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ABSTRACT

Simeprevir (also known as TMC435 or TMC435350) is a novel hepatitis C protease inhibitor. A validated high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the sensitive and selective quantification of simeprevir in human EDTA plasma is described. During assay development, special attention was given to light instability of the drug in plasma and blood. The method consisted of precipitation of plasma proteins with acetonitrile after which the supernatant was analyzed using electrospray LC–MS/MS. The linearity was confirmed in the concentration range from 2.00 to 2000 ng/mL, with 50-fold dilution extending to 100,000 ng/mL. The precision of this assay, expressed as CV, ranged between 4.4% and 8.5% over the entire concentration range with assay accuracy between −0.3% and 8.5%. The method was applied successfully in many clinical studies to document the pharmacokinetics of simeprevir in plasma from healthy volunteers and patients.

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1. Introduction

Hepatitis C is one of the most common viral liver diseases (after hepatitis B) and is caused by the hepatitis C virus (HCV). It is estimated that 170 million patients worldwide and about 2% of the population in developed countries and up to 9% in developing countries are chronically infected with HCV [1]. The majority of acute HCV infections become chronic, some of which progress towards liver cirrhosis or hepatocellular carcinoma [2–6]. However, HCV is an asymptomatic, slowly progressive disease evolving over 10–20 years. In some cases, hepatitis C can remain asymptomatic even after significant liver damage has occurred.

Since the discovery of the virus, there have been many advances in hepatitis C research. The current standard of care is pegylated interferon alpha 2a and -alpha 2b in combination with ribavirin, which has sustained a viral response rate of 40–50% in genotype 1 HCV–infected patients, which accounts for the majority of the hepatitis C population in the United States and Japan, and of 80–90% in patients infected with genotype 2 or 3 HCV [7–12]. Moreover, this therapy may be poorly tolerated and can cause serious adverse effects during the 48-weeks treatment. This means that more effective therapeutic drugs with fewer side effects such as skin problems, weight loss, depression, anaemia, neutropenia, and thrombocytopenia and shorter treatment durations are needed for patients infected with HCV.

Simeprevir is a novel, oral, selective, highly potent, HCV NS3/4A-protease inhibitor. Its efficacy and safety have been demonstrated in clinical phase I–III trials. In order to assess the human pharmacokinetics of simeprevir, a validated, robust and selective bioanalytical assay was required allowing the quantification of simeprevir; both when dosed alone or in combination with the standard of care (pegylated interferon α in combination with ribavirin).

The assay described was validated according to current Guidelines on bioanalytical method validation [13,14] and considering the proceedings from the Crystal City III conference report [15]. Initially, a bioanalytical assay was developed and validated using liquid–liquid extraction as sample clean-up. In order to increase the bioanalytical throughput and to facilitate sample clean up when analysing thousands of samples from phase II and phase III studies, the sample preparation was modified to protein precipitation.

2. Experimental

2.1. Chemicals

Simeprevir (2R,3αR,10Z,11αS,12αR,14αR)-N-[(cyclopropylsulfonyl)−2−[(2−4-isopropyl−1,3-thiazol−2−yl)−7−methoxy−8−methyl−4−quinolinyl][oxy]−5−methyl−4,14-dioxo−2,3,3a,4,5,6,7,8,9,11a,12,13,14,14a-tetradecahydrocyclopenta[c]cyclopenta[g][1,6]diazacyclotetradecine−12a−(1H)−carboxamide (C₃₈H₄₇N₅O₇S₂, MW 749.94,
Fig. 44 and software from trometer (13C1C37H44D3N5O7S2) SIL-HTc was obtained (Millipore).

Fig. 1. Chemical structure of (A) simprevir and (B) STIL internal standard.

Table 1

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.2</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>2.00</td>
<td>1.2</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>2.10</td>
<td>1.7</td>
<td>1</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>2.90</td>
<td>1.7</td>
<td>1</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>2.91</td>
<td>1.2</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>4.00</td>
<td>1.2</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
</tbody>
</table>

starting from method development till analysis of study samples in PhIII studies.

Calculations were done using Watson LIMS (Thermo Electron Corporation) version 7.2 or 7.3.

Calibration curves were created by plotting the log-transformed peak area ratios against the nominal log-transformed simprevir standard concentrations. Final concentrations were obtained by back-calculation from the calibration curves.

