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Validated stability-indicating HPLC method for the determination of dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB) and its degradation products

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ABSTRACT

High-performance liquid chromatographic method was developed for the quantitative determination of dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB) and its degradation products. Forced degradation studies were performed on bulk sample of DDB using acid (1N hydrochloric acid), alkaline (0.1N sodium hydroxide), oxidation (0.33% hydrogen peroxide), heat (70 °C) and photolytic degradation. The chromatographic method was fine tuned using the samples generated from forced degradation studies. Good resolution between the peaks corresponds to degradation products and the analyte was achieved on 5 μm ODS column (Luna, Phenomenex, USA). The mobile phase consists of a mixture of acetonitrile and water (60:40, v/v). Quantitation was achieved with UV detection at 235 nm based on peak area. The proposed HPLC method was utilized to investigate the kinetics of acidic, alkaline and oxidative degradation processes of DDB at different temperatures and the apparent pseudo first-order rate constant, half-life and activation energy were calculated. The pH-rate profiles of degradation of DDB in Britton–Robinson buffer solutions within the pH range 2–11 were studied. The developed method was validated with respect to linearity, accuracy, precision, robustness and forced degradation studies prove the stability-indicating power of the method.

1. Introduction

Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB) is a synthetic compound derived from Schizandrin C, a component of *Fructus schizandrae*. DDB protects liver against carbon tetrachloride-, D-galactosamine-, thioacetamide-, and prednisolone-induced hepatic injury in experimental animals, although the exact mechanism is not well characterized [1,2]. This compound has also been reported to be effective in improving liver function of patients with chronic hepatitis [3].

DDB is not official in any pharmacopoeia. In the literature, liquid chromatography/electrospray ion trap mass spectrometry (LC/ESI-MS/MS) method was used to investigate the metabolism and excretion of DDB in both rats and human plasma [4]. Microbore liquid chromatography and column switching method was developed for the analysis of DDB in human plasma [5]. Also, HPLC was used for determination of DDB in human serum [6,7]. In addition, separation of DDB enantiomers and its analogues was achieved using HPLC chiral column [8,9].

The International Conference on Harmonization (ICH) guideline entitled 'stability testing of new drug substances and products' requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance [10]. Hydrolytic, oxidative, heat and photolytic stability are required. DDB is an ester-type drug, which can be easily degraded. No method has been reported in the literature for the determination of DDB and its degradation products. Therefore, it was thought necessary to study the stability of DDB towards acidic, basic, heat, oxidative and photolytic degradation processes. The aim of this work was to develop stability indicating method for determination of DDB and its degradation products using HPLC to investigate the kinetics of the acidic, basic and oxidative degradation processes, and to calculate the activation energy for DDB degradation. The pH-rate profile of degradation of DDB in Britton–Robinson buffer solutions within the pH range 2–11 were studied.

2. Experimental

2.1. Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system

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controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20-μl loop and a SPD-10AVP UV–vis detector, separation and quantitation were made on a 250 mm × 4.6 mm (i.d.), 5 μm ODS column (Luna, Phenomenex, USA). The detector was set at λ 235 nm. Data acquisition was performed on class-VP software.

The IR spectrophotometer used was a Bruker Vector 22, Germany.

NMR spectra were recorded on a Varian Gemini 200 NMR spectrometer (200 mHz), USA.

2.2. Materials and reagents

Pharmaceutical grade of DDB (the Kyung-Dong Pharmaceutical Co., Kyungkido, Korea) was used and certified to contain 99.8%. Acetonitrile used was HPLC grade (BDH, Poole, UK). Sodium hydroxide, phosphoric, methanol, hydrochloric, citric and boric acids were analytical grade.

DDB pills (Beijing Pharmaceutical Factory, P.R. China) (Batch No. 1012005) contains 1.5 mg DDB per pill were used.

2.3. HPLC conditions

The mobile phase was prepared by mixing acetonitrile and water in a ratio 60:40 (v/v). The flow rate was 1.5 ml min^{-1}. All determinations were performed at ambient temperature. The injection volume was 20 μl.

2.4. Preparation of the degradation products

2.4.1. Preparation of the alkali-induced degradation product

500 mg of DDB was firstly dissolved in 50 ml methanol and refluxed with 50 ml 0.2 M sodium hydroxide at 100 °C for 1 h. Subsequently the pH of the solution was adjusted to 2.3 using 1 M hydrochloric acid to precipitate the degradation product of DDB. The precipitate was filtered, washed with 1 M hydrochloric acid and dried under vacuum and protected from air and light. The dried precipitate was analyzed by IR and NMR and found to be 4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylic acid (DG_1) as a degradation product of DDB in alkaline medium.

2.4.2. Preparation of the acid-induced degradation product

500 mg of DDB was firstly dissolved in 50 ml methanol and refluxed with 50 ml 2 M hydrochloric acid at 100 °C for 14 h. Subsequently the pH of the solution was adjusted to 2.3 using 2 M sodium hydroxide to precipitate the degradation product of DDB. The same procedure for separation of DG_1 previously described under preparation of the alkali-induced degradation product was followed.

2.4.3. Preparation of the oxidative degradation product

Accurately weighed 500 mg of DDB were dissolved in 90 ml methanol. Subsequently, 1 ml of hydrogen peroxide 33.3% (v/v) was added and the solution was completed to 100 ml with methanol and refluxed at 100 °C for 2 h. The solvent was evaporated and the residue was dried under vacuum and protected from air and light. HPLC analysis of the dried precipitate indicate the occurrence of one major component that is identified by IR and NMR as dimethyl-3,3′-dihydroxy-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DG_2) and three minors peak (m_1, m_2, m_3). Further purification of (DG_2) was carried out using a 250 mm × 10 mm (i.d.), 5 μm a reversed phase C_{18} semi-preparative HPLC column (Luna, Phenomenex, USA) using acetonitrile:water (60:40, v/v) as an eluent at a flow rate of 2 ml min^{-1} using UV detection at 235 nm. The purified compound was analyzed by IR and NMR and found to be dimethyl-3,3′-dihydroxy-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DG_2) as a oxidative degradation product of DDB.

