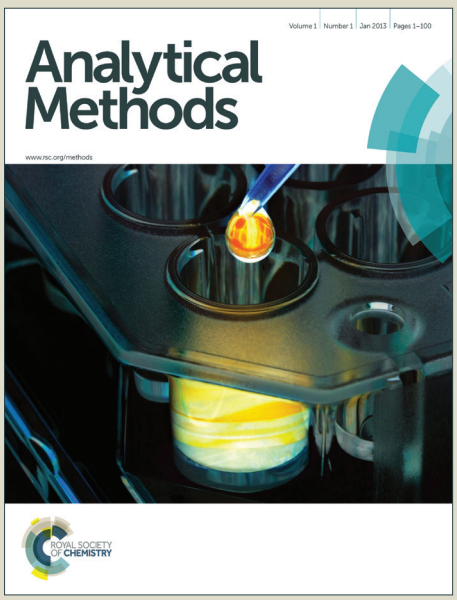
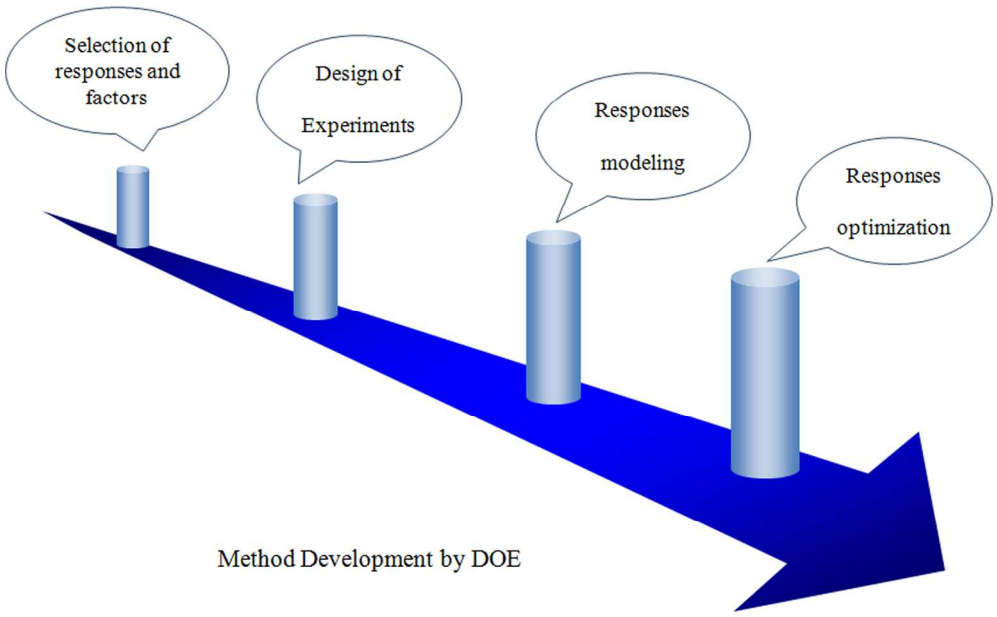
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods



227x143mm (300 x 300 DPI)

Development and validation of a UPLC method for screening potentially counterfeit anti-hypertensive drugs using design of experiment

Chiguru Vishnuvardhan[a], R. Srinivas[a,b*], N. Satheeshkumar[a*]

[a] *Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education and Research, Hyderabad (NIPER-H), Balanagar, Hyderabad 500037, India.*

[b] *National Center for Mass Spectrometry, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500607, India.*

***Corresponding Authors**

Dr. R. Srinivas., PhD
National Center for Mass Spectrometry, Indian Institute of Chemical Technology
Tarnaka, Hyderabad 500607, India.
srini@iict.res.in

&

Dr. N. Satheesh Kumar, M. Pharm., Ph.D.,
Assistant Professor
Department of Pharmaceutical Analysis
National Institute of Pharmaceutical Education and Research [NIPER]
Balanagar, Hyderabad-500037
India
Phone: +91 40 23073741 / 40
Fax: +91 40 23073751
E.mail: satish@niperhyd.ac.in

Abstract

An isocratic reversed phase ultra performance liquid chromatography (RP-UPLC) method was developed for screening counterfeit medicines with UV detection at 210 nm. Chromatographic separation was performed on a Waters BEH C-18 column (50 × 2.1 mm, i.d., 1.7 µm particle size) with isocratic elution of mobile phase containing a mixture of acetonitrile-methanol-ammonium formate buffer (0.01 M) (31: 30.5: 38.5 v/v/v) with a flow rate at 0.4 mL min^{-1} and pH 3 (adjusted with formic acid). A mixture design methodology was selected for the optimization and validation of the mobile phase composition. It was a trade-off between the experimental designs by graphical optimization of the technique using an overlay plot. In addition, the method validation was done as per the ICH guidelines using linearity, accuracy, precision, system suitability and robustness as parameters. The developed method was found to be sensitive, simple and highly robust for routine analysis and counterfeit detection of selected four drugs.

Key words: RP-UPLC, Hypertensive drugs, Counterfeit detection, Design Expert Software (Stat-Ease Inc., Minneapolis, Minnesota).

1. Introduction

Pharmaceutical counterfeiting is a enhancing problem in the world, especially in developing countries including India.[1] No country is free of counterfeit and substandard drugs. Although it is strenuous to obtain precise figures, estimates put counterfeits at more than 10% of the global medicines market. The world health organisation (WHO) has defined counterfeit drugs as those which are deliberately mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products with the correct ingredients or with the wrong active ingredients, without active ingredients, with insufficient active ingredient or with fake packing. Drug quality is presently renewed international attention. Over the past decade, there has been an escalation in public consciousness of the existence of counterfeit and substandard drugs,[2] which have been increasingly reported in developing countries where regulations are ineffective.[3, 4] To enforce effective countermeasures against counterfeit and substandard drugs, there is a requirement for more data to define the extent of the problem. The concern of poor quality drugs has been discussed more in the mass media including news papers than in biomedical literature. The U.S. center for medicine in the public interest assessed that the counterfeit drug market is worth about $ 75 billion a year. About 15 per cent of all medicines sold worldwide are counterfeits. In developing nations of Africa, Asia and Latin America, it's about 30 per cent. It's a dangerous business, one that kills an approximated 700,000 people a year.[12] That's because a lot of medicines for curing life-threatening diseases such as malaria and tuberculosis, hypertension etc. are fakes. [12] High blood pressure (BP) is a major public health issue in India and its pervasiveness is rapidly increasing among both urban and rural populations.[5] In fact, hypertension is the most extensive chronic disease in India. The prevalence of hypertension ranges from 20-40% in urban adults and 12-17% among rural adults. The number of people with

hypertensions projected to increase from 118 million in 2000 to 214 million in 2015, with nearly equal number of men and women.[5] A survey of 26,000 adults in south India showed a hypertension prevalence of 20%, but 67% of those with hypertension were incognizant of their diagnosis. Majority of hypertensive subjects still remain undetected and the control of hypertension is also incompetent. This calls for urgent prevention and control measures for hypertension. Therefore, it is especially important to ensure the quality of anti-hypertensive drugs. All four drugs (telmisartan, olmesartan, irbesartan and azilsartan medoxomil potassium) chosen in this study are commonly used in clinic for hypertension treatment. Their structures are shown in Fig.1. They decrease the blood pressure by antagonising angiotensin II receptor. Telmisartan (TEL), olmesartan (OLME) and irbesartan (IRBE) have been widely used because of their acceptable price and good curative effect. Azilsartan medoxomil potassium (AZIL) is relatively expensive, so it has potentially to be counterfeited by cheaper ones. Considering counterfeit products mainly present in rural areas, a simple and accurate method for screening medicines should be developed.

