Blank plasma

LLOQ (0.05 μg/mL)
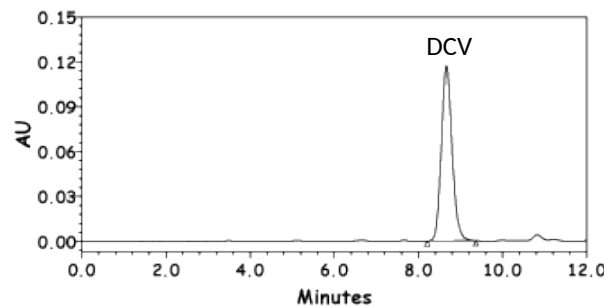
ULOQ (10.0 μg/mL)
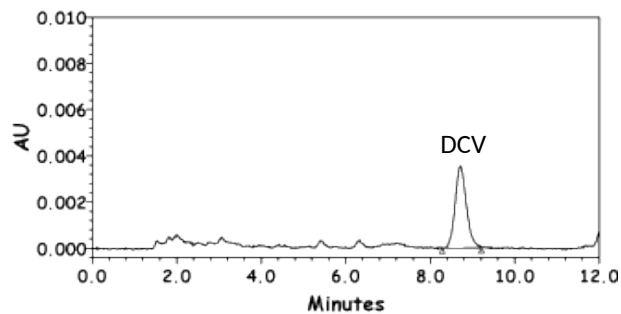
Patient plasma sample (0.35 μg/mL)

Development and validation of a simple and robust HPLC method with UV detection for quantification of the hepatitis C virus inhibitor daclatasvir in human plasma

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1   **Abstract**

2   Daclatasvir is an inhibitor of hepatitis C virus NS5A protein that is used for the therapy of chronic

3   hepatitis. So far, published methods for analysis of daclatasvir in plasma are exclusively based on

4   mass spectrometry, which is not always available in standard clinical laboratories. Thus, we

5   wished to develop and validate a simple, but still reliable and sensitive high-performance liquid

6   chromatography (HPLC) assay with UV detection for the quantification of daclatasvir, feasible for

7   a wide-spread clinical routine use. The method consisted of solid-phase extraction of daclatasvir

8   using Waters Oasis HLB 1cc cartridges, reversed-phase liquid chromatography with a Waters

9   XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column and a mobile phase of ammonium acetate

10 buffer (pH 5.0, 10 mM) and acetonitrile (56:44, v/v), and UV detection at 318 nm. This assay

11 proved to be sensitive (lower limit of quantification of 0.05 μg/mL), linear (correlation coefficients

12 ≥ 0.997), specific (no interference with various potentially co-administrated drugs), reproducible

13 (both intra-day and inter-day coefficients of variation ≤ 8.9%), and accurate (deviations ranged

14 from − 2.2 to 8.0% and from −6.5 to 9.2% for intra-day and inter-day assays, respectively). The

15 method was applied to therapeutic monitoring of patients undergoing daclatasvir therapy for

16 hepatitis C and showed to be reliable and robust. Thus, this method provides a simple, sensitive,

17 precise, and reproducible assay for dosing daclatasvir that can be readily adaptable to routine use

18 by clinical laboratories with standard equipment. In addition, the stability of daclatasvir in plasma

19 was evaluated under various conditions, including after the heating procedure required for

20 inactivation of infectious viruses and in different light exposure conditions. These studies

21 evidenced photo-instability of the compound under sunlight exposure over time. Thus, blood

22 sampling and the whole handling procedure have to be performed quickly and with minimal light

23 exposure.

24

25 *Keywords*: Daclatasvir; BMS-790052; HCV NS5A inhibitor; Solid-phase extraction; HPLC-UV;

26 Therapeutic drug monitoring.

1. Introduction

Hepatitis C virus (HCV) infection is the leading cause of progressive chronic liver disease, which can lead to the development of severe liver damages, including cirrhosis and hepatocellular carcinoma. About 130–150 million people worldwide are chronically infected with HCV, thus resulting in a major global health problem [1]. Until 2011, the treatment options available for HCV infection were restricted to the combination of pegylated alpha interferon (PEG-INF α) with ribavirin [2]. This therapy exhibited limited efficacy, especially in patients infected with genotype 1 and was associated with severe side effects [2]. The introduction of the first-generation directly acting antivirals (DAAs), telaprevir and boceprevir, in combination with PEG-INF α /RBV led to an improvement of the response rates in patients with chronic HCV genotype 1 infection. Despite the increased efficacy of these new drugs, the association with PEG-INF α and ribavirin severely limited the tolerability of this therapy and consequently their clinical utility. However, the recent approval of next-generation DAAs, including daclatasvir (DCV), has completely revolutionized the treatment of HCV infection by providing new highly effective IFN-free regimens for all HCV genotypes.