2.4.4. Effect of electrical light on stability of DDB

Accurately weighed 100 mg of DDB were dissolved in 100 ml methanol. The solution was exposed to electrical light of tungsten lamp (40 W) for 7 days.

2.4.5. Effect of dry heat on stability of DDB

Susceptibility of the drug to dry heat was studied by exposing the drug to 70 °C for 7 days. Powder and aqueous solution (without pH adjustment) were tested.

2.5. Standard solutions and calibration

Stock standard solutions were prepared by dissolving 25 mg of DDB, DG_1 and DG_2 separately in 25 ml of methanol. The standard solutions were prepared by dilution of the stock standard solutions with the mobile phase to reach concentration ranges of 1–40, 0.05–40, 0.1–40 μg ml^{-1} for DDB, DG_1 and DG_2, respectively.

Triplicate 20 μl injections were made for each concentration and chromatographed under the conditions described above. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph.

2.6. Sample preparation

Fifty pills were weighed and finely powdered. A portion of the powder equivalent to about 25 mg of DDB was accurately weighed, dissolved and diluted to 50 ml with methanol. The sample solution was filtered. Further dilutions of the sample solution were carried out with the mobile phase to reach the linearity range specified for DDB. The general procedures described under calibration were followed and the concentration of DDB was calculated.

2.7. Kinetic investigation of acidic and alkaline degradation

Accurately weighed 50 mg of DDB was dissolved in 250 ml methanol. Separate 2 ml aliquots of above solution were transferred into separate stoppered conical flasks and mixed with 2 ml of 0.2 M sodium hydroxide or 2 M hydrochloric acid. The flasks were placed in a thermostatic oven at different temperatures (90, 85, 80, 70, 60 °C for acidic degradation and 80, 70, 60, 50, 40 °C for alkaline degradation) for different time intervals. At the specified time intervals, the contents of the flasks were neutralized to pH 7.0 using predetermined volumes of 1 M sodium hydroxide and 0.1 M hydrochloric acid solutions. The contents of the flasks were transferred into 10 ml volumetric flasks and diluted to volume with mobile phase. Aliquots of 20 μl of each solution were chromatographed under the conditions described above and the concentration of the remaining DDB was calculated at each temperature and time interval.

2.8. Kinetic investigation of oxidative degradation

Accurately weighed 50 mg of DDB was dissolved in 200 ml methanol. Subsequently, 2.5 ml of hydrogen peroxide 33.3% (v/v) were added and the volume was completed to 250 ml with methanol. For each temperature investigation, separate 2 ml aliquots of the above solution were transferred into separate covered conical flasks. The flasks were placed in thermostatic oven at different temperatures (90, 85, 80, 70, and 60 °C) for different time

intervals. At specified time intervals, the contents of the flasks were quantitatively transferred to 10 ml volumetric flasks and diluted to volume with mobile phase. Aliquots of 20 μl of each solution were chromatographed under the conditions described above and the concentration of the remaining DDB was calculated at each temperature and time interval.

2.9. pH-rate profile

Accurately weighed 40 mg of DDB was transferred into 100 ml volumetric flask, dissolved and diluted to volume with methanol. Separate 1 ml aliquot of DDB solution and 2 ml of Britton–Robinson buffer solutions [11] were transferred into stoppered conical flasks. The pH values of the buffer solutions used for measurement of the pH-rate profile of the degradation of DDB are within pH 2–11 in one unit pH intervals. The flasks were placed in a thermostatic oven at 80 °C for different time intervals. At the specified time intervals the contents of flasks were neutralized using 1 M sodium hydroxide or 1 M hydrochloric acid solutions. The contents of flasks were transferred into 10 ml volumetric flasks and diluted to volume with the mobile phase. Aliquot of 20 μl of each solution was chromatographed under the conditions described above and the concentration of the remaining DDB was calculated at each pH value and time interval.

3. Results and discussion

3.1. Identification of the degradation products

When DDB was boiled with 0.1 M sodium hydroxide for 1 h or 1 M hydrochloric acid for 14 h, 4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylic acid (DG_1) could be isolated from the reaction mixture as degradation product of DDB. However, when boiled with 0.33% hydrogen peroxide, dimethyl-3,3′-dihydroxy-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DG_2) could be isolated from the reaction mixture as major degradation product of DDB and minor degradation products (m_1, m_2, m_3) are formed (Fig. 1) which was difficult to be isolated from the reaction mixture. Also, degradation was observed on exposing the drug to dry heat at 70 °C for 1 week (powder and aqueous solution (without pH adjustment), where DG_1 is the only observed degradation product (Fig. 2). However, no degradation was observed on exposing the drug to electrical light of tungsten lamp (40 W) for 7 days.

The suggested pathways for the degradation of DDB in 0.1 M sodium hydroxide, 1 M hydrochloric acid and in the presence of hydrogen peroxide are presented in Scheme 1.

The assignments of the degradation products DG_1 and DG_2 as 4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylic acid and dimethyl-3,3′-dihydroxy-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate, respectively, were based on the comparison of the IR and NMR spectral data of the purified specimens, separated from the degradation reaction, with those of the intact DDB.

