



RESEARCH ARTICLE

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Development of HPLC Method for Stress Testing of Combination of Two Drugs Using Design of Experiment Concept

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ABSTRACT

This study was conducted to develop, an High Performance Liquid Chromatography using photodiode array detector (HPLC-PDA) method to analyse the samples generated by the stress testing of antifilarial combination (albendazole and diethylcarbamazine citrate) in the solution state. The concept of Quality by Design (Design of Experiment, DoE) approach was used for the development. For the separation of the drugs and its degradation products (DPs), DoE was applied in two stages, i.e., primary parameter stage where factors having major effect were selected. This stage gives us CQA (Critical Quality Attribute) which along with minor factors affecting were varied to get the secondary design. For each of the stage a different design was selected; for primary stage IV optimal design (Response Surface Method) was selected whereas for secondary stage, Taguchi orthogonal array design was selected. The major primary parameters affecting the HPLC method as screened by preliminary studies were the buffer pH, organic modifier (methanol or acetonitrile), initial hold time (start of gradient) and gradient time. The primary stage was completed successfully. The results were compiled in form of resolution of peak from next peak and analysed by DoE. The process fixed the values for buffer pH (4.38), organic modifier (acetonitrile) and gradient time (30 min). The CQA from primary run was initial hold time. This parameter along with other parameters: initial and final concentration of organic modifier, buffer type (phosphate or acetate), buffer strength (mM) and oven temperature were further varied and samples withdrawn were analysed. The data of secondary design was compiled in the form of resolution (R), analysed by Design Expert and final value for secondary parameter for HPLC method were fixed. The resolution of the peaks for some secondary runs was sufficient reflecting some type of interaction between the drugs and/or degradation products.

Keywords: Albendazole, diethylcarbamazine, solution-state stability, HPLC, stress testing.

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INTRODUCTION

The drugs selected are from the drugs categorised under the group of infectious diseases termed "Neglected Tropical Diseases" (NTD).^[1] These diseases

are predominantly found in low- income population of most of the developing countries. The regions of highest prevalence of these diseases are mostly tropical and subtropical areas of African, Asian, as well as

American continents. The major concern of these diseases is the presence of multiple infections leading to severe pain may/may not be followed by permanent disability and even leading to death. [1] The other major drawback is disfigurement which leads to social stigma, especially for females. [1] The drugs belong to category of antifilarial drugs. [1-3] The drug for treatment and dosage regimen for these diseases was not systemized and many different regimens were followed. So, World Health Organisation (WHO) in 2017 issued, "Guideline for Alternative Mass Drug Administration Regimen to Eliminate Lymphatic Filariasis". [4] Filariasis (lymphatic dwelling filariae) is infection caused by nematodes (worms) belonging to family Filarioidea. The class encompasses organisms *Wuchereria bancrofti* (90%), *Burgias malayi* and *Burgias timori*. The two eye infections caused by *Loa loa* and *Onchocerca volvulus* (river blindness) are also included. [1-2] The drug combination albendazole and diethylcarbamazine is one of the recommendations for filariasis when it is not co-endemic with eye infections. [3] The structures of both the drugs are shown in Fig. 1. [5-6]

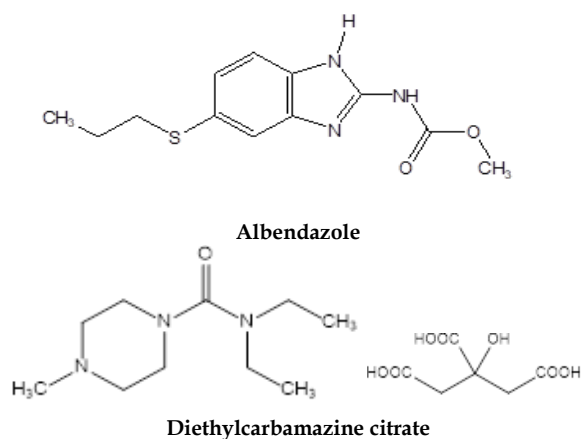


Fig. 1: Structures of the two drugs

According to ICH Q 1 A (R2) [7] guidelines on "Stability Testing of New Drug Substances and Products", stress testing is defined as "Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved." These studies are very useful to finalize storage conditions as well as the manufacturing conditions of drug or its combinations. They are also helpful in selecting excipients to be used in the manufacture of dosage forms. The drugs are put on stress testing (forced degradation) studies by exposure to extremes of hydrolytic (at different pH), light and oxidative conditions. [8-12]

