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# LCMS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTIFICATION OF AMPRENAVIR IN HUMAN PLASMA

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## ABSTRACT

A simple, rapid, sensitive, selective and high performance liquid chromatography method with MS/MS was developed and validated for determination of Amprenavir in human plasma. Extraction from the plasma was by solid phase extraction (SPE) extraction procedure. Amprenavir-D4 was used as an internal standard (IS). The chromatographic separation was performed by using an Quattro Premier XE LC-MS/MS coupled with 2695 HPLC separation module, a Purospher star, RP 18e (5µm), Hibar 150 × 4.6 mm coloumn and an SPD-M20A PDA detector. Data acquisition was done by using Mass Lynx V 4.1 software. A binary mixture of 0.3% formic acid (3.0 mL of formic acid solution into 1000 mL volumetric flask and made up the volume with Milli-Q water) and acetonitrile in the ratio of 20:80% v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 1.0 mL/min. was found to be suitable in the study range of 0.8 -1.5 mL/min. Detection was carried out by using mass detectors. Mass spectroscopy function parameter were set at MRM 506.26/245.11 for analyte and MRM 510.26/245.11 for internal standard respectively. The % CV and mean accuracy for analyte at LLOQ level were found to be 3.37 and 92.12 respectively, calibration curves analyzed were found to be linear for the standards concentration ranging from 40.176-16096.225 ng/mL. The correlation coefficient (r) was observed to be ≥ 0.9987 during the course of validation. The % mean accuracy for the CC standards were ranged from 97.22 - 103.99 and the % CV were ranged from 0.53 - 5.80. The validated method is suitable to support a wide range of therapeutic drug monitoring and pharmacokinetic studies.

**Keywords**: Bionalysis, Amprenavir, Pharmacokinetics, Human Plasma, LC-MS/MS.

## INTRODUCTION:

Amprenavir is a second generation human immunodeficiency viral protease inhibitor. The drug has demonstrated both additive and synergistic pharmacological activity against HIV in combination therapies involving both protease and reverse transcriptase inhibitors. The goal of this work was to develop a bioanalytical LCMS/MS assay to amprenavir. It was chemically (3S)-oxolan-3-ylN-[(2S,3R)-3-hydroxy-4-[N-(2-methylpropyl)(4-aminobenzene) sulfonamido]-1-phenylbutan-2-yl]carbamate. Given by molecular formula as $C_{25}H_{35}N_3O_6S$. It has a molecular weight of 505.627 g/mol.

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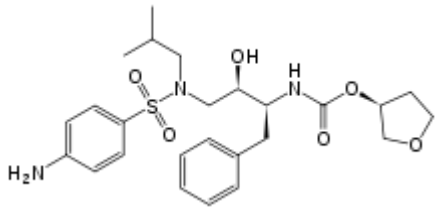
It is poorly soluble in water and readily soluble in organic solvents. The mechanism of this action of this drug include inhibition of HIV viral proteinase enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles. Amprenavir is a protease inhibitor 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 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 is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The 2 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.



**Structure of Amprenavir.**

This paper describes a novel and selective approach, which enables the determination of Amprenavir with good accuracy at low drug concentrations in plasma using liquid chromatography coupled to tandem mass spectrometry (MS/MS) below the clinically relevant range of concentrations encountered in patients.

## MATERIALS AND METHODS:

The materials and methods include 0.3% Formic acid, 10 mM Ammonium formate pH 3.0 extraction buffer, Water, Acetonitrile, Ethyl acetate, Methanol. Mobile phase of Acetonitrile/ Formic acid Buffer (80:20%, V/V) needle wash of Acetonitrile/Water (80/20%, V/V) and seal wash of Acetonitrile/Water (20/80%, V/V). ISTD stock solution (1.000 mg/mL) of Amprenavir-D4. ISTD Working Solution (700ng/mL), CC Stock Solution (2 mg/mL). Amprenavir stock solution range of 1996.800 to 800000.000 ng/mL using human Plasma as the diluent. Spiked Calibration Curve Plasma Standards ranging from 39.936 to 16000.000 ng/mL.

**Diluent:**

Using a calibrated pipette transferred 1.0 mL of formic acid solution into 10 mL volumetric flask and made up the volume with milli-Q grade water. Mixed well and stored at room temperature.

