Journal of Chromatography B, 877 (2009) 4001–4006

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Highly sensitive determination of HCV protease inhibitors boceprevir (SCH 503034) and telaprevir (VX 950) in human plasma by LC–MS/MS

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ARTICLE INFO

Article history:
Received 27 July 2009
Accepted 7 October 2009
Available online 14 October 2009

Keywords:
Tandem mass spectrometry
Boceprevir
Telaprevir
Antivirals

ABSTRACT

The purpose of this study was to develop a specific and highly sensitive method based on fast sample preparation and LC–MS/MS techniques for the determination of the HCV protease inhibitors boceprevir (SCH 503034) and telaprevir (VX 950) in human plasma. Boceprevir, telaprevir and the internal standard dimethylcelecoxib were separated on a Luna C18 column (150 mm × 2.0 mm I.D., 5 μm particle size) under gradient conditions with a mobile phase A consisting of water/ammonia solution (25%) (100:0.05, v/v) and mobile phase B consisting of methanol/ammonia solution (25%) (100:0.05, v/v) and a chromatographic run time of 11 min. The lower limit of quantification (LLOQ) of boceprevir and telaprevir is 0.25 pg on column (25 pg/mL at injection volume of 10 μL). The method possesses a reliable calibration range of 0.025–2.5 ng/mL. Due to the dilution of real life plasma samples by a factor of 10 during the precipitation process the method is suitable to quantify boceprevir and telaprevir at a concentration range of 0.25–25 ng/mL. Variations in accuracy and intraday and interday precision ($n = 6$ for each concentration) were <15% over the whole range of calibration. For the first time, a rapid, specific, sensitive, accurate and reproducible LC–MS/MS method in human plasma has been developed and validated. It is suitable to quantify the concentrations of the hepatitis C virus protease inhibitors boceprevir and telaprevir in human plasma.

1. Introduction

Chronic hepatitis C virus (HCV) infection remains a global health threat with approximately 175 million carriers worldwide [1]. Currently, treatment options consist of pegylated interferon-alfa co-administered with the nucleoside analog ribavirin for 16–72 weeks, depending on HCV genotype, baseline viral load and initial virological response to therapy. Serious adverse effects and limited sustained virological responses of 50% (genotype 1) with this therapy warrant the need for novel HCV therapies [2]. Structure determination of hepatitis C virus (HCV) key replication enzymes such as the HCV protease NS3/NS4 has enabled the design and synthesis of new HCV specific drugs. Specifically Targeted Antiviral Therapy against hepatitis C virus (STAT-C) stands for a new era in the treatment of patients with chronic hepatitis C. Various STAT-C compounds including boceprevir (SCH 503034) and telaprevir (VX 950) have already entered clinical trial phases. These compounds are blocking the NS3/4A protease of the hepatitis C virus which is located in the N-terminal domain of the HCV NS3 protein [3]. The NS3 protease is a serine protease essential for HCV polyprotein processing and therefore viral replication. NS3 forms a stable complex with the NS4A protein, which the protease requires as a cofactor, along with a structural zinc molecule, for optimal activity [3]. Boceprevir binds reversibly to the NS3 protease active site and has potent activity in the replicon system alone [4] and in combination with interferon alfa-2b and ribavirin. During boceprevir or telaprevir monotherapy multiple resistance mutations were selected [5,10]. Therefore, combination therapy with pegylated interferon-alfa, ribavirin or other direct antiviral drugs seems mandatory to avoid development of resistance. The HCV SPRINT-1 study assessed safety and efficacy of boceprevir 800 mg every 8 h plus peginterferon alfa-2b (1.5 μg/kg/week) and ribavirin in the treatment of naïve patients with chronic hepatitis C genotype 1 infection [6] with response rates up to 75%. Telaprevir is another peptidomimetic inhibitor of the NS3/4A serine protease, whose safety and antiviral activity was tested in several clinical studies (PROVE1/2) with sustained response rates up to 68% in the treatment of naïve genotype 1 patients [7,8].

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doi:10.1016/j.jchromb.2009.10.013

So far, only one method has been developed for the measurement of the diastereomers of boceprevir in monkey plasma [11] and no method has been published for the measurement of telaprevir in biological fluids. For the first time, we present a specific and highly sensitive LC–MS/MS method for the determination of boceprevir or telaprevir in human plasma. The LLOQ (25 pg/mL at injection volume of 10 μL) of boceprevir or telaprevir in the described LC–MS/MS-assay is 0.25 pg on column in human plasma for both substances.

