# Quantification of fosamprenavir in spiked human plasma using liquid chromatography–electrospray ionization–tandem mass spectrophotometry–application to pharmacokinetic study

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**Background**
Fosamprenavir (FSV) is used for the treatment of HIV infections. It is a prodrug of the protease inhibitor and antiretroviral drug amprenavir.
**Aim**
This research work described about the estimation of FSV in spiked human plasma using electrospray ionization, LC-MS/MS technique, and its application to pharmacokinetic study in rabbits.
**Materials and methods**
Liquid–liquid extraction technique was used for the extraction of FSV in spiked human plasma. The separation was achieved using ZORBAX SB-C18 column with 4.6 mm internal diameter with 5 μm particle size using acetonitrile: 5 mmol/l ammonium acetate in water (85 : 15, v/v) as a mobile phase. Positive ion mode was selected for the product ion mass spectra, *m/z* 585.6–418.2 for FSV and *m/z* 589.2–469.1 for FSV-deuterated (internal standard), The US Food and Drug Administration guidelines were adopted for successful validation of the developed method.
**Results**
The retention time of FSV was found to be 1.51 min, for FSV-deuterated it was 1.62 min, with a runtime of 2.5 min. The present method exhibits excellent intraday and interday accuracy with %nominal 95–98.4% and precision percentage coefficient variation up to 3% in all quality control (QC) levels. The developed method demonstrated excellent matrix and analyte selectivity (%interference=0), matrix effect (matrix factor 2.09 at lower quantitation limit and 1.14 at high QC level) and satisfactory stability study results in all types (%nominal 94.03–100.80%). The linearity range was found to be 0.510–200.185 ng/ml with a correlation coefficient ($r^2$) of 0.998. The calculated accuracy and precision values in the ruggedness study were within 15–20% in all QC levels. The percentage coefficient variation of the pharmacokinetic study on rabbit plasma samples was also conducted and the parameters of FSV showed $T_{max}$ of 2 h and the mean $C_{max}$, $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow \alpha}$ for test formulation were 98.6, 351.3, and 354.9, respectively.
**Conclusion**
This method was successfully optimized, validated, and applied favorable for the pharmacokinetic study of marketed formulation in rabbit blood samples in a single oral human-equivalent dose. The applicability of the developed method undoubtedly can further extend during preclinical and clinical trials.

**Keywords:**
fosamprenavir, LC-MS/MS, method development, pharmacokinetics

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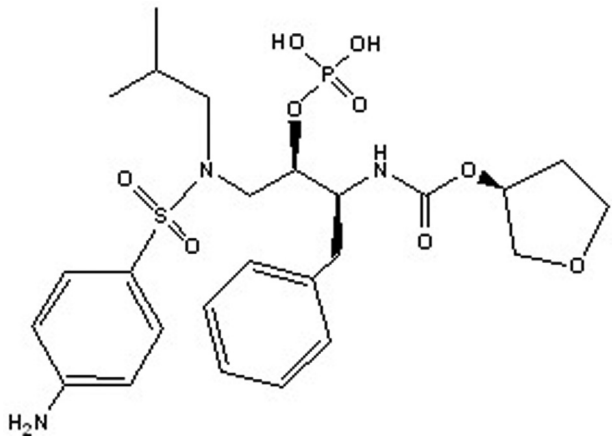
## Introduction

Fosamprenavir (FSV) is a prodrug of amprenavir (APV) and active-site inhibitor of HIV protease, rapidly and extensively metabolized to APV by the enzymes by the enzymes of the gut epithelium [1–3]. FPV has demonstrated antiviral efficacy, durability, and tolerability in antiretroviral therapy in HIV-positive and PI-experienced subjects [4]. FSV calcium, (1*S*,2*R*)-3-[[(4-aminophenyl)sulfonyl] (2-methylpropyl)amino]-1-(phenylmethyl)-2-(phosphonooxy)-propyl]carbamic acid C-[(3*S*)-tetrahydro-3-furanyl]ester calcium salt (Fig. 1), is the phosphate ester prodrug of HIV PI APV [5–7]. FSV was first approved by the Food and Drug Administration in 2003 [8] and then by the European Medicines Agency in 2004. Extensive

**Figure 1**



Chemical structure of fosamprenavir.

literature search showed one dissolution method [9], electrochemical evaluation method [10], ultraviolet, high-performance thin-layer chromatography [11], and stability indicating high-performance liquid chromatography (HPLC) method [12,13] and HPLC method for the estimation of drug from pharmaceutical formulations [14–19]. In few methods pharmacokinetic study of FSV was included but that is also with the ritronavir [20–22]. No LC-MS/MS method was reported for the estimation of FSV alone in spiked human along with pharmacokinetic profile. Hence, the present research work was planned to developed an LC-MS/MS method as per the US Food and Drug Administration bioanalytical method development [23] for the quantification of FSV alone and the application of the developed method to quantify the marketed FSV in rabbit plasma. Therefore, based on empirical evidences the authors established that this present method is easy, stable, reliable, and a validated method for the quantification of FSV in spiked human plasma using liquid chromatography tandem mass spectroscopy and its successful application to pharmacokinetic study using the rabbit model.

## Materials and methods

### Apparatus and software

The HPLC system with an autosampler was a Shimadzu LC-20ADvp (Shimadzu, Kyoto, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API-4000 Tandem mass spectrometer. The autosampler was SIL-HTC from Shimadzu. The solvent delivery module was LC-20AD from Shimadzu. Chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems, Concord, Ontario, Canada).

### Chemicals and reagents

FSV and FSV-D4 (IS) were procured from Hetero Drugs Ltd (Hyderabad, India) and formic acid was procured from Merck Specialities Pvt Ltd (Mumbai, India). Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in the laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J.T. Baker (USA).

### Calibration curve and quality control samples

The calibration curve (CC) and quality control (QC) samples of FSV were prepared from the aqueous dilutions separately. Stock solutions (10 000 ng/ml) were prepared by dissolving 1 mg of FSV in 2 ml of acetonitrile and make 100 ml with the diluent (mobile phase). From the stock solution, aqueous CC dilutions were made and from the aqueous CC samples plasma-spiked CC sample were prepared by diluting 0.15 ml aliquot from the various aqueous dilution. A measure of 0.85 ml of human plasma matrix was added to each tube and volumes were made up to 5 ml with a diluent to obtain the final CC sample concentration 0.5105–200.1853 ng/ml of FSV. The QC samples were also prepared in the same way from various aqueous QC dilutions to obtain the final concentration at a lower limit of quantitation (LLOQ) (0.512 ng/ml), lower quantitation limit (LQC) (1.506 ng/ml), medium quality control (MQC)-1 (40.165), MQC-2 (100.414), high-quality control (HQC) (170.193) levels.