The IR spectrum (KBr) of DDB was characterized by the absorption frequency of C=O ester band at 1716 cm^{-1}. On the other hand the IR spectrum (KBr) of DG_1 revealed the OH association, C=O stretching and in-plane C–O bending of the COOH at 2371–3421, 1672 and 1306 cm^{-1}, respectively. Moreover, the spectrum lacked the characteristic ester C=O. However, the IR spectrum (KBr) of DG_2 was characterized by the absorption frequency of C=O ester band at 1729 cm^{-1} and OH group at 3436 cm^{-1}.

The NMR spectrum of DDB in dimethylsulphoxide was characterized by the appearance of the protons of the ester methyl chain

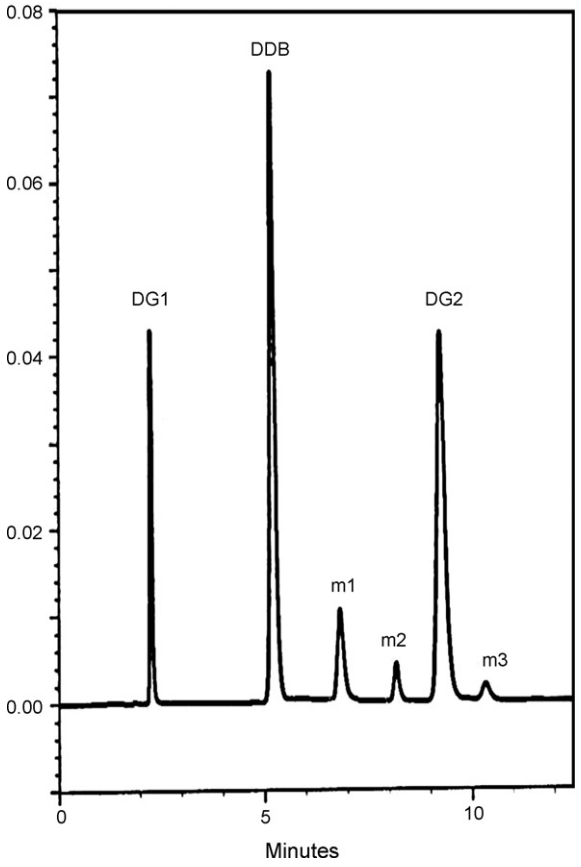


Fig. 1. HPLC chromatogram of 20 μl injection of laboratory-prepared mixture of DDB and its degradation products DG_1, DG_2, m_1, m_2, and m_3.

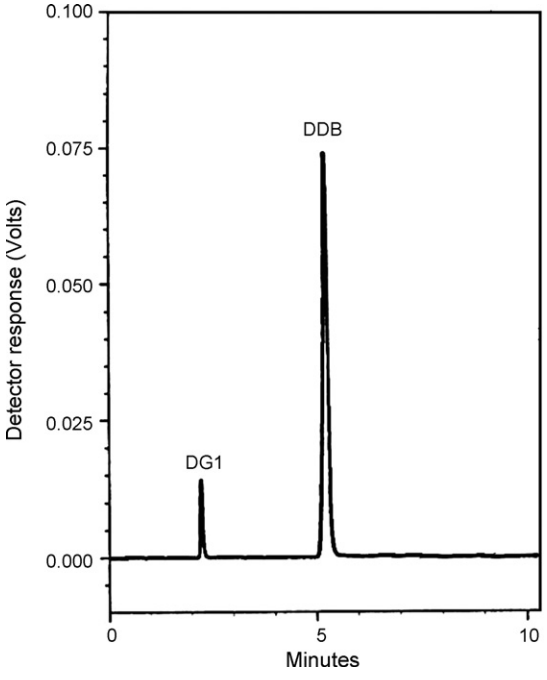
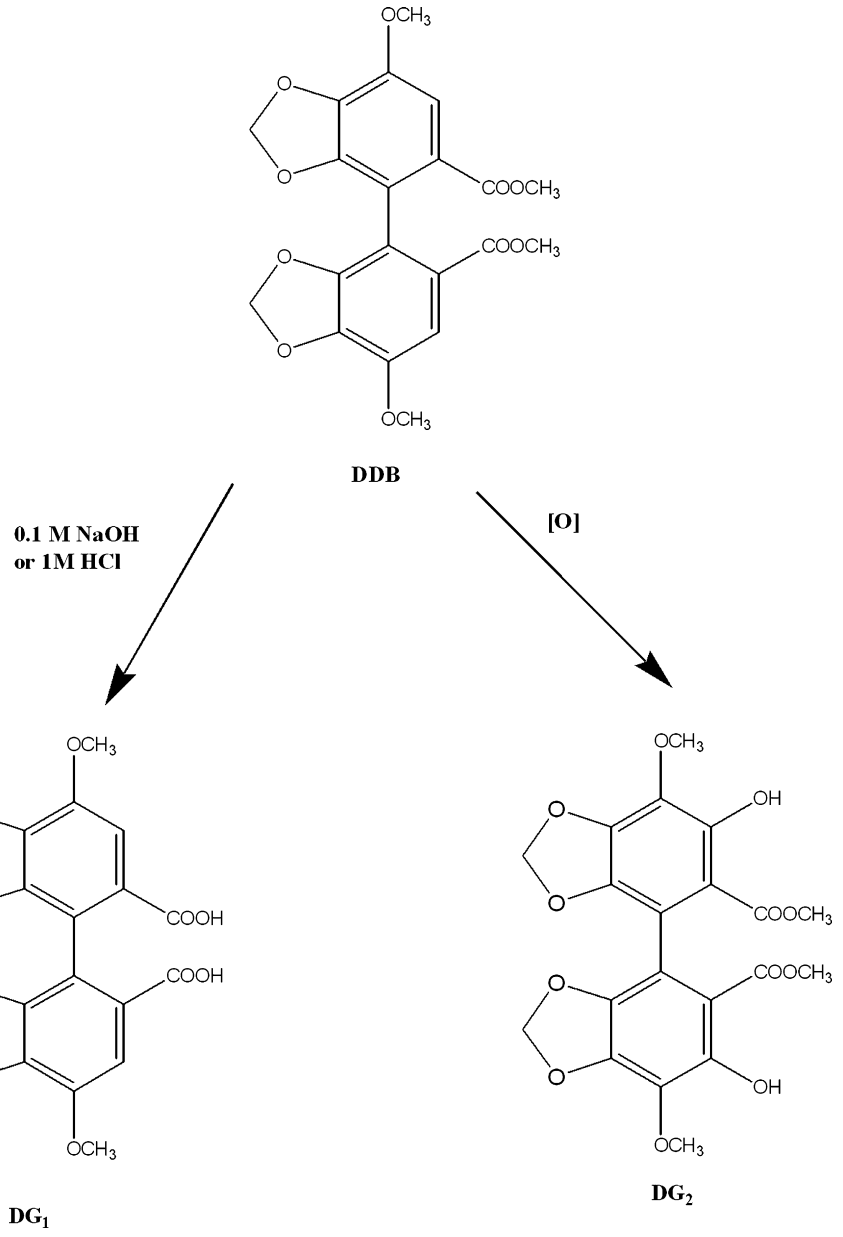


