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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT OF A FAST LIQUID CHROMATOGRAPHY METHOD WITH A CHEMOMETRIC APPROACH BASED ON BOX-BEHNKEN DESIGN FOR THE DETERMINATION OF ANTIDEPRESSANTS IN PHARMACEUTICAL FORMULATIONS

FARMASÖTİK FORMÜLASYONLARDAKİ ANTİDEPRSANLARIN TAYİNİ İÇİN BOX-BEHNKEN TASARIMINA DAYANAN KEMOMETRİK YAKLAŞIM İLE HIZLI SIVI KROMATOGRAFİ YÖNTEMİNİN GELİŞTİRİLMESİ

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ABSTRACT

Objective: *The objective of this work was to develop a liquid chromatographic method for the quantification of antidepressants, namely duloxetine (DXN), fluoxetine (FXN), citalopram (CIT), paroxetine (PXN), and sertraline (SRN), by a chemometric approach based on Box-Behnken design.*
Material and Method: *After initial experiments to determine significant parameters, a Box-Behnken design consisting of 17 experiment sets was carried out. All separations were conducted using an Agilent Poroshell 120 EC-C18 analytical column (75 mm × 4.6 mm × 2.7 µm).*
Result and Discussion: *The optimum levels of pH, acetonitrile ratio, and flow rate were determined with the desirability function as 2.7, 38%, and 1.1 ml/min, respectively. The differences (<8%) between predicted optimum responses and experimentally obtained results proved the model's suitability. Limits of detection and limits of quantification values were in the ranges of 0.17-0.29 µg/ml and 0.53-0.89 µg/ml, respectively. The feasibility of the technique was proven by analyzing PXN and DXN formulations.*
Keywords: *Antidepressants, design of experiments, liquid chromatography*

ÖZ

Amaç: *Bu çalışmanın amacı, duloksetin (DXN), fluoksetin (FXN), sitalopram (CIT), paroksetin (PXN) ve sertralin (SRN) adlı antidepresanların tayini için Box-Behnken tasarımına dayalı kemometrik bir yaklaşımla sıvı kromatografik bir yöntem geliştirmektir.*
Gereç ve Yöntem: *Önemli parametreleri belirlemek için yapılan ilk deneylerden sonra, 17 deney setinden oluşan bir Box-Behnken tasarımı gerçekleştirilmiştir. Tüm ayırma işlemleri bir Agilent Poroshell 120 EC-C18 analitik kolon (75 mm × 4.6 mm × 2.7 µm) kullanılarak gerçekleştirilmiştir.*
Sonuç ve Tartışma: *pH, asetonitril oranı ve akış hızının optimum seviyeleri, arzu edilebilirlik fonksiyonu ile sırasıyla 2.7, %38.2 ve 1.1 ml/dak olarak belirlenmiştir. Tahmin edilen optimum yanıtlar ile deneysel olarak elde edilen sonuçlar arasındaki farklar (<%8) modelin uygunluğunu kanıtlamıştır. Tespit ve tayin limitleri sırasıyla 0.17-0.29 µg/ml ve 0.53-0.89 µg/ml aralığındadır. Yöntemin uygulanabilirliği, PXN ve DXN'nin formülasyonlarının analiz edilmesiyle kanıtlanmıştır.*
Anahtar Kelimeler: *Antidepresanlar, deneysel tasarım, sıvı kromatografisi*

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INTRODUCTION

Depression, a chronic disorder with substantial social and economic effects, is often characterized by a lack of interest, reduced energy levels, guilt-ridden emotions, changes in sleep or eating patterns, and impaired concentration. Chronic, recurring, and widespread challenges in psychosocial and occupational functioning are commonly linked to major depressive disorder (MDD) and can have severe consequences such as long-term incapacity and potentially fatal illness [1]. On a global level, there are over 320 million individuals affected by MDD. The lifetime prevalence is 26.1% and 14.7% for female and male adults in the US, respectively [2]. In this picture, due to its rapid dissemination, MDD is expected to rank as the second leading cause of work incapacity for individuals of all genders and age ranges [3,4].

While the exact origins and processes of MDD remain unclear, several theories have been proposed to elucidate the underlying molecular mechanisms, including the monoamine, neuroplasticity, glutamate, cholinergic/adrenergic, and stress-induced hypothalamic-pituitary-adrenal axis hypotheses [5].

Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), classified as second-generation antidepressants, are now the most commonly prescribed antidepressant groups worldwide. The advent of these drugs has brought about a major change in the way depression is treated, as they are both highly effective and less likely to produce negative side effects, unlike their predecessors with poor tolerability profiles [6]. SSRIs target serotonin transporters to prevent the reabsorption of serotonin, resulting in higher levels of active serotonin in the synapses. Their impact on norepinephrine and dopamine transporters is minimal. The best-known members of this group are citalopram (CIT), escitalopram, fluoxetine (FXN), fluvoxamine, paroxetine (PXN), sertraline (SRN), and vilazodone. Through selective inhibition of the reuptake process, SNRIs effectively prolong the presence of serotonin and norepinephrine in the synaptic cleft, thus modulating their respective physiological effects. Venlafaxine, desvenlafaxine, milnacipran, levomilnacipran, and duloxetine (DXN) belong to this group. Elevated extracellular serotonin and norepinephrine concentrations result in improved neurotransmission, thus relieving central nervous system dysfunctions [1,7].

Due to the widespread use of SNRIs and SSRIs, reliable techniques are required for rapid and accurate quality control (QC) of their formulations. Liquid chromatography and spectrometry are the most widely used analytical methodologies for quantifying SSRIs and SNRIs [1]. In addition, gas chromatography [8], thin-layer chromatography [9], electrochemical [10], and electrophoretic methods [11] have been reported.

The most commonly used approach in developing HPLC methods is trial and error, which involves altering one variable at a time while maintaining the others constant. Though this approach may achieve the desired separation, there is no assurance of attaining the actual optimum conditions. Conventional stepwise optimization is not only time, money- and labor-consuming but also unpredictable and even unsuccessful in correcting errors [12]. In order to surmount this challenge, a considerable amount of factors must be meticulously determined, examined, and managed. In addition, the experimental variables are interdependent, and the step-by-step approach generates a large amount of raw data that is highly challenging to interpret to achieve optimum conditions [13]. In this context, chemometric tools combined with appropriate statistical analysis methods have gained widespread acceptance nowadays as they provide numerous benefits, including fewer experiments, reduced use of reagents, and decreased time spent in the laboratory [13,14].

Design of Experiments (DoE) is an experimental setup in which several factors are evaluated simultaneously by conducting a predefined number of experiments at predetermined levels [12]. DoE is frequently used to optimize the operating conditions of various analytical processes, achieve high extraction yields, and improve separation efficiency with minimum effort, time, and resources [14]. Chromatographic optimization typically involves utilizing response surface methodology that relies on various approaches, including Box-Behnken design (BBD) and central composite design (CCD) [15,16].

Despite the proven efficiency of DoE in chromatographic method optimization, there is a scarcity of research on this topic in existing literature. Carlsson and Norlancler developed an HPLC method for

the chiral separation of CIT, desmethyl-CIT, and didesmethyl-CIT using a surface-centered CCD [17]. The effects of methanol ratio, buffer amount, oven temperature, and pH on separation were successfully optimized. Hasnain et al. optimized an HPLC method for the determination of FXN in plasma by BBD [18]. The effect of organic solvent ratio, mobile phase pH, and flow rate on peak area and separation power were investigated. In another study, Houbart et al. optimized LC parameters using DoE to determine FXN and norfluoxetine in rat plasma, considering resolution (*Rs*), run time, and sensitivity [19]. To enhance the *Rs* between FXN and norfluoxetine while minimizing analysis time, a D-optimal experimental design was employed to optimize the chosen chromatographic factors.

To our knowledge, no study has employed the DoE approach to optimize the chromatographic separation of DXN, FXN, CIT, PXN, and SRN. Therefore, this work aims to optimize a reliable HPLC technique for the quantification of DXN, FXN, CIT, PXN, and SRN. DoE approach based on BBD with desirability function was used for the first time to optimize the chromatographic separation of these substances.

