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Validation and Application of a Liquid Chromatography-Tandem Mass Spectrometry Method To Determine the Concentrations of Sofosbuvir Anabolites in Cells

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Sofosbuvir (SOF) is a highly efficacious and well-tolerated uridine nucleotide analog that inhibits the hepatitis C virus (HCV) NS5B polymerase enzyme. SOF is administered as a prodrug, which undergoes intracellular phosphorylation by host enzymes to a monophosphate, diphosphate, and finally a pharmacologically active triphosphate. In order to fully understand the clinical pharmacology of SOF, there is a great need to determine the intracellular phosphate concentrations of the drug. We describe the validation and utilization of a method to characterize SOF's disposition into various *in vivo* cell types, including hepatocytes, peripheral blood mononuclear cells (PBMC), and red blood cells (RBC). Standard bioanalytical validation criteria were applied to lysed cellular matrices, with a validated linear range of 50 to 50,000 fmol/sample for each phosphate moiety. The assay was utilized to collect the first data demonstrating concentrations of phosphorylated anabolites formed in PBMC, hepatocytes, and RBC *in vivo* during SOF therapy. Median concentrations in PBMC were 220 (range, 51.5 to 846), 70.2 (range, 25.8 to 275), and 859 (range, 54.5 to 6,756) fmol/10^6 cells in the monophosphate, diphosphate, and triphosphate fractions, respectively. In contrast, RBC triphosphate concentrations were much lower than those of PBMC, as the median concentration was 2.91 (range, 1.14 to 10.4) fmol/10^6 cells. The PBMC triphosphate half-life was estimated at 26 h using noncompartmental approaches, while nonlinear mixed-effect modeling was used to estimate a 69 h half-life for this moiety in RBC. The validated method and the data it generates provide novel insight into the cellular disposition of SOF and its phosphorylated anabolites *in vivo*.

The treatment of hepatitis C virus (HCV) has undergone tremendous change in recent years (1). Interferon and, in some cases, ribavirin have been replaced with all-oral, well-tolerated, and highly effective direct-acting antiviral agents (1). One agent in particular, the uridine analog nucleotide prodrug sofosbuvir (SOF) (Fig. 1a), has demonstrated pan-genotypic efficacy against HCV by inhibiting the viral NS5B polymerase (2, 3). SOF is a highly potent antiviral, reducing HCV RNA by 4.65 logs when given as monotherapy for just 7 days (4). SOF is also very well tolerated, with fewer than 2% of patients discontinuing sofosbuvir treatment due to adverse events during clinical trials (5). Thus, SOF has become a critical component of many HCV treatment regimens (6). While cure rates with many SOF-based HCV regimens are high (>90%), there are differences in response to treatment depending on which agents SOF is combined with, the duration of therapy, and the patient population being studied (1). Thus, there remains a need to understand the contribution of SOF pharmacology to the likelihood of cure in SOF-based HCV treatment for a variety of patient populations.

SOF is administered as a phosphoramidate prodrug of the uridine nucleotide analog GS-331007 monophosphate (MP) (2). The prodrug is designed to facilitate the intracellular penetration of the drug into target tissue, specifically hepatocytes, where HCV replicates (2). Once inside cells, the prodrug is rapidly metabolized by the high levels of human cathepsin A and/or carboxylesterase 1 in hepatocytes and other cellular matrices to GS-331007 MP (also known as GS-606965) (2, 3). GS-331007 MP is then phosphorylated by uridine monophosphate-CMP kinase to the GS-331007 diphosphate (DP) form, which is then phosphorylated by nucleotide diphosphate kinase to the triphosphate (TP) (Fig. 1b) moiety (also known as GS-461203) (2, 3). The intracellular phosphorylated anabolites resulting from SOF administration are therefore labeled GS-331007 phosphates for the purposes of this article. These phosphate forms are ion trapped within the cell, conveying a unique pharmacological profile for the drug in tissue.

Previous studies have described the pharmacokinetics (PK) of the SOF prodrug and its GS-331007 metabolite in plasma (Fig. 1c), but none have reported intracellular concentrations of SOF and its anabolites *in vivo* (2–4). However, as the activity of SOF is dependent on GS-331007 TP (007-TP) concentrations, it is critical to evaluate these concentrations and associate them with clinical outcomes of SOF therapy in diverse populations (viral genotype, stage of liver disease, etc.) and clinical scenarios (i.e., drug interactions, hepatic or renal impairment, etc.). *In vivo* intracellular phosphate concentrations have previously shown great utility in predicting the effect of other antivirals (7–9); however, studies of this nature for SOF have yet to be done. The most direct measure of SOF concentrations at the site of antiviral activity is hepatocytes purified from liver tissue samples; however, these samples are difficult to obtain (require the removal of liver tissue during either biopsy or transplant), process (previous work has suggested

