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PAPER

Quantitative performance of online SPE-LC coupled to Q-Exactive for the analysis of sofosbuvir in human plasma

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A quantitive strategy of sofosbuvir (SF) in human plasma has been developed with an online solid phase extraction (SPE)-liquid chromatography (LC) coupled with high resolution mass spectrometer (HRMS) system using MS/MS targeted ion fragmentation scan (t-MS^2), targeted-selected ion monitoring mode (t-SIM) and full MS-SIM (F-SIM) mode respectively. The sample was pretreated automatically and analyzed within 10min via an online SPE system equipped with Oasis®HLB (2.1*20mm, 5μm) SPE column and LC system with ZORBAX SB-C18 (4.6*250, 5μm) analytical column. A Q-Exactive hybrid quadrupole-Orbitrap HRMS was utilized for positive identification and quantification. Method detection limits ranged between 0.5 and 2000 ng L^{-1} in human plasma for both t-MS^2 and t-SIM mode, between 2 and 2000 ng L^{-1} for F-SIM mode. In all three modes, the overall intra-day and the inter-day variations were less than 8.07%. The recovery of three methods was in the range of 92.06-107.20% with RSD% less than 3.90%. The matrix effect of three methods was in the range of 94.82-101.89% with RSD% less than 7.69%. The optimized two methods (t-MS^2 and t-SIM) demonstrated good performance in terms of specificity, lowest limit of quantification (LLOQ), linearity, recovery, precision and accuracy.

1. Introduction

Bioanalysis was one of the crucial processes in the area of pharmaceutical research including pharmacokinetics (PK), therapeutic drug monitoring (TDM) etc. Bioanalysis of drug in matrix, such as plasma, serum, urine and tissues, has contributed substantially to establish dosage schemes, to reveal metabolic variability and to minimize adverse effects.[1] Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely used as the most reliable technology platform in the field of qualitative and quantitative bioanalysis.[2-7] The MS/MS triple quadrupole (QqQ) has revolutionized trace analysis in complex samples and replaced many traditional techniques based on fluorescence, ultraviolet, single stage mass spectrometry detectors.[8] Through the analysis of the precursor ions and product ions of target compounds, QqQ could achieve remarkable sensitivity and quantification capability. However, one of the major drawbacks of conventional QqQ system is its relatively low resolution that could not differentiate a target compound from an interfering compound with a decimal difference. The inherent limitation of LC-MS/MS is its total dependence on the separation of the target molecule from interfering compounds by LC.[9] Metabolites with similar molecular masses to their parent analytes always perform as a common source of interference.[10] For instance, a metabolite with a gain of 1Da could be attributed to an enzymatic conversion of a primary amide on the parent compound to its carboxylic acid counterpart. This metabolite with carboxylic acid group is isobaric with the [M+1] isotope of the parent compound containing amide group.[11] This phenomena could interfere the quantification of the metabolite. To overcome the aforementioned limitations, the fast development of high resolution mass spectrometer (HRMS) has been offering new possibilities. Accurate mass measurement with high resolution could narrow down the number of possible molecular formulas that might be represented by a particular mass so as to avoid mass false positives.[8] The selectivity could be significantly improved by high resolving power (RP) of HRMS. Various analyzers with high resolution power including time of flight (TOF) instruments and, Orbitrap technology etc. have been developed and commercialized.[12-13] When coupled HRMS with a quadrupole mass filter, high sensitivity and high selectivity could be achieved through combining the high resolving power of HRMS with the selectivity of the quadrupole. Q-Exactive is a hybrid benchtop Orbitrap mass spectrometer coupled with quadrupole. Q-Exactive, which is equipped with a higher-energy collision cell (HCD). The trapped ions by C-trap may be subjected to HCD, generating specific fragment ions.[14] The device could achieve a mass accuracy lower than 2 ppm and resolving power up to 140,000 full width at half maximum (FWHM) at *m/z* 200.[15] The device allows not only the high resolution full scan (FS) but also MS/MS targeted ion fragmentation scan (t-MS^2). An increasing number of applications of LC-HRMS utilizing an Orbitrap mass spectrometer for the analysis of complex samples have been

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recently published, including plasma,[12] kidney,[13] manure,[15] urine,[16] milk,[17] fruits and vegetables,[18] et al.

The matrix of biological samples may contain various components such as salts, proteins and phospholipids. Biological matrix likely causes LC column efficiency degradation, ion source contamination, signal interference and matrix effects, resulting in poor bioanalysis of samples.[19] Co-eluting matrix components could lead to enhance or suppress analyte ion intensity. This is referred as matrix effects.[20] In order to prolong service life of LC column and MS instrument, as well as generate reliable and reproducible data, sample clean-up is crucial before bioanalysis. Liquid-liquid extraction (LLE), protein precipitation (PP) and offline-solid-phase extraction (SPE) have been considered as conventional sample preparation techniques.[1] However, all these methods could be time-consuming and inconvenient due to heavy involvement of manual processes. In addition, the manual processes involved in these methods are prone to generate errors, which may lead to high variation of quantification. Recently, online SPE utilizing column-switching techniques coupled LC-MS/MS has rapidly gained acceptance in bioanalytical applications to automate the sample clean-up process. The integrated process to separate target compound from matrix could not only reduce both time and manpower required for offline techniques but also increase the data quality of the bioanalysis.[21-23] Up to now, online SPE system coupled with Orbitrap has been used to analyse small molecule in aqueous matrices.[24-26] To our knowledge, an analysis platform for the analysis of drug concentration in plasma with integrated online SPE-HPLC and Q-Exactive has not yet been reported in literature.

