6-2023

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Bull. Pharm. Sci., Assiut University, Vol. 46, Issue 1, 2023, pp. 157-172.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/
e-mail: bullpharm@aun.edu.eg

DEVELOPMENT OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF NEBIVOLOL HYDROCHLORIDE, VALSARTAN AND HYDROCHLOROTHIAZIDE IN CO-ADMINISTRATED ANTIHYPERTENSIVE AGENTS AND HUMAN PLASMA

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Reversed phase high performance liquid chromatography combined with photodiode array (HPLC-PDA) detector method was developed for simultaneous determination of two binary antihypertensive combinations; either Nebivolol (NEB) with Hydrochlorothiazide (HCZ) or with Valsartan (VAL) in pharmaceutical formulation and spiked human plasma. The method was successfully applied to separate the three drugs on one chromatogram under the same chromatographic conditions that used for two binary combinations. The separation was established on Xterra C_{18} column using gradient mobile phase which composed of acidified water of pH = 3.0 (adjusted with 0.1% orthophosphoric acid) and acetonitrile with flow rate of 1.0 mL/min. The drugs were determined at 280 nm. Concentration range of each analyte was shown linearity at 5.0–70.0 µg/mL for NEB, 8.0–60.0 µg/mL for HCZ and 5.0-80.0 µg/mL for VAL. The results of method validation were displayed according to ICH guidelines. The green assessment of the proposed has been carried out using two different tools.

Keywords: *Nebivolol, Valsartan, Hydrochlorothiazide, Antihypertensive drugs, RP-HPLC, Green assessment.*

INTRODUCTION

Nebivolol hydrochloride[a] (NEB) is 1-(6-fluoro-3,4-dihydro-2H-chromen-2-yl) -2 -[[2-(6-fluoro-3,4-dihydro-2H-chromen- 2 -yl) -2-hydroxyethyl] amino] ethanol;hydrochloride[1,2]. Nebivolol has two racemic enantiomers (SRRR and RSSS NEB) with chiral centers[3]. Furthermore, NEB is cardioselective beta 1 adrenergic receptor blocker and induced vasodilator nitric oxide-mediate by activating endothelial nitric oxide synthase enzyme through b3 agonism, so hypertension and heart failure were treated by using Nebivolol[4]. Valsartan[b] (VAL) is (2S)-3-methyl-2-[pentanoyl-[[4-[2-(2H-tetrazol-5-yl) phenyl] phenyl]methyl]amino]butanoic acid[5], inhibits angiotensin II Receptor type 1 Moreover, VAL is used for treating high blood pressure, heart failure and diabetic nephropathy[6]. Hydrochlorothiazide[c] (HCZ) is 6-chloro-1,1-dioxo-3, 4- dihydro - 2H - 1λ6,2,4 - benzothiadiazine -7-sulfonamide[7]. Mechanism of electrolyte reabsorption in distal renal tubular was affected by HCZ which increases excretion of sodium and chloride in urine. HCZ is used for treatment hypertension and edema associated with congestive heart failure[8]. The previous literature review displayed other various analytical methods have been discussed for simultaneous determination of two binary mixtures (NEB with HCZ) and (NEB with VAL). These methods involved UV spectrophotometric methods[9&10]and high

Received in 8/10/2022 & Accepted in 23/11/2022

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performance chromatographic methods [11&2].This proposed work aimed for separation of NEB, HCZ and VAL in one chromatogram under the same chromatographic condition that used for separation two binary mixtures (NEB with VAL) and (NEB with HCZ). Although, there is no dosage form in the pharmaceutical Egyptian market contains the three combined drugs together; co-administration of NEB and VAL was recommended to be used in long term treatment of hypertension, in addition to HCZ [13]. The current presented work shows a challenging separation of the three co-administered drugs. In addition, laboratory prepared mixtures and pharmaceutical dosage forms were quantified with ratio 1:5 for (NEB, HCZ) and 1:16 ratio for (NEB, VAL). Each combination was successfully separated using reversed phase high performance liquid chromatography. This method was also applied for simultaneous determination of (NEB with HCZ), (NEB with VAL) and (NEB, HCZ and VAL) in spiked human plasma. Further, the green assessment of the suggested method has been assessed using two different tools: the analytical Eco- Scale and the GAPI tools.