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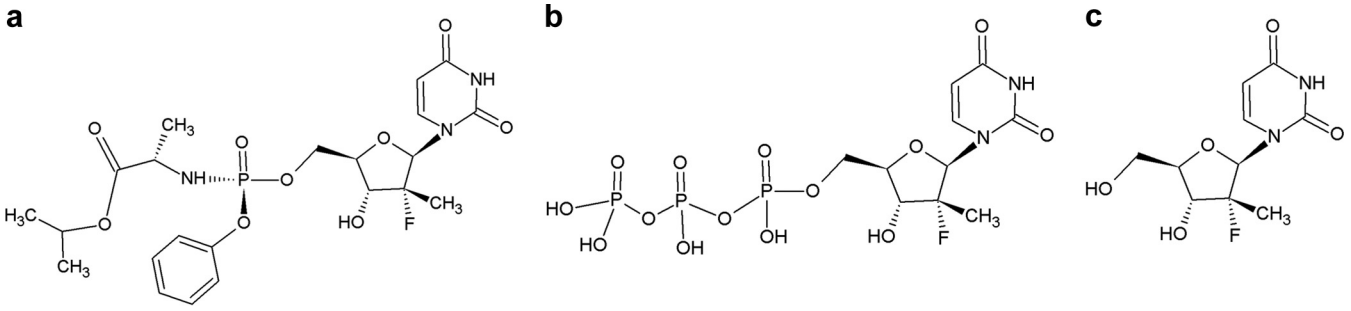

FIG 1 (a) The chemical structure of SOF. (b) The chemical structure of the pharmacologically active 007-TP anabolite. (c) The chemical structure of the base nucleoside GS-331007.

rapid degradation of endogenous liver nucleotides upon removal of tissue), and interpret (drug concentrations may not be homogenous throughout the tissue) (10, 11). Thus, there are currently few reports of hepatocellular SOF concentrations (12, 13). We therefore sought to develop a method that could be used to determine *in vivo* intracellular concentrations in hepatocytes and in more accessible cellular matrices, including peripheral blood mononuclear cells (PBMC) and red blood cells (RBC). An understanding of the cellular pharmacology of SOF in different cell types, including blood and liver, is needed to inform the relevant PK distribution of SOF *in vivo*.

The measurement of intracellular nucleoside analog phosphates from cellular matrices like PBMC and RBC poses significant technical challenges (14). Previous analytical methods have quantified intracellular phosphate concentrations for other nucleos(t)ide analogs (15–19); however, none have described the quantification of GS-331007 phosphates. Many of these published methods utilize direct methodologies, which require ion-pairing chromatography for separation of the MP, DP, and TP fractions, which can result in decreased method sensitivity and increased analytical noise (20, 21). We therefore sought to develop an indirect method, in which the phosphate anabolites are first fractionated and then dephosphorylated to the base nucleoside (GS-331007) prior to quantification, in an effort to increase the sensitivity and reduce analytical noise through enhanced sample cleanup. This report (i) describes the validation of a novel bioanalytical method to determine phosphorylated concentrations of GS-331007 that can be applied to a variety of cellular matrices, (ii) evaluates the clinical application of the method by determining for the first time GS-331007 phosphate concentrations in human PBMC, RBC, and hepatocytes resulting from SOF therapy, and (iii) describes the pharmacokinetic disposition of SOF in PBMC and RBC *in vivo*.

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MATERIALS AND METHODS

Chemicals and materials. Chemicals were acquired from the following manufacturers: ApexBio Technology LLC, Houston, TX, USA (GS-331007, molecular weight [MW] = 260.2); Alsachim SAS, Illkirch, France (GS-331007-$^{13}C^{2}H_{3}$ as an internal standard [GS-331007 IS], MW = 264.2); and Gilead Sciences, Foster City, CA, USA (007-TP, MW = 500.2). Analytical grade reagents were obtained from Fisher Scientific, Fairlawn, NJ, USA (methanol, formic acid, and potassium chloride); Sigma-Aldrich Chemical, St. Louis, MO, USA (alkaline phosphatase and sodium acetate); and JT Baker, Phillipsburg, NJ, USA (acetonitrile). Ultrapure (UP) water was prepared in-house from deionized water with a Barnstead Nanopure system (Thermo Fisher Scientific, Waltham, MA, USA). Consumables included Waters Sep-Pak Accell Plus QMA cartridge (3 ml/500 mg; Water Corporation, Milford, MA, USA), Phenomenex Strata-X-CW 33-μm polymeric weak cation mixed-mode cartridge (3 ml/200 mg; Phenomenex, Inc., Torrance, CA, USA), and blood products for the lysed cellular matrix (Bonfils, Denver, CO, USA).

Sample preparation. Blood samples were collected into EDTA anticoagulant tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), followed by separation of blood cell types using a Ficoll gradient and centrifugation. PBMC fractions underwent an RBC lysis step using an RBC lysis medium (Gibco; Invitrogen, Carlsbad, CA, USA). Purified cellular fractions were counted, lysed using cold 70:30 methanol-water, and stored at −80°C until analysis. Blank PBMC were harvested from leukocyte reduction filters, processed to a concentration of 10×10^6 cells/ml, and then used for working standard samples and quality control (QC) sample preparation.

Purified hepatocyte samples were harvested from the liver tissue of subjects undergoing orthotopic cadaveric liver transplantation using procedures developed in our laboratory. Biopsy samples were soaked in a warm perfusion medium and dissociated by collagenase digestion. Dissociated cells were then filtered through a 100-mm cell strainer and rinsed with wash medium. Cell samples were centrifuged, and the pellet was resuspended in wash medium for cell counting. Isolated hepatocytes were then lysed with 70:30 methanol/water and were then stored at −80°C until analysis.

Standard and quality control preparation. Standard preparation stocks were created in methanol from the GS-331007 reference standard at a concentration of 3,843 pmol/μl (stored at −20°C) and were then diluted in UP water to working standards ranging between 50 and 50,000 fmol/sample (stored at 4°C). A sample was defined as 20 μl working standard spiked into 2 ml of 1 M KCl resulting from a blank lysed cellular matrix (2×10^6 cells) carried through the strong anion exchange extraction and dephosphorylation processes described below.