In our research group, we have successfully developed various methods for bioanalysis of plasma samples using online-SPE coupled with API 4000+ triple quadrupole mass.[4-5] With an aim to explore the possibility using HRMS as an alternative to MS/MS for bioanalysis of plasma samples, we attempted to establish such a platform cascaded online SPE system with LC-Q-Exactive. To verify the feasibility of our quantification strategy, sofosbuvir (SF) was chosen as the target drug for bioanalysis. Approved by US FDA in 2013, SF, as the active principal ingredient of Sovaldi and Harvoni, has become a breakthrough in the cure of Hepatitis C virus (HCV) infection.[27-28] Previously, the available treatment for patients with HCV infection is a combination therapy using pegylated interferon (IFN) and ribavirin (RBV).[29] Unfortunately, the treatment has only moderate efficacy and is poorly tolerated by patients due to significant adverse effects.[30] SF was designed as a phosphoramidate prodrug of cytidine nucleoside analog (Fig. 1), and provided a higher cure rate with fewer side effects. A reliable analysis method has been of importance for both drug development and clinic research. Until now, there is only a single report on determination method of SF in biological matrices with liquid-liquid extraction using UPLC-MS/MS.[31] In the clinic research, the concentration of SF and its metabolite GS-331007 in the plasma were used as surveillance data.[32] Minimal accumulation and linear pharmacokinetics of GS-331007 and SF were observed when SF was administrated with multiple dosed.[33] Therefore, only SF was selected for our study. An UltiMate 3000*2 Dual-gradient HPLC system was introduced in the sample pre-treatment and separation of analyte via online-SPE-HPLC, and then Q-Exactive was used in the identification and quantification of the product ion with t-MS^2, t-SIM and F-SIM modes. The quantification performance of three modes was studied. The validation of methods was assessed by the determination of the method linearity, lowest limit of quantification (LLOQ), accuracy, extraction recovery and matrix effect.

2. Materials and methods

2.1. Chemicals and reagents

SF (purity > 99%) was synthesized and purified according to literature.[28] Chemical structure of SF is shown in Fig. 1. Carbamazepine (CBZ, purity 99.0%), purchased from Shanghai Oriental Pharmaceutical Science and Technology Co. Ltd (Shanghai, China), was used as the internal standard (IS). Acetonitrile and methanol were HPLC grade purchased from Fisher Scientific (Whitby, ON, Canada), and water used in the experiment was prepared by a Milli-Q Ultrapure purification water system (Bedford, MA, USA). Ammonium acetate and acetic acid were purchased from J&K (Beijing, China). Chromatographic mobile phases were prepared daily. Under the approval of the ethical committee of Tianjin AnDing Hospital, all blank plasma samples were kindly donated by informed consents of healthy volunteers from Tianjin AnDing Hospital, collected in K_2EDTA-treated tubes and stored at -80 ºC.

The heated electrospray ionization (HESI) source of Q-Exactive was tuned and calibrated once a week. The component of positive ion calibration solution are singly-charged ions. The ions are as follows: n-butylamine (*m/z* 74), caffeine (*m/z* 195 and its fragment *m/z* 138), Ultramark 1621 (*m/z* 1022, 1122, 1222, 1322, 1422, 1522, 1622, 1722, 1822), and MRFA (*m/z* 524).[17]

2.2 Stock and working solutions

The standard stock solutions of SF and CBZ were prepared by dissolving accurately weighted SF and CBZ in methanol at the concentration of 1 mg mL^{-1}. Then the stock solution of SF was diluted with methanol:water (50:50, v/v) for concentration series of 40,000 ng mL^{-1}, 20,000 ng mL^{-1}, 10,000 ng mL^{-1}, 4,000 ng mL^{-1}, 1,000 ng mL^{-1}, 400 ng mL^{-1}, 100 ng mL^{-1}, 40 ng mL^{-1} and 10 ng mL^{-1}. The stock solution of CBZ was diluted with methanol:water (50:50, v/v) to 500 ng mL^{-1} as internal standard working solution. All the standard stock solutions were stored at -20 ºC and the working solutions were stored at 4 ºC.