**0.3% Formic Acid Buffer:**

Using measuring cylinder, measured and transferred 3.0 mL of formic acid solution into 1000 mL volumetric flask and made up the volume with milli-Q grade water.

**10 mM Ammonium formate pH 3.0 extraction buffer:**

Weighed accurately about 630.60 mg of ammonium formate and transferred it quantitatively to a 1000 mL volumetric flask and made up the volume with milli-Q grade water. Mixed well, adjusted the pH to 3.0 by adding dilute formic acid solution (10% V/V) drop wise.

**Mobile phase preparation [Acetonitrile/ Formic acid Buffer (80:20%, V/V)]:**

Measured and transferred 800 mL of acetonitrile and 200 mL of mobile phase buffer to an appropriately sized clean and pre-labeled bottle.

**Needle wash [Acetonitrile/Water (80/20%, V/V)]**

800 mL of acetonitrile and 200 mL of milli-Q grade water was measured and transferred to an appropriately sized reagent bottle.

**Seal wash [Acetonitrile/Water (20/80%, V/V)]**

Measured and transferred 200 mL of acetonitrile and 800 mL of milli-Q grade water to an appropriately sized reagent bottle.

**Reconstitution solution [Acetonitrile/Mobile Phase Buffer (80:20, V/V)]:**

Measured and transferred 80 mL of acetonitrile and 20 mL of mobile phase buffer to a 100 mL beaker.

**Internal standard solution preparation:**

**Preparation of ISTD stock solution (1.000 mg/mL):**

Amprenavir-D4 standard equivalent to 1.0 mg of Amprenavir-d4 was weighed and transferred into a 1.0 mL volumetric flask. The standard was dissolved with methanol and made up the volume with the same solvent. It was mixed well, and stored at 5 ± 3ºC.

**Preparation of ISTD Working Solution (700ng/mL):**

Using a calibrated pipette, 1.50 mL of ISTD stock solution (1.0 mg/mL) was pipetted into a 50.0 mL volumetric flask, and made up the volume with the diluent. It was mixed well and stored at 5 ± 3ºC.

**Preparation of calibration standard:**

**Preparation of CC Stock Solution (2 mg/mL**):

Amprenavir standard equivalent to 10.0 mg of Amprenavir was weighed and transferred into a 5 mL volumetric flask. It was dissolved in methanol and made up the volume using the same solvent. It was mixed well and stored the solution at 2-8ºC.

**Preparation of CC Spiking Solutions:**

The calibration curve dilutions were prepared from Amprenavir stock solution as per the table given below in the concentration range of 1996.800 to 800000.000 ng/mL using human Plasma as the diluent. These dilutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.

**Spiked Calibration Curve Plasma Standards:**

The above calibration curve dilutions (CC spiking solutions) were used to spike the screened blank human plasma matrix to prepare the plasma calibration curve standards ranging from 39.936 to 16000.000 ng/mL 0.3 mL aliquots of the above plasma calibration curve standards were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at -20°C

**Preparation of Quality Control (QC) Samples:**

**Preparation of QC Stock Solution (3.0 mg/mL)**

Amprenavir standard equivalent to 15.0 mg of Amprenavir was weighed and transferred into a 5 mL volumetric flask. It was dissolved in methanol and made up the volume using the same solvent. It was mixed well and stored the solution at 2-8 ºC.

**Preparation o QC spiking solution:**

The quality control dilutions (QC spiking solutions) from Amprenavir stock solution were prepared as per the table given below in the concentration range from 2012.511 to 680400.000 ng/mL using Human Plasma as the diluent. These dilutions (QC spiking solutions) were subsequently used for spiking the screened blank plasma.

**Spiked QC Plasma Samples**

The above quality control dilutions (QC spiking solutions) were used to spike the screened blank human plasma to prepare the plasma quality control plasma samples ranging from 40.250 to 13608.000 ng/mL as per the table given below.

0.3 mL aliquots of the above plasma quality control samples were taken in pre labeled polypropylene vials which were then capped properly and stored in a freezer at –20°C.

**Preparation of standard blank samples:**

Prepared the standard blank by spiking the diluent in screened human plasma as described in the following table

0.300 mL aliquots of the above plasma blank samples were taken in pre labeled polypropylene vials which were then capped properly and stored in a freezer at –$20^0$C.

