# Bio-Analytical Analysis of Sofosbuvir and Velpatasvir in Human Plasma by using LC-MS/MS

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**ABSTRACT**

The Sofosbuvir (SOFOS) and Velpatasvir (VELPA) virus particles may be quickly and accurately detected in human plasma using LC-MS/MS. Ledipasvir A was used as a reference standard in an easy extraction technique. An efficient approach for purifying analytes from human plasma was protein precipitation with acetonitrile. The used a C18 Zorbax eclipse plus (4.6 mm ID x 50 mm) column with a mobile phase of 1% formic acid in acetonitrile to separate the important components. The volumetric ratio was fixed at 50:50 v/v, and the flow rate was kept at 0.55 mL/min. The method's effectiveness was confirmed by physical factors.

**Keywords:** Sofosbuvir, Velpatasvir, Ledipasvir, Bio-analysis, LC-MS/MS, Pharmacokinetics

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## 1. Introduction

Hepatitis C is a liver disease caused by an infection with the hepatitis C virus (HCV). These pathogenic bacteria enter the body through the blood. Risky medical operations, blood transfusions, and infectious needles containing small amounts of blood are the most common ways that the disease can spread.[1] The annual death toll from hepatitis C is close to approximately 290 thousand. Because of the virus, the person has an increased risk of getting cirrhosis and hepatocellular carcinoma, two forms of liver cancer.[2] The best antiviral drugs for hepatitis C are direct acting antivirals (DAAs), while there are a few alternatives. The most effective treatment options for chronic hepatitis C are these drugs.[3] Velpatasvir isn't the only anti-viral drug that can help slow down liver damage; sofosbuvir is another one. The combination treatment has helped many people with chronic hepatitis C.[4]

Hepatitis C (HCV) is treated with sofosbuvir, a drug that kills viruses. It stops the activity of NS5B nucleotide polymerase, which means it affects the NS5B protein, a key part of the HCV virus protease.[5] Sofosbuvir specifically works by resembling the natural nucleotides that the virus uses to make its RNA and that are then added to the final virion as part of the replication process. Infusing extra blocks stops the cycle of virus self-replication. Sofosbuvir stops viruses from copying themselves by using the nucleoside analogue triphosphate GS-461203. GS-461203 stops the virus from replicating by adding more blocks to the building chain once it is attached to the viral RNA molecule. HCV reproduction depends on NS5B, and its catalytic centre is very similar to that of HCV type 1-6. According to this, the heart of the enzyme stays the same in all the main forms of the virus. This creates what is called Pan Genotypic activity, which is a large pool of different genotypes of HCV. That's exactly why Sofosbuvir is thought to be helpful in treating HCV.

Sofosbuvir is one of the best NS5B inhibitors because it is hard for viruses to become resistant to it and there isn't much chance that resistance-related changes will happen.[6] It can also be used to treat different types of HCV. Cathepsin A, CES1, and HENT1 are the main enzymes that break it down, while the CYP450 enzyme system breaks down many other drugs. This means that Sofosbuvir interacts differently with drugs that use the CYP450 pathways. This makes it less likely that drugs will interact with each other, which makes it safer. Because of how it works with metabolism, it is an easy and successful treatment, especially for people who are taking other medicines for other health problems. This makes it even more appealing for treating HCV.

Velpatasvir is a new NS5A inhibitor of the second generation that is used to treat the Hepatitis C virus (HCV).[7] There are six different types of HCV that this drug can work against, so it can be used to treat all of them. It's interesting that the drug's mean 50% effective concentrations (EC50) in genotypes 1–6 were found to be between 6 and 120 pM. This shows that the drug is strong. Additionally, velpatasvir can work against genotype 1 NS5A resistance-associated substitutions (RAS), which is a type of change that might make some medicines less effective. Cytochrome P450 enzymes, such as CYP2B6, CYP2C8, and CYP3A4, break down velpatasvir the most. There are many other drugs that depend on these enzymes for digestion, so Velpatasvir might not work well with medicines that rely on these pathways. So, it will be important to keep an eye out for any possible drug combinations when Velpatasvir is given with other medicines as part of a routine.