Fig. 2. Typical HPLC chromatogram of 20 μl injection of DDB and its dry heat induced degradation product (DG_1).



Scheme 1. Suggested pathway for the degradation of DDB in 0.1 M NaOH, 1 M HCl or in 0.33% hydrogen peroxide.

at δ 3.590 ppm (singlet, 6H, CH_3–COO); the protons of methoxy groups at δ 3.903 ppm (singlet, 6H, OCH_3), protons of dimethylene groups at δ 5.993–6.025 ppm (doublet, 4H, O–CH_2–O–) and aromatic protons at δ 7.262 ppm (singlet, 2H, aromatic C_3–*H*, C'_3–*H*). By contrast the NMR spectrum of DG_1 in the same solvent lacked the characteristic ester methyl chain protons signals and showed the protons of methoxy groups at δ 3.881 ppm (singlet, 6H, OCH_3), protons of dimethylene groups at δ 5.955–5.992 ppm (doublet, 4H, O–CH_2–O–) and aromatic protons at δ 7.269 ppm (singlet, 2H, aromatic C_3–*H*, C'_3–*H*). However, the NMR spectrum of DG_2 in the same solvent lacked the aromatic protons characteristic and showed the protons of hydroxyl groups at δ 3.363 ppm (singlet, 2H, O*H*), the protons of hydroxyl groups at δ 3.363 ppm were exchangeable with deuterated water (D_2O), the ester methyl chain at δ 3.624 ppm (singlet, 6H, CH_3–COO), methoxy groups at δ 4.053 ppm (singlet, 6H, OCH_3) and protons of dimethylene groups at δ 6.079–6.156 ppm (doublet, 4H, O–CH_2–O–).

3.2. Assay parameters

The UV absorption spectra of DDB and its degradation products DG_1 and DG_2 are overlapped (Fig. 3). The simultaneous determination of DDB, DG_1 and DG_2 by conventional, derivative and derivative ratio spectrophotometry is hindered by strong spectral overlap throughout the wavelength range. The method used to resolve a complex mixture of such compounds is mainly HPLC.

The developed HPLC method has been applied for the separation of DDB, DG_1, DG_2 and other minors degradation products (m_1, m_2, m_3). To optimize the HPLC assay parameters, the mobile phase composition was studied. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile–water (60:40, v/v) at ambient temperature. Increasing acetonitrile concentration to more than 80% led to inadequate separation of DDB and its degradation products. At lower acetonitrile concentration (<40%) separation was occurred but with excessive tailing for DDB and DG_2. Quantita-

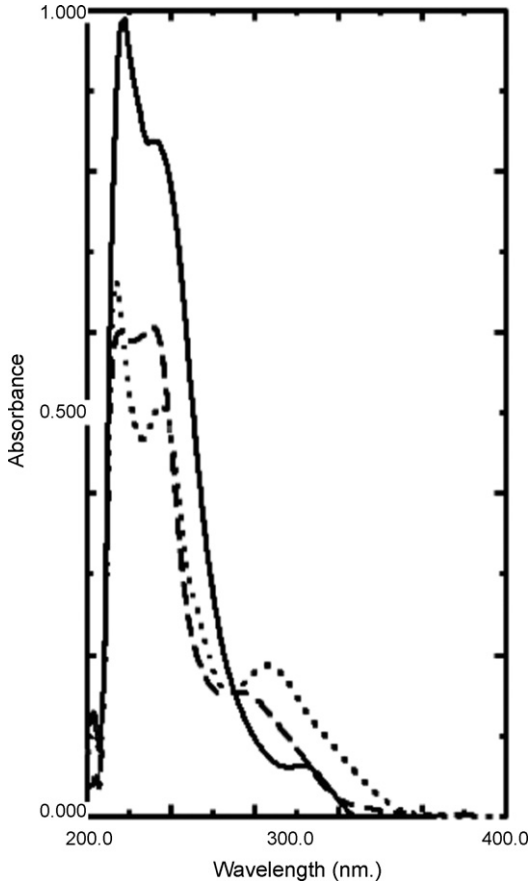


Fig. 3. UV absorption spectra of 10 μg ml^{-1} of DDB (- - -), 10 μg ml^{-1} of DG_1 (—) and 10 μg ml^{-1} of DG_2 (– – –) in methanol.

tion was achieved with UV detection at 235 nm based on peak area. The specificity of the HPLC method is illustrated in Fig. 1 where complete separation of DDB and its degradation products was noticed. The average retention time ± S.D. for DG_1, DDB, m_1, m_2, DG_2 and m_3 were found to be 2.3 ± 0.005, 5.2 ± 0.006, 6.9 ± 0.004, 8.2 ± 0.006, 9.3 ± 0.006 and 10.3 ± 0.006 min, respectively, for 10 replicates.

