Original Research Paper

Acta Chromatographica 27(2015)3, 477–493
DOI: 10.1556/AChrom.27.2015.3.6

Validated Selective HPLC–DAD Method for the Simultaneous Determination of Diclofenac Sodium and Lidocaine Hydrochloride in Presence of Four of Their Related Substances and Potential Impurities

T.S. BELAL[1,*], M.M. BEDAIR[1], A.A. GAZY[1], AND K.M. GUIRGUIS[2]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, University of Alexandria, Elmessalah 21521, Alexandria, Egypt
[2]Pharmaceutical Chemistry Department, Faculty of Pharmacy and Drug Manufacturing, Pharos University in Alexandria, Canal El-Mahmoudia Street, Alexandria, Egypt
*E-mail: tbelaleg@yahoo.com

Summary. This study presents a selective high-performance liquid chromatography (HPLC) with diode array detection (DAD) method for the simultaneous estimation of diclofenac sodium and lidocaine hydrochloride in presence of four of their related substances and potential impurities, namely, 2,6-dimethylaniline (DMA), 2,6-dichloroaniline (DCA), N-phenyl-2,6-dichloroaniline (PDCA), and N-chloroacetyl-N-phenyl-2,6-dichloroaniline (CPDCA). Some of these related substances are reported as degradation products as well. Effective chromatographic separation was achieved using Waters Symmetry C18 column, (3.9 × 150 mm, 5 µm particle size) with gradient elution of the mobile phase composed of 0.05 M orthophosphoric acid and acetonitrile. The gradient elution started with 5% (by volume) acetonitrile, ramped up linearly to 65% in 5 min then kept constant till the end of the run. The mobile phase was pumped at a flow rate of 1.5 mL min^{-1}. The multiple wavelength detector was set at 220 nm, and quantification of both drugs was based on measuring their peak areas. The retention times for lidocaine and diclofenac were about 5.5 and 9.5 min, respectively. The reliability and analytical performance of the proposed HPLC procedure were statistically validated with respect to system suitability, linearity, ranges, precision, accuracy, specificity, robustness, detection, and quantification limits. Calibration curves were linear in the ranges of 10–200 µg mL^{-1} for both drugs with correlation coefficients not less than 0.9998. The proposed method proved to be selective by resolution of the two drugs from their related substances and potential impurities. The validated HPLC method was successfully applied to the analysis of this binary mixture in the combined formulation (ampoules dosage form), and the assay results were favorably compared with a previously reported HPLC method. The proposed method made use of DAD as a tool for peak identity and purity confirmation.

Key Words: diclofenac sodium, lidocaine hydrochloride, HPLC–DAD, related substances, potential impurities

Introduction

Diclofenac sodium (DC) *(Fig. 1)* is a phenylacetic acid derivative nonsteroidal anti-inflammatory drug. It is used for the relief of pain and inflammation in various conditions: musculoskeletal and joint disorders such as rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis; periarticular disorders such as bursitis and tendinitis; soft-tissue disorders such as sprains and strains; and other painful conditions such as renal colic, acute gout, dysmenorrhea, and migraine [1]. DC is an official drug in both the British Pharmacopoeia (BP) [2] and the United States Pharmacopeia (USP) [3] where assay of the raw material is carried out by potentiometric nonaqueous titration. Different dosage forms are assayed in both pharmacopeias using high-performance liquid chromatography (HPLC) methods, while the prolonged-release capsules are assayed spectrophotometrically at 275 nm in the BP [2]. Moreover, the quantification of DC in its various drug formulations and/or biological samples has been addressed in many reports. Liquid chromatography using various detection modes has been widely applied. Examples of these reports are HPLC with ultraviolet (UV) detection [4, 5], HPLC with electrochemical detection [6], and HPLC with mass spectrometric detection [7]. In addition, other analytical techniques involved the use of potentiometric membrane sensors [8], differential-pulse voltammetry [9], spectrophotometry [10], spectrofluorimetry [11], Raman spectroscopy [12], gas chromatography–mass spectrometry (GC–MS) [13], high-performance thin-layer chromatography (HPTLC) [14], and capillary electrophoresis (CE) [15].

Lidocaine hydrochloride (LD) *(Fig. 1)* is a local anesthetic of the amide type. It is used for infiltration anesthesia and regional nerve blocks. LD is included in some injections to prevent pain, itching, and other local irritation [1]. The BP [2] and the USP [3] suggest several procedures for the assay of LD powder and dosage forms. Most BP procedures depend on titrimetry, while HPLC is described for the assay of the ointment form. On the other hand, HPLC is predominant in the USP monographs of LD and its dosage forms, while titrimetric procedures are used for the semisolid topical preparations (ointment and jelly). Several reports can be found in the scientific literature for determination of LD. Examples of the published analytical methods are electrochemical methods [16], spectrophotometry [17], capillary zone electrophoresis with diode array detection (DAD) [18], CE with electrochemiluminescence detection [19], GC with nitrogen phosphorus detection [20], and HPTLC [21]. Liquid chromatography has been utilized for the estimation of LD. Examples of the reported LC methods are ultra-performance liquid chromatography (UPLC) [22], HPLC with amperometric

detection [23], liquid chromatography–mass spectrometry (LC–MS/MS) [24], and HPLC with UV detection [25, 26].