Starting off— The discovery of direct-acting antivirals changed the way people were treated for Hepatitis C Virus (HCV) infections, making treatment plans easier to follow and more efficient.[8] The main topic of this story is on how the Pan Genotypic regimen of sofosbuvir and velpatasvir was created. This regimen not only treats all common HCV genotypes, but it also has a high SVR and is simple and easy to use. This mix has a high sustained viral response (SVR), which means that there is no virus in the blood after treatment. A high rate of SVR is a key indicator of treatment success and is linked to eradicating the virus for good and better patient results.[9] The results show that the sofosbuvir-velpatasvir combination will work very well to treat HCV cases.

However, a search of the literature showed that not many studies have used LC with a UV monitor to find both sofosbuvir and velpatasvir in plasma at the same time.[10] On the other hand, different ways have been outlined to find sofosbuvir in bodily fluids. The goal of this study was to create the first HPLC method for isolating and measuring sofosbuvir and velpatasvir in human plasma so that they could be used for bioequivalence and pharmacokinetic studies. It was best to be able to find both drugs at the same time in

the plasma samples that were separated. This could be done in a single integration and run, which is very important for quickly analyzing bigger groups of samples, especially for absorption studies. The EMA (European Medicines Agency) standards were used to thoroughly test the analysis method to make sure it gave accurate and reliable results. This gave trust in the method's strength and ability to be repeated, making it useful in the fields of pharmacology and clinical research.

## 2. Experimental

### 2.1 Materials and reagents

All of these drugs, standards, and solutions were lab-grade and used in the work. NAC Pharmaceutical Pvt. Ltd. sold both Sofosbuvir and Velpatasvir in Hyderabad, Telangana, India. They were both 99.74 percent pure. Some of the things that were needed were acetonitrile, methanol, water, and super-gradient grade formic acid. Coir acetate membrane screens (0.22 µm) were made with Chrom Tech. Background: For this study, samples of human plasma were taken both before and during the research. This made it possible to test the diagnostic method, which turned out to work for this study.

### 2.2. Instrumentation

These group of things was all made by Agilent: the degasser, the mixer, the auto-sampler (model 1260 series), and the detector (triple quadrupole, model 6410A). To get the information and figure out how much it was, Mass Hunter software was used. The research used an ultrasonic blender (Elma, Germany) and a swirling mixer (Stuart, England). For data research and drug tests, WinNonlin 8.0 was used.

### 2.3. LC–MS/MS conditions

The separation technique made use of a reversed-phase Agilent C18 Zorbax Eclipse Plus column (5 µm, 100 × 4.6 mm). The solution containing 0.1% formic acid, water, acetonitrile, and methanol was pumped at a flow rate of 0.55 mL/min. Using a volume of 10µL, the chromatographic separation was carried out at 40°C. Under the specified conditions, the retention times for sofosbuvir, velpatasvir, and the internal standard were 2.04 min, 1.80 min and 2.02 min, respectively, with a total run time of 2.8 minutes. For the mass spectrometry data, we used the MRM (positive ion multiple reaction) mode. The test utilized an annual protective nitrogen gas flow rate of 11 L/min and all analytes were given a 200 ms rest interval. Force spray gas, weighing 45 pounds, was activated. Four thousand volts jolted the containers. It was found that sofosbuvir and velpatasvir had two separate voltages. Voltages of 135 V were used by the fragmentor and 160 V were used by the IS. The contact energies used for the administration of sofosbuvir, velpatasvir, and IS (Ledipasvir) were 20 V, 50 V, and 25 V, respectively. We kept the m/z for sofosbuvir at 530.3/243.1, the m/z for velpatasvir at 883.4/643, and the internal standard (IS) for amount determination at 889.2/732.3.

### 2.4. Preparation of Standard Solutions

**Solution A**: 10 mg of sofosbuvir was accurately weighed and dissolved in 100 mL of methanol to obtain a concentration of 100 µg/mL.

**Solution B**: 0.5 mL of Solution A was further diluted to 10 mL with methanol, resulting in a concentration of 5000 ng/mL.

**Solution C**: 10 mg of velpatasvir was accurately weighed and dissolved in 100 mL of methanol to obtain a concentration of 100 µg/mL.

**Solution D**: 0.4 mL of Solution C was further diluted to 10 mL with methanol, resulting in a concentration of 4000 ng/mL.