2. Experimental

2.1. Materials

Acetonitrile (LC–MS grade), water (LC–MS grade), methanol (LC–MS grade), ammonia solution (25%) were purchased from Roth GmbH (Karlsruhe, Germany). The protease inhibitor VX 950 (purity >97% (HPLC)) as standard substance was obtained from partners of the DRUGPHARM platform within the 6th framework Network of Excellence viRgil. SCH 503034 (purity >97% (HPLC)) as standard substance was obtained from Schering Plough (Kenilworth, USA). Dimethylcelecoxib (purity >99.5% (HPLC)), used as internal standard was synthesized from Laboratorien Berlin-Adlershof GmbH (Berlin, Germany). Blank human plasma was a gift from the Blutspendedienst Hessen (Deutsches Rotes Kreuz, Frankfurt am Main, Germany).

2.2. Instrumentation

Sample analysis was performed by using liquid chromatography–electrospray-ionization-tandem mass spectrometry (LC–ESI-MS/MS). The LC–MS/MS system consisted of an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative ESI mode, an Agilent 1200 binary HPLC pump (G1312B) and degasser (G1379B) (Agilent, Waldbronn, Germany) and an HTC Pal autosampler (Chromtech, Idstein). A cooling stack was used to store the samples at 4 °C in the autosampler. High purity nitrogen for the mass spectrometer was produced by a NGM 22-LC/MS nitrogen generator (cmc Instruments, Eschborn, Germany).

2.3. Preparation of stock solutions and calibration curve

The stock solutions of boceprevir (SCH 503034) and telaprevir (VX 950) were prepared at a concentration of 1 mg/mL in methanol. Dilutions prepared in acetonitrile at concentrations of 0.1 mg/mL, 10 μg/mL and 1 μg/mL were used as working standards to prepare the calibration standards. Appropriate amounts of the working standards were added into 1.5 mL test tubes. The working standards were prepared at the concentration levels of 0.5, 1, 1.5, 2, 5, 10, 20, 30, 40, 50 ng/mL in acetonitrile. For each batch of measurement the working standards were freshly prepared. The stock solution (1 mg/mL) of the IS was prepared in methanol and was further diluted with acetonitrile to 8 ng/mL and used for all analyses. All solutions were stored at −70 °C except the working standard of the IS which was kept at 4 °C.

2.4. Sample extraction

Boceprevir and telaprevir were extracted from human plasma by precipitation without an evaporation and reconstitution process. Therefore, samples were prepared by mixing 100 μL blank human plasma with 50 μL of working standard (0.5–50 ng/mL in acetonitrile), 100 μL internal standard (8 ng/mL dimethylcelecoxib in acetonitrile) and 750 μL acetonitrile to obtain calibration standards from 0.025 to 2.5 ng/mL and with a final concentration of dimethylcelecoxib of 0.8 ng/mL. The mixture was then vortexed for 1 min and centrifuged for 3 min at 13,000 × rpm. Finally, the supernatant was transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC–MS/MS system.

Double blank samples (DB) were not spiked with working standard or with internal standard solution. Blank samples (B) were spiked with internal standard solution (dimethylcelecoxib 8 ng/mL) but not with working standard solution. Boceprevir and telaprevir in study plasma samples are extracted by mixing 100 μL plasma with 50 μL acetonitrile instead of working standard, 100 μL internal standard (8 ng/mL dimethylcelecoxib in acetonitrile) and 750 μL acetonitrile. The further extraction process works as aforementioned.

2.5. LC–MS/MS conditions

For the chromatographic separation, a reversed phase Luna C18 column with a precolumn was used (150 mm × 2.0 mm I.D., 5 μm particle size, 100 Å pore size, phenomenex, Aschaffenburg, Germany). A linear gradient was employed at a flow rate of 0.4 mL/min. Mobile phase A was water/ammonia solution (25%) (100:0.05, v/v) and mobile phase B was methanol/ammonia solution (25%) (100:0.05, v/v). Directly after injection of the sample the gradient started from 95/5 A/B to 5/95 A/B within 3 min. Then for 4 min the mobile phase was held at 5/95 A/B. To re-equilibrate the column the gradient composition shifted back to 95/5 A/B within 0.5 min and was held for 3.5 min. Total run time was 11 min. Injection volume of samples was 10 μL. Retention times of boceprevir, telaprevir and IS were 6.50 min ± 0.01 min, 6.63 min ± 0.01 min and 6.47 min ± 0.01 min (mean ± S.D.), $n = 10$, respectively.

