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Improvement and Application HPLC-UV Method for detecting of N-drugs in Pharmaceutical Formulations

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ABSTRACT

The ultraviolet-visible high-performance liquid chromatography (HPLC–UV) coupled method was developed to detect five pharmaceutical compounds: indapamide (INDP), clomipramine (CMI), promethazine HCl (PMH), lisinopril (LSP) and trifluoperazine HCl (TFPH). The mobile phase consisted of 70% acetonitrile and 30% water, ensuring specific retention times (tR) for each compound. Analytical validation parameters were calculated from a calibration curve, including sensitivity, limit of detection (LOD) and linearity of the method. The LOD values of N-drugs were calculated as 6.24 µM for INDP, 2.19 for CMI, 10.57 for PMH, 6.68 for LSP and 1.25 for TFPH. Peak deconvolution or spectral deconvolution of standard solutions of drug mixtures was used to separate overlapping peaks in a dataset and extract information about individuals. Chromatographic separation of the standard mixture of pure compounds achieved a good resolution (R_s), ensuring the effective separation of the mixture components. However, other mixtures did not achieve satisfactory resolution due to retention time interference. Overall, the HPLC–UV method demonstrated good sensitivity and selectivity for the detection of the chosen pharmaceutical compounds.

KEYWORDS
Chromatogram, deconvolution, limit of detection, pharmaceutical analysis, retention time, sensitivity

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1. Introduction

Pharmaceutical compounds containing nitrogen atoms (N-atoms) are used to treat various diseases. Indapamide (INDP) is prescribed to manage hypertension, while lisinopril (LSP) is used for heart failure (Goa *et al.*, 1997; Thomas, 1985). Promethazine HCL (PMH) is utilized to prevent nausea and vomiting associated with certain conditions (Shearer and Miller, 1976). Trifluoperazine HCl (TFPH) is employed to help reduce hallucinations (Vardanyan and Hruby, 2006). Clomipramine (CMI) is frequently prescribed for the treatment of obsessive–compulsive disorder (Van-Scheyen and Van-Kammen, 1979). Various analytical methods have been developed to determine the concentration of pharmaceutical compounds containing N-atoms in formulations. Bakir *et al.* developed a spectrofluorometric method for assessing pharmaceutical compounds containing N-atoms, utilizing their rapid reaction with Eosin Y. The limits of detection (LODs) were found to be 2.07 µmol/L for INDP, 1.36 µmol/L for CMI, 3.02 µmol/L for PMH, 3.52 µmol/L for LSP and 2.09 µmol/L for TFPH (Alkulaib *et al.*, 2024). Reverse-phase high-performance liquid chromatography (RP-HPLC) was employed to quantify the concentration of INDP in oral antihypertensive tablets and bulk dosage forms. In a mobile phase consisting of a 60:40 ratio of methanol and phosphate buffer (pH: 5.8), the dynamic range for INDP was 0.2–1.2 µg/mL, with an R^2 value of 0.999 (Agrawal, 2021). A simultaneous quantification of CMI and its active metabolite in plasma was performed using a liquid chromatography coupled with mass spectrometry technique (Mohiuddin *et al.*, 2021). Solid-phase extraction, combined with ultra-performance liquid chromatography, was employed to detect perindopril (PP) and INDP (IP) in human plasma, with a LOD of 8.6 ng/mL for INDP (Palakeeti *et al.*, 2021). CMI and its major N-demethylated and hydroxy metabolites were detected in ng/ml using a sensitive ultraviolet-visible high-performance liquid chromatography (HPLC–UV) coupling method. The method employed a modified column to separate the compounds from the plasma. A mobile phase consisting of 10 mM K_2HPO_4, acetonitrile and methanol in a 35:25:40 v/v/v ratio was used (Pirola *et al.*, 2002). LSP and amlodipine (AML) were detected in solid tablet forms using an RP-HPLC method. The methodology achieved speed, cost-effectiveness, short response time and high resolution (Pawar *et al.*, 2021). An HPLC–UV coupled system with a modified column was used to detect TFPH and its photodegradation products. The mobile phase consisted of acetonitrile, 2-tetrabutylammonium hydroxide and o-phosphoric acid in a ratio of 50.5:0.83:0.1 (v/w/w; Shetti and Venkatachalam, 2010). An HPLC-ACE 5 C18 analytical column (12.5 × 4.6 mm) was used to analyze perindopril, INDP (IND) and AML in the TRIPLIXAM® sample. The LODs were in the ppm concentration range (Özsar and Altınöz, 2024).