**Solution F**: An accurately weighed 10 mg of ledipasvir was transferred into a 100 mL volumetric flask and diluted to volume with acetonitrile to prepare **Solution E** with a concentration of 100 µg/mL. Next, 20 mL of Solution E was diluted to 100 mL with methanol, resulting in **Solution F** with a concentration of 20,000 ng/mL. All solutions were stored at -20°C, protected from light, until the time of analysis.

### 2.5. Calibration and Quality control samples preparation

There were eight non-zero calibrators, one blank sample, and one zero sample (a matrix sample used only with the internal standard, or IS). In order to create calibration standards (CS), 50 µL of IS stock solution (Solution F) was combined with 400 µL of drug-free K2EDTA human plasma. Afterwards, 50 µL of each diluted solution (Solutions A-D) containing sofosbuvir and velpatasvir was added. It ranged from 5 to 5000 ng/mL for sofosbuvir and 10 to 1500 ng/mL for velpatasvir. The checkers contained this. Four analyte quality control (QC) samples were prepared at four levels high (QCH), medium (QCM2, QCM1) and low (QCL) at concentrations 15+30, 550+200, 2000+600, 4000+1200 for QCL, QCM2, QCM1 and QCH, respectively. The quantities discovered using QCM2 were substantially within the bioanalytical

sample range. That is why QCM2 was incorporated to guarantee accurate sample collection. All spiked plasma samples were thoroughly mixed using a vortex for 1 minute prior to use.

**2.6. Sample preparation**

The mixing process required 50 µL of IS solution F, 0.5 mL of plasma, and 1 mL of acetonitrile. After then, the ingredients were whisked together for 1.5 minutes. A cold centrifuge was used to spin them at 4500 rpm for a duration of 12 minutes. To evaluate the most transparent organic layer in the supernatant, a tiny volume (10 µL) was cautiously transferred to the chromatograph without diluting it.

**3. Results and Discussion**

Once the new combination treatment enclose was approved, it was time to come up with a good bioanalytical way to test the drugs that actually work. It is used to treat hepatitis C virus (HCV) and is made up of sofosbuvir and velpatasvir. With just one extraction method and one chromatography run, the key goal was to find a reliable and quick way to measure both drugs at the same time. A lot of samples had to be looked at, like in bioequivalence studies. These methods were very helpful because they cut down on processing time by a huge amount without changing how accurate the analysis was or how regular the results were.

**3.1. Method development**

With the two antiviral drugs' stated Cmax values as a guide, the regression ranges for the measurements were confirmed to be 5 – 5000 ng/mL for Sofosbuvir and 10 – 1500 ng/mL for Velpatasvir.

**3.1.1. Development of Mass Spectrometry**

The goal when setting up MS/MS values was to get the best answer for analytes with the least amount of MRM (multiple reaction monitoring) channel cross-talk. Ionizing the three drugs with an ESI source was used to find them in MRM mode. The following changes were watched: 530.3/243.1 for Sofosbuvir, 883.4/643 for Velpatasvir, and 889.2/732.3 for the Internal standard (IS).

**3.1.2. Optimization of Sample extraction**

Solid-phase extraction, liquid-liquid extraction, and direct precipitation with organic solvents are some of the ways we looked at to get rid of material. Protein precipitation was used even though all three options gave pretty good results because it was simple, quick, and reliable. These steps gave the best peaks and a little better perception. Many things were tried, and the best result for both sofosbuvir and velpatasvir was found when acetonitrile was used as a precipitant. You could get the same results again and again. Since it makes it easier for phospholipids to mix, acetonitrile is the most common chemical solvent used for protein precipitation. In mass spectrometry [8,9], matrix effects are mostly caused by phospholipids, so this is important.

**3.1.3. Optimization of chromatographic conditions**

Several tests were done with the C18 Zorbax Eclipse Plus (5cm, 100 x 4.6 mm, USA) and the set phase. The research looked at various mobile phases that included ammonium formate buffer (0.1% formic acid), ammonium acetate buffer mixed with methanol or acetonitrile in an isocratic mode. In the test, acetonitrile and methanol worked best as an organic stabilizer when mixed together. More research was done on different mixes of the watery and organic stages. The best mobile phase was found to be 0.1% formic acid in 30:60:10 water, acetonitrile, and methanol, based on the findings above.