Daclatasvir, formerly termed BMS-790052, is an inhibitor of HCV nonstructural protein 5A (NS5A) replication complex [3,4]. DCV targets NS5A with great potency, and it is thought to act on HCV by interfering with the assembly of the replication complex, more specifically inhibiting conformational change of NS5A through impairing phosphatidylinositol-4-kinase III α activation [5]. Although quite potent, resistant variants have emerged during DCV monotherapy in patients with genotype 1a and 1b [6], thus it is administered in combination therapies with sofosbuvir with or without ribavirin.

The increasing number of therapeutic options already available and that will be available soon for HCV-infected patients, poses a significant challenge in the choice and the management of the HCV regimen treatment, especially in case of problematic patients or in comorbidities. In this regard, therapeutic drug monitoring (TDM) represents a very useful tool to evaluate drug efficacy

1   and to prevent adverse events, in order to optimize the therapy. Daclatasvir is a substrate of

2   cytochrome P450 (CYP) enzyme 3A and an inhibitor of P-glycoprotein (P-gp), organic anion

3   transporting polypeptide (OATP) 1B1/3, and breast cancer resistance protein (BCRP) [4]. Drug-

4   drug interactions with DCV have been observed with moderate and strong CYP 3A inducers and

5   strong inhibitors, which significantly affect DCV concentrations [7]. As an example, dose

6   modifications of DCV in patients treated with atazanavir or efavirenz are necessary [8]. Thus,

7   TDM data on DCV levels can guide the clinicians to adjust the drug dosage or switch the treatment

8   regimen, in order to guarantee the effectiveness of therapy.

9   To our knowledge, the methods published so far for DCV quantification in plasma samples

10 are only based on liquid chromatography coupled to mass spectrometry [e.g., 9-13]. However,

11 these methods could not be suitable for standard clinical laboratories that do not to dispose of such

12 an equipment. In the context of a large-scale pharmacokinetic feasibility analysis, alternative

13 analytical methods using UV detection, which employ standard equipment and are simpler,

14 cheaper, and easier to set up, are urgently needed.

15   Here, we report the development and validation of the first HPLC-UV method for the

16 measurement of DCV in human plasma. This method was validated according to the US Food and

17 Drug Administration (FDA) guidelines [14] and it was found to be sensitive, accurate, precise, and

18 reproducible to meet the requirements of routine TDM laboratories. Moreover, the stability of

19 DCV was assessed under different common conditions to which the drug can be subjected during

20 the sample handling and analysis in the TDM process. In particular, this work provides, for the

21 first time, evidences concerning the stability of DCV in plasma under the heating procedure which

22 is required to inactive the HCV and/or HIV particles and the photodegradation of DCV when the

23 samples were exposed at the sunlight.

24

25

26

2. Material and methods

2.1 Chemicals and reagents

DCV was purchased from Alsachim (Illkirch, France). Acetonitrile for HPLC (Gradient Grade, LiChrosolv) was from Merck (Darmstadt, Germany), methanol for chromatography was from Carlo Erba (Milan, Italy). All other chemicals were of analytical grade and purchased from J. T. Baker (Deventer, The Netherlands). Ultrapure water was produced by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Control human plasma (with K_3EDTA as anticoagulant) was obtained from Roche (Milan, Italy; human plasma included as a negative control in the TaqScreen West Nile Virus COBAS kit).

2.2 Equipment and chromatographic conditions

The chromatographic system consisted of an Alliance 2695 Separation Module equipped with an online degasser and an automatic injector thermostated at 4°C, and a 2996 Photodiode Array Detector coupled with the Empower data acquisition software (version 2.0) (Waters, Milford, MA, USA). Chromatographic separation was achieved on an XTerra RP18 (150 mm × 4.6 mm, particle size 3.5 μm; Waters) analytical reversed-phase column equipped with an XTerra RP18 (20 mm × 3.9 mm, particle size 5 μm; Waters) guard column, both operating at 40°C. The autosampler was maintained at 4°C. The mobile phase consisted of a mixture of acetonitrile-ammonium acetate buffer (pH 5.0, 10 mM) (44:56, v/v) and was filtered through a 0.22 μm nylon filter membrane (Millipore, Bedford, MA, USA) before use. After 10 min of isocratic condition, the mobile phase was ramped to 100% acetonitrile in 1 min and maintained for 5 min to wash out any remaining plasma components. The gradient composition was then shifted back to the starting condition in 1 min and maintained for 6 min to re-equilibrate the column. The flow rate was 1.0 mL/min, and the total assay run-time was 23 min. Eluents were detected at a wavelength of 318 nm. As an alternative, an entirely isocratic method could be applied as well. However, in this case a longer run-time (about 35 min) is necessary to remove all late-eluting plasma contaminants

1 from the column. An Extraction Manifold (Waters) liquid handling system was used to perform

2 the sample preparation. A DRI-BLOCK DB-3 evaporator (Techne, Stone, United Kingdom) was

3 used for drying the SPE eluates.

