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Werner J. Heinz*, Dieter Kuschak, Diana Schirmer, Anna Grau, Daniela Keller and Hartwig Klinker

HPLC method for the determination of the S- and R-diastereomers of telaprevir for treatment of patients with hepatitis C

Etablierung einer HPLC-Methodik für die Bestimmung der S- und R-Diastereomere von Telaprevir für die Therapie der Hepatitis C

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Abstract: Telaprevir (TVR) was approved by the FDA in May 2011 for the treatment of hepatitis C. This protease inhibitor converts into two diastereomers with significant difference in antiviral activity. Clinical efficacy has been correlated with serum concentrations. Therefore, a sensitive and selective high-performance liquid chromatographic method for the simultaneous determination of both clinically relevant diastereomers of TVR was developed. Linearity ranged from 20 to 10,000 ng/mL. The coefficients of variation were <7.3%, and accuracy was between −4.0 and 5.4%. In 105 clinical samples, both diastereomers of TVR had a high degree of correlation to each other, but concentrations showed a broad range and an increase during therapy.

Keywords: drug monitoring; hepatitis C; high-performance liquid chromatography; telaprevir.

Zusammenfassung: Telaprevir (TVR) wurde im Mai 2011 von der FDA für die Behandlung der Hepatitis C zugelassen. Dieser Protease Inhibitor wechselt zwischen zwei diastereomeren Konfigurationen mit deutlich unterschiedlicher antiviraler Aktivität. Erste Daten legen eine Korrelation der klinischen Effektivität mit den Serumkonzentrationen nahe. Daher wurde eine sensitive und selektive Methode mittels Hochdruckflüssigkeitschromatographie für die gleichzeitige Bestimmung beider klinisch relevanter Diastereomere von TVR entwickelt. Diese Bestimmung ist linear für den Bereich von 20 bis 10,000 ng/mL. Der Variationskoeffizient war kleiner 7,3% und die Genauigkeit liegt zwischen −4,0 und 5,4%. In 105 klinischen Proben zeigte sich ein hoher Grad der Korrelation zwischen beiden TVR Diastereomeren. Dabei hatten die TVR Spiegel eine hohe Schwankungsbreite und stiegen im Verlauf der Therapie an.

Schlüsselwörter: Telaprevir; Hochdruckflüssigkeitschromatographie; Therapeutische Drug Monitoring; Hepatitis C.

Introduction

Hepatitis C virus infection affects about 180 million people worldwide and is a cause of significant morbidity and mortality including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1]. Adding telaprevir (TVR), one of the first approved hepatitis C protease inhibitors, to combination therapy of ribavirin and pegylated interferon raised the rate of sustained virological response (SVR) by about 25% for treatment-naïve patients with genotype 1 hepatitis C [2]. In addition, duration of treatment can be reduced by 50% for most patients.

TVR inhibits the serine-dependent NS3-4A protease. TVR is only slightly soluble in water, its weight is

***Correspondence: Werner J. Heinz,** Division of Infectious Diseases, Department of Internal Medicine II, University of Wuerzburg Medical Center, Oberduerrbacher Str. 6, 97080 Wuerzburg, Germany, Tel.: +49 931 201 40044, Fax: +49 931 201 640044, E-Mail: Heinz_W@ukw.de
Dieter Kuschak, Diana Schirmer, Anna Grau and Hartwig Klinker: Department of Internal Medicine II, University of Wuerzburg Medical Center, Oberduerrbacher Str. 6, 97080 Wuerzburg, Germany
Daniela Keller: Statistics and Counseling, Jakob-Stoeßel-Str. 7, 97262 Hausen, Germany

679.8 g/mol and the formula is $C_{36}H_{53}N_7O_6$ (see Figure 1). In plasma, S-telaprevir (S-TVR, previously known as VX-950) interconverts to its diastereomer R-telaprevir (R-TVR, previously known as VRT-127384). Drug absorption is dependent on consumption of a fat-containing meal. Its short elimination half-life necessitates a twice or three times daily administration. Because TVR is a substrate and an inhibitor of cytochrome p450 (Cyp) 3A4 and of the membrane transporter p-glycoprotein, there is a broad range of potential and known interactions with impact on the bioavailability of TVR [3, 4].