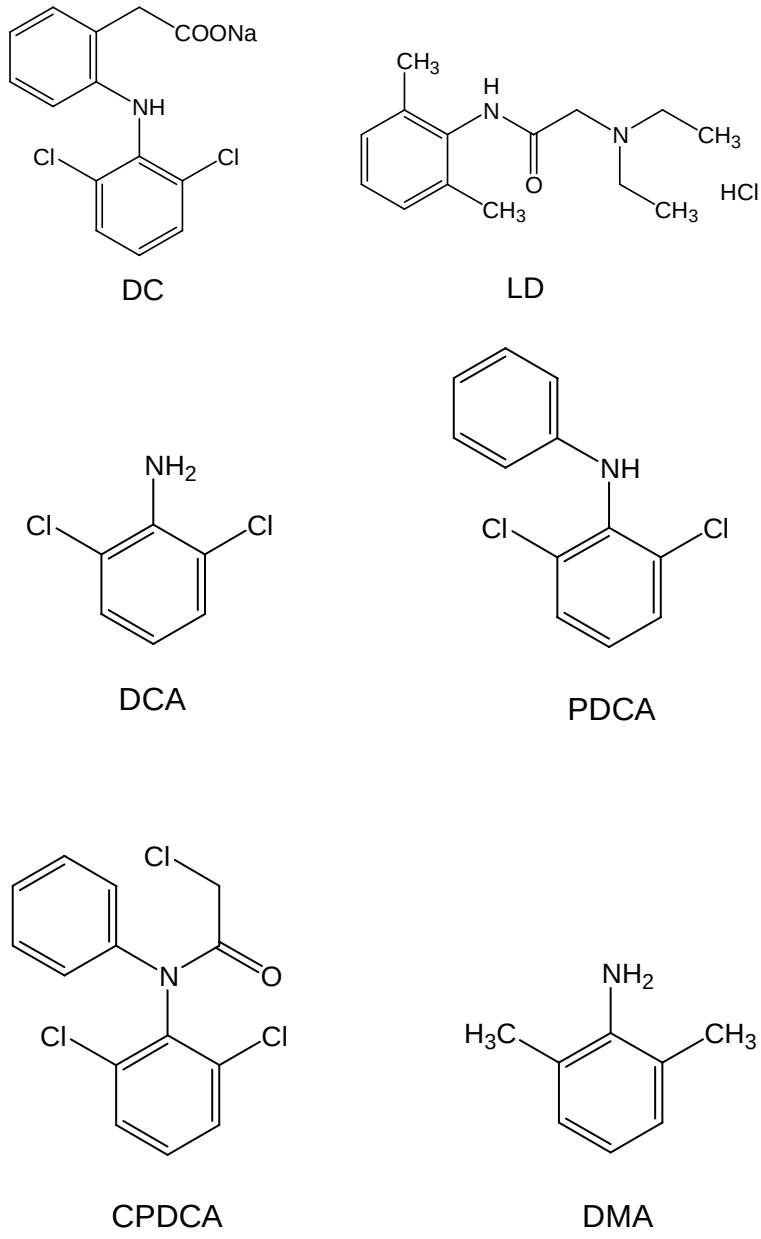

Fig. 1. Structures of diclofenac sodium (DC), lidocaine hydrochloride (LD), and the related substances 2,6-dichloroaniline (DCA), N-phenyl-2,6-dichloroaniline (PDCA), N-chloroacetyl-N-phenyl-2,6-dichloroaniline (CPDCA), and 2,6-dimethylaniline (DMA)

DC has several related substances and potential impurities that should be monitored. From these related substances, the three compounds, 2,6-dichloroaniline (DCA), N-phenyl-2,6-dichloroaniline (PDCA), and N-chloroacetyl-N-phenyl-2,6-dichloroaniline (CPDCA), can be considered precursors or intermediates during synthesis of DC [27, 28]. Moreover, previous studies reported that DCA is actually one of the degradation products of DC, and it is formed upon stress oxidative conditions [29, 30]. *Figure 1* shows the chemical structures of the related substances involved in this study. Very few reports can be found in the scientific literature for the determination of the drug in presence of these particular related substances. HPLC with UV detection has been used for the assay of DC in presence of PDCA and CPDCA [31]. Recently, HPLC with DAD has been applied for the stability indicating determination of DC in presence of its forced-degradation products as well as the three related substances DCA, PDCA, and CPDCA [32]. The related substance 2,6-dimethylaniline (also known as 2,6-xylidine, DMA) *(Fig. 1)* is well known as a potential impurity and a degradation product for LD [2, 33]. Several methods have been reported describing the stability-indicating analysis of LD in presence of its related substance (DMA). These reports suggested the use of HPLC with UV detection [34, 35], HPLC with amperometric detection [36], thin-layer chromatography (TLC)–densitometry [37], and derivative spectrophotometry [37]. Recently, several HPLC–DAD methods have been published for the stability-indicating assay of LD in presence of its related substance in several pharmaceutical combinations [38–40]. DC and LD have been coformulated in ampoules dosage form for the treatment of exacerbation of inflammatory and degenerative forms of rheumatism, rheumatoid arthritis, osteoarthritis, and other painful conditions. The simultaneous determination of the binary combination of DC and LD has been addressed in very few analytical reports. These reports proposed several reversed-phase HPLC methods [41, 42] and a micellar electrokinetic chromatographic (MEKC) method [43]. Reviewing the literature exposed that there were no reports for the simultaneous selective determination of DC and LD in presence of their related substances and potential impurities. This work introduces the development, validation, and application of a simple, selective, and reliable HPLC–DAD method for the simultaneous determination of DC and LD in bulk form and in their combined dosage form. The method was validated for its specificity by resolution of the parent drugs from the related substances DMA, DCA, PDCA, and CPDCA.