HPLC methods have been reported for the simultaneous analysis of irbesartan and telmisartan[6], olmesartan and irbesartan[7]. There is no published method for simultaneous determination of these four drugs. It is the objective of this research to develop and validate a simple UPLC method for the separation and simultaneous determination of the four different anti-hypertensive drugs. The major advantage of the proposed method is that four commonly used anti-hypertensive drugs can be separated on an isocratic solvent system within less time (2 min). Although a combination of these active ingredients would not normally be present in the same formulation, it could afford a useful method for screening potentially counterfeit drugs.

The present work targets to provide a new method to develop mobile phase composition using the most relevant experimental mixture design methodology. Rational practice of experimental mixture design in analytical method development helps to establish a robust mobile phase composition. The literature already address the utility of experimental design methodology in LC analytical methods, such as the application of Box-Behnken design (BBD) for the optimization of mobile phase composition for lenalidomide[8] and fractional factorial design (FFD) and central composite design (CCD) in the screening and optimization of bioanalytical methods[9]. Among the several types of experimental design methodologies (BBD, CCD, FFD), mixture design is the most appropriate methodology for experiments with at least two independent variables or factors having constraints and more than one response.[10, 11] As there is no report of developed analytical method using experimental design techniques, it was envisaged to develop and validate a simple and fast analytical method by UPLC to identify and quantify them by optimization of the mobile phase composition using Design Expert Software (Version 7.0.0; Stat-Ease Inc., Minneapolis, Minnesota). This work portrays the validation parameters stated by the ICH guidelines [13] to achieve an analytical method with acceptable characteristics of suitability, reliability and feasibility.

2. Experimental

2.1 Standards and reagents

Olmesartan (99.80%), Irbesartan (99.91%), Telmesartan (99.75%) and Azilsartan medoxomil potassium (99.57%) were obtained as gratis samples from Daiichi Sankyo Pharma Pvt. Ltd. (Gurgaon), Aurobindo Pharma Ltd. (Hyderabad), Mylan Laboratories Ltd. (Hyderabad) and Takeda Pharmaceutical Company Ltd. (Mumbai) respectively. Ammonium formate, ammonium

acetate and ammonium bicarbonate were of analytical grade. Acetonitrile and methanol were of HPLC grade and purchased from Merck Chemicals Ltd. (Mumbai, India). High purity water was prepared by passing through a Millipore Milli-Q plus system (Milford, MA, USA) and was used to prepare buffer solutions.

2.2 Instrumentation

Method development and validation was performed on an Acquity UPLC^{TM} system (Waters, Milford, USA). The system consisted of a quaternary solvent manager, a sample manager and a photo diode array detector. The output signal was monitored and processed using the Waters Empower 3 software. The column used was Waters BEH C-18 column (50 × 2.1 mm, i.d., 1.7 µm particle size). A mixture of acetonitrile-methanol-ammonium formate buffer (10 mM) (31: 30.5: 38.5) was used as the mobile phase with a flow rate of 0.4 mL min^{-1}, pH 3.0 (adjusted with formic acid) and UV detection at 210 nm. Prior to injection of the drug solution, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The data were acquired, stored and analysed with Empower v.3 software.

2.3 Method development as per mixture design

The mobile phase comprising a mixture of acetonitrile, methanol and ammonium formate buffer (10 mM) of pH 3.0 (adjusted with formic acid) was selected for mobile phase optimization. The concentration of mobile phase components *viz.* acetonitrile (X_1), methanol (X_2) and formate buffer (X_3) were selected as independent variables, whereas resolution between OLME and IRBE (Y_1, critical pair) and retention time of AZIL (Y_2) were chosen as response variables and parameters of robustness testing, to measure the elution performance of the mobile phase. For optimization of D-optimal mixture design a methodology was employed using Design-Expert 7.0

software (Stat-Ease Inc., USA). Table 1 shows the levels of mobile phase components, employed in the optimization at a fixed flow rate of 0.4 mL min^{-1} and pH 3.0 (adjusted with formic acid). A total 14 experimental runs obtained from the design mixture were subjected to experiment in order to generate the response variables (Y_1 and Y_2), as summarized in Table 2. Statistical analysis was performed using ANOVA to calculate the significant difference in the mobile phase compositions obtained from the design matrix. The response surface methodology (RSM) was selected to analyze the effect of independent variables on the responses. Further, the effect of interactions among the independent variables and responses were observed by scrutinizing the linear polynomial equations generated by the multiple linear regression analysis (MLRA) method. A linear model equation generated by the design is portrayed below as eqn (1). The equation indicates coefficients (β_0 to β_7) of various model terms including the two factor and three factor interaction terms. It helps to anticipate plausible interactions among the critical factors selected for the development of the UPLC method. Finally, the optimum mobile phase composition was trade-off using the graphical optimization procedure.

$$Y=\beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_4X_1X_2+ \beta_5X_2X_3+ \beta_6X_2X_3+ \beta 7X_1X_2X_3 \qquad (1)$$

2.4 Preparation of the standard solution

A stock solution (1 mg mL^{-1}) of mixture of TEL, OLME, IRBE and AZIL was prepared by dissolving 10 mg of each drug in a 10 mL volumetric flask using acetonitrile: methanol (50:50) as diluent. From this stock solution a working standard solution of 100 µg mL^{-1} strength was prepared. All other solutions were prepared by further diluting the working standard solution.