MATERIAL AND METHOD

Chemicals and Materials

All chemicals used were of analytical grade. Acetonitrile, methanol, phosphoric acid, sodium dihydrogen phosphate, and NaOH were products of Sigma Aldrich (St. Louis, MO, USA). DXN, FXN, CIT, PXN, and SRN were kindly supplied by companies Santa Farma (Şişli / İstanbul), Abdi İbrahim (Sarıyer / İstanbul), Nobel (Ümraniye / İstanbul), Ali Raif (Kağıthane / İstanbul), and Sanovel (Silivri, İstanbul), respectively. Paxera 10® tablets, which were labeled to contain 10 mg PXN, and Duloxx 30® capsules, labeled to contain 30 mg DXN, were purchased from a local pharmacy.

Preparation of Standard Solutions

Using methanol as the solvent, separate stock solutions were prepared for each analyte at 1000 µg/ml. Mixed standard solutions between 2 and 50 µg/ml were prepared by appropriately diluting stock solutions with water. The solutions were stored in a refrigerated environment at a temperature of 4°C and shielded from any light exposure.

Instrumentation and Apparatus

A Prominence-20 series HPLC instrument with an SPD-20A diode array detector (DAD) was used for all experiments. LCsolution 1.25 (Shimadzu, Japan) software was utilized for the system control and data acquisition. Sample and solution preparation involved an HI 2211 pH meter from Hanna Instruments, a magnetic stirrer, a vortex mixer, and an ultrasonic bath from Isolab Laborgerte.

Chromatographic Conditions

Agilent Poroshell 120 EC-C18 analytical column (75 mm × 4.6 mm × 2.7 µm) was utilized for analyses. A mobile phase of acetonitrile and phosphate buffer (20 mM, pH 2.7) (38.2:61.8 %v/v) was used. Flow rate was adjusted to 1.1 ml/min. All analyses were carried out at 25ºC. DAD was operated at 220 nm. 10 µl of standard solutions or samples were injected into the system.

Analysis of Commercial Formulations

For the assay of the pharmaceutical products, five Paxera 10® tablets or five Duloxx 30® capsules were randomly selected and weighed. Afterward, these tablets or capsules were homogenized, and an amount equivalent to 12.5 mg of PXN or 25 mg of DXN was transferred to a 25 ml volumetric flask and completed to volume with methanol. The suspensions were ultrasonicated for 10 min to dissolve the analytes. The tablet and capsule suspensions were diluted 25 and 50-fold, respectively, with the mobile phase to achieve an analyte concentration of 20 µg/ml. The final solution was filtered using a 0.45 µm nylon syringe filter and transferred to a vial for further HPLC analysis.

Optimization Approach

The optimization approach focused on evaluating the influence of 3 variables: pH, acetonitrile

ratio, and flow rate utilizing the BBD. The desirability function was employed to simultaneously optimize *Rs* between critical peak pairs, capacity factor (*k*) of the first peak in the chromatogram, and peak symmetry. The experimental data were evaluated by the software Design Expert Version 11.1.2 (Stat-Ease, USA).

Method Validation

Validation studies were carried out following the recommendations of the International Conference on Harmonization (ICH) and official pharmacopeias. A 10 µg/ml mixed standard solution of all analytes was analyzed 11 times for the system suitability test (SST). Linearity was verified by quadruplicate analysis of standard solutions prepared at six levels (2, 5, 10, 20, 35, 50 µg/ml). QC solutions at three different concentrations (15, 20, and 25 µg/ml) were analyzed to examine the intra- and inter-day accuracy and repeatability of the method. Four analyses were performed in the same day at each level for intra-day experiments, whereas twelve analyses were carried out on three days for the evaluation of inter-day experiments. Relative standard deviation (RSD) and % accuracy were used to express the results of repeatability and accuracy, respectively. The limits of detection (LOD) and quantification (LOQ) were statistically estimated as previously reported [20]. Peak purity values obtained for formulation analyses were evaluated to demonstrate the selectivity of the method.

RESULT AND DISCUSSION

Optimization of Chromatographic Conditions

Conventional HPLC method development typically relies on a step-by-step methodology, which can be time-consuming, solvent-intensive, and costly due to the large number of experimental runs required. In contrast, the DoE enables the concurrent manipulation of numerous variables and rapid optimization of chromatographic conditions by considering the interactions between significant factors and their collective effects on the response. For this work, the DoE approach using the BBD was preferred as it requires fewer experiments, avoids edge parameter combinations, and provides flexibility in exploring quadratic response surfaces [16,21]. BBD was used to identify the shortest run time that allows for satisfactory separation of antidepressants and proper retention of the first-eluting analyte (CIT). Initial studies showed that gradient elution is not necessary to obtain the baseline separation of analytes in acceptable run times. In this manner, isocratic elution was preferred due to its ease of application and not requiring conditioning between consecutive injections.