Experimental

Instrumentation

The HPLC–DAD system consisted of Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) (auto injector, quaternary pump, vacuum degasser and diode array, and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software. The column used was Waters Symmetry C18 (3.9 × 150 mm, 5 μm particle size).

Materials and Chemicals

DC and LD were kindly supplied by Alexandria Company for Pharmaceuticals, Alexandria, Egypt. The two related substances, PDCA and CPDCA, were kindly donated by Pharco Pharmaceuticals Company, Alexandria, Egypt. The related substances DCA and DMA were purchased form Sigma-Aldrich, St. Louis, MO, USA. HPLC-grade acetonitrile and methanol (Solvents Documentation Syntheses (SDS), Peypin, France), analytical grade of orthophosphoric acid, and high purity distilled water were used. The pharmaceutical preparation assayed in the study is Olfen® ampoules (Medical Union Pharmaceuticals, Abu Sultan, Ismailia, Egypt under license from Mepha Ltd., Basel, Switzerland, BN. 122805) labeled to contain 75 mg DC and 20 mg LD per ampoule.

General Procedure

A gradient mobile phase system consisting of (A) 0.05 M orthophosphoric acid and (B) acetonitrile was used. The separation was achieved with a linear gradient program as follows: 5% v/v B at zero time; from 0 to 5 min, ramp up to 65% v/v B; and from 5 to 15 min, holding 65% v/v B. After 15 min, the gradient program was returned to the initial conditions and the analytical column was reconditioned for 3 min. The flow rate was 1.5 mL min^{-1} all over the run. The injection volume was 20 μL. The eluant was monitored by the diode array detector from 190 to 400 nm, and chromatograms were extracted at the wavelength 220 nm. All determinations were performed at 25°C.

DC (1000 μg mL^{-1}) and LD (1000 μg mL^{-1}) stock solutions were prepared in distilled water. The working solutions were prepared by dilution of aliquots of the stock solutions with distilled water to reach the concentration range 10–200 μg mL^{-1} for both DC and LD. Triplicate injections were

made for each concentration and chromatographed under the previously described HPLC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration graphs.

Stock solutions of the four related substances (1000 μg mL^{-1}) were separately prepared in HPLC-grade methanol. Aliquots of these stock solutions were added to aliquots of the two parent compounds stock solutions, a volume of methanol (2 mL) was added to avoid precipitation of some components of the mixture, and the solution was diluted to volume with distilled water. This mixture was chromatographed under the previously described LC conditions.

Assay of Ampoules Dosage Form

The contents of three ampoules were mixed. The stock sample solution was prepared by transferring 1 mL of the mixture into a 10-mL volumetric flask, and then the solution was completed to volume with distilled water (3750 μg mL^{-1} DC and 1000 μg mL^{-1} LD). Aliquots of this stock sample solution were diluted with distilled water to obtain final concentrations within the specified range and then treated as under "General Procedure," and recovered concentrations were calculated from the corresponding external standard solutions. For standard addition assay, sample solutions were spiked with aliquots of standard solutions of the two drugs to obtain total concentrations within the previously specified range and then treated as under "General Procedure." Recovered concentrations were calculated by comparing the analyte response with the increment response attained after addition of the standard.

Results and Discussion

Optimization of Chromatographic Conditions

A gradient liquid chromatographic method coupled with DAD was developed to provide a suitable procedure for the routine quality control analysis of binary mixture of DC and LD. The developed method was carefully designed and optimized to separate the cited drugs from their related substances as well. The most important aspect in HPLC method development is the achievement of sufficient resolution of the target drugs from all other compounds present in the sample with acceptable peak symmetry in a reasonable analysis time. To achieve this goal, several experiments were carried out in order to optimize both the stationary and mobile phases. For optimization of the stationary phase, several reversed phase columns (Waters

Symmetry C8 (3.9 × 150 mm), Waters Symmetry C18 (3.9 × 150 mm), and Zorbax Eclipse XDB-C18 (4.6 × 150 mm)) were tested. All these columns managed to resolve the two analytes; however, the best clear separation between all the eluting peaks including the four related substances, symmetric peaks, and relatively shorter retention times was attained by using the Waters Symmetry C18 column; hence, it became the column of choice for this study.

Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers. The best mobile phase combination was 0.05 M orthophosphoric acid solution and acetonitrile. Methanol was tried as an organic modifier, and phosphoric acid solution was substituted by other aqueous phases such as water or acetic acid solution. In these trials, LD and DC suffered from increased retention times and some chromatograms showed broad peaks; additionally, poor separation was frequently observed between the analytes peaks and those of their related substances. Isocratic elution of different proportions of 0.05 M phosphoric acid and acetonitrile either did not provide adequate baseline separation between all the eluting peaks or resulted in longer retention times and broad asymmetric peaks; therefore, gradient elution was adopted. The gradient elution starts with high aqueous ratio and low acetonitrile ratio to ensure adequate separation between LD and its related substance (DMA), and then the organic modifier ratio increases linearly up to a certain value to allow separation of the remaining peaks in reasonable retention times. Several gradient programs were tried, and the best compromise among adequate resolution, reasonable retention times, and tolerable peak asymmetry was achieved using a gradient system starting with 5% (by volume) acetonitrile ramped up linearly to 65% in 5 min and then maintained at this percentage afterwards till the end of the run. Flow rate was kept constant at 1.5 mL min^{-1} all over the run, and column temperature was adjusted at 25 °C. It is noteworthy to mention that the applied gradient program produced almost stable baseline without excessive drift or deformation.