**3.2. Bio-analytical method pre-study validation**

**3.2.1. Lower Limit of Quantitation (LLOQ):**

It stands for "Lower Limit of Quantitation." The LLOQ is the smallest amount of a biomarker that can be tested still be accurate. It is best if the rate of change is less than 20%, according to the rule. The EMA says it should be less than 5% chance (LLOQ). LLOQs for this method were 5 ng/mL for sofosbuvir and 10 ng/mL for velpatasvir. The rates of change were 9.92% for sofosbuvir and 6.71% for velpatasvir. The average lowest concentration of sofosbuvir was 95.60%, and the average lowest concentration of velpatasvir was 98.46%. This proved that both methods always and correctly worked with low amounts. (Figure 5)

**3.2.2. Linearity:**

A sofosbuvir calibration value was between 5 and 5000 ng/mL, and a velpatasvir calibration value was between 10 and 100 ng/mL. The calibration curves are made by plotting the area ratios (the area of sofosbuvir or velpatasvir split by the area of Ledipasvir) against the amounts of sofosbuvir or velpatasvir. Linear regression with weights ($1/X^2$) was used on the data. You can see a summary of the regression factors for sofosbuvir and velpatasvir in Tables 1 and 2. To make sure the uniformity, the amounts of the calibration standards that were estimated backwards were used. At the LLOQ, the gaps between what happened and

what was predicted were less than 20%. For standards other than the LLOQ, they were less than 15% (Table 3 and 4). This proves the method is straight-forward and correct across the whole testing range.

**3.2.3. Selectivity**:

A method is selective if it can clearly measure and separate the analyte(s) from other things in the matrix, such as toxins, degradants, metabolites, and matrix parts. Plasma samples from six different people who had not been taking any drugs were tested to see if they interacted with sofosbuvir, velpatasvir, or an internal standard (IS). This was done to see how specific the test was. There wasn't a big difference between the drugs and IS at the ion channels or retention times in any of these samples. Figure 6 shows a normal MRM chromatogram of a drug-free blank plasma sample that was taken out. As you can see from the chromatogram, there aren't any other spots that get in the way of the target analytes' retention times. This is strong proof that the method we created can only measure the target analytes in the living sample.

**3.2.4. Extraction Recovery**:

To find out how much of each drug was retrieved, the mean peak areas of three QC samples that had been taken out were compared to the mean peak areas of three standards that had been made at the same concentration. 83.35% of people who were given sofosbuvir were given QCL, 81.23% were given QCM, and 80.57% were given QCH. They were able to get 81.00% of the velpatasvir back in QCL, 79.76% in QCM, and 80.94% in QCH. It took 81.18% on average to make money with IS bonds. When different concentrations were used, the results show that the extraction method always works the same way.

**3.2.5. Matrix Effect**:

Researchers looked at the matrix effect using six different blank plasma matrices and two plasma samples that were high in lipids and hemolysin. With each sample and the internal standard (IS), the matrix factor (MF) was found. The pure analyte solution is subtracted from the peak area that contains the matrix components to get this result. This estimate was made for both the low QC sample and the high QC sample. Figuring out the IS-normalized MF: Meaning of analyte: Meaning of IS was done with this formula. The IS-normalized MF for all eight lots in the matrix had a variation (CV) of 15% or less. The sofosbuvir CV was 12.07% for QCL and 2.75% for QCH, according to the findings. For velpatasvir, it was 9.0 percent for QCL and 1.1 percent for QCH. The sample was cleaned and processed correctly, and the analytes used had little matrix effect and the right ion channels, so there was no cross-talk.

**3.2.6. Within and Between Runs Accuracy and Precision**:

Six results for the LLOQ, QCL, QCM, and QCH were gathered in three different runs to see how exact and accurate the method was. Averagely, the accuracy should be within 20% of the stated concentration at the LLOQ level. At other stages of quality control, it should be within 15% of the actual concentration on average. As a result, the correct method produced within-run mean precisions of X% for sofosbuvir and Y% for velpatasvir, as well as between-run mean precisions of A% for sofosbuvir and B% for velpatasvir. As a result, the new method could be used to measure sofosbuvir and velpatasvir more exactly (Table 5 and 6).