### Chromatographic conditions

Chromatographic separation was performed on a ZORBAX SB-C18 column with 4.6 mm internal diameter with 5 μm particle size using acetonitrile: 5 mmol/l ammonium acetate in water (85 : 15, v/v) as a mobile phase. An isocratic elution technique was adopted with a flow rate of 600 μl. Injection volume was 20 μl. The retention time of FSV was 1.51 min and for deuterated fosamprenavir (FSV-D4) (IS) it was 1.62 min with a total runtime of 2.5 min.

### Mass spectrometric conditions

The LC eluent was split (75%) and ~0.25 ml/min was introduced and quantitation was achieved with MS/MS detection in a negative ion mode for the analytes and IS using an MDS Sciex API-4000 mass spectrometer (Concord, Ontario, Canada) equipped with electrospray ionization, operating in a positive ion mode, at 800°C. The source parameters: were capillary voltage, 3.5 kV; cone voltage, 35 kV; and source temperature, 4000°C. The compound parameters viz. the declustering potential, collision

energy, entrance potential, and collision cell exit potential for FSV and FSV-D4 are cited in Table 1. Detection of the ions was carried out in the multiple-reaction monitoring mode, by monitoring the transition pairs of m transitions of *m/z* 585.6–418.2 for FSV, as shown in Fig. 2 and *m/z* 589.2–469.1 for IS, as shown in Fig. 3.

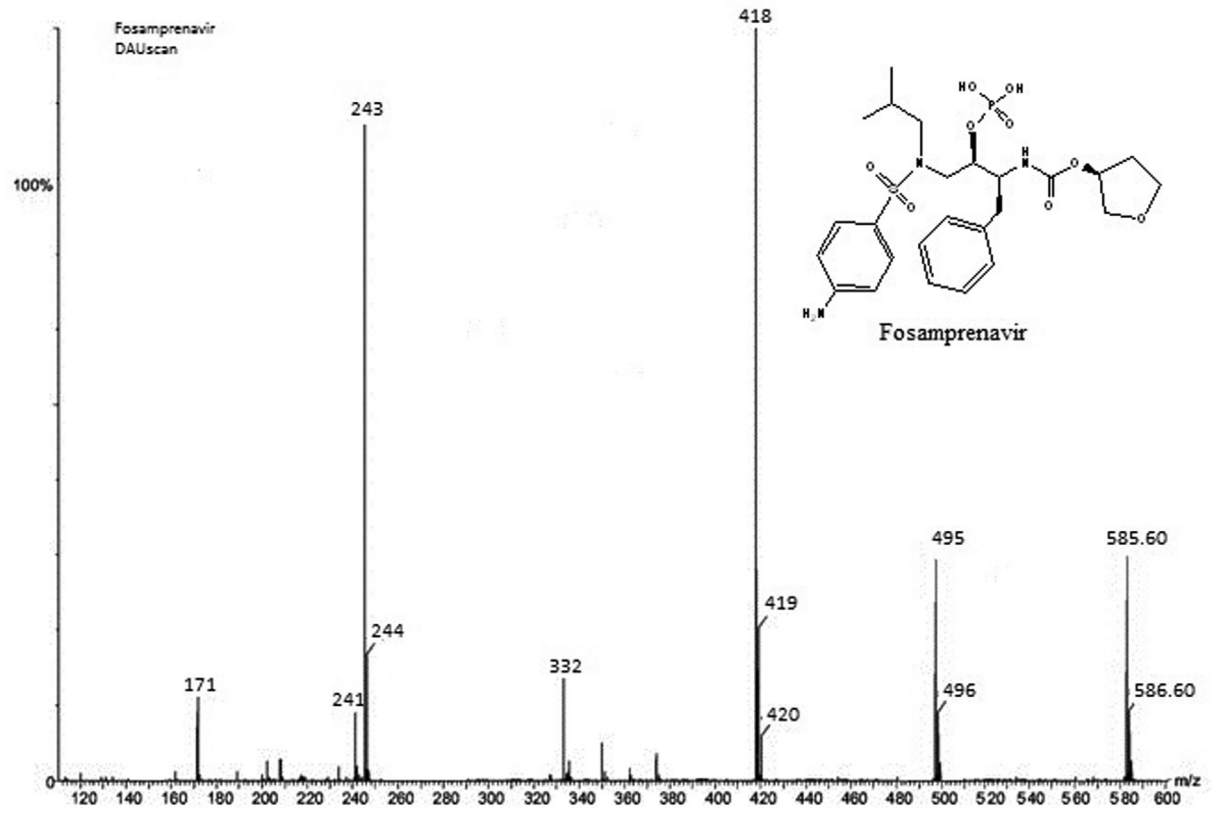
### Sample preparation method

A simple liquid–liquid extraction technique was adopted for sample preparation. The plasma samples of the analytes and internal standard for CC and QC samples were thawed at room temperature and the samples were vortexed to ensure complete mixing. A measure of 250 µl of FSV plasma sample was pipetted

