Objective: The main aim of the present study was to develop a sensitive liquid chromatography–electrospray ionization–tandem mass spectrometric technique for the quantification of amprenavir in human plasma.

Methods: Chromatographic separation was achieved on a reversed-phase Symmetry C18 (50 mm×4.6 mm, 3.5 μm) column with isocratic elution by acetonitrile and 0.1% v/v formic acid in the ratio of 90:10 v/v as mobile phase. Chromatographic peaks were resolved with 0.7 ml/min flow rate. Drug was extracted with ethyl acetate solvent by liquid–liquid extraction method. Monitoring of transition of m/z 506.2 and 71.0 for amprenavir and 628 and 421 for methyl-indinavir was made on multiple reaction monitoring. Calibration curve of amprenavir was linear over 1–600 ng/ml concentration range with regression coefficient (r²) value of >0.99. The % relative standard deviation values were <8.5% for interday and intraday precision and accuracy. The method has excellent recovery, and the percentage recovery values of lower quality control (QC), median QC, and higher QC samples were 101.86%, 102.8%, and 99.28%, respectively.

Results: Calibration curve of amprenavir was linear over 1–600 ng/ml concentration range with regression coefficient (r²) value of >0.99. The % relative standard deviation values were <8.5% for interday and intraday precision and accuracy. The method has excellent recovery, and the percentage recovery values of lower quality control (QC), median QC, and higher QC samples were 101.86%, 102.8%, and 99.28%, respectively.

Conclusion: The drug was stable for more time at variable stability conditions, and method was successfully applicable to regular analysis of amprenavir in biological matrices.

Keywords: Amprenavir, Protease inhibitor, Liquid chromatography tandem-mass spectrometry, Food and Drug Administration guidelines and dilution integrity.

INTRODUCTION

Amprenavir is a protease inhibitor with activity against human immunodeficiency virus type-1 (HIV-1). Protease inhibitors block the part of HIV called protease. HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Amprenavir chemically designated as (3S)-oxolan-3-yl N-[(2S,3R)-3-hydroxy-4-[N-(2-methylpropyl)][4-amino benzenesulfonamido]-1-phenylbutan-2-yl] carbamate with molecular formula C₃₅H₃₅N₆O₅S (Fig. 1) and molecular weight 505.626 g/mol [1-3]. Amprenavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Amprenavir inhibits the HIV viral protease enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles. Hepatic amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The two major metabolites result from oxidation of the tetrahydrofuran and aniline moieties. Glucuronide conjugates of oxidized metabolites have been identified as minor metabolites in urine and feces [4,5].

Drug literature review discloses only few analytical quantification methods for the amprenavir in bulk, formulations, and biological matrices. The reported analytical techniques were high-performance liquid chromatography (HPLC) [6], spectroscopic [7], and liquid chromatography tandem-mass spectrometry (LC-MS/MS) [8]. Goal of the research was to develop a fast and sensitive LC-MS/MS technique for the quantification of amprenavir in human plasma samples and application of method validation as per the regulatory guidelines.

METHODS

Chemicals and reagents

Amprenavir (purity: 99.87%) was obtained from MSN Laboratories, India. Internal standard (IS) (methyl-indinavir) of 99.81% was acquired from hetero drugs Pvt. Ltd., Hyderabad, India. Acetonitrile of HPLC grade and formic acid of analytical grade were bought from J.T. Baker, Mumbai, India. In the present research work, water used from Milli-Q water purification system installed in the laboratory obtained from Bengaluru, India.

LC-MS/MS system

A modular LC system (Shimadzu, Japan) equipped with a DGU-20A3 solvent degasser, binary LC-20A3 prominence pump, column temperature ovens (CTO)-ASVP oven for column, and high-throughput SILHTC autosampler were utilized for the present research. Chromatography was achieved on a reversed-phase (RP) Symmetry C18 (50 mm×4.6 mm, 3.5 μm) with isocratic elution by acetonitrile and 0.1% v/v formic acid in the ratio of 90:10 v/v as mobile phase. Chromatographic peaks were resolved by the mobile phase with a flow rate of 0.7 ml/min. Amprenavir and methyl-indinavir internal standard were separated in the total run time of 5 min. The autosampler temperature and analytical column temperatures were kept at 5°C and 35°C, respectively.