Several studies could demonstrate a relationship between serum drug concentration and clinical efficacy of TVR [5–7]. R-TVR has a clearly reduced antiviral activity compared to S-TVR (about 1:30) [8]. This necessitates differential determination of both diastereomers. Up to now, only methods including mass spectrometry (MS) for determination of both diastereomers of TVR have been established [9, 10]. Unfortunately, this technique is associated with high costs and is not generally available. Therefore, the aim of the present study was to develop a high-performance liquid chromatography (HPLC)-based method with UV detection for determination of both S-TVR and R-TVR separately and its evaluation in clinical practice.

Materials and methods

S-TVR and R-TVR were provided by Janssen Pharmaceutica NV (Beerse, Belgium), the internal standard (IS) diazepam by Sequoia research Products Ltd. (Pangbourne, UK) and bovine serum albumin (BSA) powder was obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of S-TVR, R-TVR and diazepam were prepared in acetonitrile:acetic acid 0.5% (v/v) (Sigma-Aldrich, Steinheim, Germany) with a concentration of 1 mg/mL each. Six combined TVR working solutions were prepared by dilution with the same solvent resulting in concentrations of 50, 200, 500, 1000, 2500 and 5000 ng/mL for both TVR isomers. Each solution was stored at –20 °C.

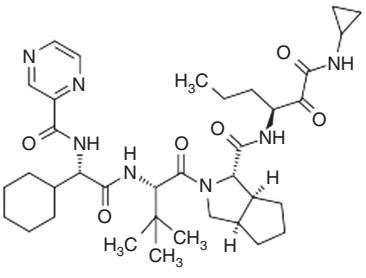

Figure 1: Chemical structure of telaprevir.

For the preparation of serum standard samples, BSA solutions (5%, wt/vol) were spiked with S-TVR and R-TVR to obtain the above-mentioned concentrations. For patient samples, blood was centrifuged by 2500 *g* for 10 min at 4 °C without delay directly after blood drawing and clotting. Samples were stored at –20 °C. Seventy-five microliters of IS was added to 1 mL of serum or BSA standard samples, mixed with 50 µL acetic acid 10% (Merck, Darmstadt, Germany); the solutions were briefly vortexed.

Samples were extracted twice with 3 mL of diethyl ether (Merck, Darmstadt, Germany) for 5 min, followed by centrifugation at 2500 *g* for 5 min. The organic layers were transferred into glass tubes and evaporated to dryness (37 °C) under a gentle stream of nitrogen. The residue was reconstituted in 200 µL solvent (H_2O+NH_4OH (25%) (100:0.05 v/v): 85% MeOH:15% ACN+NH_4OH (25%) (100:0.05 v/v) 1:1) and washed for 5 min with 1.0 mL n-hexane (Merck, Darmstadt, Germany), followed by another centrifugation at 2500 *g* for 5 min. The organic layers were discarded, and the samples were transferred to autosampler vials with glass micro inserts for HPLC analysis.

The HPLC system (Beckman-Coulter, Krefeld, Germany) consisted of a 126 solvent pump, a 168 UV-VIS photodiode array detector, a 508 autosampler, a column oven Jetstream 2plus and a 32 Karat software version 5.0. Chromatographic separation was performed by a LUNA C18(2)-HST 100×2 mm/2.5 µ column protected by a guard column C18 4×2 mm (Phenomenex, Aschaffenburg, Germany) at 40 °C using a column thermostat. A linear gradient was employed at a flow rate of 0.2 mL/min, with mobile phase A: H_2O+NH_4OH (25%) (100:0.05 v/v): 85% MeOH:15% ACN+NH_4OH (25%) (100:0.05 v/v) 90:10 and mobile phase B: H_2O+NH_4OH (25%) (100:0.05 v/v): 85% MeOH:15% ACN+NH_4OH (25%) (100:0.05 v/v) 10:90). A 50 µL aliquot was injected into the chromatograph. The flow rate was 0.2 mL/min, and detection was carried out at 270 nm. For calculation of linearity, variability, lower limit of detection and quantification, Valistat 2.0 software (Arvecon, Walldorf, Germany) was used. Precision and accuracy were evaluated with three combined quality control samples in six separate runs. Concentration of S-TVR and R-TVR were 200, 1000 and 2500 ng/mL.

For clinical validation, serum samples were obtained from patients on TVR treatment for chronic hepatitis C, after patient had signed informed consent, approved by the responsible ethics committee of the University of Wuerzburg medical faculty.