**Table 1 LC-MS/MS operating conditions (positive ion mode)**

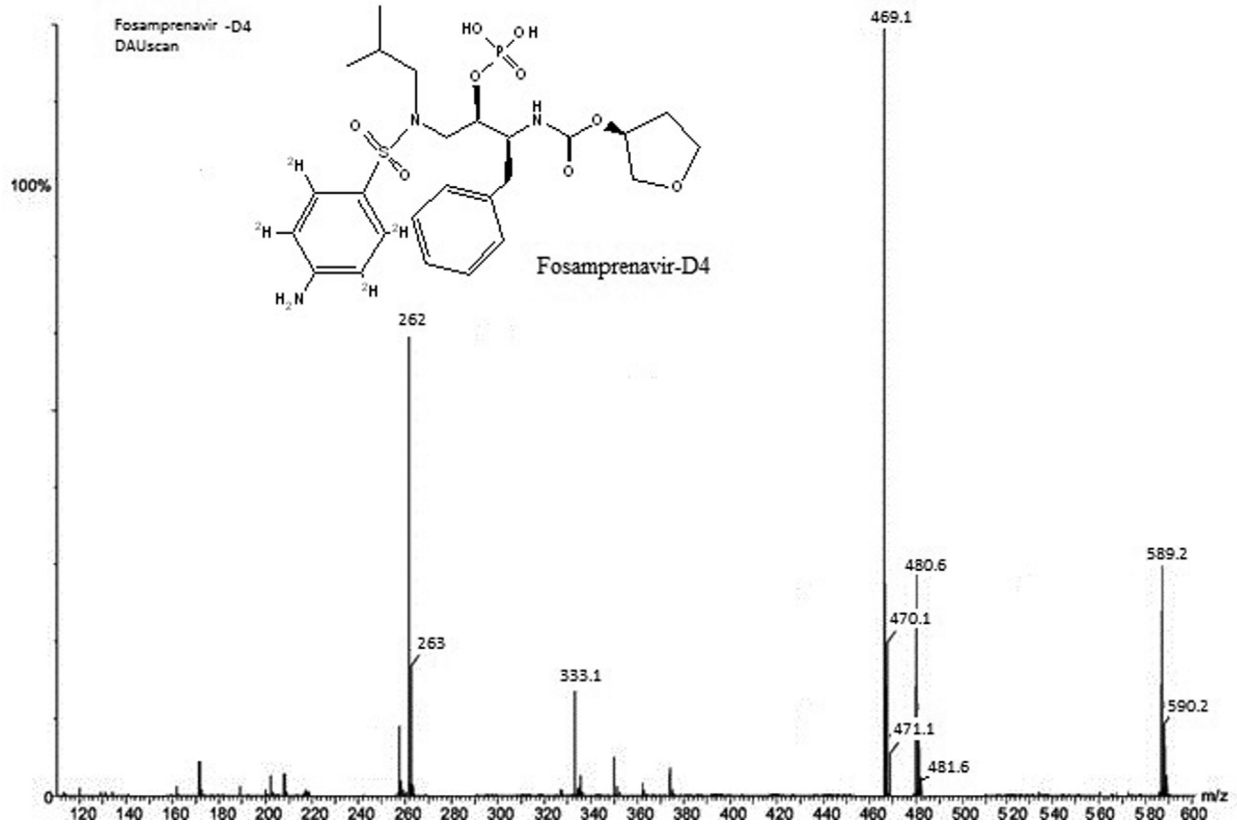
| Parameters | Fosamprenavir | Fosamprenavir-D4 (IS) |
|---|---|---|
| MRM (*m/z*) (Da) | | |
| Parent | 586.1 | 589.2 |
| Daughter | 418 | 469.1 |
| Ion spray (V) | 4000 | 5300 |
| Source temperature (deg.) | 400 | 400 |
| Dwell time (ms) | 200 | 200 |
| Curtain gas 1 (psi) | 20 | 20 |
| Declustering potential (V) | 80 | 80 |
| Capillary (kV) | 3.50 | 3.50 |
| Cone (V) | 35 | 35 |
| Collision energy | 30 | 30 |
| Collision exit potential | 20 | 18 |
| Extractor (V) | 5 | 5 |
| RF lens (V) | 0 | 0 |
| Desolvation temperature (deg.) | 800 | 800 |
| Desolvation gas flow (l/h) | 75 | 75 |

**Figure 2**




Product ion mass spectra of fosmaprenavir.

**Figure 3**




Product ion mass spectra of fosamprenavir-D4 (internal standard).

out and placed into a 15 ml Stoppard tube and 50 µl of internal standard FSV-D4 (50 ng/ml) was added to each Stoppard tube and vortexed the tubes except blank plasma samples where 25 µl of the diluent (mobile phase) was added. A quantity of ml of tert-butyl methyl ether was added as an extracting solvent to each tube and was shaken for 20 min on a reciprocating shaker at 200 rpm. The samples were then centrifuged at 2000 rpm for 10 min at 25°C. The supernatant layer was transferred into a prelabeled tubes and evaporated to dryness under nitrogen gas at 40°C. All the samples were reconstituted with 300 µl of mobile phase and transferred into autosampler loading vials and injected into the LC-MS/MS system.

### Pharmacokinetic study design

Six male albino rabbits (weighing about 2.5 kg) were selected as the animal model. The age of the rabbits was 8–12 weeks. The rabbits selected for the study had no medication for 2 weeks prior to the study. Twelve hours before drug administration, food was withdrawn from the rabbits until 24 h postdosing, while water was available for rabbits throughout the study. A tablet powder (FSV 700 mg from Mylan Pharmaceuticals) equivalent to 65 mg based on the animal surface area was administered to rabbits using a balling gun. The orally administered rabbit dose which was the human-equivalent FSV dose has been calculated as per the US-FDA guidelines for equivalent dose calculations [24]. The study protocol was approved by the Institutional Animal Ethics Committee of Balaji Institute of Pharmaceutical Sciences (Narsampet, India) with a CPCSEA no. 1694/PO/Re/S/13/CPCSEA. Blood samples (0.6 ml) were withdrawn from the marginal ear vein before dosing (zero time) and at time intervals of 0.25, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 h after administration. For each animal the total number of blood samples drawn during the study was 13. EDTA disodium salt was used as an anticoagulant. Plasma was separated by centrifugation at 5000 rpm for 10 min and the resulting plasma sample from each blood sample was divided into two aliquots and stored in suitably labeled polypropylene tubes at −20°C until used. All the plasma samples were analyzed under the construction of standard CC of FSV in rabbit's plasma. The FSV concentrations in the rabbit plasma samples were calculated using the CC, obtained after linear regression of the peak area ratio (FSV/FSV-D4) versus the concentration of FSV.

### Pharmacokinetic analysis

Single-dosage pharmacokinetic parameters were calculated using a PK solver tool from the plasma drug concentration–time data by noncompartmental

methods. The maximum plasma concentration ($C_{max}$) and time to maximum plasma concentration ($T_{max}$) were obtained directly from the observed concentration–time profiles. Linear trapezoidal rule was used to estimate the area under the plasma concentration versus time curve [area under curve (AUC)] from 0 to the last measurable concentration ($AUC_{0-t}$). The area under the plasma concentration versus time curve from 0 to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t}+ C_t/K_e$, where $C_t$ was the last measurable concentration. $K_e$ was the elimination rate constant. The terminal elimination half-life ($t_{1/2}$) was calculated as $0.693/K_e$.

### Validation

#### *Matrix effect*

It was determined at LQC and HQC level in eight replicates for FSV and internal standard. One set each of hemolytic and lipemic plasma samples was spiked with blank matrices (hemolytic) and another set each of LQC and HQC samples spiked with internal standard with lipemic plasma, were prepared. Six replicates of aqueous samples equivalent to final LQC and HQC concentrations were prepared. IS-normalized matrix factor was calculated by dividing the peak response area in the presence of matrix ion and mean peak area response ratio in the absence of matrix ion. The variability in IS-normalized factor was measured by the coefficient of variation which should be less than 15%.

#### *Linearity*

Linearity of the developed method was assessed in the concentration range of 0.15–200.18 ng/ml. Ten CC samples have been prepared by spiking with human plasma and processed. The CC has been constructed by using factor 1/(concentration ratio)$^2$ of the drug to an internal standard concentration to produce best fit for the concentration versus response relationship. The acceptance criteria for the linearity, $r^2$ (coefficient of correlation) should be at least 0.98.