The analytical method for the drug combination of albendazole (ALB) and diethylcarbamazine (DEC) and separation of their degradation products is not well

documented in literature, only some studies giving the stability indicating assay for the drugs are available. Keeping all the above in mind, this study was planned for development of method of analysis using HPLC-PDA. The stress conditions selected were hydrolytic (acidic, basic and neutral) and oxidative stress using hydrogen peroxide as reagent. The DoE concept was only applied to analytical method but not the stress conditions as highlighted in many publications. [13-19]

Materials and Methods

Drugs and reagents

Pure albendazole (ALB) and diethylcarbamazine (DEC) were obtained from Mahalaxmi Chemicals (Hyderabad, India). Analytical reagent (AR) grade sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from LOBA Chemie Pvt. Ltd. (Mumbai, India), hydrogen peroxide (H₂O₂) from S.D. fine-chem Ltd. Boisar, India). Buffer salts and all other chemicals of AR grade were bought from local suppliers. HPLC grade acetonitrile (ACN) and methanol (MeOH) were procured from Fischer Scientific (Mumbai, India). HPLC grade water for studies was obtained from water purification unit (Milli-Q, Millipore Corporation, Germany).

Apparatus and equipment

Separation behaviour of the drugs and their degradation products formed were studied using a Agilent LC liquid chromatography system that was equipped with a photodiode detector (1260 DAD VL), and was controlled by EZ chrome Elite software, version 3 (all from Agilent, Germany). The column used was Inertsil ODS-3 C-18 (250 nm × 4.6 nm, i.d., particle size 5μ).

Precision water bath equipped with MV controller (Julabo, Seelbach, Germany) was used for generating samples for solution degradation studies. A pH/Ion analyzer (Seven Easy, Mettler Toledo, Schwerzenbach, Switzerland) was used adjust the pH of buffer solutions. Other small equipment used was sonicator (YJ 5200 DT, Citizen Scale India Pvt. Ltd., India), precision analytical balance (XP 205 and AG204, Mettler Toledo, Schwerzenbach, Switzerland) and autopipettes (Eppendorf, Hamburg, Germany).

Solution-state stress studies

The degradation studies were conducted for the hydrolytic stress under acidic (1N HCl), basic (1N NaOH) and neutral (50:50, ACN: H₂O) according to the conditions stated in table 1 and for oxidative stress two concentrations of H₂O₂ (5% and 15%) were selected. [3, 9-10] The solution of both the drugs was prepared at 2 mg/ml. For the individual drug studies, the volume of 25 ml was drawn of the drug and was mixed with 25 ml of the stressor. In case of combination, the individual drug volume of 12.5 ml was withdrawn, mixed and then added 25 ml of the stressor to the mixture. [17-19] Studies were carried on the solution where the stressor drugs and stressor concentration was in the ratio of 1:1. All the samples were maintained at 80°C for the period

of the study. The sample of 3 ml was withdrawn after fixed intervals. Replicates of all the possible sets were also prepared and simultaneously subjected to same study conditions.

Preparation of samples for HPLC analyses

The samples withdrawn were diluted with equal amount of diluent. The final sample of each study condition were mixed and then diluted further to get concentration of 1 mg/ml. All the final generated samples were filtered through 0.22µ membrane filter before injecting in HPLC system.

