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Article

Development of a validated stability-indicating HPLC-DAD method for dasabuvir and the characterization of its degradation products using LC-QToF-MS/MS

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A stability-indicating HPLC-DAD method was developed and validated for the simultaneous determination of dasabuvir and its degradation products in the pharmaceutical formulation. The proposed method utilized a Symmetry® C18 (4.6 x 75 mm, 3.5 µm) column, and the mobile phase consisted of an isocratic elution of formic acid (0.1%) and acetonitrile (55:45, *v/v*), at a flow of 1 mL min^{-1}; analytes were detected at 244 nm. Dasabuvir was submitted to different stress degradation conditions, such as acidic, alkaline, neutral, thermal, oxidative and photolytic, and the structural elucidation of degradation products was performed using LC-QToF-MS/MS. The HPLC-DAD stability-indicating method was validated for selectivity, linearity, limit of detection and quantification, accuracy, precision and robustness, according to ICH guidelines. Dasabuvir produced two degradation products (DP_1 and DP_2) from the alkaline stress conditions, which were characterized in negative ion mode. Dasabuvir was linear in the range 9.78 to 136.92 µg mL^{-1}, and DP_1 and DP_2 were linear in the range 2.9 to 20.2 µg mL^{-1} and 1.3 to 14.9 µg mL^{-1}, respectively. The recovery ranged between 99.16 and 100.86%, while precision ranged from 1.02 to 2.89%. As the method can effectively separate the dasabuvir from its degradation products and quantitate them, it may be employed as a stability-indicating method for the pharmaceutical formulation.

Keywords: Dasabuvir. Degradation products. Liquid chromatography. Stability study.

INTRODUCTION

Dasabuvir (DBV), N-[6-[5-(3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinyl)-3-(1,1-dimethylethyl)-2-methoxyphenyl]-2-naphthalenyl]-methanesulfonamide, is a second-generation direct-acting antiviral, taken orally for the treatment of hepatitis C virus (Figure 1). It acts as an non-nucleoside inhibitor of non-structural protein 5B (NS5B) polymerase, which is responsible for viral replication, and has been used in combination therapy with ombitasvir, paritaprevir and ritonavir for the treatment of the genotype 1 infection (AbbVie, 2019).

The efficacy and safety of medicines is directly related to their stability. Impurities and related substances may compromise the pharmacological effect and, in some cases, cause the occurrence or increase of adverse effects (Maggio, Vignaduzzo, Kaufman, 2013). Thus, the impurity content of the active pharmaceutical ingredient must be evaluated to ensure that safe limits are not exceeded. It is possible to evaluate the content of these substances using a stability-indicating analytical method (SIAM), which, by definition, is a validated analytical method capable of unequivocally quantifying the drug in the presence of all of its degradation products (DPs), as well as any matrix components expected in the formulation (Bakshi, Singh, 2002).

According to the literature, the most commonly used technique for DBV analysis is liquid chromatography (LC) coupled to a UV/Visible (UV-Vis) detector and/or mass spectrometry (MS). DBV determination is often performed

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simultaneously with other drugs used in combination therapy, aiming at different matrices. Some studies have targeted the quantification of DBV and several direct-acting antivirals in the aspirate of the human liver by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Ocque *et al.*, 2017), while other authors have assessed the pharmacokinetics of DBV in human plasma by UPLC-MS/MS and high performance chromatography-tandem mass spectrometry (HPLC-MS/MS) (Ariaudo *et al.*, 2016; Polepally *et al.*, 2016) and the content in the pharmaceutical formulation by high performance liquid chromatography coupled to a diode-array detector (HPLC-DAD) (Al-Zoman, Maher, Al-Subaie 2017). However, a SIAM for the determination of DBV and its DPs in both the pharmaceutical formulation and the raw material has not yet been reported in the literature.

The aim of the present study was to obtain the degradation profile of DBV through forced stress degradation, to develop and validate a stability-indicating HPLC-DAD method for the determination of DBV and its potential DPs in the pharmaceutical formulation and the raw material, and to characterize the DPs using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QToF-MS/MS). It is expected that this study will contribute to a rapid method, capable of separating and quantitating the active pharmaceutical ingredient and its DPs, as a way of monitoring the stability of the medicine to ensure its safety and quality to the patient.

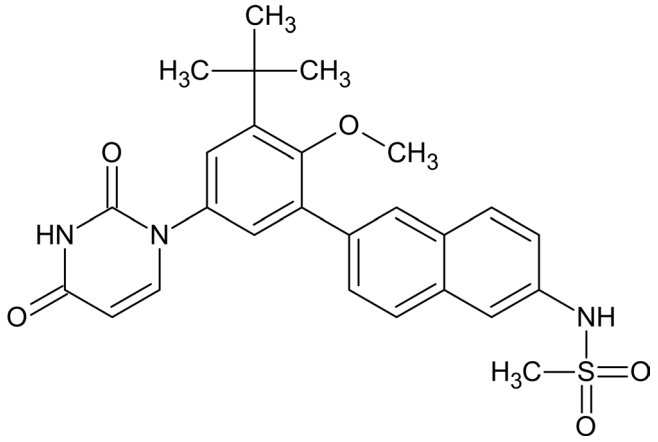

FIGURE 1 - The chemical structure of dasabuvir.

MATERIAL AND METHODS

Material

Pharmaceutical grade dasabuvir (97.8%) was purchased from Nanjing Kaimubo Pharmatech Ltd (Nanjing, Jiangsu, China). HPLC-grade acetonitrile was purchased from JT Baker (Phillipsburg, New Jersey, USA), and ultrapure water was obtained by a Milli-Q® purification system from Millipore (Milford, Massachusetts, USA). Analytical grade hydrochloric acid, ammonium hydroxide, hydrogen peroxide, dimethyl sulfoxide and formic acid (98%) were purchased from Sigma (St. Louis, Missouri, USA).