#### *Recovery studies*

It was conducted to prove the extraction efficiency of the developed analytical process, by comparing the peak response from extracted and nonextracted samples. Six LQC, MQC, and HQC samples were prepared freshly and these samples were processed by adding internal standard and injected. For the preparation of nonextracted samples, 18 blank matrix samples were spiked with six sets of LQC, MQC, and HQC with internal standard and injected. The mean overall %recovery was calculated, and the percentage of difference should not be more than 25% between the highest and lowest %recovery value.

#### *Precision and accuracy*

Intraday precision and accuracy were determined by analyzing QC standards (1.5062, 100.41, and 170.19 ng/ml) and LLOQ_QC standard (0.5121 ng/ml) five times a day randomly; interday precision and accuracy were determined from the analysis of each QC standards (1.5062, 100.41, and 170.19 ng/ml) and LLOQ_QC standards (0.5121 ng/ml) once on each of the five different days. The acceptance criteria for accuracy (%nominal) is ±15 and ±20% only for LLOQ_ level and for precision percentage coefficient variation (%CV) it should be within ±15% and only for LLOQ_ sample it is ±20%.

### Matrix selectivity and specificity

Matrix selectivity was evaluated by analyzing the plasma of six different lots, including one hemolytic and one lipemic plasma to investigate the interference at the retention time of the FSV and internal standard. The interference at the retention time of internal standard was evaluated against the response of the extracted internal standard in LLOQ_ sample. The response of the interfering substance will be acceptable if it is less than 20% of the mean drug response in LLOQ_sample and is considered less than 5% in the case of internal standard.

### Carryover effect

This study was conducted to investigate the effect of carrying analytes and internal standard in different sections of the system. For this study, six replicates of the extracted blank matrix and six replicates of the extracted high concentration of analytes at the CC range upper limit of quantitation (ULOQ), six extracted LLOQ_ concentrations of the analytes and internal standard were prepared and injected as per the following sequence; first six injections was extracted LLOQ, followed by extracted ULOQ, and extracted blank plasma. ULOQ_ and blank plasma samples were injected alternatively. Response of interfering peaks in blank samples at the retention time of the analytes must be up to 20% of average drug response and for internal standard must be up to 5% of the average internal standard response in LLOQ_ samples.

### Ruggedness

To investigate the ruggedness of the developed method, one precision and accuracy batch of samples were processed and analyzed with different columns of the same make and with different reagent lots.

### Stability studies

The stability study (wet extract, benchtop freeze–thaw, autosampler, short-term, and long-term stability) of FSV was performed using freshly prepared CC samples and QC samples. The samples were prepared and analyzed at low, middle, and high level. Concentration response linearity data were collected and used to calculate the concentration of stability samples.

#### *Room temperature stability study*

This study was performed by considering the stock solution prepared for at least a period of 6 h. Fresh stock solution of analyte and internal standard were prepared. Six replicates of fresh and comparison samples were injected immediately and the percentage of stability was calculated. It must be between 95–105% and %CV should be less than 10%.

#### *Refrigerator stock solution stability*

Six replicates of the stock solution was prepared for this study and stored in the refrigerator at 2–8°C for 4 days. On the evaluation day, a fresh standard stock solution was prepared (comparison sample) equivalent to the final MQC concentration of the analytes with a final concentration of internal standard in a reconstituted solution. All the comparison and stability samples were injected. Percentage of stability was also calculated for the analytes and internal standard and it must be between 95–105% and %CV must be less than 10%.

#### *Benchtop stability*

Six sets of LQC and HQC samples were removed from the deep freezer and placed unprocessed for a period of 12 h. Six sets of fresh QC samples (low, middle, and high) and calibration samples were prepared. Benchtop stability samples were analyzed along with fresh samples. the concentration was calculated from the linearity data.

#### *Autosampler stability*

For this study, six sets of QC samples were prepared in LQC and HQC levels and were kept in an autosampler for 3 days. The concentrations of all the stability samples were calculated against the freshly prepared spiked CC and QC samples at all three levels.

#### *Freeze–thaw stability*

This study was conducted by four freeze–thaw cycles. Six replicates of LQC and HQC samples were collected from the deep freezer and after 24 h the first six samples were withdrawn and thawed at room temperature and refrozen again. In a similar way, the remaining samples were also withdrawn after the next 12 h followed by another 12 h and refrozen again. Stability samples were analyzed by quantifying along with freshly spiked calibration samples and QC samples at three levels.

#### *Wet extract stability*

Wet extract stability study was conducted with six replicates of LQC and HQC samples. The samples were kept for 1 day at room temperature (20±5°C). Then the samples were injected with freshly spiked CC and QC samples at low, middle, and high levels. The amount of analytes in stability samples were calculated in comparison with freshly prepared samples.

#### *Short-term stability at –200°C*

For this study, six sets of QC samples at low and high levels were prepared and stored in a deep freezer at −20°C after spiking. At the day of evaluation, the samples were processed along with freshly prepared QC samples in all levels and CC samples. The concentration of the stability samples were calculated in comparison to freshly prepared samples.

#### *Long-term stability at –700°C*

The long-term stability study was investigated with LQC and HQC samples. The prepared samples were kept for 30 days at −70°C. On the day of evaluation all samples were processed with freshly prepared CC and QC samples. All the stability samples were quantified from the CC data. For all stability samples mean % nominal concentration at each QC level must be between 85 and 115% and the precision must be up to 15% of the %CV. At least 67% of the stability QC samples should be within 15% of their respective nominal values.