4

5 **2.3 Preparation of stock solutions, working solutions, calibration standards and quality control**

6 **(QC) samples**

7 Since possible degradation of DCV under high-intensity light conditions was previously

8 reported [15], all solutions and samples containing DCV were protected from daylight during

9 preparation, storage, and analysis. Stock solutions of DCV were prepared at 1 mg/mL in 100%

10 methanol. The stock solution was diluted further to obtain working solutions at the concentration

11 levels of 500, 250, 100, 50, 25, 10, 5 and 2.5 μg/mL in methanol. The stock and working solutions

12 were stored at –20°C in the dark. Plasma calibration standards at 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05

13 μg/mL were freshly prepared by 1:50 dilution of the respective working solution in control human

14 plasma and analyzed on the same day. Of note, the total added volume of organic solvent in all

15 samples corresponded to 2% of biological sample in accordance to the FDA guidelines [14]

16 recommending that non-biological matrix (i.e., organic solvent) should correspond to only ≤ 2% of

17 the volume of final biological samples. Quality control (QC) samples at the lower limit of

18 quantification (LLOQ = 0.05 μg/mL), low (0.2 μg/mL), medium (1 μg/mL), high (7.5 μg/mL), and

19 the upper limit of quantification (ULOQ = 10 μg/mL) concentration levels were prepared by

20 diluting the working solutions in plasma. The QC samples were prepared in batches at the same

21 occasion, stored at –20°C in the dark, and then thawed and thermized at 60°C for 60 min on the

22 day of analysis.

23

24 **2.4 Sample pretreatment and preparation**

25 Daclatasvir being sensitive to and degraded by sunlight (see Section 3.3.1), special care is

26 required for blood sampling and for the whole handling procedure. To this end, all manipulations

1 have to be conducted quickly and with minimal light exposure. Patients plasma samples were

2 obtained after centrifugation of blood samples (5 mL), collected in tubes with K_3EDTA as

3 anticoagulant and wrapped in aluminum foil to totally protect them from light, centrifuged at 3,000

4 rpm for 10 min at 4°C (Sigma Centrifuge, Model 2K15) and then stored at −80°C in the dark until

5 analysis. On the day of analysis, plasma samples were thawed and heated at 60°C for 60 min.

6 Sample clean-up was achieved by an SPE procedure as follows. Two hundred and fifty µL of

7 acetonitrile were added to 250 µL of heat-inactivated plasma samples, vortexed for ~ 1 min, and

8 then centrifuged at 13,000 rpm for 5 min at room temperature (RT) to allow the protein

9 precipitation. Supernatants were transferred in clean tubes and diluted with 500 µL of water.

10 Oasis HLB 1cc cartridges (Waters, Milford, MA, USA) were pre-conditioned with 1 mL of

11 methanol, equilibrated with 1 mL of water and then loaded with the sample mixtures.

12 Subsequently, the cartridges were washed with 1 mL of 60% methanol (v/v). The analytes were

13 successively eluted with 1 mL of methanol. The eluates were collected and evaporated to dryness

14 under a N_2 stream at 40°C (out of the light). The dried residues were reconstituted with 62.5 µL of

15 mobile phase and centrifuged at 13,000 rpm for 5 min at RT. The supernatants were kept at 4°C in

16 the autosampler and 20 µL of these were injected onto the HPLC system.

17

18 *2.5 Method validation*

19   The validation of the assay was performed according to the guidelines for Bioanalytical

20 Method Validation recommended by the FDA [14]. Assay validation involved the determination

21 of recovery, linearity, selectivity, specificity, accuracy, precision, limit of detection (LOD), and

22 LLOQ.

23

24 *2.5.1 Recovery*

25   The overall recovery of DCV from human plasma was determined at the LLOQ, low-,

26 medium-, high-QC, and ULOQ level. The peak area response of extracted QC samples (four

samples for each concentration level) was compared to that of unextracted standards obtained by injecting the corresponding concentration of DCV in mobile phase (four samples for each concentration level). The extraction recovery was calculated as previously reported [16], using the ratio of the response and the concentration factor of the assay (250:62.5, since during the SPE procedure 250-µL plasma samples are extracted, evaporated to dryness and then reconstituted with 62.5 µL of mobile phase) and was expressed as a percentage of the response of the calculated amount of DCV diluted in mobile phase and directly injected onto the HPLC, which corresponds to 100% recovery.