The system suitability test results of the developed method are presented in Table 1.

3.3. Analysis of pharmaceutical product

The proposed HPLC method was applied to the determination of DDB in freshly prepared pills. Seven replicates determinations were made. Satisfactory results were obtained for DDB in a good agreement with the label claims (Table 2). Typical chromatogram obtained for the quantitative analysis of the DDB in freshly prepared pills was very similar to that presented in Fig. 1, except that the degradation products could not be detected. The results of analysis of DDB in pharmaceutical product, obtained by the proposed method, were compared with that obtained by the published HPLC method [7] for determination of DDB. Statistical comparison of the results was performed with regards to accuracy and precision using Student's *t*-test and the *F*-ratio at 95% confidence level (Table 2). There was no significant difference between the results.

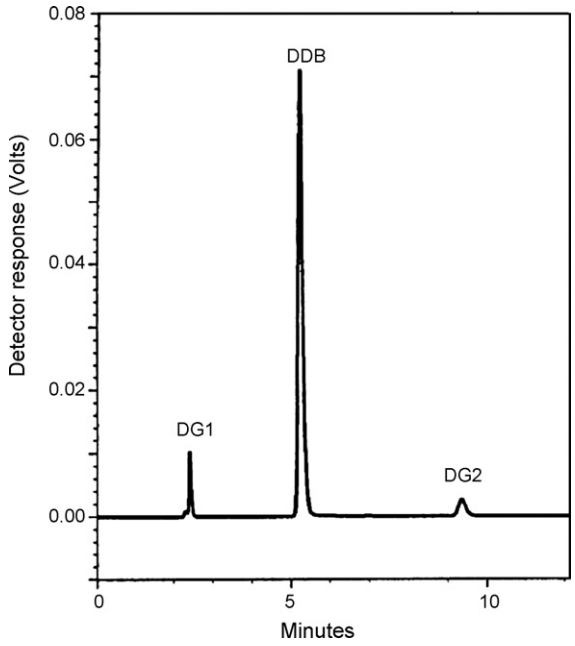


Fig. 4. Typical HPLC chromatogram of expired DDB pills containing DDB and its degradation products (DG_1 and DG_2).

Expired batch of DDB pills® stored at ambient temperature under normal conditions was analyzed by the proposed HPLC method. The DG_1 and DG_2 as degradation products of DDB were found clearly (Fig. 4). The mean percentage of DDB ± S.D. (n = 7) was found to be 92.1 ± 0.72% and the mean concentration ± S.D. (n = 7) of DG_1 and DG_2 were found to be 63 ± 0.65 and 54 ± 0.49 μg/pill, respectively.

3.4. Kinetic investigation

The kinetics of degradation of DDB were investigated in 0.33% hydrogen peroxide, 0.1 M sodium hydroxide and 1 M hydrochloric acid since the decomposition rate of DDB at lower strengths of hydrochloric acid were too slow to obtain reliable kinetic data. A regular decrease in the concentration of intact DDB with increasing time intervals was observed. At the selected temperatures, the acidic, alkaline and oxidative degradation processes followed pseudo first-order kinetics (Fig. 5). From the slopes of the straight lines, it was possible to calculate the apparent first

Table 1
The system suitability test results of the developed method for determination of DG_1, DDB, m_1, m_2, DG_2 and m_3

Compound	Retention time (min)	Capacity factor (k')	Selectivity α	Resolution R_s [15,16]	Tailing factor [15,16]	%R.S.D. of retention time	Plate count
DG_1	2.3	0.92	3.62 (a_1)	2.84 (b_1)	1.01	0.22	5,102
DDB	5.2	3.33	1.43 (a_2)	2.01 (b_2)	1.10	0.12	7,025
m_1	6.9	4.75	1.23 (a_3)	2.30 (b_3)	1.15	0.06	9,148
m_2	8.2	5.83	1.16 (a_4)	1.88 (b_4)	1.17	0.07	15,544
DG_2	9.3	6.75	1.12 (a_5)	2.77 (b_5)	1.13	0.06	10,409
m_3	10.3	7.58			1.19	0.06	14,695

The retention time of unretained peak is 1.2 min. a_1, b_1: are α and R_s calculated for DG_1–DDB. a_2, b_2: are α and R_s calculated for DDB–m_1. a_3, b_3: are α and R_s calculated for m_1–m_2. a_4, b_4: are α and R_s calculated for m_2–DG_2. a_5, b_5: are α and R_s calculated for DG_2–m_3.

