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A UHPLC-MS/MS method for the quantification of direct antiviral agents simeprevir, daclatasvir, ledipasvir, sofosbuvir/GS-331007, dasabuvir, ombitasvir and paritaprevir, together with ritonavir, in human plasma

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(Article begins on next page)

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3 **and paritaprevir, together with ritonavir, in human plasma.**

4
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20

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24

25

26 **Abstract**

27 To date, the new standard for treatment of chronic hepatitis C is based on the administration of
28 novel direct acting antivirals. Among these, sofosbuvir, simeprevir, daclatasvir, ledipasvir,
29 dasabuvir, ombitasvir and paritaprevir already entered the clinical use. Anyway, since few
30 pharmacokinetic studies have been conducted on these drugs in a “real life” context poor
31 knowledge is available about their optimal therapeutic range. Without this background, therapeutic
32 drug monitoring is not applicable for treatment optimization. Up to now, a few methods are
33 reported to quantify these drugs in human plasma, and none of them in a simultaneous way. The
34 aim of this work was to develop and validate a simple, fast and cheap, but still reliable UHPLC-
35 MS/MS method for the quantification of these drugs, feasible for a clinical routine use.

36 Solid phase extraction was performed using HLB C18 96-well plates. Chromatographic separation
37 was performed on a BEH C18 1.7 μm , 2.1 mm \times 50 mm column, settled at 50°C, with a gradient run
38 of two mobile phases: ammonium acetate 5mM (pH 9,5) and acetonitrile, with a flow rate of 0.4
39 mL/min for 5 minutes. Tandem-mass detection was carried out in positive electrospray ionization
40 mode.

41 Both inter and intraday imprecision and inaccuracy were below 15%, as required by FDA
42 guidelines, while both recoveries and matrix effects resulted within the acceptance criteria. The
43 method was tested on 80 patients samples with good performance.

44 Being robust, simple and fast and requiring a low plasma volume, this method resulted eligible for a
45 clinical routine use.

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1. INTRODUCTION

To date, HCV infection affects more than 130 million people worldwide, being an important cause of liver cirrhosis, hepatocellular carcinoma and liver transplantation [1].

HCV is a single strand RNA virus, belonging to the Flaviviridae family with a genome coding for 10 proteins, among which protease NS3-4A and polymerases NS5A/NS5B have been identified as druggable targets.

Up to 2011, the standard for treatment of HCV infection consisted in the combination of ribavirin (RBV) and pegylated-interferon α (Peg-IFN α). More recently, the better knowledge of viral life cycle and of its enzymes lead to the development of new direct acting antivirals (DAAs) [2].

The first generation of protease inhibitors, telaprevir (TVR) and boceprevir (BOC), has been approved in 2011 for use in combination with Peg-IFN α /RBV and, despite the enhanced response rate, these agents caused important side effects: anemia, neutropenia, and fatigue for BOC and anemia, skin rash, and anorectal symptoms for TVR [2, 3].

The goal of the research of new therapies is to develop drugs with pangenotypic activity, high genetic barrier and fewer side effects for patients with HCV.

These drugs include Nucleoside Inhibitors (NIs) and Non-Nucleoside Inhibitors (NNIs) of viral polymerase NS5A/5B and Protease Inhibitors (PIs) [4].

Among NIs, sofosbuvir (SOF) is currently the most used, because of its high pangenotypic effectiveness, alone or in combination with Peg-IFN α /RBV or with PIs (such as simeprevir, SMV) or NNIs (as ledipasvir, LDV, or daclatasvir, DAC), with or without RBV. An issue emerging in the evaluation of SOF kinetics is its fast metabolism to its main plasma metabolite (>90%), GS-331007, which can be considered a good marker for SOF plasma exposure [5].

Other than SOF-based regimens, therapeutic alternatives include the co-administration of SMV or DAC with Peg-IFN α /RBV and, more recently, the single tablet formulation of ritonavir-boosted ombitasvir and paritaprevir (OMV and PAR, a NNI and a PI, respectively), with or without dasabuvir (DBV, a NI) and/or RBV [4]. However, poor knowledge about these drugs

76 pharmacokinetics in plasma and, going further, about the possible correlation between plasma
77 concentrations and therapeutic response and/or toxicity is currently available.