2.5.2 Accuracy, precision, and limit of quantification

Intra-day and inter-day precision and accuracy values were determined by assaying plasma samples spiked with five different concentrations of DCV, corresponding to the LLOQ, low-, medium-, high-QC, and ULOQ four times on the same day and on four separate days. Precision was expressed as a percentage of the coefficient of variation (CV), while the accuracy was expressed as the percentage of deviation between nominal and measured concentration (% bias). The criteria for acceptability of intra-day and inter-day precision and accuracy were set at ≤15% at each QC level except at LLOQ, where the values should not deviate by more than 20%, as requested by FDA guidelines [14].

2.5.3 Specificity and selectivity

In order to investigate the potential interferences from endogenous substances, ten different lots of human plasma were evaluated as blank and at LLOQ level of spiked DCV. Chromatograms of the drug-free plasma samples were compared with the corresponding spiked plasma to check for the absence of analytic interferences from endogenous substances. Possible interferences due to potentially co-administered drugs to the patients were also investigated by analyzing both patient samples and blank plasma spiked with the following drugs: abacavir, acyclovir, ampicillin,

1 amprenavir, atazanavir, carbenicillin, chloramphenicol, daptomycin, didanosine, efavirenz,

2 erythromycin, fluconazole, foscarnet, ganciclovir, gentamicin, imipenem, indinavir, interferon,

3 kanamycin, lamivudine, linezolid, lopinavir, nevirapine, posaconazole, ribavirin, rifampicin,

4 ritonavir, saquinavir, simeprevir, sofosbuvir, sofosbuvir metabolite GS-331007, stavudine,

5 streptomycin, teicoplanin, telaprevir, tenofovir, tetracycline, tigecycline, vancomycin,

6 voriconazole, zalcitabine, and zidovudine.

7

8 *2.6. Stability assessment*

9   Stability tests were performed under different conditions simulating those to which a clinical

10 sample may be exposed during routine analysis. Studies of the stability of DCV in plasma

11 included: (a) after the thermization process (60°C for 60 min), (b) after three freeze-thaw cycles;

12 (c) storage at RT for 48 h; and (d) storage at −80°C for 5 months. Moreover, light stability of

13 DCV at RT for 15, 30, 60, 90, 120, 240, and 480 min was assessed in the 3 following light

14 conditions: complete darkness (tubes wrapped in aluminum foil), minimal light exposure (with no

15 artificial light and lit naturally through closed shutters), and sunlight exposure (to daylight at a

16 window bench in a sunny day). In addition, the stability of dried extracts (i.e., after SPE) at −20°C

17 for 120 h and of extracts reconstituted in mobile phase and kept at 4°C for 96 h in the autosampler

18 was analyzed. For each tested condition, six series of LLOQ, low-, medium-, high-QC, and

19 ULOQ plasma samples were prepared. Three series were immediately analyzed, while the three

20 remaining series were subjected to the storage or treatment conditions under examination. The

21 DCV concentrations in both groups of samples were compared. For all stability studies, DCV was

22 considered as stable if the difference between these percentages was lower than 15%, as indicated

23 in the FDA guidelines for bioanalytical methods validation [14].

24

25

26

3. Results and discussion

3.1 Sample preparation and chromatographic conditions

A good drug recovery in sample preparation is important for quantification of DCV in human plasma at low concentration levels. Different approaches were tried such as liquid–liquid extraction with different solvents (i.e., methanol, chloroform, ethylacetate, ether, dichloromethane, and dichloroethane) and acidic precipitation techniques (e.g., precipitation with perchloric acid) for determination of DCV, but they gave low recovery or low clean-up efficiency thus resulting in significant interference from background plasma peaks (data not shown). Deproteinization using acetonitrile followed by a simple SPE procedure employing Waters Oasis HLB 1cc cartridges was applied as a fast and reliable technique for determination of DCV in human plasma samples. Previously, a very low recovery (around 30%) following SPE of DCV from plasma was reported [9], but this result could be due to the different cartridge used for SPE (ISOLUTE C8 from International Sorbent Technology, UK) and most likely, to the fact that DCV is ~99% bound to human plasma proteins [17]. For this reason, the SPE was preceded by a deproteinization step with acetonitrile. This strategy was previously applied successfully by us to improve the extraction recovery of simeprevir, another anti-HCV drug extensively bound to plasma proteins [18]. In addition, the standard SPE procedure recommended by the producer for these cartridges was modified by introducing a washing step with 60% methanol instead of the suggested 5% concentration to improve the sample clean-up without decreasing the drug recovery. This allowed obtaining a high and constant recovery of DCV from human plasma in all the tested concentration range (see Section 3.2.2).