2.5 Estimation of drugs in pharmaceutical dosage forms

The developed UPLC method was used for the determination of selected drugs in pharmaceutical formulations. Marketed formulations, Olmetor (20 mg), Telsite (20 mg) and Irbest (300 mg) were procured from local pharmacy shop and evaluated for the amount present in the formulation. Twenty tablets were weighed and powdered in a mortar and pestle. Accurately measured powder equivalent to 10 mg of each drug was transferred into a 50 mL volumetric flask containing 45 mL of diluent (acetonitrile: methanol (50:50)). As AZIL formulation is not available in India, API powder spiked with commonly used excipients equivalent to 10 mg of AZIL was transferred to the above volumetric flask. Then it was sonicated for 15 min, to ensure complete solubility of the drugs. Finally, the volume was adjusted up to 50 mL with diluent. The resulting solution was thoroughly mixed and filtered through a 0.22 µm syringe filter. From this 50 µg mL^{-1} concentration solution was prepared by suitable dilution. The sample (2 µL) was analysed in triplicate and the mean values of peak areas were determined and the drug contents were quantified using the regression equation obtained from the calibration curve.

3. Results and discussion

3.1 Method development

Initial screenings were performed on an Acquity BEH C18 column 2.1 mm × 50 mm, 1.7 µm. For the screenings mobile phases were used consisting of combinations of three buffers: an ammonium formate buffer of pH 3.0 (adjusted with formic acid), an ammonium acetate buffer of pH 5.0 (adjusted with acetic acid), an ammonium bicarbonate buffer of pH 7.5 (adjusted with acetic acid) and two organic modifiers: acetonitrile and methanol. The screenings were performed at a flow rate of 0.4 mL min^{-1} and detection wavelength of 210 nm. Column oven

temperature was set at 30 ^{0}C and the injection volume on 2 µL. Based on the visual inspection (peak symmetry and peak separation), it was confirmed that the best initial separation was obtained using acetonitrile, methanol and ammonium formate buffer (pH 3.0). The suitability of mobile phase composition was identified by varying ratios of acetonitrile, methanol and formate buffer using the Design Expert software. Further, to identify the robust and optimized mobile phase composition, RSM was employed to analyze the effect of various independent variables on the selected responses. It helped in the identification of the positive and/or negative interactions amongst the independent variables. Fig. 2A and B portray the response surfaces and depict the relationship between the independent variables (X_1, X_2 and X_3) on robustness parameters *i.e.*, resolution between OLME and IRBE (Y_1) and retention time of AZIL (Y_2). For better discerning of the effect of interaction among the independent variables and responses, linear polynomial equations were studied. The linear polynomial equations generated from ANOVA are depicted below.

$$Y_1 \text{ (resolution)} = 1.49X_1 + 2.25X_2 + 3.23X_3 + 0.44X_1X_2 - 0.26X_1X_3 + 1.11X_2X_3 - 2.03X_1X_2X_3$$

$$Y_2 \text{ (retention time)} = 1.33X_1 + 1.78X_2 + 7.71X_3 - 0.29X_1X_2 - 7.43X_1X_3 - 3.31X_2X_3$$

From the polynomial equations, it has been observed that all three independent variables with a positive sign designated a positive effect on both the observed responses. Positive interaction terms indicate the combined effect of independent variables on response variables. In case of response (Y_1), two of the interaction terms were positive i.*e.,* the effect of concentration of X_1 and X_2 and concentration of X_2 and X_3, where as for the effect of concentration of X_1 and X_3 and concentration of X_1, X_2 and X_3 it was found to be negative. However, for response (Y_2), all three interaction terms were negative. The optimum mobile phase composition was selected from

design technique by a graphical optimization trade-off technique obtained from the overlay plot (Fig. 3). The optimized mobile phase contained acetonitrile (31%), methanol (30.5%) and ammonium formate buffer (38.5%). The chromatogram of blank (Fig. 4a) and synthetic mixtures containing four drugs (Fig. 4b), developed in the optimum mobile phase composition was obtained from experimental design. The optimized mobile phase produced good resolution in chromatographic elution.

3.2 Validation

The analytical performance parameters such as system suitability, linearity, range, precision, accuracy, limit of detection, limit of quantification, specificity and robustness were validated according to International Conference on Harmonization ICH Q2B[13] guidelines.

3.2.1 System suitability study

The system suitability as assessed by six replicate analysis of 50 µg mL^{-1} concentration of all drugs. % RSDs (relative standard deviation) for peak areas, resolution, retention time and tailing factor were tabulated in Table 3. The %RSD values of peak area were found to be below 0.21 *i.e.,* within the limit (2%) indicating the suitability of the method development.

3.2.2 Linearity and range

The linearity of the method used for each anti-hypertensive drug was evaluated on a standard curve of peak area versus the concentration of analyte. The calibration curve (5 points) was constructed by injecting 40, 45, 50, 55 and 60 µg mL^{-1} concentration of solution and evaluated by its correlation coefficient. The peak areas of the drugs were linear in the range of 10-100 µg mL^{-1}. The correlation coefficients (r^2) of the calibration curves obtained from the regression line

were above 0.998, which demonstrates the excellent relationship between peak area and concentration. The statistical data of regression analysis are summarised in Table 4.

3.2.3 LOD and LOQ

The limit of detection (s/n=3) and limit of quantitation (s/n=10) were determined from signal to noise ratio values by injecting a series of 6 diluted solutions with known concentrations (Fig. 4c). The results were depicted in Table 4. Accuracy and precision (n=3) was measured at LOQ level and results were depicted in Table 8.

3.2.4 Accuracy

The accuracy study was performed by the standard addition method using three different solutions containing 40, 50 and 60 µg mL^{-1}. Triplicate samples of each concentration level (n=3) were prepared and the recovery at each level (n=3) and mean recovery (n=9) were determined. The obtained % recovery values were within the range (98.25-100.32) satisfying the acceptance criteria for the study (Table 5).

3.2.5 Precision

The precision of the method was checked by injecting six individual preparations (n=6) of 100% solution i.e., 50 µg mL^{-1}. Percentage RSD for peak areas of each drug was calculated. The intermediate precision (ruggedness) of the method was also evaluated by a different analyst and different instrument in the same laboratory. The percentage RSD of areas of each drug was within 0.6, confirming the good precision at low level of the developed analytical method. The precision data are summarized in Table 5.

3.2.6 Specificity

Specificity was examined by using different excipients without active substances and verifying the absence of interferences. As there was no interference from excipients, it was concluded that the method was specific. Specificity at LOQ level was also checked and found that it is specific.

3.2.7 Robustness

The study of robustness was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of resolution between OLME and IRBE and retention time of AZIL. As total run time of a method depends on retention time of last peak and resolution between critical pair (OLME and IRBE) was 2, so they were taken into consideration. The factors chosen for this study were the flow rate (mL min^{-1}) and pH of the mobile phase. None of the parameters exceeded the limit and therefore showed that the study variable factors did not affect selected response factors (Table 6).

3.2.8 Analysis of the marketed tablets.

The UPLC method developed is sensitive and specific for the quantitative determination of all four selected drugs. The method has been validated for different parameters, hence it has been applied for the estimation of the drug in pharmaceutical dosage forms. Each sample was analysed in triplicate after extracting the drug. The amount of drug present was within the specified range (95-105%). None of the ingredients interfered with the analyte peak when compared with chromatogram. The results are given in Table 7 which shows the high percentage recoveries and low RSD (%) values.