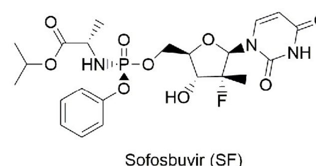

Fig. 1. The structure of sofosbuvir

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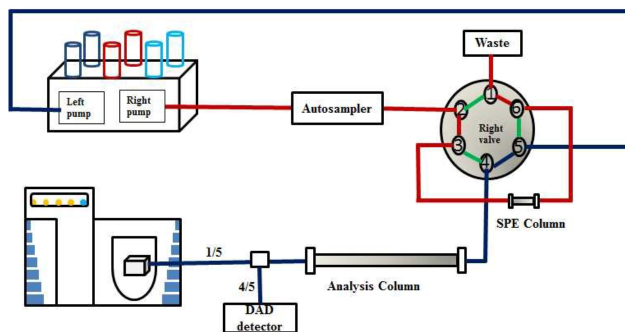

Fig. 2. The schematic diagram of online- SPE-HPLC-HRMS system.

Table 1. Online-SPE and HPLC conditions[a]

Time (min)	SPE cartridge			Analytical column		
	Flow rate (mL min^{-1})	A (%)	B (%)	Flow rate (mL min^{-1})	A (%)	B (%)
0	1	2	98	1	40	60
3	1	2	98	1	40	60
6	1	100	0	1	70	30
7	1	100	0	1	70	30
7.5	1	2	98	1	40	60
10	1	2	98	1	40	60

[a]A, acetonitrileB, 5 mM ammonium acetate buffer (pH=3.5, adjusted by acetic acid)

Table 2. The parameters of the three modes

Mode	RP	AGC	Maximum IT (ms)	Scan range (*m/z*)
t-MS^2	17,500	2×10^5	100	243.00756
t-SIM	70,000	5×10^4	200	530.16982
F-SIM	70,000	3×10^6	200	100-600

Calibration standard solutions were prepared by spiking the diluted working standard solutions into blank human plasma (1:19, v/v) to give concentration series of 0.5 ng mL^{-1}, 2 ng mL^{-1}, 5 ng mL^{-1}, 20 ng mL^{-1}, 50 ng mL^{-1}, 200 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1} and 2000 ng mL^{-1}. 10 µL of IS working solution was added to 200 µL human plasma samples. Then the samples were vortexed for 30 sec, and centrifuged at 13000 rpm for 15 min at 4℃. Quality control (QC) samples were prepared at 0.5 ng mL^{-1} (low quality control, LQC), 200 ng mL^{-1} (middle quality control, MQC), 2000 ng mL^{-1} (high quality control, HQC) for SF.

2.3. Online SPE and LC parameters

A Thermo Scientific Dionex Ultimate 3000 pump coupled with a Thermo Scientific Dionex Ultimate 3000 RS column compartment and a Thermo Scientific Ultimate 3000 autosampler controlled by Chromeleon 7.2 Software (Thermo Scientific Fisher, Waltham, MA and Dionex Softron GMbH Part of Thermo Fisher Scientific, Germany) were used for analysis.

Sample pre-treatment was performed with an online SPE methodology with a SPE cartridge Oasis®HLB (2.1*20mm, 5µm) from Waters. The system setup for online SPE consisted of three steps (Fig. 2). In the loading step, the plasma sample was injected directly into the SPE cartridge and the biological matrix was washed out in 2 min with acetonitrile-5 mM ammonium acetate buffer 2/98 (v/v, pH 3.5) at a flow rate of 1 mL min^{-1}. SF was retained on the SPE cartridge. In the transfer step, SPE cartridge was coupled with the analytical column. SF was transferred from SPE cartridge to the analytical column by isocratic elution consisted of acetonitrile-5 mM ammonium acetate buffer 40/60 (v/v, pH 3.5) at a flow rate of 1 mL min^{-1} for 1 min. In the separation step, the SPE cartridge and analytical column were switched by valve to parallel state once again. The analytes were separated by gradient elution (showed in Table 1) at a flow rate of 1 mL min^{-1}. This kind of technological process allows shortening the cycle time, which in our approach is only 10 min/sample.

A C18 (Agilent ZORBAX SB-C18, 4.6 * 250 mm, 5 µm particles) column was used for chromatographic separation of SF and IS at 30 ºC. Mobile phase A was acetonitrile whereas mobile phase B was 5 mM ammonium acetate buffer (pH 3.5). A flow rate of 1 mL min^{-1} and an injection volume of 10 µL were chosen. Through a tuneable flow divider valve, 4/5 liquid passed through Dionex Ultimate 3000 diode array detector, while 1/5 entered into Q-Exactive.

2.4 Electrospray ionization source

A HESI-II in positive mode was used for the ionization of the SF. Source parameters were as follows: the spray voltage +3.5 KV, S-lens voltage 50 V, aux gas heater temperature 300 ºC and capillary temperature 320 ºC. Ultrapure liquid nitrogen was used as ion source gas and collision gas, the sheath gas flow rate and aux gas flow rate were 35 and 10 (arbitrary units), respectively. The parameters of different scan modes were shown in Table 2.

2.5 Data analysis and method validation

Xcalibur 3.0 (Thermo Fisher Scientific, San Jose, CA, USA) was used for instrument control and data acquisition. Positive identification of analyte was based on the accurate mass of the analyte with less than ±5 ppm error, retention time comparison within ± 0.03 min. Massfrontier 7.0 (HighChem, Ltd, Bratislava, Slovakia) was used to obtain the fragmentation pattern and accurate mass of the product ions in positive mode.