Method development and optimization

The forced degraded sample solutions were preliminary screened for degradation behaviour. After the initial screening, the samples parameters-concentration of stressor, time period of study- were finalized. This also gave us knowledge regarding HPLC parameters effects. The finalized samples were then subjected to DoE. The number of runs and parameters for each run were calculated from the Design expert software (trial version 9). The limit for variable primary run parameters selected (organic modifier, buffer pH, initial hold time, gradient time) [20-23] are shown in table 3. The suggested 32 runs were determined using IV optimal design. The actual run details of variable parameters were as shown under factors heading in table 6. The fixed parameters were initial organic modifier, final organic modifier, flow rate, buffer type, buffer strength, injection volume and oven temperature the detail is given in table 2. [24-27] All the analysis were performed using PDA detector and the wavelength finally selected was 220 nm for DEC and all its combinations.

The compilation of results and statistical treatment of data obtained from study of primary run were done. The results were analysed, and the parameters optimized for this primary stage were pH of the buffer, gradient time, organic modifier concentration and initial hold time. These parameters were used as fixed parameters in secondary run and other parameters varied in the secondary design are as compiled in table 4. The initial hold time was identified as CQA which was further again studied in secondary stage. The seven parameters selected were subjected to Taguchi orthogonal design and the runs are as compiled in table 5. The samples were subjected to the runs of secondary study and the results obtained were compiled and subjected to DoE.

RESULTS

The results of samples subjected to chromatographic study for preliminary screening were the basis for subsequent selection of parameters for primary selection as well as secondary screening. The parameters detailed in table 2 were the parameters most affecting the method development. So, they were selected for primary and secondary based on major or minor effects. The summary of all the run for primary parameters and resolution (R) of peak from next peak

in each run are enumerated in table 6. The overlay chromatograms of the some of the runs are also shown in fig. 2. The data obtained was subjected to analysis and evaluation by Design Expert software and results obtained are shown in fig. 3. The optimized condition for primary parameters were buffer pH at 4.38, gradient time to be 30 min, ratio of organic modifier ACN: MeOH; 100:0, and initial hold time for the method being 0 min.