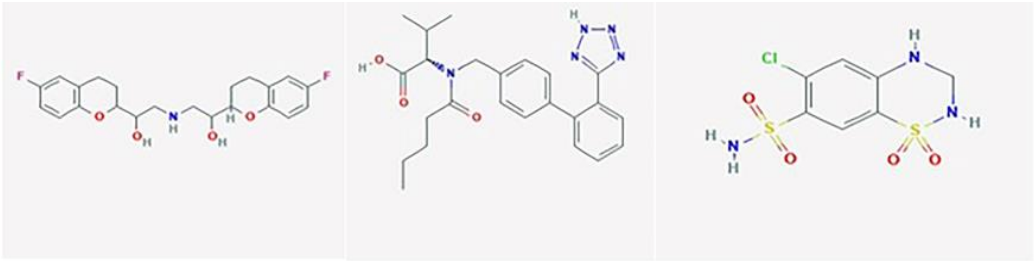
Experimental

Instruments

Chromatographic analysis was carried out using waters 2690 Alliance HPLC. Instrument equipped with vacuum degassing for mobile phase and pump with low mixing system. Separation was occurred on Xterra C_{18} column with dimensions (100 mm × 4.60 mm, 5.0 µm) as a stationary phase. Photodiode array detector (Waters 996 PDA) with wide range (200.0-600.0 nm) were applied. The software was Waters Empower. Elmasonic S 60 H water bath sonicator (Germany) was used for solvent degassing. PH was adjusted by using pH-meter (Jenway digital pH- meter 3310, Dunmow, Essex, United Kingdom). Deionized water was attained from a Milli-Q water purification system (Thermo scientific Barnstead Smart 2 Pure 3 UV, Hungary). Human plasma samples were mixed using vortex (VELP Scientifica, Europe), centrifuged using centurion K241R centrifuge (UK). Afterwards, rotary vacuum concentrator connected to vacuum pump (DVP TYRO 12, Germany) was used.

Chemicals and Reagents

- Nebivolol hydrochloride (NEB), valsartan (VAL) and hydrochlorothiazide (HCZ) contain 99.90% of pure raw material drugs were kindly donated from the national organization for drug control and research (NODCAR).
- Nevilob plus ® tablets (Batch No. 1933063) labeled to contain 5 mg Nebivolol and 25 mg Hydrochlorothiazide (Marcyrl, Cairo, Egypt) was purchased from the local market.
- Nebilet tablets (Batch No. 01506B) labeled to contain 5 mg Nebivolol and Tareg tablets (Batch No.062023) labeled to contain 80 mg valsartan (were used for synthetic tablet preparation). Both dosage forms were purchased from the local market.
- HPLC grade methanol, acetonitrile, methanol and (85%) orthophosphoric acid from Fisher Scientific (Loughborough, Leicestershire, UK), were used.
- Deionized water was obtained from Milli-Q water purification system.
- Blank human plasma was obtained from VACSERA, National Blood Bank, Egypt.



(a) (b) (c)

Fig. 1 The chemical structures of (a) NEB, (b) VAL and (c) HCZ.

Chromatographic Conditions

Separation was carried out on Xterra C_{18} column with dimensions (100 mm × 4.60 mm, 5.0 µm) as a stationary phase. Acidified water of pH= 3.0 (adjusted using 0.1% orthophosphoric acid) and acetonitrile were used as mobile phase. Gradient elution was started from 0-3.0 mins (90:10, *v/v*), 4.0-8.0 mins (20:80, *v/v*)) and 9.0-13.0 mins (90:10, *v/v*) ratios. The mobile phase was subjected to degassing for 20.0 minutes. Then the HPLC pump was equipped to deliver mobile phase with 1.0 mL/min flow rate to the column of which temperature was maintained at ambient temperature. The injection volume was 10.0 µL. Photodiode array was set at 280 nm for determination of NEB, HCZ and VAL.