**Instrumentation:**

| Spiking solution ID | Spiking volume (mL) | volume of matrix (mL) | Final Volume (mL) | Spiked CC ID |
|---|---|---|---|---|
| Diluent | 0.200 | 9.800 | 10.000 | STD Blk |

| | |
|---|---|
| HPLC System | Alliance LC |
| Deep Freezer | (-86) Deg C Deep Forma, Thermo scientific |
| Microbalance | XP 205, Mettler Toledo |
| Vibramax | Heidolph |
| Vacuum pump | Millipore |
| Refrigerator | Samsung |
| PH meter | Orion |
| Micropipettes, Multipette and Micro tips | Brand and Eppendorf |
| Vortexer | GV lab, Gilson® |
| Solid phase extraction chamber | Water's |
| MCX 30mg/1CC cartridges | Oasis; Water's |
| Poly propylene tubes | Torson's |
| Water Purification System | Elix 10 / Milli-Q gradient |
| Ultra sonicator | Power Sonic510, (Hwashin echnology) |
| Nitrogen Evaporator | Zymark Turbovap LV station, Caliper |

**Optimized Chromatographic conditions are as follows**

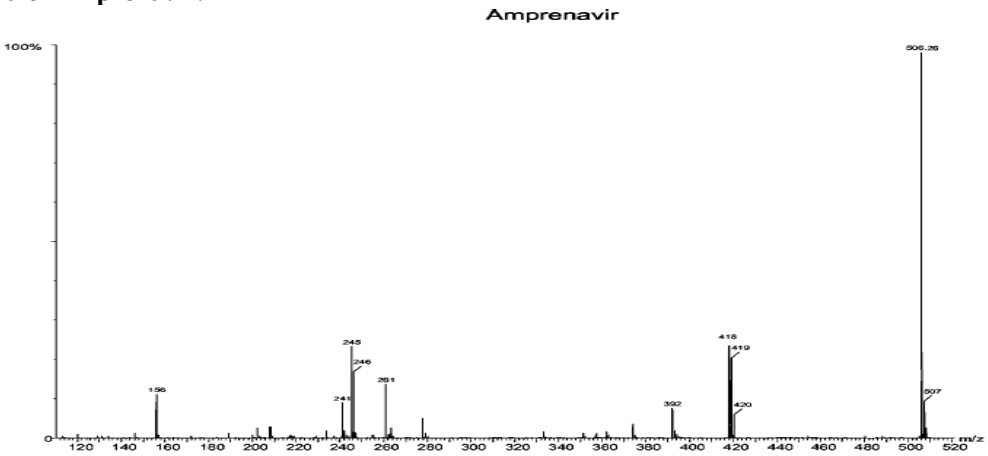
| Parameter | Value |
|---|---|
| Column | Purospher star, RP 18e Hibar (150 × 4.6 mm, 5µm) |
| Mobile phase | Acetonitrile /Mobile Phase buffer (80/20 V/V) |
| Flow rate | 1.0 mL/min |
| Run time | 2.50 min |
| Column oven temperature | 40 ± 2ºC |
| Auto sampler temperature | 5 ± 3ºC |
| Volume of injection | 5µL |
| Detection | Mass detector |
| Retention time of Amprenavir | 1.86 min. |
| Retention time of Amprenavir-d4 | 1.85 min. |

**Mass Instrumentation and parameters:**

The parameters of mass spectrometer are given in the table below.