Quality control (QC) preparation stocks were created in UP water from 007-TP reference powder at a concentration of 1,444 pmol/μl and were then diluted to concentrations of 150, 1,500, and 40,000 fmol/sample (QL, QM, and QH, respectively) in blank 70:30 PBMC lysate (stored at −80°C). For QCs, a sample was defined as 200 μl (2×10^6 PBMC) of lysate added to the strong anion exchange cartridge following the extraction procedure described below.

GS-331007 IS was prepared in methanol at 3,785 pmol/μl and was then diluted into UP water to a concentration of 125 fmol/μl to make the working IS. A 20-μl volume of working IS was added to each sample, resulting in an IS concentration of 2,500 fmol/sample.

TABLE 1 Accuracy and precision of calibration standards from 4 analytical runs

Parameter	Nominal value according to phosphate concn (fmol/sample):									
	50	100	250	500	1,000	2,500	5,000	10,000	25,000	50,000
%CV	2.8	5.3	4.5	2.8	0.6	0.9	1.5	0.9	0.8	0.4
%Dev[a]	2.0	0.8	−4.0	1.3	−0.9	0.7	0.2	0.9	−1.0	0.3
No.	4	4	4	4	4	4	4	4	4	4

[a] Percentage deviation.

Extraction. Separation of intracellular 007-MP, 007-DP, and 007-TP fractions utilized Waters QMA strong anion exchange solid-phase extraction (SPE) cartridges, with each fraction collected into a separate tube. This extraction included cartridge preparation (1 × 2.0 ml UP water, 1 × 1.5 ml 1 M KCl, and 1 × 2 ml 5 mM KCl), followed by sample addition and separation of the anabolites using a potassium chloride concentration gradient (MP, 5 ml × 75 mM KCl; DP, 7 ml × 90 mM KCl; TP, 2 ml × 1 M KCl) (19). Each fraction was dephosphorylated to the GS-331007 base nucleoside using an excess of alkaline phosphatase, which was allowed to incubate for 30 min at 37°C. Following dephosphorylation, samples were desalted and concentrated with Phenomenex Strata-X-CW (mixed-mode weak cation exchange) SPE cartridges using an extraction process optimized for GS-331007. The process first added IS (20 μl) to all but the blank without IS sample and was followed by the addition of working standard solutions (20 μl) to the extracted blank cellular lysate samples designated for standard samples. The Strata-X-CW SPE was prepared (1 × 2.0 ml methanol and 1 × 2.0 ml UP water) and was followed by sample application, which consisted of the entire fraction collected during the strong anion exchange SPE extraction. Cartridges were subsequently washed (2 × 2.0 ml UP water and 1 × 0.25 ml methanol), and analytes were then eluted (3 × 0.5 ml methanol). Samples were dried at 50°C for 25 min under nitrogen in a Zymark TurboVap (Zymark Corp., Hopkinton, MA, USA) and were then reconstituted using 100 μl UP water. The sample was vortex mixed and transferred to a 150-μl low volume insert, and 30 μl was injected onto the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Analytical instrumentation. Samples were analyzed by LC-MS/MS. The high-pressure liquid chromatography (HPLC) system consisted of a Thermo Scientific Accela pump (San Jose, CA, USA), an Eppendorf CH-30/CH-50 heater/controller (Hauppauge, NY, USA), and a CTC Analytics HTC PAL autosampler (Zwingen, Switzerland) equipped with a 100-μl sample syringe and a 50-μl PEEK sample loop. A Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer coupled with a HESI II probe was used for detection. Data were captured with Xcalibur 2.2 SP1.48 software.

A 2.0 by 150 mm Phenomenex Develosil 3-μm RP-aqueous C_{30} 140-Å analytical column (Phenomenex, Torrance, CA, USA) was used for chromatographic separations. The mobile phase consisted of 10% acetonitrile and 0.1% formic acid in UP water at an isocratic flow of 200 μl/min. The column was maintained at 40°C, and the sample temperature was maintained at 20°C. Each injection was followed by a strong and weak needle wash (50% acetonitrile–50% UP water and 10% methanol–90% UP water, respectively).

The MS/MS source was operated in positive mode, with the detector in simultaneous reaction monitoring (SRM) ionization mode. The spray voltage was 4,000 V, vaporizer temperature was 250°C, sheath gas (nitrogen) was 40 arbitrary units, auxiliary gas (nitrogen) was 20 arbitrary units, capillary temperature was 225°C, chromatography filter peak width was 10.0 s, collision gas (argon) pressure was 1.0 millitorr, S lens was 46 V, collision energy was 13 V, Q1 and Q3 peak widths were 0.7 full width at half maximum (FWHM), scan width was 0.002 *m/z*, scan time was 0.200 s, and centroid data type was collected. The run time was 6 min. Parent/product transitions were 261.2/113.1 for GS-331007 and 265.2/113.1 for GS-331007 IS.

