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Determination of GS-441524 in The Polyethylene Glycol Preparation by HPLC Coupled with High Resolution Mass Spectrometry

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Abstract—**GS-441524, a nucleoside analogue molecule, is both intermediate and metabolite of remdesivir. Because of proved effectiveness in animals, GS-441524 is expected to be a better potential drug than remdesivir in the treatment of Covid-19. Since GS-441524 is not easily soluble in pure water, polyethylene glycol (PEG) was used as the auxiliary solvent. The developed LC-MS method utilized a UHPLC coupled with a TOF mass spectrometer and developed a gradient elution to efficiently separate GS-441524 and other PEG-400 molecules. The chromatographic separation completed within 6 minutes. GS-441524 was identified in peak at 0.9 min explicitly. No interferent was co-eluted. UV detection was not proved suitable for the determination of GS-441524 under this condition. The correlation coefficient R^2 (0.9970) indicated a good linear relationship in range of 0.1 ~ 10 μg/mL. The LOD and LOQ of the method was 0.05μg/mL and 0.1 μg/mL respectively. A concrete sample was accuracy analyzed. The developed LC-MS method provided a simple, rapid, accurate and precise analysis protocol for the determination of GS-441524 in preparation system with PEG-400 and water as solvent.**

Index Terms—**GS-441524, PEG-400, HPLC-MS, Nucleoside analogue, Determination.**

I. Introduction

Nucleoside analogues are small-molecule antiviral drugs that directly inhibit viral transcription and replication by targeting viral RNA-dependent RNA polymerase (RdRp) [1]. Remdesivir (GS-5734, Gilead Sciences), one of the nucleoside analogues [2], was proved to be a broad-spectrum antiviral against such as Ebola Virus (EBOV) [3] and a number of other RNA viruses [4][5].

Severe acute respiratory coronavirus type 2 (SARS-CoV-2, COVID-19) appeared in December 2019 has become a worldwide pandemic [2]. Remdesivir is a relatively new experimental broad-spectrum antiviral drug and is highly anticipated for its moderate anti-Covid-19 effect. Its parent nucleoside GS-441524 has also received attention. Both of remdesivir and GS-441524 were found to be metabolized into an active nucleoside triphosphate [6]. GS-441524 is an active metabolite of remdesivir and also an important intermediate for the synthesis of remdesivir [7]. It itself is also a small molecule nucleoside analogue and a precursor molecule for the synthesis of nucleoside triphosphate with pharmacological activity. The molecular structure of it is presented in Fig. 1.

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The pharmacokinetic analysis of remdesivir proved that it was prematurely hydrolyzed to GS-441524. GS-441524 is the predominant metabolite reaching the lungs [8].

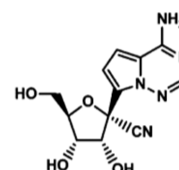

Fig. 1. Molecular structure of GS-441524

GS-441524 inhibits viral RNA polymerases [9] and has broad spectrum activity against a variety of viruses. For examples, the filoviruses including EBOV[10][11] and MARV, the coronaviruses including SARS-CoV [12][13] and MERS-CoV [14][15], the paramyxoviruses including respiratory syncytial virus, Nipah virus [16], and Hendra virus [17] are all inhibited by remdesivir or its metabolite GS-441524.

GS-441524 has been reported to have a strong inhibitory effect on feline infectious peritonitis (FIP) virus, with a half-maximal effect concentration (EC_{50}) of 0.78 μM [5][18]. Pharmacologically, GS-441524 acts as an alternative substrate for viral RNA polymerase, causing the termination of viral RNA strand polymerization [19]. Known as a nucleoside triphosphate competitive inhibitor, it exhibits potent antiviral activity against many other RNA viruses.

Due to its ease of synthesis and effectiveness in animals, GS-441524 is expected to be a better potential drug than remdesivir in the treatment of Covid-19 [8]. The argument lies in the following reasons. The plasma half-life of GS-441524 is longer than remdesivir [20]. GS-441524 shows an excellent effect on feline coronavirus [21][22], meanwhile shows comparable efficacy in cell-based models of primary human lung and cat cells infected with coronavirus [5][23]. Although feline coronavirus (FCoV) and the coronavirus SARS CoV-2 belong to different genera, coronavirus A and coronavirus B, they share some common virological and epidemiological characteristics [24].