DAD enhances the power of HPLC and is an elegant option for assessing method specificity by monitoring the recorded spectra during peak elution. Quantification was achieved using the DAD based on peak area measurement. DC exhibits a broad absorption band all over the range 200–310 nm with maximum absorption at 276 nm. On the other hand, LD is considered a weak UV absorbing compound. It shows considerable absorbance only in the short UV region below 230 nm; consequently, 220 nm was found suitable to record all chromatograms in this study and to quantify both DC and LD; in addition, it was found suitable for monitoring of related substances peaks. The previously described chromatographic conditions showed excellent separation of the two analytes DC and LD together with

their related substances. *Figure 2* shows a typical chromatogram for separation of this complex mixture. Retention times, capacity factors, theoretical plates, resolution values, and other system suitability parameters were calculated, and they were found acceptable *(Table I)*.

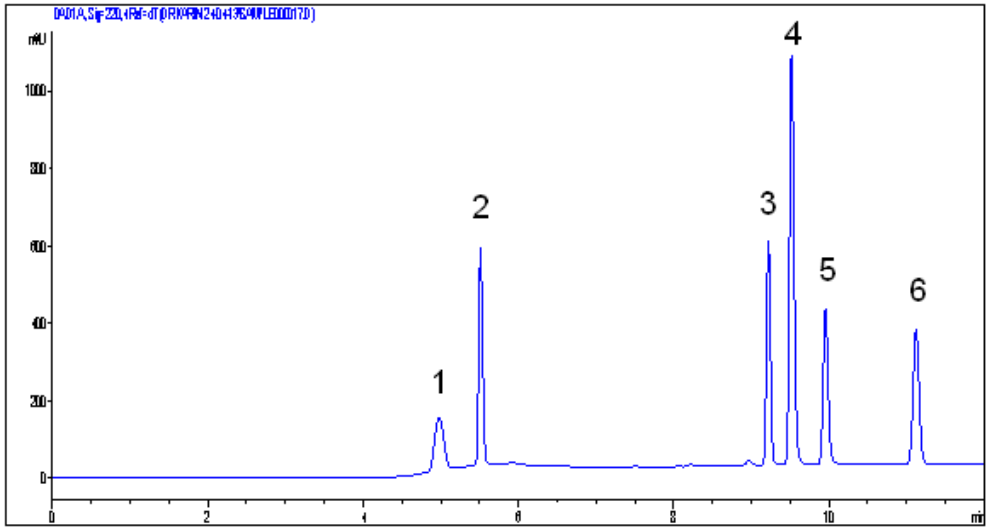

Fig. 2. HPLC chromatogram at 220 nm of a mixture containing:
(1) 50 μg mL^{-1} 2,6-dimethylaniline (DMA); (2) 100 μg mL^{-1} lidocaine hydrochloride (LD); (3) 50 μg mL^{-1} 2,6-dichloroaniline (DCA); (4) 100 μg mL^{-1} diclofenac sodium (DC); (5) 50 μg mL^{-1} N-chloroacetyl-N-phenyl-2,6-dichloroaniline (CPDCA); (6) 50 μg mL^{-1} N-phenyl-2,6-dichloroaniline (PDCA)

Table I. System suitability parameters for the separated compounds in this study

Compound	t_R ± SD (min)	Capacity factor (k')	Theoretical plates (N)	Symmetry	Selectivity (α)	Resolution (R_s)
DMA	4.98 ± 0.010	5.95	6793	1.07		
LD	5.52 ± 0.009	6.69	53710	0.90	1.12	3.16
DCA	9.23 ± 0.005	11.87	152632	0.89	1.77	39.05
DC	9.53 ± 0.007	12.28	127081	0.81	1.04	2.96
CPDCA	9.96 ± 0.003	12.89	119558	0.79	1.05	3.93
PDCA	11.12 ± 0.004	14.52	98692	0.94	1.13	9.06

Validation of the Proposed Method

The proposed HPLC method was validated as per the International Conference on Harmonization (ICH) guidelines on validation of analytical procedures [44].

Linearity and Concentration Ranges

Linearity of the proposed HPLC procedure was evaluated by analyzing a series of different concentrations for each of the two analytes. Linear regression equations were generated by least squares treatment of the calibration data. Under the optimized conditions described above, the measured peak areas were found proportional to concentrations. *Table II* presents the performance data and statistical parameters including linear regression equations, concentration range, correlation coefficients, standard deviations of the intercept (S_a), slope (S_b), and standard deviations of residuals ($S_{y/x}$). Regression analysis shows good linearity as indicated from the correlation coefficient values (>0.9998). In addition, deviation around the slope can be further evaluated by calculation of the percentage relative standard deviation (RSD %) of the slope (S_b %) which were found to be less than 1.0%.

Table II. Analytical parameters for the determination of DC–LD mixture using the proposed HPLC–DAD method

Parameter	DC	LD
Concentration range (μg mL^{-1})	10–200	10–200
Intercept (a)	39.99	22.55
S_a[a]	27.78	13.98
Slope (b)	42.59	18.30
S_b[b]	0.27	0.14
RSD % of the slope	0.63	0.77
Correlation coefficient (r)	0.99988	0.99983
$S_{y/x}$[c]	46.91	23.60
F[d]	24847	18121
Significance F	4.40×10^{-12}	1.13×10^{-11}
LOD[e] (μg mL^{-1})	0.63	1.21
LOQ[f] (μg mL^{-1})	2.10	4.03

[a]Standard deviation of the intercept.
[b]Standard deviation of the slope.
[c]Standard deviation of residuals.
[d]Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).
[e]Limit of detection.
[f]Limit of quantification.