Acetonitrile and methanol are the most frequently utilized strong organic solvents in reversed-phase liquid chromatography separations. Instead of methanol, acetonitrile was opted as the strong organic solvent, considering the relatively smaller particle size of the employed analytical column, which could have generated high backpressures exceeding the pressure limit of conventional HPLC systems when methanol with high viscosity was used in the mobile phase composition. Following initial experiments, the main parameters affecting the separation were identified as the acetonitrile ratio (%B), pH, and flow rate. Since the initial experiments showed limited effects of temperature and buffer concentration, they were excluded from the DoE to reduce the number of experiments. *Rs* of FXN-SRN, *Rs* of CIT-PXN, *k* of CIT, and average *T* were selected as the responses.

Table 1 displays the BBD matrix and the corresponding experimental results. In order to calculate the experimental error, the experiments were repeated five times at the center point, while all other runs were randomly conducted without duplication.

Multiple regression analysis was used to fit the experimental data to a quadratic polynomial model. The resulting equations, representing the corresponding relationships, are as follows:

$$Y1 = 2.9534 - 0.06625A - 4.61425B + 0.14425C + 0.02325AB - 0.01025AC + 0.12975BC + 0.147925A^2 + 2.13742B^2 - 0.159575C^2$$

$$Y2 = 1.479 + 0.18975A - 0.098125B + 0.121375C + 0.02675AB + 0.00325AC - 0.0145BC + 0.22725A^2 - 0.419B^2 - 0.044C^2$$

$$Y3 = 0.8838 + 0.04625A - 1.4585B + 0.01625C - 0.03925AB - 0.00225AC - 0.03575BC + 0.073475A^2 + 0.940475B^2 - 0.018525C^2$$

$$Y4 = 1.62476 + 0.1672A + 0.061325B + -0.004575C + 0.0249AB + 0.0011AC + 0.03525BC + 0.060995A^2 - 0.104655B^2 - 0.011355C^2$$

where Y1, Y2, Y3, and Y4 are the responses of *Rs* between CIT and PXN, *Rs* between FXN and SRN, *k* of CIT, and average *T*, respectively. The three chromatographic parameters are pH, the percentage of acetonitrile in the mobile phase, and flow rate, represented by A, B, and C, respectively.

Table 1. The experimental results for BBD

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Std order	**Run order**	**A: pH**	**B: B%**	**C: Flow rate (ml/min)**	***Rs* of CIT-PXN**	***Rs* of FXN-SRN**	***k* of CIT**	**Average *T***
11	1	3.75	30	1.2	9.59	1.33	3.30	1.45
6	2	5	40	0.6	2.73	1.76	0.98	1.85
4	3	5	50	0.9	0.62	1.45	0.42	1.87
7	4	2.5	40	1.2	3.17	1.55	0.90	1.49
9	5	3.75	30	0.6	9.57	1.02	3.16	1.50
15	6	3.75	40	0.9	2.92	1.47	0.88	1.62
13	7	3.75	40	0.9	2.93	1.47	0.88	1.62
8	8	5	40	1.2	3.00	1.96	0.97	1.81
3	9	2.5	50	0.9	0.70	1.04	0.39	1.46
2	10	5	30	0.9	9.73	1.47	3.48	1.64
14	11	3.75	40	0.9	2.97	1.48	0.88	1.62
17	12	3.75	40	0.9	2.97	1.48	0.88	1.62
16	13	3.75	40	0.9	2.95	1.47	0.88	1.62
5	14	2.5	40	0.6	2.85	1.36	0.89	1.53
1	15	2.5	30	0.9	9.90	1.17	3.29	1.34
10	16	3.75	50	0.6	0.01	0.72	0.38	1.49
12	17	3.75	50	1.2	0.55	0.98	0.37	1.58

ANOVA was used for the statistical evaluation of the models, and the results are displayed in Tables S1-4. The p-value for all models was equal to or less than 0.0001, indicating statistical significance. The goodness of fit of the proposed equation can be evaluated by the regression coefficient (R^2), which helps estimate the predictive power of the model [22]. The fit of the data was considered adequate considering high R^2 and adjusted R^2 values (Tables S1-4).