For the chromatographic separation of DCV, different HPLC columns, including a Waters Atlantis dC18 (150 mm × 3.9 mm, 5 μm) column, a Symmetry C18 (75 mm × 4.6 mm, 3.5 μm) column, and an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column, and different mobile phases were tested. In general, better separation of DCV was observed under acidic chromatographic conditions, wherein the drug is protonated. The best chromatographic conditions were achieved

1 using an isocratic mode of acetonitrile:ammonium acetate buffer (pH 5.0, 10 mM) (44:56, v/v) at a

2 flow rate of 1.0 ml/min on an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm; Waters) column. In these

3 conditions, DCV eluted at 8.7 min with an optimal separation from plasma endogenous peaks (see

4 Fig. 1). To shorten the total run time and allow faster analysis of multiple samples, after 10 min of

5 isocratic condition the mobile phase was ramped to 100% acetonitrile in 1 min and maintained for

6 5 min to wash out any remaining contaminants. Detection of DCV was performed at 318 nm, as

7 DCV presents an absorbance maximum at this wavelength (Fig. 2) and a cleaner baseline at the

8 retention time of DCV and a higher signal-to-noise ratio were obtained in these conditions than at

9 different wavelengths. Fig. 1 shows the chromatograms of (A) drug-free human plasma; (B) blank

10 plasma spiked with DCV at LLOQ, (C) blank plasma spiked with DCV at ULOQ, and (D) plasma

11 sample from a subject after administration of 60 mg daclatasvir.

12

13 *3.2 Method validation*

14 *3.2.1. Linearity and sensitivity*

15   An eight-point calibration standard curve of DCV in plasma, ranging from 0.05 to 10 μg/mL,

16 was prepared in triplicate in five independent runs. The calibration curves were linear over the

17 validated concentration range and the correlation coefficients were at least 0.997. The LLOQ of

18 DCV was 0.050 μg/mL. A typical chromatogram of an LLOQ sample is shown in Fig. 1B. This

19 LLOQ is below the C_{trough} values usually observed in patients (mean level of 0.198 μg/mL, ranging

20 from 0.083 to 0.414 μg/mL) [12] and thus provides sufficient sensitivity for routine analysis of

21 human plasma samples in the clinical setting. The LOD, defined as the concentration giving a

22 signal-to-noise ratio of 3, was 0.020 μg/mL.

23

24 *3.2.2. Extraction recovery*

1   The mean extraction recovery for DCV in the tested concentration range (0.05-10 μg/mL)

2   varied from 93.8 to 99.7%, with the CV ranging from 2.6 to 4.8%. These results indicate that the

3   sample extraction procedure results in clean extracts and good recovery to obtain the required

4   sensitivity for the assay.

5

6   *3.2.3. Precision and accuracy*

7   The precision (expressed as % CV) and accuracy (expressed as % bias) data are reported in

8   Table 1. The CVs in the intraday and interday assays ranged from 2.6 to 5.5% and 3.8 to 8.9%,

9   respectively. The intra-day and inter-day deviations (% bias) from the nominal concentrations

10   were always ≤8.0% and ≤9.2%, respectively. Thus, both precision and accuracy were <15%,

11   according to guidelines [14]. These results demonstrate that the method developed here achieves a

12   high degree of reproducibility and accuracy.

13

14   *3.2.4. Selectivity and specificity*

15   The sample extraction and chromatographic analysis were developed to produce a selective

16   assay for the analyte. Ten different lots of commercial control human plasma were carefully

17   evaluated for interference in the assay. A typical chromatogram of blank plasma (Fig 1A) shows

18   that no significant interfering peaks from human plasma were found at the retention time of DCV

19   and at the detection wavelength of 318 nm.

20   The interference from a number of potentially co-administered drugs (see Section 2.5.3) was

21   investigated by determining their retention times at the concentration of 10 μg/ml. No interference

22   was detected between the drugs tested and DCV. Furthermore, absence of chromatographic

23   interference was investigated during routine analysis of plasma samples from patients treated with

24   DCV. The absence of analytic interference was also confirmed by the use of the peak purity

25   checking system and the library matching of the Empower software.

26

1   *3.3 Stability data*

2   *3.3.1. Light stability*

3   Although a European Medicines Agency (EMA)'s report stated that daclatasvir is susceptible to

4   degradation in solution at high-intensity UV and visible light [15], to the best of our knowledge no

5   detailed investigation on DCV photo-stability in human plasma under common light conditions

6   that clinical samples can experience has been yet published. Indeed, only a few data, apparently

7   discrepant with the EMA's report, have been published on stability of DCV in plasma following

8   exposure to light. In fact, recent studies reported that the drug is stable in plasma stored for 113 h

9   under normal light condition [10] and for 24 h under ambient light (bench top) [13]. These

10   apparent discrepancies put the photo-stability of daclatasvir in question and demanded further

11   investigation. The stability of DCV in plasma was tested over 8 h (T0, 15 min, 30 min, 60 min, 90

12   min, 2 h, 4 h, and 8 h) in the 3 following light conditions: complete darkness, minimal light

13   exposure, and sunlight exposure, and compared to those measured on fresh spiked plasmas that

14   were immediately analyzed. A maximum duration of 8 hours was selected to coincide with

15   regular daily working time. Light stability studies were performed on plasma samples spiked with

16   DCV at the LLOQ, low-, medium-, high-QC, and ULOQ level; Fig. 3 shows the results from

17   medium-QC samples, similar results were obtained with the other tested DCV concentrations.