2.3. Assay history

Originally, the assay consisted of a liquid–liquid extraction, chromatographic separation on a RP-18 stationary phase, followed by MS/MS detection. For the reasons mentioned above, liquid–liquid extraction was replaced by protein precipitation. Method robustness was further improved by preparing calibration standards in bulk that were stored frozen until use (instead of daily spiking calibration samples from standard solutions), and by changing HPLC conditions. Cross validation of the assays was done using independent QCs. All validated assays complied with the acceptance criteria applicable for regulated bioanalysis. Procedural details of the final assay are given in following paragraphs.

2.4. Assay description

A 100 μL aliquot of the samples was transferred to a 96-well round deep-well microplate (Porvair) and spiked with 100 μL methanol and 50 μL STIL-IS (200 ng/mL methanol). Proteins were then precipitated with 350 μL acetonitrile, followed by vigorous mixing for 3 min using a VX-2500 Multitube Vortexer and centrifugation for 10 min at 5000–6000 g. Two μL from the supernatant was injected on the LC–MS/MS system.

Chromatographic separation was achieved using a gradient mobile phase on a reversed phase 4.6 mm × 30 mm column packed with 3.5 μm C18-Xbridge (Waters) operating at 40 °C. Details are given in Table 1.

Detection was done by a Triple Quadrupole Mass Spectrometer API-4000 with a Turbolonspray™ interface. The Turbolonspray™ ionsource was set at 500 °C with an ionization voltage of 5 kV. The nebulizing gas (N2) and curtain gas flows (N2) were set at 50 and 40 l/min, respectively, and the declustering potential at 40 V. Collision-induced fragmentation at Q2 occurred at a collision energy of 47 eV. The dwell time was 300 ms and mass analysers Q1 and Q3 were operated at unit mass resolution. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode (m/z 750.3 → 315.0 for simprevir and at m/z 754.3 → 319.0 for the STIL-IS).

The compound eluted at a retention time of approximately 2.11 min and showed to be selective towards circulating metabolites. In Fig. 2, a product ion scan of simprevir, acquired in positive ion mode by infusing the standard solution at a concentration of 20 μg/mL, and a chromatogram of simprevir and its STIL-IS of a real patient sample, taken at 6 h post-dose are shown.

The LC–MS/MS assay for determination of simprevir in human EDTA plasma was transferred to 2 different contract labs and was used to analyze samples originating from phase I, phase II and
phase III clinical studies. Cross-validation of the assay, using the same set of QCs, was performed between the reference lab and the 2 other labs.

2.5. Light sensitivity evaluation

During method development, photochemical degradation of simeprevir in blood and plasma under daylight conditions was observed. Light sensitivity tests were designed to cover the anticipated conditions that samples of pharmacokinetic studies may experience: the photochemical degradation of simeprevir in human blood and plasma samples was evaluated when protected from light and exposed to day light for 4 h. In addition, a Suntest CPS+ instrument [16] was used to enable photochemical stability testing under accelerated and standardized conditions. Analyses were done in single. The instrument was operated under the following conditions: 550 W/m², black standard temperature (BST) 22 °C, phase time 180 min. Under these conditions, 0.5 h in the Suntest corresponds to 2 h near the window on a sunny summer’s day in the Belgian lab (which is located at a longitude of 51° N) for blood and for plasma, 1 h in the Suntest corresponds to 12 h near the window [17].

2.6. Stock stability in methanol

The stability of simeprevir in methanol was evaluated on a HPLC-UV (Agilent/HP-1100) system. The stock solutions (20 μg/ml) were stored during the described time intervals (see Table 5) and analyzed in single using a generic HPLC-UV method. Five μL from
the stock solution was injected. Chromatographic separation was achieved on a reversed phase 4.6 mm × 100 mm column packed with 3 μm C18 BDS-Hypersil (Alltech). Elution conditions are given in Table 2.

UV detection occurred at a wavelength of 210 nm (DAD).

3. Results and discussion
3.1. Assay validation

The method was validated in terms of linearity and range, accuracy, precision, LLOQ, specificity/selectivity, matrix effect and incurred sample reproducibility (ISR) in accordance with procedures that reflect the FDA and EMA guidelines for bioanalytical method validation [13–15,18].

Stock solutions were prepared by dissolving the compound (or STL IS) in methanol. Two separate stock solutions were prepared from different weighings, one for the calibration curves and the other for the quality control samples.

The calibration range was 2.00–2000 ng/mL. Quality control samples were prepared at 4 concentration levels. The internal standard working solution was prepared at a concentration of 200 ng/mL.

All the solutions were prepared in amber glassware and stored in a –20 °C freezer.