4. Conclusion

An UPLC method was developed for the determination of olmesartan, irbesartan, telmisartan and azilsartan medoxomil potassium in different formulations. The method is characterised by good linearity, precision and accuracy. The use of a single method for analysing the four most employed anti-hypertensive drugs will be highly advantageous as it would provide scope to screen potentially different counterfeit drugs. The method would help one to identify quality of the formulations and hence potential substitution of the more expensive active substance with the cheaper one. The access to good quality medicines is a right of all people in the world and hence counterfeiting of life saving medicines should be treated as a crime towards the humanity. Counterfeiting pharmaceuticals is a worldwide phenomenon particularly in developing countries, the need for the development and validation of suitable methods for analysing medicines will play important role in eradicating pharmaceutical counterfeiting. The developed method could be useful for screening of counterfeit or substandard preparations of four anti-hypertensive drugs by the national quality control laboratories in developing countries for the analysis of medicines of dubious origin. A future development of this work will be the extension of the method to other anti-hypertensive drugs and the statistical evaluation of drugs in a number of developing countries.

Acknowledgements

The authors wish to thank Dr. Ahmed Kamal, Project Director, National Institute of Pharmaceutical Education & Research, Hyderabad for his constant encouragement. One of the author Mr. Ch. Vishnuvardhan thankful to Department of Pharmaceuticals, Ministry of chemicals and fertilizers, New Delhi, India for providing a research fellowship.

References

1. A. Y. Khan and N. Ghilzai, *Indian. J. Pharmacol.*, 2007, **39**, 206-207.
2. R. Verduin-Muttiganzi and G. Verduin-Muttiganzi, *Trop. Med. Int. Health.*, 1998, **3**, 602-602.
3. B. Pecoul, P. Chirac, P. Trouiller and J. Pinel, *J AM MED A*., 1999, **281**, 361-367.
4. D. B. Menkes, *BMJ.*, 1997, **315**, 1553-1158.
5. R. Gupta and S. Guptha, *Indian. J. Med. Res.*, 2010, **132**, 531-542.
6. M. H. Hani, *Pharm. Anal. Acta.*, 2012, **3**,1-11.
7. N. Sultana, M. S. Arayne, S. S. Ali and S. Sajid, *Se Pu.*, 2008, **26**, 544-549.
8. M. S. Hasnain, S. Rao, M. K. Singh, N. Vig, A. Gupta, A. Ansari, P. Sen, P. Joshi and S. A. Ansari, *Analyst.*, 2013, **138**, 1581-1588.
9. L. Živanović, A. Ličanski, M. Zečević, B. Jocić and M. Kostić, *J. Pharm. Biomed. Anal.*, 2008, **47**, 575-585.
10. A. C. Atkinson and R. D. Tobias, *J. Chromatogr. A.*, 2008, **1177**, 1-11.
11. A. Alves de Almeida and I. Spacino Scarminio, *J. Sep. Sci.*, 2007, **30**, 414-420.
12. The globe and mail, http://www.theglobeandmail.com/life/health-and-fitness/trade-in-counterfeit-medicines-needs-an-international-fix/article622372/ , (accessed January 2014).
13. International Conference on Harmonization Q2 (R1) Guideline on Validation of Analytical Procedures: Text and Methodology, Geneva, Switzerland, 2005

Table legends

Table 1 Selected levels of mobile phase as independent variables

Table 2 Experimental design matrix representing the runs of mobile phase composition and observed responses

Table 3 System suitability results (n=5)

Table 4 Results of the linearity, LOD and LOQ

Table 5 Accuracy and precision results for determination of four anti-hypertensive drugs

Table 6 Chromatographic conditions investigated during robustness testing

Table 7 Assay results of drugs

Table 8 Accuracy and precision results at LOQ level

Table 1 Selected levels of mobile phase as independent variables

Coded factor	Level	X_1 (Acetonitrile %)	X_2 (Methanol %)	X_3 (Buffer %)
-1	Low	25	20	40
1	High	35	30	50

Table 2 Experimental design matrix representing the runs of mobile phase composition and observed responses

Experimental runs	Acetonitrile (X_1)	Methanol (X_2)	Formate buffer (X_3)	Resolution (Y_1)	Retention time (Y_2)
1	25.0	30.0	45.0	2.83	3.00
2	35.0	25.0	40.0	1.84	1.33
3	27.5	30.0	42.5	2.42	2.10
4	25.0	25.0	50.0	3.15	5.00
5	30.0	30.0	40.0	2.10	1.62
6	35.0	20.0	45.0	2.02	1.94
7	30.0	30.0	40.0	2.10	1.62
8	30.0	25.0	45.0	2.40	2.39
9	35.0	22.5	42.5	1.93	1.59
10	32.5	20.0	47.5	2.31	2.67
11	25.0	30.0	45.0	2.81	2.91
12	30.0	20.0	50.0	2.58	3.84
13	30.0	25.0	45.0	2.38	2.38
14	25.0	25.0	50.0	3.15	5.00

Table 3 System suitability results (n=5)

Parameter	OLME	IRBE	TEL	AZIL
Retention time	0.69	0.88	1.19	1.52
Resolution	-	2.01	3.04	2.61
Tailing factor	0.94	1.08	1.29	1.18
% RSD of area (n=5)	0.12	0.10	0.21	0.14

Table 4 Results of the linearity, LOD and LOQ

Analyte	Equation	r^2	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)	Range ($\mu g\ mL^{-1}$)
Olmesartan	y= 9717.9x+22782	0.9981	0.20	0.55	0.55-100
Irbesartan	y= 11713x+16412	0.9995	0.15	0.45	0.45-100
Telmisartan	y= 17731x-8733.4	0.9999	0.10	0.30	0.30-100
Azilsartan	y= 13024x+845.4	0.9987	0.25	0.80	0.80-100

Table 5 Accuracy and precision results for determination of four anti-hypertensive drugs

Analyte	Spiked concentration ($\mu g\ mL^{-1}$)	Mean measured concentration ($\mu g\ mL^{-1}$)	Accuracy (%)	Intraday precision (%)	Intermediate precision (%)
Olmesartan	40.12	39.76	99.10	0.16	0.23
	50.05	50.10	100.09	0.20	0.36
	60.16	60.10	99.90	0.10	0.25
Irbesartan	40.06	39.75	99.22	0.31	0.45
	50.12	50.01	99.78	0.12	0.22
	60.11	59.90	99.65	0.16	0.35
Telmisartan	40.20	39.50	98.25	0.50	0.45
	50.19	50.06	99.74	0.42	0.60
	60.03	59.75	99.53	0.39	0.45
Azilsartan	40.12	40.25	100.32	0.22	0.31
	50.31	50.16	99.70	0.16	0.19
	60.15	60.29	100.23	0.27	0.35