## Results

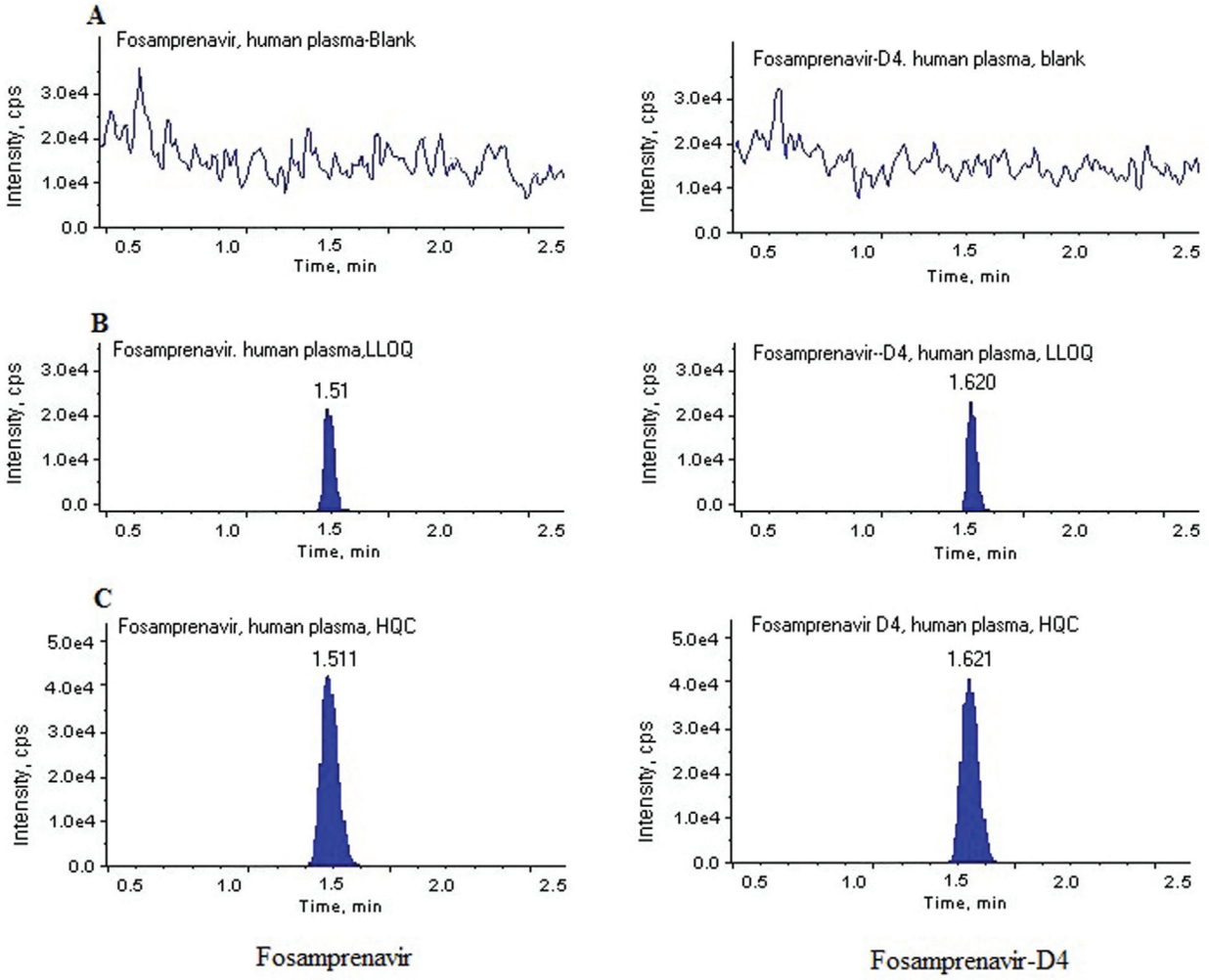
### Mass spectrometry

The parameters of mass has been optimized after proper tuning in both positive and negative ion mode FSV and internal standard FSVD-4. Finally the positive ion mode with multiple-reaction monitoring was selected for specificity. All optimized mass parameters are cited in Table 1. Protonated analyte (FSV) and internal standard $[M+H]^+$ ion were the parent ion in the quadropole-1 segment and that was used as a precursor ion to obtain quadropole-3 product ion spectra as shown in Fig. 4.

### Method development

Several trials have been conducted to achieve the optimized chromatographic conditions using different mobile phases, columns, etc. Several combinations of

**Figure 4**




MRM chromatograms of fosamprenavir, human blank plasma (a), LLOQ samples with internal standard (right panel) (b), HQC samples with internal standard (right panel) (c). HQC, high-quality control; LLOQ, lower limit of quantitation.

acetonitrile and buffers have been tested during initial trials, different types of columns like C18, C8, of waters, hypersil, and kromasil have been used and finally ZORBAX SB-C18 column with 4.6 mm internal diameter with 5 µm particle size using acetonitrile: 5 mmol/l ammonium acetate in water (85 : 15, v/v) as a mobile phase has been selected. In the above-specified condition the peak shape was satisfactory with reasonable retention times for the analyte (FSV) and internal standard even at very low QC samples. Initially several compounds were investigated to select a suitable internal standard, finally FSV-D4 was selected because the retention time and other values were very selective, and reproducible with FSV. Under this optimized condition, the retention time of FSV was found to be 1.51 min, for FSV-D4 it was 1.62 min as shown in Fig. 4.

### Prevalidation and validation

The obtained response for blank samples was found 0, so the calculated % carryover is also 0, which is satisfied well with the acceptance criteria. The 'internal standard normalized factor' was calculated for the investigation of matrix effect for the analyte. The %CV of the IS-normalized factor was found to be 2.09 and 1.14 for FSV LQC and HQC samples, which satisfied the acceptance criteria. Interday precision for LLOQ, LQC, and HQC samples of FSV was found 3.07, 1.96, 2.38, 1.83, and 1.77. Interday accuracy for LLOQ, LQC, MQC-1, MQC-1, and HQC were found to be 97.54, 100.35, 99.12, 102.02, and 97.86 for FSV. Intraday accuracy and precision results for FSV was 3.13, 5.52, 1.74, 1.52, and 1.67 for all the levels. The accuracy values of 99.21, 103.8, 99.84, 103.35, and 97.72 for all QC levels were found within acceptance criteria shown in Table 2. In the study of matrix selectivity, the response of interfering peaks at the retention time of FSV and IS was found 0% of the mean drug response. Analyte selectivity study results show that there was no interference at the retention time of both

**Table 2 Accuracy and precision data of fosamprenavir in the human plasma**

| | Blonanserin measured concentration (ng/ml) | | | | |
|---|---|---|---|---|---|
| Quality control levels | Day[a] | Mean | SD | %CV | %Nominal |
| *Interday* | | | | | |
| LLOQ (0.5121 ng/ml) | 1 | 0.50510 | 0.0170 | 3.38 | 98.63 |
| | 2 | 0.49512 | 0.0023 | 2.46 | 97.86 |
| | 3 | 0.51265 | 0.0125 | 3.89 | 98.12 |
| | 4 | 0.48634 | 0.0534 | 2.57 | 97.13 |
| LQC (1.5062 ng/ml) | 1 | 1.51049 | 0.037 | 2.51 | 100.26 |
| | 2 | 1.52345 | 0.021 | 1.35 | 100.68 |
| | 3 | 1.50353 | 0.034 | 2.22 | 100.12 |
| | 4 | 1.52703 | 0.033 | 1.78 | 100.84 |
| MQC-1 (40.1657 ng/ml) | 1 | 39.81815 | 0.648 | 1.63 | 99.21 |
| | 2 | 40.74510 | 0.003 | 2.34 | 99.84 |
| | 3 | 38.80612 | 0.044 | 3.02 | 98.87 |
| | 4 | 39.84835 | 0.056 | 2.54 | 99.15 |
| MQC-2 (100.4143 ng/ml) | 1 | 103.29150 | 1.649 | 1.60 | 102.87 |
| | 2 | 102.89443 | 1.345 | 1.79 | 102.15 |
| | 3 | 101.76253 | 1.119 | 1.55 | 101.50 |
| | 4 | 101.56350 | 1.146 | 2.38 | 102.32 |
| HQC (170.1937 ng/ml) | 1 | 165.70903 | 2.918 | 1.76 | 97.48 |
| | 2 | 168.99805 | 2.330 | 1.56 | 98.12 |
| | 3 | 169.94458 | 1.793 | 1.33 | 98.76 |
| | 4 | 167.41902 | 2.338 | 2.45 | 96.87 |
| *Intraday* | | | | | |
| LLOQ | | 0.50807 | 0.015 | 3.13 | 99.21 |
| LQC | | 1.56469 | 0.086 | 5.52 | 103.8 |
| MQC-1 | | 40.10331 | 0.698 | 1.74 | 99.84 |
| MQC-2 | | 103.77931 | 1.580 | 1.52 | 103.35 |
| HQC | | 166.31386 | 2.772 | 1.67 | 97.72 |