For the validation of the regression model, the calculation of accuracy and precision and the establishment of the lower and upper limits of quantification (LLOQ and ULOQ), QC s were analyzed in 6-fold in three independently processed analytical runs, together with a human plasma spiked calibration curve.

The specificity of the analytical method was investigated by extraction and analysis of blank human EDTA plasma samples from six different non-pooled sources and six spiked samples at LLOQ level to assess potential interference from endogenous substances. The apparent response (peak area) at the retention time of semprevir in the 6 blank samples was compared with the mean peak area of TKMC435 in the 6 lower limit of quantification (2.00 ng/mL) samples.

The effect of the human EDTA plasma matrix on positive ionization with the Turbospray® device was investigated by the analysis of blank non-pooled plasma extracts of 6 different sources spiked (before extraction) at two concentration levels (at 4.00 ng/mL and at 2000 ng/mL).

An overview of the results of all parameters validated is given in Table 3.

Incurred sample reproducibility (ISR) was performed in several clinical studies. In all cases, the study samples selected for ISR were re-analyzed as soon as possible after generation of the first results.

Calculation of each individual re-analysis result was expressed as bias (%), where the first obtained result was the reference value:

\[
\text{Bias}[\%] = \left( \frac{100 \times \text{concentration}_{\text{re-analysis}}}{\text{concentration}_{\text{original analysis}}} \right) - 100
\]

ISR was proven when the bias was between 80.0% and 120.0% for at least 67% of all analyzed ISR study samples. The type of studies for ISR purpose was selected according to the EMA [14] and EBF [19] recommendations: 10% of the available PK samples per study were re-analyzed. For each selected study, samples around Cmax and from the elimination phase were chosen.

ISR was performed in the first in human study, a special population (healthy Japanese subjects) study, Japanese food effect study, renal impairment study, DD1 study, bioavailability study and Phase IIb study.

The preset ISR criterion was met in all selected studies.

In addition to the validated parameters described above, selectivity of the assay in the presence of co-medication, used in the semprevir DDI Ph1 studies was tested. The assay showed to be selective towards all tested comedication.

3.2. Light sensitivity of semprevir

As photochemical degradation of compounds can vary depending on season, latitude, weather conditions (sunny versus cloudy) etc., it is important to test photochemical degradation under well controlled conditions which are representative for the clinical site where samples are collected and the lab where samples are analyzed.

The different conditions tested along with the results are displayed in Table 4. A human blood and plasma sample incubated under yellow light conditions was used as reference.

The results revealed that semprevir in blood is stable for 4 h when exposed to day light and for 30 min under Suntest conditions, indicating the effectiveness of red blood cells in absorbing radiation and thus preventing or significantly slowing down decomposition of semprevir.

In plasma, 4 h of testing under daylight did not reveal any photochemical degradation, but only 70.9% of semprevir could be recovered when exposed for 1 h to the standardized conditions in the Suntest. Therefore, the clinical sites were instructed to handle plasma samples under yellow light conditions and to store the plasma samples in brown polypropylene tubes; blood samples do not need to be protected from light as long as they are processed within 4 h of collection.

Table 3
Overview of results of validated parameters.

<table>
<thead>
<tr>
<th>Range (ng/mL)</th>
<th>Inter-run accuracy (2% bias)/precision (SCV)</th>
<th>Selectivity</th>
<th>Matrix effect</th>
<th>Extraction recovery (%)</th>
<th>Dilution ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00–2000</td>
<td>2.00 ng/mL: 8.5%/8.5%</td>
<td>Interference ≤ 15.0%</td>
<td>Simeprevir: 5.00 ng/mL: 96.9</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.81 ng/mL: 7.1%/5.5%</td>
<td>= 20.0%</td>
<td>100 ng/mL: 96.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>76.1 ng/mL: –0.3%/4.8%</td>
<td>≤ 5.0%</td>
<td>2000 ng/mL: 100.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1560 ng/mL: 0.0%/4.4%</td>
<td></td>
<td>2000 ng/mL: 100.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Selectivity evaluated on LLOQ samples and contribution of analyte in blank matrix.

<sup>2</sup> Contribution of STL internal standard in blank matrix.

Table 4
Stability results of semprevir in human plasma and blood under different light conditions.

<table>
<thead>
<tr>
<th>Human blood (%)</th>
<th>Human plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference sample</td>
<td>100</td>
</tr>
<tr>
<td>0.5 h suntest</td>
<td>94.7</td>
</tr>
<tr>
<td>1 h suntest</td>
<td>NT</td>
</tr>
<tr>
<td>3 h suntest</td>
<td>NT</td>
</tr>
<tr>
<td>4 h day light</td>
<td>88.6</td>
</tr>
</tbody>
</table>

NT: not tested.