In DoE, lack-of-fit (LOF) is a statistical measure that assesses how well a model fits the observed data. The use of LOF can help determine whether the model predictions significantly differ from the observed data. The LOF F-value is determined by dividing the discrepancy between the observed measurements and the model-predicted values by the variability among replicate measurements. In this manner, a statistically significant LOF may occur because of the improved precision of the central points and the presence of error at axial points [23]. The RSD values of 5 replicates calculated for 4 responses at the central point were ≤0.76% (Table 1). Results show that the LOF was due to the low variability at the center point.

Figure 1 depicts the response surface plots as defined by the regression model. Results indicate that the most significant factor for *Rs* of CIT-PXN was %B, which was strongly associated with a substantial decrease in the response (Figure 1A). The critical peak pair in the chromatogram was FXN-SRN. The mobile phase pH was the most significant parameter for the *Rs* of FXN-SRN, which increased with the increase in the pH from 2.5 to 5. The parameter %B had a dual effect on *Rs* of FXN-SRN. Figure 1B shows that *Rs* of FXN-SRN initially increased with %B up to 40%, and then slightly decreased with further increase in acetonitrile ratio. The *k* of CIT was most significantly affected by the %B in an inversely proportional manner, while the effects of pH and flow rate were limited (Figure 1C). A severe peak tailing was observed for all analytes with the increase in the mobile phase pH (Figure 1D), which

can be attributed to interactions between the negatively charged free surface silanols of the silica stationary phase and the positively charged analytes [24].