Instrumentation and chromatographic conditions

The analysis was conducted using an Agilent 1100 series HPLC system (Agilent Technologies, California, USA) coupled to a diode-array detector (DAD), and the chromatograms were recorded and analysed through an Agilent ChemStation® version B.04.03[16]. Chromatographic separation was achieved using a Symmetry® C18 (4.6 x 75 mm, 3.5 µm) column, with a mobile phase consisting of A) formic acid (0.1%) pH 2 and B) acetonitrile (55:45, *v/v*), with a flow rate of 1 mL min^{-1} at 25º C; the injection volume was 10 µL, and the analytes were detected at 244 nm using a DAD.

The structural elucidation of DPs was carried out using a Waters ACQUITY UPLC® H-Class system coupled to a Waters Xevo G2-S QToF mass spectrometer (Waters Corporation, Massachusetts, USA), and the MS data were recorded and analysed using Waters MassLynx® NT4.1. LC analysis was conducted using the same chromatographic conditions as in the developed HPLC method, and analytes were detected in positive and negative electrospray ionization (ESI), which was set to a spray voltage of ±3 kV, a sampling cone of 40 kV, and 400 L h^{-1} of desolvation gas. The vaporizer temperature and source temperature were set to 350 °C and 150 °C, respectively.

DBV standard and sample preparation

DBV was accurately weighed and diluted in dimethyl sulfoxide (DMSO) in order to obtain a 1 mg mL^{-1} DBV standard solution. For the stress degradation assays, the DBV standard and placebo product (a blend of excipients, as specified by the manufacturer, considering the usual content of the tablet) (AbbVie, 2019; EMA, 2015) were dissolved in a mixture of DMSO and stressing agent (1:1, *v/v*) to obtain a 1 mg mL^{-1} stress solution (see section below titled Stress degradation assays). Aliquots were withdrawn from the stressed samples, neutralized, if necessary, filtered through a 0.22 µm polytetrafluorethylene (PTFE) syringe filter, diluted with mobile phase to a concentration of 100 µg mL^{-1} and subjected to chromatography.

The extraction of DBV and DPs from the pharmaceutical formulation involved solubilizing the sample with DMSO in a ratio of 1:1 (*m/v*) in relation to DBV. The mixture was sonicated for 5 min and filtered through a 0.22 µm PTFE syringe filter. The extracted solution was diluted with mobile phase to achieve a concentration of 100 µg mL^{-1} DBV and then submitted to chromatography.

Stress degradation assays

The degradation profile of DBV was obtained using a solution containing the DBV standard and the placebo product, which was submitted to stress degradation conditions, such as acidic, alkaline, oxidative, photolytic and thermal (Brazil, 2019; ICH, 2003). To assess the stability of DBV against the stressing agents, screening tests were carried out in 1M HCl, 1M NH_4OH, water at 80 °C (for hydrolytic stress), 3% H_2O_2 at room temperature (for oxidative stress), solid state at 80 ºC (for thermal stress) and exposed to a light source option 2 (for photolytic stress), accordingly to the ICH recommendations (ICH, 1996).

All of the stress conditions were monitored for seven days, with experiments carried out in triplicate. Student's t-tests were performed to compare the mean DBV content initially and on the final day of the stress assays, where a statistical significance (*p-value* < 0.05) indicated that DBV was unstable in that condition. Those conditions in which DBV was shown to be unstable were repeated, and the content decay was evaluated daily for one week, in order to establish the end-point of the degradation assay and to prevent the consumption of the DP of interest to ensure that the levels of any DPs are appropriately assessed.

Characterization of degradation products

The observed DP peaks in the different stress degradation assays were assessed by MS. In order to elucidate the identity of the main DPs, the fragmentation profile of DBV was compared to the DP profiles; by evaluating the mass difference of the ion fragments, it was possible to suggest the most probable molecular loss. Confirmation of the monoisotopic mass of the structures obtained were calculated using Chemsketch® C50E41 (ACD/Labs, inc., Toronto, Canada).

Isolation of the degradation products

In order to isolate the DPs from the stressed samples, the eluted fractions were collected at the retention time of each compound, from the LC PEEK tube after the detector. The purity of the collected fractions was confirmed by subjecting the samples to MS and evaluating the existence of a single mass. The purified samples were dried in a CentriVap vacuum concentrator (Labconco Co., Missouri, USA) and subsequently used in the SIAM development and validation.

Stability-indicating method development

The chromatographic conditions used to monitor the stress assays were adapted from the Al-Zoman, Maher and Al-Subaie (2017) method. The method was optimized using the mixture of DBV and excipients, which was spiked with all of the DPs detected in the degradation assays. Chromatographic parameters, such as flow, mobile phase pH, organic modifier, column temperature and wavelength detection were modified to achieve suitable selectivity and peak resolution (> 1.5) of DBV and its DPs.

Once the method was optimized for the analytes, the following system suitability test (SST) parameters were fixed: retention factor (k'), tailing factor (T), resolution (Rs) and theoretical plates (n).

Method validation

The optimized method was validated according to ICH and ANVISA guidelines for the validation of analytical procedures (ICH, 1994; Brazil, 2017). The following parameters were assessed: selectivity, linearity, limit of detection (LoD) and limit of quantification (LoQ), accuracy, precision and robustness. All statistical analyses were carried out using Minitab® 17.1.0 (LEAD Technologies, North Carolina, USA) and Prism® 8 (GraphPad, Inc, California, USA).

Selectivity

The selectivity was evaluated by considering the retention times of the peaks of interest, analysing the DBV sample spiked with all of the DPs. Analysis of the placebo and blank samples were performed to verify the presence of any chromatographic peak with the same retention time as the analytes. The similarity of the spectral profile was further evaluated from three different samples, using the ChemStation® software peak purity tool. The sample was considered pure where the spectral profile had spectral similarity superior to 98%.

