

A Validated LC Method for Determination of 2,3-Dichlorobenzoic Acid and its Associated Regio Isomers

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Received 16 March 2011; revised 10 May 2011

A simple, selective and sensitive gradient reversed-phase liquid chromatography method has been developed for the separation and determination of 2,3-dichlorobenzoic acid, which is an intermediate of the lamotrigine drug substance, and its regio isomers. The separation was achieved on a reversed-phase United States Pharmacopeia L1 (C-18) column using 0.01M ammonium acetate buffer at pH 2.5 and methanol (50:50 v/v) mixture as mobile phase A and a methanol and water mixture (80:20 v/v) as mobile phase B in a gradient elution at flow rate 1.2 mL/min with ultraviolet detection at 210 nm. The method is found to be selective, precise, linear, accurate and robust. It was used for quality assurance and monitoring the synthetic reactions involved in the process development of lamotrigine. The method is found to be simple, rapid, specific and reliable for the determination of unreacted levels of raw materials and isomers in reaction mixtures and finished product lamotrigine. The method was fully validated as per International Conference of Harmonization guidelines and results from validation confirm that the method is highly suitable for its intended purpose.

Introduction

Analytical method development and validation play important roles in the discovery, development and manufacture of pharmaceuticals. Isocratic and gradient reversed-phase (RP) liquid chromatography (LC) have evolved as the primary techniques for the analysis of non-volatile active pharmaceutical ingredients and their associated impurities. For LC methods used in pharmaceutical analysis, the stability-indicating ability is a critical aspect that needs to be addressed in method development and validation. The high-performance liquid chromatography (HPLC) detector of choice for the development of many types is the photodiode array (PDA) detector because it can be used for both quantitative and qualitative analysis. The use of a PDA detector to determine peak purity of the active main peak in stressed samples greatly facilitates the development of stability-indicating assays.

2,3-Dichlorobenzoic (2,3-DCBA) (Figure 1A) is a chemical used in the synthesis of a drug substance in the laid development phase. Although 2,3-DCBA is a well-known bulk chemical/intermediate stage moiety manufactured commonly for the bulk/pharmaceutical drug industry, some references can be found in literature regarding the separation and detection of its related isomers and impurities (1–5).

2,3-DCBA is the key starting material (main moiety) for the synthesis of lamotrigine. 2,3-DCBA has five positional isomers

(Figures 1B–1F). Lamictal (lamotrigine), an antiepileptic drug (AED) of the phenyltriazine class, is chemically unrelated to existing antiepileptic drugs. Its chemical name is (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, its molecular formula is C₉H₇N₅Cl₂, and its molecular weight is 256.09. Lamotrigine is a white to pale cream-colored powder with a pKa of 5.7. Lamotrigine is very slightly soluble in water (0.17 mg/mL at 25°C) and slightly soluble in 0.1M HCl (4.1 mg/mL at 25°C).

2,3-DCBA is the control in 2,3-dichloro-benzoic acid, and the control of these isomers is critical in controlling the same analogues in the lamotrigine drug substance. Hence, it is necessary to develop a simple quantitative method for the estimation of 2,3-DCBA and its associated isomers.

This study reports a simple and effective analytical method for the development and validation of 2,3-DCBA and its regio isomers. This method is also capable of detecting all regio isomer analog impurities. The method for identification of the process impurities (regio isomers) of 2,3-DCBA synthesized in our group according to the International Conference on Harmonization (ICH) guidelines (6–8) is also discussed.

Experimental

Chemicals

2,3-DCBA and its regio isomers were received from the process research department of Dr. Reddy's Laboratories (Hyderabad, India) (Figure 1). Merck HPLC-grade methanol gradient grade, analytical grade glacial acetic acid (Merck, Darmstadt, Germany) and analytical grade ammonium acetate (Fluka) were used as received. Water purified by a Millipore system (in-house) was used for making the solutions.