Linearity can be further guaranteed by the analysis of variance (ANOVA) test. The most important statistic in this test is the *F* value which is the ratio of the mean of squares due to regression divided by the mean of squares due to residuals. High *F* values reveal an increase in the mean of squares due to regression and a decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the steeper is the regression line. The smaller the mean of squares due to residuals, the less is the scatter of experimental points around the regression line. Consequently, regression lines with high *F* values (low significance *F*) are much better than those with lower ones. Good regression lines show high values for both *r* and *F* statistical parameters [45].

Detection and Quantification Limits

The limit of detection (LOD) is defined as the concentration of the analyte which has a signal-to-noise ratio of 3:1. For the limit of quantification (LOQ), the ratio considered is 10:1. The LOD and LOQ values of DC and LD were calculated using the signal-to-noise ratio method *(Table II)*. Both LOD and LOQ values confirm the sensitivity of the proposed HPLC procedure.

Precision and Accuracy

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels for each compound using three replicate determinations for each concentration within 1 day. Similarly, the

Table III. Precision and accuracy for the analysis of DC and LD in bulk form using the proposed HPLC–DAD method

Compound	Type of analysis	Nominal value (μg mL^{-1})	Found ± SD[a] (μg mL^{-1})	RSD (%)[b]	E_r (%)[c]
DC	Within-day	20	19.68 ± 0.19	0.97	−1.60
		100	100.59 ± 0.77	0.77	0.59
		150	149.70 ± 1.26	0.84	−0.20
	Between-day	20	19.89 ± 0.27	1.36	−0.55
		100	101.09 ± 0.94	0.93	1.09
		150	150.30 ± 2.14	1.42	0.20
LD	Within-day	20	19.90 ± 0.16	0.80	−0.50
		100	100.89 ± 1.11	1.10	0.89
		150	150.46 ± 0.87	0.58	0.31
	Between-day	20	19.94 ± 0.21	1.05	−0.30
		100	101.27 ± 1.51	1.49	1.27
		150	148.79 ± 2.02	1.36	−0.81

[a]Mean ± standard deviation for three determinations.
[b]% Relative standard deviation.
[c]% Relative error.

between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on 3 days. Recoveries were calculated using the corresponding regression equations, and they were satisfactory. The RSD % and percentage relative error (E_r %) did not exceed 2.0% proving the high precision and accuracy of the developed method for the estimation of the analytes in their bulk form *(Table III)*.

Selectivity and Specificity

Method selectivity was examined by preparing several laboratory-prepared mixtures of the two compounds at various concentrations within the specified linearity range. These mixtures were of different ratios both above and below the normal ratio expected in the combined dosage form. The laboratory-prepared mixtures were analyzed according to the previously described procedure. The analysis results including RSD % and E_r % values shown in *Table IV* were satisfactory, thus, validating the selectivity, precision, and accuracy of the developed method and demonstrating its capability to resolve and quantify the analytes in different ratios. Specificity is defined as the ability to access unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Method specificity was already demonstrated by the successful resolution of both DC and LD from their related substances and potential impurities *(Fig. 2)*.

Table IV. Determination of DC–LD laboratory-prepared mixtures using the proposed HPLC–DAD method

Nominal value (μg mL^{-1})		Found ± SD[a] (μg mL^{-1})		RSD (%)[b]		E_r (%)[c]	
DC	LD	DC	LD	DC	LD	DC	LD
200	20	197.82 ± 2.30	20.14 ± 0.37	1.16	1.84	−1.09	0.70
100	20	98.77 ± 0.96	20.24 ± 0.32	0.97	1.58	−1.23	1.20
80	20	80.50 ± 0.67	20.28 ± 0.27	0.83	1.33	0.63	1.40
80	40	80.72 ± 0.98	39.96 ± 0.56	1.21	1.40	0.90	−0.10
100	100	99.85 ± 1.37	98.31 ± 0.73	1.37	0.74	−0.15	−1.69
40	80	39.48 ± 0.53	79.97 ± 0.88	1.34	1.10	−1.30	−0.04

[a]Mean ± standard deviation for five determinations.
[b]% Relative standard deviation.
[c]% Relative error.

Robustness

The robustness of an analytical procedure is a measure of its capability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage [44]. Robustness was examined by making small changes in acetonitrile content in the mobile phase (±3%), flow rate (±0.05 mL min^{-1}), column temperature