The mass spectrometer was operated in the negative ion mode with an ionspray voltage of −4100 V at 550 °C. Ion source gas 1 (GS1) was set to 60 psi, ion source gas 2 (GS2) to 90 psi and curtain gas to 15 psi. Multiple reaction monitoring (MRM) was used for quantification. The mass transitions used for quantification were m/z 518 → 419 (collision energy (CE) −28 eV) for boceprevir, m/z 678 → 320 (−38 eV) for telaprevir and m/z 394 → 330 (−32 eV) for the internal standard dimethylcelecoxib with a dwell time of 120 ms. Control transitions were m/z 518 → 195 (−30 eV) for boceprevir, m/z 678 → 235 (−54 eV) for telaprevir and m/z 394 → 69 (−54 eV) for the internal standard, dimethylcelecoxib. The product ion spectra of boceprevir and telaprevir are shown in Fig. 1 and representative chromatograms are shown in Figs. 2–4. All quadrupoles were working at unit resolution. Quantitation was performed with Analyst Software V1.4.2 (Applied Biosystems, Darmstadt, Germany) using a matrix matched calibration. Ratios of analyte peak area and internal standard peak area (y-axis) were plotted against ratios of analyte and internal standard concentrations (x-axis) and calibration curves were calculated by least square regression with $1/x^2$ weighting.

3. Results and discussion

3.1. Method development and optimization

To assess the optimal compound-dependent parameters such as declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP), tuning solutions of 10 ng/mL in methanol were infused into the mass spectrometer with an infusion rate of 10 μL/min. Intense molecular peaks and significant fragments were found in the negative ionization mode. Additionally, source dependent parameters such as collision gas (CAD), curtain gas (CUR), ion source gas 1 (GS1), ion source gas 2 (GS2), ionspray voltage (IS) and temperature (TEM) were optimized by flow injection. At last, HPLC conditions were adjusted.

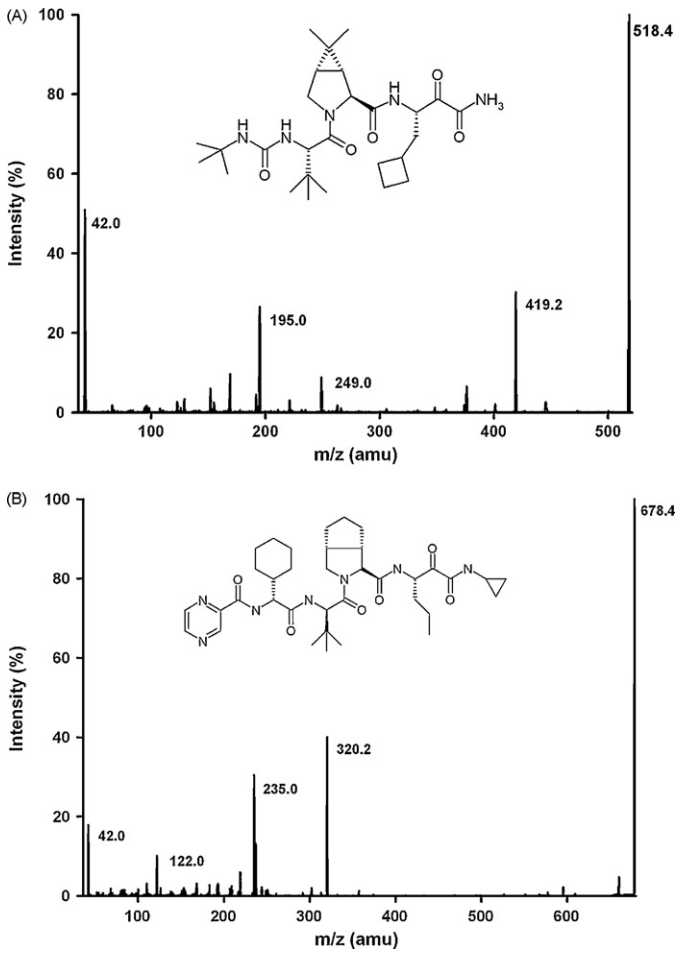

Fig. 1. Structures and product ion spectra of boceprevir (a) and telaprevir (b).

3.1.1. Internal standard

The best way to cope with sample matrix effects and variations during extraction process is to use a stable isotope labelled analyte as internal standard. Since no such internal standard is commercially available, an alternative had to be found. Such a substance should match the chromatographic retention, recovery and ionization properties of boceprevir and telaprevir. Dimethylcelecoxib was found to fulfill these criteria sufficiently.