Procedures

Preparation of standards' solutions

Stock solutions of NEB, HCZ and VAL with concentrations of 1.0 mg/mL were prepared accurately by weighing 100 mg of each drug separately then transferred each one separately into 100.0 mL volumetric flask. 20.0 mLs of methanol were added for dissolving the drugs then complete the volume with the same volume. The working solutions of NEB, HCZ and VAL with final concentrations of 100.0 µg/mL were obtained by taking 10.0 mLs from the stock solution of each drug, then to be transferred into 100.0 mL volumetric flasks and methanol was added up to the final mark.

Calibration Curves Development

Aliquots equivalent to (50.0 to 700.0 µg of NEB), (80.0 to 600.0 µg of HCZ) and (50.0 to 800.0 µg of VAL) were accurately taken from their working solutions into 10-mL volumetric flasks. Volumes were completed using methanol. Final ranges of concentrations were: 5.0–70.0 µg/mL for NEB, 8.0–60.0 µg/mL for HCZ and 5.0-80.0 µg/mL for VAL. 10.0 µL of each analyte was injected into RP-HPLC. The area under the curve of each analyte was plotted against its concentration then the corresponding calibration curve was constructed for each drug.

Validation of analytical method

The developed method has been validated regarding to ICH guidelines with respect to linearity, accuracy, precision, and limit of detection, limit of quantitation, specificity and robustness.

Linearity

The linearity of the chromatographic method for NEB, HCZ and VAL were assessed by analyzing five different concentrations of each drug. The linear relationship between different concentrations and its area under the curve were determined.

Accuracy

Accuracy was determined by using 40.0, 50.0 and 70.0 µg/mL for NEB, 30.0, 40.0 and 60.0 µg/mL for HCZ and 10.0, 50.0 and 80.0 µg/mL for VAL). After construction of the calibration curves, the found concentrations were calculated using the corresponding regression equations. The recovery% and SD were determined.

Precision

Intraday precision was evaluated by analyzing 40.0, 50.0 and 70.0 µg/mL for NEB, 30.0, 40.0 and 60.0 µg/mL for HCZ and 10.0, 50.0 and 80.0 µg/mL for VAL. In addition, interday precision was applied by analyzing 5.0, 10.0 and 40.0 µg/mL for NEB, 10.0, 40.0 and 60.0 µg/mL for HCZ and 10.0, 50.0 and 80.0 µg/mL for VAL). The corresponding RSD% values were calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The calculated LOD and LOQ values were based on standard deviation and slope of response in the calibration curves of each drug.

Specificity

Specificity was evaluated through determination % Recovery of each drug in laboratory mixtures and in presence of the co-formulated substances in the corresponding dosage forms. In addition to the purity of peaks of the cited drugs were investigated.

Robustness

In this method, the flow rate was altered by ± 0.30. While, the wavelength was changed by ± 2 nm.

Assay of Laboratory Prepared Mixtures

Aliquots of (NEB with HCZ) and (NEB with VAL) were transferred from the corresponding working solutions into a series of 10.0 mL volumetric flasks to prepare different laboratory mixtures containing various ratios of the studied drugs. The prepared mixtures were prepared to fit in the linearity range and in the ratios of dosage forms. Afterwards, volumes were completed using methanol. Then 10.0 µL was injected to the column. Consequently, % recovery and standard deviation of each drug was calculated from the corresponding regression equation.