78 Analyses on previous anti-HCV drugs, as RBV, BOC and TVR, already revealed in the past years
79 the relationship between plasma concentration of these drugs and some of adverse effects, such as
80 anemia, or therapeutic failure [6, 7].

81 Indeed, the therapeutic drug monitoring (TDM) of anti-HCV drugs plasma concentration could
82 represent a useful tool for the clinicians to evaluate drug efficacy and to prevent adverse events, in
83 order to optimize the therapy. Treatment optimization through TDM is already reported to improve
84 the quality of life and the efficacy of the therapy itself, but also it could lead to a cost saving,
85 reducing side effects and consequent clinical cost for patient's care, in many different contexts .

86 At the moment only few methods have been developed for the quantification of some of the new
87 DAAs, and not altogether [8-12]. For these reasons, a robust quantification method for all the
88 currently used drugs is currently needed. Therefore, the aim of this work was to develop and
89 validate a new high-throughput UHPLC-MS/MS method for the simultaneous quantification in
90 human plasma of SOF/GS-331007, SMV, DAC, LDV, OMV, PAR and DBV, together with RTV,
91 eligible for a wide routine use following FDA guidelines [13].

92

93 2. EXPERIMENTAL

94

95 2.1 Chemicals

96 DAC and [¹³C₂, ²H₆]-DAC (d-DAC), SMV, SOF and its metabolite GS-331007, DBV, OMV and
97 PAR were all purchased from Alsachim (Illkirch Graffenstaden, France); LDV was purchased from
98 Selleckchem (Munich, Germany). Acetonitrile (ACN) HPLC grade and Methanol HPLC grade
99 were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-
100 DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). 6,7-Dimethyl-2,3-di(2-
101 pyridyl) quinoxaline (QX), RTV and formic acid were obtained from Sigma-Aldrich (Milan, Italy).
102 Blank plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria
103 Hospital (Turin, Italy). Ammonium acetate and DMSO were purchased from Sigma Aldrich.

104

105 2.2 Chromatographic conditions

106 Chromatographic analysis was performed on a Shimadzu Nexera X2 ® LC system coupled with a
107 LC-8050 ® tandem mass spectrometer (Shimadzu, Kyoto, Japan). Chromatographic separation was
108 performed on an Acquity UPLC BEH C18 column, (2.1 mm×50 mm, 1.7 µm; Waters, Milan, Italy)
109 maintained at 50°C through the column oven.

110 Compounds separation was obtained through a gradient (Table 1) of mobile phases A (Ammonium
111 acetate 5mM buffer, ph 9,5) and B (ACN) at flow rate of 0.4 mL/min and a time run of 5 minutes.
112 Auto-sampler was settled at 4°C and the injection volume was 0.3 µL, with a sampling rate of 1
113 µL/sec. Data processing and system control was managed through the LabSolution ® software
114 (Shimadzu, Kyoto, Japan) version 1.0.

115

116 2.3 Stock Solutions, Standards and Quality Controls

117 Stock solutions of DAC, SMV, LDV, SOF/GS-331007, DBV, OMV and PAR were prepared in
118 DMSO at a concentration of 1 mg/mL and stored at -80 °C. QX, RTV and d-DAC stock solutions

119 (1 mg/mL) were prepared in pure methanol and stored at 4°C until analysis. Internal standard
120 working solution (IS) was made with QX and d-DAC (both at [0.625 µg/mL]) in water:methanol
121 (50:50 v:v) at the time of the analysis.

122 The highest standard sample (STD 9) and the three quality controls, high (H), medium (M) and low
123 (L), were prepared by spiking blank plasma with stock solutions; Lower STDs were prepared by
124 serial 1:1 dilution from STD 9 to STD 1 with blank plasma, in order to obtain 9 different spiked
125 concentrations plus a blank sample (STD 0). STDs and QC were stored at -80°C.

126 Calibration ranges and QCs concentrations for all drugs are listed in Table 2.

127

128

129 **2.4 Sample extraction**

130 HLB C18 96 wells plates were chosen for the samples extraction. Each well (cartridge) was
131 activated with 1 mL of pure methanol and equilibrated with 1 mL of water, in a positive pressure-96
132 manifold ® (Waters, Milan, Italy).