All statistical calculations were performed with Statistical Product and Service Solutions (SPSS) for Windows, version 21.0 (SPSS, Chicago, IL, USA). The *t*-tests of mean

concentrations and correlations were considered statistically significant at p values ≤0.05.

Results

Chromatography and detection

A representative HPLC chromatogram for S-TVR and R-TVR is shown in Figure 2. Peak shape separation from endogenous compounds and separation of both diastereomers from each other were optimized by using a C18 (Luna HST 100×2 mm/2.5 µ) column protected by a security guard column. Retention times for IS, S-TVR and R-TVR were approximately 7.0, 13.4 and 14.4 min. Neither in the blank plasma sample nor in any of the 105 clinical samples were interfering endogenous peaks detectable.

Linearity, accuracy and precision of the assay

Linearity was evaluated over a concentration range of 20–10,000 ng/µL with a correlation >0.9995 for both isoforms of TVR. The rate of recovery was 84% for both S-TVR and R-TVR and did not change significantly in

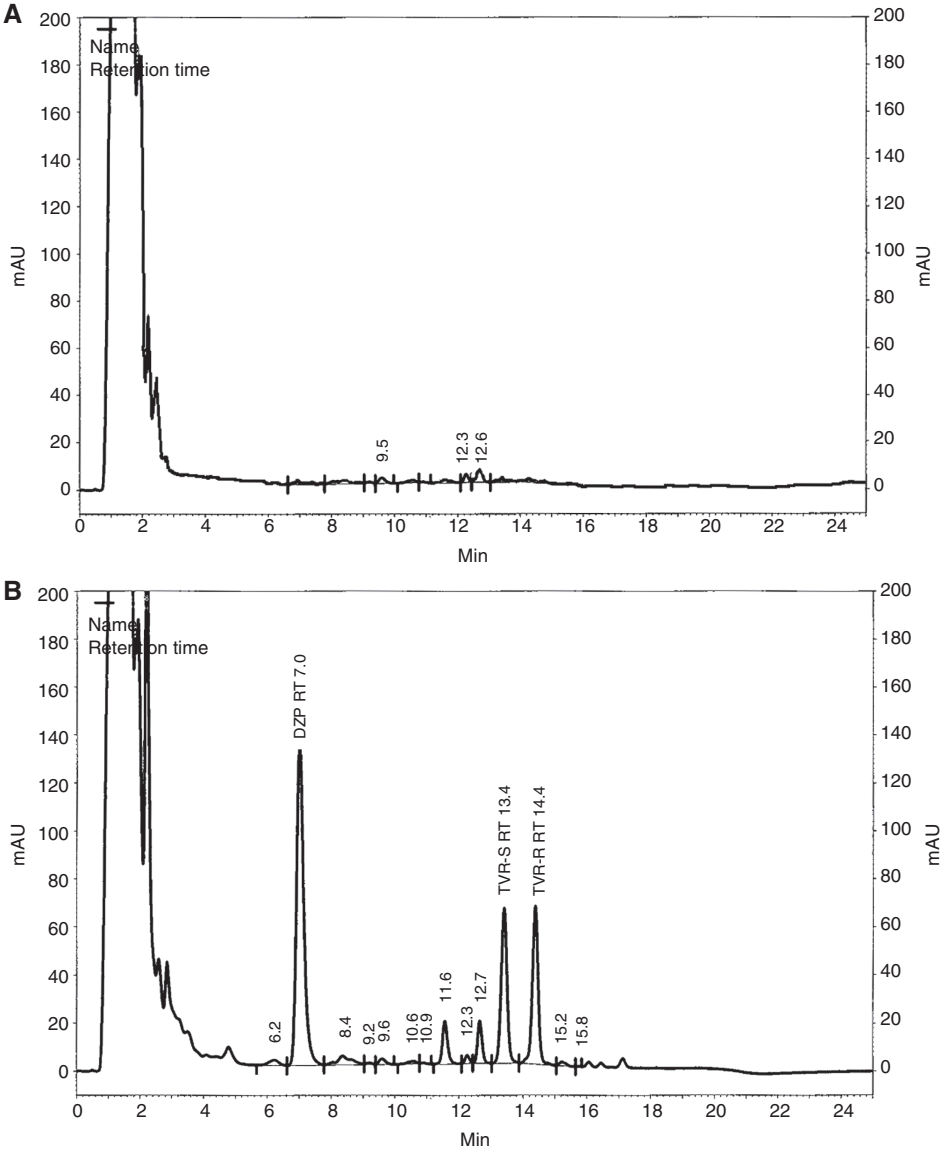

Figure 2: Representative HPLC chromatograms (at 270 nm) of (A) a blank BSA sample; (B) serum sample from a patient treated for hepatitis C, with the internal standard diazepam (DZP), S-TVR (1281 ng/mL) and R-TVR (1531 ng/mL).