Application to Pharmaceutical Preparation

Ten tablets of Nevilob plus ® tablet labelled to contain 5 mg NEB and 25 mg HCZ were accurately weighed, average weight of one tablet has been calculated then the film coat was removed by rubbing with methanol. The tablets were crushed to a fine powder. An equivalent weight of 5 mg NEB and 25 mg HCZ were weighed and transferred into beaker. 50.0 mLs of methanol was added. The solution was sonicated for 30.0 min and transferred to 100.0 mLs volumetric flask. The flask was completed with methanol. The solution was filtered with 0.22 µm syringe filter. The final stock sample concentration was 50.0 µg/mL of NEB and 250.0µg/mL of HCZ. Aliquots of 90.0 µg and 100.0 µg of NEB with their corresponding of 450.0 µg and 500.0 µg of HCZ were transferred from their stock sample solution to 10.0 mL volumetric flasks. Volumes were completed with methanol to obtain linear concentrations of 9.0 µg/mL and 10.0 µg/mL of NEB with 45.0 µg/mL and 50.0 µg/mL of HCZ. Synthetic tablets of (NEB/VAL) mixture was simulated through combining Nebilet 5 mg ® tablets labelled to contain 5 mg of NEB and Tareg 80 mg ® tablets labelled to contain 80 mg of VAL as it does not found in local market. Five tablets of Nebilet and Tareg separately were accurately weighed and grinded into fine powder. To simulate one tablet, an equivalent weight of 5 mg of NEB and 80 mg of VAL were weighed separately from each tablet powder and transferred into beaker. 50.0 mLs of methanol were added for dissolving drugs. The solution was sonicated for 30.0 mins and transferred to 100.0 mL volumetric flask. The flask was completed with methanol. The solution was filtered with 0.22 µm syringe filter. The final stock sample concentration was 50.0 µg/mL of NEB and 800.0 µg/mL of VAL. Aliquots of 50.0 µg of NEB with their corresponding of 800.0 µg of VAL were transferred from their stock sample solution to 10.0 mL volumetric flasks. Volumes were completed with methanol to obtain linear concentrations of 5.0 µg/mL of NEB with 80.0 µg/mL of VAL. Furthermore, spiking was applied by taking 2.50 µg/mL from NEB and VAL sample stock added to 5.0 µg/mL of pure NEB into 10.0 mL volumetric flask to obtain final concentrations 7.50 µg/mL NEB and 40 µg/mL VAL to fit the linearity range. Finally, aliquots of 10.0 µl were injected on C_{18} stationary phase. % recovery and standard deviation of each sample was calculated from the corresponding regression equation.

Application to spiked human plasma

0.50 mL were taken from stock standard solutions which equivalent to (5.0, 10.0, 20.0, 25.0 and 40.0 µg of HCZ), (5.0, 10.0, 15.0, 25.0 and 30.0 µg of NEB) and (10.0, 20.0, 25.0, 40.0 and 80.0 µg of VAL), each aliquot was added separately to 0.50 mL of plasma in 10.0 mL centrifuge tubes then 1.50 mL of acetonitrile was transferred to precipitate the plasma protein. The solutions were mixed by vortex mixer for 5.0 minutes at 3000 rpm. After that the mixtures have been centrifuged for 15.0 minutes at 6000 rpm. The supernatant was taken and evaporated by using the rotary

vacuum concentrator at 60 °C and 1500 rpm for 4.0 hours. Samples were reconstituted in 1.0 mL of methanol to reach the required final concentrations of 5.0-40.0 µg/mL for HCZ, 5.0-30.0 µg/mL for NEB and 10.0-80.0 µg/mL for VAL. Similarly the blank human plasma was prepared. Plasma was spiked separately with the dosage forms of 5 mg NEB: 25 mg HCZ and 5 mg NEB: 80 mg VAL. Then spiking with combined dosage form of NEB and HCZ with the dosage form of Valsartan was carried out to prove the separation capability of the proposed method to determine the three drugs in their corresponding pharmaceutical preparations. Aliquots of 10.0 µl were injected on C_{18} stationary phase. The area under the curve was plotted against its corresponding concentration to construct calibration curve.The accuracy and precision were determined.

RESULTS AND DISCUSSION

Results

The proposed work represents an accurate, simple developed and validated method of HPLC–PDA for quantitative determination of two binary mixtures (NEB with HCZ) and (NEB with VAL) in bulk and dosage forms. Moreover, challenging separation of the three drugs in one chromatogram under the same chromatographic conditions was attained that enables this method to be used for routine analysis in quality control laboratories.