18   DCV was very stable in the complete light-free conditions up to 8 h (CV% ≤ 9.2%). In minimal

19   light exposure, daclatasvir was also very stable al least up to 8 h (CV% ≤ 8.9%). In contrast, upon

20   exposure to sunlight the drug concentration started to decrease at 90 min (reduction of ~23%), and

21   degradation reached the maximum at 4 h (reduction of ~40%); this 40% decrease in concentration

22   then remained stable up to 8 h (CV% ≤ 7.8%). In the next future, we plan to identify and

23   determine the structure of the degradant(s) that are formed when plasma samples containing DCV

24   are exposed to sunlight. The apparent discrepancies between our data and those previously

25   published by others on photo-stability of DCV in plasma [10,13] could be explained by the

26   different light conditions to which plasma samples were subjected. In fact, as we also show,

DCV is stable upon exposure to minimal/normal daylight conditions (i.e., no direct exposure to sunlight), while is degraded when exposed to sunlight.

3.3.2. Stability under thermal virus inactivation

Since heat treatment (56–60°C for 30–60 min) is recommended to inactivate HCV and/or HIV particles in biological samples for safety issues [19,20], we evaluated the stability of DCV in plasma samples heated at 60°C for 60 min as previously reported [21]. As shown in Table 2, such a thermization procedure does not affect DCV concentrations within the considered concentration range.

3.3.3. Other stability studies

We also investigated the DCV stability in human plasma under other conditions that clinical samples commonly experience. After three freeze–thaw cycles (24 h at -−80°C to RT) on plasma samples spiked with DCV at 0.05, 0.2, 1, 7.5, 10 μg/mL, the concentrations of DCV were similar to those measured on fresh spiked plasmas. Moreover, no evidence of DCV decomposition was found during short-term (48 h) storage of plasma samples at RT and long-term storage at−80°C for 5 months (Table 2). These data confirm the good stability of DCV in human plasma both at room temperature and at −80°C reported in previous studies [10-13]. In addition, the stability of DCV both in dried extracts and in processed samples reconstituted in mobile phase was assessed. After 5 days at −20°C or 4 days of storage in the autosampler at 4°C, processed samples were within ± 15% of their nominal concentrations (Table 2), indicating that DCV is stable both in dried and in reconstituted extracts under the tested conditions.

3.4 Analysis of patient samples

Concentration of DCV in plasma was determined with the currently reported method in samples from patients to assess the applicability of the method. A typical HPLC profile of a

plasma sample obtained in a patient receiving a conventional daily oral dose of 60 mg of DCV is

shown in Fig. 1D. This plasma was obtained at steady state, just before the morning intake of the

drug (Ctrough). This method is currently used as a part of our routine TDM of DCV for both

HCV-infected and HCV/HIV-coinfected patients [22]. These data confirm the clinical

applicability of the present analytical method.

4. Conclusions

A simple, precise, selective, and sensitive HPLC-UV assay for the quantification of DCV in

plasma has been developed and validated. The applicability of the method and the appropriateness

of the validated concentrations ranges have been demonstrated in the analysis of plasma samples

of HCV-infected subjects. So far, analytical methods developed for pharmacokinetic

investigations of DCV were all based on liquid chromatography–tandem mass spectrometry

methods (LC–MS/MS) and required costly MS equipment [9-13], which limited the feasibility of

routine DCV analysis. This HPLC assay with UV detection is clearly applicable to routine TDM

of DCV in plasma in conventional hospital laboratories wherein LC–MS is not available.

Analysis of the stability of daclatasvir under different common conditions to which the drug

can be subjected during routine TDM showed that DCV concentration remains stable both in

plasma and in processed samples stored in various conditions as well as in samples undergoing

thermal virus inactivation for 60 min at 60°C or three freeze–thaw cycles. Noteworthy, as shown

in Fig. 3, an important feature that must be kept in mind, irrespective of the analytical method, is

the photo-instability of the compound upon exposure to sunlight, which requires blood sampling

and the whole handling procedure to be performed quickly and with minimal light exposure.

In conclusion, the present HPLC method and the results of the stability experiments could be

useful for dosing DCV and properly handling DCV-containing plasma samples both in routine

clinical surveys and in pharmacokinetic–pharmacodynamic study programs.