Table 2
Determination of DDB, DG_1 and DG_2 in laboratory-prepared mixtures and commercial pharmaceutical product using the proposed HPLC method

	Mean found ± S.D.[a]			
	Proposed HPLC method			Published method [7]
	DDB	DG_1	DG_2	
Laboratory-prepared mixture	100.4 ± 0.51	100.8 ± 0.65	99.9 ± 0.71	
DDP pills	101.6 ± 0.65			101.3 ± 0.75
t	0.80			(2.18)[b]
F	1.33			(4.28)[b]
Recovery[c]	100.7 ± 0.61	101.1 ± 0.80	100.5 ± 0.55	

[a] Mean and S.D., percentage recovery from the label claim amount.
[b] Theoretical values for *t* and *F*.
[c] For standard addition of 50% of the nominal content.

order degradation rate constant and the half-life at each temperature for acidic, alkaline and oxidative degradation processes of DDB (Table 3). Plotting $\log K_{obs}$ values versus $1/T$, the Arrhenius plots (Fig. 6) were obtained, which were found to be linear in the temperature range 60–90 °C for the acidic, 40–80 °C for alkaline degradation and 60–90 °C for oxidative degradation. The activation energy was calculated for DBB and found to be 19.91 kcal mol^{-1} for acidic degradation process, 8.75 kcal mol^{-1} for alkaline degradation process and 16.43 kcal mol^{-1} for oxidative degradation of DDB.

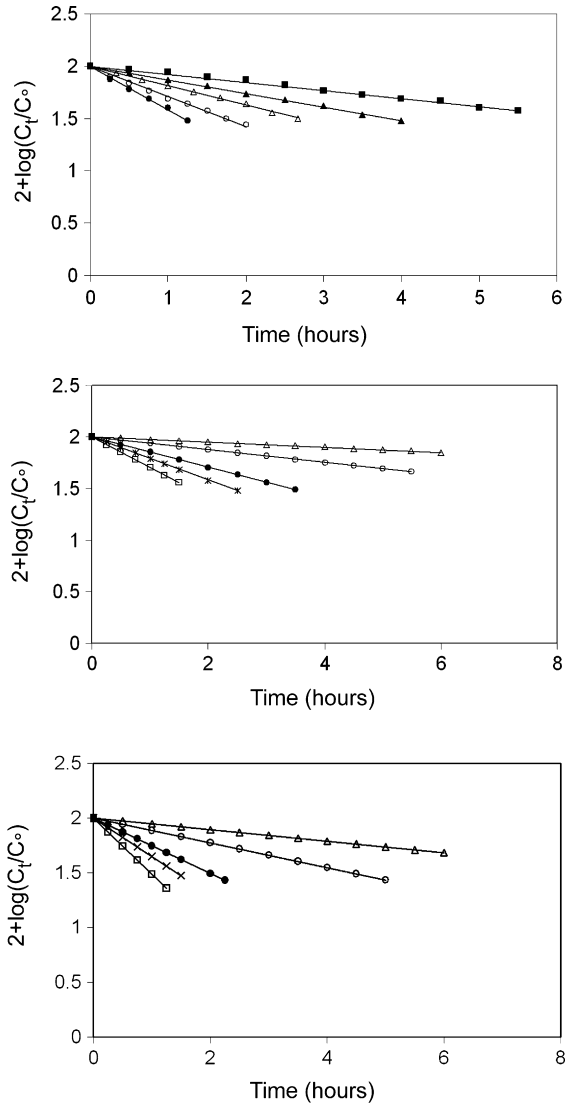


Fig. 5. Pseudo first-order plots for the degradation of DDB in 0.1 M sodium hydroxide (a), in 1 M hydrochloric acid (b) and in 0.33% hydrogen peroxide (c) at various temperatures using the proposed HPLC method. Key: 40 (■), 50 (▲), 60 (Δ), 70 (○), 80 (●), 85 (×) and 90 (□); C_t, concentration at time *t*, and C_0, concentration at zero time.

Table 3
Degradation rate constant (K_{obs}) and half-life ($t_{1/2}$) for DDB in 1 M hydrochloric acid, 0.1 M sodium hydroxide and 0.33% hydrogen peroxide determined by the proposed HPLC

Temperature (°C)	K_{obs} (h^{-1})	$t_{1/2}$ (h)
In 1 M hydrochloric acid		
60	0.058	11.989
70	0.143	4.853
80	0.338	2.048
85	0.482	1.437
90	0.684	1.014
In 0.1 M sodium hydroxide		
40	0.184	3.761
50	0.299	2.318
60	0.427	1.623
70	0.629	1.102
80	0.932	0.744
In 0.33% hydrogen peroxide		
60	0.121	5.721
70	0.260	2.663
80	0.583	1.189
85	0.806	0.860
90	1.182	0.586

The pH-rate profiles of degradation of DDB in Britton–Robinson buffer solutions were studied at 80 °C (Fig. 7). Britton–Robinson buffer solutions were used throughout the entire pH range in order

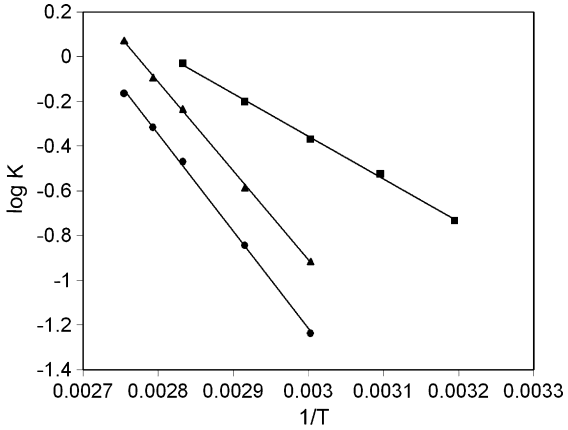


Fig. 6. Arrhenius plots for the degradation of DDB in 0.1 M sodium hydroxide (■), 1 M hydrochloric acid (●) and in 0.33% hydrogen peroxide (▲) using the proposed HPLC method.