In t-MS^2 mode, the presence of a product ion (*m/z* 243.07756) was used for confirmation and quantification. In t-SIM and F-SIM mode, precursor ion (*m/z* 530.16982) was used for quantification. The mass tolerance window (MTW) was set to 5 ppm for the three analysis modes.

2.5.1 Linearity and LLOQ. Nine-point calibration curves were obtained by passing the online SPE method on SF human plasma samples spiked between 0.5 and 2000 ng mL^{-1} and analysed in quintuplicates.

Least squares linear regression model y=ax±b weighted by 1/x^2 was used to fit calibration curves, in which y is peak area ratio of SF and IS, a is slope of the calibration curve, b is the y

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axis intercept and x is the analyte concentration. The linear range was set as 0.5-2000 ng mL^{-1}.

2.5.2 Accuracy and precision. Accuracy values were determined by the relative standard deviation (RSD%). Intra-day precision was evaluated by analysis of the same spiked sample at three different concentrations (0.5, 200 and 2000 ng mL^{-1}) five times on a single workday. Inter-day precision was calculated at three different concentrations (0.5, 200 and 2000 ng mL^{-1}) prepared daily for a period of 3 days.

2.5.3 Extraction recovery and matrix effect. The extraction recoveries of SF were assessed by comparing the mean peak area of the QC samples spiked before SPE with the mean peak area of corresponding concentration samples spiked after SPE. The matrix effect of SF were determined by the method of Matuszewski,[20] comparing the mean peak area of the three different concentration samples (0.5, 200 and 2000 ng mL^{-1}) against the corresponding extracted samples through HPLC analysis.

2.5.4 Stability study. The stability of SF in plasma was evaluated by analysing the three different concentration samples (0.5, 200 and 2000 ng mL^{-1}) from long-term (frozen at the -80 ºC), short-term (at room temperature 24 h) storage and freeze-thaw cycles.

3. Results and discussion

3.1 Optimization of SPE and LC parameters

In online SPE process, one of the most important parameters is the selection of SPE cartridge. The goals of SPE are the separation of analyte from biological matrix and removal of interfering substances. Three cartridges were evaluated for their efficiency in online SPE procedure: Oasis®HLB cartridge (2.1*20mm, 5μm), CAPCELL MF Ph-1 cartridge (4.0*10mm, 5 μm) and HyperSep Hypercarb cartridge (2.1*20mm, 30μm).

To compare the performance of different SPE cartridges, 10 μL standard sample (2000 ng mL^{-1}) was loaded on a cartridge directly linked to flow divider valve and rinsed with pure water at 1 mL min^{-1} flow rate. As the result shown in Fig. 3, Oasis and Hypercarb exhibited satisfactory retentive efficiency compared to CAPCELL MF Ph-1.

After the endogenous matrix was clean up, SF was eluted from SPE and analysed via an analytical column with acetonitrile-water (40/60). According to Fig. 4, Hypercarb gave a tailing shape peak of SF, while HLB gave a relative better peak. Eventually, HLB cartridge was selected for its trapping efficiency and elution ability for further research.

Different ratio of water-acetonitrile solution was used to optimize the elution efficiency of endogenous matrix with 1 mL min^{-1} flow rate. The monitored wavelength of diode array detection (DAD) was set at 210 nm, different proportions were evaluated as listed in Fig. 5. The loading time was refined to 2 min, within which endogenous matrix was eluted at the extreme using acetonitrile-5 mM ammonium acetate buffer (pH 3.5) (2/98, v/v). To ensure SF sufficiently eluted from SPE cartridge and entered into the analysis column, the transfer time was optimized to 1 min and the transfer mobile phase was 5 mM ammonium acetate buffer (pH 3.5)-acetonitrile (60/40, v/v).

3.2. HRMS parameters optimization

Q-Exactive was operated in t-MS^2, t-SIM and F-SIM positive modes. In t-MS^2 mode, Q-Exactive carried out target MS/MS fragmentation scan using HCD. In t-SIM and F-SIM mode, Q-Exactive performed full MS and target MS scan without HCD fragmentation, respectively. The data of the three modes was collected simultaneously when sample was analysed.