Table 6 Chromatographic conditions investigated during robustness testing

Variable	Range investigated	Resolution (Y_1)	Retention time (Y_2)
Flow rate	0.35	2.23	1.73
	0.4	2.09	1.52
	0.45	1.82	1.38
pH	2.8	1.92	1.41
	3	2.09	1.52
	3.2	2.18	1.65

Table 7 Assay results of drugs

Parameter	OLME	IRBE	TEL	AZIL
Mean recovery %	99.23 ± 0.13	100.25 ± 0.25	98.56 ± 0.19	99.46 ± 0.31
% RSD (n=3)	0.36	0.45	0.26	0.14

Table 8 Accuracy and precision results at LOQ level

Analyte	Spiked concentration (µg mL^{-1})	Mean measured concentration (µg mL^{-1})	Accuracy (%)	Precision (% RSD)
Olmesartan	0.55	0.53	96.36	1.20
Irbesartan	0.45	0.42	93.33	0.98
Telmisartan	0.30	0.28	93.33	1.56
Azilsartan	0.80	0.77	96.25	1.29

Figure legends

Fig. 1 Chemical structure of OLME, IRBE, TEL and AZIL

Fig. 2 (A) 3D response surface for the effect of independent variables (X_1, X_2, X_3) on response variable Y_1 (resolution between OLME and IRBE); (B) 3D response surface for the effect of independent variables (X_1, X_2, X_3) on response variable Y_2 (retention time of AZIL).

Fig. 3 Overlay plot of the experimental design indicating the optimum mobile phase composition.

Fig. 4a Blank Chromatogram

Fig. 4 b Typical chromatogram of synthetic mixtures containing four drugs

Fig. 4 c Typical chromatogram of synthetic mixtures containing four drugs at LOQ level

Fig. 1 Chemical structure of OLME, IRBE, TEL and AZIL

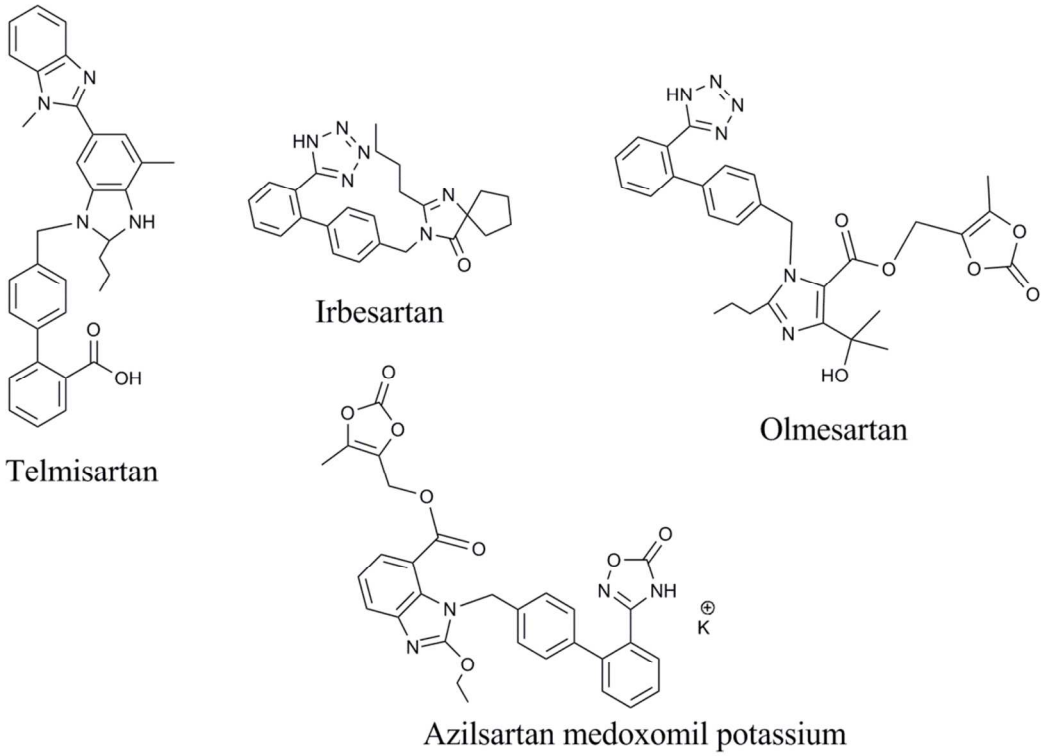
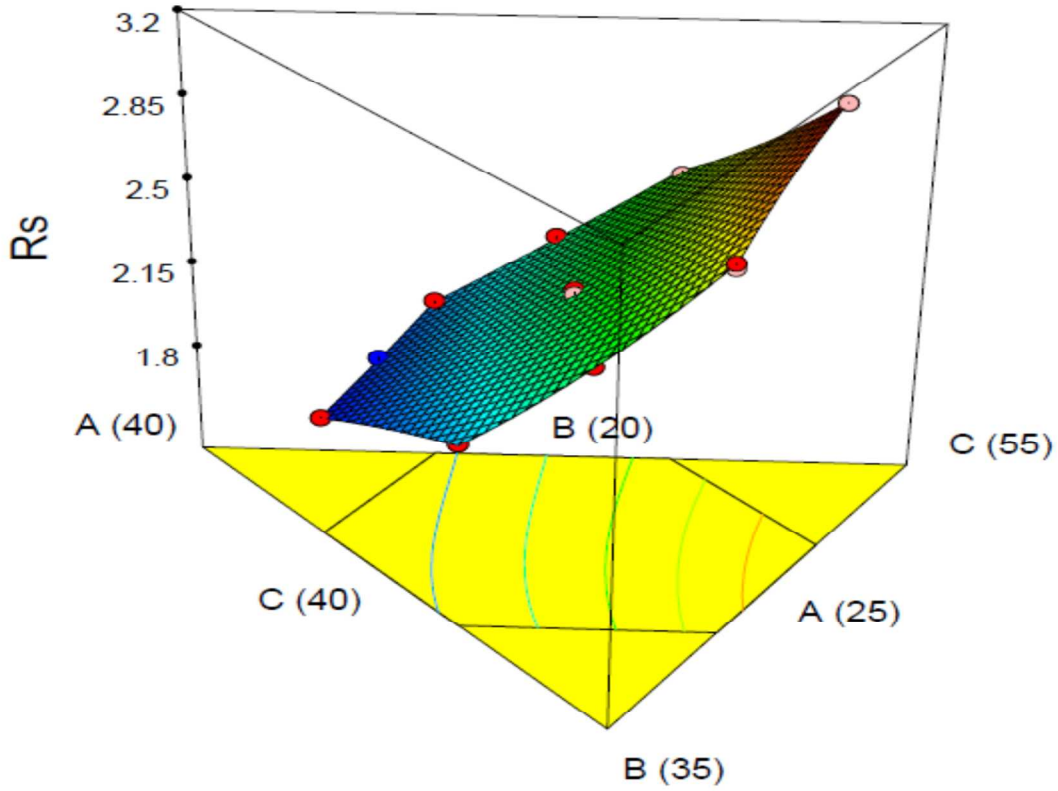


Fig. 2 (A) 3D response surface for the effect of independent variables (X1, X2, X3) on response variable Y1 (resolution between OLME and IRBE); (B) 3D response surface for the effect of independent variables (X1, X2, X3) on response variable Y2 (retention time of AZIL).