133 Two hundred microliters of plasma were diluted 1:2 with H₂O 1% phosphoric acid, added with 40
134 µL of IS working solution and centrifuged at 21000 x g for 10 minutes: then, the supernatants were
135 loaded into the corresponding wells. After a washing step with 200 µL of pure water, the samples
136 were eluted in a 96 well 2 mL collection plate with 500 µL of methanol:ACN 90:10 (vol:vol): 0.5
137 µL of the resulting extracts have been injected in the chromatographic system.

138

139 **2.5 Mass conditions**

140 Tandem mass spectrometric detection was carried-out through electrospray ionization source set in
141 positive ionization mode (ESI+) for all the considered analytes.

142 Ionization conditions were optimized by directly injecting solutions containing each single drug,
143 prepared in a mixture of the two mobile phases (A and B) 50:50 (vol:vol), bypassing the column

144 (Fast Injection Analysis, FIA): the optimization process was automatically performed using the
145 “optimization for method” function of the chromatographic system.

146 The optimized instrument parameters were as follows: capillary voltage 4 kV, nebulizing gas flow 3
147 L/min, drying gas flow 10 L/min, heating gas flow 10 L/min, interface temperature 300 °C, heating
148 block temperature 400 °C, desolvation line temperature 250 °C.

149 The ion monitoring was performed by positive electrospray ionization (ESI+) in multiple reaction
150 monitoring (MRM) mode, with the mass transitions reported in table 3. Each drug was monitored at
151 two different transitions (except for simeprevir): the first was used to quantify (quantification trace)
152 and the second as confirmation (secondary ion trace, not reported).

153

154 **2.6 Real samples collection**

155 To obtain a confirmation of the applicability of this method to clinical routine this was tested by use
156 on 80 real plasma samples from 40 HCV+ patients (2 samples for each patient) in treatment with
157 the novel DAAs, all giving informed consent as requested by the local Ethical Committee
158 guidelines, in the context of the approved “Kineti-C” clinical study (protocol number 186/2014).

159 Blood samples from patients were collected in lithium heparin tube (7 mL) at the end of dosing
160 interval (Ctrough) and plasma was obtained after centrifugation at 1400 g for 10 min at +4°C
161 (Jouan Centrifuge, Model BR4i, Saint-Herblain, France). Plasma samples were immediately stored
162 at -80°C until analysis.

163

164 **2.7 Specificity and selectivity**

165 Interference from endogenous compounds was investigated by analysis of 6 different blank plasma
166 samples. A possible “interfering peak” has been considered as a peak which exhibited a retention
167 time within 0.1 minutes from the analytes ones.

168

169 **2.8 Accuracy, precision, calibration and limit of quantification**

170 Inter-day precision and accuracy were determined by assaying QC samples in double replicate in 6
171 different validation sessions. Intra-day precision was evaluated in 5 intra-day replicates for each QC
172 level. Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day
173 and intra-day precision were expressed as the relative standard deviation (RSD) at each QC
174 concentration. Calibration curves were obtained by processing chromatograms of STDs by peak
175 areas. “Quadratic through zero” regression models were used for all curves, in order to compensate
176 a slight saturation phenomenon. The fitting to the calibration model was evaluated up to ten times
177 concentration of STD 9 for each drug.

178 The limit of detection (LOD) in plasma was defined as the concentration that yields a signal-to-
179 noise ratio of 3:1. Percent deviation from the nominal concentration (measure of accuracy) and
180 RSD (measure of precision) of the concentration considered as the limit of quantification (LOQ)
181 had to be < 20%, and it was considered as the lowest calibration standard, as requested by FDA and
182 EMA guidelines.

183

184 **2.9 Recovery**

185 Recovery was evaluated in six different sessions by comparing the peak areas obtained from the
186 three QC samples with peaks corresponding to dry extracts of blank plasma, reconstituted with
187 spiked solutions at the same concentrations (post-extraction addition).