The eluents of the LC system were infused into the electrospray ionization source operated with positive ionization method. Starting 0.5 min eluent was avoided from the chromatographic system to evade unnecessary impurities from the various salts existed in the human plasma samples.
In the mass system, following conditions were applied: Gas 1 – nitrogen (40 psi), gas 2 – nitrogen (40 psi), temperature of ion source – 400°C, curtain gas – nitrogen (25 psi), and voltage of ion spray – 5000 V. Monitoring of transition of m/z 506.2 and 71.0 for amprenavir and 628 and 421 for methyl-indinavir was made on multiple reaction monitoring. The mass conditions are presented in Table 1.

### Sample preparation
To 250 µl of spiked plasma, 50 µl of methyl-indinavir (1 µg/ml) was mixed and sonicated for 15 s. To the resulting solution, 500 µl of ethyl acetate was added and vortexed for 5 min, followed by centrifuged at 5000 rpm for 10–20 min at 5°C. The organic phase was dried in a lyophilizer. The final residue was dissolved in 200 µl of mobile phase and transferred into a pm-labeled autosampler vials and infused into an LC-MS/MS system.

### Preparation of standard stock and calibration standards (CCs)
Amprenavir and IS stock solutions were processed in 60% methanol at concentration level of 1000 µg/ml. Quality control (QC) and CC solutions were prepared by spiking blank human plasma a sample from the amprenavir stock solution. CC solutions of eight concentration levels were prepared to produce the final concentrations of 2.0, 4.0, 20.0, 40.0, 100.0, 200.0, 300.0, and 600.0 ng/ml. Lower QC (LQC) standard, median QC (MQC) standard, and higher QC (HQC) standards were QC sample solutions and were prepared to produce the concentrations of 4, 200, and 500 ng/ml, respectively. All the stock, CC and QC solutions were stored at −20°C till the method of analysis.

### Validation
The method of analysis was assessed by validation parameters such as sensitivity, precision, linearity, recovery, dilution integrity, accuracy, matrix effect (ME), and stability. Three QC samples of LQC, MQC, and HQCs as well as lower limit of quantification (LLOQ) were employed and analyzed in method validation [9-12].

### Precision and accuracy (PA)
Intraday and interday PA were examined as a part of PA parameter. Intraday PA was evaluated by injecting QC solutions (2, 200, and 500 ng/ml) and LLOQ (1.0 ng/ml) in 5 replicates in a day arbitrarily. Interday PA was evaluated by injecting the same QC and LLOQ solutions once in a day for 5 different days. The % relative standard deviations (RSDs) for LQC, MQC, and HQCs should be ≤±20% for LLOQ and ≤±15% for the remaining QC standards [13].

### Linearity
CC standards (non-zero) of 8 different concentrations at 2.0, 4.0, 20.0, 40.0, 100.0, 200.0, 300.0, and 600.0 ng/ml were prepared and analyzed in three separate runs. Linearity curve (peak area ratio of amprenavir and methyl-indinavir peaks against nominal concentration) was plotted by least squares linear regression and reciprocal of the squared concentration (1/x²) used as a weighting factor. Deviation should be within ±20% for LLOQ and ±15% for remaining concentrations.

### Specificity and selectivity
Method selectivity was analyzed by comparing the chromatograms obtained from blank and spiked solutions. Method specificity was analyzed by infusing six different lots of blank plasma solutions to ensure no endogenous compounds interfere with amprenavir and IS.

### Recovery and ME
Amprenavir recovery was evaluated by paralleling the mean peak areas of extracted and un-extracted samples at low, medium, and high QC standard levels. At each concentration level, percentage recoveries were calculated, and finally, overall mean recovery was calculated. The ME was analyzed by paralleling the un-extracted samples with post-extracted samples [14-16].

### Stability
Stability was studied at LQC, MQC, and HQC levels. It includes bench-top, freeze-thaw, autosampler, and long-term stabilities. The bench-top stability was evaluated for 5 h at ambient temperature (25°C). Freeze and thaw stability was analyzed by storing the QC samples at −70°C for at least 3 h, and for thaw cycle, keep the solutions at room temperature. Repeat the freeze and thaw cycles for 3 times. The autosampler stability was analyzed by placing the QC samples in autosampler at 10°C for 8 h. Long-term stability was evaluated by placing the QC samples in a freezer at −70°C for 3 months [17-19].