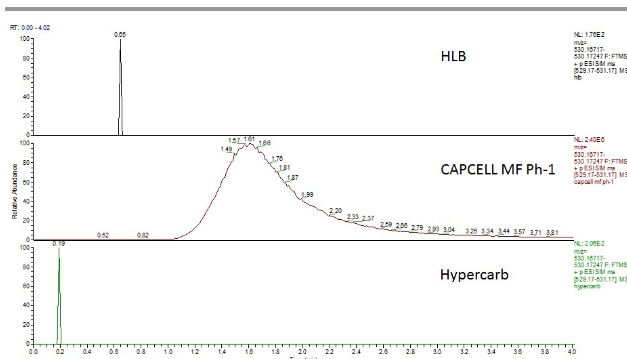

Fig.3. The retention effect of the different SPE cartridges

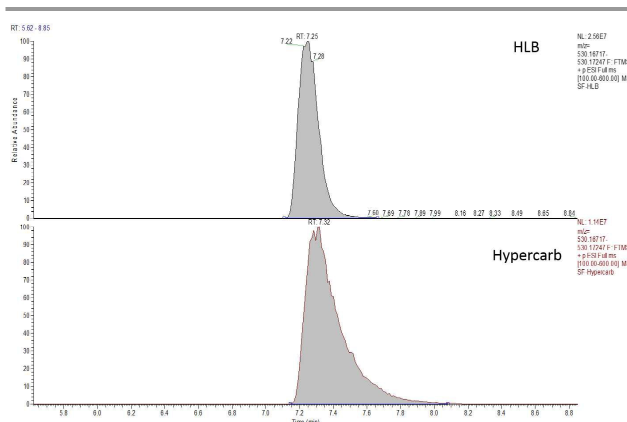

Fig. 4. The eluting effect of SF in HLB and Hypercarb cartridge

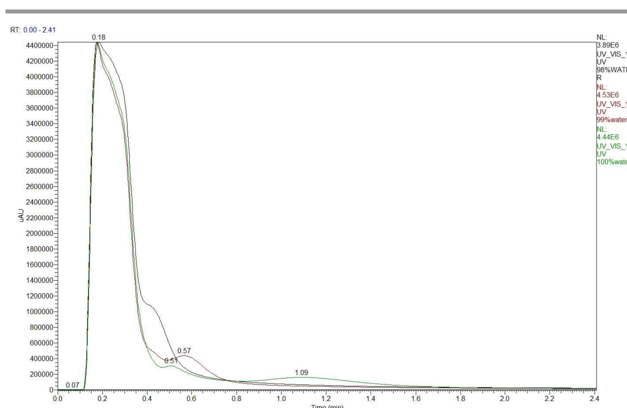

Fig. 5. The eluting effect of endogenous matrix at different conditions**.** Black line: 98% water, red line: 99% water, green line: 100% water

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	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	NCE
1	530.16982				Positive			16
2	237.10224				Positive			50
3								

Fig. 6. The parameters of the t-MS^2 in inclusion list

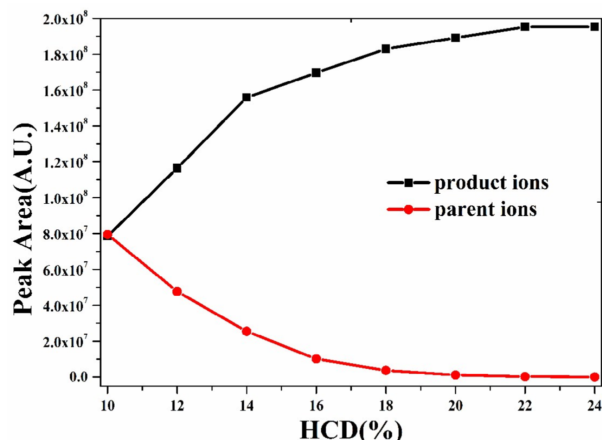

Fig. 7. SF fragmentation with increasing HCD percentage

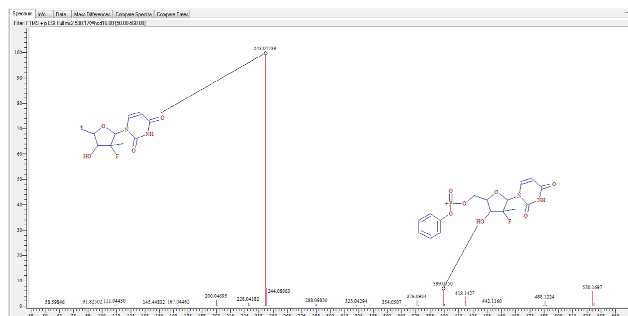

Fig. 8. The retrieval result of MS^2 of SF by Mass Frontier

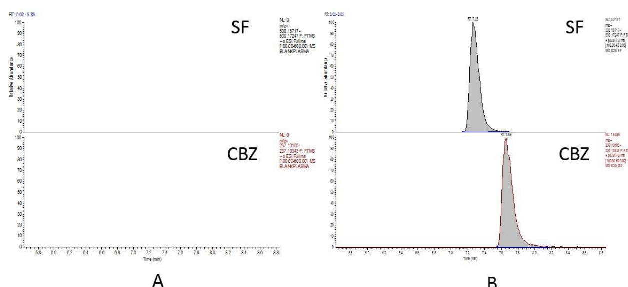

Fig. 9. The chromatograms of blank plasma (A), spiked plasma with SF and CBZ (B)