Whether as a synthetic intermediate or metabolite of remdesivir, or directly as a drug itself, the content of GS-441524 is required to be accurately determined in the field of pharmaceutical analysis [25][26]. There were reports that introduced LC-MS/MS technique for determination of GS-441524. Xiao and coworkers validated a LC-MS/MS method for determination of remdesivir and its metabolites GS-441524 and GS-704277 in acidified human plasma [2].

Alvarez and coworkers quantified the plasma remdesivir and GS-441524 using the similar approach [7]. Avataneo and coworkers developed a UHPLC coupled tandem MS method for quantification of remdesivir and GS-441524 [27]. A two-dimensional LC-MS/MS method was also reported to simultaneously quantify seven repurposed COVID-19 drugs [18].

Since GS-441524 is not easily soluble in pure water, a more empirical pharmaceutical preparation method is to dissolve GS441 into polyethylene glycol (PEG) firstly. Polyethylene glycol is a commonly used solvent in pharmaceutical preparations. However, polyethylene glycol is a polymer, it contains a wide molecular size distribution. This distribution may take in difficulties into determination of the analytes or other solute molecules. Especially in chromatography methods, quantitative analysis usually cannot be achieved because of the overlap of polyethylene glycol series molecules and analyte molecules.

Hitherto, there is no report on the determination method of the content of remdesivir intermediate GS-441524 in the preparation system with polyethylene glycol and water. The main objectives of this study were to provide a simple, rapid, accurate and precise analysis method for the determination of GS-441524 in preparation system with polyethylene glycol and water as solvent.

II. MATERIALS AND METHODS

A. *Reagents*

The standard of GS-441524 (purity greater than 98.2%) was obtained from Hycultec (Beutelsbach, Germany). HPLC grade methanol and acetonitrile were bought from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (>98%) used as mobile phase modifier was purchased from Merck (Darmstadt, Germany). Pure water was prepared through a water purifier (Milli-QDirect8, Millipore, France). Dimethyl sulfoxide (DMSO) and PEG-400 were purchase from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other reagents used in study were all of analytical grade.

B. *Apparatus*

An Agilent 1290 infinity system (Made in Germany) was employed as a UHPLC device, which consisted of a binary pump, an autosampler, a column thermostat, and a DAD detector. The mass spectrometer was an AB Sciex Triple TOF 5600$^+$ (Manufactured in Singapore) with a DuoSpray ion source. Analytical balance model in SartoriusBSA224S (Beijing, China) and ultrasonic cleaner model in KQ100DE (Kunshan, China) was employed to accelerate sample dissolution.

C. *Concrete sample*

The simulated concrete sample was made in our laboratory according to the rule of double-blind experiment. The procedure was concisely described as that 30 mg GS-441524 was ultrasonically dissolved into 1.0 mL PEG-400. The solution was then diluted with 1.0 mL water to form the simulated concrete sample.

D. *Sample preparation*

A mixture of acetonitrile/water (1:9, v/v) was prepared beforehand to serve as the dilution solution.

100 µL of the concrete sample was accurately measured and diluted 10000 times step by step with the dilution solution to prepare the test sample. The test sample solution was filtered through 0.22 µm microporous membrane before measurement.

To prepare stock standard solution, 20 mg of GS-441524 standard was accurately weighed and dissolved in 0.5 mL DMSO assisted with ultrasonic bath. The stock solution was then diluted step by step with the dilution solution to produce a series of standards solutions. The concentrations of GS-441524 in series solutions were from 0.05 µg/mL to 10.0 µg/mL. All solutions were filtered by 0.22 µm microporous membrane to be tested.

E. *Instrumental conditions*

The liquid chromatographic separation was performed on a column of C_{18} (2.1×50 mm i.d., 1.7 µm) (Waters Co., Milford, MA, USA). The mobile phase A was a formic acid/water (0.2%, v/v) solution. The mobile phase B consisted of a 0.2% (v/v) formic acid/acetonitrile solution. In order to remove bubbles in the mobile phase, ultrasonic degassing treatment was carried out for more than 30 minutes. The mobile phase flow rate was set at 0.2 mL/min for all experiments. The injection volume was 2.0 µL. The column temperature was set at 38 °C. The DAD worked in scan mode which wavelengths ranged in 190～400 nm.

The ratio of phase A and phase B was adjusted according to the optimization progress.

The MS analysis was operated in ESI$^+$ mode where the ion source temperature was set at 550 °C. The ionization voltage was set at +5 kV with 55 psi nebulizing gas, 55 psi auxiliary gas, and 35 psi curtain gas. The TOF scan range of m/z 100-1000 was recorded.

