Therefore we aimed to develop a simple, economical and reliable plasma using lopinavir (LPV) as internal standard.

Chromatographic method for the determination of ritonavir (RTV) in hospital laboratories and also requires high analytical cost. Sophisticated equipments may not be available in conventional MALDI-QqQ-MS/MS [30, 31] have also been published and these desorption/ionization-triple quadrapole tandem mass spectrometry methods for quantification of antiretroviral drugs by tandem mass undesirable outcome may result from a failure to achieve effective patients develop drug resistance and metabolic complications. This easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies of RTV in HIV-infected patients with TB.

Keywords: Ritonavir, Pharmacokinetics, HPLC, Plasma extraction, HIV

INTRODUCTION

The clinical treatment of patients with human immunodeficiency virus (HIV-1) infection has been advanced by the development of highly active antiretroviral therapy (HAART). However, some patients develop drug resistance and metabolic complications. This undesirably outcome may result from a failure to achieve effective antiretroviral drug plasma concentrations.

Therefore monitoring plasma drug concentrations is essential to ensure optimal drug efficacy, to prevent viral resistance, to manage drug interactions, to avoid adverse effects, and to assess non adherence [1-2]. Ritonavir (RTV), an antiretroviral medication along with other medications to treat HIV/AIDS. Ritonavir, originally developed as an inhibitor of HIV protease, is widely used as a booster for other protease inhibitors. Additionally, because RTV is metabolized by cytochrome P450 enzymes, monitoring may be useful for evaluation of drug-drug interactions, such as those with a potent CYP 3A4 inducer or inhibitor that has been shown to increase or decrease the RTV plasma concentration.

In recent years, several HPLC methods for simultaneous determination of antiretroviral drugs in plasma have been published [3-23]. Most of the methods using HPLC, though they are reliable and sensitive, have some critical disadvantages involving solid phase extraction, special columns, gradient elution programs, cumbersome operation procedures, larger sample volumes, multiple wavelengths, special detectors and/or longer run time. Other methods for quantification of antiretroviral drugs by tandem mass spectrometry (LCMS/MS) [24-29] and matrix-assisted laser desorption/ionization-triple quadrapole tandem mass spectrometry (MALDI-QqQ-MS/MS) [30, 31] have also been published and these sophisticated equipments may not be available in conventional hospital laboratories and also requires high analytical cost. Therefore we aimed to develop a simple, economical and reliable chromatographic method for the determination of ritonavir (RTV) in plasma using lopinavir (LPV) as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

RTV (Code No: RD322) and LPV (Code No: R8FB6) were kindly provided by Cipla Ltd. Acetonitrile used was of HPLC grade (Merck, India). Sodium Acetate, methyl tert butyl Ether and Sodium hydroxide from Qualigens (India) were used. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma from healthy volunteers was obtained from Lions Blood Bank, Chennai, India.

Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), diode array detector (SPD-M10Avp) and autosampler with built-in system controller (SIL-HTA). ClassVP-LC workstation was used for data collection and acquisition. The analytical column was a C8, 250 x 4.6 mm ID, 5 µm particle size (Lichrospher 100 RP-8e, Merck, Germany) protected by a compatible guard column.

Chromatographic conditions

The mobile phase consisted of 20 mM Sodium Acetate in water, pH 4.8 (adjusted with 1N HCl) and acetonitrile (55:45 v/v). Prior to preparation of the mobile phase, the aqueous Sodium acetate and acetonitrile were degassed separately using a Millipore vacuum pump and filtered through 0.4 µm nylon membrane filter. The UV detector was set at a wavelength of 212 nm. The chromatogram was run for 13 min at a flow rate of 1.5 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) vs. Concentration curve. The linearity was verified using estimates of coefficient of determinants (r) and correlation coefficient (R2) values.

Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving RTV in methanol. The working standards of RTV in concentrations ranging from 0.5 to 10.0µg/ml were prepared in human plasma.

ABSTRACT

Objective: An accurate, simple, and rapid HPLC-UV based method for the quantitative determination of ritonavir (RTV) in human plasma is developed.

Methods: The method involved deproteinization of the sample with 0.125N NaOH and methyl tertiary butyl ether and evaporated to dryness. The residue was reconstituted with mobile phase (20 mM Sodium Acetate and Acetonitrile–55:45 v/v). The analysis was done using C8 column (250 x 4.6 mm ID) and detection at a wavelength of 212 nm

Results: The method range was linear over the range 0.5–10.0 µg/ml as derived using calibration curve method. Mean intra-and inter-day variations over the ranges of the standard curves were less than 10 % and mean extraction recoveries from human plasma ranged from 96 to 110 %.

Conclusion: A rapid and accurate method for quantitation of RTV in plasma was validated. The assay spans the concentration range of clinical interest. The easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies of RTV in HIV-infected patients with TB.