1 **Conflict of interest**

2 The authors disclose no conflicts.

3

4 **Acknowledgements**

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1   **Figure Legends**

2

3   Fig. 1. Representative chromatograms of (A) blank plasma sample, (B) spiked plasma sample

4   containing DCV 0.05 μg/mL (LLOQ), and (C) spiked plasma sample containing DCV 10 μg/mL

5   (ULOQ), and (D) plasma sample from a HCV/HIV co-infected patient treated with DCV in

6   combination with sofosbuvir and ribavirin. The measured concentration of DCV was 0.35 μg/mL.

7

8   Fig. 2. The UV spectrum of DCV.

9

10   Fig. 3. Effect of sunlight exposure on the photo-stability of daclatasvir. Stability results of

11   daclatasvir in human plasma under different light conditions. Stability of DCV in plasma was

12   tested at different time-points in the 3 following light conditions: complete darkness, minimal light

13   exposure, and sunlight exposure. Details are described in the Material and methods section.

14   Stability (%) indicates relative concentration of DCV in each test sample compared to that of

15   freshly prepared samples as determined by HPLC analysis. Daclatasvir is considered to be stable

16   when the deviation of the test sample compared to the reference sample is less than 15.0%.

17   Reported data represent the means ± SD of data derived from four experiments in triplicate.

18

Table 1

Intra-day and inter-day accuracy and precision results.

Nominal concentration (µg/mL)	Intra-day (*n* = 4)			Inter-day (*n* = 4)		
	Mean measured concentration (µg/mL)	Accuracy[a] (% bias)	Precision (% CV)	Mean measured concentration (µg/mL)	Accuracy (% bias)	Precision (% CV)
0.05 (LLOQ)	0.054	8.0	5.5	0.047	-6.0	8.9
0.2 (low QC)	0.207	3.5	4.1	0.187	-6.5	6.4
1.0 (medium QC)	0.978	-2.2	2.6	1.092	9.2	7.1
7.5 (high QC)	7.577	1.0	3.8	7.329	-2.3	3.8
10.0 (ULOQ)	9.786	-2.1	4.8	10.371	3.7	5.9

[a]Accuracy = [(measured concentration – nominal concentration)/nominal concentration] x 100

Table 2

Stability of DCV in plasma samples and in dried or reconstituted extracts under different treatment/storage conditions.

	Nominal concentration (µg/mL)				
	0.05	**0.2**	**1.0**	**7.5**	**10.0**
(A) In plasma heated at 60°C for 60 min					
Mean measured conc. at *t=0* (µg/mL)	0.055	0.187	1.109	7.678	9.877
Mean recovered conc. (µg/mL)	0.052	0.210	1.112	7.499	9.688
Deviation (%)	-5.4	12.3	0.3	-2.3	-1.9
CV (%)	6.8	4.3	5.3	1.9	2.1
(B) In plasma subjected to three freeze/thaw cycles					
Mean measured conc. at *t=0* (µg/mL)	0.048	0.211	1.006	7.655	10.333
Mean recovered conc. (µg/mL)	0.053	0.188	1.100	7.621	10.446
Deviation (%)	10.4	-10.9	9.3	-0.4	1.1
CV (%)	6.7	8.1	6.3	1.4	0.9
(C) In plasma stored at room temperature for 48 h					
Mean measured conc. at *t=0* (µg/mL)	0.044	0.212	1.040	7.488	11.016

Mean recovered conc. (µg/mL)	0.048	0.208	1.089	7.611	10.566
Deviation (%)	9.1	-1.9	4.7	1.6	-4.1
CV (%)	5.5	6.3	2.6	5.2	2.3

(D) In plasma stored at – 80°C for five months

Mean measured conc. at $t=0$ (µg/mL)	0.059	0.219	0.966	7.596	9.866
Mean recovered conc. (µg/mL)	0.055	0.211	0.988	7.623	9.542
Deviation (%)	-6.8	-3.7	2.3	0.4	-3.3
CV (%)	8.5	6.2	2.4	1.8	4.1

(E) In dried extracts stored at – 20°C for 120 h

Mean measured conc. at $t=0$ (µg/mL)	0.048	0.198	1.010	7.455	10.242
Mean recovered conc. (µg/mL)	0.051	0.203	0.999	7.388	10.983
Deviation (%)	6.3	2.5	-1.1	-0.9	7.2
CV (%)	7.6	1.6	0.9	3.3	2.2