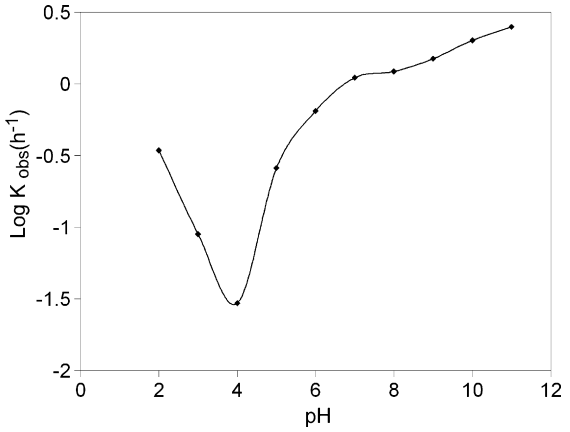


Fig. 7. pH-rate profile for the degradation of DDB in Britton–Robinson buffer at 80 °C.

Table 4
Degradation rate constant (K_{obs}) and half-life ($t_{1/2}$) for DDB in Britton–Robinson buffer at different pH values at 80 °C

pH	K_{obs} (h^{-1})	$t_{1/2}$ (h)
2.0	0.149	2.016835
3.0	0.039	7.75546
4.0	0.013	23.50874
5.0	0.112	2.681924
6.0	0.281	1.07277
7.0	0.480	0.626378
8.0	0.530	0.567758
9.0	0.650	0.462941
10.0	0.875	0.344057
11.0	1.084	0.277492

to avoid possible effects of different buffer species. The apparent first order degradation rate constant and the half-life were calculated for each pH value (Table 4). DDB were found to be most stable at a pH of 4.0.

3.5. Validation of the method

3.5.1. Linearity

The linearity of the HPLC detector response for determination of DDB, DG_1 and DG_2 was evaluated by analyzing a series of different concentrations of each compound. Seven concentrations were chosen, ranging between 1–40, 0.05–40, 0.1–40 μg ml^{-1} for DDB, DG_1 and DG_2, respectively.

Each concentration was repeated three times; this approach provided information on the variation in peak area between samples of same concentration. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p < 0.05$) different from zero. Characteristic parameters for regression equations obtained by least squares treatment of the results are given in Table 5.

Upon AMC (Analytical Methods Committee), a value of regression coefficient close to unity is not necessarily the outcome of a linear relationship and in consequence the test for the lack of fit should be checked [12] (Table 6). This test evaluates the variance of the residual values [13]. The calculated values were lower than the tabulated ones ($\alpha = 0.05$), linearity thus being demonstrated.

3.5.2. Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level were evaluated by one-way ANOVA. An 8 days × 2 replicates design was performed. Statistical comparison of the results was performed using the *p*-value of the *F*-test. Three univariate analyses of variance for each concentration level were made. Since the *p*-value of the *F*-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level.

3.5.3. Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in pharmaceutical product, to give accurate, precise and linear results. The calibration range of each compound is given in Table 5.

3.5.4. Detection and quantitation limits

According to ICH recommendations [14], the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and are given in Table 5.

3.5.5. System suitability tests

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. The following parameters are used for system suitability evaluation [15,16].

3.5.5.1. Capacity factor (k′). Capacity factor (retention factor) is a measure of the retention time of a compound in the sample with a given combination of mobile phase and column. It is defined as $k'_{(A)} = (t_A - t_0)/t_0$ in which t_A is the retention time of the compound and t_0 refers to retention time for an unretained compound. t_0 can

Table 5
Characteristic parameters for the regression equations of the proposed HPLC method for determination of DDB, DG_1 and DG_2

Parameters	DDB	DG_1	DG_2
Calibration range (μg ml^{-1})	1–40	0.05–40	0.1–40
Detection limit (μg ml^{-1})	0.020	0.015	0.014
Quantitation limit (μg ml^{-1})	0.07	0.05	0.047
Regression equation (*Y*)[a]			
Slope (*b*)	29.41×10^3	34.20×10^3	30.47×10^3
Standard deviation of the slope (S_b)	2.69×10^2	219.38	186.53
Relative standard deviation of the slope (%)	0.92	0.64	0.61
Confidence limit of the slope[b]	29.14×10^3–29.68×10^3	33.99×10^3–34.40×10^3	30.30×10^3–30.65×10^3
Intercept (*a*)	19.78×10^3	28.71×10^2	-22.19×10^2
Standard deviation of the intercept (S_a)	22.30×10^3	53.71×10^2	31.11×10^2
Confidence limit of the intercept[b]	-18.83×10^2–41.44×10^3	-20.97×10^2–78.38×10^3	-50.97×10^2–6.58×10^2
Correlation coefficient (*r*)	0.9997	0.9997	0.9998

[a] $Y = a + bC$, where *C* is the concentration in μg ml^{-1} and *Y* is the peak area.
[b] 95% confidence limit.

Table 6
ANOVA (showing lack of fit calculation) for DDB, DG_1 and DG_2

Compound	Source of variation	Sum of squares	Degree of freedom	Mean sum of squares	*F*-Ratio
DDB	Total	1.40×10^{12}	21	6.67×10^{10}	1.35
	Regression	1.40×10^{12}	2	7.60×10^{11}	
	Residual	8.41×10^{8}	19	4.40×10^{7}	
	Replicate	2.54×10^{8}	7	3.62×10^{7}	
	Lack of fit	5.88×10^{8}	12	6.90×10^{7}	
DG_1	Total	2.36×10^{12}	21	1.12×10^{11}	1.55
	Regression	2.36×10^{12}	2	1.18×10^{12}	
	Residual	8.32×10^{7}	19	4.38×10^{6}	
	Replicate	2.27×10^{8}	7	3.25×10^{6}	
	Lack of fit	6.05×10^{7}	12	5.04×10^{6}	
DG_2	Total	1.12×10^{12}	21	5.36×10^{10}	0.94
	Regression	1.12×10^{12}	2	5.62×10^{10}	
	Residual	3.61×10^{7}	19	1.90×10^{6}	
	Replicate	1.38×10^{7}	7	1.97×10^{6}	
	Lack of fit	2.23×10^{7}	12	1.86×10^{6}	