Method Development

Optimized chromatographic conditions were attained through using Xterra C_{18} column with dimensions (100 mm × 4.60 mm, 5.0 µm) via gradient elution to separate a ternary mixture of NEB, VAL and HCZ and binary mixtures of NEB and VAL and NEB and HCZ. To optimize the developed method conditions, different mobile phase compositions such as 0.02 M phosphate buffer with methanol and acetonitrile with isopropanol in different ratios and different pH values were tried in an isocratic mode, causing salting out of the buffer. Aqueous solution (Deionized water with 0.1% phosphoric acid) to be adjusted at pH 3.0 and be pumped with acetonitrile in gradient elution started from 0-3.0 mins (90:10, v/v), 4.0-8.0 mins (20:80, v/v) and 9.0-13.0 mins (90:10, v/v) ratios at a flow rate of 1.0 mL/min resulting in a good separation and well resolution using gradient elution during the analysis of the two binary mixtures. Moreover, it gave an optimum resolution of the three peaks of NEB, HCZ and VAL. Photodiode array detector (PDA) was set at 280 nm to obtain sufficient peak intensity for the two binary mixtures. Chromatogram with sufficient resolution is obtained; HCZ retention time was at 5.0 mins, NEB retention time was at 7.0 mins and VAL retention time was at 8.0 mins as shown in Fig.2. The late elution of VAL was due to its hydrophobicity in comparison to NEB and HCZ leading to higher retention on the reversed phase stationary phase (C_{18} column).

Method Validation

The validation parameters of the suggested method include linearity, accuracy, precision, limit of detection, limit of quantification, specificity and robustness have been studied as recommended by ICH Q2[14].

Linearity

The linearity ranges were found to be 5.0–70.0 µg/mL for NEB, 8.0–60.0 µg/mL for HCZ and 5.0-80.0 µg/mL for VAL with correlation coefficients of 0.9996, 0.9991 and 0.9995 for NEB, HCZ and VAL; respectively, under the optimum chromatographic conditions, as shown in **Table 1.**

Accuracy

The suggested method was found to be accurate by calculating the recovery% and SD of three different concentrations of standard solutions of the studied drugs. The results are presented in **Table 1.**

Precision

Intraday precision was evaluated by analyzing 3 different concentrations of each drug in triplicate in the same day. Furthermore, interday precision was assessed by analyzing 3 different concentrations of each drug in triplicate over 3 consecutive days. The RSD% values were less than 2.0 % which indicates the method's precision as presented in **Table 1.**

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOD were calculated. The results are shown in **Table 1.**

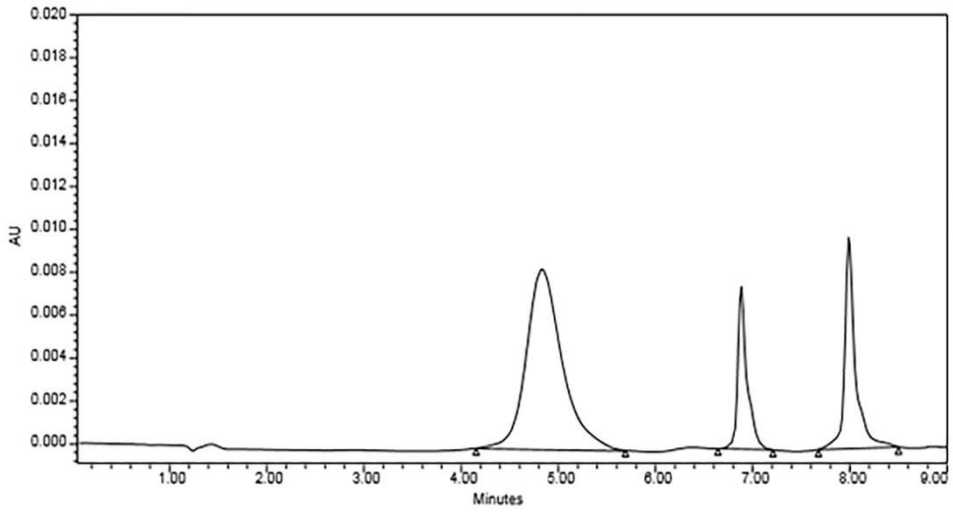


Fig.2: Chromatogram shows the separation of the three drugs, retention time of 20.0 µg/mL HCZ was at 5.0 mins, retention time of 20.0 µg/mL NEB was at 7.0 mins and retention time of 20.0 µg/mL VAL was at 8.0 mins under the mentioned chromatographic conditions.