Chromatographic conditions and equipment

The stationary phase used was a Cosmosil MS-II RP18, 250 × 4.6 mm, 5 mm particles. The mobile phase A contained a mixture of 0.01 mM of ammonium acetate in 1000 mL of water, pH 2.5: methanol (50:50, v/v) and mobile phase B contained a mixture of water: methanol (20:80, v/v). The flow rate was 1.2 mL/min. The HPLC gradient program was set as described in Table I and the delayed time to next injection was 5.0 min. The column temperature (COT) was maintained at 20°C and the detection was monitored at a wavelength of 210 nm. The injection volume was 10 mL, and methanol was used as diluent.

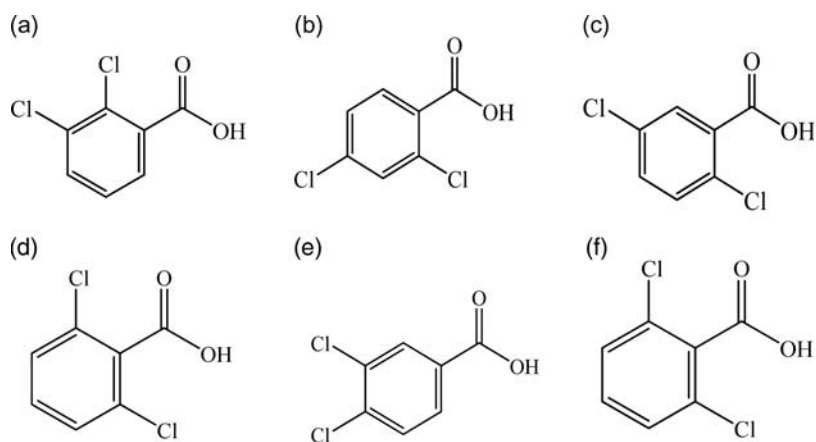


Figure 1. The positional isomeric impurities of 2,3 Dichlorobenzoic acid, (a) 2, 3-Dichlorobenzoic acid (2, 3-DCBA), (b) 2, 4-Dichlorobenzoic acid (2, 4-DCBA), (c) 2, 5-Dichlorobenzoic acid (2, 5-DCBA), (d) 2, 6 (Dichlorobenzoic acid (2, 6-DCBA), (e) 3, 4-Dichlorobenzoic acid (3, 4-DCBA), (f) 3, 5-Dichlorobenzoic acid (3, 5-DCBA).

Table I

Finalized gradient programme

S.No.	Time	Flow	% Pump A	% Pump B
1	0.01	1.2	95	5
2	25	1.2	95	5
3	55	1.2	70	30
4	60	1.2	70	30
5	61	1.2	95	5
6	65	1.2	95	5

The LC systems used for method development and method validation were a Waters 515 binary pump, 717 plus autosampler with a 2996 PDA detector and an Agilent (Wilmington, DE) 1100 system equipped with a PDA detector and variable wavelength detector. The output signal was monitored and processed using Waters (Milford, MA) Empower-1 software with a P4 system.

Preparation of solutions

Test sample/standard preparations

Twenty milligrams of the 2,3-DCBA test sample/standard was placed in a 10 mL volumetric flask, dissolved and diluted to the mark with methanol. Further dilutions made for limit of detection (LOD) and limit of quantitation (LOQ), and complete method validations.

HPLC method development

The HPLC procedure was developed to resolve the possible regio isomers and process impurities from the 2,3-DCBA. Various mobile phase compositions, columns of different packing materials (C18, C8 and phenyl) and configurations (15 and 25 cm columns) were attempted to obtain good peak shapes and to resolve the peaks of all regio isomers and its impurities in 2,3-DCBA in single run. The composition of mobile phases A and B (as described previously) were found to be appropriate for the separation of all process impurities and its regio isomers.

Optimization of gradient elution

A gradient elution system was designed as described previously. Changes in the composition of the gradient program and the experimental conditions were chosen within the optimum region predicted by the gradient mode. Because of the late-eluting isomers, the buffer concentration was chosen according to separation of all isomers. 2,3-DCBA, the related region isomers and impurities were all baseline separated. Gradient elution provides several advantages: (1) higher separation selectivity, (2) better peak shapes, (3) higher sensitivity, (4) shorter retention times and (5) mass compatibility; the detection limit for 2,3-DCBA isomers was largely enhanced by using the gradient elution.