**3.2.7. Dilution Integrity Test**:

We made (ACE) samples by adding plasma that had 9200 ng/ml of Sofosbuvir and 2700 ng/ml of Velpatasvir. This was done to see how well weakened samples can work above the ULOQ (Upper Limit of Quantitation) without losing accuracy and precision. These things were done with reduction factors of two and four. Both drugs were found to be accurate and precise within the acceptable range of ±15%. It was done on six samples for each reduction factor at the same time as the calibration curve standards. It was shown that very accurate measurements could be taken of both sofosbuvir and velpatasvir at both dilution factors. Figuring out the amounts of the samples that had been diluted also proved that the process worked well.

**3.2.8. Stability**:

Testing the stability of a bioanalytical technique is a crucial part of confirming its efficacy. In order to assess the stability of sofosbuvir and velpatasvir in different conditions, it was required to examine both the original and processed QCL and QCH samples of the drugs. A new calibration curve was used to test the QC samples, and the outcomes were compared to the predicted values. We looked studied how the MATRIXX analytes held up in the short term at room temperature, how they held up in the long term at -70 ± 5°C, how stable the auto-sampler was after preparing a sample, how freeze-thaw cycles affected them, and how different people kept their stock solutions.

Scientific method may be useful in many dealing and keeping problems, as they shown.

**4. Conclusions**:

To determine the simultaneous concentrations of sofosbuvir and velpatasvir in human plasma, the researchers in this study developed a reliable LC-MS/MS technique. Its simple mobile step, fast analysis, and isocratic response prove that it is effective. There's no expense, it works, and it's good for the environment. You shouldn't be bothered by it and it is unrelated. Come up with a term. It may be "types," "notation," or anywhere else. No matter how many times you apply this removal process, it will always be effective.

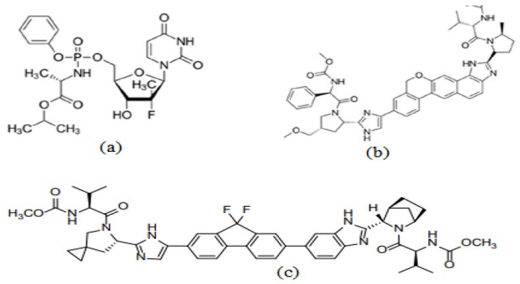
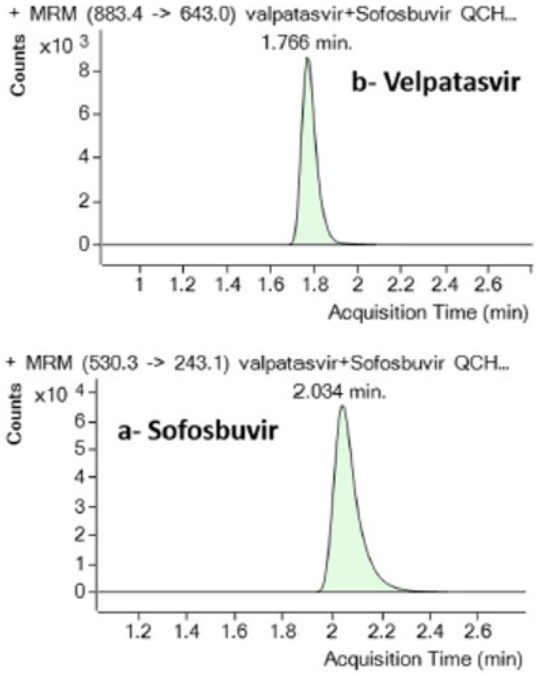