III. RESULTS AND DISCUSSION

A. *Optimization of the separation process*

For the optimization of LC separation, the concrete sample was employed as the model sample. When methanol worked as organic phase, 0.2% formic acid solution worked as aqueous phase, analytes separation can be achieved within 8 minutes after chromatographic condition optimized. The above empirical conditions refer to the isocratic elution with the mobile phase ratio of 1:9 (v/v, organic/aqueous). Although the chromatographic separation process was completed within 8 min, there were overlapping peaks in chromatogram. The retention time (RT) of GS-441524 was about 1.46 min. At this retention time, some polyethylene glycol molecules were co-eluted.

Table 1. Optimized gradient time table

Time（min）	A*（%）	B**（%）
0	90	10
0.8	90	10
0.9	85	15
3	85	15

5	0	100
5.2	0	100
5.3	90	10
6	90	10

A*: formic acid/water (0.2%, v/v); B **: formic acid/acetonitrile (0.2%, v/v)

When organic phase was acetonitrile, better separation for GS-441524 and PEG was observed. In order to separate as many as different molecules of PEG so it can make the chromatogram more specific, a gradient elution was applied. After gradual optimization, the chromatographic condition was determined. The adopted gradient program was shown in Table 1.

Under this gradient condition, the rapid separation and satisfied resolution were achieved simultaneously. Typical chromatogram of concrete sample was shown in Fig. 2.

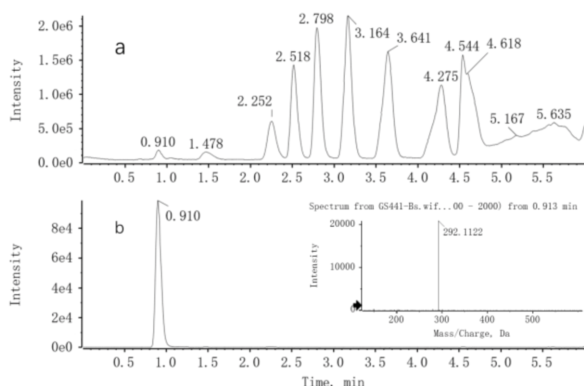

Fig. 2 (a)The TIC of concrete sample and (b)The EIC of GS-441524 (m/z 292.1)

As shown in Fig. 2a, there is not any interference in the TIC chromatogram. It infers that no molecule of PEG-400 or other impurity co-eluted with GS-441524. The mass spectrum at 0.91 min was shown as inset in Fig.2. The only peak of m/z 292.1 is the $[M+H]^+$ of GS-441524.

The EIC chromatogram of m/z 292.1 showed only one peak (Fig. 2b). It reconfirmed the specificity of the developed method for GS-441524. Also, good quantification in EIC is easy to accomplished.

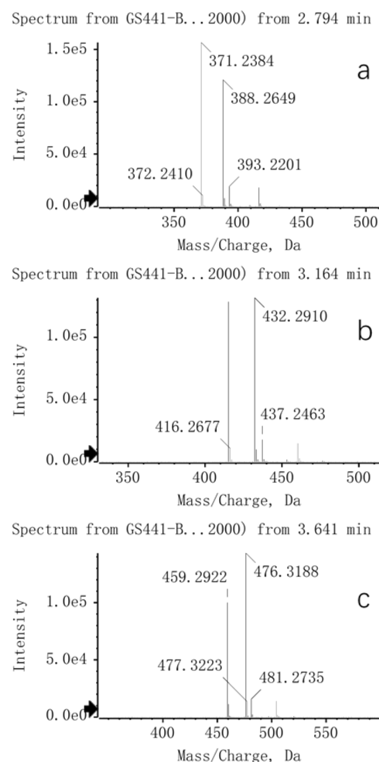

Fig. 3. Spectra of series PEG-400 at (a)RT 2.79 min, (b) RT 3.16 min, (c) RT 3.64 min

In TIC chromatogram, the peak at 0.910 min is assigned to GS-441524. The rest series of peak were assigned to each monomer of PEG-400. The identification is confirmed with the mass spectrum of each peak.

Other peaks in the total ion current chromatogram are the distribution peaks of PEG molecules of different sizes. Take the three most abundant peaks as examples. The mass spectra at retention times 2.79 min, 3.16 min, and 3.64 min are shown in Fig 3a, 3b, 3c respectively. They exhibit typical characteristics of a series polymer molecules that with series weight.