Table V. Evaluation of robustness of the proposed HPLC–DAD method

Parameter	Compound	Retention time ± SD	RSD %	Resolution (R_s ± SD)	Peak area ± SD	RSD %
Acetonitrile percentage in the mobile phase ± 3%	DMA	4.98 ± 0.029	0.58			
	LD	5.51 ± 0.043	0.78	3.28 ± 0.11	1926.2 ± 17.72	0.92
	DCA	9.24 ± 0.230	2.49	39.81 ± 0.86		
	DC	9.55 ± 0.303	3.17	3.02 ± 0.50	4422.3 ± 45.87	1.04
	CPDCA	9.99 ± 0.376	3.76	3.93 ± 0.32		
	PDCA	11.18± 0.439	3.93	9.11 ± 0.80		
Flow rate ± 0.05 mL min^{-1}	DMA	4.98 ± 0.155	3.11			
	LD	5.51 ± 0.153	2.78	3.27 ± 0.11	1935.7 ± 37.55	1.94
	DCA	9.23 ± 0.184	1.99	39.69 ± 0.73		
	DC	9.53 ± 0.182	1.91	2.97 ± 0.05	4422.4 ± 97.05	2.20
	CPDCA	9.96 ± 0.198	1.99	3.92 ± 0.08		
	PDCA	11.13 ± 0.235	2.11	9.08 ± 0.15		
Column temperature ± 2 °C	DMA	4.97 ± 0.033	0.66			
	LD	5.51 ± 0.022	0.40	3.28 ± 0.17	1905.8 ± 12.31	0.65
	DCA	9.22 ± 0.034	0.37	39.67 ± 0.54		
	DC	9.52 ± 0.033	0.35	2.95 ± 0.06	4443.1 ± 23.60	0.53
	CPDCA	9.96 ± 0.038	0.38	3.91 ± 0.03		
	PDCA	11.12 ± 0.056	0.50	9.09 ± 0.03		
Working wavelength ± 1 nm	DMA	4.98				
	LD	5.52		3.16	1954.1 ± 68.43	3.50
	DCA	9.22		39.05		
	DC	9.52		2.96	4428.7 ± 100.91	2.28
	CPDCA	9.96		3.93		
	PDCA	11.12		9.06		
Phosphoric acid conc. ± 0.01 M	DMA	4.97 ± 0.015	0.30			
	LD	5.50 ± 0.018	0.33	3.23 ± 0.07	1906.3 ± 29.03	1.52
	DCA	9.22 ± 0.002	0.02	39.83 ± 0.70		
	DC	9.52 ± 0.006	0.06	2.99 ± 0.05	4387.7 ± 24.83	0.57
	CPDCA	9.96 ± 0.003	0.03	3.93 ± 0.06		
	PDCA	11.12 ± 0.003	0.03	9.09 ± 0.04		
ACN Source (SDS, France and Merck Specialities Private Ltd., Mumbai, India)	DMA	4.97 ± 0.023	0.46			
	LD	5.51 ± 0.006	0.11	3.18 ± 0.03	1913.6 ± 9.28	0.49
	DCA	9.22 ± 0.003	0.03	39.43 ± 0.54		
	DC	9.52 ± 0.002	0.02	2.99 ± 0.04	4388.0 ± 40.20	0.92
	CPDCA	9.95 ± 0.006	0.06	3.92 ± 0.02		
	PDCA	11.12 ± 0.004	0.04	9.10 ± 0.05		

(±2 °C), working wavelengths (±1 nm), concentration of phosphoric acid solution (±0.01 M), source of acetonitrile (SDS, Peypin, France or Merck Specialities Private Ltd., Mumbai, India), and recording the chromatograms of a standard mixture of both target compounds together with the studied related substances. These variations did not have any significant effect on retention times of the eluting peaks or the measured responses (peak areas) of DC and LD. *Table V* shows the effects of the studied variations on retention times of all six peaks and peak areas of the parent compounds (DC and LD). Additionally, these minor experimental changes did not affect the resolution between any of the separated compounds. A minimum resolution value of 1.5 is usually regarded as sufficient for the baseline separation of closely eluted peaks. Resolution between any two successive peaks was never less than 2.55, which implies excellent baseline separation.

Stability of Solutions

The stability of DC and LD working solutions as well as the sample solutions in distilled water was examined, and no chromatographic changes were observed within 6 h at room temperature. The same applies for mixture solutions of both drugs with their related substances. Also, the stock solutions of all investigated compounds were stable for at least 1 week when stored refrigerated at 4 °C. Retention times and peak areas of the drugs remained unchanged, and no degradation was observed during these periods.

Analysis of Pharmaceutical Dosage Form

The developed method was applied for the assay of this drug combination in its commercial formulation (Olfen® ampoules). The very simple sample preparation involves only dilution of the ampoules with water to reach concentration levels within the specified range prior to direct injection to the column. A representative chromatogram obtained from the ampoules solution is shown in *Fig. 3*. The active ingredients eluted at their specific retention times, and no interfering peaks were observed from any of the inactive ingredients of the assayed ampoules. The DAD enables peak purity verification where no signs of coelution from any of the inactive components were detected. Recoveries were calculated using both external standard and standard addition methods. Assay results revealed satisfactory accuracy and precision as indicated from % recovery, SD, and RSD % values *(Table VI)*. Furthermore, a reference reversed-phase HPLC method [42] was applied for the simultaneous determination of DC and LD in their combined

formulation. Recovery data obtained from the developed HPLC–DAD method were statistically compared with those of the reference method using the Student's *t*-tests and the variance ratio *F*-tests. In both tests, the calculated values did not exceed the theoretical ones at 95% confidence level which indicated that there were no significant differences between the recoveries obtained from both methods *(Table VI)*. It is evident from these results that the proposed method is applicable to the assay of DC and LD in ampoules dosage form with minimum sample preparation and satisfactory level of selectivity, accuracy and precision.