Clinical application. To determine the clinical application of the above-described method, the validated assay was utilized to determine 007-TP concentrations *in vivo*. Specifically, PBMC, RBC, and hepatocyte samples were collected from HCV-infected subjects who provided written, informed consent to participate in Institutional Review Board (IRB)-approved studies. All subjects received 400 mg SOF daily as part of their therapy. PBMC and RBC samples were utilized to determine PK parameters describing drug disposition into these cell types; however, half-life was not determined in hepatocytes due to the limited number of samples. PBMC PK parameters were determined using noncompartmental analyses, while RBC 007-TP PK was determined using a one-compartment model in NONMEM version 7.2 (ICON Development Solutions, Ellicot City, MD, USA). In the one-compartment model, k_a represents the aggregate rate of formation of 007-TP, volume is a scalar, and k_e represents the rate of elimination of 007-TP from the RBC. Covariates, including sex, race, HCV genotype, fibrosis stage, liver decompensation, and concomitant medications, were analyzed for association with 007-TP concentrations in the two cell types using either unpaired *t* tests (dichotomous covariates) or Pearson rho correlations (continuous covariates) in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Finally, sustained virologic response (SVR) was tested for association with 007-TP in RBC with regressions in SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Method validation. The following experiments were evaluated, using acceptance criteria of ±15% for accuracy (compared to nominal as the percentage of deviation [%dev]) and precision (as the percent coefficient of variation [%CV]) determinations at all concentrations except the lower limit of quantification (LLOQ), where ±20% was allowed.

(i) Extraction process. The recovery of the 007-TP moiety off of the Waters QMA SPE is expected to be similar to that of uridine, which was determined to be 94.4%. Alkaline phosphatase was used to dephosphorylate the individual nucleotide fractions, and peak area responses were equivalent whether samples were incubated for 10, 30, or 60 min (%CV, ≤5.4%) and were not different from those for nominal concentrations (%dev, ≤±6.1%), demonstrating a consistent recovery from the dephosphorylation step.

TABLE 2 Interassay and intra-assay accuracy and precision of quality control samples prepared at known concentrations

Assay type	Parameter	Nominal value according to phosphate concn (fmol/sample):			
		50	150	1,500	40,000
Interassay	%CV	10.0	6.2	1.9	0.9
	%Dev	−1.7	−4.2	−3.4	−1.5
	No.	17	18	18	18
Intra-assay ($n = 3$)	%CV	3.6 to 7.8	3.5 to 9.0	1.1 to 2.1	0.7 to 1.3
	%Dev	−8.7 to 10.6	−6.2 to −1.4	−4.8 to −2.7	−1.8 to −1.3

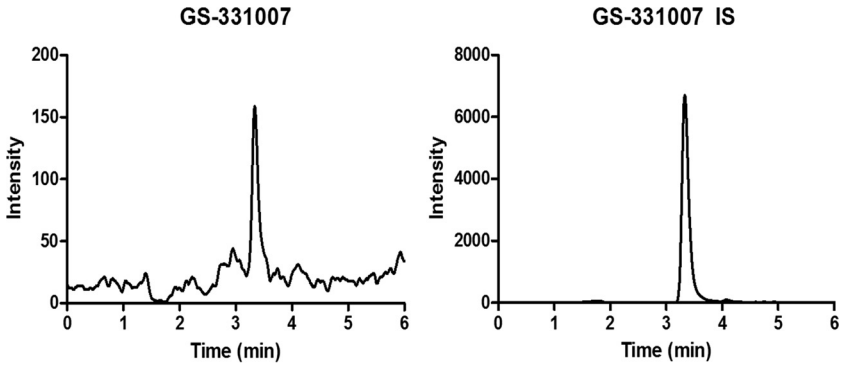

FIG 2 Typical chromatogram showing the response generated by a sample at the lower limit of quantitation (50 fmol/sample). The left portion of the chromatogram is GS-331007, and the right represents GS-331007 IS.

Sample desalting and concentration were optimized for GS-331007 on a Strata-X-CW cartridge. Matrix effect, recovery, and process efficiency were determined following the experiments described by Matuszewski et al. (22). Briefly, three sets of samples were prepared in 5 replicates at concentrations of 250, 2,500, and 10,000 fmol/sample: set 1 consisted of neat samples prepared in UP water (the reconstitution matrix), set 2 consisted of 5 different lots of lysed PBMC matrix carried through the entire extraction process prior to the addition of the working standard stock (postextraction spike), and set 3 consisted of extracted samples generated in 5 different lots of lysed PBMC matrix. The matrix effect was determined by comparing the response of set 1 to those of set 2 samples, recovery was determined by comparing set 2 to set 3 samples, and overall process efficiency was determined by comparing set 1 to set 3 samples. Matrix effect on GS-331007 was found to be −12.4%, recovery of GS-331007 was determined to be 90.8%, while the process efficiency was 79.5% and 83.8% for GS-331007 and GS-331007 IS, respectively. Finally, unweighted linear regression slopes were generated for each of the 5 different lots of lysed PBMC matrix based on the peak area ratio of the three concentrations tested. The slopes of these regressions were precise (%CV, 0.22%), demonstrating the lack of significant matrix effects between different lots of lysed PBMC matrix.

(ii) Accuracy and precision. Accuracy and precision were determined by replicate analysis ($n = 6$) at each of the QC levels described above, in addition to a QC prepared at the LLOQ level (50 fmol/sample) in three separate analytical runs. Standard curve performance in these runs was also analyzed.

Standard curves were best fit by linear regression with 1/concentration weighting and were linear from 50 to 50,000 fmol/sample. Standard performance in four runs is shown in Table 1. Accuracy was within ±4.0% and precision was ≤5.3% for back-calculated standards. The calibration curve slopes were precise (%CV, 2.4%), and R^2 was ≥0.9998.

Intra-assay and interassay accuracy and precision based on the QCs are shown in Table 2. Intra-assay accuracy was within ±10.6% and precision was within 9.0%, while interassay accuracy was within ±4.2% and precision was within 10.0% for all QC levels, including the LLOQ. One LLOQ QC (on validation run 3) was removed from the analysis, as it tested as an outlier ($P < 0.01$). The 150 fmol/sample QC in validation run 1 approached acceptance criteria (%dev = −13.3%); thus, it was removed from analysis, remade, and evaluated in three subsequent validation runs (as a result, four total validation runs were performed). A typical chromatograph for the 50 fmol/sample LLOQ is shown in Fig. 2.