System suitability criteria

The standard solution was used as the system suitability solution; the system suitability solution was determined from the principle peak of the standard solution. The acceptance criteria of the theoretical plates (N) was not less than 6000 plates and United States Pharmacopeia (USP) tailing (T) was not more than 1.5.

Purity estimation of 2,3-DCBA

For purity estimation of 2,3-DCBA, a 2.0 mg/mL sample was injected without exceeding the linear range of the primary component (2,3-DCBA). The sensitivity towards isomers and impurities was thus increased; the response of each impurity was recorded. Weight percentage of each isomer present in the sample was calculated by area normalization method because all regio isomers share the same response of 2,3-DCBA, therefore, the responses of all regio isomers are as per pharmacopeia guidelines; i.e., between 0.9 and 1.1. Hence, all isomers falls under the same range (0.98–1.02). Therefore, this wavelength was selected for analysis.

Method Validation

System suitability criteria

System suitability solution was prepared as described previously. The system suitability test solution was injected and the chromatographic parameters, theoretical plates and USP tailing factor for the principle peak of 2,3-DCBA were evaluated to prove system suitability (9).

Limit of detection and limit of quantification The LOD and LOQ for 2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA isomers were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentration.

Linearity of response

Detector response linearity for 2,3-DCBA was assessed by injecting eight separately prepared solutions covering the range LOQ–200% of the normal sample concentration (2 mg/mL). A good linear relationship between the sample concentration and the detector response (peak area) of 2,3-DCBA was obtained [the correlation coefficient (r^2) was calculated]. Good linearity was also obtained for the isomers 2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA, which allows the determination of these isomers by an area normalization procedure.

Precision

The precision of the related substance method was checked by injecting six individual preparations ($n = 6$) of 2,3-DCBA spiked with 0.15% each isomer (2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA) w.r.t analyte concentration, and calculated % RSD for Retention time and Area %.

Accuracy

Standard addition and recovery experiments were conducted to determine accuracy of the method for the quantification of isomers in the 2,3-DCBA sample. The study was carried out in triplicate at 50, 100 and 150% of the all isomers with regard to analyte concentration (2 mg/mL). The percentage of recoveries was calculated for 2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA isomers.

Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered and theoretical plates and tailing factor for the principle 2,3-DCBA peak was evaluated. The flow rate of the mobile phase was 1.2 mL/min. To study the effect of flow rate on the theoretical plates and USP tailing, it was changed by 0.2 units from 1.2 mL to 1.0 and 1.4 mL/min. The pH of the mobile phase was 2.5. To study the effect of pH on the theoretical plates and USP tailing, it was changed by 0.2 units from 2.3 to 2.5 and 2.7.

The column oven temperature was 20°C. To study the effect of column oven temperature on the theoretical plates and USP tailing, it was changed by 5.0°C units from 15 to 20 and 25°C.

Solution stability and mobile phase stability

2,3-DCBA solutions prepared in diluent were injected at 0, 6 and 24 h, the impurity content was calculated (2,4-DCBA, 2,5-DCBA, 2,6-DCBA, 3,4-DCBA and 3,5-DCBA isomers) and the consistency was checked in the percentage area of the principal peak at each interval. The prepared mobile phase was kept constant during the study period.