B. Detection method

In addition to mass spectrometers, diode array detector (DAD) is also one of the commonly used detectors for chromatography. In order to investigate the response of ultraviolet (UV) detection, the absorption spectrum of GS-441524 was recorded in Fig. 4.

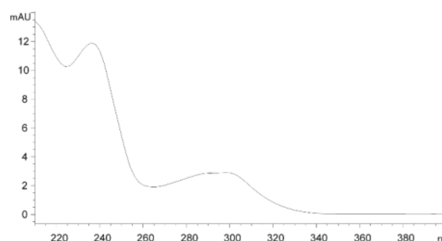

Fig. 4. The UV absorption spectrum of GS-441524

The wavelengths of 240 nm and 300 nm are the strong absorption peaks in GS-441524 UV spectrum. Under the same separation conditions as Fig.2, the chromatogram of UV detection was recorded in Fig.5. During chromatographic process, when 300 nm was set, the absorbance of GS-441524

was weak. As shown in Fig. 5a, the peak height of GS-441524 (RT 0.79 min) was only 9.9 mAU. Subsequently, more peaks of elution substances were displayed. When 240 nm was selected, the peak height of GS-441524 was 30.0 mAU (Fig. 5b). Since less impurity was detected, it may result in incorrect high values of the target compound.

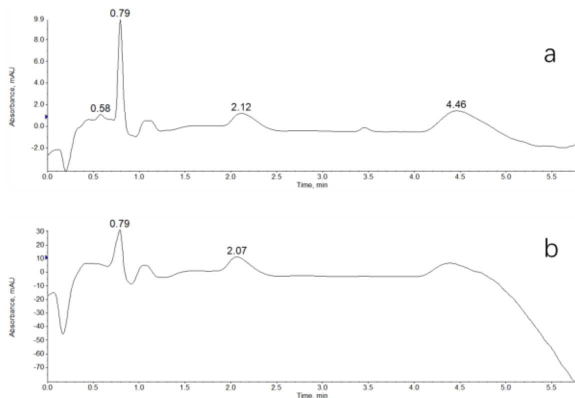

Fig. 5. Chromatogram of concrete sample extracted from DAD results at UV wavelength (a)300 nm, (b)240 nm.

In both cases, GS-441524 did not obtain a good peak shape. Accurate results cannot be obtained by relying solely on the UV detector. Therefore, the relatively easy protocol is to employ a mass spectrometer as the detector. Furthermore, the chromatographic peak area in the EIC is a more sophisticated parameter for the quantitative detection.

C. Linear range investigation

The series of calibration standards of 0.1 μg/mL, 2.0 μg/mL, 4.0 μg/mL, 6.0 μg/mL, 8.0 μg/mL and 10 μg/mL were analyzed in batches. For each solution, 2 μL was injected into the LC-MS system to get determination according to the method described above. The measurement of each solution was repeated three times. The total ion current was recorded and then the m/z 292.1 ion was extracted to generate the EIC (XIC) chromatogram. The typical chromatogram was shown in Fig. 2b.

The peak area in EIC of GS-441524 (RT: 0.91 min) was integrated. The average peak area versus concentration was illustrated in Fig. 6. A linear regression was calculated.

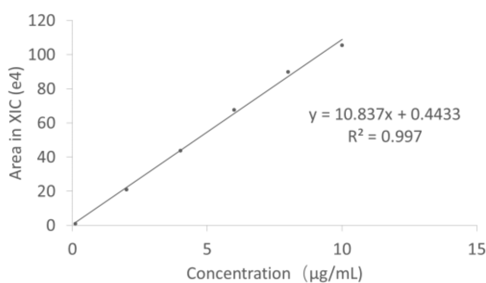

Fig. 6. The linear relationship of peak area against concentration of GS-441524

In the investigated concentration range, the linear regression equation y = 10.837x + 0.4433 was fitted by the least-squares algorithm. The correlation coefficient R^2 (0.9970) indicated that the quantification method for GS-441524 was good in linear relationship in range of 0.1 ~ 10 μg/mL.

D. Precision

Precision of the method were evaluated by analyzing the prepared concrete sample solution. For instrumental analysis, sampling volume was 2 μL. The determination repeated 9 times continually. The average value and relative standard deviation (RSD) were calculated with the peak (m/z 292.1) area in EIC. The results are listed in Table 2.