%CV, percentage coefficient variation; HQC, high-quality control; LLOQ, lower limit of quantitation; LQC, lower quantitation limit; MQC, medium quality control. [a]Each day includes six replicates.

analytes in the presence of internal standard and also found 0% interference at the retention time of internal standard in the presence of FSV. A regression equation with a weighing factor of 1/(concentration ratio)$^2$ of drug to the internal standard concentration shows the best fit relationship with a correlation coefficient ($r^2$) of 0.998 in the concentration range of 0.510–200.185 ng/ml for FSV, as shown in Table 3. The mean overall recovery of FSV was found to be 94.75% with a precision range of 1.76–4.20% and a percentage difference of 4.34. Internal standard mean overall recovery was found to be 101.36% which satisfied the acceptance criteria. In ruggedness study within the batch precision of LLOQ, LQC, MQC, and HQC ruggedness FSV samples were found to be 3.24, 0.78, 0.646, and 0.381%. The accuracy values for FSV were 99.53, 98.68, 98.10, and 99.82%.

**Table 3 Calibration curve concentrations and obtained percentage accuracy of individual concentration**

| Calibration curve concentrations (ng/ml) | Mean of back calculated concentrations[a] (ng/ml) | SD | % Accuracy |
|---|---|---|---|
| 0.510 | 0.5204 | 0.0041 | 101.36 |
| 1.020 | 0.9926 | 0.0054 | 97.15 |
| 2.552 | 2.5064 | 0.0199 | 100.17 |
| 5.104 | 4.9591 | 0.0034 | 98.00 |
| 10.209 | 10.3544 | 0.0053 | 101.00 |
| 30.027 | 30.2733 | 0.0177 | 102.20 |
| 60.055 | 59.4865 | 0.0597 | 90.00 |
| 120.111 | 115.5854 | 0.0311 | 89.46 |
| 160.148 | 178.9084 | 0.0256 | 105.00 |
| 200.185 | 192.6631 | 0.0567 | 98.80 |

[a]Mean of three runs of each concentration.

### Stability study

The results of room temperature (20±5°C) stability study was found within the acceptance criteria as the calculated %stability for FSV and internal standard were 98.4, and 98.8. In refrigerator stock solution stability study (2–8°C), the calculated %stability for FSV was found to be 97.23 and for internal standard it was 98.68. The results of benchtop stability study was found to be 94.03 and 99.44% as the mean % nominal of LQC and HQC samples. After 72 h of suitable stability period in an autosampler, the % nominal (accuracy) was found 100.80 and 95.91% for FSV at LQC and HQC level. The four cycles of

freeze–thaw stability sample shows %nominal of 98.06 and 98.56% in LQC and HQC samples of FSV. In 24 h wet extract stability study, the % nominal value for FSV was found to be 98.33 and 94.11% in LQC and HQC, satisfied acceptance criteria for stability study. The results of short-term stability samples satisfied the acceptance limit as the calculated %nominal for FSV was 98.28 and 96.47.76% at the LQC and HQC levels. The mean % nominal for long-term stability (30 days at −70°C) were 94.57% for LQC and 98.45% for HQC stability samples of FSV. The summary of all stability study results are demonstrated in Table 4.

### Results of pharmacokinetic studies

In pharmacokinetic study, mean plasma concentrations of FSV at various time intervals was calculated and found to be 0.0, 10.33, 18.33, 42.83, 60.67, 91.67, 98.67, 95.83, 82.83, 52.00, 11.67, 2.667, 0.00, and 0.00 ng/ml at 0, 0.15, 0.50, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 h of the study. The details are given in Table 5. The drugs were eluted at their corresponding retention time and no metabolites were found with a runtime of 2.5 min. The pharmacokinetic profile of FSV was reported in a previous research work by Ford *et al.* [25], who conducted the study using FSV 1400 mg and found $AUC_{0\rightarrow 24}$ (μg h/ml)=20.67, $AUC_{0\rightarrow\infty}$=22.05 μg h/ml, $C_{max}$=4.73 μg/ml, $T_{max}$=1.5 h. In comparison to the previous work, we found that the pharmacokinetic parameters of FSV (65 mg equivalent tablet powder) was calculated from the plasma concentration–time curves, as shown in Fig. 5, using PK solver 2 (a menu-driven adding program for MS excel). Also, the area under the plasma concentration–time curve from 0 to 12 h ($AUC_{0-12}$) was calculated using the trapezoidal rule. FSV showed $T_{max}$=2 h and the mean $C_{max}$, $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\alpha}$ for the test formulation is 98.6, 351.3, and 354.9, respectively. The results are presented in Table 6.