Table 1: Validation parameters of the proposed method.

parameters	NEB	HCZ	VAL
Range of linearity	5.0-70.0 µg/mL	8.0-60.0 µg/mL	5.0-80.0 µg/mL
Slope	6397.2	17040	5048.7
Intercept	-9140.5	22723	-5720.3
Correlation coefficient	0.9996	0.9991	0.9995
Accuracy% [a] (mean ± SD)	99.73 ± 0.89	99.85 ± 1.10	100.83 ± 1.45
LOD (µg/mL)	2.01	2.67	2.79
LOQ (µg/mL)	6.08	8.08	8.44
Precision Intraday %R.S.D[b]	0.82	1.0	1.29
Precision Interday %R.S.D[b]	1.25	1.11	1.32

[a] Data presented as mean recovery ± standard deviation.
[b] % Relative standard deviation.

Specificity

Specificity is the ability of the analytical method to detect the analyte of interest in presence of excipients. The suggested method was applied for the determination of two binary mixtures one of NEB with HCZ and the other of NEB with VAL. No additional peaks are shown in the obtained chromatograms. Furthermore, the suggested method could effectively separate each drug in presence of others as presented in Fig.3 (a, b, c, d and e). Regarding dosage form of (2.50 µg/ml NEB and 40.0 µg/ml VAL) spiking was necessary to enrich the concentration of nebivolol using 5.0 µg/ml of nebivolol standard solution. Results displayed no interference from any other component in the dosage forms as well as shown in **Tables** 2, 3, 4 and 5. Moreover, the purity of peaks were obtained from the mathematic analysis of the software. The purity of the peaks of cited drugs were 99.90%.

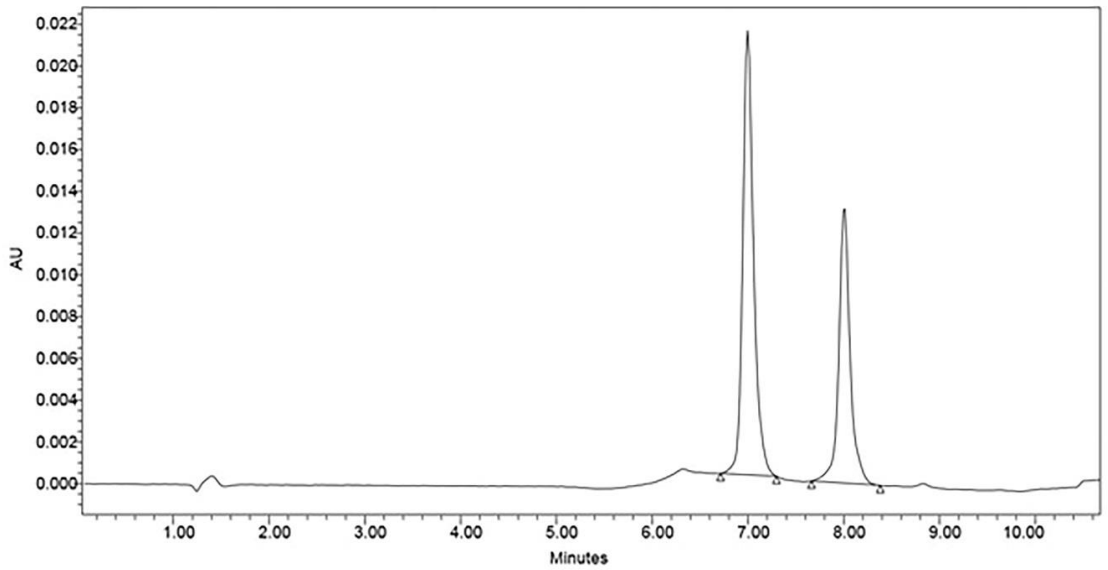


Fig.3: (a) Chromatogram of laboratory prepared mixture of 50.0 µg/mL NEB and 50.0 µg/mL VAL.