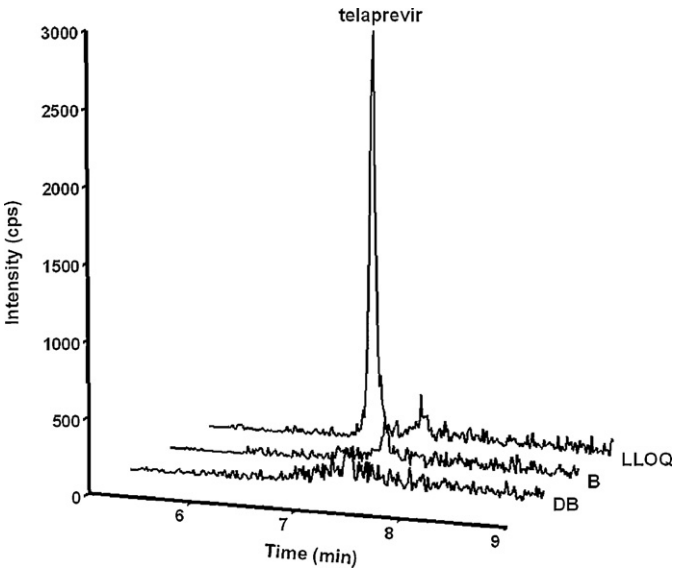

Fig. 3. Representative chromatograms for telaprevir (MRM transition m/z 678 → 320) from a double blank (without IS and analyte), blank (with IS and without analyte) and calibration standard plasma sample containing 0.25 pg telaprevir on column (LLOQ).

3.2. Method validation

Method validation was performed according to the FDA recommendations [9].

3.2.1. Accuracy of extracted samples

Assay accuracy was calculated with six different standard series in the range from 0.025 to 2.5 ng/mL. Each calibration curve was prepared with drug free human plasma. For calculation, ratios between peak areas and concentrations of boceprevir or telaprevir

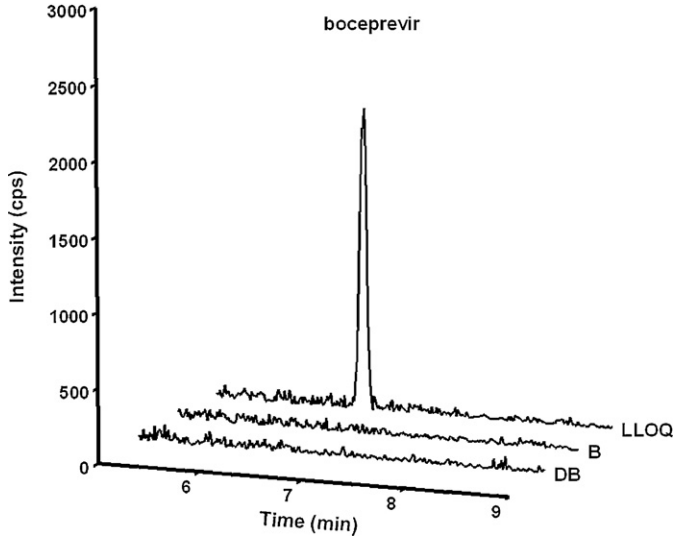

Fig. 2. Representative chromatograms for boceprevir (MRM transition m/z 518 → 419) from a double blank (without IS and analyte), blank (with IS and without analyte) and calibration standard plasma sample containing 0.25 pg boceprevir on column (LLOQ).