**Table 4 Stability data of fosamprenavir**

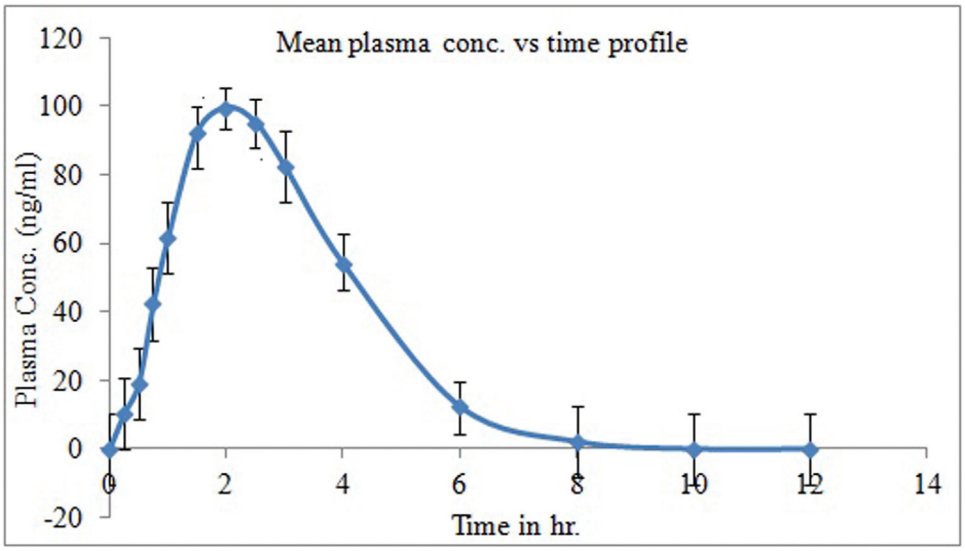
| Quality control levels | Type of stability | Fosamprenavir | | | |
|---|---|---|---|---|---|
| | | Mean[a] | SD | %CV | %Nominal |
| LQC (1.5062 ng/ml) | Benchtop | 1.42402 | 0.01452 | 2.93 | 94.03 |
| | Freeze–thaw | 1.48435 | 0.00756 | 2.87 | 98.06 |
| | Autosampler | 1.51738 | 0.02342 | 4.66 | 100.80 |
| | Wet extract | 1.48234 | 0.01323 | 5.54 | 98.33 |
| | Short term | 1.47352 | 0.00775 | 3.67 | 98.28 |
| | Long term | 1.41885 | 0.03432 | 9.04 | 94.57 |
| HQC (170.1937 ng/ml) | Benchtop | 171.356 | 0.65879 | 5.04 | 99.44 |
| | Freeze–thaw | 167.342 | 0.46320 | 6.79 | 98.56 |
| | Autosampler | 162.340 | 0.35460 | 3.80 | 95.91 |
| | Wet extract | 160.447 | 0.28799 | 2.60 | 94.11 |
| | Short term | 164.321 | 0.75695 | 6.34 | 96.47 |
| | Long term | 168.432 | 0.65789 | 2.54 | 98.45 |

%CV, percentage coefficient variation; HQC, high-quality control; LQC, lower quantitation limit. [a]Mean of six replicates.

**Table 5 Plasma drug concentrations of animals at various time intervals**

| Time (h) | Plasma drug concentration (ng/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Rabbit 1 | Rabbit 2 | Rabbit 3 | Rabbit 4 | Rabbit 5 | Rabbit 6 | Average | SD |
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.25 | 10.23 | 9.32 | 8.04 | 14.98 | 12.33 | 9.10 | 10.33 | 2.25 |
| 0.5 | 18.57 | 14.09 | 12.32 | 23.89 | 22.10 | 21.09 | 18.33 | 4.5 |
| 0.75 | 38.47 | 32.00 | 41.36 | 45.10 | 49.46 | 52.09 | 42.83 | 7.36 |
| 1 | 59.98 | 61.09 | 67.91 | 61.01 | 57.21 | 59.23 | 60.67 | 3.44 |
| 1.5 | 83.12 | 92.09 | 94.45 | 94.33 | 98.35 | 89.35 | 91.67 | 5.16 |
| 2 | 91.43 | 101.74 | 103.63 | 95.21 | 105.11 | 97.12 | 98.67 | 5.28 |
| 2.5 | 104.23 | 89.87 | 98.76 | 90.32 | 92.25 | 102.13 | 95.83 | 6.4 |
| 3 | 88.09 | 81.76 | 69.93 | 82.03 | 87.91 | 90.35 | 82.83 | 7.63 |
| 4 | 51.63 | 56.46 | 34.02 | 57.64 | 58.21 | 62.02 | 53 | 9.96 |
| 6 | 16.12 | 10.43 | 8.56 | 7.45 | 13.02 | 16.32 | 11.67 | 3.93 |
| 8 | 2.66 | 3.51 | 1.65 | 2.76 | 3.75 | 5.98 | 2.667 | 1.37 |
| 10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

**Figure 5**




Plasma concentration versus time profile of fosamprenavir.

**Table 6 Calculated mean values of pharmacokinetic parameters for test animals**

| Parameters | Unit | Value |
|---|---|---|
| Lambda (*z*) | 1/h | 0.747365665 |
| $t_{1/2}$ | h | 0.927453873 |
| $T_{max}$ | h | 2 |
| $C_{max}$ | ng/ml | 98.66666667 |
| $T_{lag}$ | h | 0 |
| Clast_obs/$C_{max}$ | | 0.027027027 |
| $AUC_{0-t}$ | ng/ml×h | 351.3333333 |
| $AUC_{0-inf\_obs}$ | ng/ml×h | 354.9014216 |
| $AUC_{0-t/0-inf\_obs}$ | | 0.989946255 |
| $AUMC_{0-inf\_obs}$ | ng/ml×$h^2$ | 1019.954343 |
| $MRT_{0-inf\_obs}$ | h | 2.873908869 |
| Vz/F_obs | mg/ng/ml | 2.639107975 |
| Cl/F_obs | mg/ng/ml/h | 1.972378687 |

## Discussion

The results from the optimization of mass parameters and LC operation conditions indicate the separation FSV in spiked human plasma with specific, good peak shape and reasonable retention times for FSV and internal standard (FSV-D4). Validation parameters were within the acceptance criteria as per the US-FDA bioanalytical method development guidelines. A study of the matrix effect has shown limited 'IS-normalized factors' for FSV which indicates that there was no such significant matrix ion effect for the analyte and internal standard. The carryover test results show 0% carryover in LLOQ and ULOQ samples of analytes and internal standard which satisfied the acceptance criteria. The matrix and analyte selectivity study results proved that the developed LC-MS/MS method was found selective because of 0% of interference. The results of interday and intraday precision and accuracy in all QC levels demonstrated that the % CV values and %nominal values were within the acceptance criteria and were found to be accurate and precise. The method was found linear in 0.51–200.18 ng/ml with a regression coefficient of more than 0.99 in FSV, which proved the linearity of the present method. The results of the mean overall recovery of FSV and internal standards in LQC, MQC, and HQC levels were found to be extremely satisfactory recovery and differences in percent of recovery were within the acceptance criteria. The calculated accuracy and precision values in ruggedness study were within 15–20% in all QC levels which were within the limits and confirms the ruggedness of the present method. The calculated % of stability values in stock solution stability at room temperature (±25°C) and refrigerator (2–8°C, 4 days) were found satisfactory and within the limit which confirms the stability of the present method. In benchtop, wet extract, freeze–thaw, autosampler, short-term and long-term (−70°C, 30 days) stability studies, and all stability samples have been compared with freshly prepared samples and concentration was back calculated from the CC sample. In all types of stability study, the mean %nominal values were found within 94–101% and the %CV values were less than 10%, which proves the stability of the developed method. The pharmacokinetic study results show that FSV maximum concentration in the plasma ($C_{max}$) is 98.6 and $T_{max}$ was achieved at 2 h. The present developed method was implemented well during the analysis of pharmacokinetic samples. The

plasma concentration and time curves for FSV was sufficiently smooth to derive all parameters (Fig. 5) The elimination half-life and clearance were well fitted with a one-compartment model. The sensitivity, selectivity, and specificity of the developed method were sufficient enough for the characterization of pharmacokinetic profile of FSV in rabbits.