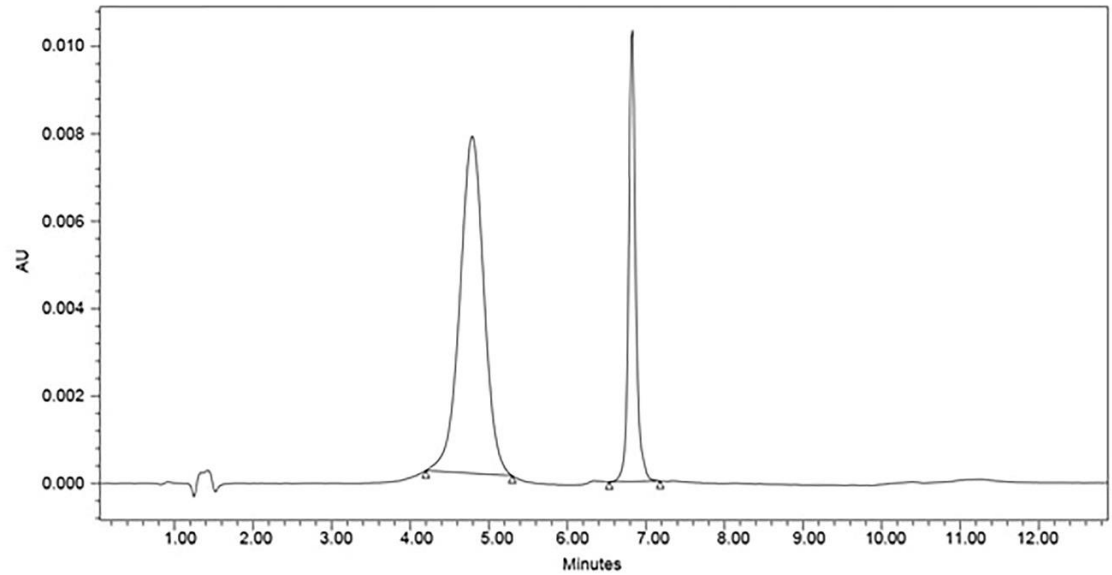


Fig.3:(b) Chromatogram of laboratory prepared mixture of 20.0 µg/mL HCZ and 25.0 µg/mL NEB.

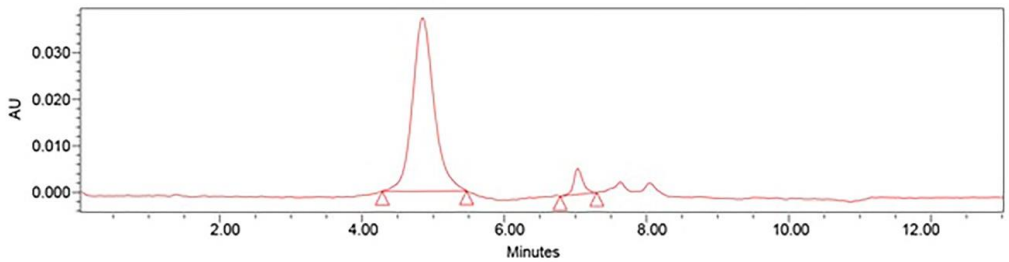


Fig.3: (c) Chromatogram of the dosage form of 50.0 µg/ml HCZ and 10.0 µg/ml NEB.

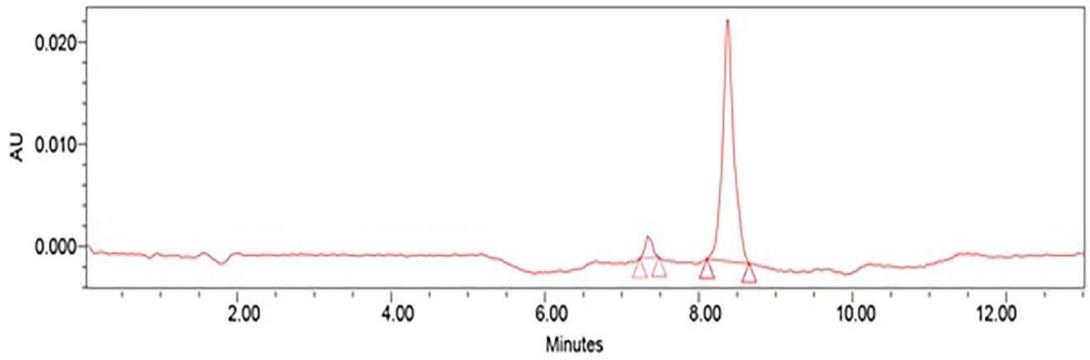


Fig.3: (d) Chromatogram of the dosage form of 5.0 µg/ml NEB and 80.0 µg/ml VAL.