188

189 **2.10 Matrix Effect**

190 The “matrix effect” was investigated on six lots of blank plasma from individual donors, as
191 requested by guidelines. Peak areas from blank extracts spiked with all analytes at three QC
192 concentrations were compared with peak areas from direct injection of standard solutions (prepared
193 in methanol:ACN 90:10 v:v) spiked with the analytes at the same concentration, as described by

194 Taylor (post-extraction addition method) [14]. The “matrix effect” was calculated as percentage of
195 deviation in peak area between these two conditions at high, medium and low levels.

196

197 **2.11 Carry-over**

198 Carry-over was investigated by comparing peak areas obtained from three blank plasma samples
199 injected after a sample containing target analytes concentration 5-fold higher than STD9.

200 A signal lower than 20% of the lower limit of quantification (LLOQ) for each target drug and a
201 value lower than 5% for the IS were considered as an acceptable carry-over.

202

203 **2.12 Stability**

204 All DAA drugs were evaluated for long-term stability in plasma samples at -80°C for 3 months, at
205 high, medium and low QC levels.

206 “Bench-top” stability was evaluated after 24h on the bench at room temperature, by comparison
207 with the signal from samples stored at -80°C and immediately extracted.

208 Autosampler stability was evaluated on QCs extracts maintained in the autosampler at 10°C for
209 24h, by comparing their concentrations with fresh extracts.

210 “Freeze and thaw” stability has been evaluated after one to three freezing and thawing cycles, by
211 comparison with freshly prepared QCs.

212 Acceptable stabilities have been considered as percent differences in concentration lower than 5%.

213

214 **3. RESULTS**

215 **3.1 Calibration curve**

216 All calibration curves for all drug shown a mean regression coefficient (r^2) ranged from 0.997 to
217 0.999. A quadratic through zero regression was chosen for all drugs, due to a slight saturation
218 phenomenon at higher concentrations.

219

220 **3.2 Specificity and selectivity**

221 No interference with other potential concomitants drugs was found in the assay. The retention time
222 of DAAs, QX and d-DAC are represented in Figure 1. No endogenous interferences were observed
223 in six different plasma lots, taking in account the analytes retention time windows. Secondary ion
224 traces were used to confirm the nature of the peaks (except for simeprevir).

225

226 **3.3 Accuracy and precision**

227 All accuracy, inter-day and intra-day precision parameters fitted the limits requested by FDA
228 guidelines at each one of the QC levels. Data are summarized in table 2.

229

230 **3.4 Lower limit of quantification (LLOQ) and limit of detection (LOD)**

231 The evaluation of LOD was performed by diluting the lowest calibration point (STD1) several
232 times. The LLOQ corresponded to the STD 1, as shown shown in table 2. Overlaid chromatograms
233 of LLOQ and of a blank sample for each drug is showed in figure 2. There was not any interfering
234 peak at the retention times of the analytes of interest.

235

236 **3.5 Recovery**

237 Mean recovery (and RSD%) for each analyte are summarized in table 4. Recovery resulted
238 reproducible for all the considered analytes.

239

240 **3.6 Matrix effect**

241 Mean matrix effect data (and RSD%) for each drug are summarized in table 4. Matrix effect
242 resulted reproducible between different plasma lots.

243

244 **3.7 Carry-over**

245 DAAs peaks observed in blank samples, injected immediately after mixes at high concentrations of
246 analytes, were all lower than the corresponding LOD.

247 Likewise, mean carry-over of IS was lower than 1%. These data showed the absence of relevant
248 carry-over.

249

250 **3.8 Stability**

251 Considering “freeze and thawing”, bench-top, autosampler and -80°C storage stability testing, all
252 the analytes showed percent degradations lower than 5%, thus resulting stable in our working
253 conditions.