(iii) Alternative matrices. As the intent of the method was to be able to quantify GS-331007 phosphates in a variety of cell types (such as RBC, dried blood spots [DBS], and purified hepatocytes), validation included an examination of lysed matrices generated from cell types other than PBMC, as well as a demonstration of the ability to quantify concentrations in the MP and DP fractions generated from the Waters QMA SPE extraction. To do this, two calibration standards (1,000 and 10,000 fmol/sample) were spiked into 3 different lysed RBC matrix lots and the MP and DP fractions generated from 3 different lysed PBMC matrix lots previously extracted through the QMA process and compared to a full calibration curve extracted from the lysed PBMC matrix TP fraction typically used for analysis.

The alternative matrices results are presented in Table 3. The accuracy of all matrices was within ±2.6%, while precision was ≤1.7%. Given the major physiological differences between PBMC and RBC (e.g., nucleated versus non-nucleated), these results support the application of this method to the quantification of GS-331007 phosphates from purified lysed RBC, DBS, and other nucleated cell types such as hepatocyte matrices as well as from the MP and DP fractions of the Waters QMA extraction process.

(iv) Specificity. Specificity was determined by injecting extracted blank lysed PBMC matrix TP fractions ($n = 6$ lots) and monitoring for GS-331007. A sample containing the high standard (50,000 fmol/sample) with no IS and a sample consisting of

TABLE 3 Accuracy and precision results for the alternative matrices anticipated to be studied using this method

Alternative matrix	Parameter	Value according to phosphate concn (fmol/sample): 1,000	10,000
RBC-TP	Accuracy (%dev)	−1.4	−1.0
	Precision (%CV)	0.7	1.1
PBMC-MP	Accuracy (%dev)	−0.5	−1.8
	Precision (%CV)	0.3	0.1
PBMC-DP	Accuracy (%dev)	−2.6	−0.8
	Precision (%CV)	1.3	1.7

TABLE 4 Accuracy and precision of samples consisting of between 0.1 and 100 million PBMC or RBC

Dilutional accuracy	Value according to no. of PBMC or RBC:					
	0.1 million PBMC	1 million PBMC	4 million PBMC	10 million RBC	50 million RBC	100 million RBC
Nominal concn (fmol/sample)	2,000	750	300	2,500	2,500	2,500
%CV	5.2	3.1	0.9	0.2	0.8	1.6
%Dev	−2.2	−1.3	0.0	2.3	−0.1	0.7

blank matrix spiked with IS were used to evaluate cross talk between GS-331007 and GS-331007 IS. Carryover was evaluated by checking for a signal in a blank water injection following the cross talk samples. Blank and blank internal standards were included with each analytical run to monitor for specific response. No GS-331007 was found in the blank lysed PBMC matrix samples, nor was any cross talk or carryover observed for either GS-331007 or the IS, showing the method to be specific for GS-331007 and GS-331007 IS.

(v) Effect of cell number. The need to assay low or high cell numbers was an important consideration for this method. As limited data exist on *in vivo* GS-331007 phosphate concentrations, it was unknown how many cells would need to be assayed in order to obtain concentrations in the reportable concentration range. Furthermore, it is anticipated that collected hepatocyte samples will be limited and may contain low cell counts, increasing the need to demonstrate the method applicability to a variety of cell counts. This was demonstrated by extracting triplicate samples at various concentrations extracted from 0.1, 1.0, 4.0, 10, 50, and 100 million cells (representing a variety of sample volumes). Results shown in Table 4 demonstrate that the method was robust for a wide range of assayed cell numbers, as accuracy was within ±2.3% and precision was within 5.2% for all tested cell counts and sample volumes.

(vi) Analyte stability. Conditional 007-TP and GS-331007 stability was determined by assessing the freeze/thaw stability, room temperature stability, and extracted sample stability using the 1,500 fmol/sample and 40,000 fmol/sample QCs. Triplicate QCs at these levels were tested in separate experiments to evaluate the stability of samples subjected to these conditions. Freeze/thaw stability was tested using four freeze/thaw cycles, room temperature stability was tested by maintaining samples at room temperature (20°C) for 4 days prior to extraction, and extracted sample stability was tested by maintaining previously extracted samples in the autosampler (20°C) for 4 days prior to reinjection, with the results from each experiment compared to nominal values and fresh triplicate QC samples at the same levels as those that were not subjected to any of the treatment conditions. Finally, triplicate GS-331007 working standards (1,000 and 10,000 fmol/sample) were maintained at room temperature (20°C) for 20 h and compared to triplicate control standards. Table 5 demonstrates that GS-331007 and 007-TP were stable under these conditions, as treated samples were within ±6.7% of nominal and ±2.3% of control for all tested conditions.

In addition to assessing conditional stability, the long term stability of GS-331007 and 007-TP was examined. Peak responses of methanolic GS-331007 prep stocks (3,843 pmol/μl) stored at −20°C for various lengths of time were compared in triplicate to those of a fresh GS-331007 preparation at the same concentration to evaluate the long term stability of GS-331007 stock solutions. Triplicate 1,500 fmol/sample QC samples that were stored at −80°C for 1 month were compared to the nominal concentration to assess long term 007-TP stability. GS-331007 preparation stocks in methanol were found to be stable for at least 6 months at −20°C (%dev, −0.4%), while 007-TP in 70:30 methanol/water was stable for at least 1 month at −80°C (%dev, −1.8%).

