□報告□

Determination of sofosbuvir via a high-performance liquid chromatography method using ultraviolet detection

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Abstract

Sofosbuvir (SOF) is a nucleotide analog drug that inhibits hepatitis C virus replication. In the present study, we aimed to develop a simple, specific, sensitive, and precise method for the quantification of SOF in water-based and human plasma samples by using a high-performance liquid chromatography method with ultraviolet detection (HPLC-UV). The analyte was extracted from 0.25 mL water or human plasma samples with an acetonitrile-based solution and analyzed on a C_{18} reversed-phase column. Sorafenib was used as an internal standard (IS). The mobile phase was composed of ammonium acetate buffer, and methanol and acetonitrile. The flow rate was 1.0 mL/min, and the UV wavelength was set at 265 nm. The retention times of SOF and IS were 7.8 min and 9.1 min, respectively. The total run time for a single analysis was 15.0 min. The calibration curve was linear within the range 0.1−10.0 μg/mL. The limit of quantitation was 0.1 μg/mL. The recovery of the drug from human plasma samples was greater than 99 %. The intra- and inter-day precision of water-based and plasma samples ranged from 1.41% to 10.06% and 0.21 to 6.53%, respectively. The intra- and inter-day accuracy of water-based and plasma samples ranged from 92.71 to 98.41% and 89.31 to 101.21%, respectively. The proposed method is very simple and allows acquiring a good recovery of the analytes. The developed HPLC-UV method may be applied to conducting pharmacokinetic studies in the determination of SOF.

Keywords：sofosbubir, hepatitis C virus, HPLC, UV detection

HPLC-UV を用いたソホスブビルの定量法の確立

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抄　録

Sofosbuvir（SOF）は，C 型肝炎ウイルスの増殖を阻害するヌクレオチドアナログ薬である．本研究において，我々は，水溶液およびヒト血漿中 SOF 濃度の簡便，特異的，高感度および精密な UV 検出を用いた高速クロマトグラフィー法による測定方法の確立を試みた．0.25 mL の水溶液またはヒト血漿からアセトニトリルを含む抽出液で抽出し，C_{18} 逆相カラムを用いて分析した．ソラフェニブを内標準物質（IS）とし，移動相は酢酸アンモニウム緩衝液，メタノールおよびアセトニトリルを用いた．流速は 1.0 mL/min，UV 検出波長は 265 nm とした．SOF と IS の検出時間はそれぞれ 7.8 min および 9.1 min，1 検体の測定時間は 15.0 min であった．検量線は，0.1−10.0 μg/mL の範囲で直線性を得た．それぞれの検出限界は 0.1 μg/mL，ヒト血漿試料からの回収率は 99％以上であった．

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水溶液およびヒト血漿中の日内，日間変動係数は それぞれ 1.41−10.06％および 0.21−6.53％であった．日内，日間精度はそれぞれ 92.71−98.41％および 89.31−101.21％であった．本方法は，非常に簡便でかつ試料から良好な回収が可能であり SOF の薬物動態研究を行うために用いることができる．

キーワード：sofosbuvir，C 型肝炎ウイルス，HPLC，UV 検出

Ⅰ．Introduction

Hepatitis C infection is a leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma[1]. Approximately 150 million people worldwide are chronically infected with hepatitis C virus (HCV), and approximately 500,000 patients die every year from the liver disease associated with HCV[2]. Nowadays, there are new treatments that can get rid of the HCV are available. Sofosbuvir (SOF) is a first specific inhibitor of the nonstructural protein 5B (NS5B) RNA-dependent RNA polymerase that is essential for viral replication[3]. SOF was approved by the United States Food and Drug Administration and the European Medical Agency and has come to be commercially available for the treatment of chronic hepatitis C and has a potent activity against all HCV genotypes[4,5]. The sustained virological response (SVR) rate of SOF–based regimen is higher than that of peginterferon plus ribavirin in HCV-infected patients[6]. The rate of SVR in co-administration of SOF with ledipasvir, NS5A inhibitor is above 95%[1]. The mean maximum plasma concentration (C_{max}) of SOF after oral administration of 400 mg single dose in HCV-infected and healthy subjects was about 0.6 μg/mL and peak plasma concentration was observed at 0.5−2 hr[7]. In addition, it was reported that C_{max} of SOF was two times higher in the co-administered with ledipasvir than SOF alone in HCV-infected patients[8]. The plasma protein binding rate of SOF is about 61−65% in human within the concentration range of 1 μg/mL to 20 μg/mL, whereas its major metabolite has minimal binding[7].