Results are expressed in % compared to the reference, semprevir is considered to be stable when the deviation of the test sample compared to the reference sample is less than 15.0%.
3.3. Stability data

Stability investigations were performed in whole blood, plasma and stock solutions as part of the validation of the bioanalytical assay for simprevir in human plasma. Stability of simprevir in the respective matrices and solvents was assessed by means of spiked samples stored under different conditions of temperature. As photochemical degradation of simprevir in plasma under daylight conditions was observed during method development, all stability experiments for method validation were performed under yellow light conditions. The results (peak area) of the stock stability were evaluated by comparison with the mean peak area results of a freshly prepared stock solution, analyzed in duplicate. Stability of the compound in solution was proven if the peak area of the test solution was between 85.0% and 115.0% of the mean peak area of the new prepared stock solution.

For the evaluation of simprevir in whole blood, the whole blood samples were centrifuged after equilibration and the mean back-calculated concentrations of the corresponding plasma samples were compared with the mean back-calculated concentrations of the reference plasma samples, which were obtained immediately after spiking of fresh blood. Stability of the compound in blood was proven if the mean measured value of the test samples was between 85.0% and 115.0% of the mean measured value of the reference samples. The stability of simprevir in human EDTA plasma was evaluated by comparing the mean back-calculated concentrations of the stored stability samples with the nominal concentrations. Stability of the compound in plasma was proven if the mean measured value of the test samples was between 85.0% and 115.0% of the spiked (nominal) value.

Post-extraction analyte stability in the autosampler was evaluated after 74 and 120 h of storage. To this end, 4 sets of QC samples (low and high concentration) were processed; 2 sets of QCs were stored at room temperature and protected from light for 74 and 120 h respectively; 2 sets of QCs were stored at refrigerator temperature for 74 and 120 h, respectively. The concentrations of the injected QCs were calculated using freshly prepared calibrator samples. Stability of the compound was proven if the mean measured value of the test samples was between 85.0% and 115.0% of the spiked (nominal) value.

The results of all stability experiments are summarized in Table 5.

### Table 5

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Temperature</th>
<th>Period</th>
<th>Low (n = 3)</th>
<th>High (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Accuracy</td>
<td>%CV</td>
</tr>
<tr>
<td>Stock solution in methanol</td>
<td>~20 °C</td>
<td>6 months</td>
<td>100.7%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>4 °C</td>
<td>1 month</td>
<td>102.2%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>3 days</td>
<td>92.6%</td>
<td>–</td>
</tr>
<tr>
<td>Plasma (human EDTA)</td>
<td>Ambient</td>
<td>72 h</td>
<td>98.5%</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Freezer/thaw</td>
<td>6 cycles</td>
<td>102.0%</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>~20 °C</td>
<td>1184 days</td>
<td>97.9%</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>~70 °C</td>
<td>64 days</td>
<td>99.2%</td>
<td>0.8</td>
</tr>
<tr>
<td>Whole blood (human EDTA)</td>
<td>4 °C</td>
<td>24 h</td>
<td>108.0%</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>24 h</td>
<td>102.9%</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
<td>2 h</td>
<td>104.3%</td>
<td>3.6</td>
</tr>
<tr>
<td>Processed sample</td>
<td>Ambient</td>
<td>120 h</td>
<td>97.3%</td>
<td>3.5</td>
</tr>
<tr>
<td>stability (PSS)</td>
<td>4 °C</td>
<td>120 h</td>
<td>97.8%</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Stock solution samples were analyzed at one concentration in single using a generic HPLC-UV method. Stability of simprevir was met under all conditions mentioned in Table 5.

4. Conclusions

A bioanalytical assay for the determination of simprevir in human plasma samples was developed and validated using LC–MS/MS and showed to be a robust, selective and sensitive assay for the quantification of simprevir in human EDTA plasma samples. Precision and bias were within ±15.0% across the whole calibration range of the method. The assay showed to be selective and specific, without interference from endogenous substances or co-medications. Sample stability was demonstrated in different matrices and under different conditions. Because of potential degradation of simprevir when exposed to daylight, the samples need to be protected from daylight during sampling, storage, shipment and analysis.

In addition, the high-throughput analysis achieved using LC–MS/MS made this assay particularly applicable for pharmacokinetic investigations in phase II and III trials, when large numbers of samples need to be processed in a biosafety lab.

References

14. EMA, Guideline on Bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), 2012.