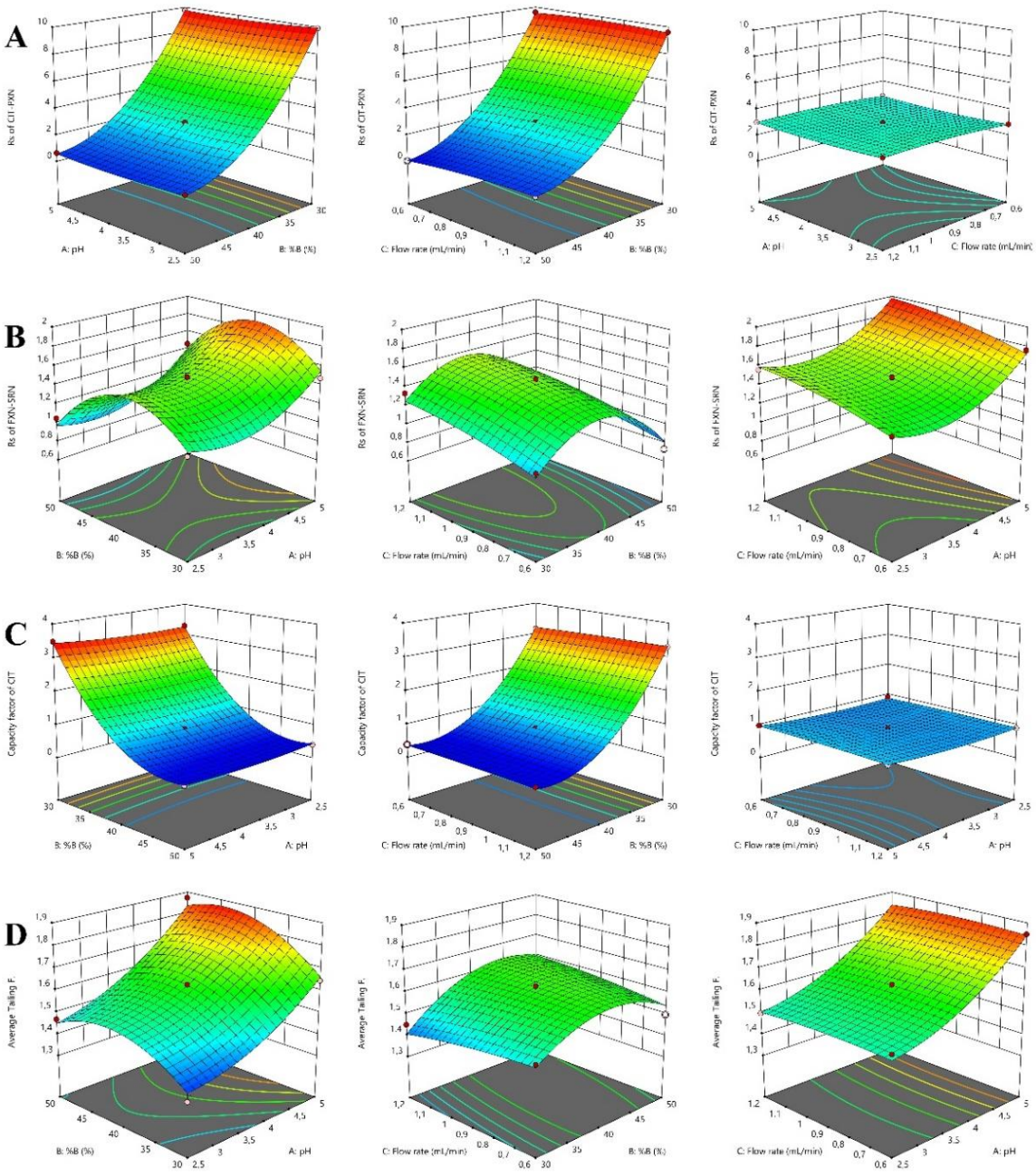

Figure 1. Response surfaces for (A) *Rs* between CIT and PXN, (B) *Rs* between FXN and SRN, (C) *k* of CIT, and (D) average *T*

The Design-Expert software's optimization tool, based on the Derringer and Suich desirability function [25], was employed to predict the most suitable conditions for the separation of antidepressants. The optimization aimed to achieve *Rs* values greater than 1.5 for CIT-PXN and FXN-SRN and peak pairs, a *k* greater than 1 for CIT, and an average *T* smaller than 1.5. The optimum values of pH, acetonitrile ratio, and flow rate were determined as 2.7, 38.2 %, and 1.1 ml/min, respectively. The optimal results for responses Y1, Y2, Y3, and Y4 were calculated as 4.1, 1.55, 1.22, and 1.5, respectively. The differences between predicted and experimentally obtained values for Y1, Y2, Y3,

and Y4 were 2,7%, 2.6%, 8.3%, and 0.61%, respectively, confirming the validity of the utilized models. Figure 2 shows the chromatogram recorded under the optimized chromatographic conditions. The retention times for CIT, PXN, DXN, FXN, and SRN were approximately 1.6, 1.9, 2.3, 2.9, and 3.2 min, respectively, resulting in a total run time of 5 min.

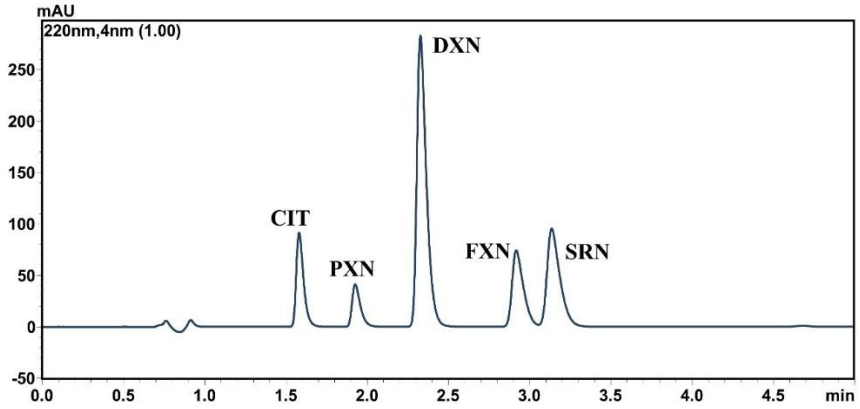

Figure 2. Chromatogram of a standard solution (10 µg/ml) of CIT, PXN, DXN, FXN, and SRN under optimized conditions at 220 nm

Method Validation

Validation studies were conducted compliant with the recommendations of ICH to demonstrate that the reported method was suitable for practical analysis. An SST was performed at the 10 µg/ml level (Table 2). The *k* of the first peak in the chromatogram, i.e., CIT, was 1.1, providing an adequate retention window for the elution of hydrophilic interferences originating from the matrix. *Rs* values obtained for all peak pairs were higher than the recommended value (1.5), except the FXN-SRN pair, for which the *Rs* was calculated as 1.4. It should be noted that peak purity index values calculated for both FXN and SRN were higher than 0.9999, demonstrating that acceptable resolution was achieved for the critical peak pair. Additionally, since the combined dosage form of these drugs is not commercially available, there is no risk of co-elution, even if a decrease in column efficiency occurs over time. The *T* values were in the range of 1.4-1.5, affirming the formation of symmetric peaks. Additionally, the RSDs were below 1%, demonstrating high precision.