A satisfactory number of scan points per chromatographic peak was important for quantification and it was depended on the parameter of automatic gain control (AGC) target (the number of ions to fill C-Trap), maximum injection time (IT) (the time of ions to reach C-Trap) and RP. In t-MS^2 mode, the AGC target was set to 2×10^5, with a IT of 100 ms. In t-SIM mode, AGC target was set to 5×10^4, with a IT of 200 ms. In F-SIM mode, AGC target was set to 3×10^6, with a IT of 200 ms. The analyzer Orbitrap has RP of 17,500, 35,000, 70,000, 140,000 FWHM. The selection of RP is crucial for the overall performance of the identification and quantification. In t-MS^2 mode, accurate mass of parent ions were selected by quadrupole before entering the HCD cell (Fig. 6), a 17,500 FWHM was sufficient to avoid false positive and provide an acceptable selectivity. Meanwhile, it also contributed to gain more scan points. In both t-SIM and F-SIM mode, the mass resolution of 70,000 and 140,000 was sufficient for the required mass accuracy with MTW of 5ppm. The resolving power is reversely correlated to the scanning speed that has significant impact on the number of scan points. Compared with 140,000, the selection of a 70,000 FWHM would has positive impact on the sensitivity by increasing the scanning speed.[34] Therefore, 70,000 FWHM was selected for quantification study in both t-SIM and F-SIM mode.

Another factor that influenced the sensitivity of t-MS^2 mode is the normalized collision energy (NCE) value of the HCD cell. The area of parent ions was decreased while the area of product ions was increased by increasing the NCE (Fig. 7). The optimal NCE for SF was 16%. Under this condition, both the parent ions and product ions could coexist, meanwhile the response of product ions was relative high. The structures of main product ions (red lines) were determined and recognized by Mass Frontier 7.0 (Fig. 8).

3.3. Method validation

Under the optimum conditions for online SPE-LC-HRMS, the proposed quantitative performances of three modes were evaluated regarding selectivity, linearity, accuracy, precision, extraction recovery and matrix effect.

3.3.1 Selectivity. The chromatograms of blank plasma, spiked plasma with SF and CBZ were shown in Fig. 9. Little interference from endogenous materials was found at the retention time of the analyte indicating that the online-SPE procedure was selective.

3.3.2 Linearity and LLOQ. The LLOQ was determined by the criteria that the analyte response is at least 10 times of baseline noise ($S/N \geqslant 10$), and defined with acceptable accuracy (within ±20%) and precision (within ±20%). In the concentrations of 0.5-2000 ng mL^{-1}, the linear relationship between the chromatographic peak area ratio and the different concentration of SF was investigated by Xcalibur 3.0. A concentration of 2000 ng mL^{-1} should be able to satisfy the bioanalysis demand of C_{max} (1823.7±639.1 ng mL^{-1}) in human plasma.[32] At the concentration of 0.5 ng mL^{-1}, the signal noise ratio (S/N) of three mode were shown in Fig. 10. The t-MS^2 mode (17) and t-SIM mode (21) were higher than F-SIM (Not able to calculate). At the concentration of 0.5 ng mL^{-1}, the result indicated the noise was so serious indeed that S/N couldn't be recognized by Xcalibur. Due to the selection of ions

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by quadrupole, t-MS^2 mode and t-SIM mode improved the sensitivity and reduced the quantification limit. In order to meet the requirement of bioanalysis, the linear range of SF was eventually achieved between 2 ng mL^{-1} to 2000 ng mL^{-1} when F-SIM mode was used for quantification. As a result, F-SIM was only used for quantification performance comparison purpose and not used for method development. In the following experiment, only the quantification ability at 200 ng mL^{-1} (MQC) and 2000 ng mL^{-1} (HQC) for F-SIM mode were compared with the other two modes. Data of linear regression equation of the analytes was listed in Table 3. Good linearity in all three quantification methods ($R^2 > 0.99$).

3.3.3 Accuracy and precision. The accuracy of SF was expressed as the ratio between nominal concentration and the mean value of the detected concentration. The precision was calculated by RSD%. The intraday and interday accuracy and precision of QC samples were shown in Table 4.

In t-MS^2 mode, intraday precision was calculated for 3 concentration levels (n = 5) and ranged from 97.37% to 107.00% while the RSD% of QC samples was in the range of 1.38% to 6.93%. Interday precision calculated for 3 levels (n = 5) ranged from 97.40% to 105.92% while the RSD% of QC samples was in the range of 3.92% to 7.20%.

In t-SIM mode, intraday precision was calculated for 3 concentration levels (n = 5) and ranged from 99.33% to 104.72% while the RSD% of QC samples was in the range of 1.20% to 4.26%. Interday precision calculated for 3 concentrations levels (n = 5) ranged from 98.27% to 105.92% while the RSD% of QC samples was in the range of 4.01% to 8.07%.

In F-SIM mode, intraday precision calculated for MQC and HQC levels (n = 5) ranged from 96.81% to 109.54% while the RSD% was in the range of 1.24% to 3.1%. Interday precision calculated for MQC and HQC levels (n = 5) ranged from 93.29% to 106.76% while the RSD% of QC samples was in the range of 3.32% to 5.42%.