A)



B)

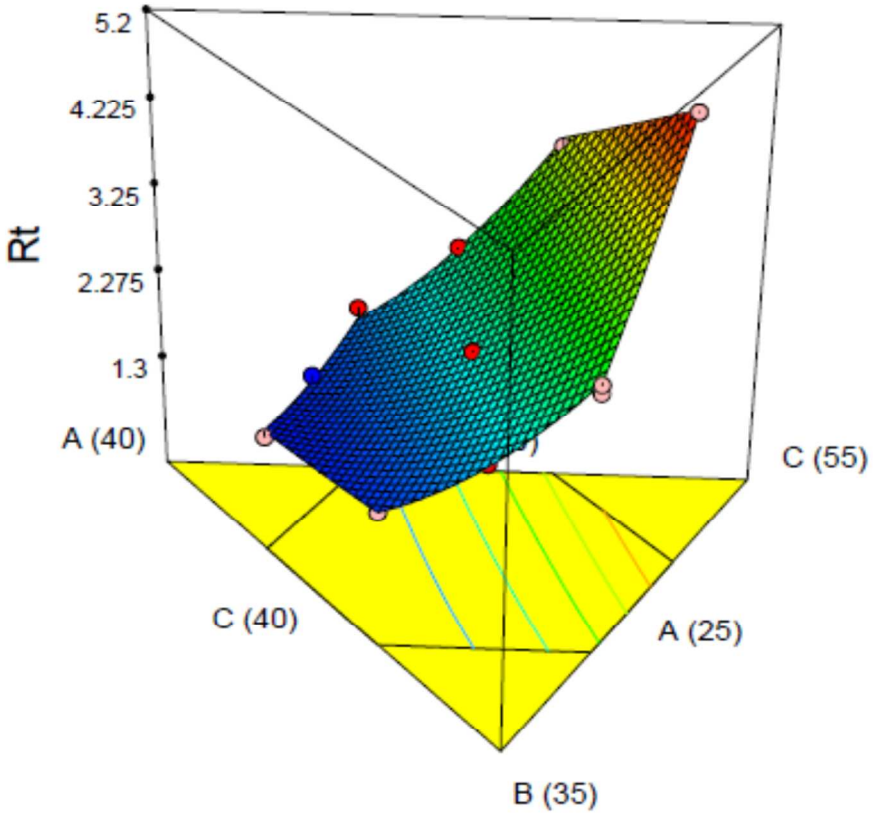


Fig. 3 Overlay plot of the experimental design indicating the optimum mobile phase composition.

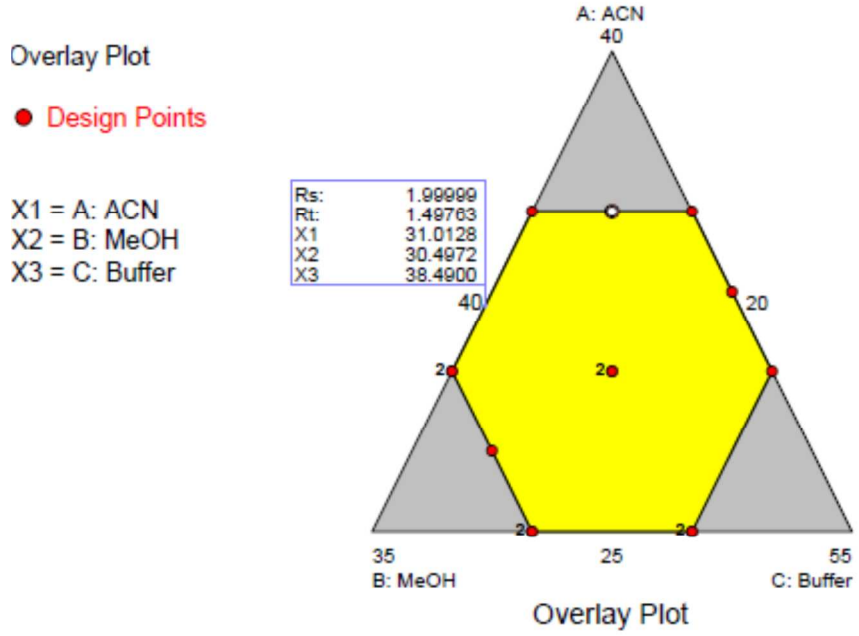


Fig. 4 a Blank Chromatogram

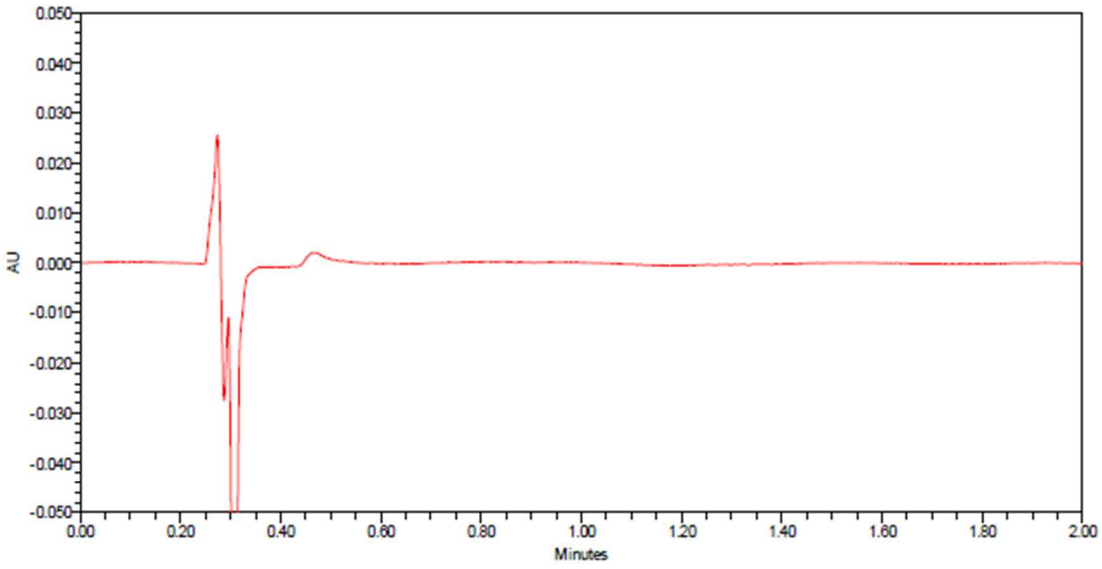


Fig. 4 b Typical chromatogram of synthetic mixtures containing four drugs (50 µg/mL each)