The results of linearity and sensitivity experiments are shown in Table 3. The developed analytical method displayed acceptable linearity across the calibration range of 2-50 µg/ml for all five analytes, with correlation coefficients (r) ≥ 0.999. LODs were in the range of 0.17-0.29 µg/ml, while LOQ values ranged from 0.53 µg/ml to 0.89 µg/ml.

Intra-day accuracies ranged from 97.7 to 102.8 %, while inter-day accuracies were between 97.1% and 102.9%, with RSD values lower than 2.4% (Table 4). The results show that the proposed technique exhibits sufficient precision and accuracy for the determination of the antidepressants.

Table 2. Results of SST for antidepressants (n = 11)

	CIT	PXN	DXN	FXN	SRN	Recommended value
Retention time (min)	1.57	1.93	2.34	2.94	3.16	-
Tailing factor (T)	1.5	1.5	1.5	1.4	1.5	<2
Capacity factor (*k*)	1.1	1.5	2.1	2.9	3.2	>1
Resolution (Rs)	-	3	3.2	4.3	1.4	>1.5
Theoretical plates (N)	2963.2	3934.5	4998.3	6410.9	5814.8	>2000
Selectivity factor (*a*)	-	1.36	1.4	1.4	1.1	>1.05
RSD% of retention time	0.31	0.35	0.37	0.38	0.38	<1
RSD% of peak area	0.19	0.33	0.12	0.20	0.22	<1

Table 3. The results of linearity and sensitivity studies for CIT, PXN, DXN, FXN, and SRN

	CIT	PXN	DXN	FXN	SRN
Linear range (µg/ml)	2-50	2-50	2-50	2-50	2-50
Slope	16012	8751.1	72645	19021	35801
Intercept	3960.4	615.1	-10788	- 1247.7	-4361.8
SE of slope	52.4	17.1	146.5	38.1	95.6
SE of intercept	1425.4	465.7	3983.8	1035.3	2600.2
Correlation coefficient (*r*)	0.9998	0.9999	0.9999	0.9999	0.9999
LOD (µg/ml)	0.29	0.17	0.18	0.18	0.24
LOQ (µg/ml)	0.89	0.53	0.55	0.54	0.73

*SE: standard error

Table 4. Validation results of intra- and inter-day precision and accuracy for CIT, PXN, DXN, FXN, and SRN

		Intra-day (n= 4)		Inter-day (n= 12)	
Analyte	**Concentration level (µg/ml)**	**Accuracy (%)**	**RSD (%)**	**Accuracy (%)**	**RSD (%)**
CIT	15	100.3	0.32	101.6	2.33
	20	100.6	0.21	101.2	1.33
	25	102.1	0.05	102.9	1.44
PXN	15	102.8	0.17	102.7	0.26
	20	101.5	0.17	101.2	0.23
	25	100.4	0.09	100.2	0.33
DXN	15	98.7	0.06	97.9	1.05
	20	97.7	0.07	97.1	0.83
	25	101.2	0.02	100.6	0.65
FXN	15	100.3	0.21	100.1	0.21
	20	98.1	0.14	97.7	0.28
	25	100.5	0.14	100.2	0.32
SRN	15	100.8	0.07	100.6	0.22
	20	98.8	0.08	98.7	0.21
	25	100.3	0.06	100.1	0.25

Analyses of the Pharmaceutical Formulations

Commercially available formulations of PXN (Paxera 10® tablets) and DXN (Duloxx 30® capsules) were analyzed to prove the feasibility of the developed technique. Paxera tablets contain excipients such as lactose, dicalcium phosphate, croscarmellose sodium, starch, magnesium stearate, and colorants, while Duloxx capsules include sugar pellet, polysorbate 80, crospovidone, hypromellose 6 CPS, talc, hypromellose acetate succinate, and triethyl citrate. Formulations were prepared for analysis as described in the "Analysis of Commercial Formulations" section. Each sample was analyzed in triplicate, and the chromatograms are presented in Figure 3. No interfering compounds were found to be eluted from the column within the retention windows of the analytes. Additionally, the peak purity indices calculated via the DAD were greater than 0.999 for both PXN and DXN, indicating the absence of impurity.