Therefore, the suggested methods aimed to detect N-drug concentrations in pharmaceutical formulations using the HPLC–UV coupled system. The chemical structures of chosen pharmaceutical compounds have primary, secondary and tertiary N-atoms. The LOD values were calculated to be 6.24 µM for INDP, 2.19 for CMI, 10.57 for PMH, 6.68 for LSP and 1.25 for TFPH. Peak deconvolution was used to separate the chromatogram graphs of the mixture standard solution of N-drugs. The vision of peak deconvolution provided an approach to accurately analyze and predict the separation resolution of N-drugs in complex mixtures. The resolution for certain mixtures of pure N-drug solutions in pharmaceutical compounds was below 1.5, indicating difficulties in effectively separating and accurately detecting the concentrations of pharmaceutical compounds within complex mixtures.

2. Experimental

2.1. Reagents and Instrument:

High-grade pharmaceutical compounds, such as INDP, TFPH, LSP, CMI and PMH, were received from Sigma Aldrich. HPLC-grade solvents were used in the experiments. INDP formula was used as Normalix SR 1.50 mg per tablet (Jazeera Pharmaceutical Industries, KSA), CMI as Anafranil 10 mg/tablet (Novartis, UK), PMH as Prometin syrup 5.0/5 ml (Kuwait Saudi Pharmaceutical Industries), LSP as Zestril 5 mg/g (Astra Zeneca, UK) and TFPH as Stellasil tablet 5mg.g^{-1} (Kahira

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Pharmaceuticals and Chemical Industries Company, Egypt), which were purchased from the medicine market in Saudi Arabia. An HPLC–UV coupled system (Agilent 1200 series, Waldbronn, Germany) with column (Zorbax Eclipse Plus C18, 50 × 2.1 mm ID, 1.8 µm) was used for determining the retention time (t_R) of the samples. Agilent ChemStation (Ver. B 4.0.2) software was used to monitor the signals.

A Stock solution of drugs (1000 µmmol L^{-1}) was prepared by dissolving the accurate weights of drugs in 10-ml organic solvents (methanol and ethanol); then, solutions of different concentrations (5.0–50 µmmol l^{-1}) of calibration plots were prepared from this stock solution.

Chromatographic conditions used a flow rate of 1.0 ml min^{-1}. The injection volume was 10 µl. UV absorption was at 252 nm or close to 255 nm. However, INDP and TFPH had a good absorbance at 240 and 255 nm, while CMI, PMH and LSP did not have a good absorbance at 218–255 nm. This indicates that CMI, PMH and LSP have smaller molar absorptivity in the solvent used. The experiments were conducted using a mobile phase composed of acetonitrile and water in a 70:30 (v/v) ratio. The runtime was set to 10 minutes and the column temperature was maintained at ambient conditions (24°C–26°C). The column was equilibrated with the mobile phase for 30 minutes before injecting the analyte. The pre-sample was filtered through a 0.45-µm membrane filter and degassed using sonication.

The robustness of the method was assessed to evaluate the effect of small, controlled variations in chromatographic conditions on drug determination. This was achieved by adjusting the mobile phase flow rate to 0.9 and 1.1 mL/min and modifying the acetonitrile concentration in the mobile phase to 68% and 72%.