Table 1: Intraassay and interassay variability: accuracy and precision for the determination of S-TVR and R-TVR concentrations in spiked samples using HPLC.

Isomer	Nominal conc., ng/mL	Intraassay (n=6)			Interassay (n=8)		
		Observed conc. and SD[a], ng/mL	Accuracy[b], %	Precision[c] (CV)	Observed conc. and SD[a], ng/mL	Accuracy[b], %	Precision[c] (CV)
S-TVR	200	202±11.3	0.9	5.6	204±11.4	1.8	5.6
	1000	1020±67.6	3.0	6.6	1008±45.1	0.8	4.5
	2500	2474±163	−1.1	6.6	2481±167	−0.8	6.7
R-TVR	200	203±10.9	1.6	5.4	211±12.6	5.4	6.0
	1000	996±72.9	−0.4	7.3	988±56	−1.2	5.7
	2500	2400±167	−4.0	7.0	2526±89	1.0	3.5

Calculation performed by mean of six and eight replicates. [a]Observed concentration±mean standard deviation (SD) as shown). [b]Accuracy (%) is calculated as [(nominal concentration−observed concentration)/observed concentration]×100. [c]Precision is expressed as the coefficient of variation (CV), calculated as (standard deviation/observed concentration)×100.

human serum. Intraassay and interassay variability was between 5.6–6.6% and 5.6–6.7% for S-TVR and 5.4–7.3% and 3.5–6.0% for R-TVR. The intraassay rate of accuracy was between –1.1 and 3.0 for S-TVR and between –0.4 and 4.0 for R-TVR. In the interassay validation, the rate of accuracy was between –0.8 and 1.8% for S-TVR and –1.2 and 5.4 for R-TVR (Table 1).

Limits of quantification

The lower limit of detection of S-TVR in serum was 4.4 ng/mL. The lower limit of quantification was reached at a concentration of 11.2 ng/mL. For R-TVR, 5.7 ng/mL was determined as the limit of detection and 11.2 ng/mL as the limit of quantification. All limits were calculated with 99% significance according to DIN32645. The upper limit of quantitation was arbitrarily set at 10,000 ng/mL.

Analysis of patient samples

For clinical validation, 105 serum samples, obtained from 18 patients after 1–12 weeks of treatment with TVR for hepatitis C, were investigated. In one sample, no TVR was detected. Median concentration per sample was 2044 ng/mL for S-TVR and 2011 ng/mL for R-TVR, and also median concentrations per patient were 2036 ng/mL for S-TVR and 1981 ng/mL for R-TVR (Table 2). Here, both diastereomers were highly correlated to each other ($p<0.001$, by Spearman regression, Figure 3). Gender and age of the patient had no significant impact on TVR levels. But for R-TVR the concentrations did show a slight but significant increase (Spearman 0.539, p 0.02) (Supplementary Figure S1 that accompanies the article at http://www.degruyter.com/view/j/labm.2015.39.issue-3/labmed-2015-0001/labmed-2015-0001.xml?format=INT) and for S-TVR (Spearman 0.384, p 0.12) a trend to higher concentrations during therapy (data not shown).

Table 2: S-TVR and R-TVR concentrations in 105 samples from 18 patients.

	R-TVR	S-TVR
Concentration/sample		
Mean±standard deviation	2264 ng/mL±1053	2390 ng/mL±1294
Median	2011 ng/mL	2044 ng/mL
Concentration/patient		
Mean±standard deviation	2122 ng/mL±988	2266 ng/mL±1216
Median	1981 ng/mL	2036 ng/mL