The results of sample analysis by the developed method are summarized in Table 5. The obtained results were found to be satisfactory and in accordance with the manufacturer's declaration. The standard addition method was utilized to evaluate the method's accuracy in the presence of other matrix components, i.e., excipients. For this purpose, known amounts of standard solutions at 10 µg/ml level were added to pre-analysed samples. Obtained recoveries were higher than 96%, demonstrating the adequate accuracy of the HPLC method.

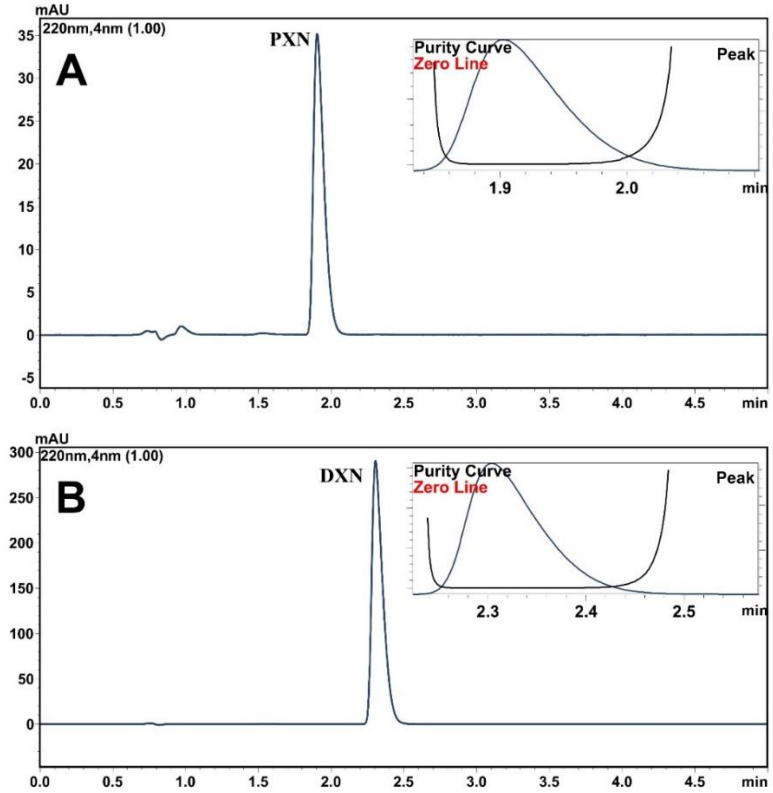

Figure 3. Representative chromatograms of Paxera® tablets (A) and Duloxx® capsules (B) at 220 nm (20 µg/ml)

Table 5. Assay results and mean recovery studies of PXN and DXN in pharmaceutical dosage forms

	Pharmaceutical formulations	
	PXN	**DXN**
Labeled claim (µg/ml)	20.00	20.0
Amount found (µg/ml)[a]	20.25	21.15
RSD (%)[a]	0.2	0.1
Bias (%)	1.25	5.7
Added (µg/ml)	10	10
Found (µg/ml)[a]	29.90	30.94
Recovery (%)	96.5	97.8
RSD% of recovery[a]	0.1	0.2
Bias (%)	-3.5	-2.2

[a] Mean of three experiments

Conclusion

A novel and reliable HPLC-DAD method was optimized for the quantification of DXN, FXN, CIT, PXN, and SRN in pharmaceutical formulations. DoE was employed for the first time to optimize the chromatographic conditions of selected analytes. Significant chromatographic parameters were determined by the preliminary trial and error experiments. Optimum levels of mobile phase pH, acetonitrile ratio, and flow rate were determined by BBD with desirability function considering the *Rs* of two peak pairs, elution of the first analyte, i.e. CIT, as well as the peak symmetry. Under optimum conditions, five antidepressants were separated under isocratic conditions within 3.5 min. The proposed technique was effectively utilized to determine PXN and DXN in commercial formulations. Acceptable

accuracy and precision were obtained by the real sample analyses. In addition, no interference from matrix components was observed. The proposed approach presents a quick, reliable, and uncomplicated substitute for QC or content uniformity testing of chosen antidepressants.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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