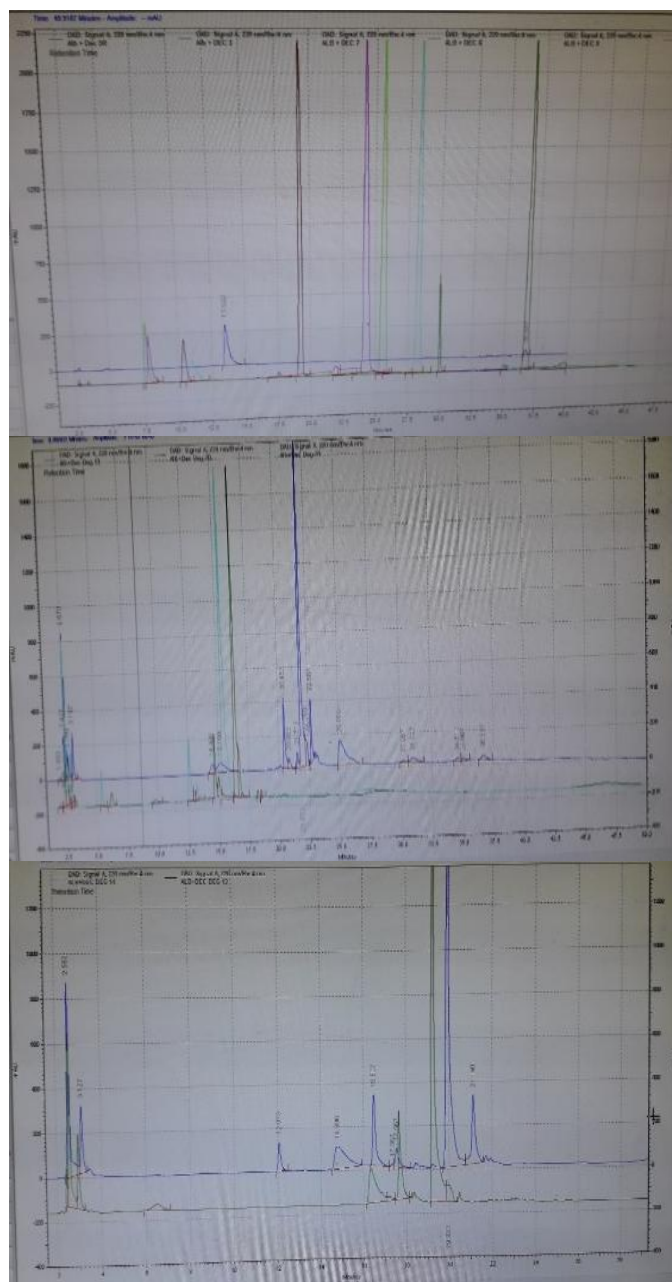


Fig. 2: Primary screening data showing overlay chromatograms of some of the runs of ALB+DEC combination.

Table 1: Levels of forced degradation parameters used for optimization of hydrolytic and oxidative conditions.

Conditions	Stressor	Concentration	Temperature (°C)
Hydrolytic	HCl	1N	80
	NaOH	1N	80
	ACN:H ₂ O	50:50, v/v	80
Oxidative	H ₂ O ₂	5% and 15%	80

Table 2: Selected primary and secondary parameters and then optimization for analytical methodology using various experimental designs.

Input method parameters	Primary parameter selection (IV-optimal design)	Secondary parameter screening (Taguchi orthogonal array)
Organic Modifier (ACN: MeOH)	100:0, 50:50 and 0:100	100:0*
Buffer pH	3-7	4.38*
Gradient time	20-40 min	30 min*
Initial hold time	0, 5 and 10 min	0 min*
Initial organic modifier	5%*	2 and 8%
Final organic modifier	75%*	60 and 80%
Flow rate	1.0 ml/min*	0.5 and 1.0 ml/min
Buffer type	Phosphate*	Phosphate and acetate
Buffer strength	10 mM*	8 and 12 mM
Injection volume	10µl*	5 and 15µl
Oven temperature	30°C*	30 and 35°C

*Parameters which are fixed in the study.

Table 3: Design Summary for primary parameters selection.

File Version	9.0.6.2
Response Surface	I-optimal
Design type	Coordinate exchange
Design model	Quadratic
Runs	32
Build time (ms)	2928.00

Table 4: Design summary for secondary parameter screening.

File Version	9.0.6.2
Design type	Taguchi OA
Study type	Coordinate exchange
Design model	Factorial
Runs	Main effect
Build time (ms)	8
	1.00

Table 5: Variable parameters selected for secondary studies.

Method	Initial organic modifier (%)	Final organic modifier (%)	Injection Volume (µL)	Buffer Type	Buffer strength (mM)	Initial hold time (min)	Oven temperature (°C)
Sec1	8	60	1	Acetate	8	15	25
Sec2	8	90	0.5	Acetate	8	5	35
Sec3	2	90	1	Acetate	12	5	25
Sec4	2	60	0.5	Acetate	12	15	35
Sec5	2	60	0.5	Phosphate	8	5	25
Sec6	2	90	1	Phosphate	8	15	35
Sec7	8	90	0.5	Phosphate	12	15	25
Sec8	8	60	1	Phosphate	12	5	35

Table 6: Variable parameters selected for primary studies.