Clinical application. PBMC samples were collected between 2.3 and 27 h postdose from 45 subjects who had been treated with SOF for a median of 29 (range, 19 to 162) days. GS-331007 phosphate concentrations in PBMC were quantifiable in all TP samples; however, 2 MP and 16 DP samples were below the limit of quantitation. For those samples that were quantifiable, median PBMC concentrations were 220 (range, 51.5 to 846), 70.2 (range, 25.8 to 275), and 859 (range, 54.5 to 6756) fmol/10^6 cells in the MP, DP, and TP fractions, respectively. The half-lives of the MP and TP moieties in PBMC were 14 and 26 h, respectively. Due to the number of DP samples below the limit of quantitation, the half-life of this moiety could not be reliably determined. Clinical covariates including sex, race, HCV genotype, fibrosis score, and liver decompensation were not associated with concentrations of GS-331007 phosphates. Increased PBMC 007-MP (85%; $P = 0.03$) and 007-TP (135%; $P = 0.01$) concentrations were observed in subjects ($n = 18$) coadministered simeprevir (Fig. 3). No such association was observed for subjects also receiving ribavirin as part of their care. Subject demographics and the determined PBMC PK parameters are summarized in Table 6, and a typical chromatograph for the clinical research of PBMC samples is shown in Fig. 4.

Two hepatocyte samples were obtained from individuals receiving SOF-based HCV treatment undergoing orthotopic cadaveric liver transplantation. These hepatocyte samples yielded concentrations of 551 and 1,839 fmol/10^6 cells (MP), 75.2 and 259 fmol/10^6 cells (DP), and 64.4 and 202 fmol/10^6 cells (TP). Due to the limited number of samples, no PK analyses were performed on these samples.

RBC samples were collected on day 3 and on weeks 4, 12, and 24 from 47 individuals participating in the National Institute of

TABLE 5 Accuracy of samples evaluated for conditional stability[a]

Sample type and parameter	Conditional stability values	
007-TP (QC) (fmol/sample)	1,500	40,000
Freeze/thaw (4 cycles)	−1.9	0.2
Room temp (4 days, 20°C)	−1.8	1.1
Extracted sample (4 days, 20°C)	−2.3	−0.2
GS-331007 (standard) (fmol/sample)	1,000	10,000
Room temp (20 h, 20°C)	0.9	1.3

[a] Data shown are the percentages of deviation between treated and control.

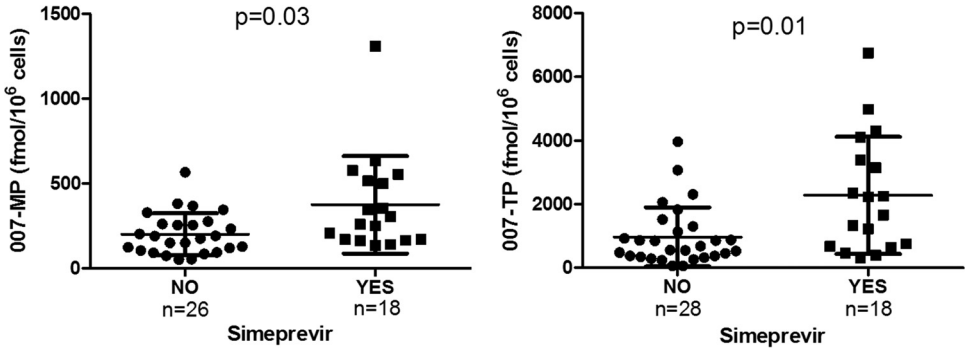

FIG 3 Coadministration of simeprevir was associated with increased concentrations of 007-MP (left, 85%) and 007-TP (right, 135%) in PBMC. Data are shown as means ± standard deviations (SD).

Allergy and Infectious Diseases SPARE trial (23). Briefly, the study was designed to investigate the efficacy of a combination of sofosbuvir plus either low-dose or weight-based ribavirin in genotype 1-infected individuals. The RBC 007-TP median steady-state concentration was 2.91 (range, 1.14 to 10.4) fmol/10^6 cells, while the mean half-life determined from the model was 69 (95% confidence interval [CI], 64 to 75) hours. Analyzed RBC samples largely yielded results near the 50 fmol/sample lower limit of quantitation, indicating that GS-331007 phosphates are present at significantly lower concentrations in this matrix compared to PBMC. 007-TP concentrations in RBC were found to be 42% lower in men than those in women ($P = 0.005$). The likelihood of SVR was increased in subjects with higher RBC 007-TP concentrations on day 3 of the study; however, this trend did not reach significance ($P = 0.13$). More significant predictors of SVR in this study were fibrosis stage of ≤2 ($P = 0.07$), female sex ($P = 0.05$), and a baseline viral load of <800,000 IU/ml ($P = 0.03$). Subject demographics and the determined RBC PK parameters are summarized in Table 6, and a typical chromatograph for clinical research RBC samples is shown in Fig. 5.