A survey of the recent literature reveals high-performance liquid chromatography method with ultraviolet detection (HPLC-UV) for SOF measurement in the pure form[9] or in tablet dosage form[10], whereas there were few studies of SOF determination in plasma samples by HPLC-UV. The simple, cost-effective and specific quantification of plasma levels of SOF is very useful and currently needed in routine clinical practice. Therefore, the aim of this study was to develop a simple and specific HPLC method with UV detection for quantification of SOF from spiked human plasma.

Ⅱ．Materials and Methods

1. Chemicals and reagents

SOF and sorafenib (SOR) (internal standard, IS) were purchased from Funakoshi Co. (Tokyo, Japan). Acetonitrile (HPLC grade), methanol (HPLC grade), ammonium acetate (analytical grade), and dimethyl sulfoxide (DMSO) (analytical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2. Apparatus and chromatographic system

The HPLC system consisted of a LC-20AD Prominence binary HPLC pump, SPD-20A Prominence UV/Vis detector, SIL-20AC Prominence autosampler, CTO-20A Prominence column oven, and DGU-20A5 Prominence degasser (Shimadzu, Kyoto, Japan). Compounds were separated using an Inertsil ODS-3 C_{18} reversed-phase HPLC column (4.6 × 250 mm, 5 μm) (GL Sciences, Tokyo, Japan). The temperature of the column was maintained at 40℃, and the detection wavelength was set at 265 nm. Total analysis time was 15.0 min, and the flow rate was set at 1.0 mL/min. The mobile phase was composed of solution A (150 mM ammonium acetate, pH 8.0) and solution B (methanol: acetonitrile 50:50). The gradient was as follows: 0−1 min: 0−60 % B; 1−3 min: 60−95 % B; 3−4 min: 95−100 % B; 4−10 min: 100 % B; 10−12 min: 100−0 % B; 12−15 min: 0 % B. The

injection volume was 50 μL. Quantitation was performed by measurement of the peak area ratios of the drug to the IS.

3. Plasma sample collection and storage

Pooled drug-free human plasma was obtained from twelve healthy volunteers at the International University of Health and Welfare. The volunteers participated in the present study of their own free will and provided written informed consent. This study was approved by the Research Ethics Committee of the Graduate School of Pharmaceutical Sciences, International University of Health and Welfare (Approval No. 14-Io-131). Whole blood (10 mL) was collected from each healthy volunteer, and all of the blood samples from twelve subjects were pooled into the heparinized tubes. Then the tubes were centrifuged at 3,000 × *g* for 10 min at 4℃. The resulting supernatant (plasma) was transferred into another tube, and frozen in − 30℃ up to the time of analysis.

4. Preparation of standard and working solutions

A standard stock solution of SOF was prepared in DMSO and stored at − 20℃ throughout the experiment. Working solutions for SOF in de-ionized water and plasma samples were prepared by further dilution of stock solution with DMSO to provide final concentrations of 0.1, 0.5, 1, 5, and 10 μg/mL. For preparing calibration curves, a volume of 5 μL of an adequate working solution was spiked into 0.25 mL of de-ionized water or blank heparinized plasma and then extracted according to the procedure described below.