254

255 **3.9 “Real” samples testing**

256 Among the 40 patients enrolled, 16 were treated with SOF+SMV, 10 with SOF+DAC, 4 with
257 SOF+LDV and 10 with DBV+OMV+PAR and RTV. 29 out of 30 patients treated with showed
258 undetectable concentrations of SOF, while GS-331007 was quantifiable in all samples, with a
259 median concentration of 319.0 ng/mL (interquartile range, IQR 193.2 – 494.0 ng/mL). All samples
260 from patients treated with SMV, DAC or LDV had quantifiable drugs concentrations, with median
261 levels of 776.5 ng/mL (IQR 284.7 – 2390.2 ng/mL), 198.0 ng/mL (IQR 83.5 – 414.5 ng/mL) and
262 135.0 ng/mL (99.7 – 385.5 ng/mL), respectively. Likewise, all samples from patients treated with
263 DBV+OMV+PAR and RTV had quantifiable concentrations, with median levels of 223.5 ng/mL
264 (IQR 120.6 – 357.0 ng/mL) for DBV, 28.0 ng/mL (IQR 14.0 – 58.2 ng/mL) for OMV, 31.2 ng/mL
265 (IQR 5 – 198 ng/mL) for PAR and 33.1 ng/mL (14.2 – 74.5 ng/mL) for RTV.

267 **4. DISCUSSION and CONCLUSION**

268 In this work the validation of a UHPLC-MS/MS method for the simultaneous quantification of the
269 main DAAs already in use for treatment of HCV infection is described. The use of the HLB 96
270 wells plates allowed a fast and clean solid phase extraction in a high-throughput, but still highly
271 sensitive and specific manner.

272 In literature we can find a few methods developed for the quantification of DAAs: one method by
273 Rezk *et al.* [11], allows the quantification of SOF and its metabolite GS-331007 in human plasma,
274 starting from 500 μ l of human plasma and operating a cumbersome liquid-liquid extraction. The
275 calibration range that Rezk *et al.* chose for the GS-331007 probably cannot cover the expected
276 concentration range in all types of patients and, anyway, it is not adequate to make AUC analyses;
277 moreover, this method started from a higher volume of sample and the extraction process could be
278 too long if the number of sample is high, as in a clinical routine context.

279 A second method, developed by Jiang *et al.* [8], included the quantification of DAC, asunaprevir
280 and beclabuvir in human plasma, using a liquid-liquid extraction followed by an evaporation step:
281 anyway, this method did not include SOF, the currently most used DAA.

282 Other available methods were developed for the quantification of some of the other DAAs in
283 different combinations, but they were validated for use on rat plasma [9, 10, 12].

284 During the method development, we tried different columns and chromatographic conditions: we
285 compared an 2.1 x 150 mm HSS T3 1.8 μ m column with acidic mobile phase to a 2.1 x 150 mm
286 BEH C18 1.7 μ m column, with basic mobile phase. We chose the second condition due to a better
287 peak shape and higher sensitivity, especially for GS-331007 and SMV. Also protein precipitation
288 was tried instead of SPE, but it did not work well for all the compounds (high matrix effect was
289 observed, data not shown). Several SPE-plates were tested with different sorbents and finally we
290 chose the HLB plate, which showed the highest (and most stable) recovery for all the compounds.

291 Although in clinical practice the trough concentration is the most used in antiviral therapy (and our
292 method has been tested on Ctough, accordingly), the choice of calibration ranges was based on the
293 reported AUCs in clinical trials, with the aim of covering all the expected concentrations in patients.
294 Considering method performance on “real” samples from patients, it was capable of successfully
295 quantifying each drug in all sample, except for SOF concentrations, which resulted almost always
296 undetectable. However, this behavior was already known in literature, as SOF is very rapidly
297 metabolized in its active triphosphate intracellular metabolite and then converted in GS-331007
298 [15]: this compound was already described as a good marker for SOF exposure and showed plasma
299 concentrations above our LLOQ in all the tested samples.

300 The choice of the ISs was based on chemical, analytical and economical criteria: QX was chosen
301 because of its relatively high LogP, its well known stability, its high recovery in our conditions and
302 its low cost, while d-DAC was used to normalize the signal for DAC, which exhibited a different
303 recovery and matrix effect respect to the other drugs and QX.

304 Concluding, the high accuracy and precision of the assay, taken together with the good and stable
305 recovery and the contained matrix effect, made this method eligible for use in clinical studies for
306 the determination of optimal therapeutic ranges for each DAA (still unknown) and, then, for a
307 future routine TDM of these drugs, becoming a useful tool for treatment management.