Linearity

Linearity was determined by analysing at least five levels of concentration distributed around the target concentration. The working range was 9.78–136.92 μg mL^{-1} for DBV, 2.9–20.2 μg mL^{-1} for DP_1 and 1.3–14.9 μg mL^{-1} for DP_2. All calibration curves were performed in triplicate. The data obtained were fitted by linear regression, determining the coefficients and significance of the equations' slopes and intercepts. The linearity of the method was further confirmed by evaluating the analysis of variance (ANOVA) for regression fit and lack-of-fit, homoscedasticity (Brown-Forsythe test) and residual normality (Anderson-Darling test), with a significance level of 5% (*p-value* < 0.05) for all tests.

Limit of detection (LoD) and limit of quantification (LoQ)

The LoD was obtained from the regression parameters for all analytes, as recommended by ICH and ANVISA (ICH, 1994; Brazil, 2017). The LoQ was fixed as the lowest concentration of the calibration curve sample that provided at least a signal-noise ratio of 10.

Accuracy and precision

The accuracy of the method was determined by the degree of agreement obtained in the method, in triplicate, calculated as the mean percentage recovery and relative standard deviation (RSD) of a mixture of placebo spiked with DBV and DPs at known concentrations, at a high, medium and low level.

The precision (repeatability) was determined by injecting three replicates of a standard sample preparation containing low, medium, and high concentrations of DBV, DP_1 and DP_2. The same protocol was followed to determine the intermediate precision, but with a different analyst and on a different day. The RSD was calculated to evaluate the repeatability, while the intermediate precision was evaluated using an F-test between the determinations, where a *p-value* > 0.05 indicated the equality of variances.

Sample preparations were spiked to achieve concentrations of 9.78, 97.8 and 136.92 μg mL^{-1} for DBV; 2.9, 9.6 and 20.2 μg mL^{-1} for DP_1; and 1.3, 5.1 and 14.9 μg mL^{-1} for DP_2.

Robustness

The robustness was assessed by subjecting the method to deliberate changes in the percentage of formic acid in the mobile phase, flow rate, column oven temperature and organic modifier. The variables monitored were the number of peaks, the number of peaks with Rs > 1.5, the number of peaks with T < 1.5, Rs 0 (DBV and anterior peak) superior to 1.5 and the percentage of DBV concentration. This evaluation was established using a fractional factorial design (2^{4-1}) and assessing the effects with a Pareto chart and half-normal plot.

RESULTS AND DISCUSSION

Stress degradation assays

The stress degradation screening tests showed that DBV was stable under neutral and acidic hydrolysis, and under oxidative, thermal and photolytic conditions (*p-value* > 0.05), but sensitive to the alkaline medium (*p-value* < 0.05). The difference in the DBV content under the various stress conditions over the course of seven days and the *p-values* are shown in Table I.

Two DPs were found in the alkaline medium (DP_1 and DP_2) during the stress tests (Figure 2). By monitoring the DBV peak area in the alkaline condition daily, it was possible to verify the DPs' maximum yield from stress conditions in two days of degradation, after to this point, the DPs were consumed in the medium.

TABLE I - The observed content of dasabuvir in stability screening tests under different conditions

Condition	T_0 (µg mL^{-1}) [a]	T_1 (µg mL^{-1}) [b]	Difference (µg mL^{-1}) [c]	p-value [d]
Acidic	95.19 ± 1.90	93.62 ± 1.92	1.91	0.49
Alkaline	97.92 ± 3.59	54.17 ± 0.98	43.74	< 0.05
Neutral	95.70 ± 2.02	96.55 ± 3.19	–0.85	0.61
Oxidative	95.98 ± 4.25	95.48 ± 4.98	0.49	0.87
Thermal	94.92 ± 1.44	95.02 ± 1.43	–0.10	0.98
Photolytic	95.89 ± 5.01	97.32 ± 1.81	–1.43	0.74

[a] T_0 relative to day 0 of stress degradation; [b] T_1 relative to day 7 of stress degradation; [c] The difference between the mean content of T_1 and T_0; [d] *P-value* obtained from Student's t-test.

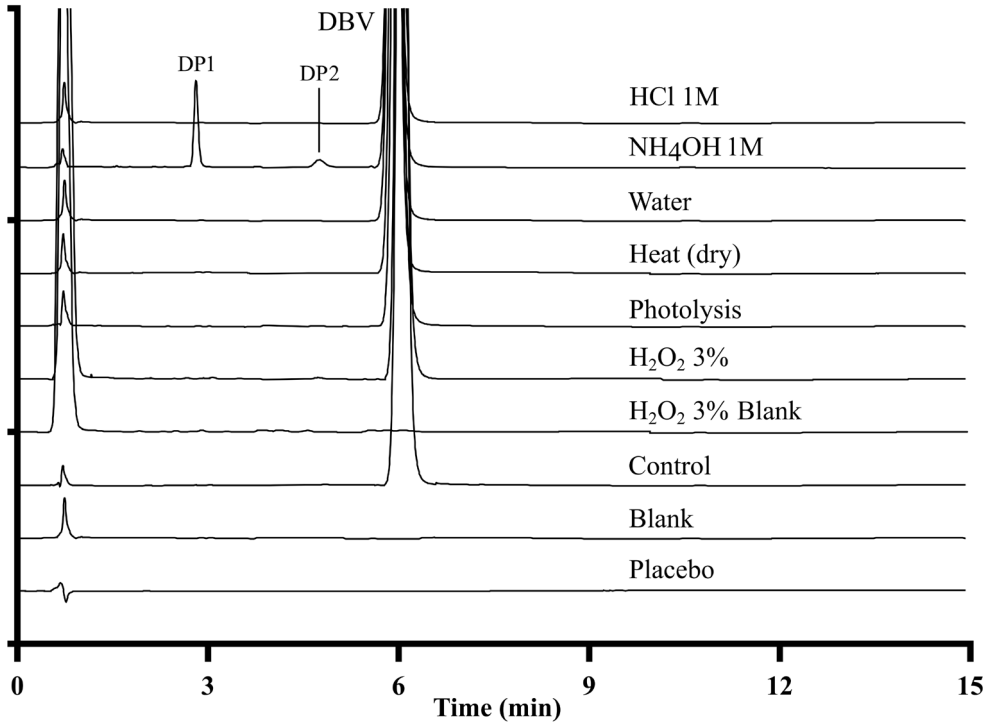

FIGURE 2 - The degradation assay screening test chromatograms at the end-point of the stress conditions. DBV - dasabuvir; DP_1 - alkaline degradation product 1; DP_2 - alkaline degradation product 2.