Overall, both t-MS^2 and t-SIM mode showed similar performance in terms of accuracy and precision at all three concentrations, while F-SIM showed comparable accuracy and precision at both MQC and HQC. Both t-MS^2 and t-SIM mode were all within the defined acceptance criteria of U. S. Food and Drug Administration (FDA).[35]

3.3.4 Extraction recovery and matrix effect. As shown in Table 5, the results of analyte extracted using online SPE gave high extraction recoveries ranging from 98.86% to 105.56%, from 97.42% to 107.20% and from 92.06% to 99.12%, for t-MS^2, t-SIM and, F-SIM mode, respectively. Matrix effect is signal suppression or enhancement effect that caused by interference and is considered tolerable if the value is in the range of 80–120%.[20] The methods gave acceptable value of the matrix effect ranging from 96.5% to 98.82%, from 97.48% to 101.10% and 94.82% to 101.89%, for t-MS^2, t-SIM and F-SIM mode, respectively. The results were within acceptable criteria and assay of the analytes in human plasma.[35]

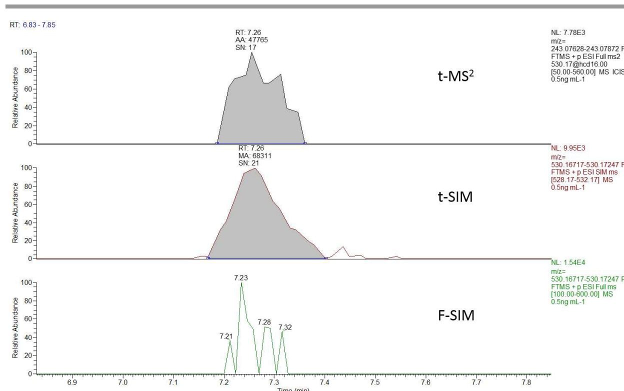

Fig. 10. The S/N of three modes at the concentration of 0.5 ng mL^{-1}

Table 3. Data of linear regression equation of SF

Mode	Linear range(ng mL^{-1})	LLOQ (ng mL^{-1})	Slope	Intercept	R^2
t-MS^2	0.5-2000	0.5	0.418886	-0.0227852	0.9988
t-SIM	0.5-2000	0.5	0.0067732	0.05638435	0.9971
F-SIM	2-2000	2	0.00742484	-0.00246331	0.9966

Table 4. Intraday and interday precision and accuracy

Mode	Nominal concentration (ng m L^{-1})	Intraday				Interday			
		N	Mean concentration found (ng mL^{-1})	Accuracy (%)	RSD (%)	N	Mean concentration found (ng mL^{-1})	Accuracy (%)	RSD (%)
t-MS^2	0.5	5	0.535	107.00	6.93	15	0.530	105.92	7.20
	200	5	194.7368	97.37	1.38	15	194.7964	97.40	5.24
	2000	5	1965.951	98.30	4.72	15	2065.84	103.29	3.92
t-SIM	0.5	5	0.524	104.72	4.26	15	0.526	105.12	8.07
	200	5	198.68	99.33	1.20	15	196.75	98.27	4.01
	2000	5	2225.92	111.20	1.20	15	2118.58	105.92	4.47
F-SIM	200	5	193.62	96.81	3.1	15	186.58	93.29	5.42
	200	5	2190.9	109.54	1.24	15	2135.29	106.76	3.32

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Table 5. Matrix effect and extraction recovery

Mode	Nominal concentration (ng mL^{-1})	Extraction recovery (%)		Matrix effect (%)	
		Mean	RSD (%)	Mean	RSD (%)
t-MS^2	0.5	103.02	3.49	96.5	4.34
	200	98.86	3.90	98.18	2.97
	2000	105.56	2.42	98.82	2.81
t-SIM	0.5	107.20	3.21	97.48	7.69
	200	103.61	3.49	99.78	1.24
	2000	97.42	1.85	101.10	3.67
F-SIM	200	99.12	2.23	101.89	2.64
	2000	92.06	2.26	94.82	1.72

Table 6. Stability study for SF under different conditions

Mode	Stability	Storage conditions	Level	Mean comparison samples (ng mL^{-1})	Mean stability samples (ng mL^{-1})	Accuracy (%)	RSD (%)
t-MS^2	Short-term	Room temperature for 6h	LQC	0.535	0.505	100.92	5.53
			MQC	194.74	208.85	104.43	4.08
			HQC	2025.95	2052.54	102.63	4.22
	Freeze-thaw	At -80 ºC for 3 cycles	LQC	0.518	0.478	95.57	5.15
			MQC	200.22	203.66	101.83	3.16
			HQC	2163.77	2007.37	100.37	3.79
	Long-term	At -80 ºC for 30 days	LQC	0.536	0.520	104.08	3.17
			MQC	185.67	187.36	93.68	3.00
			HQC	2067.79	1925.48	96.27	4.77
t-SIM	Short-term	Room temperature for 6 h	LQC	0.524	0.552	110.37	2.16
			MQC	198.68	205.92	102.95	1.93
			HQC	2225.92	2211.06	110.55	2.83
	Freeze-thaw	At -80 ºC for 3 cycles	MQC	0.515	0.477	95.47	3.45
			MQC	204.24	202.21	101.11	1.27
			HQC	2027.66	2112.31	105.62	2.03
	Long-term	At -80 ºC for 30 days	LQC	0.529	0.537	107.32	4.07
			MQC	187.35	203.72	101.86	2.73
			HQC	2002.13	1916.44	95.82	1.72
F-SIM	Short-term	Room temperature for 6 h	MQC	193.62	195.50	97.75	1.63
			HQC	2190.91	2178.60	108.93	1.63
	Freeze-thaw	At -80 ºC for 3 cycles	MQC	184.40	187.93	93.97	1.78
			HQC	2152.96	2157.92	107.90	2.82
	Long-term	At -80 ºC for 30 days	MQC	191.72	197.52	98.76	3.32
			HQC	2062.00	1972.66	98.63	2.15