308

309

310

311 **Bibliography**

312

- 313 [1] WHO, Hepatitis C: Fact Sheet no. 164, 2014, <http://www.who.int/mediacentre/factsheets/fs164/en/>.
- 314 [2] A.A. Butt, F. Kanwal, Boceprevir and telaprevir in the management of hepatitis C virus-infected patients,
315 Clin Infect Dis, 54 (2012) 96-104.
- 316 [3] I.M. Jacobson, J.G. McHutchison, G. Dusheiko, A.M. Di Bisceglie, K.R. Reddy, N.H. Bzowej, P. Marcellin,
317 A.J. Muir, P. Ferenci, R. Flisiak, J. George, M. Rizzetto, D. Shouval, R. Sola, R.A. Terg, E.M. Yoshida, N. Adda,
318 L. Bengtsson, A.J. Sankoh, T.L. Kieffer, S. George, R.S. Kauffman, S. Zeuzem, Telaprevir for previously
319 untreated chronic hepatitis C virus infection, N Engl J Med, 364 (2011) 2405-2416.
- 320 [4] EASL, EASL Recommendations on treatment of hepatitis C 2015., 2015,
321 <http://www.easl.eu/medias/cpg/HEPC-2015/Full-report.pdf>.
- 322 [5] B.J. Kirby, W.T. Symonds, B.P. Kearney, A.A. Mathias, Pharmacokinetic, Pharmacodynamic, and Drug-
323 Interaction Profile of the Hepatitis C Virus NS5B Polymerase Inhibitor Sofosbuvir, Clin Pharmacokinet, 54
324 (2015) 677-690.
- 325 [6] L. Boggione, A. De Nicolo, C.S. Cardellino, T. Ruggiero, V. Ghisetti, G. Cariti, G. Di Perri, A. D'Avolio,
326 Relationship between the early boceprevir-S isomer plasma concentrations and the onset of breakthrough
327 during HCV genotype 1 triple therapy, Clin Microbiol Infect, 21 (2015) 205 e201-203.
- 328 [7] A. De Nicolò, L. Boggione, A. Ciancio, J. Cusato, S. Strona, C.S. Cardellino, A.M. Abdi, G. Cariti, G. Troshina,
329 G.P. Caviglia, A. Smedile, M. Rizzetto, G. Di Perri, A. D'Avolio, Telaprevir-S isomer enhances ribavirin
330 exposure and the ribavirin-related haemolytic anaemia in a concentration-dependent manner, Antiviral
331 Res, 109 (2014) 7-14.
- 332 [8] H. Jiang, H. Kandoussi, J. Zeng, J. Wang, R. Demers, T. Eley, B. He, R. Burrell, J. Easter, P. Kadiyala, J.
333 Pursley, L. Cojocar, C. Baker, J. Ryan, A.F. Aubry, M.E. Arnold, Multiplexed LC-MS/MS method for the
334 simultaneous quantitation of three novel hepatitis C antivirals, daclatasvir, asunaprevir, and beclabuvir in
335 human plasma, J Pharm Biomed Anal, 107 (2015) 409-418.
- 336 [9] H. Kandoussi, H. Jiang, J. Zeng, N. Zheng, P. Kadiyala, T. Eley, B. He, T. Garimella, R. Demers, L. Cojocar,
337 A.F. Aubry, M.E. Arnold, A highly sensitive and selective LC-MS/MS method to quantify asunaprevir, an HCV
338 NS3 protease inhibitor, in human plasma in support of pharmacokinetic studies, J Pharm Biomed Anal, 119
339 (2015) 145-151.
- 340 [10] C. Pan, Y. Chen, W. Chen, G. Zhou, L. Jin, Y. Zheng, W. Lin, Z. Pan, Simultaneous determination of
341 ledipasvir, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS and its application to a
342 pharmacokinetic study, J Chromatogr B Analyt Technol Biomed Life Sci, 1008 (2015) 255-259.
- 343 [11] M.R. Rezk, E.B. Basalious, I.A. Karim, Development of a sensitive UPLC-ESI-MS/MS method for
344 quantification of sofosbuvir and its metabolite, GS-331007, in human plasma: Application to a
345 bioequivalence study, J Pharm Biomed Anal, 114 (2015) 97-104.
- 346 [12] X. Shi, D. Zhu, J. Lou, B. Zhu, A.R. Hu, D. Gan, Evaluation of a rapid method for the simultaneous
347 quantification of ribavirin, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS, J Chromatogr B
348 Analyt Technol Biomed Life Sci, 1002 (2015) 353-357.
- 349 [13] FDA, Guidance for Industry Bioanalytical Method Validation, (2013).
- 350 [14] P.J. Taylor, Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-
351 electrospray-tandem mass spectrometry, Clin Biochem, 38 (2005) 328-334.
- 352 [15] C.T. de Kanter, J.P. Drenth, J.E. Arends, H.W. Reesink, M. van der Valk, R.J. de Kneegt, D.M. Burger, Viral
353 hepatitis C therapy: pharmacokinetic and pharmacodynamic considerations, Clin Pharmacokinet, 53 (2014)
354 409-427.