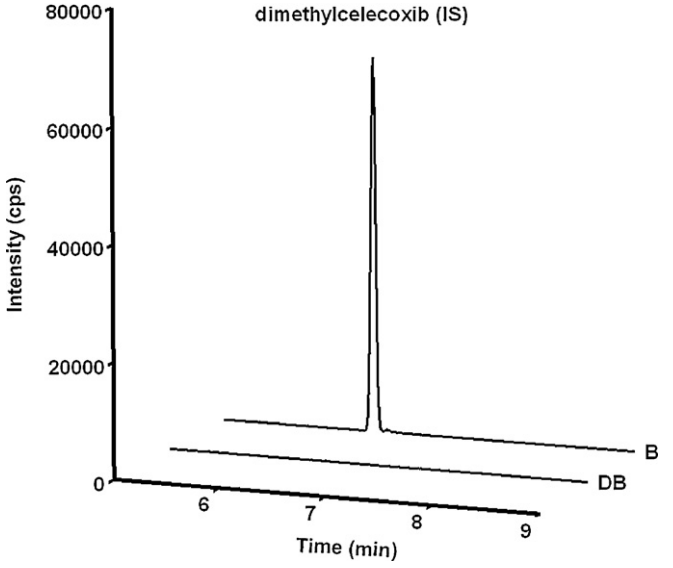

Fig. 4. Representative chromatograms for dimethylcelecoxib (IS, MRM transition m/z 394 → 69) from a double blank (without IS and analyte) and blank (with IS and without analyte) standard plasma sample containing 0.8 ng/mL dimethylcelecoxib.

Table 1
Accuracy of extracted standard samples in human plasma.

Nominal concentration (ng/mL)	Telaprevir			Boceprevir		
	Mean ± S.D. (%) (n = 6)		R.S.D. (%)	Mean ± S.D. (%) (n = 6)		R.S.D. (%)
	ng/mL	%		ng/mL	%	
Double blank	No peak			No peak		
Blank	No peak	–	–	No peak	–	–
0.025	0.025 ± 0.0	99.2 ± 1.0	1.0	0.025 ± 0.0	99.8 ± 0.8	0.8
0.05	0.051 ± 0.0	102.3 ± 2.0	2.0	0.050 ± 0.0	100.2 ± 1.9	1.9
0.15	0.143 ± 0.0	95.3 ± 1.8	1.9	0.149 ± 0.0	98.9 ± 2.6	2.6
0.25	0.263 ± 0.0	105.3 ± 1.5	1.4	0.257 ± 0.0	102.8 ± 1.0	1.0
0.5	0.514 ± 0.0	102.8 ± 3.6	3.5	0.502 ± 0.0	100.3 ± 2.6	2.6
0.75	0.757 ± 0.0	100.9 ± 2.4	2.4	0.743 ± 0.0	99.1 ± 1.9	1.9
1	0.906 ± 0.0	90.6 ± 4.1	4.5	0.969 ± 0.0	96.9 ± 2.3	2.4
1.5	1.538 ± 0.0	102.3 ± 1.2	1.2	1.520 ± 0.0	101.5 ± 1.7	1.6
2	2.015 ± 0.0	100.6 ± 1.4	1.4	2.002 ± 0.0	100.0 ± 1.3	1.3
2.5	2.513 ± 0.0	100.6 ± 1.3	1.3	2.507 ± 0.0	100.4 ± 1.5	1.5
	Mean ± S.D. (%)	100.0 ± 4.2		Mean ± S.D. (%)	100.0 ± 1.6	

Accuracy was determined using six standard curves prepared with six different human plasmas. No peaks of boceprevir/telaprevir were detectable in unspiked plasma samples (double blank, blank, extracted blank plasma + 8 ng/mL dimethylcelecoxib; S.D., standard deviation; R.S.D., relative standard deviation).

and internal standard, dimethylcelecoxib, were used. Best values were obtained with weighted least square regression (weighting factor $1/x^2$). Mean accuracy (% ± S.D. (%)) of the assay was found to be 100.0 ± 1.6 for boceprevir and 100.0 ± 4.2 for telaprevir over the calibration range of 0.025–2.5 ng/mL. Detailed data are given in Table 1.

3.2.2. Precision of extracted samples

Intraday precision of the assay was determined using four concentrations (0.025, 0.5, 1 and 2.5 ng/mL). Each sample was then analyzed six times in a row. Interday precision was measured at three different days. The precision determined at each concentration level (0.025, 0.5, 1, 2.5 ng/mL) showed a mean accuracy value (% ± S.D. (%)) of intraday precision of 101.2 ± 3.5 for boceprevir and 100.5 ± 5.9 for telaprevir and a mean interday precision value of 99.1 ± 5.6 for boceprevir and 100.2 ± 6.4 for telaprevir (Table 2).

3.2.3. Relative and absolute recovery

Extraction efficacy of boceprevir/telaprevir and dimethylcelecoxib was determined at three different concentrations (0.05, 0.75 and 2.5 ng/mL). Relative recovery was calculated by comparing the mean peak areas of six extracted standards with the mean peak areas of six blank extracted and subsequently spiked samples. Relative recovery was constant over the calibration range. Mean relative recovery (% ± S.D. (%)) was 101.2 ± 7.7 for boceprevir, 98.6 ± 4.7 for telaprevir and 100.3 ± 3.0 for dimethylcelecoxib. Absolute recovery was ascertained by comparing mean peak areas of extracted samples with matrix free solvent standards in acetonitrile. Absolute recovery was constant over the calibration range. Mean absolute recovery (% ± S.D. (%)) was 99.4 ± 2.9 for boceprevir, 104.6 ± 2.4 for telaprevir and 105.4 ± 2.7 for dimethylcelecoxib (Table 3).