3.3.5 Stability study. SF in human plasma was stable at room temperature for 6 h and at -80 ºC for 30 days. Freeze-thaw stability study at -80 ºC for 3 cycles was carried out. Data of the stability experiments are listed in Table 6. The result showed that the analyte was stable under the above conditions.

According to the guideline of US FDA summarized in the Table 7,[35] the bioanalytical method of Sofosbuvir is valid.

3.4 Analysis of Online SPE-LC-HRMS

An online SPE-LC coupled with Q-Exactive has been established for bioanalysis of SF in human plasma. The integration of SPE with the bioanalysis could fully automate the process of sample pretreatment and matrix removal. The benefit of online-SPE is not only the time saving and easiness of application but also the overall quality of the bioanalysis such as accuracy, precision, recovery and matrix effect. Q-Exactive is a hybrid bench-top instrument that combine quadrupole with Orbitrap technology. The high resolving power of accurate mass provides high security of identification compared to

Table 7. The guideline of US FDA in Bioanalytical Method Validation

	LLOQ	Other concentrations
Accuracy	80-120%	85-115%
Precision	±20%	±15%
Sensitivity	S/N>5	-
Calibration Curve	At least six non-zero samples	
Selectivity	No interference in blank samples	
Matrix effect	85-115%	
Recovery	Consistent, precise and reproducible	
Reproducibility	Intraday and interday	
Stability	±15% (room temperature, long-term and freeze-thaw, etc)	

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conventional mass spectrometer. Three modes of quantification (t-MS^2, t-SIM and F-SIM) have been used for bioanalysis of SF in human plasma. Generally, all three methods gave a better LLOQ than published LC-MS/MS method[31] (0.5 ng mL^{-1}, 0.5 ng mL^{-1}, 2 ng mL^{-1} vs. 10 ng mL^{-1}). Among three quantification modes, t-MS^2 mode and t-SIM mode improved the sensitivity and reduced the quantification limit through the selection of ions by quadrupole. Indeed, t-MS^2 and t-SIM modes are especially suitable for known compounds. F-SIM mode has the ability to obtain wealth of ions information about compound residues or metabolites in a single analysis which could be particularly useful in the case of bioanalysis with limited information. The quantification performance of online-SPE-LC-Q-Exactive could well meet the requirement of bioanalysis of SF in human plasma.

4. Conclusion

In this work, HRMS was explored as an identification and quantification approach for bioanalysis of drugs in human plasma. Online-SPE-LC was coupled with Q-Exactive for bioanalysis of SF in order to achieve automation of sample pretreatment and high efficiency of identification and quantification. Quantification performance of t-MS^2, t-SIM and F-SIM mode has been studied. Bioanalysis methods of SF in human plasma that meet the defined acceptance criteria have been achieved with both t-MS^2 and t-SIM quantification in a linear range of 0.5-2000 ng mL^{-1}. It suggests that HRMS could provide an alternative to MS/MS for bioanalysis in identification and quantification of drugs in biological matrix. Compared with the published method, our online-SPE-LC-HRMS is featured as easy to use (no manual sample pretreatment process) and high sensitivity (LLOQ 0.5 ng mL^{-1}). The established online SPE-LC-HRMS could provide a sensitive, accurate and reliable method for the bioanalysis of SF in human plasma.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 program, Grant Nos. 2013CB911104, 2013CB911100), the National Natural Science Foundation of China (Grant No. 21572116), the Fundamental Research Funds for the Central Universities (Grant No. 20141081129) and the Tianjin Science and Technology Program (Grant Nos. 13JCYBJC24300, 13JCQNJC13100).

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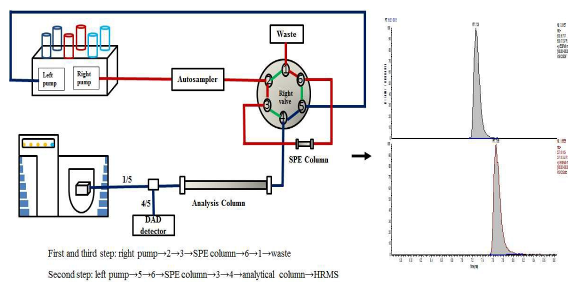
Text:

A quantitative strategy of sofosbuvir in human plasma was developed with online-SPE-LC-HRMS using t-MS^2, t-SIM and F-SIM mode.

Color graphic:



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