5. Extraction of sofosbuvir from plasma samples

To 0.25 mL of water or plasma samples containing SOF, 1 mL of IS-containing acetonitrile (giving a concentration 2 μg/mL in the final sample) was added. The mixture was vortexed and centrifuged (14,000 × *g*) at 4℃ for 5 min. The organic layer was transferred into another glass tube and evaporated to dryness at 40℃ under a stream of nitrogen. The extracted sample was reconstituted with 150 μL of solvent mixtures of acetonitrile: water (80:20, v/v). Finally, the reconstituted solution was transferred to an injection vial using 0.45 μm pore size Cosmonice Filter W (Nacalai Tesque, Inc., Kyoto, Japan).

Ⅲ. Results

1. Chromatographic characteristics

To analyze SOF by HPLC-UV, SOR was chosen as an IS, because the UV wavelength of maximum absorbance is similar for both. The chromatographic condition in the present study can completely separate SOF and SOR. Symmetrical peaks were observed for SOF and IS in both water-based and human plasma samples. The retention times of SOF and IS were 7.8 min and 9.1 min, respectively. Typical chromatograms from water-based samples and human plasma samples are illustrated in Figure 1 (a) and

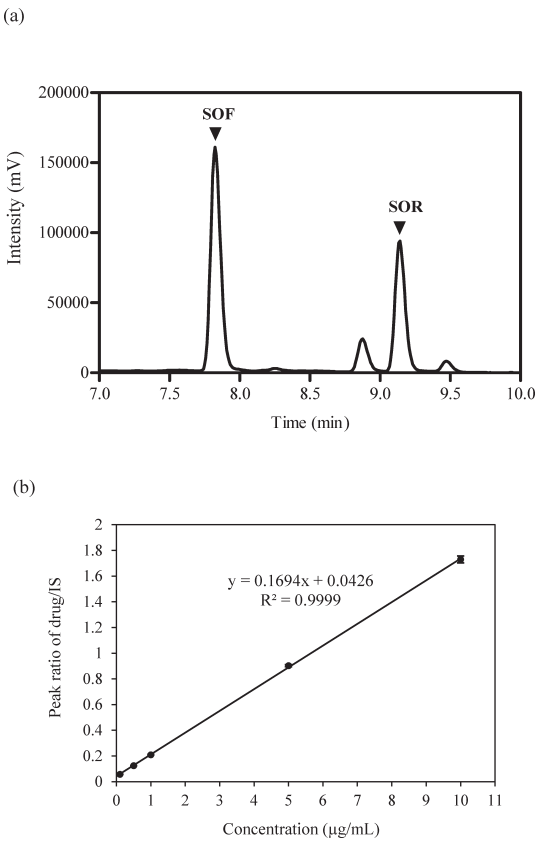

Figure 1　Chromatograms of water-based samples with 10 μg/mL SOF and 2 μg/mL SOR (IS) (a), and its calibration curve in the concentration range of 0.1−10.0 μg/mL (b).

Figure 2 (a), respectively.

2. Linearity

The calibration curve (n=3) for water-based samples was constructed by plotting the peak area of SOF to IS against the SOF concentration, and linear regression analysis showed the curve to be well-fitted over the range 0.1−10.0 μg/mL (R^2 = 0.9999). The calibration curve is shown in Figure 1 (b). The calibration standard containing 0.1−10.0 μg/mL (n=3) was prepared by spiking 0.25 mL human plasma samples with working solutions of SOF. There was good linearity in the examined concentration range, with a correlation coefficient of 0.9996 (Figure 2 (b)).