Characterization of degradation products

The end-point samples from the alkaline stress tests containing the DPs were analysed in a mass spectrometer, with ESI in both positive and negative mode. Even with the addition of formic acid as an additive in the positive mode, the negative mode produced a higher molecular ion and fragment ion intensity for all analytes. The use of formic acid was maintained, since it assisted in providing peak resolution and a low tailing factor in the chromatographic peaks.

The MS spectra and the most probable molecular loss of the DBV and DPs are shown in Table II. The exact mass of DBV corresponding to $[M–H]^-$ has a *m/z* of 492.1597 (-0.8127 error ppm). During evaluation of the fragmentation profile of DBV, it was possible to observe the abundant fragmentation of *m/z* 477.1358 (methyl, Δ *m/z* 15), *m/z* 462.1130 (two methyls, Δ *m/z* 30), *m/z* 435.0885 (tert-butyl, Δ *m/z* 57) and *m/z* 399.1577 (methanosulfonamide, Δ *m/z* 93), followed by the less abundant *m/z* 449.1548 (amide, Δ *m/z* 43), *m/z* 414.1804 (methanosulfonyl, Δ *m/z* 78), *m/z* 384.1358 (methyl and methanosulfonamide, Δ *m/z* 108) and *m/z* 356.1061 (methanosulfonyl and tert-butyl, Δ *m/z* 136). The position of the functional groups are shown in Figure 3.

The exact mass of DP_1 and DP_2 were *m/z* 397.1586 and *m/z* 440.1644, respectively. Both fragmentation profiles showed a similar molecular loss to that of DBV, with the main DP_1 and DP_2 fragments corresponding, respectively, to *m/z* 382.1345 and 425.1405 (related to methyl loss), *m/z* 367.1115 and 410.1181 (two methyl loss), *m/z* 340.0873 and 383.0938 (tert-butyl loss) and *m/z* 304.1576 and 347.1628 (methanosulfonamide loss).

Based on the fragmentation profiles of the DPs, it is suggested that the alkaline hydrolysis occurs in the uracil ring portion for both DPs, since the majority of the fragmentation pattern is similar to that of DBV, except for the loss of amide (Δ *m/z* 43), which was absent in the DP_1 profile, suggesting that most of the functional groups are still present on the DP structures. It was possible to propose the structure of both DPs according to the mass error (Table II); these structures provided an accurate agreement in relation to the observed (error ppm < 5). The IUPAC names are [3-tert-butyl-5-(6-methanesulfonamidonaphthalen-2-yl)-4-methoxyphenyl]urea (DP_1) and N-[6-(5-amino-3-tert-butyl-2-methoxyphenyl)naphthalen-2-yl]methanesulfonamide (DP_2) (Figure 4). Figure 5 shows the hydrolysis reaction under alkaline conditions.

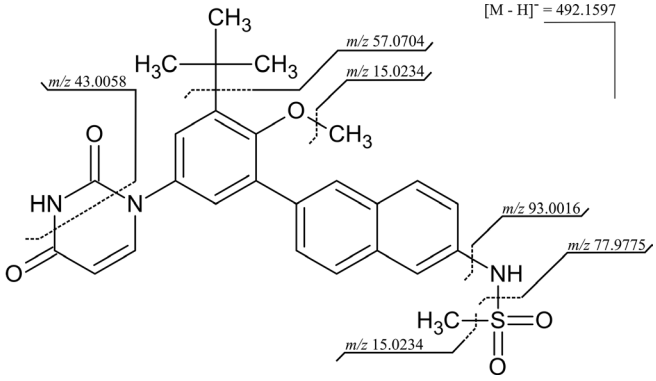

FIGURE 3 - The structure of dasabuvir and its most probable fragments.

TABLE II - The fragmentation profile of dasabuvir and its degradation products obtained from LC-QToF-MS/MS

ID	Ret. time (min)	Observed *m/z*	Calculated *m/z*	Error (ppm)	MS/MS fragment ions	Mass difference	Most probable composition for the mass difference
DBV	6.14	492.1597	492.1593	–0.8127	-	-	-
					477.1358	15.0234	CH_3
					462.1130	30.0469	$2CH_3$
					449.1548	43.0049	NHCO
					435.0885	57.0704	$C3CH_3$
					414.1804	77.9775	SO_2CH_3

					399.1577	93.0016	$NHSO_2CH_3$
					384.1358	108.0119	$NHSO_2CH_3$-CH_3
					356.1061	136.0558	SO_2CH_3-$C3CH_3$
DP_1	2.02	397.1587	397.1586	0.2518	-	-	
					382.1345	15.0237	CH_3
					367.1115	30.0473	$2CH_3$
					340.0873	57.0715	$C3CH_3$
					319.1807	77.9779	SO_2CH_3
					304.1576	93.0011	$NHSO_2CH_3$
					289.1338	108.0249	$NHSO_2CH_3$-CH_3
					261.1027	136.0559	SO_2CH_3-$C3CH_3$
DP_2	4.84	440.1649	440.1644	1.1335	-	-	
					425.1405	15.0245	CH_3
					410.1181	30.0468	$2CH_3$
					397.1591	43.0058	NHCO
					383.0938	57.0711	$C3CH_3$
					347.1628	93.0021	$NHSO_2CH_3$
					332.1398	108.0251	$NHSO_2CH_3$-CH_3
					304.1565	136.0079	SO_2CH_3-$C3CH_3$

DBV = dasabuvir; DP_1 = alkaline degradation product 1; DP_2 = alkaline degradation product 2.