Regarding the procedure for pharmaceutical formulation, five tablets were finely grounded using a mortar. A measured portion of the powder was dissolved and sonicated in methanol for 10 minutes. The solution was then centrifuged at 1800 rpm/5 min and the residue was filtered and then washed with methanol; then, the filtrate was diluted into 100 mL of methanol. For a syrup sample, the volume of 1.0 mL was diluted with methanol into 10 mL to prepare 0.1 mg/ml. The homogenous solution was obtained by sonication for 15 mins. A 10-µL aliquot of the solution was injected into the chromatograph.

2.2. Theoretical Considerations:

Retention time (t_R) refers to the duration a solute spends in the column. It depends on the interaction of the analyte with the stationary phase (Ahuja and Dong, 2005). The retention factor (k) is the relative ratio time of the solute spent in the stationary compared with mobile phases (see Eq. 1).

$$k = \frac{t_R - t_M}{t_M}, \qquad \text{Eq. 1}$$

Where the retention times (t_R) and (t_M) of analyte and mobile phases.

Column efficiency (N) expresses the theoretical layers per column (see Eq. 2.).

$$N = 5.545\left(\frac{t_R}{W_{0.5}}\right)^2 \qquad \text{Eq. 2}$$

Where (N) is theoretical layers (N/m), (t_R) is retention time and ($w_{0.5}$) is the half-height width of a peak (Jennings *et al.*, 1997; Robards and Ryan, 2021).

3. Results and Discussion

The mobile phase was selected based on the physicochemical properties of the pharmaceutical drugs to achieve optimal separation. Various solvent mixtures, including water and acetonitrile, were evaluated for their effectiveness. A mixture of acetonitrile and water was chosen as the mobile phase to provide the best resolution and a short runtime for the N-drugs (Beasley *et al.*, 2005; Chaudhary *et al.*, 2016; Hang *et al.*, 2006; Mostafavi *et al.*, 2010; Thumma *et al.*, 2008). The mobile phase composition was selected considering the clarity and symmetry of the peaks. The pharmaceutical compounds were successfully separated in 5 minutes using gradient elution with a mobile phase composed of 30% water and 70% acetonitrile. The selected wavelengths for the N-drugs were applied in the range of 200–440 nm (Beasley *et al.*, 2005; Chaudhary *et al.*, 2016; Hang *et al.*, 2006; Mostafavi *et al.*, 2010; Thumma *et al.*, 2008).

Table 1: LODs, LOQs and Precision of the Proposed Method (n = 5)