Run	Variable factor				Responses										
	Buffer pH	Gradient Time (Min)	ACN: MeOH (%)	Initial Hold time (Min)	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
1	3	20	0:100	10	0	0	0	0.78	0.95	0	0	0	0	0	0
2	7	26.7	100:0	0											
3	7	20	50:50	0	0	2.39	2.5	6.63	-4.78	5.88	0	2.94	6.47	0	0
4	7	40	100:0	10		2.95	2.7	2.03	0	1.25	0	7.69	4.47	4.91	0
5	4.5	31	50:50	5	0	2.19	1.55	2.17	0	1.98	0	1.89	1.72	3.06	0
6	7	40	0:100	5		3.1	23.06	4.33	0.09	8.22	0	2.66	2.19	0	2.76
7	4.5	22.9	50:50	0	0	2.38	2.48	5.72	4.26	4.26	5.69	0	2.94	7.39	0
8	5	20	100:0	10	0	1.12	0	14.18	3.04	3.42	0	4.58	5.29	0	7.09
9	6.1	33.3	50:50	0	0	2.51	1.23	0	3.61	26.29	39.75	4.95	3.54	0	9.89
10	3	40	100:0	0	0	2.44	2.67	14.25	0	0	57.05	0	1.49	7.41	0
11	7	26.7	50:50	10	1.2	0	2.77	0	0	15.58	36.56	0	1.2	7.6	0
12	7	26.7	100:0	0	-1.25	2.96	15.1	0	36.2	0	3	1.24	7.57	0	0
13	3	20	0:100	0	1.15	2.76	3.14	13.17	1.61	6.16	2.65	1.61	11.56	4.83	1.62
14	3	20	100:0	5	0	2.4	2.51	5.24	8.87	3.65	4.13	1.59	7.14	1.3	4.93
15	4.5	31	0:100	0	0	2.68	1.32	15.32	-27.1	2.27	0	9.33	0	1.53	3.8
16	3	33.3	100:0	10	0	0	2.01	-4.89	0	15.05	7.49	1.64	5.26	2.44	0
17	3	40	0:100	5	-1.18	1.69	3.26	0	4.63	1.4	0	5.74	13.85	0	-0.39
18	3	20	50:50	5	0	3.64	4.51	2.35	20.64	-6.74	4.58	0	6.91	0	0
19	5	40	100:0	5	0	1.15	2.91	7.22	8.22	5.46	0	2.21	17.37	1.25	5.46
20	7	20	100:0	5	-1.2	-2.81	3.2	0	0	12.06	12.13	1.51	5.62	0	0
23	7	20	0:100	10	2.79	1.18	0	-3.95	0	8.72	0	1.68	0	1.89	0
26	3	40	50:50	10	0	1.19	16.86	8.1	0	8.1	11.04	0	1.46	2.47	0
28	5	20	0:100	5	0	3.8	47.78	4.73	0	2.92	0	4.53	9.47	0	4.5
29	7	40	50:50	5	2.44	3.45	21.22	21.22	0	31.15	0	1.61	6.3	2.48	0
30	3	34.1	50:50	0	0	1.93	5.88	13.1	0.91	0	0	0	0	0	11.98
31	5	40	0:100	10	0	3.65	48.87	5.31	0	3.34	0	4.98	11.99	0	6.04
32	7	40	0:100	0	0	4.96	26.67	0	0	41.1	0	4.27	4.59	0	7.42

The optimized conditions were fixed parameters for secondary studies except hold time which needed to be further varied along with other parameters as given in table 2. The results of secondary run were compiled in

table 7 and the chromatograms are shown in fig. 4. The chromatograms of all the 8 runs are shown in fig. 4 where the upper curve shows the chromatogram of the pure drug combination followed by their stressed

sample chromatogram at same run condition. PDA detector screening chromatogram for one of the run is

shown in fig. 5.

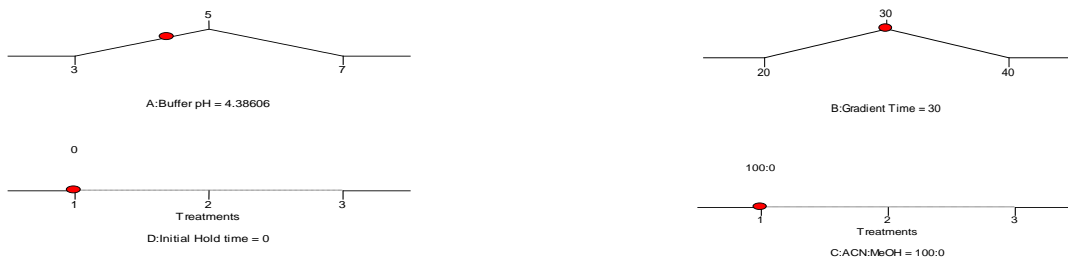


Fig. 3: Finalised condition of variable parameters from the primary screening from compiled data and after analysis by DoE.