3.2.4. Stability of boceprevir and telaprevir

Each stability test (freeze/thaw, short-term, long-term, and autosampler) of boceprevir and telaprevir was determined with six standard samples at two different concentrations (0.05 and 2 ng/mL), which were prepared with six different blank human plasmas. The sample extraction was arranged as described below.

The freeze/thaw stability of the analytes was evaluated by analysing spiked and extracted samples at time t = 0 and after the third freeze and thawing cycle. One cycle included storing at −70 °C for 24 h and unassisted thawing at room temperature for 30 min. The samples were measured against a freshly prepared calibration curve. Boceprevir and telaprevir were stable under the freeze/thaw conditions described above.

To assess short-term stability a series of six extracts at each concentration (0.05 and 2 ng/mL) were stored at −70 °C for 24 h. After thawing for 4 h at room temperature the samples were mea-

Table 2
Intra- and interday precision of extracted standard samples in human plasma.

Nominal concentration (ng/mL)	Telaprevir		Boceprevir	
	Mean (ng/mL) ± S.D. (%) (n = 6)	R.S.D. (%)	Mean (ng/mL) ± S.D. (%) (n = 6)	R.S.D. (%)
Intraday				
0.025	0.03 ± 0.0	5.5	0.03 ± 0.0	2.95
0.5	0.53 ± 0.0	0.7	0.52 ± 0.0	0.59
1	0.97 ± 0.05	4.8	0.97 ± 0.02	1.77
2.5	2.51 ± 0.13	5.2	2.5 ± 0.05	1.84
Nominal concentration (ng/mL)	Telaprevir		Boceprevir	
	Mean (ng/mL) ± S.D. (%) (n = 18)	R.S.D. (%)	Mean (ng/mL) ± S.D. (%) (n = 18)	R.S.D. (%)
Interday				
0.025	0.03 ± 0.0	8.2	0.02 ± 0.0	7.0
0.5	0.50 ± 0.03	6.3	0.49 ± 0.02	4.8
1	0.96 ± 0.04	3.9	0.96 ± 0.03	3.6
2.5	2.56 ± 0.10	3.9	2.54 ± 0.14	5.5

Intraday precision was determined on three different days with four concentrations of boceprevir/telaprevir (0.025, 0.5, 1, 2.5 ng/mL). Each sample was analyzed six times in a row. Final concentration of dimethylcelecoxib in each sample was 0.8 ng/mL (S.D., standard deviation; R.S.D., relative standard deviation).

Table 3
Relative and absolute recoveries of extracted samples in human plasma.

Nominal concentration (ng/mL)	Telaprevir		Boceprevir		Dimethylcelecoxib	
	Mean (%) ± S.D. (%) (n = 6)	R.S.D. (%)	Mean (%) ± S.D. (%) (n = 6)	R.S.D. (%)	Mean (%) ± S.D. (%) (n = 6)	R.S.D. (%)
Relative recovery						
0.05	93.3 ± 5.3	5.7	110.1 ± 8.1	7.3	102.2 ± 1.4	1.3
0.75	99.9 ± 3.8	3.9	96.9 ± 2.9	3.0	96.9 ± 2.9	3
2.5	102.5 ± 14.9	14.4	96.6 ± 13.4	13.9	101.9 ± 15.2	14.9
Mean (%) ± S.D. (%)	98.6 ± 4.7		101.2 ± 7.7		100.3 ± 3.0	
Absolute recovery						
0.05	104.7 ± 5.0	4.8	99.1 ± 5.5	5.6	104.2 ± 2.3	2.2
0.75	107.0 ± 3.5	3.3	102.5 ± 3.2	3.1	108.5 ± 2.8	2.6
2.5	102.2 ± 1.9	1.9	96.8 ± 2.1	2.1	103.5 ± 2.5	2.4
Mean (%) ± S.D. (%)	104.6 ± 2.4		99.4 ± 2.9		105.4 ± 2.7	

Relative (as compared to blank extracted and subsequently spiked matrix samples) and absolute recovery (as compared to spiked matrix free samples) for boceprevir/telaprevir and dimethylcelecoxib of six different plasma samples. Final concentration of dimethylcelecoxib in each sample was 0.8 ng/mL (S.D., standard deviation; R.S.D., relative standard deviation).

sured against a freshly prepared calibration curve. Boceprevir and telaprevir were stable under those conditions.