## Conclusion

Empirical evidences from the results demonstrated that the method is satisfactorily validated, more feasible, and cost-effective because of the utilization of convenient liquid–liquid drug extraction and limitable validation parameters. The obtained plasma concentration versus time profile was very selective for the drug, and the obtained pharmacokinetic parameters indicate the specific applicability of the developed method. Hence, the present developed first reporting the bioanalytical method for the estimation of FSV alone in spiked human plasma can undoubtedly be highly applicable for the quantitative analysis of FSV during clinical trials and toxicological study in human and other experimental animals.

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### Conflicts of interest

There are no conflicts of interest.

## References

1 Seminari E, Bona AD, Gentilini G, Galli L, Schira G, Gianotti N, *et al.* Amprenavir and ritonavir plasma concentrations in HIV-infected patients treated with fosamprenavir/ritonavir with various degrees of liver impairment. J Antimicrob Chemother 2007; 60:831–836.

2 Furfine ES, Baker CT, Hale MR, Reynolds DJ, Salisbury JA, Searle AD, *et al.* Preclinical pharmacology and pharmacokinetics of GW433908, a water-soluble prodrug of the human immunodeficiency virus protease inhibitor amprenavir. Antimicrob Agents Chemother 2004; 48:791–798.

3 Wood R, Arasteh K, Stellbrink HJ, Teofilo E, Raffi F, Pollard RB, *et al.* Six-week randomized controlled trial to compare the tolerabilities, pharmacokinetics, and antiviral activities of GW433908 and amprenavir in human immunodeficiency virus type 1-infected patients. Antimicrob Agents Chemother 2004; 48:116–123.

4 Gathe JC, Wood R, Schurmann D, Bellos NC, DeJesus E, Gladysz A, *et al.* SOLO: 48 week efficacy and safety comparison of once-daily fosamprenavir/ritonavir versus twice-daily nelfinavirin native HIV-1 infected patients. AIDS 2004; 18:1529–1537.

5 Corbett AH, Kashuba ADM. Fosamprenavir Vertex Pharmaceuticals/GlaxoSmithKline. Curr Opin Investig Drugs 2002; 3:384–390.

6 Falcoz C, Jenkins JM, Bye C, Hardman TC, Kenney KB, Studenberg S, *et al.* Pharmacokinetics of GW433908, a prodrug of amprenavir, in healthy male volunteers. J Clin Pharmacol 2002; 42:887–898.

7 Furfine ES, Baker CT, Hale MR, Reynolds DJ, Salisburu JA, Searle AD, *et al.* Preclinical pharmacology and pharmacokinetics of GW433908, a water-soluble prodrug of the human immunodeficiency virus protease inhibitor amprenavir. Antimicrob Agents Chemother 2004; 48:791–798.

8 FDA; Food and Drug Administration, Orange Book. Approved drug products with therapeutic equivalence evaluations, 2009.

9 Rossi RC, Dias CL, Bajerski L, Bergold AM, Froehlich PE. Development and validation of discriminating method of dissolution for fosamprenavir tablets based on in vivo data. J Pharm Biomed Anal 2011; 54:439–444.

10 Gumustas M, Ozkan SA. Electrochemical evaluation and determination of antiretroviral drug fosamprenavir using boron-doped diamond and glassy carbon electrodes. Anal Bioanal Chem 2010; 397:189–203.

11 Pekamwar SS, Bhavar GB, Aher KB, Kakad SJ. Validated UV spectrophotometric and HPTLC method for determination of fosamprenavir calcium in pharmaceutical formulation. Austin J Anal Pharm Chem 2015; 2:1049.

12 Chilukuri M, Reddy PN, Reddy KH. Development and validation of stability indicating RP-HPLC method for estimation of fosamprenavir calcium in pure and pharmaceutical dosage forms. Chromatogr Sci 2014; 52:781.

13 Mallikarjuna Rao N, Gowrisankar D. Development and validation of stability indicating RP-HPLC method for estimation of fosamprenavir calcium in pure and pharmaceutical dosage forms. Asian J Chem 2015; 27:3484–3488.

14 Pekamwar SS, Bhavar GB, Aher KB, Kakad SJ. Development of high performance liquid chromatography (HPLC) method for the estimation of fosamprenavir calcium in pharmaceutical formulation. Austin Chromatogr 2015; 2:1035.

15 Aboul-Enein HY, Ali I. Determination of tadalafil in pharmaceutical preparation by HPLC using monolithic silica column. Talanta 2005; 65:276–280.

16 Ali I, Gaitonde VD, Aboul-Enein HY, Hussain A. Chiral separation of β-adrenergic blockers on CelluCoat column by HPLC. Talanta 2009; 78:458–463.

17 Ali I, Saleem K, Hussain I, Vinay DG, Aboul-Enein HY. Polysaccharides chiral stationary phases in liquid chromatography. Sep Purif Rev 2009; 38:97–147.

18 Ali I, Aboul-Enein HY. Impact of immobilized polysaccharide chiral stationary phases on enantiomeric separations. J Sep Sci 2006; 29:762–769.

19 Aboul-Enein HY, Ali I. Optimization strategies for HPLC enantioseparation of racemic drugs using polysaccharides and macrocyclic glycopeptide antibiotic chiral stationary phases. Farmaco 2002; 57:513–529.

20 Shelton MJ, Wire MB, Lou Y, Adamkiewicz B, Min SS. Pharmacokinetic and safety evaluation of high-dose combinations of fosamprenavir and ritonavir. Antimicrob Agents Chemother 2006; 50:928–934.

21 Luber AD, Brower R, Kim D, Silverman R, Peloquin CA, Frank I. Steady-state pharmacokinetics of once-daily fosamprenavir/ritonavir and atazanavir/ritonavir alone and in combination with 20 mg omeprazole in healthy volunteers. HIV Med 2007; 8:457–464.

22 Perez-Elias MJ, Morellon ML, Ortega E, Hernandez-Quero J, Rodriguez-Torres M, Clotet B, *et al.* Pharmacokinetics of fosamprenavir plus ritonavir in human immunodeficiency virus type 1-infected adult subjects with hepatic impairment. Antimicrob Agents Chemother 2009; 53:5185–5196.

23 US Food and Drug Administration, Guidance for Industry (2013). Bioanalytical method validation. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf. [Accessed 12 March 2018].

24 Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016; 7:27–31.

25 Ford SL, Wire MB, Lou Y, Baker KL, Stein DS. Antimicrob Agents Chemother 2005; 49:467.