TABLE 6 Study demographics and pharmacokinetic data for GS-331007 phosphates in PBMC and RBC

Demographic[a]	PBMC	RBC
No. of subjects	45	47
HCV genotype (no.)		
1	29	47
2	5	
3	8	
4	3	
Sex (no.)	28 men, 17 women	32 men, 15 women
Race (no.)	5 African-American, 40 non-African-American	37 African-American, 10 non-African-American
No. fibrosis HAI of ≥3	32	14
No. decompensated cirrhosis	22	
007-MP		
C_{ss} (fmol/10^6 cells)	220 (51.5–846)[b]	
$t_{1/2}$ (hours)	13.8[c]	
007-DP		
C_{ss} (fmol/10^6 cells)	70.2 (25.8–275)[b]	
007-TP		
C_{ss} (fmol/10^6 cells)	859 (54.5–6756)[b]	2.91 (1.14–10.4)[b]
$t_{1/2}$ (hours)	26.0[c]	69 (64–75)[d]

[a] HAI, histology activity index; C_{ss}, concentration at steady state; $t_{1/2}$, half-life.
[b] Data indicate median (range).
[c] Data indicate mean.
[d] Data indicate mean (95% CI).

Conclusions. This report describes the development, validation, and application of a new LC-MS/MS method to quantify GS-331007 phosphate concentrations in lysed cellular matrices. The method was accurate and precise throughout the linear range of the assay. Furthermore, the assay was validated for use with a variety of cell matrices and numbers, and GS-331007 was found to be stable under a variety of conditions. The multiple cell types validated for use with this assay demonstrate the broad applicability of this method for characterizing the disposition of SOF within blood and tissues of interest, including the liver. The validated method was used to quantify 007 MP, DP, and TP concentrations in PBMC, RBC, and hepatocytes from patients receiving SOF-based HCV treatment, providing an initial look at the intracellular disposition of SOF in humans.

Due to the limited *in vivo* data on the intracellular pharmacology of SOF in the literature, the needed calibration range was unknown prior to method development. We anticipated from *in vitro* data that concentrations would likely be sufficient to warrant a picomole per sample calibration range (13); therefore, we first used a calibration range of 0.5 to 200 pmol/sample, similar to the calibration curves used for emtricitabine, lamivudine, and ribavirin (19, 24). However, the first analysis of unknown samples collected from study subjects demonstrated that this was not sufficiently sensitive, as a majority of the TP samples were found to have low picomole per sample or high femtomole per sample concentrations (below the limit of quantitation to approximately 4.5 pmol/sample), while all but 2 MP and DP fractions were below the 0.5 pmol/sample limit. From these results, we found that a 10-fold increase in sensitivity to an LLOQ of 0.05 pmol/sample (50 fmol/sample) was necessary. This was accomplished by increasing the injection volume from 10 μl during development to the 30-μl volume used for validation. This change greatly increased signal, with only a minor increase in the accompanying noise, and thus increased sensitivity at the low end. However, this change led to a broader peak width, which suggested that refining the LC conditions might generate an even greater signal improvement. We therefore increased the amount of organic content in the mobile phase, which decreased the retention time and peak width of GS-331007 and benefited sensitivity at the low end. These changes successfully increased sensitivity to the targeted 50 fmol/sample LLOQ, as evidenced by the high level of interassay accuracy

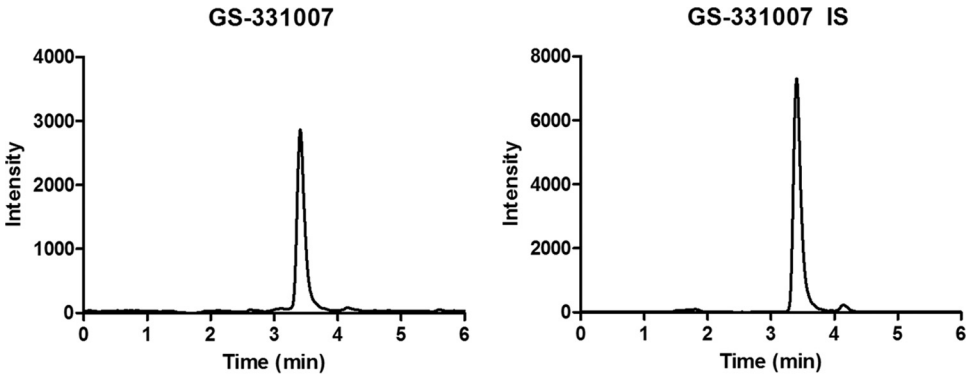

FIG 4 Typical subject PBMC sample chromatogram, resulting in a concentration of 482 fmol/10^6 cells. The left portion of the chromatogram is GS-331007, and the right represents GS-331007 IS.

(−1.7%) and precision (10.0%) demonstrated at this LLOQ. As a result, only 18 PBMC samples (2 MP and 16 DP) were found to be below the limit of quantitation out of the 138 MP, DP, and TP fractions analyzed.

This method achieved high recovery and process efficiency, in part due to a desalting and concentration extraction process that was developed in the laboratory for the extraction of endogenous 2-deoxynucleosides (25). In contrast to our previously validated method for other nucleos(t)ide analogs, which utilized Strata-X SPE, the current extraction process used Strata-X-CW cartridges. The original method development with the Strata-X SPE was targeted toward quantifying the anti-HIV nucleos(t)ide analogs tenofovir, zidovudine, lamivudine, and emtricitabine and focused on optimizing sensitivity for tenofovir (19). It was found that the Strata-X cartridge provided the best retention and, thus, the best recovery and greatest sensitivity for tenofovir, and it was, thus, selected for the extraction cartridge. As GS-331007 was the only analyte of interest for this method and it does not present the same challenges as tenofovir, we were able to optimize this extraction method for a high recovery and process efficiency for GS-331007. This was accomplished using a Strata-X-CW cartridge with an extraction process that was simple (using only water and methanol), generated a clean sample, and resulted in high (91%) recovery of GS-331007. The high recovery and good process efficiency (80%) of the optimized Strata-X-CW extraction process aided the sensitive determination of GS-331007 phosphate concentrations in lysed cellular matrices. Additionally, the method sensitivity benefitted from the fractionation and dephosphorylation of the different phosphate moieties present in each sample and, therefore, eliminated the need for ion-pairing chromatography, which can reduce sensitivity, cause analytical noise, and increase system contamination (20, 21).