**Figure 1:** Chemical structures of Sofosbuvir (a) and Velpatasvir (b) and Ledipasvir (c)



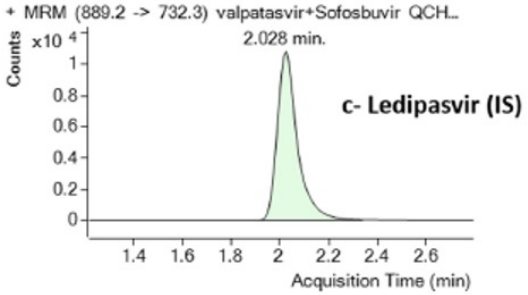




**Figure 2**: Typical MRM chromatograms of QCH of Sofosbuvir (a), Velpatasvir (b) and Ledipasvir IS (c).

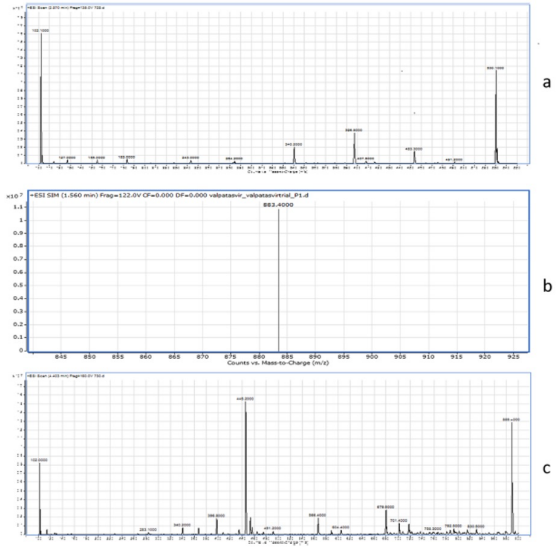



**Figure 3:** ESI-MS precursor ion scan of Sofosbuvir (a), Velpatasvir (b) and Ledipasvir (c).

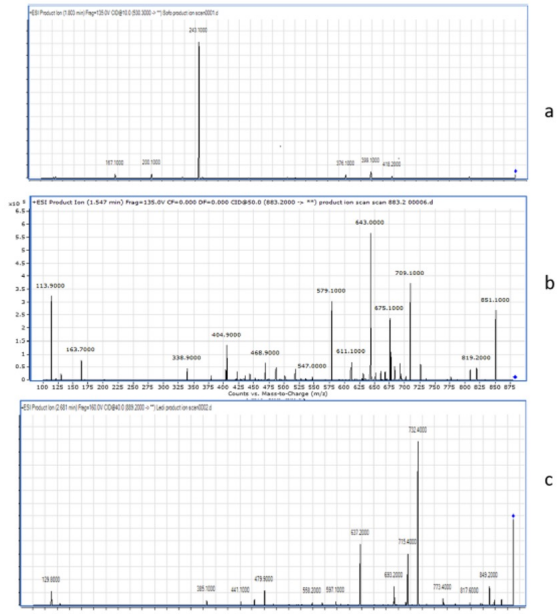
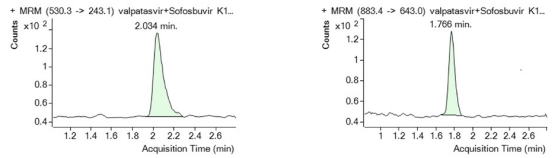



**Figure 4**: ESI-MS daughter ion scan of Sofosbuvir (a), Velpatasvir (b) and Ledipasvir (c).




(a) Sofosbuvir (b) Velpatasvir

**Figure 5:** Typical LLOQ chromatograms of Sofosbuvir (a) and Velpatasvir (b).

## 5. Results Summary:

**Table 1: Summary of Pre-study Validation Calibration Curves' Parameters for Sofosbuvir**

| Cal. No | Y-intercept | Slope | Coefficient of determination ($R^2$) |
|---|---|---|---|
| 1 | 0.0014 | 0.0007 | 0.9968 |
| 2 | 0.0014 | 0.0007 | 0.9951 |
| 3 | 0.0008 | 0.0007 | 0.9962 |
| 4 | 0.0014 | 0.0008 | 0.9949 |
| 5 | 0.0010 | 0.0008 | 0.9918 |
| 6 | 0.0016 | 0.0008 | 0.9906 |
| n | 6 | 6 | 6 |
| mean | 0.0013 | 0.0008 | 0.9942 |
| SD | 0.0003 | 0.00005 | 0.0025 |
| CV% | | 6.6578 | 0.2484 |

**Table 2: Summary of Pre-study Validation Calibration Curves' Parameters for Velpatasvir**

| Cal. No | Y-intercept | Slope | Coefficient of determination (R2) |
|---|---|---|---|
| 1 | 0.0012 | 0.0007 | 0.9969 |
| 2 | 0.0010 | 0.0007 | 0.9958 |
| 3 | 0.0013 | 0.0006 | 0.9980 |
| 4 | 0.0014 | 0.0008 | 0.9968 |
| 5 | 0.0013 | 0.0008 | 0.9963 |
| 6 | 0.0011 | 0.0008 | 0.9968 |
| n | 6 | 6 | 6 |
| mean | 0.0012 | 0.0007 | 0.9968 |
| SD | 0.0002 | 0.00006 | 0.0007 |
| CV% | | 8.8854 | 0.0738 |