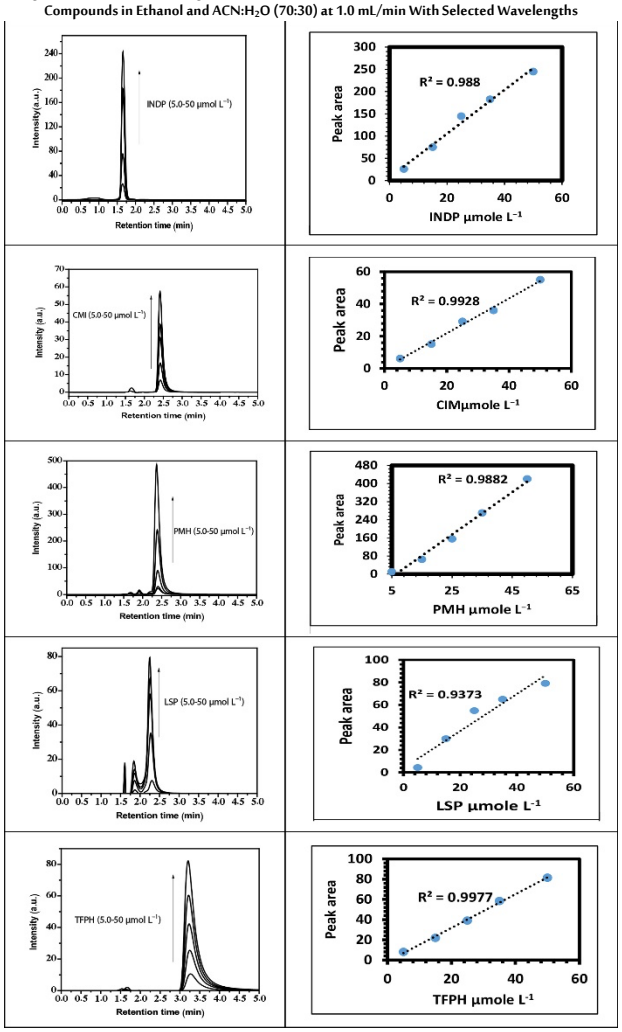
N-drugs	λ_{max}^{abs} (nm)	LOD	LOQ	Precision %RSD		Ref,
				Repeatability (%RSD)	Reproducibility (%RSD)	LOD
INDP	240.0	6.24	18.19	1.01	1.52	0.1 ppm (Özsar and Altınöz, 2024)
CMI	252.0	2.19	6.65	1.61	1.57	0.507 µg/ml (Vijayavaani et al., 2024)
PMH	255.0	10.57	32.04	0.55	0.55	10–100µg/ml (Pippalla et al., 2024)
LSP	218.0	6.68	20.26	4.07	4.70	1.11 µg/ml (Pawar et al., 2024)
TFPH	255.0	1.25	3.79	0.91	0.94	3.41µg/ml (Chauhan et al., 2024)

*** The concentration in µmol L^{-1}; recovery, RSD (%); replicate times, n = 5.**

3.1. Validation of the Analytical Method:

Linearity of the calibration curve: The linear range of observation was 5.0–50 µmmol.L^{-1} of N-drug solutions. The calibration graphs were plotted between the peak area vs. concentration (figure. 1) and the data are represented in table 1.

Figure 1: (a) HPLC Chromatogram and (b) Calibration Curve of 5.0–50 µmol L^{-1} Pharmaceutical Compounds in Ethanol and ACN:H_2O (70:30) at 1.0 mL/min With Selected Wavelengths



The LODs and limits of quantification (LOQs) were calculated as LOD

= 3.3σ/s and LOQ = 10σ/s, where (σ) is the standard deviation of peak area and (s) is the slope of the calibration curve.

Table 1 shows that the LOD values of N-drugs were calculated to be 6.24 µM for INDP, 2.19 for CMI, 10.57 for PMH, 6.68 for LSP and 1.25 for TFPH. The chemical structure and geometric positioning of the N-atom improved drug detection at low LODs by affecting molecular reactivity, polarity and detection system interactions. LSP showed a low LOD value due to the geometry of the N-atom in its primary, secondary and tertiary positions. Table 1 presents the reference methods for detecting N-drugs. Furthermore, the current method demonstrated good sensitivity.

To confirm that the method was suitable for sample analysis, a system suitability test was performed at optimized chromatographic conditions. The retention time, resolution (between adjacent peaks), peak symmetry and theoretical layers in the column were calculated (table 2). The effective column efficiencies were calculated as 97.37 for INDP, 103.32 for CMI, 86.57 for PMH, 61.45 for LSP and 56.61 for TFPH in the analysis of drug molecules. The column provided narrow, sharp peaks with excellent resolution. Furthermore, the low mobile phase consumption was achieved at 1.0 mL/min and 10.0 µL sample volume. The column efficiencies (N) are presented in Table 2, where the order of efficiency of CMI > INDP > PMH > LSP > TFPH was observed.