(F) In reconstituted extracts stored at 4°C for 96 h

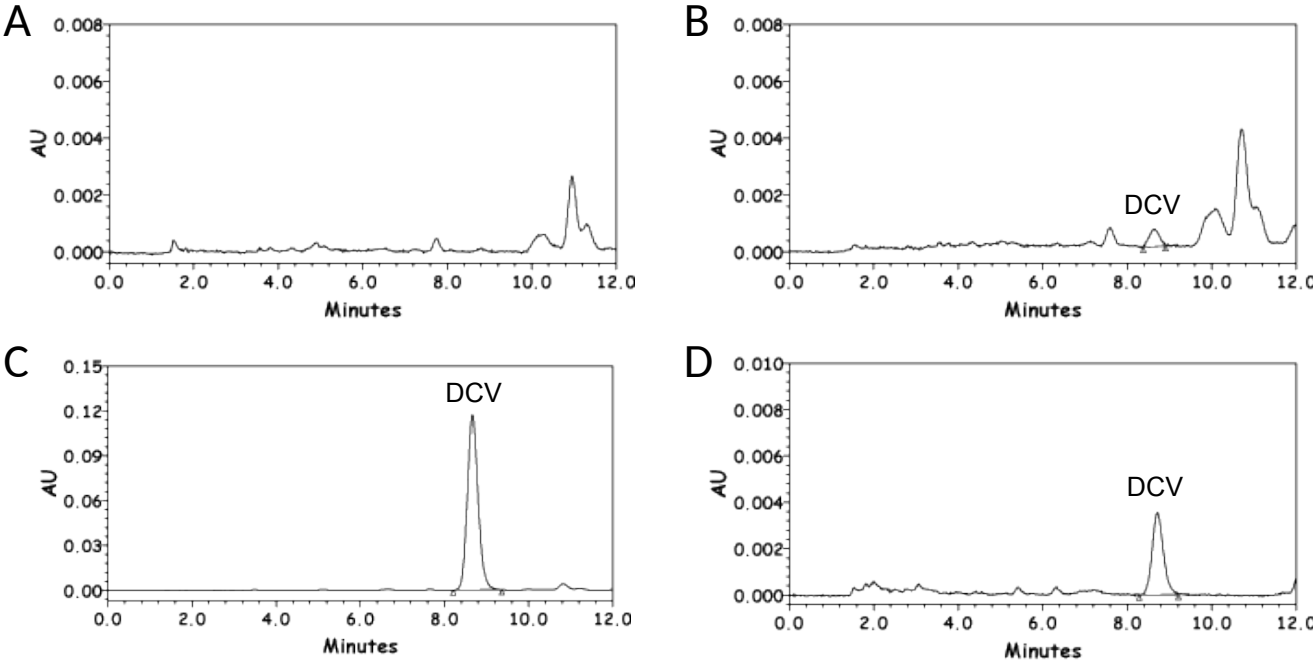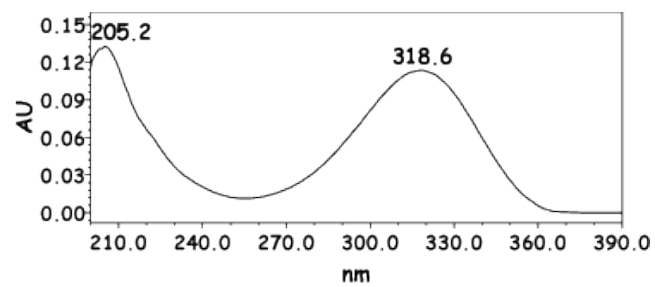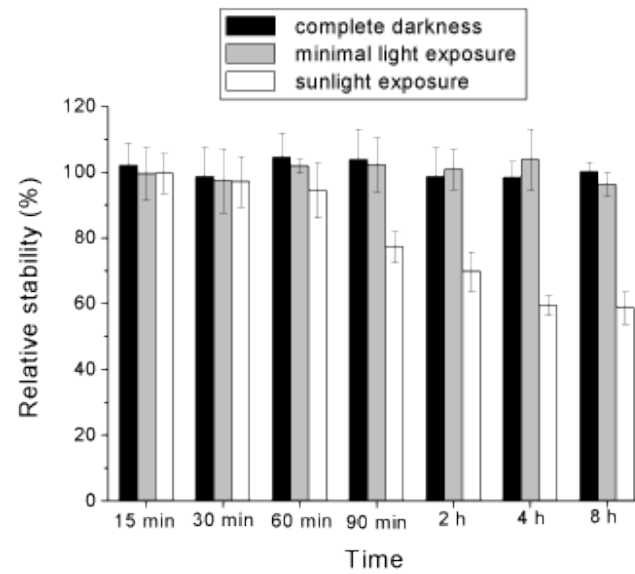|					
Mean measured conc. at $t=0$ (µg/mL)	0.059	0.220	0.933	7.711	9.653
Mean recovered conc. (µg/mL)	0.063	0.208	0.974	7.622	9.833
Deviation (%)	6.8	-5.5	4.4	-1.2	1.9
CV (%)	9.5	6.2	0.8	1.5	2.1

Figure 1

Figure 2

Figure 3

Highlights

• The first HPLC-UV method was developed for the determination of DCV in human plasma.

• This method was applied to quantification of daclatasvir in clinical samples.

• Daclatasvir is degraded by sunlight.