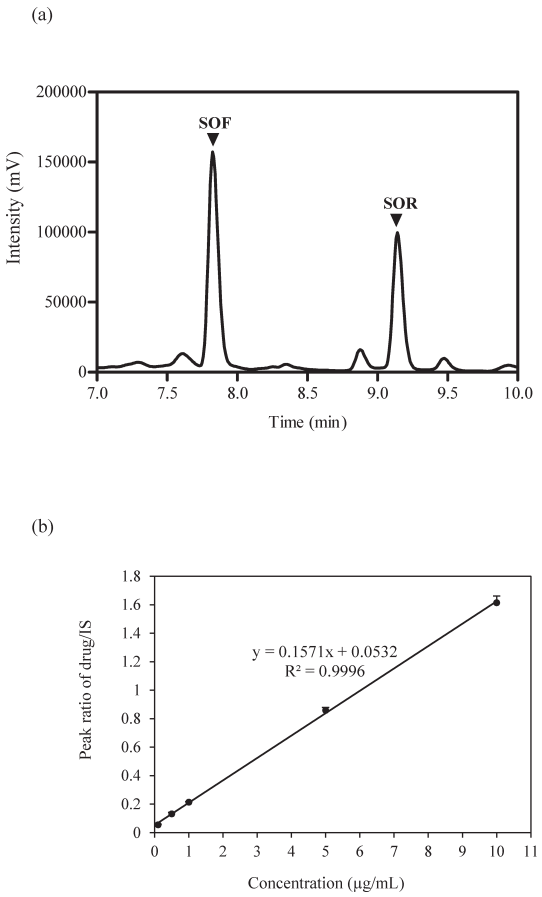

Figure 2　Chromatograms of human plasma spiked with 10 μg/mL SOF and 2 μg/mL SOR (IS) (a), and its calibration curve in the concentration range of 0.1−10.0 μg/mL (b).

3. Accuracy and precision

The intra- and inter-day precision and accuracy were determined by spiking three concentration levels (0.5, 1, and 5 μg/mL) into water-based samples and plasma samples, and evaluating duplicate samples on the same day and on three consecutive days, respectively. The precision was expressed as the coefficient of variation value (CV%). The CV% of the intra-day precision for water-based and plasma samples ranged from 1.41 to 10.06% and 0.21 to 6.53%, respectively. The CV% of the inter-day precision for water-based and plasma samples ranged from 1.77 to 5.08% and 1.55 to 2.79%, respectively. The accuracy was calculated as the percentage of the nominal concentration. The mean accuracy of water-based samples ranged from 92.71 to 98.41% and plasma samples ranged from 89.31 to 101.21%. The results of water-based and human plasma samples are shown in Table 1 and Table 2, respectively.

4. Limit of quantitation and detection

The limit of quantitation (LOQ) of SOF, defined as the lowest concentration that could be measured with accuracy and precision, i.e., within ± 20% of the actual value, was 0.1 μg/mL for both water-based samples and plasma

Table 1　Results of intra- and inter-day variability of SOF in water-based samples.

Spiked amount of SOF (μg/mL)	Detected amount (μg/mL) Mean ± SD	Intra-day CV (%)	Intra-day Accuracy (%)	Inter-day CV (%)	Inter-day Accuracy (%)
0.5	0.49 ± 0.01	5.44	98.06	2.95	98.41
1	0.96 ± 0.05	10.06	97.36	5.08	95.59
5	4.64 ± 0.09	1.41	94.02	1.77	92.71

SD: standard deviation; CV: coefficient of variation

samples. The limit of detection (LOD) was based on standard deviation (SD) of response and slope: LOD = 3.3 δ /S, where S is the slope of the calibration curve and δ is SD of the response[11]. LOD of SOF calculated from the calibration curve of water-based samples and plasma samples were 0.18 μg/mL and 0.32 μg/mL, respectively.

5. Recovery

The recovery of SOF was assessed by comparing the response of multiple extracted samples (0.5, 1, and 5 μg/mL) to the response of the pure standard at the same concentration level. The recovery of SOF from plasma samples was greater than 99 % for all tested concentrations (Table 3).