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INTRODUCTION

The clinical treatment of patients with human immunodeficiency virus (HIV-1) infection has been advanced by the development of highly active antiretroviral therapy (HAART). However, some patients develop drug resistance and metabolic complications. This undesirable outcome may result from a failure to achieve effective antiretroviral drug plasma concentrations.

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Sample preparation

To 200 µl each of calibration standards and test samples, 20 µl of LPV (internal standard) was added at a concentration of 100 µg/ml. This was mixed with 400 µl of 0.125N NaOH and 2.0 ml of methyl tertiary butyl ether, the contents were vortexed vigorously, and centrifuged at 5,000 rpm for 5 min. 1.5 ml of the clear supernatant was taken into the test tube, evaporated to dryness in a nitrogen evaporator (Turbo vap) for 20 min. The dried residue was reconstituted in 100 µl of mobile phase; 75 µl was injected into the HPLC column.

Accuracy and linearity

The accuracy and linearity of RTV standards were evaluated by analyzing a set of standards ranging from 0.5 to 10.0µg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

Precision

In order to evaluate the precision of the method, patient samples at three different concentrations of RTV (0.73, 1.15 and 3.34 µg/ml) were analysed in duplicate on three consecutive days.

Recovery

Varying concentrations of RTV (1.5, 3.5, and 7.5 µg/ml) were prepared in drug-free human plasma and extracted as described above along with the internal standard. The percentage of the drug recovered from the plasma samples was determined by comparing the peak height ratio after extraction with those of unextracted methanolic solutions containing same concentrations of RTV as in plasma. Recovery experiments were carried out on three different occasions.

Specificity

Co elution from endogenous compound was investigated by analysing blank plasma samples obtained from six each of male and female subjects. Interference from certain antiretroviral drugs, namely, nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir at a concentration of 10 µg/ml and anti-tuberculosis drugs such as, isoniazid, pyrazinamide, rifampicin, ethambutol, streptomycin was also evaluated.

Limit of detection (LOD) and lower limit of quantitation (LLOQ)

The (LOD) and (LLOQ) were calculated using slope analysis of 0.5-20 ng/ml for RTV using the formula LOD = 3 x S.D/Slope and LOQ = 10 x S.D/slope.

Pharmacokinetic application of methods to human plasma samples

Serial blood samples at pre-dosing (0 hour) and at 1, 2, 4, 6, 8 and 12 h post dosing were drawn in heparinized containers from six HIV-infected adult patients with tuberculosis who were receiving ritonavir (100 mg daily) as part of the treatment regimen.

All the blood samples were centrifuged and plasma was separated and stored at 200C until assay. Estimation of plasma RTV was undertaken within 24 to 48 h of blood collection. Informed written consent was obtained from the parent before blood draws were made.

RESULTS AND DISCUSSION

Method development

The analytical method developed was based on the protocol set out in the International Conference on Harmonization (ICH) guidelines [32]. Under the chromatographic conditions described above, peaks of RTV was presented in separate retention time with other elutants as seen in the chromatograms representing high and low concentrations (fig. 1a, b). The retention times of RTV and the internal standard (LPV) were 11.0 and 11.4 min respectively. Blank plasma samples did not give any peak at the retention times of RTV (fig. 1c).

No endogenous substances or antiretroviral drugs such as nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir or first-line anti-tuberculosis drugs such as isoniazid, pyrazinamide, ethambutol and streptomycin was coeluted as cross checked using pure standards with the RTV chromatogram.

Ritonavir concentrations ranging from 0.5-10.0 µg/ml were checked for linearity. The calibration curve parameters of RTV from six individual experiments for standard concentrations ranging from 0.5 to 10.0 µg/ml showed a linear relationship between peak height ratio and concentrations. The mean (+SD) correlation coefficient, slope and intercept values were 0.9997, 0.266 and -0.0232 respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma RTV are shown in fig. 2. The within-day and between-day percent variation (CV) for...
standards containing 0.5 to 10.0 µg/ml ranged from 3.4 to 7.9% and 2.0 to 10.3% respectively (table 1).

Table 1: Linearity and reproducibility of plasma RTV standards

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Mean peak height ratio±SD (RSD %)</th>
<th>Intraday (n=6)</th>
<th>Inter day (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrations (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.0</td>
<td>2.747±0.055(2.0)</td>
<td>2.65±0.090(3.4)</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>1.267±0.032(2.5)</td>
<td>1.287±0.095(7.4)</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>0.62±0.026(4.2)</td>
<td>0.62±0.027(4.3)</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.223±0.023(10.3)</td>
<td>0.25±0.017(6.8)</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>0.117±0.012(10.3)</td>
<td>0.127±0.010(7.9)</td>
</tr>
</tbody>
</table>

Data given is the mean of 6 samples±Standard deviation and Relative Standard deviation in the parantheses,