Table 2: The System Suitability for the N-Drugs

	INDP	CMI	PMH	LSP	TFPH
t_R(min)	1.65	2.42	2.40	2.30	3.24
Asymmetry S	0.29	0.06	1.4	1,2	0.07
Theoretical plates	1600	833	800	10	392
$W_{0.5}$	0.09	0.12	0.15	0.20	0.31
N	97.37	103.32	86.57	61.45	56.61

Accuracy, intraday and interday precision: For the pre-analyzed standard and sample solution, the standard solutions 30, 50 and 70 µmol L^{-1} were used. The %RSD values were 0.55%–1.61%, meeting the acceptable precision (UNODC., 2009,). The average % recoveries obtained for pure compounds were 97.90%–99.70%, while those of the pharmaceutical formulations were 82.93% and 100.03% for tablet and syrup dosage forms, which is within the acceptable range (Rao, 2018).

The robustness of the method was tested and no significant change in the retention time of N-drugs was observed when the composition and flow rate of the mobile phase were altered. The low %RSD values were below 0.05%, further confirming the robustness of the method.

The specificity of the method was examined in the presence of interferences. It is important to note that the chromatogram of the excipient solution showed no peaks, indicating that no interference from the excipients (additives in pharmaceutical formulation) was observed. The low LOD, LOQ, precision and recovery values demonstrated high sensitivity, accuracy and precision for determining N-drugs in standard solutions. Table 3 shows the good performance and acceptable levels for all the selected N-drugs. The chromatographic method achieved suitability and sensitivity.

Table 3: Results of the % Recovery Studies of the Developed HPLC Method (n = 5)

N-drugs (µmol L^{-1})	Pre-analyzed standard			Pre-analyzed tablet and syrup*		
	Spiked amount	Recovery (%)	Av. recovery ± SD (%)	Spiked amount	Recovery (%)	Av. recovery ± SD (%)
INDP	30.0	94.50	97.90 ± 4.30	30.0	81.47	84.89 ± 4.30
	50.0	96.50		50.0	83.49	
	70.0	102.70		70.0	89.70	
CIM	30.0	97.80	99.16 ± 1.60	30.0	98.1	99.46 ± 0.1.61
	50.0	98.70		50.0	99.0	
	70.0	101.0		70.0	101.3	
PMH	30.0	99.30	99.70 ± 0.55	30.0	99.61	100.03 ± 0.55
	50.0	99.50		50.0	99.84	
	70.0	100.35		70.0	100.65	
LSP	30.0	94.84	98.02 ± 3.99	30.0	79.75	82.93 ± 3.99
	50.0	96.75		50.0	81.66	
	70.0	102.49		70.0	87.40	
TFPH	30.0	98.79	99.53 ± 0.9	30.0	98.66	99.40 ± 0.91
	50.0	99.26		50.0	99.13	
	70.0	100.54		70.0	100.41	

***The concentration in µmol L^{-1}; recovery in %; mean ± SD; replicate times, n = 5.**

3.2. Analysis of the Mixture of N-drugs:

The difference in retention times between the two peaks divided by the combined widths of the elution peaks was used to measure the resolution of separation (see Eq. 3).

$$\text{Resolution} = 2\,\Delta tr\,/(w_1 + w_2) = \Delta tr\,/\,W_{av} \quad \text{Eq. 3}$$

(Δtr) is the difference between the retention time of two signals and (W_{av}) is the average baseline width. Good resolution is more than > 1.5.

The selectivity (α) is the ability to differentiate between sample components based on the chromatographic system (see Eq. 4).

$$\alpha = \frac{K_2}{K_1} = \frac{(t_{R2} - t_M)}{(t_{R1} - t_M)} \quad \text{Eq. 4}$$

(K_2) and (K_1) are the retention factors of (2) and (1).

This is where R = 1.5 gives an essentially complete separation because the overlap is about 0.3%.

=1.0, 4% overlap of each compound.

=R < 1.0, not acceptable.