Ⅳ. Discussion

In the present study, a simple, sensitive and reliable HPLC-UV method for determination of SOF in water and human plasma has been developed and validated. Ariaudo *et al*. reported that the quantification of SOF and other anti-HCV drugs in biological fluids is essential to therapeutic monitoring of drug levels and clinical pharmacokinetic studies[12]. The determination of SOF can be the best accomplished using ultra-performance liquid chromatography-tandem mass spectrometry[12,13], as this technique has the highest detection sensitivity. However, its application in some clinical laboratories has remained limited because the costs of this instrument and maintenance are relatively expensive. On the other hand, UV detection is the most commonly used in HPLC and is popular for its simplicity and readily available. This proposed HPLC-UV can be an

Table 2　Results of intra- and inter-day variability of SOF in spiked human plasma samples.

Spiked amount of SOF (μg/mL)	Detected amount (μg/mL) Mean ± SD	Intra-day		Inter-day	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
0.5	0.47 ± 0.02	1.08	89.31	1.55	94.14
1	0.95 ± 0.03	0.21	92.70	2.79	95.22
5	4.91 ± 0.24	6.53	101.21	2.54	98.28

SD: standard deviation; CV: coefficient of variation

Table 3　Recovery data of SOF from spiked human plasma samples.

S. No.	Amount of SOF (μg/mL)					
	0.5		**1**		**5**	
	Aqueous area ratio	Extracted area ratio	Aqueous area ratio	Extracted area ratio	Aqueous area ratio	Extracted area ratio
1	0.12	0.12	0.19	0.20	0.84	0.91
2	0.13	0.12	0.22	0.20	0.86	0.83
3	0.12	0.13	0.19	0.21	0.81	0.80
4	0.13	0.13	0.20	0.20	0.84	0.80
5	0.12	0.13	0.21	0.20	0.83	0.86
6	0.12	0.13	0.20	0.22	0.84	0.80
Mean	0.12	0.12	0.20	0.20	0.84	0.83
SD	0.00	0.01	0.01	0.01	0.02	0.04
% CV	2.87	4.42	5.26	3.66	1.91	5.11
% Mean recovery	101.17		102.08		99.89	

SD: standard deviation; CV: coefficient of variation

alternative method to performing pharmacokinetics studies and therapeutic drug monitoring of SOF in human plasma.

Good resolution and reasonable retention times were observed for SOF and IS in this study. The total run time for each analysis was less than 15 min that allows the detection of many samples in a day. The required plasma volume was only 0.25 mL when compared with 0.5 mL for other method[13]. The extraction procedure for the recovery of SOF and IS were very simple, consistent and reproducible. We employed acetonitrile-based solution to extract the analytes from human plasma. Under this condition, the majority of protein was precipitated and the peaks of SOF and IS were free of interference from endogenous components in plasma. The chromatograms of SOF and SOR (IS) which were obtained under this assay condition were symmetrical and sharp.

The linearity mean of the calibration curves were excellent for both water-based and human plasma. The precision and accuracy of the method developed were within the acceptable limits for bioanalytical assays[11]. The detection limit of SOF is sufficient for the determination of plasma concentration of SOF in clinical studies as the proposed calibration ranges was based on the reported C_{max} in clinical trials[7,8]. The recovery of SOF using described procedure was reliable and consistent. When compared with a previous study, in which SOF was detected in human plasma using HPLC with photodiode arrays[14], the present method facilitates complete recovery of SOF in spiked plasma samples.

The simple and cost-effective HPLC-UV method is eligible for the determination of SOF in pharmacokinetic studies and can be applied for the analysis of SOF in routine quality control.

Ⅴ. Conclusion

A simple, sensitive, precise and reliable HPLC-UV method for determination of SOF in aqueous-based and human plasma over the concentration range 0.1−10.0 μg/mL have been validated and developed. The present method used a simple extraction procedure for the recovery of SOF. The procedure does not require any crucial experimental circumstances, and the reagents used in the method are cost-effective and readily available. The quantification of SOF in a small volume of human plasma was successfully achieved by the proposed method without any interference. Therefore, the proposed HPLC-UV method is appropriate for the analysis of SOF in biological fluids.

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Conflict of interest

The authors declare that they have no conflict of interest.

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