355

356

357

358 **Figure Legends**

359

360 **Figure 1:** Overlaid MRM chromatograms for each analyte from the injection of STD 9.

361

362 **Figure 2:** Overlaid peaks from analysis of LLOQ and blank plasma extracts for each analyte.

Table 1: Chromatographic gradient of mobile phases A (Ammonium acetate 5mM pH 9.5) and B (acetonitrile).

Time (min)	Solvent B %	Time (min)
0.00	3	0.00
0.50	8	0.50
0.60	55	0.60
1.80	70	1.80
2.00	95	2.00
3.50	95	3.50
3.60	3	3.60
5.00	3	5.00

Table 2: Summary of calibration ranges, quality controls concentrations and validation parameters.

	ULOQ LLOQ ng/mL		LLOD ng/mL		QC H (n=6)		QC M (n=6)		QC L (n=6)					
	ng/mL	ng/mL	CONC. Accuracy (%)	Precision (%)	CONC. Accuracy (%)	Precision (%)	CONC. Accuracy (%)	Precision (%)	CONC. Accuracy (%)	Precision (%)				
			ng/mL	<i>intraday interday</i>	ng/mL	<i>intraday interday</i>	ng/mL	<i>intraday interday</i>	ng/mL	<i>intraday interday</i>				
DAC	3000	11,7	2000	1,3%	2,9%	4,2%	600	-0,9%	4,1%	5,8%	80	6,3%	3,8%	7,5%
SOF	3000	11,7	2000	1,3%	6,7%	9,9%	600	-0,9%	6,9%	14,2%	80	11,2%	6,3%	13,5%
SMV	8000	31,2	6000	-2,3%	8,3%	10,8%	1000	-4,8%	10,1%	14,4%	150	-9,0%	13,0%	13,8%
LDV	3000	11,7	2000	3,5%	5,4%	8,1%	600	4,9%	4,7%	14,1%	80	8,2%	10,4%	11,7%
OMV	1000	3,9	800	8,2%	12,7%	10,6%	20	2,3%	14,1%	13,8%	2	-4,8%	15,5%	15,8%
DBV	3000	11,7	2000	-2,3%	5,9%	7,3%	600	-5,6%	6,2%	11,8%	80	-6,9%	13,8%	15,0%
PAR	3000	11,7	2000	0,5%	10,6%	12,7%	700	-0,6%	9,4%	13,4%	90	7,8%	13,8%	13,6%
RTV	3000	11,7	2000	11,6%	4,5%	5,8%	500	1,9%	4,3%	9,7%	70	-3,3%	3,6%	8,6%
GS-331007	10000	39,1	6000	-6,6%	12,6%	14,4%	1000	-2,7%	10,0%	13,0%	150	2,2%	15,9%	13,5%

Table 3: Summary of the monitored quantification traces for the considered analytes

ANALYTE	Precursor (m/z)	Quantification trace (m/z)	CE
DAC	739.40	565.10	45
SOF	530.05	243.05	22
SOF-G	258,1	239,1	8
SMV	751.15	314.90	33
LDV	890.30	733.00	41
OMV	895.40	588,3	50
DBV	492.15	476.85	29
PAR	766.40	571.05	21
RTV	720.95	295.95	20
IS QX	312.60	246.90	35
IS d-DAC	747.40	569.00	45

Table 4: Mean recovery and matrix effect (and Relative Standard Deviations) for each drug.