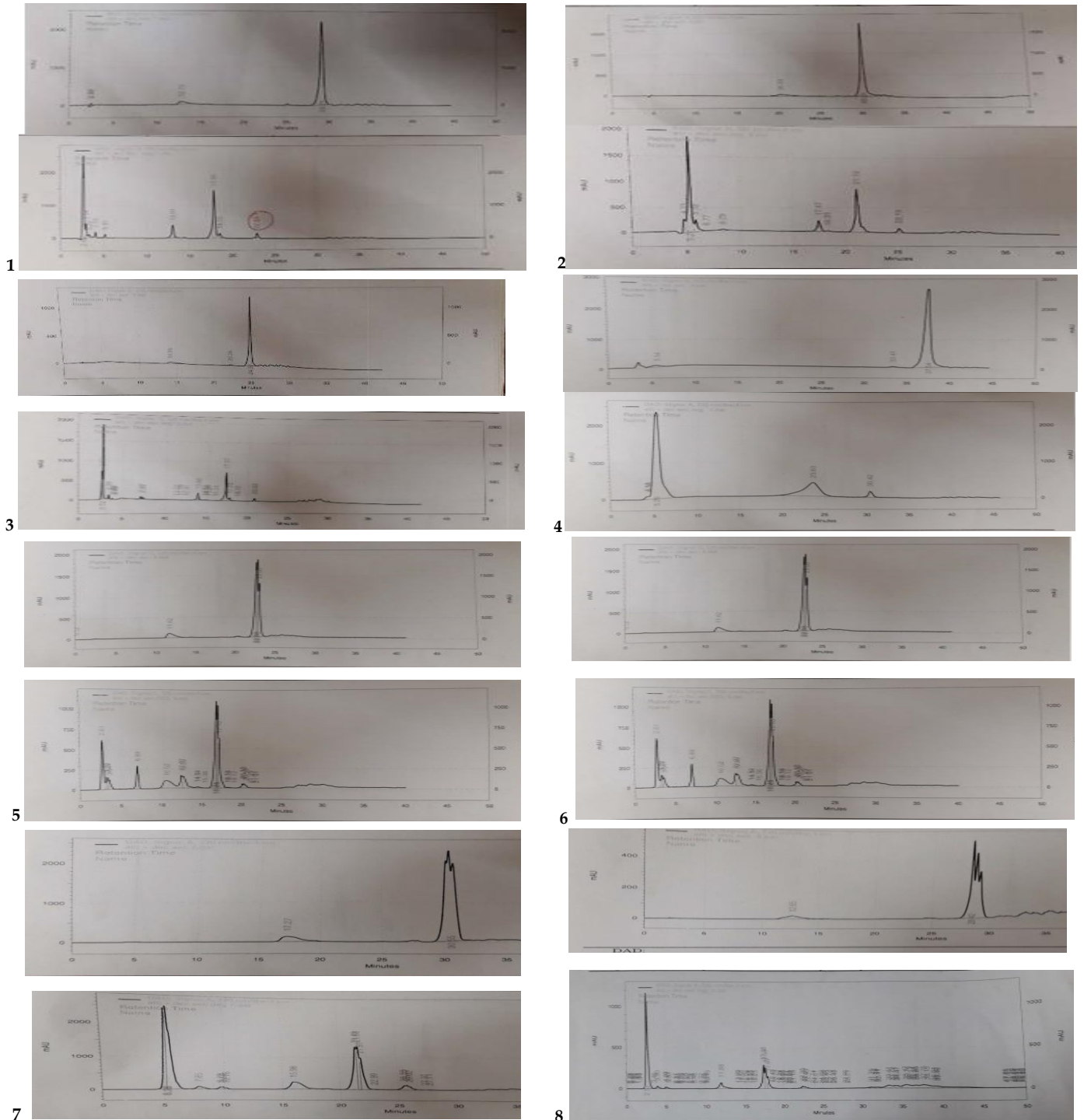


Fig. 4: Secondary run chromatograms for all the 8 runs (1-8) as given detailed in table 5. The data is aligned with the combination (ALB+DEC pure drugs) run on top and that of the stressed samples below it.