### Dilution integrity
The sample solution above the upper limit of calibration range was prepared and evaluated for PA parameters. The percentage nominal concentration must be ±15%.

### Ruggedness
Method ruggedness was assessed by processing QC standards for one PA batch utilizing different columns of the same composition by different analysts. The %RSDs for LQC, MQC, and HQCs should be ≤±20% for LLOQ and ≤±15% for the remaining QC standards.

### RESULTS AND DISCUSSION
The LC-MS/MS chromatograms of amprenavir – blank and LLOQ and LQC, MQC, and HQC concentration levels – are shown in Figs. 2 and 3.

### Method validation
**Specificity**
From the Figs. 2 and 3, system chromatographic conditions were clearly separating amprenavir and IS from endogenous and other plasma

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**Table 1: Mass conditions for amprenavir and IS**

| Component         | Precursor ion (m/z) | Product ion (m/z) | DP (V) | EP (V) | CEP (V) | CE (V) | CXP (V) |
|-------------------|---------------------|------------------|--------|--------|---------|--------|---------|
| Amprenavir        | 506.2               | 71.0             | 40     | 8      | 25      | 30     | 5       |
| Methyl-indinavir  | 628                 | 421              | 100    | 8      | 25      | 82     | 5       |

DP: Declustering potential, EP: Entrance potential, CEP: Collision cell entrance potential, CE: Collisional energy, CXP: Collision cell exit potential, IS: Internal standard
The amprenavir-LLOQ peak response is more than 20% of the interference peak response, and methyl-indinavir peak response is more than 5% from the interference peak response.

**PA**

Amprenavir interday and intraday PA were analyzed, and the %RSD values are calculated for the same and are tabulated in Table 2.

### Table 2: Amprenavir precision and accuracy data

| Nominal concentration (ng/ml) | Intraday | Interday |
|------------------------------|----------|----------|
|                              | Accuracy (%) | %RSD | Accuracy (%) | %RSD |
| 2                            | 104.6     | 4.5    | 107.5        | 5.2 |
| 4                            | 89.6      | 3.9    | 91.6         | 6.1 |
| 200                          | 105.2     | 2.5    | 101.2        | 4.2 |
| 500                          | 109.8     | 8.1    | 96.4         | 2.9 |

n=6 replicates (for precision), RSD: Relative standard deviation

**Linearity**

Amprenavir calibration graph was linear in the concentration range of 2–600 ng/ml with regression equation of $Y=0.4528X+3.145$. The regression coefficient ($r^2$) value is more than 0.99 which was acceptable as per the Food and Drug Administration (FDA) regulatory guidelines [18].

**Recovery and ME**

The method has excellent recovery and the percentage recovery values were 101.86%, 102.8%, and 99.28% for LQC, MQC, and HQC samples, respectively. The data for amprenavir recovery are tabulated in Table 3. The ME was evaluated at LQC and HQC level, and the calculated % coefficient of variation (CV) values was 4.59% and 3.68%, respectively.

**Dilution integrity**

Dilution integrity of amprenavir was performed and evaluated. The percentage nominal was within the limit (±15%), and the estimated precision was ≤15%. It shows that the drug can be diluted to 20 times and the results will be reproducible.