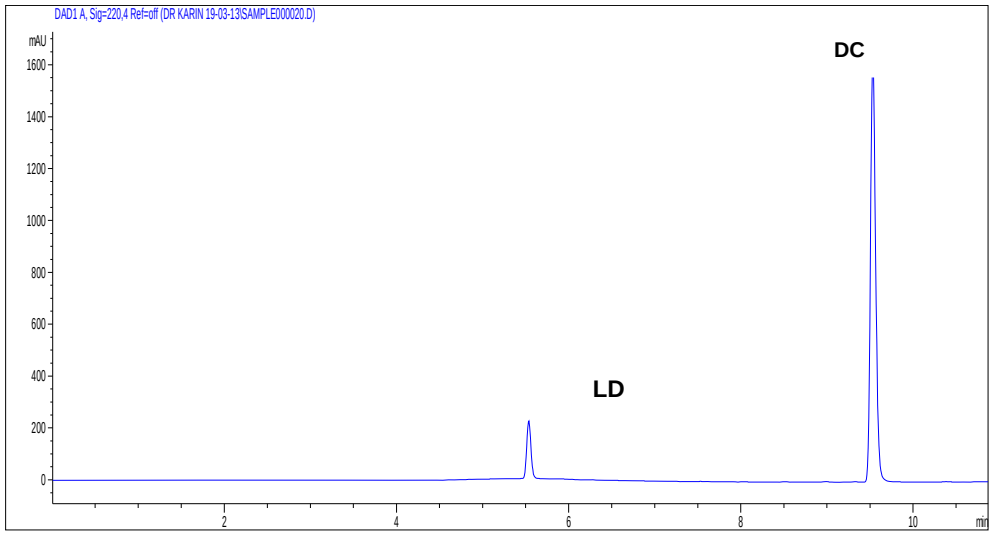

Fig. 3. HPLC chromatogram at 220 nm of a solution containing 150 μg mL^{-1} DC and 40 μg mL^{-1} LD obtained from Olfen® ampoules

Table VI. Application of the proposed HPLC–DAD method to the analysis of DC–LD mixture in ampoules dosage form

Parameters	External standard		Reference method	
	DC	LD	DC	LD
% Recovery ± SD[a]	100.54 ± 1.19	98.92 ± 1.26	100.74 ± 1.61	99.30 ± 1.53
RSD %[b]	1.18	1.27	1.60	1.54
t	0.22	0.43		
F	1.83	1.48		
Parameters	Standard addition			
	DC	LD		
% Recovery ± SD[a]	99.81 ± 1.67	99.15 ± 1.41		
RSD %[b]	1.67	1.42		

[a]Mean ± standard deviation for five determinations.
[b]% Relative standard deviation.
Theoretical values for *t* and *F* at *P* = 0.05 are 2.31 and 6.39, respectively.

Conclusion

This study described a simple, selective, and reliable HPLC–DAD procedure for the assay of DC and LD in their combined pharmaceutical dosage form. Simplicity was illustrated by the minimum requirement of organic solvents since water was used as a solvent for preparation of working standard and sample solutions. This suggests that the proposed method is cost-effective and environment-friendly. Also, the gradient program involves a single step of linear change of the mobile phase composition without variations in flow rate or wavelength. The described method is much more selective and superior over all previously reported analytical methods for DC–LD mixture [41–43]. A significant advantage in the study is the separation of both analytes from four of their related substances and potential impurities. Excellent resolution between all the separated peaks was achieved in a reasonable run time (less than 12 min). In addition, the described method can be considered stability indicating because at least two of the studied related substances are degradation products for their parent drugs. Nevertheless, a more comprehensive stability-indicating method is still required to ensure the assay of both drugs in presence of all their forced degradation products, and this can be the target for a future work. The diode array detector used in this study is superior compared to the universal UV detector used in the previously published HPLC methods [41, 42] since the DAD has the advantage of being a tool for peak identity and purity confirmation; however, the developed method can be adapted to conventional HPLC with UV detection which is the most popular in quality control laboratories. Regarding sensitivity, the developed method is comparable or even advantageous over several previous methods of analysis for the cited drugs [4, 25, 26, 32, 34, 35, 38–40, 42, 43]. Reliability was guaranteed by testing various validation parameters of the method and the successful application to commercial ampoules dosage form.

References

[1] S.C. Sweetman (Ed) Martindale: The Complete Drug Reference, 36th edn, The Pharmaceutical Press, London, UK, 2009, pp. 44–47, 1862–1866

[2] The British Pharmacopoeia, Her Majesty's Stationary Office, London, 2013, pp. 702–703, 1334–1335, 2797–2801, 3080–3082

[3] The United States Pharmacopeia, 36th edn, The National Formulary, 31th edn, The Official Compendia of Standards, United States Pharmacopoeial Convention, Inc., Asian Edition, Rockville, MD, 2013, pp. 3220–3223, 4112–4115

[4] T. Korodi, K. Bukowski, and B. Lachmann, Pharmazie, **67**, 980–983 (2012)

[5] L.H. Emara, N.F. Taha, A.A. El-Ashmawy, H.M. Raslan, and N.M. Mursi, J. Liq. Chromatogr. Relat. Technol., **35**, 2203–2216 (2012)