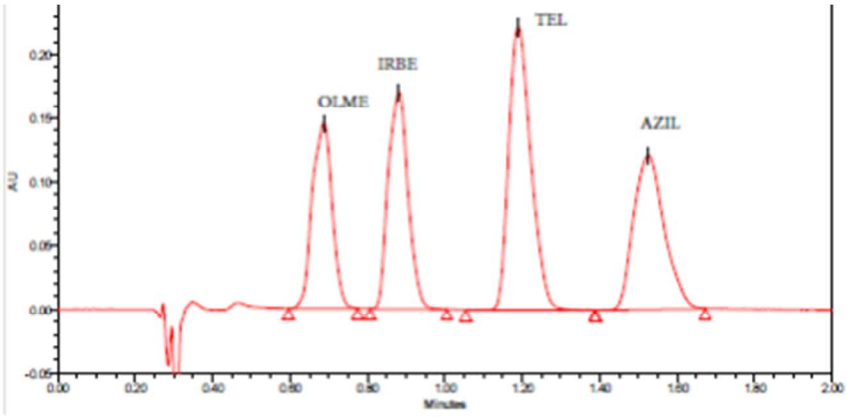
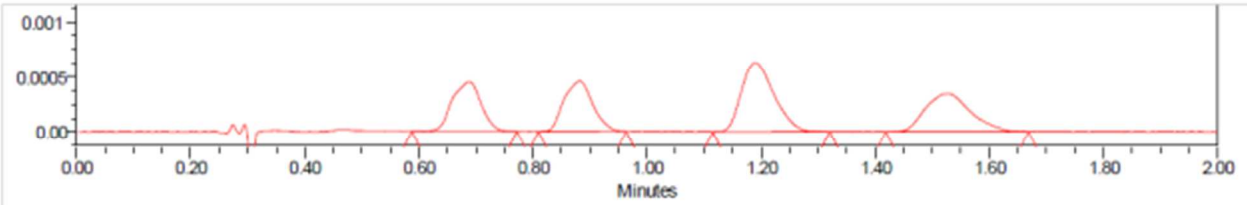
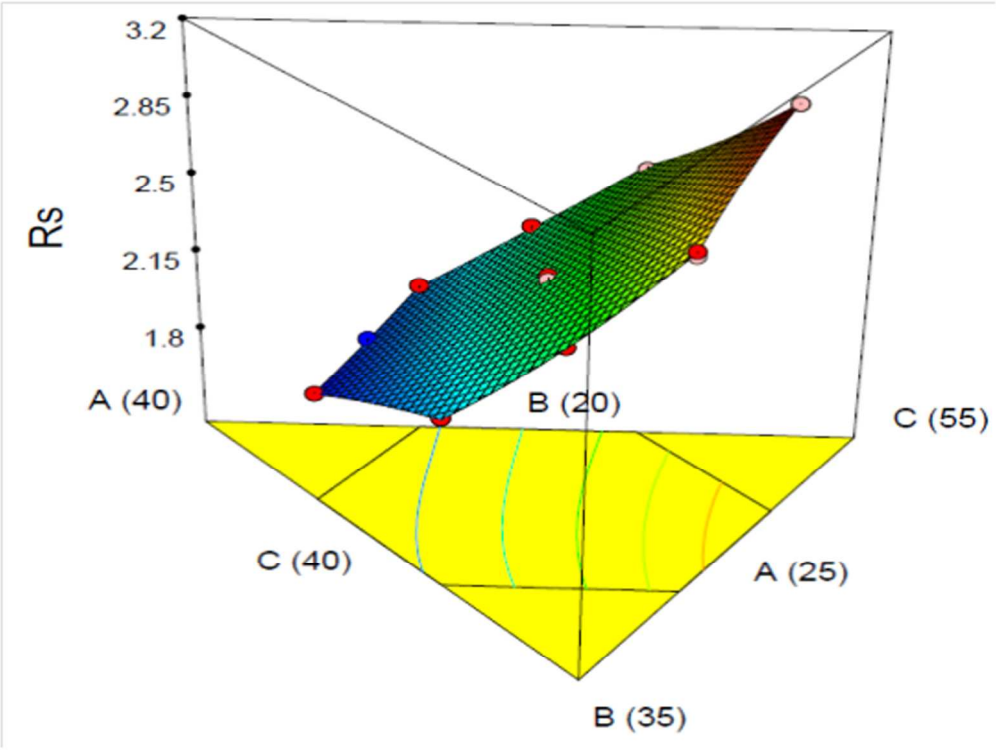
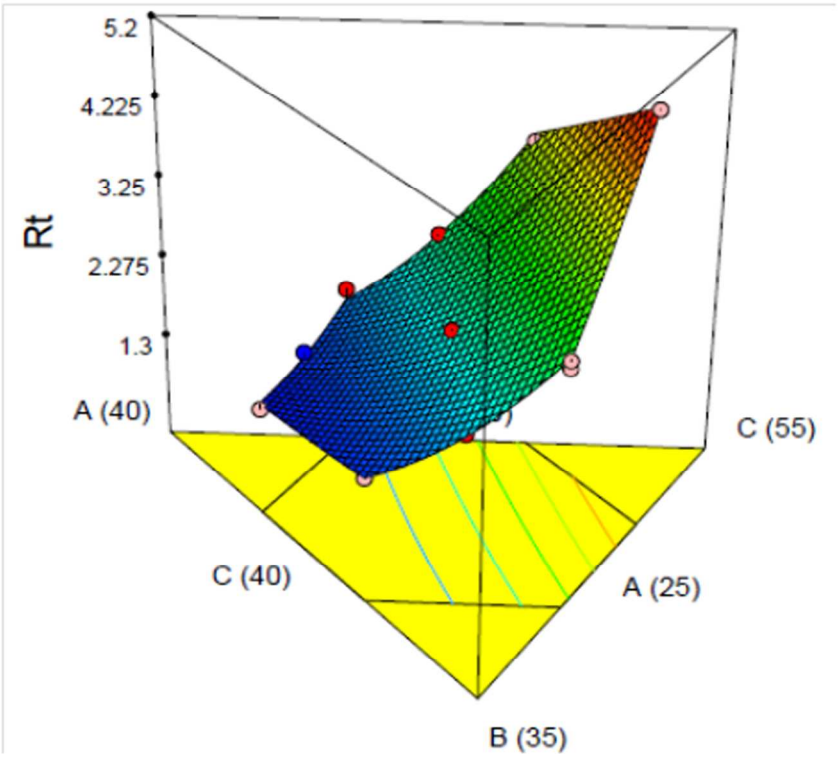