Table 2. Precision results

No.	Peak area	average	RSD
1	425581.245	420912.1446	1.98%
2	429805.7751		
3	403783.8389		
4	421484.3569		
5	428077.2019		
6	411058.1218		
7	421616.4256		
8	422469.3849		
9	424332.9517		

The RSD value (1.98%) indicating that the method for determining the content of GS-441524 in PEG-400 solution is good in precision and reproducibility.

E. Recovery

In order to evaluate the accuracy of the proposed method, the recovery of the method is validated. The concrete sample was employed as the bulk sample. The GS-441524 standard substance was added to prepare the spiked sample. The additional amount in the final solution was calculated to be 0.50 μg/mL. 5 parallel measurements were performed. The results are listed in table 3.

Table 3. Recovery results

No.	recovery rate	average	RSD
1	100.6%	98.5%	1.47%
2	103.2%		
3	96.8%		
4	99.2%		
5	99.4%		

Average recovery rate (98.5%) and relatively small RSD (1.47%) indicated a good accuracy of the method.

F. Limit of detect and Limit of Quantification

The ratio of peak height to baseline noise (signal-to-noise ratio, S/N) greater than 3/1 in the EIC of GS-441524 (m/z 292.1) was used as the limit of detect (LOD) criterion. For practical application purposes, the lowest concentration sample tested in this research was a 0.05 μg/mL solution. The S/N ratio of 0.05 μg/mL sample in EIC was far greater than 3/1. Technically, the concentration of 0.05 μg/mL was adopted as the LOD of the method. The limit of quantification (LOQ) was estimated to be 0.1 μg/mL (S/N > 10/1), the lowest concentration investigated in the linear range.

G. Determination of concrete sample

Although the concrete sample had been employed for the method validation, the exact concentration was not tested and calculated. In order to determine the value, a batch of analysis was conducted explicitly. The test sample (2.0 μL) was introduced. The analysis was repeated three times. The average peak area corresponding to RT 0.91 min in EIC was substituted into the linear regression equation. The calculated concentration of GS-441524 in the simulated concrete sample was 15.12 mg/mL.

H. Stability

To investigate the stability, the concrete sample was diluted 10000 times to be test sample. The test Samples were stored at 4 °C for 0, 6, 12, 24 and 48 hours respectively. According to the proposed LC-MS method, the standard linear curve was re-constructed in each batch. The content of GS-441524 in each sample was analyzed in triplicate and calculated. The results are shown in Table 4.

Table 4. Stability results

Time (h)	Concentration (μg/mL)	average	RSD
0	1.493	1.4962	1.06%
6	1.471		
12	1.498		
24	1.511		
48	1.508		

The relative standard deviation of the content result (RSD = 1.06%) was acceptable. It can conclude that the LC-MS method developed in this study is good in stability in 48 hours when the candidate sample being stored at 4 °C.

IV. CONCLUSION

GS-441524 is an active metabolite of remdesivir and also an important intermediate for the synthesis of remdesivir. It itself is also a small nucleoside analogue molecule and a precursor molecule for the synthesis of nucleoside triphosphate with pharmacological activity. Because of the proved effectiveness in animals, GS-441524 is expected to be a better potential drug than remdesivir in the treatment of Covid-19. Since GS-441524 is not easily soluble in pure water, polyethylene glycol (PEG) was used as the auxiliary solvent. The wide molecular size distribution takes in difficulties into determination of the analytes. The reason is the co-elution of distributed series PEG molecules and analyte molecules in chromatography.

The developed LC-MS method utilizes a HPLC coupled with a TOF mass spectrometer. When acetonitrile was employed as the organic phase, better separation between GS-441524 and PEG was observed. In order to separate as many as different molecules of PEG so it can make the chromatogram more specific, a gradient elution was optimized. All separation for GS-441524 and PEG was achieved within 6 min. GS-441524 was identified in peak at 0.9 min explicitly. No interferent was co-eluted. The peak of variety of PEG-400 molecules was also be screened. UV detection was not proved suitable for the determination of GS-441524 in PEG preparations.

The correlation coefficient R^2 (0.9970) indicated that the developed method for GS-441524 quantification possesses a good linear relationship in the range of 0.1-10 μg/mL. The robustness of method was studied by precision, accuracy and stability. The LOD and LOQ was 0.05μg/mL and 0.1 μg/mL respectively. The method was applied to a concrete sample and the defemination was accuracy and satisfied.

This study provided a simple, rapid, accurate and precise analysis method for the determination of GS-441524 in preparation system with PEG-400 and water as solvent.

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