[6] A. Chmielewska, L. Konieczna, A. Plenis, M. Bieniecki, and H. Lamparczyk, Biomed. Chromatogr., **20**, 119–124 (2006)
[7] R.W. Sparidans, J.S. Lagas, A.H. Schinkel, J.H.M. Schellens, and J.H. Beijnen, J. Chromatogr. B, **872**, 77–82 (2008)
[8] A.O. Santini, H.R. Pezza, and L. Pezza, Talanta, **68**, 636–642 (2006)
[9] A.A. Ensafi, M. Izadi, and H. Karimi-Maleh, Ionics, **19**, 137–144 (2013)
[10] M.E. El-Kommos, N.A. Mohamed, and A.F. Abdel Hakiem, J. AOAC Int., **96**, 737–744 (2013)
[11] S.T. Ulu, J. Food Drug Anal., **19**, 94–101 (2011)
[12] R. Szostak and S. Mazurek, Drug Test. Anal., **5**, 126–129 (2013)
[13] B. Yilmaz, Chromatographia, **71**, 549–551 (2010)
[14] D.S. Patel, A.D. Captain, P.P. Prajapati, and H.G. Shah, Int. J. PharmTech Res., **5**, 147–154 (2013)
[15] R.R. Cunha, D.T. Gimenes, R.A.A. Munoz, C.L. Do Lago, and E.M. Richter, Electrophoresis, **34**, 1423–1428 (2013)
[16] R.T.S. Oliveira, G.R. Salazar-Banda, V.S. Ferreira, S.C. Oliveira, and L.A. Avaca, Electroanalysis, **19**, 1189–1194 (2007)
[17] V.M. Ivanov, E.M. Adamova, and V.N. Figurovskaya, J. Anal. Chem., **65**, 912–920 (2010)
[18] M. Lombardo-Aguei, C. Cruces-Blanco, and A.M. Garcia-Campana, J. Chromatogr. B, **877**, 833–836 (2009)
[19] H. Sun, L. Li, and M. Su, Chromatographia, **67**, 399–405 (2008)
[20] N. Raikos, G. Theodoridis, E. Alexiadou, H. Gika, H. Argiriadou, H. Parlapani, and H. Tsoukali, J. Sep. Sci., **32**, 1018–1026 (2009)
[21] P.V. Devarajan, M.H. Adani, and A.S. Gandhi, J. Pharm. Biomed. Anal., **22**, 685–690 (2000)
[22] E. Dinç and D. Baleanu, J. AOAC Int., **95**, 903–912 (2012)
[23] M. Jadach, A. Błazewicz, and Z. Fijalek, Acta Pol. Pharm., **69**, 397–403 (2012)
[24] E. ter Weijden, M.P.H. van den Broek, and F.F.T. Ververs, J. Chromatogr. B, **881–882**, 111–114 (2012)
[25] B. Jancic-Stojanović, A. Malenović, S. Marković, D. Ivanović, and M. Medenica, J. AOAC Int., **93**, 102–107 (2010)
[26] M. Pendela, G. Kahsay, I. Baekelandt, A. Van Schepdael, and E. Adams, J. Pharm. Biomed. Anal., **56**, 641–644 (2011)
[27] C.M. Adeyeye, P.K. Li, and K. Florey (Ed), Analytical Profiles of Drug Substances, vol. 19, Academic Press, Inc., London, UK, 1990, pp. 123–144
[28] Pharmaceutical Manufacturing Encyclopedia, 3rd edn, vol. 2, William Andrew Publishing, Norwich, NY, USA, 2007, pp. 1278–1279
[29] J. Hofmann, U. Freier, M. Wecks, and S. Hohmann, Appl. Catal., B, **70**, 447–451 (2007)
[30] D. Vogna, R. Marotta, A. Napolitano, R. Andreozzi, and M. d'Ischia, Water Res., **38**, 414–422 (2004)
[31] T. Kubala, B. Gambhir, and S.I. Borst, Drug Dev. Ind. Pharm., **19**, 749–757 (1993)
[32] R.A. Shaalan and T.S. Belal, Sci. Pharm., **81**, 713–731 (2013)
[33] M.F. Powell, Pharm. Res., **4**, 42–45 (1987)
[34] M.A.A. Mohammad, Chromatographia, **70**, 563–568 (2009)
[35] A. Plenis, L. Konieczna, N. Miekus, and T. Baczek, Chromatographia, **76**, 255–265 (2013)

[36] Z. Fijalek, E. Baczynski, A. Piwonska, and M. Warowna-Grzeskiewicz, J. Pharm. Biomed. Anal., **37**, 913–918 (2005)
[37] S.M. Riad, M.A.A. Mohammad, and A.O. Mohamed, Bull. Fac. Pharm. (Cairo Univ.), **46**, 35–48 (2008)
[38] R.A. Shaalan, and T.S. Belal, J. Chromatogr. Sci., **48**, 647–653 (2010)
[39] T.S. Belal, R.A. Shaalan, and R.S. Haggag, J. AOAC Int., **94**, 503–512 (2011)
[40] T.S. Belal and R.S. Haggag, J. Chromatogr. Sci., **50**, 401–409 (2012)
[41] L. Zhang and Y. Chen, Yaowu Fenxi Zazhi, **22**, 407–409 (2002)
[42] M.E. El-Kommos, N.A. Mohamed, and A.F. Abdel Hakiem, J. Liq. Chromatogr. Relat. Technol., **35**, 2188–2202 (2012)
[43] M.E. El-Kommos, N.A. Mohamed, and A.F. Abdel Hakiem, J. Pharm. Anal., **3**, 53–60 (2013)
[44] ICH, Validation of Analytical Procedures: Text and Methodology, Q2(R1), International Conference on Harmonisation, November 2005
[45] J.N. Miller and J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 4th edn, Pearson Education Limited, England, 2000, pp. 126–137

Accepted by DA