Fig. 4 c Typical chromatogram of synthetic mixtures containing four drugs at LOQ level

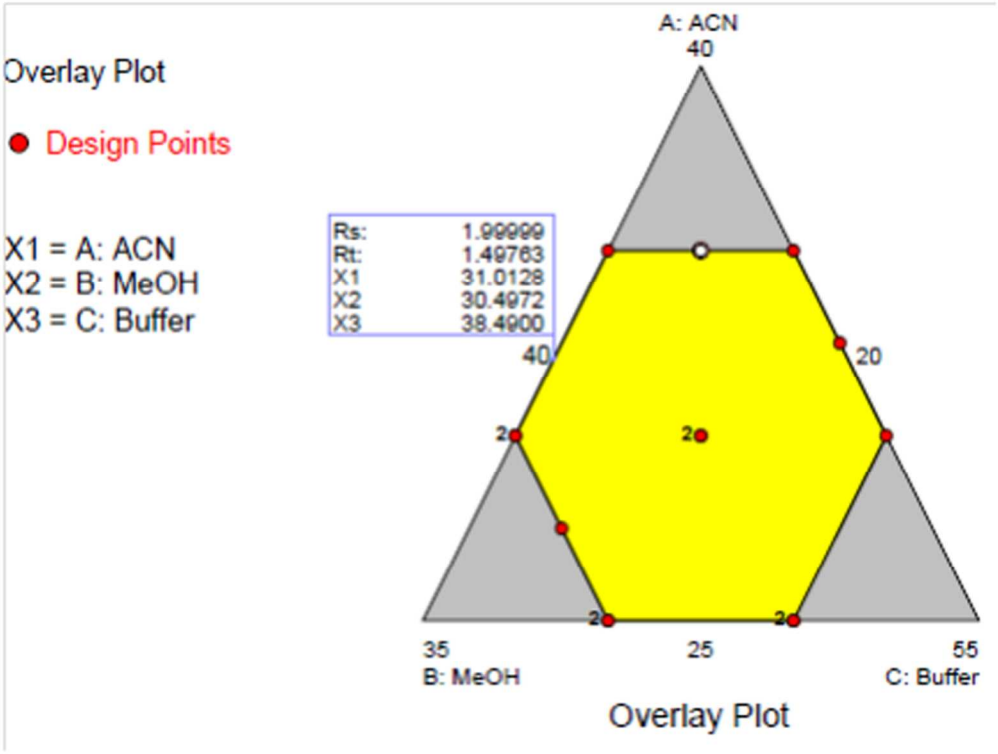




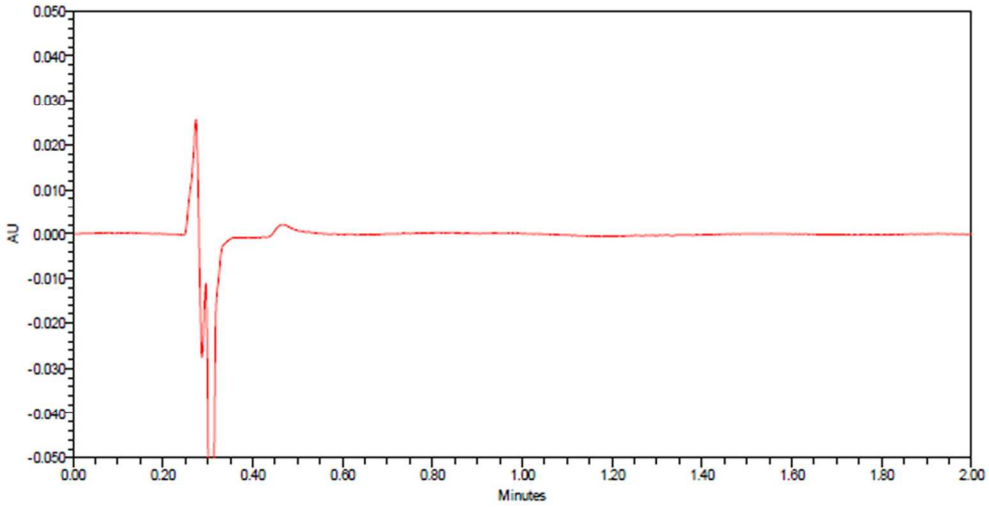
528x397mm (300 x 300 DPI)



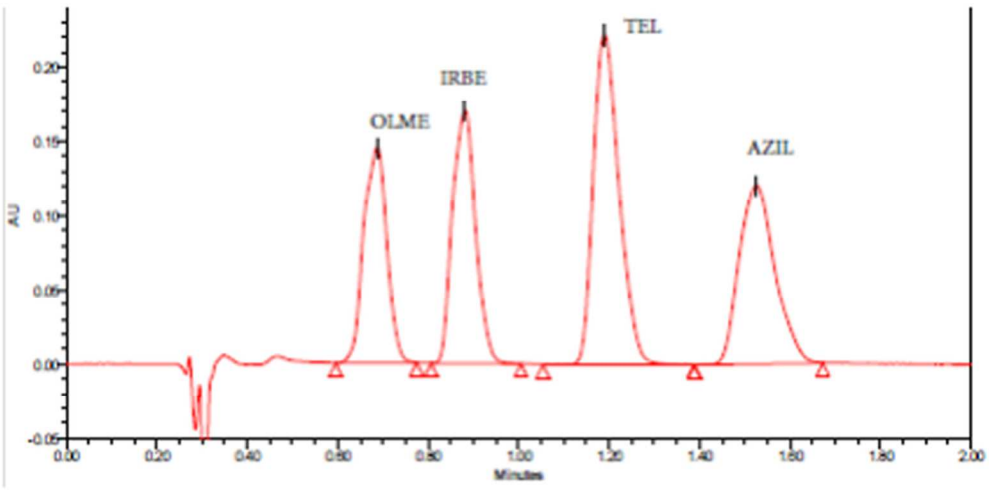
143x108mm (300 x 300 DPI)



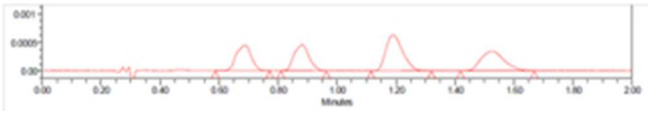
114x86mm (300 x 300 DPI)



87x44mm (300 x 300 DPI)



74x36mm (300 x 300 DPI)



27x4mm (300 x 300 DPI)