Results and Discussion

HPLC method development and optimization

Sample preparation largely influences the success of any chromatographic experiment. In the present study, all the samples were dissolved in methanol diluent due to the higher solubility of the investigated compounds and because mobile phase co-solvents are the same as methanol. The RP-LC method was chosen and optimized with a view to develop a single method for all the regio isomers and 2,3-DCBA. In the present investigation, different columns of different packing materials, USP L1, L8 and L40 (C-18, C-8, phenyl), and configurations (15 and 25 cm columns) were tested to obtain sharp peaks and resolve the peaks of all possible regio isomers (Figures 1B–1F) from the 2,3-DCBA peak. The isocratic method was initially tried with different column stationary phases (CSP) and different mobile phases compositions [organic modifiers like acetonitrile, methanol and tetrahydrofuran (THF)], but no combinations of 3,4-DCBA and 3,5-DCBA were eluted up to 60 min (Figure 2A). To further investigate the gradient program, the change in gradient composition and corresponding change in selectivity were studied. Initially, a mixture of acetate buffer and methanol was used in various mobile phase gradient compositions on an Inertsil ODS-3V, 250 × 4.6 mm, 5 mm particles. The mobile phase A contained a mixture of 0.01 mM of ammonium acetate in 1000 mL of water, pH 2.5, acetonitrile (50:50, *v/v*) and mobile phase B contains a mixture of water: acetonitrile (10:90, *v/v*). The flow rate was 1.2 mL/min. The HPLC gradient program was set as described in the following. Time in minutes per percent of solution B: 0.01/5, 10/15, 20/15, 25/25, 30/35, 45/35, 50/5 and 55/5. The COT was maintained at 25°C and the detection was monitored at a wavelength of 210 nm. Poor selectivity was observed and not considered for further analysis. Two isomers were co-eluted (3,5-DCBA and 3,4-DCBA) when using Inertsil, Hypersil BDS and Phenyl columns (Figure 2B). Later, acetonitrile was replaced with methanol, which provides better selectivity through hydrogen bonding and H-donor and H-acceptor properties. When analyzing 2,3-DCBA and its isomers using a Cosmosil MS-II RP-18, 25 cm × 4.6 mm, 5 mm particles, mobile phase A was a mixture of 0.01 mM of ammonium acetate in 1000 mL of water, pH 2.5, methanol (50:50, *v/v*) and mobile phase B contained a mixture of water: methanol (20:80, *v/v*) at a flow rate of 1.2 mL/min. The LC gradient program was set as described in the following. The time in minutes per percent of solution B: 0.01/5, 10/5, 20/15, 25/15, 45/40, 50/5 and 55/5 with UV 210 nm. A blank interference was observed and peaks that were slightly broad in nature. Further experimentation was continued by changing only the gradient composition and 1.2 mL/min flow rate, and the gradient program was set as described in the following. The time in minutes per percent of solution B: 0.01/5, 25/5,