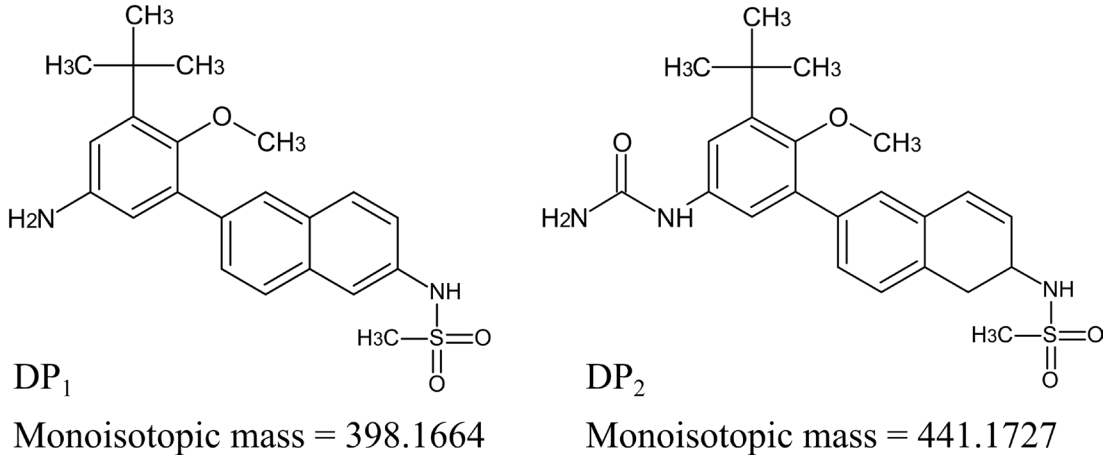

FIGURE 4 - The structure of [3-tert-butyl-5-(6-methanesulfonamidonaphthalen-2-yl)-4-methoxyphenyl]urea (DP_1) and N-[6-(5-amino-3-tert-butyl-2-methoxyphenyl)naphthalen-2-yl] methanesulfonamide (DP_2).

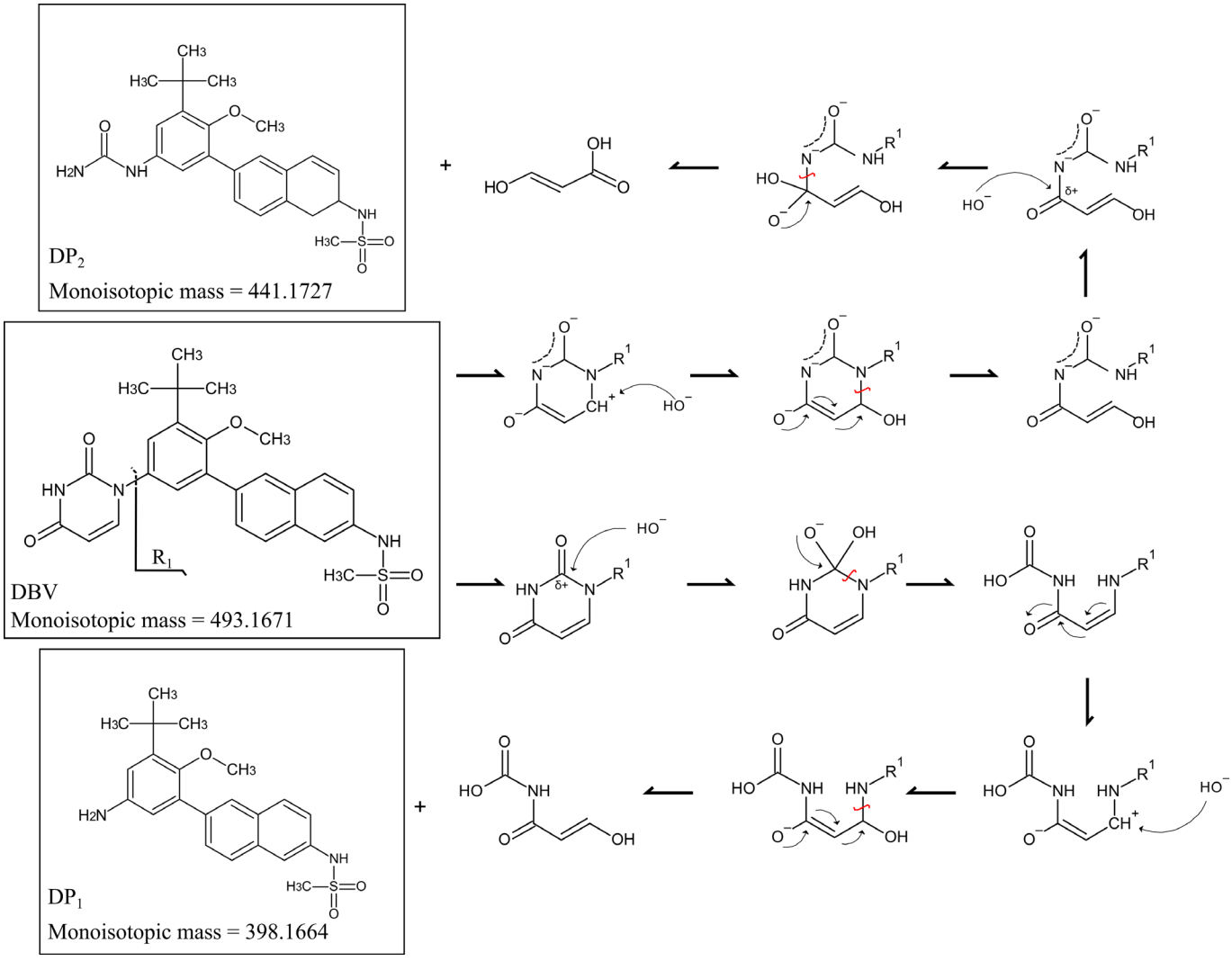

FIGURE 5 – The hydrolysis reaction of dasabuvir under alkaline conditions.