DRUGS	Mean Recovery	Recovery RSD	Mean Matrix Effect	Matrix effect RSD
DAC	99%	13.3%	14%	2.5%
SOF	95%	10.2%	7%	1.3%
GS-331007	100%	5.6%	2%	0.9%
SMV	50%	13.2%	1%	2.9%
LDV	88%	4.7%	6%	3.5%
DBV	80%	4.3%	4%	5.8%
OMV	62%	7.5%	24%	6.2%
PAR	52%	13.2%	6%	3.5%
RTV	91%	3.1%	7%	1.8%
IS QX	86%	1.5%	8%	2.5%
IS d-DAC	99%	11.2%	14%	2.1%

Figure 1
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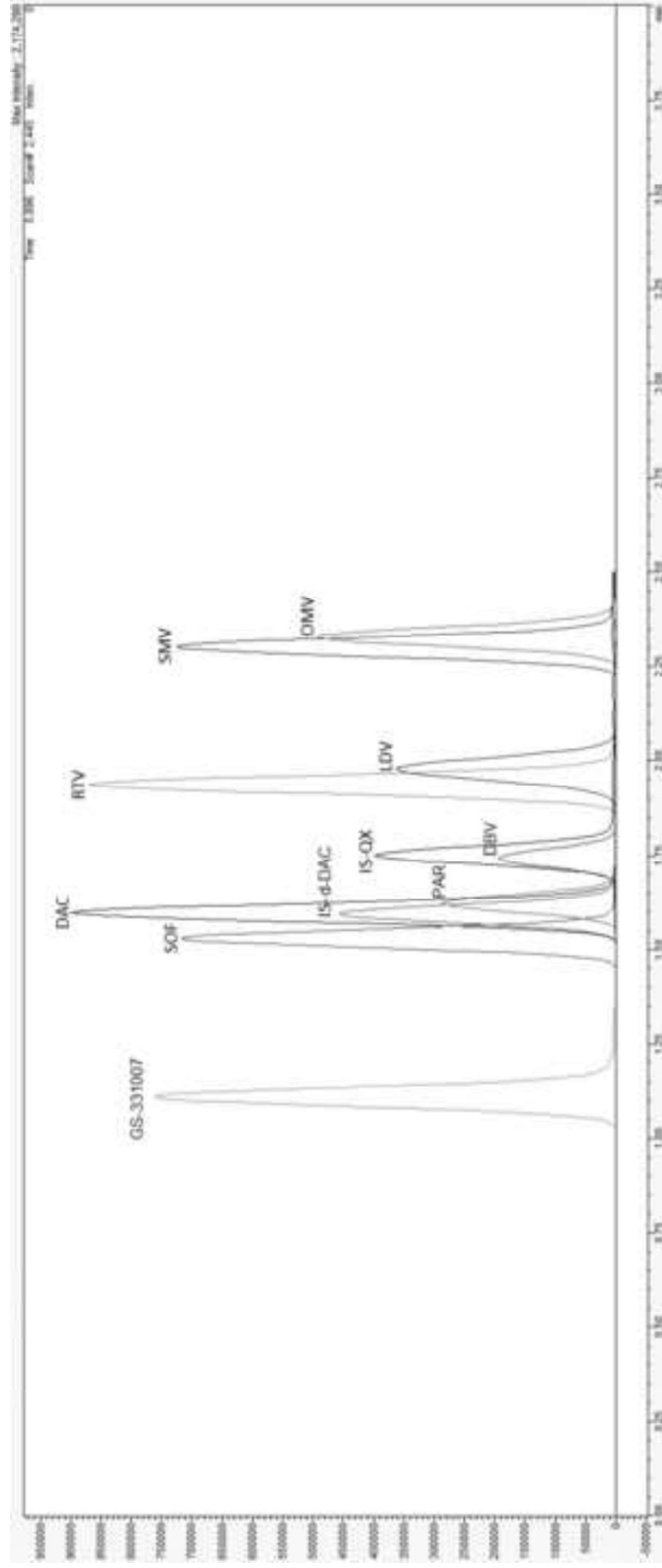


Figure 2
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