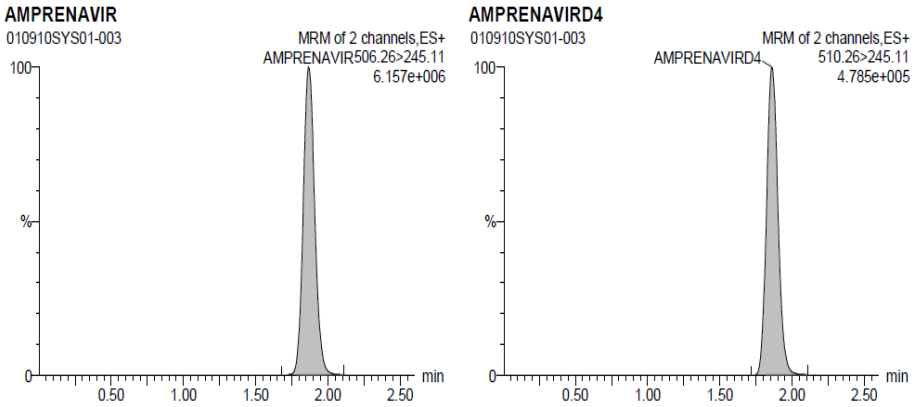
| ES + Source Parameter | Settings | Analyser Parameter | Settings |
|---|---|---|---|
| Capillary (kV) | 3.00 | LM Resolution 1 | 13.0 |
| Cone (V) | 22 | HM Resolution 1 | 13.0 |
| Extractor (V) | 5 | Ion Energy 1 | 0.5 |
| RF Lens (V). | 0.0 | Entrance | -1 |
| Source Temp ($^0$ C) | 100 | Collision | 20 |
| Desolvation Temp ($^0$ C) | 450 | Exit | 0 |
| Cone Flow (L/h) | 50 | LM Resolution 2 | 13.0 |
| Desolvation Flow (L/h) | 800 | HM Resolution 2 | 13.0 |
| Collision gas Pressure | $3.5\times e^{-3}$ - $4.5\times e^{-3}$ | Ion Energy 2 | 1.0 |
| | | Multiplier | 650 |

**Mass spectra of Amprenavir:**




**MRM Acquisition parameters:**

| Parameter | Setting (Analyte) | Setting (ISTD) |
|---|---|---|
| MS function | MRM 506.26/245.11 | MRM 510.26/245.11 |
| Dwell | 0.200 | 0.200 |
| Cone voltage (V) | 22 | 22 |
| Collision energy | 20 | 20 |




**Chromatograms of Amprenavir and Internal standard**

**Extraction Process of Plasma Samples and Their Drying**

**Sample Preparation & Extraction Procedure**

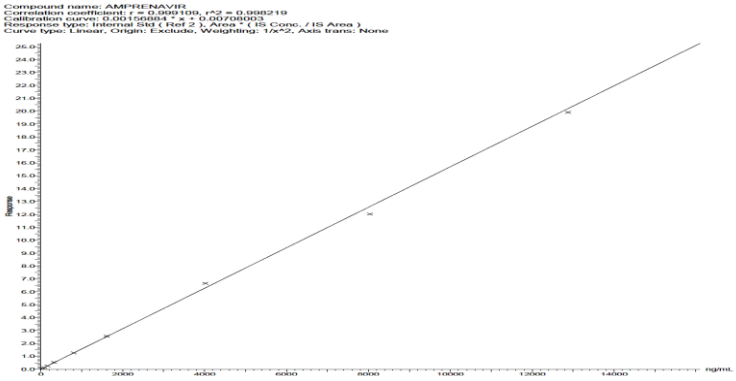
Required number of plasma samples were taken from the deep freezer, thawed them at room temperature and vortex the tubes to mix. Transferred 200 µL of plasma into pre-labeled tubes. 50.0 µL of 3.0 µg/mL ISTD working solution to all the vials excepting the STD Blank and vortexed for about 15 seconds. 100.0 µL of extraction buffer was added to all the vials and vortex for about 30 seconds. 2.5 mL ethyl acetate was added to the all vials and vortex for a period of 20

mins, in rotospin at 40 rpm. All the vials were centrifuged at 4500 rpm, at 4°C for 5 min. About 2.0 mL of supernatant was transferred into pre-labeled tubes and the supernatant solutions were evaporated to dryness under nitrogen at 40 ± 5°C. 400.0 µL of reconstitution solution was added to all the tubes and vortexed for about 30 sec. Appropriate volumes of the reconstituted solutions were transferred into pre-labeled auto-sampler vials and 10 µL was injected into LC-MS/MS.

**Procedure for Un-Extracted Sample Preparation**: 40.0µL of respective spiking solutions were taken in pre-labeled tubes. 500.0µL of 3.0 µg/mL ISTD dilution was added and vortexed to mix. 3.460mL of reconstitution solution was added and vortexed to mix. Appropriate volumes were transferred into pre-labeled auto-sampler vials and 10.0 µL was injected into LC-MS/MS.