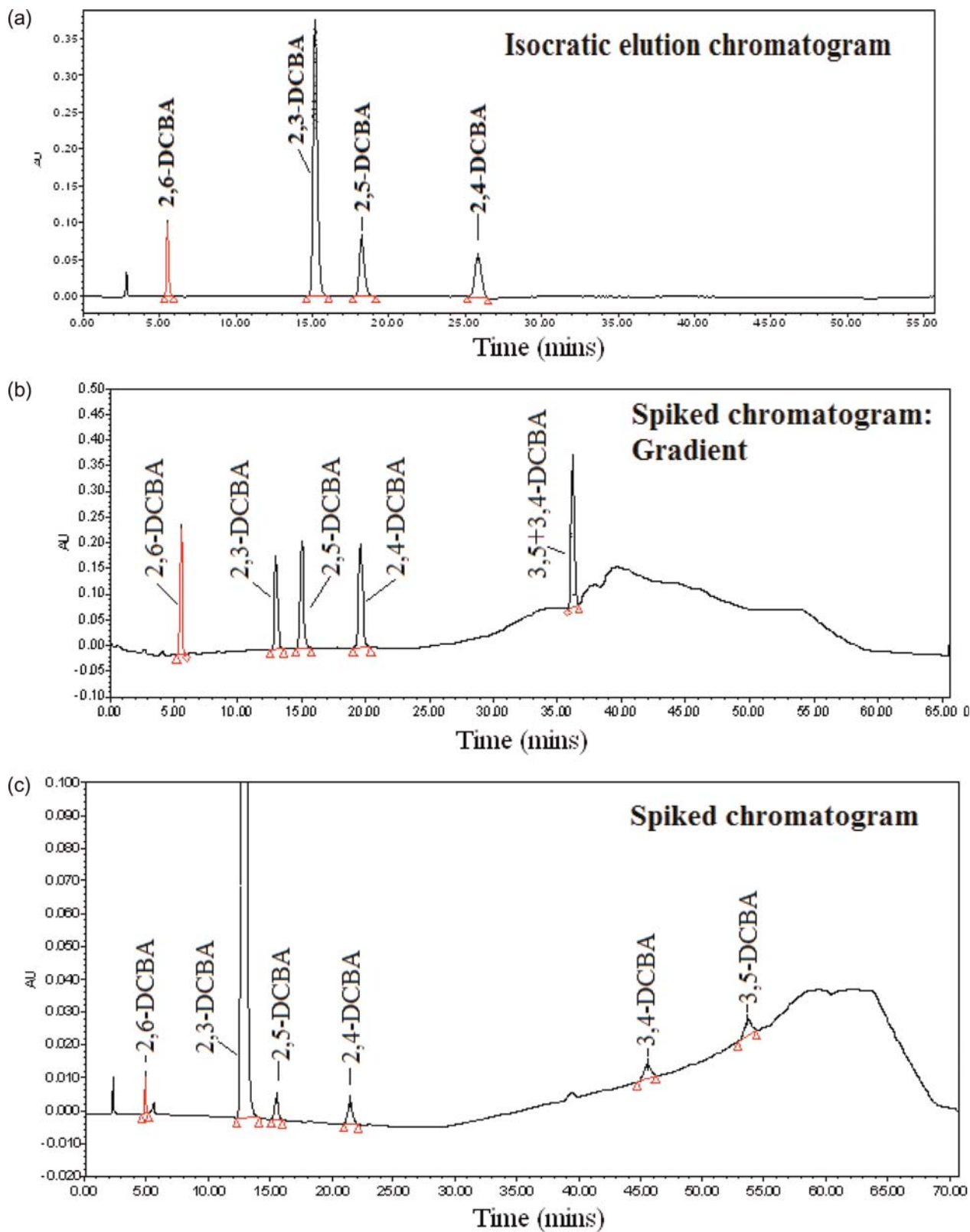


Figure 2. Typical blend chromatogram of 2,3-Dichlorobenzoic acid and its isomers (a) Typical chromatogram of Isocratic elution, (b) Typical chromatogram of gradient elution, (c) Spiked typical chromatogram.

55/30, 60/30, 61/5 and 65/5. The peak shapes and the resolution is very good between all regio isomers and the 2,3-DCBA (Figure 2C). The peaks were identified by injecting and comparing the retentions with those of authentic standards. Reproducible peak shapes were obtained under the optimum conditions. The USP tailing factor (T) > 0.95 and number of theoretical plates (N) > 19000 were found for all peaks. The relative retentions of 2,5-DCBA (RRT approximately 1.11), 2,4-DCBA (RRT approximately 1.55), 3,4-DCBA (RRT approximately 3.29), 2,6-DCBA (RRT approximately 0.35) and 3,5-DCBA (RRT approximately 3.88) were observed. The UV detector was set at 210 nm for both detection and quantification. This was selected based on the observations that the detector response was higher than the determinations made at other wavelengths for all compounds.

Method validation

The described method was extensively validated for purity by HPLC determination.

Limit of detection and limit of quantification

The LOD of 2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA isomers was 0.079, 0.088, 0.088, 0.097 and 0.106 mg/mL, respectively, with regard to 2,3-DCBA concentration for a 10-mL injection volume.

The LOQ of 2,6-DCBA, 2,5-DCBA, 2,4-DCBA, 3,4-DCBA and 3,5-DCBA isomers were 0.26, 0.29, 0.30, 0.32 and 0.35 mg/mL, respectively, with regard to 2,3-DCBA concentration for a 10-mL injection volume.