Long-term stability of boceprevir and telaprevir was analyzed at 0.05 and 2 ng/mL. For each concentration and each time (time zero (t = 0) and after 15, 30, 90 and 120 days), a series of six samples established with six different blank human plasmas were prepared. The spiked plasma samples were aliquoted and stored under conditions of −70 °C. A freshly prepared standard row was used for each measurement. No difference was observed to freshly prepared calibration standards.

The autosampler stability was performed by measuring samples of two different concentrations (0.05 and 2 ng/mL). After storing in the autosampler of the analytical system at 4 °C the measurement was repeated after 24 h. All measured stability samples were constant over the calibration range (Table 4).

3.2.5. Matrix effects

Matrix and suppression effects were assessed with 18 extracted blank matrix samples at three different concentrations (0.05, 0.75 and 2.5 ng/mL) from 6 different human plasmas, which were reconstituted with a corresponding amount of analyte in acetonitrile. The mean peak areas of all samples were compared with the mean peak areas of 18 matrix free standards in acetonitrile. The ratio was within the acceptable limits (85–115%). No significant ion suppression or enhancement was observed at the expected retention time of the targeted ions.

3.3. Discussion

Among all new compounds of STAT-C therapy, the clinical evaluation process of the protease inhibitors boceprevir and telaprevir is most advanced. The clinical approval of boceprevir and telaprevir will likely take place in 2011. Current pharmacokinetic studies are still strictly monitored by investigators. Therefore, little information has been published so far and more combined data on pharmacokinetics, pharmacodynamics, and the emergence of resistant strains is required.

Boceprevir is manufactured and applied in humans as a 1:1 mixture of SCH 534129 ((R)-isomer) and SCH 534128 ((S)-isomer) [11]. To date, it is unknown, if the two diastereomers have different pharmacological activities.

The first analytic mass spectrometric determination of diastereomers of boceprevir was described in monkey plasma, with a concentration range of 1–2500 ng/mL [11]. Our method has several advantages as compared to this method: (i) we use human plasma because the designated indication of boceprevir is STAT-C therapy in humans, (ii) we were not forced to use ion-pairing reagents with respect to the possible source pollution and high background contribution effects and (iii) our method is very sensitive giving the opportunity to use very little plasma volumes (10 μL or less). So far, it is not described that diastereomers of boceprevir differ in their pharmacology, therefore a diastereomeric separation was not included in our method development. Furthermore, the different composition of monkey and human plasma probably

Table 4
Stabilities for telaprevir and boceprevir samples in human plasma.

Nominal concentration (ng/mL)	Telaprevir		Boceprevir	
	Mean (%) ± S.D. (%) (n = 8)	R.S.D. (%)	Mean (%) ± S.D. (%) (n = 8)	R.S.D. (%)
Freeze/thaw stability				
0.05	101.5 ± 4.4	4.3	99.1 ± 11.1	11.2
2	101.8 ± 6.5	6.4	96.7 ± 4.5	4.7
Short-term stability				
0.05	105.9 ± 5.0	4.7	105.1 ± 3.3	3.1
2	104.3 ± 3.5	3.4	101.7 ± 3.1	3.0
Long-term stability				
0.05	94.4 ± 5.4	5.8	92.4 ± 5.2	5.6
2	95.5 ± 5.9	6.2	95.8 ± 4.8	5.0
Autosampler stability				
0.05	100.8 ± 6.9	6.8	102.4 ± 2.2	2.2
2	103.9 ± 2.1	2.0	101.2 ± 2.0	2.0

Recovery of six different plasma samples after performing, freeze/thaw stability, short-term stability, long-term stability and autosampler stability for boceprevir and telaprevir (S.D., standard deviation; R.S.D., relative standard deviation).

plays an important role for the stability of the antiviral compounds, since the described bench-top instability of boceprevir in monkey plasma was not seen in our study with human plasma.