The critical value of *F*-ratio is 3.57 at $\alpha = 0.05$.

be calculated by observing the initial baseline deflection of the trace above and below the baseline, caused by the difference in sample solution composition and the mobile phase. When an entail baseline deflection of this shape is observed, it is safe to assure that this correspond to t_0 [17]. In the present work, t_0 was 1.2 min. For an optimum separation, retention factor should be in the range of $0.5 < k' < 10$. Calculated k' values are represented in Table 1.

3.5.5.2. Selectivity factor (α). Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. For two components A and B it is defined as $\alpha = k'_B/k'_A$ (k' is the respective capacity factor). Therefore, it is the ratio of the relative retentions of the two compounds. In the present study the calculated selectivity parameter for separation of each compound is represented in Table 1.

3.5.5.3. Resolution (R). Resolution is a measure of the degree of separation between adjacent peaks. For two compounds A and B in a chromatographic run it is expressed as $R = 1.18\,(t_{r,b} - t_{r,a})/(w_{0.5,a} + w_{0.5,b})$ in which $t_{r,a}$ and $t_{r,b}$ = retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks, and $w_{0.5,a}$ and $w_{0.5,b}$ = peak widths at half-height. A value of 1.5 for resolution implies a complete separation of two compounds [15,16]. In this work, the resolution value for the separation of each compound was greater than 1.5 and is represented in Table 1.

3.5.5.4. Column efficiency (number of theoretical plates). In a particular separation, column efficiency refers to the performance of the stationary phase. It means how well the column is packed. There are several methods to measure the column efficiency, which may or may not be affected by chromatographic anomalies, such as tailing or fronting. In the present study the number of theoretical plates was calculated using the following equation: $N = 5.54(t_R/w_{0.5})^2$ in which $w_{0.5}$ is width of the peak at half-height and t_R is retention time along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component. The *N* values for each compound are represented in Table 1.

3.5.5.5. Tailing factor (T). Tailing factor refers to peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. Therefore, tailing factor should be calculated using the following equation for chromatographic peaks: $T = w_{0.05}/2d$ in which $w_{0.05}$ is the width of the peak at one-twentieth of the peak height and *d* is distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height. A tailing factor of 1 refers to a symmetric peak. The calculated value for the tailing factor for each compound is in the acceptable range of $0.8 \leq T \leq 1.5$ [15,16] and is represented in Table 1.

3.5.5.6. Relative standard deviation of peak area of six injections of 1 μg ml^{-1} of standards. The USP defines the relative standard deviation (R.S.D.) of peak area for repetitive injections as one of the parameters that can determine system suitability prior to analysis [16]. For an assay method, the R.S.D. typically should be less than 1% for these repetitive injections. The mean of peak area ± R.S.D. of six injection of 1 μg ml^{-1} of each of DDB, DG_1 and DG_2 were found to be $33.80 \times 10^3 \pm 0.87$, $34.85 \times 10^3 \pm 0.91$ and $30.16 \times 10^3 \pm 0.85$, respectively.

3.5.6. Selectivity

Method selectivity was achieved by preparing different mixtures of DDB, DG_1 and DG_2 within the linearity range concentration. The laboratory-prepared mixtures were analyzed according to the previous procedure described under the proposed HPLC method. Satisfactory results were obtained (Table 2), indicating the high selectivity of the proposed method for determination of DDB, DG_1 and DG_2.

3.5.7. Accuracy

The accuracy study was performed by addition of known amounts DDB, DG_1 and DG_2 to a known concentration of the commercial pills (standard addition method). The resulting mixtures were assayed and results obtained for DDB, DG_1 and DG_2 were compared with expected results.

The excellent recoveries of standard addition method (Table 2) suggest that good accuracy of the proposed HPLC method.

3.5.8. Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered and the resolution between DDB, DG_1 and DG_2 was evaluated. The flow rate of the mobile phase was 1.5 ml min^{-1}. To study the effect of flow rate on the resolution, it was changed by 0.2 units from 1.3 to 1.7 ml min^{-1}, while the mobile phase components were held constant as stated in Section 2.3. The effect of the percent of organic strength on resolution was studied by varying acetonitrile from 57 to 63%. In all the deliberate varied chromatographic conditions (flow rate,

percentage organic strength), the resolution between DDB and its degradation products was not altered, illustrating the robustness of the method.

3.5.9. Stability

The stability of DDB, DG_1 and DG_2 standard solutions in the mobile phase were evaluated by leaving the standard solutions in tightly capped volumetric flasks, protected from light, on a laboratory bench and in the refrigerator. The studied compound solutions in mobile phase exhibited no chromatographic changes for 24 h when kept at room temperature and for 3 days when stored refrigerated at 5 °C.

4. Conclusion

The proposed HPLC method provides simple, accurate and reproducible quantitative analysis for the determination of DDB and its degradation products (DG_1, DG_2) in pharmaceutical product, without any interference from the excipients. The proposed method can be applied in laboratories lacking sophisticated instruments such as GC–MS or LC–MS. The suggested method can be simply applied to accelerated stability study to predict expiry dates of pharmaceuticals. DDB is rapidly degraded in alkaline medium and in the presence of hydrogen peroxide while it is more stable in acidic medium. The optimum stability of DDB was found to be at pH 4.0.

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