The analyses of LOD and LOQ infer that the method is highly sensitive toward the isomers present in 2,3-DCBA.

Linearity

Linearity was checked for all isomers (2,4-DCBA, 2,5-DCBA, 2,6-DCBA, 3,4-DCBA, 3,5-DCBA isomers to the 0.15% limit of each isomer with respect to the concentration of 2,3-DCBA) at eight different concentration levels ranging from LOQ, 25, 50, 75, 100, 125, 150 and 200%. The equation for calibration Y-intercept, slope and correlation coefficient is equal to 0.999. The cross-validated R^2 was also calculated and found to be more than 0.997, indicating good linearity. The results are summarized in (Table II).

Precision

Method repeatability/intermediate precision

The system repeatability was determined from six replicate injections of a sample solution of 2,3-DCBA at the analytical concentration of approximately 2.0 mg/mL by two different analysts using different chromatographic systems on different days and different lots of equivalent columns. The RSD values of repeatability/intermediate precision show that the proposed method provides acceptable precision, because they were found to be in the range of 1.4 to 7.9% (Table II).

Table II
Summary of analytical method validation of 2,3-DCBA and its regio isomers

Test parameter	2,6-DCBA	2,5-DCBA	2,4-DCBA	3,4-DCBA	3,5-DCBA
Precision (%RSD)	1.5	2.2	3.2	3.9	7.9
LOD ($\mu\text{g/mL}$)	0.079	0.088	0.088	0.097	0.106
LOQ ($\mu\text{g/mL}$)	0.26	0.29	0.30	0.32	0.35
Intermediate precision (%RSD)	1.4	3.2	5.6	2.9	4.3
Linearity	0.9991	0.9995	0.9994	0.9996	0.9993
Accuracy (in %)	93.2–105.5	97.6–105.1	94.9–104.9	92.6–104.8	90.7–109.8
Robustness	Rs between 2,3-DCBA and 2,5-DCBA > 2.5	Rs between 2,3-DCBA and 2,5-DCBA > 2.5	Rs between 2,3-DCBA and 2,5-DCBA > 2.5	Rs between 2,3-DCBA and 2,5-DCBA > 2.5	Rs between 2,3-DCBA and 2,5-DCBA > 2.5
Solution stability	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr
Mobile phase stability	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr	Stable up to 24hr

Accuracy/recovery

Accuracy of the method for assay determination was checked by spiking pre-analyzed samples with three different concentration levels; i.e., at 1.5, 3.0 and 4.5 mg/mL (50, 100 and 150 % with regard to 0.15% of test concentration) of all regio isomers of 2,3-DCBA, and the mixtures were analyzed by proposed method ($n = 3$). The mean recovery shows that the proposed method provides acceptable recovery, because it was found to be in the range of 90.7 to 109.8%. The mean recovery data obtained for each level and the percentage recoveries are tabulated in (Table II).

Solution stability and mobile phase stability

Consistency was observed in regio isomers levels (spiked samples) and the principal peak area at each interval. The solution stability and mobile phase stability experiment data confirm that sample solutions and mobile phase were stable up to 24 h.

Robustness

During the robustness study, the USP tailing factor and USP theoretical plates of 2,3 DCBA were greater than 0.94 and 22540, respectively; resolution between 2,3-DCBA and 2,5-DCBA was greater than 2.5, illustrating the robustness of the method.

Conclusion

A simple, rugged and robust gradient RP-HPLC method was developed for the separation and quantitative determination of 2,3-DCBA, its regio isomers and related impurities. The method is precise, accurate and selective. The method was completely validated and showed satisfactory data for all tested method validation parameters. The developed method is stability-indicating and can be used to assess the stability of bulk samples of 2,3-DCBA and to monitor the synthetic procedures of 2,3-DCBA.

Acknowledgment

The authors wish to thank the management of Dr. Reddy's Laboratories Ltd. for supporting this work. Cooperation from colleagues and of Research & Development and Analytical Research & Development of Dr. Reddy's Laboratories Ltd. is appreciated.

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