SOF was designed to specifically target hepatocytes through the phosphoramidate prodrug moiety, which is rapidly metabolized by enzymes that are highly expressed in the liver (2). Subsequent to the metabolism of the phosphoramidate moiety, the drug is then phosphorylated to the active TP form. We, therefore, anticipated that phosphate concentrations in hepatocytes would be much higher than those found in PBMC, with the majority of the drug in the 007-TP form. However, hepatocyte concentrations in our 2 patients with end-stage liver disease did not support this hypothesis, as the MP concentrations of the two samples were higher than the TP concentrations and were comparable to the concentrations observed in PBMC. Previous work on liver sample processing procedures suggested that endogenous nucleotides in hepatocytes may degrade once the liver is removed (10), which would result in an underestimation of hepatocellular phosphorylated anabolites *in vivo*. More work is needed to evaluate and optimize liver sample processing procedures in order to better characterize hepatocellular nucleotide phosphorylation and SOF disposition *in vivo* in patients with end-stage liver disease.

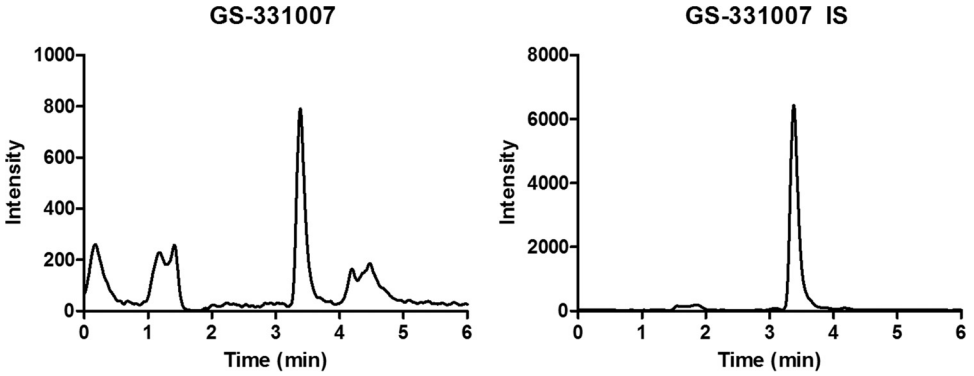

FIG 5 Typical subject RBC sample chromatogram, resulting in a concentration of 3.15 fmol/10^6 cells when 100 ×10^6 cells were assayed. The left portion of the chromatogram is GS-331007, and the right represents GS-331007 IS.

The application of this method provides insight into the pharmacology of SOF in blood cells. Specifically, data collected from this method were utilized to determine the PK of 007-TP in PBMC and RBC, to explore the clinical covariates associated with 007-TP in these cell types, and finally to evaluate the potential for relationships between these concentrations and the efficacy of SOF. PBMC and RBC concentrations were determined to persist with relatively long half-lives (26 and 69 h, respectively), which may aid in the understanding of the clinical forgiveness of SOF in patients who are nonadherent. Second, the data suggest that PBMC 007-TP concentrations were significantly increased (135%, $P = 0.01$) in those subjects also receiving simeprevir as part of their HCV therapy, although further investigation is needed to determine the clinical significance of this finding. The interaction between simeprevir and SOF has previously been observed in plasma, as the maximum concentration (1.91-fold) and the area under the concentration-time curve (3.16-fold) for plasma SOF were found to be higher in individuals receiving simeprevir (26); however, this is the first demonstration that this interaction occurs intracellularly. Finally, day 3 RBC 007-TP demonstrated a trend toward association ($P = 0.13$) with the increased likelihood of achieving sustained virologic response in genotype 1-infected individuals receiving SOF plus ribavirin. This supports the possibility of using clinically accessible matrices such as RBC for predicting the efficacy of SOF-based therapy and for determining if a patient requires an adjustment to the dosing regimen or a lengthened duration of treatment. Together, these early results using data generated by this assay support the critical need to understand the cellular disposition of SOF and its anabolites.

The intracellular concentrations determined from these studies offer a first glance at factors that may influence the outcomes in SOF-based therapy in various patient populations and clinical scenarios. First, these data show that intracellular SOF pharmacology is cell dependent, as 300-fold higher 007-TP concentrations were observed in PBMC compared to those in RBC, indicating that the cellular transport and/or phosphorylation of SOF and its anabolites are distinct between these cell types. Furthermore, 007-MP concentrations were higher than 007-TP concentrations in hepatocytes but not in PBMC, which provides more evidence that phosphorylation rates are cell specific; however, more work is needed to confirm the hepatocellular concentrations. This supports the critical need to understand the cellular pharmacology in hepatocytes in contrast to more accessible cellular matrices such as RBC and PBMC to determine if these cell types can be utilized as a marker of SOF efficacy.

In conclusion, a method for determining GS-331007 phosphate concentrations from a variety of cellular matrices was validated, applied to clinically derived samples, and utilized to provide the first data on the intracellular pharmacokinetics of this widely used agent.

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