Stability-indicating method development

Al-Zoman, Maher and Al-Subaie (2017) used a Waters Symmetry® C18 column (150 x 4.5 mm, 3.5 µm) with a mobile phase of acetonitrile and 10 mM potassium dihydrogen orthophosphate at a ratio of 65:35 (*v/v*) pH 7, delivered at 1 mL min^{-1}. As an alternative, a Waters Symmetry® C18 column (75 x 4.6 mm, 3.5 µm) was used in this study; however, this column provided a retention time of 1.4 min for DBV, even after reproducing all other chromatographic conditions. Thus, the retention time and concentration of DBV were optimized by changing the chromatographic parameters, to assist in monitoring the emergence of potential DPs during the screening stress assays.

After method optimization in the presence of DPs, the mobile phase proportion of 55:45 (*v/v*) provided a DBV retention time of 6.14 min, compared to 2.02 and 4.84 min for DP_1 and DP_2, respectively. The buffer of the mobile phase was changed to acidification with formic acid at 0.1% (pH 2.6), justified by the incompatibility between the MS and precipitable salts; this change maintained the SST parameters and the retention of DBV and its DPs. Screening of the wavelength showed optimal detection at 244 nm for the simultaneous determination of DBV and its DPs. The temperature was fixed at 25 ºC. Elution was also tested using gradients; however, this elution mode had no benefits in terms of the resolution, tailing and retention time. Moreover, the gradient elution mode resulted in an increase of analysis time, since equilibration to the initial

composition extends the run-time. The chromatographic conditions were fixed using a Symmetry® C18 (4.6 x 75 mm, 3.5 µm) column with a mobile phase consisting of an isocratic elution of formic acid (0.1%) and acetonitrile (55:45, *v/v*), at a flow of 1 mL min^{-1}, and analytes were detected at 244 nm. The chromatogram for the stability-indicating method is shown in Figure 6.

Once the SIAM had been developed, the SST parameters were evaluated and fixed, to ensure the adequacy of the system for this analysis. The parameters for retention time, retention factor, tailing factor, resolution and theoretical plates are shown in Table III.

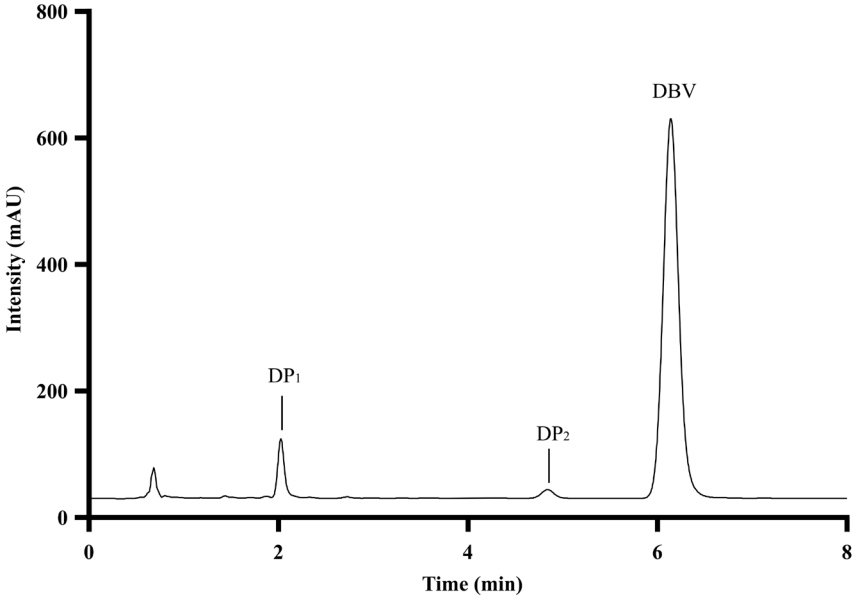

FIGURE 6 - The chromatogram of the dasabuvir stability-indicating method. DBV - dasabuvir; DP_1 - alkaline degradation product 1; DP_2 - alkaline degradation product 2.

TABLE III - System suitability test parameters of the stability-indicating method

Compound	Rt (min)	k'	T	Rs	Theoretical plates
DP_1	2.02	1.96	1.236	11.619	4109
DP_2	4.84	6.08	1.012	3.082	6630
DBV	6.14	7.98	1.079	4.804	6578

DBV = dasabuvir; DP_1 = alkaline degradation product 1; DP_2 = alkaline degradation product 2. Rt = retention time; k' = capacity factor; T = tailing factor; Rs = resolution

Method validation

Selectivity

The method proved to be selective, since there was no chromatographic peak overlap in the analysis of the placebo, blank and DBV sample spiked with DPs, and each compound had a distinct retention time. Moreover, using the peak purity tool, the similarity of the DBV, DP_1 and DP_2 UV spectra was 99.96% (± 0.02), 98.17% (± 1.69) and 98.30% (± 2.86), respectively (Figure 7). These determinations were superior to 98%, which ensured the selectivity of each compound.

