritonavir and combination with ashwagandha in healthy rats (n = 6)

Group	SGOT (U/L)	SGPT (U/L)	Serum creatinine levels (mg/dL)
Control	17.83 ± 0.75	11 ± 1.78	1.93 ± 0.08
Ritonavir	33.33 ± 2.16***	25.83 ± 1.72***	2.61 ± 0.39**
Ritonavir + Ashwagandha (1^{st}day)	26.33 ± 1.63**	24.66 ± 1.75**	2.41 ± 0.36*
Ritonavir + Ashwagandha (9^{th} day)	17.5 ± 1.04	10.83 ± 1.47	3.06 ± 0.33***

***$p < 0.0001$, ** $p < 0.01$ compared to Control.