The hypothesis of mixing previously uncombined drugs aims to understand the chemical and pharmacological interactions and estimate their concentrations while considering potential new effects. The peak deconvolution effectively resolved overlapping signals, enabling a more accurate detection of coupled active pharmaceutical ingredient quantities. Additionally, this improvement in deconvolution enhanced drug formulations, helping to minimize complications and reduce overall healthcare costs. The R resolution results of specific mixtures of standard solutions were evaluated to determine the effectiveness of the chromatographic separation. The deconvolution process for overlapping peaks was applied to isolate and extract information from the hidden peaks in chromatographic data (Verfaillie *et al.*, 2024). For these mixtures, the resolution between individual compounds was assessed to ensure the clear separation of each component. The accurate quantification and reliable analysis of the sample mixture must be confirmed, as shown in Figure 2. However, the resolution values of certain mixtures, as presented in Table 4, were observed to be lower than 1.5, so the identification and separation analysis of these mixtures were difficult to achieve (Bernard *et al.*, 2008).

Figure 2: HPLC Chromatogram for 50 µmol L^{-1} Mixtures of Pure Form of INDP (Black Line), CMI (Red Line), PMH (Blue Line), LSP (Green Line) and TFPH (Pink Line) at the Flow Rate 1.0 mL/min With 70:30 ACN:H_2O

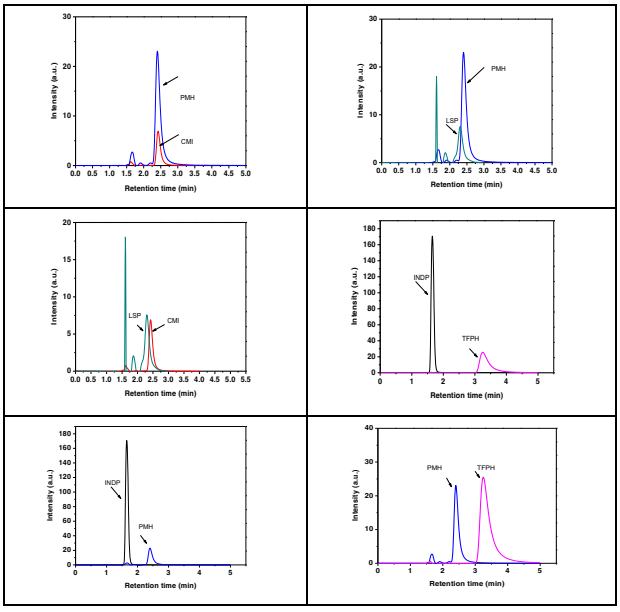


Table 4: HPLC Resolution of the Standard Solution of Pure Pharmaceutical Compounds

Mixture	t_1, t_2 (min)	k_1, k_2 (retention factors)	α (selectivity factor)	Rs = resolution
INDP/PMH	1.65, 2.40	0.65, 0.40	0.62	03.65
PMH/TFPH	2.40, 3.24	0.40, 1.24	3.10	02.12
CMI/TFPH	2.42, 3.24	0.42, 1.24	2.95	02.18
CMI/LSP	2.42, 2.30	0.42, 0.30	0.71	0.418
CMI/PMH	2.42, 2.40	0.42, 0.40	0.95	0.083
PMH/LSP	2.40, 2.30	0.40, 0.30	0.75	0.325

4. Conclusion

The HPLC–UV coupled method was developed to determine the N-drug concentrations in their formula. The wavelength and composition of the mobile phase were studied to obtain a good chromatogram. The mobile phase composition was 70% ACN:30% H_2O. The LOD values of N-drugs were calculated to be 6.24 μM for INDP, 2.19 for CMI, 10.57 for PMH, 6.68 for LSP and 1.25 for TFPH. The suggested method achieved good sensitivity and selectivity for the determination of the cited pharmaceutical compounds in the formulations of tablets and syrup. The deconvolution process of the overlapping peaks of the N-drug mixture was used to calculate the resolution factor. In conclusion, the resolution was less than 1.5 in certain mixtures of pharmaceutical compounds, suggesting challenges in separating and detecting the concentrations.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

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Conflicts of Interest

No conflicts of interest exist.

Biographies

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