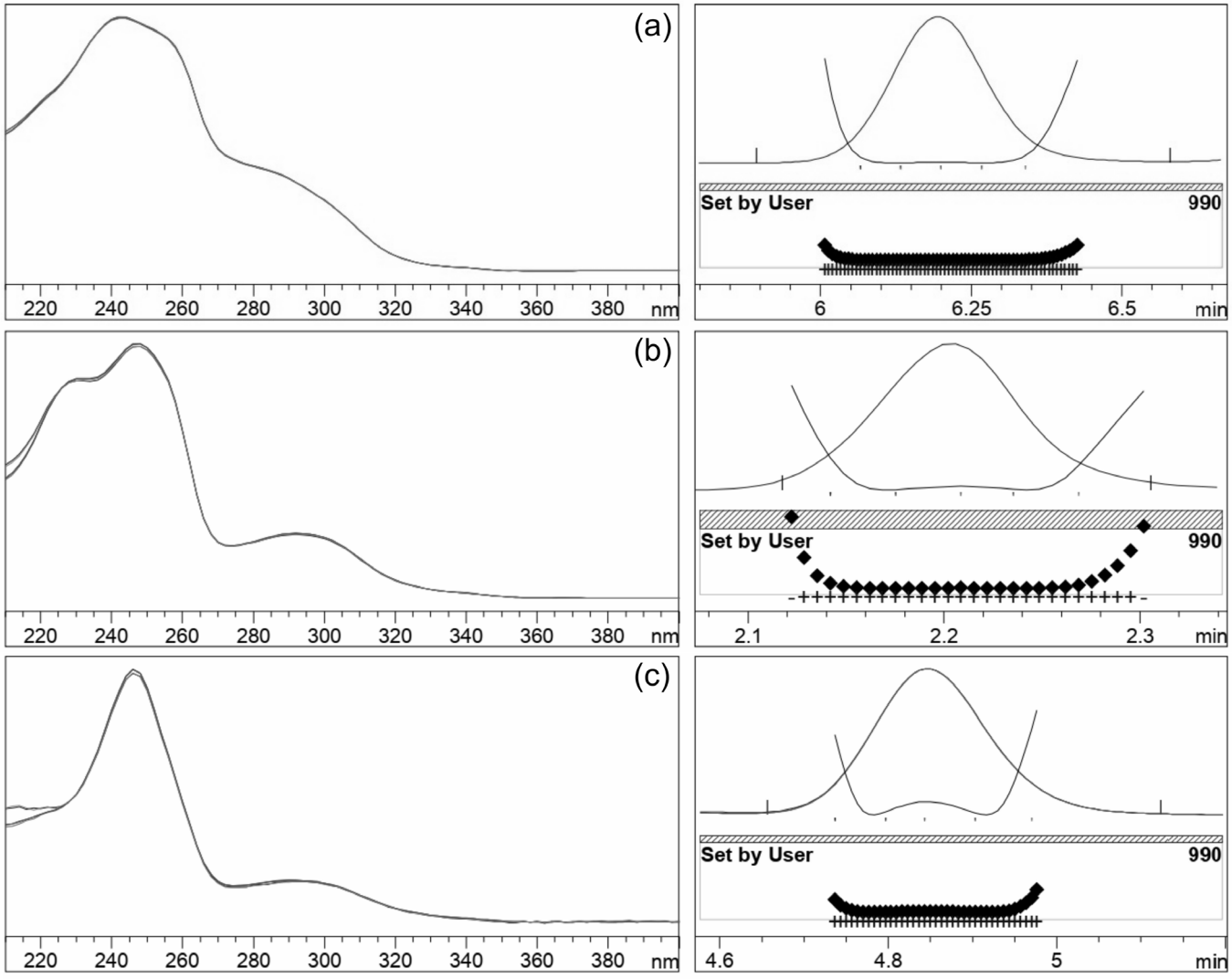

FIGURE 7 - UV spectrum and peak purity of dasabuvir and degradation products. (a) dasabuvir; (b) alkaline degradation product 1; (c) alkaline degradation product 2.

Linearity

The regression analysis was shown to be linear in the concentration range 9.78–136.92 µg mL^{-1} for DBV, 2.9–20.2 µg mL^{-1} for DP_1 and 1.3–14.9 µg mL^{-1} for DP_2. The coefficient of determination was superior to 0.98 and the regression fit gave a *p-value* < 0.05 for all analytes, suggesting the significance of the linear model. The lack-of-fit parameter (*p-value* > 0.05) showed that the error was random. At the proposed concentrations, the data was shown to be homoscedastic (*p-value* > 0.05), and with normal residues (*p-value* > 0.05). All slopes were different from zero (*p-value* < 0.05), and the intercepts were equal to zero (*p-value* > 0.05). The regression analysis parameters are shown in Table IV.

TABLE IV - Regression analysis data for dasabuvir and its degradation products

Parameter	DBV	DP_1	DP_2
Range (μg mL^{-1})	9.78–136.92	2.9–20.2	1.3–14.9
Slope (b) •	64.29 ± 0.18	2.59 ± 0.01	12.47 ± 0.42
Intercept (a) •	1.72 ± 14.79	–0.25 ± 0.23	0.86 ± 3.28
R-Squared	0.9998	0.9991	0.9819
Slope significance ▪	< 0.001	< 0.001	< 0.001
Intercept significance ▪	0.8660	0.3040	0.8350
Regression fit ▲	< 0.001	< 0.001	< 0.001
Lack-of-fit ▲	0.9020	0.1110	0.8190
Homoscedasticity ▼	0.9579	0.4756	0.5199
Residual normality ♦	0.9830	0.5740	0.1360

• Regression coefficients shown as mean ± SD (n = 3); ▪ Coefficient significance obtained from t-test; ▲ Regression fit and lack-of-fit obtained from ANOVA; ▼ Homoscedasticity obtained from Brown-Forsythe test; ♦ Residual normality obtained from Anderson-Darling test. *P-value* with a significance < 0.05 for all tests. DBV = dasabuvir; DP_1 = alkaline degradation product 1; DP_2 = alkaline degradation product 2;

Limit of detection (LoD) and limit of quantification (LoQ)

The LoD values calculated from the regression curves were 0.8102 μg mL^{-1} for DBV, 0.2930 μg mL^{-1} for DP_1 and 0.8680 μg mL^{-1} for DP_2. Likewise, the calculated LoQ were 2.4551 μg mL^{-1} for DBV, 0.8878 μg mL^{-1} for DP_1 and 2.6303 μg mL^{-1} for DP_2. However, for DP_1 and DP_2, the LoQ were fixed at 2.9 μg mL^{-1} and 1.3 μg mL^{-1}, since their signal-noise ratios were 12.69 and 10.52, respectively, at these concentrations. All LoQs were accurate and precise.

Accuracy and precision

Accuracy was assessed as the mean recovery of the spiked samples. The average percentage recovery ranged from 99.17 to 100.86%, with standard deviations below 1.97%. The repeatability was less than 1.93 for DBV, 2.63 for DP_1 and 2.89 for DP_2, at all levels. The intermediate precision for all analytes and levels, using the F-test, showed that the precisions were similar over different days (*p-value* > 0.05). The accuracy and precision data are shown in Table V, demonstrating that the method is exact and precise in the proposed range.