The sparse information in humans include mean trough plasma concentrations (±S.D.) of telaprevir ranging from 719 ± 120 ng/mL in the breakthrough group, 827 ± 417 ng/mL in the plateau group, and 1064 ± 353 ng/mL in the continuous decline group in HCV genotype 1 infected patients [10] (data provided by the investigator Vertex, Cambridge, USA). These high plasma concentrations indicate that there is probably an additional dilution step of real life samples necessary to determine telaprevir by our method (calibration range 0.25–25 ng/mL). However, one aspect during method development was to establish a highly sensitive method which may find application also in future approaches in quantification of intracellular concentrations of boceprevir and telaprevir.

4. Conclusion

For the first time, a LC–MS/MS method combined with plasma precipitation was developed and validated for the determination of boceprevir and telaprevir in human plasma in the concentration range of 0.25–25 ng/mL. This method offers advantages of fast extraction process, low sample volume, high sensitivity and selectivity. The LLOQ of boceprevir and telaprevir was found to be 0.25 pg on column (25 pg/mL at injection volume of 10 μL).

Funding

The present study was supported by a DFG grant (Klinische Forschergruppe, KFO 129) and the Dr. Robert Pfleger Stiftung, Bamberg.

Conflict of interest

Prof. Zeuzem is a consultant, clinical investigator and has served on the Speaker's Bureau for Roche Pharmaceuticals, Nutley, NJ, Schering-Plough, Kenilworth, NJ and Vertex, Cambridge, MA. Prof. Sarrazin provides research support for Vertex and Schering-Plough. Other authors: none to declare.

Acknowledgements

We thank the viRgil Drugpharm (LSHM-CT-2004-503359) and Schering Plough for generously providing VX 950 and SCH 503034, respectively.

References

[1] T. Poynard, M.F. Yuen, V. Ratziu, C.L. Lai, Lancet 362 (2003) 2095.
[2] M.W. Fried, M.L. Shiffman, K.R. Reddy, C. Smith, G. Marinos, F.L. Gonçales Jr., D. Häussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, J. Yu, N. Engl. J. Med. 347 (2002) 975.
[3] S.H. Chen, S.L. Tan, Curr. Med. Chem. 12 (2005) 2317.
[4] B.A. Malcolm, R. Liu, F. Lahser, S. Agrawal, B. Belanger, N. Butkiewicz, R. Chase, F. Gheyas, A. Hart, D. Hesk, P. Ingravallo, C. Jiang, R. Kong, J. Lu, J. Pichardo, A. Prongay, A. Skelton, X. Tong, S. Venkatraman, E. Xia, V. Girijavallabhan, F.G. Njoroge, Antimicrob. Agents Chemother. 50 (2006) 1013.
[5] S. Susser, C. Welsch, Y. Wang, M. Zettler, F.S. Domingues, U. Karey, E. Hughes, R. Ralston, X. Tong, E. Herrmann, S. Zeuzem, C. Sarrazin, Hepatology. (2009) Aug 4. [Epub ahead of print].
[6] P. Kwo, E. Lawitz, J. McCone, E. Schiff, J. Vierling, D. Pound, M. Davis, J. Galati, S. Gordon, N. Ravendhran, L. Rossaro, F. Anderson, I. Jacobson, R. Rubin, K. Koury, C. Brass, E. Chaudhri, J. Albrecht, J. Hepatol. 50 (2009) S4.
[7] C. Hézode, N. Forestier, G. Dusheiko, P. Ferenci, S. Pol, T. Goeser, J.P. Bronowicki, M. Bourlière, S. Gharakhanian, L. Bengtsson, L. McNair, S. George, T. Kieffer, A. Kwong, R.S. Kauffman, J. Alam, J.M. Pawlotsky, S. Zeuzem, N. Engl. J. Med. 360 (2009) 1839.
[8] J.G. McHutchison, G.T. Everson, S.C. Gordon, I.M. Jacobson, M. Sulkowski, R. Kauffman, L. McNair, J. Alam, A.J. Muir, N. Engl. J. Med. 360 (2009) 1827.
[9] FDA, Guidance for Industry—Bioanalytical Method Validation, 2001.
[10] C. Sarrazin, T.L. Kieffer, D. Bartels, B. Hanzelka, U. Müh, M. Welker, D. Wincheringer, Y. Zhou, H.M. Chu, C. Lin, C. Weegink, H. Reesink, S. Zeuzem, A.D. Kwong, Gastroenterology 132 (2007) 1767.
[11] G. Wang, Y. Hsieh, K.C. Cheng, R.A. Morrison, S. Venkatraman, F.G. Njoroge, L. Heimark, W.A. Korfmacher, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 852 (2007) 92.