Table 2: Precision of plasma RTV assay

<table>
<thead>
<tr>
<th>S. No.</th>
<th>mean±2SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.72±0.04</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.17±0.04</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3.21±0.10</td>
</tr>
</tbody>
</table>

The values given are the mean of 3 concentrations with standard deviation

The reproducibility of the method was further evaluated by analyzing three plasma samples containing different concentrations of RTV. The RSD for these samples ranged from 2.6 to 5.0% (table 2). The % variations from the actual concentrations ranged from 92 to 104%. (LOD) and lower (LLOQ) were 140 ng/ml and 0.4 µg/ml respectively. The percent recovery of RTV from plasma ranged from 98 to 108% (table 3). The mean concentrations of RTV in serial blood samples collected at predosing (0 hour) and at 1, 2, 4, 6, 8, 12 and 24 h post dosing were 0.17, 0.63, 1.38, 1.60, 1.22, 0.91, 0.40 and 0.09 µg/ml respectively (fig. 3).

Table 3: Recovery

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Base</th>
<th>Added (µg/ml)</th>
<th>Actual (µg/ml)</th>
<th>Obtained (µg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.5</td>
<td>1.5</td>
<td>1.59</td>
<td>106</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>2.5</td>
<td>3.5</td>
<td>3.85</td>
<td>110</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>2.5</td>
<td>7.5</td>
<td>7.24</td>
<td>96</td>
</tr>
</tbody>
</table>

DISCUSSION

LPV/RTV is a major antiretroviral in salvage regimens, has been proven effective in Chinese HIV-positive adults with first-line treatment failure [33-35]. Thus, the establishment of a quantitative method for determination of RTV in clinical pharmacokinetic studies and therapeutic drug monitoring is necessary.

There have been recent reports of HPLC methods for the simultaneous determination of antiretroviral drugs which includes RTV. However, these methods have several disadvantages in terms of cost performance [24-29], time consumption [4-20], necessary equipments [30, 31], the use of expensive disposable solid phase extraction cartridges [8] and gradient elution control.

We describe the development, validation and application of a simple HPLC method for quantitative determination of RTV in human plasma using LPV as internal standard. The principal advantages of our method are a rapid liquid-liquid drug extraction from plasma in a simple one-step sample preparation and using a small sample volume (200 microlitres) without any loss of analyte, an isocratic elution on a reversed phase C8 column and a shorter run time when compared to the existing methods.

The developed method was simple, specific and did not require any expensive equipment or extra processing steps. A gradient elution program is commonly used for determination of LPV and RTV [36-38]. However, this requires binary pump system, a difficult elution procedure, long run time and results in consumption of large volume of reagent. Under isocratic conditions, as described here, the run time was short (15 min) and there was no need for the analytical column to be washed or re-equilibrated between runs. This condition provided good resolutions for the analyte peaks and separated them from endogenous interferences in plasma and companion antiretroviral drugs.

The method was quite robust as evidenced by a discrete peak denoting RTV. The percent variations (within-day and between-day) for the standards were below 10%. The method reliably yielded an acceptable range of recovery. Further, data from the accuracy and precision experiments yielded satisfactory results.

In view of its potent anti-retroviral activity, RTV is used in the treatment regimen along with other anti-retroviral and anti-tuberculosis drugs in HIV positive patients with and without tuberculosis, who fail to first-line anti-retroviral drugs. It therefore becomes necessary to rule out interference of both these classes of drugs in the assay of RTV and establish the specificity of the method. The method was highly specific for RTV; drugs such as nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir or anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin or any endogenous substance interfered in the assay.
When this method was applied to estimate RTV in plasma samples collected serially at different time points from adult HIV-TB patients who received 100 mg of RTV along with LPV and other anti-tuberculosis and second line anti-retroviral drugs, we obtained a mean peak concentration of 1.59 µg/ml, which was within the therapeutic range of RTV (9).

Thus the method spans the range of clinical interest and could be applied to pharmacokinetic studies in both adults and children. Most of the methods published were simultaneous determination of anti-retroviral drugs which requires longer run time and permits quantitation of limited samples. The method has the potential to implement at low budget hospital environment, as it does not require high operational cost instruments such as NMR or tandem mass spectrometers.

CONCLUSION
A sensitive, specific and validated method for quantitative determination of RTV in plasma is described. This simple, rapid, accurate and reproducible method utilizes a single step direct extraction without involvement of expensive solid phase cartridges. The chromatogram yields a well-resolved peak for RTV with good intra-and inter-day precision. This simple HPLC method can be conveniently used as a routine clinical application and enables study of the drug pharmacokinetics in conventional hospital and research laboratories.

CONFLICT OF INTERESTS
Declared none

REFERENCES
17. La Porte CIL, Colbers EPF, Bertz R, Vonken DS, Wikstrom K. Pharmacokinetics of adjusted-dose lopinavir-ritonavir