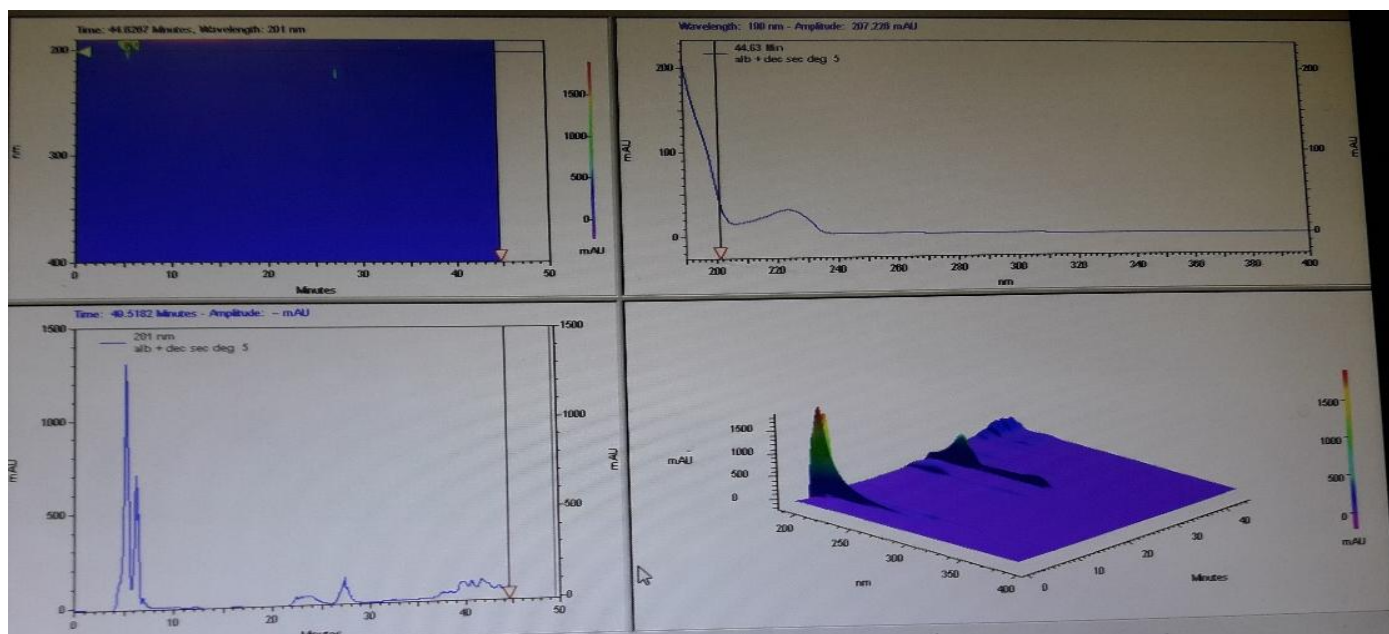


Fig. 5: Data showing the PDA detector screening data for one of the secondary screening run of ALB + DEC combination.

Table 7: Compiled results of secondary runs showing the resolution of peak relative to next adjacent peaks.