### Table 3: Recovery data for amprenavir

| ID | LQC | MQC | HQC |
|----|-----|-----|-----|
|    | Un extracted (Area ratio) | Extracted (Area ratio) | %Recovery | Un extracted (Area ratio) | Extracted (Area ratio) | %Recovery | Un extracted (Area ratio) | Extracted (Area ratio) | %Recovery |
| 1  | 0.213 | 0.251 | 117.85 | 0.648 | 0.634 | 98.451 | 0.897 | 0.885 | 98.66 |
| 2  | 0.246 | 0.235 | 95.59  | 0.612 | 0.65  | 106.20 | 0.905 | 0.879 | 97.12 |
| 3  | 0.254 | 0.249 | 98.03  | 0.598 | 0.635 | 106.18 | 0.856 | 0.985 | 115.07 |
| 4  | 0.217 | 0.215 | 99.08  | 0.688 | 0.643 | 93.45  | 0.956 | 0.912 | 95.39 |
| 5  | 0.214 | 0.219 | 102.34 | 0.675 | 0.684 | 101.33 | 0.965 | 0.845 | 87.56 |
| 6  | 0.243 | 0.239 | 98.35  | 0.598 | 0.665 | 111.20 | 0.895 | 0.912 | 101.89 |
| Mean | 0.23 | 0.24 | 101.86 | 0.64 | 0.6525 | 102.80 | 0.912 | 0.903 | 99.28 |
| SD  | 0.017 | 0.014 | 7.42  | 0.02 | 0.017 | 5.80  | 0.037 | 0.04 | 8.301 |
| %CV | 7.29 | 5.83 | 7.28  | 5.68 | 2.62 | 5.64  | 4.11 | 4.77 | 8.36 |

LQC: Lower quality control, MQC: Median quality control, HQC: Higher quality control

Fig. 2: Amprenavir chromatograms (a) blank and (b) spiked lower limit of quantification samples
Stability
All the QC standards were exposed to different stability conditions and evaluated to analyze the stability of amprenavir. From evaluated %CV stability data, the drug was stable for more time at variable conditions such as bench-top stability (<10.8%), freeze-thaw stability (<9.5%), autosampler stability (<8.9%), and long-term stability (<8.4%), and the values are presented in Table 4.

Ruggedness
Method ruggedness for amprenavir was performed and evaluated. The % RSD values are calculated for the same and are tabulated in Table 5.

### Table 4: Stability for amprenavir

| Drug    | Concentration (ng/ml) | Bench-top stability | Autosampler stability | Freeze and thaw stability | Long-term stability |
|---------|-----------------------|---------------------|-----------------------|---------------------------|---------------------|
|         | Mean±SD (ng/ml)       | %CV                 | Mean±SD (ng/ml)       | %CV                       | Mean±SD (ng/ml)     | %CV                 |
| Amprenavir | 4                     | 4±0.5               | 4±0.3                 | 4±0.2                     | 4±0.2               | 10.7                |
|         | 200                   | 200±17.0            | 200±11.0              | 200±8.0                   | 200±10.0            | 9.6                 |
|         | 500                   | 500±20              | 500±8.0               | 500±7.3                   | 500±11.0            | 6.6                 |

SD: Standard deviation, CV: Coefficient of variance

### Table 5: Amprenavir ruggedness data

| Nominal concentration (ng/ml) | Analyst-1 and column-1 | Analyst-2 and column-2 |
|------------------------------|------------------------|------------------------|
|                              | Accuracy (%) | %RSD (%) | Accuracy (%) | %RSD (%)         |
| 2                            | 106.3        | 5.2      | 102.6        | 6.8              |
| 4                            | 98.4         | 2.9      | 94.6         | 5.9              |
| 200                          | 105.8        | 6.8      | 97.2         | 3.8              |
| 500                          | 104.2        | 2.6      | 106.9        | 4.5              |

n: 6 replicates, RSD: Relative standard deviation
CONCLUSION
A bioanalytical LC-MS/MS method for the amprenavir was developed and validated with methyl-indinavir as IS. This method has excellent recovery, accuracy, and precision compared with existed methods for the analysis of drug in human plasma samples. The drug was extracted from plasma samples by liquid–liquid extraction method with ethyl acetate as an extraction solvent. The drug was eluted within 5 min using RP Symmetry C18 (50 mm×4.6 mm, 3.5 µm) column with isocratic elution by acetonitrile and 0.1% v/v formic acid in the ratio of 90:10 v/v as mobile phase. The developed technique was validated according to the FDA regulatory guidelines, and all the validation parameters were within the acceptable range. The developed technique was effectively applied to routine analysis of amprenavir in plasma samples.

AUTHORS’ CONTRIBUTIONS
All authors contribute equally to this manuscript.

CONFLICTS OF INTERESTS
The authors declare that there are no conflicts of interests regarding the publication of this paper.

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