TABLE V - The accuracy and precision data for dasabuvir and its degradation products

Parameter	Level	DBV	DP_1	DP_2
Accuracy (mean % ± SD)	High	99.16 ± 1.15	99.36 ± 0.88	99.17 ± 1.29
	Medium	100.86 ± 1.21	99.70 ± 1.52	100.05 ± 0.93
	Low	99.36 ± 1.97	99.58 ± 0.76	100.53 ± 1.32
Precision (RSD)	High	1.02	2.63	1.37
	Medium	1.93	2.48	2.89
	Low	1.74	2.58	1.93

TABLE V - The accuracy and precision data for dasabuvir and its degradation products

Parameter	Level	DBV	DP_1	DP_2
Intermediate precision (*p-value*) [a]	High	0.9329	0.3692	0.3860
	Medium	0.6944	0.5618	0.8999
	Low	0.1848	0.3240	0.2352

[a] Intermediate precision *p-value* obtained from the F-test. DBV = dasabuvir; DP_1 = alkaline degradation product 1; DP_2 = alkaline degradation product 2.

Robustness

The Pareto charts and half-normal plots for deliberate changes in the temperature, mobile phase pH, flow rate and the percentage of organic modifier did not show any significant effect (Figure 8), suggesting that the method was robust enough to withstand these deliberate changes. The graph for the number of peaks, the number of peaks with Rs > 1.5 and the number of peaks with T < 1.5 were not plotted, since there was zero effect in relation to these robustness factors.

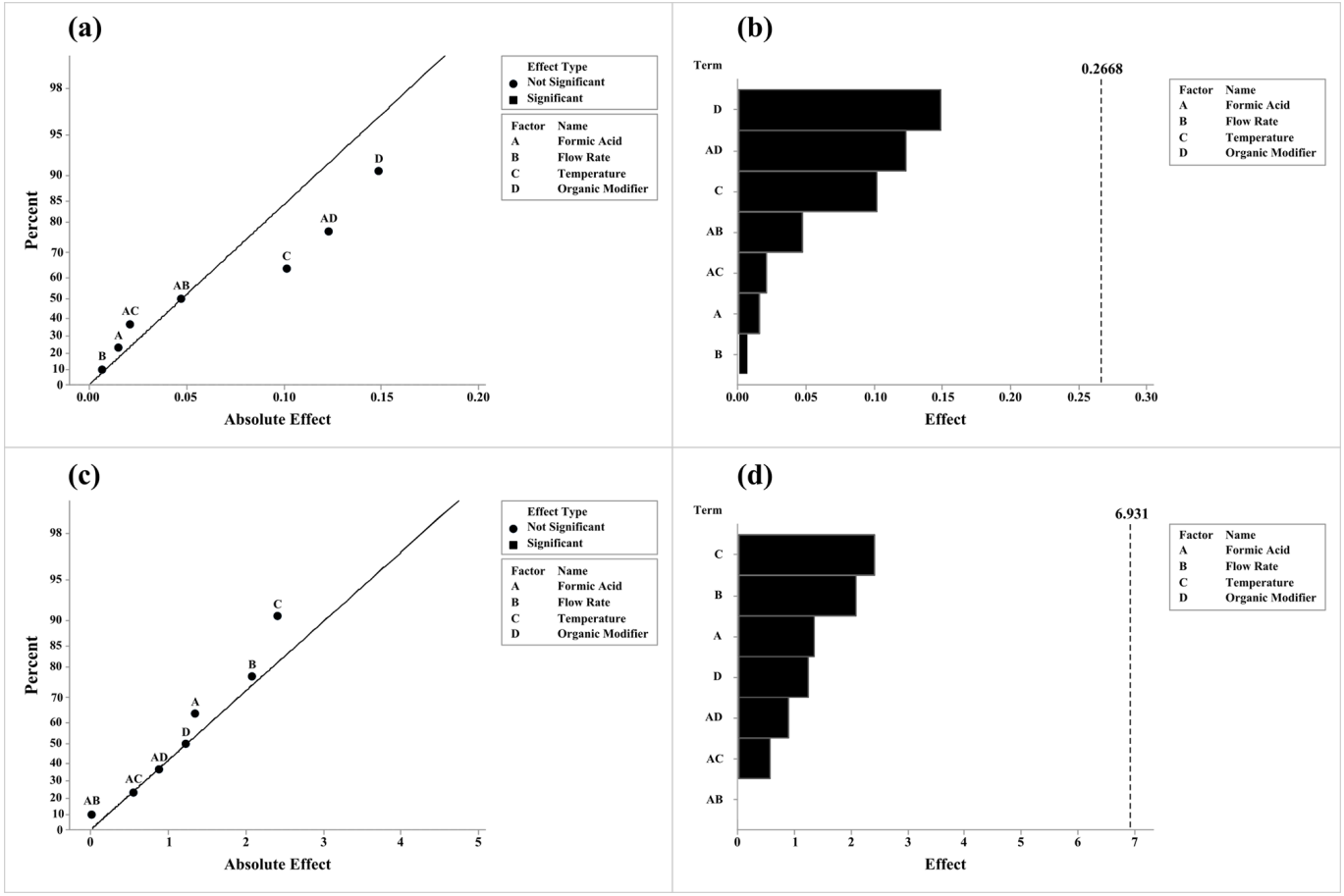

FIGURE 8 - Half-normal plots and Pareto chart of effects. (a) Half normal plot (DBV resolution); (b) Pareto chart (DBV resolution); (c) Half normal plot (DBV concentration); (d) Pareto chart (DBV concentration).

CONCLUSION

Dasabuvir was found to be sensitive to alkaline stress conditions, providing two different degradation products that were not previously reported. Both DPs were structurally elucidated by LC-QToF-MS/MS, with a mass error inferior to 1.13, with the structures being compatible with the fragmentation profile. An analytical stability-indicating method by HPLC-DAD was developed and validated to confirm its linearity, accuracy, precision, sensitivity and robustness for DBV and its DPs. Thus, the method is suitable for monitoring the stability of DBV in the pharmaceutical formulation and the raw material, as a way of ensuring quality and safety to the patient.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

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