Run	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Sec1	0	4	3	2	1	4	6	7	0	5	9
Sec2	0	5	4	2	2	7	8	9	8	9	0
Sec 3	0	7	1	7	8	9	7	9	9	8	7
Sec 4	0	6	5	7	8	7	9	7	5	5	4
Sec 5	0	8	8	6	5	6	5	9	7	4	3
Sec 6	0	6	4	5	2	5	8	9	9	7	3
Sec 7	0	5	6	4	1	4	9	3	6	9	7
Sec 8	0	4	8	3	4	9	6	4	3	6	9

The HPLC secondary studies data was compiled and further analyzed by the Design Expert software to get the optimization parameters (fig. 6). The Initial organic modifier at 8%, final organic modifier at 80%, flow rate of 1.0 ml was the optimized condition for the first three varied factors. The buffer type optimized was acetate buffer, helpful when further doing LC-MS studies. The strength of the buffer finalized was 12 mM selected from 8 mM and 10 mM. The oven temperature also affected the resolution, so it was finalized at 35°C.

DISCUSSION

Systematic forced degradation studies on combination of albendazole and diethylcarbamazine were performed keeping in mind the various guidelines already discussed namely ICH Q1A(R2) guideline, WHO guidelines. [7-8, 27] An HPLC with PDA detector method was developed using DoE, for best results many of the effectors of performance were evaluated. DoE was applied in two stages as if method was developed in one stage would lead a method which will a huge number of parameters which will be tough to handle. Some to divide it method was developed in two stages, primary for selection of parameters and secondary for screening. [17]

The design applied was under category RSM (response surface methodology) while for secondary stage was orthogonal array design. The number of runs in primary was 32, in some of runs the peaks were

exchanged so a negative sign was put and for some peak it was not resolved so a 0. The first peak was taken as 0 but where it was exchanged a negative value.

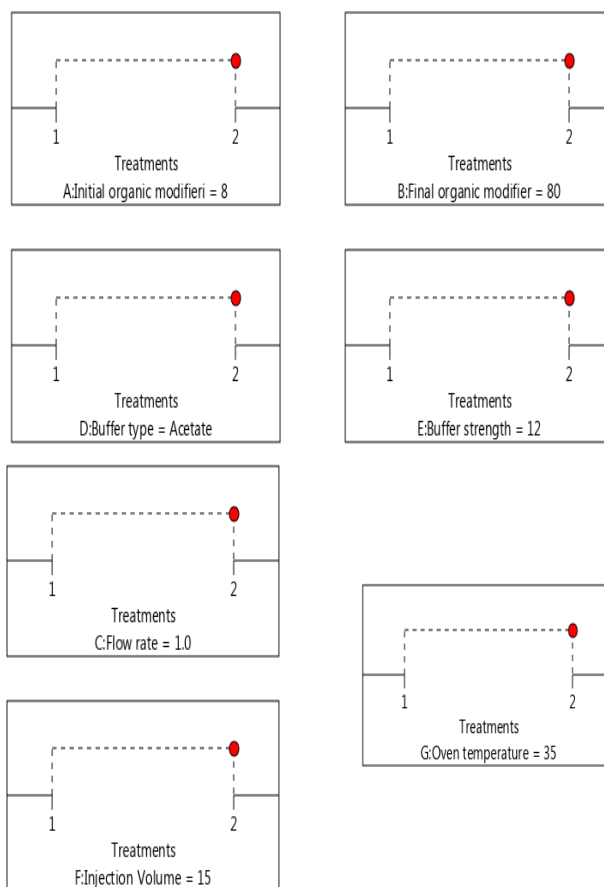


Fig. 6: Results of analysis of secondary data by DoE software and the optimised conditions from 8 runs.

The overlay chromatogram of primary peak highlighted that most of peak were well resolved and first three peaks were much affected by change in chromatographic conditions. [20] These peaks can be due

to H₂O₂ and citrate salt which is salt form of diethylcarbamazine. There are chances that the salt can under harsher conditions react with DEC or may be ALB leading to formation of some of the degradation products, that so under particular conditions. The will lead to variation in the peak and some interactions. As per the reported literature and its metabolism albendazole is more prone to oxidation. [5] There are reported literatures of formation of disulphide bond [28] as well as hydrazide bond [29] under some conditions. This is the possibility in albendazole.

The final method was optimized using acetate buffer in the mobile phase, so that this method can be extended to mass systems when and if need arise. This can be helpful technique for the characterisation of the DPs.

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