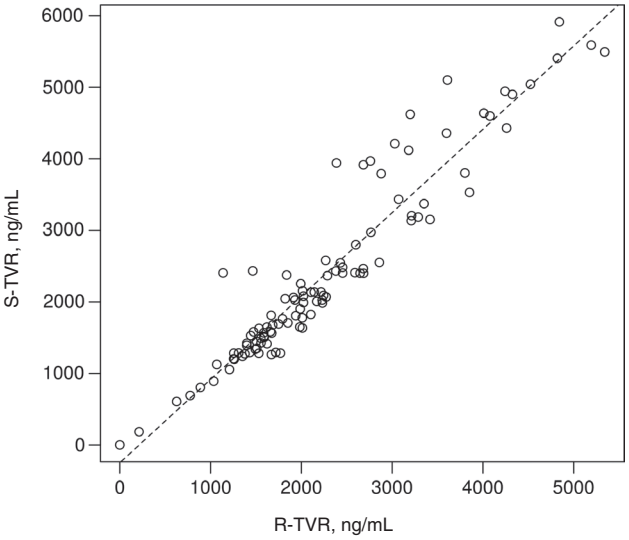

Figure 3: Plot of S-TVR vs R-TVR concentrations in all 104 samples (Spearman correlation of the data of 17 patients: $\rho=0.9463$, $p<0.001$).
One sample/patient without detectable S-TVR and R-TVR concentration was excluded.

Discussion

Here we describe the first HPLC assay in combination with UV detection for the determination of both clinically active isomers of TVR. This method has a high rate of precision and accuracy and is able to determine S-TVR and R-TVR concentrations between 20 and 10,000 ng/μL. The assay has been verified in 105 clinical samples, and no interfering signal or compound could be detected. Due to its use as internal standard, the test is interfered in case of concomitant use of diazepam and potentially also of other closely related benzodiazepines.

This HPLC-UV assay has a broader range for quantification and compatible accuracy and precision compared to three MS-based methods for determination of TVR concentration [9, 11, 12]. Another reported that MS-based method does have a remarkably low limit and range of quantification (0.025–2.5 ng/mL), outside clinical relevance [10]. MS is associated with higher costs, and MS facilities are not generally available in standard hospital laboratories. So, for many centers, the HPLC assay with UV detection presented here is not only a convenient and accessible method but also a reliable option for therapeutic drug monitoring of serum concentrations of both TVR diastereomers.

The only previously reported HPLC-UV assay has a remarkable lower interassay precision (up to 13.0%) and accuracy (up to 8.13%) and most importantly determines TVR without differentiation of the diastereomers [13]. Little is known on interconversion of S-TVR and R-TVR, including timing, proportion and stability of each isoform. No fixed correlation of TVR diastereomers, instead variation in the rate of epimerization has been reported. This interconversion of TVR changes over time, in relation to the pH of the medium, and is different in human plasma, rat plasma or buffer. But differentiation of both isoforms is important, as the antiviral activity of S-TVR is 30-fold higher than the activity of R-TVR [8], mandating a separate determination at least of S-TVR.

The survey on S-TVR and R-TVR levels in 18 patients treated for hepatitis C demonstrates a high intraindividual range for both diastereomers. Although TVR levels might increase during therapy, they still have a broad range. Many parameters with impact on TVR drug serum concentrations, including concomitant intake of food and CYP 3A4 or PGP inhibiting or inducing medication, have already been identified.

Furusyo et al. [5] could demonstrate significantly higher TVR mean concentrations on day 3 in patients achieving SVR compared to those who did not. Another study comparing twice and three times daily intake of TVR did show higher mean $AUC_{24\,h}$ for TVR in patients with SVR (89.787 h*ng/mL) compared to those without SVR (79.001 h*ng/mL) [6]. In these trials, patients were receiving TVR in combination with pegylated interferone and ribavirin, which may influence outcome. In an early phase I study, patients did show three different groups of viral response. After an initial decline, viral load did increase again (TVR 719 ng/mL) and did reach a plateau (827 ng/mL) or continuously declined (1064 ng/mL). Differences in mean TVR plasma trough concentration were significant between the groups (p 0.032) but did not correlate with the dose, emphasizing the impact of TVR drug monitoring [7].

Taking into account the potential relevance of TVR levels to achieve SVR, determination of TVR concentrations might help to avoid failure of this complex and cost-intensive therapy.

Conclusions

Here we present a validated, reliable and convenient HPLC-based method with UV detection for determination of both diastereomers S-TVR and R-TVR. Calibration for a range of concentrations of 20–10,000 ng/μL for both isoforms is appropriate for clinical drug monitoring. This and the practicability of the assay are demonstrated by 105 samples from patients treated for chronic hepatitis C in our outpatient care unit. The described assay can be helpful to further investigate epimerization of TVR and help to better understand interconversion of an antiviral compound with different activities of the isoforms.

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