**Method Validation:**

System suitability experiment was performed by injecting 6 consecutive injections using aqueous standard mixture equivalent to MQC1 concentration of the calibration curve. The % CV of peak area ratio (analyte area/ISTD area) and %CV of retention times for analyte and ISTD were within acceptance criteria. The carryover effect due to the auto sampler was investigated by injecting a sequence of unextracted samples consisting of RS, AQ ULOQ, RS, AQ LLOQ and extracted samples containing standard STD Blk, ULOQ, STD Blk and LLOQ. No significant carry over observed during this experiment. The specificity of the LC-MS/MS method was established by screening the standard blanks of different lots of human plasma. 10 different lots of plasma were screened for the specificity experiment. Out of 10, 7 lots were of intended anticoagulant plasma i.e. $K_2$EDTA (In-house ID: P-381, P-383, P-386, P-391, P-393, P-394, P-395), one hemolytic plasma (In-house ID: P-327), one lipidemic plasma (In-house ID: P-259) and one lot containing Heparin as anticoagulant (In-house ID: P-338). All the investigated human plasma lots were found to be free of significant interferences at the retention time of drug. The Sensitivity of the method was evaluated by analyzing 6 LLOQ samples (40.176 **ng/mL).** The % CV and mean accuracy for analyte at LLOQ level were found to be 3.37 and 92.12 respectively. The matrix effect for the LC-MS/MS method was assessed by using chromatographically screened 8 different lots of normal plasma (P-381, P-383, P-391, P-393, P-394 and P-395), one haemolytic plasma (In-house ID: P-327) and one lipidemic plasma (In-house ID: P-259).With each lot of plasma, samples concentration equivalent to HQC and LQC of Amprenavir were prepared and injected. The % CV of back calculated concentrations for the HQC and LQC samples of all the investigated lots were found to be 2.29 and 4.01 respectively. The % mean accuracy of back calculated concentrations for the HQC and LQC samples of all the investigated lots were found to be 94.43 and 99.90 respectively. The linearity of the method was determined by using a $1/X^2$ weighted least square regression analysis of standard plots associated with a ten-point standard curve. All the 3 calibration curves analyzed were found to be linear for the standards concentration ranging from 40.176-16096.225 ng/mL. The correlation coefficient (r) was observed to be ≥ 0.9987 during the course of validation. The % mean accuracy for the CC standards were ranged from 97.22 - 103.99 and the % CV were ranged from 0.53 - 5.80.




The % mean recovery at HQC, MQC1, MQC2 and LQC levels were found to be 63.80, 59.07, 56.61 and 57.53 respectively. Over all % mean recovery and % CV at all QC levels were found to be 59.254 and 5.39 respectively. The overall % mean recovery for internal standard was found to 64.71. The dilution integrity of the method was evaluated by preparing the DI spiking solution (1961250.428 ng/mL) which is approximately equivalent to 2.5 times of the highest CC spiking solution which were spiked in the screened plasma to get a DIQC concentration (39225.009 ng/mL). DIQC sample is further diluted by 1/5 and 1/10 times.

Freeze thaw stability of the spiked quality control samples was determined after four freeze thaw cycles stored at -28 ± 5°C. The % mean stability for HQC and LQC was found to be 99.91 and 100.85 respectively. Bench top stability of the spiked quality control samples was determined for a period of 14 hours 02 minutes stored at room temperature. The % mean stability for HQC and LQC was found to be 99.75 and 100.06.

**CONCLUSION:**

Based on the experiments done during the course of validation, it can be concluded that the intended method is validated for the estimation of

Amprenavir in human plasma over the concentration range of 40.176 - 16096.225 ng/mL using Amprenavir-$d_4$ as internal standard. The precision and mean accuracy are within the acceptable limits. Consistent recoveries are observed for HQC, MQC1, MQC2 and LQC. The method is specific enough in the presence of $K_2$EDTA anticoagulant. The method is precise and accurate enough to dilute the samples, if necessary. it is concluded that the intended analyte is stable in all the preformed experiments and the stability experiments preformed are within the acceptance limits.

This method was proved to be rugged by using different analyst and different column

By considering the above conclusions on the method and stability of the analyte, this method shall be used for subject sample analysis. This method can be used for quantification of Amprenavir in human plasma for Bioequivalence studies.

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