**Table 3: Results of back-calculated standards from Calibration curves for Sofosbuvir**

| Cal No | 5 | 10 | 100 | 500 | 1000 | 2500 | 4500 | 5000 |
|---|---|---|---|---|---|---|---|---|
| Mean | 5.02 | 10.34 | 95.44 | 444.01 | 975.65 | 2578.19 | 4994.38 | 5186.91 |
| CV (%) | 2.25 | 8.91 | 2.74 | 3.15 | 3.07 | 2.34 | 1.98 | 2.27 |
| Accuracy | 100.32 | 103.37 | 95.44 | 88.80 | 97.57 | 103.13 | 110.99 | 103.74 |

**Table 4: Results of back-calculated standards from Calibration curves for Velpatasvir**

| Cal No. | 10 | 20 | 50 | 100 | 400 | 800 | 1300 | 1500 |
|---|---|---|---|---|---|---|---|---|
| Mean | 9.98 | 20.76 | 45.71 | 99.22 | 406.44 | 811.54 | 1373.19 | 1456.17 |
| CV (%) | 2.31 | 3.78 | 1.90 | 2.30 | 1.57 | 2.36 | 2.61 | 2.14 |
| Accuracy | 99.82 | 103.78 | 91.42 | 99.22 | 101.61 | 101.44 | 105.63 | 97.08 |

**Table 5: A Summary of the Priori Validation results for Sofosbuvir**

| Parameter | Item | Results | | |
|---|---|---|---|---|
| Calibration | | 5-5000 ng/ml | | |
| Lower Limit | | 5 ng/ml | | |
| | | QCL | Q | QC |
| Between- runs | Accuracy | 97.38 | 97. | 101. |
| Between- runs | Coefficients of | 5.44 | 8.11 | 4.39 |
| Within-run accuracy | Accuracy [Range from | 93.21-100.22 | 92.89-100.97 | 93.91-105.84 |

| | | | | |
|---|---|---|---|---|
| Within-run precision | Coefficients of variation % | 4.66-5.52 | 6.36-8.71 | 4.19-4.40 |
| Recovery of | QC mean % | 83.35 | 81.23 | 80.57 |
| Short-term | Accuracy | 107.8 | -- | 110.00 |
| Long-term | Accuracy | 100. | -- | 103. |
| Freeze and | Accuracy | 96.11 | -- | 103. |
| Post-prepara | Accuracy | 103 11 | -- | 103 |
| Stock solution stability | Stability % | 6 hrs at room | 97.10 | |
| | | 10 days | 103.55 | |
| Stock solution stability | Stability % | 6 hrs at room | 85.38 | |
| | | 16 days at -20°C | 102.56 | |

**Table 6: A Summary of the Priori Validation results for Velpatasvir**

| Parameter | Item | Results | | |
|---|---|---|---|---|
| Calibration Curve Range | | 10-1500 ng/ml | | |
| Lower Limit of Quantitation | | 10 ng/ml | | |
| | | QCL | QCM | QCH |
| Between- runs accuracy | Accuracy | 96.46 | 101.65 | 94.61 |
| Between- runs precision | Coefficients of variation %. | 9.28 | 12.45 | 8.22 |
| Within-run accuracy | Accuracy % [Range from lowest to highest values] | 93.76-100.78 | 98.97-105.35 | 89.30-100.41 |
| Within-run precision | Coefficients of variation % [Range from lowest to highest values] | 2.77-3.79 | 4.90-7.97 | 2.13-3.63 |
| Recovery of analyte | QC mean % recovery | 81.00 | 79.76 | 80.94 |
| Short-term stability of analyte in matrix at room temperature (24 hours) | Accuracy | 99.69 | -- | 91.99 |
| Long-term stability of analyte in matrix at -70°C (116 days) | Accuracy | 98.49 | -- | 103.57 |
| Freeze and thaw stability of analyte in matrix at -70°C | Accuracy | 92.06 | -- | 90.32 |
| Post-preparative stability (24 hours) | Accuracy | 97.20 | -- | 89.92 |
| Stock solution stability of the drug | Stability % | 6 hrs at room temp | 98.17 | |
| | | 10 days at -20°C | 100.62 | |
| Stock solution stability of the internal standard | Stability % | 6 hrs at room temp | 85.38 | |
| | | 16 days at -20°C | 102.56 | |

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