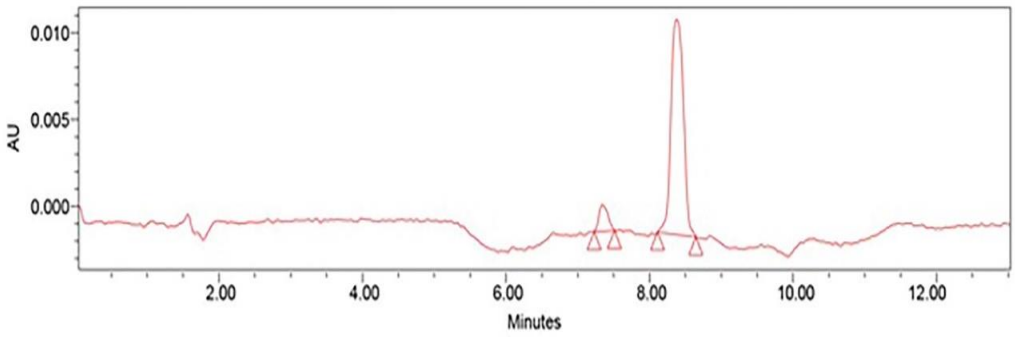


Fig.3: (e) Chromatogram of the dosage form of 2.50 µg/ml NEB spiked with 5.0 µg/ml of NEB standard solution and 40.0 µg/ml VAL.

Table 2: HPLC determination of laboratory-prepared mixtures of NEB and HCZ.

Analyte conc. in laboratory prepared mix, µg/mL		**Recovery%**	
NEB	HCZ	NEB	HCZ
10.0	10.0	100.54	98.14
[a]5.0	[a]25.0	100.48	99.79
[a]10.0	[a]50.0	99.73	100.16
Mean ± SD		100.25 ± 0.45	99.36 ± 0.89

[a]Data represent the ratio of analyte concentrations present in the pharmaceutical formulations.

Table 3: HPLC determination of laboratory-prepared mixtures of NEB and VAL.

Analyte conc. in laboratory prepared mix, µg/mL		**Recovery, %**	
NEB	VAL	NEB	VAL
[a]5.0	[a]80.0	100.81	99.29
10.0	10.0	99.76	100.86
Mean ± SD		100.29 ± 0.74	100.08 ± 1.11

[a]Data represent the ratio of analyte concentrations present in the pharmaceutical formulations.

Table 4: HPLC determination of NEB and HCZ in pharmaceutical dosage form.

Dosage form µg/mL		Recovery%	
NEB	HCZ	NEB	HCZ
[a] 9.0	[a] 45.0	99.42	100.28
[a]10.0	[a]50.0	100.26	99.91
Mean ± SD		99.84 ± 0.59	100.10 ± 0.26

[a]Nebilet plus ® tablet dosage form contains 5 mg Nebivolol and 25 mg Hydrochlorothiazide.

Table 5: Determination of NEB and VAL in pharmaceutical synthetic dosage form.

Dosage form µg/mL		Recovery%	
NEB	VAL	NEB	VAL
[a]5.0	[a]80.0	101.26	98.10
[a,b]7.50	[a,b]40.0	100.11	100.17
Mean ± SD		100.95 ± 0.44	98.81 ± 1.00

[a] Nebilet tablet contains 5 mg Nebivolol and Tareg tablet contains 80 mg.
[b] Spiking Technique (2.50 µg of Nebivolol from Nevilob plus dosage Form added to 5.0 µg of pure Nebivolol).

Robustness

The flow rate was altered by ± 0.30 mL/min. While, the wavelength was changed by ± 2 nm. The proposed method was robust against any studied change, as shown in the **Table 6.**

Application to spiked human plasma

The drugs were extracted from plasma by applying sample preparation technique which leads to decrease the effect of plasma protein and other plasma matrix components that could interfere with determination of the studied drugs.1.50 mL of acetonitrile was added to 0.50 mL plasma for precipitation of the plasma protein[15]. %Recoveries were found to be 97.27%, 99.74% and 98.21% for HCZ, NEB and VAL dosage forms respectively. % Recovery was calculated from regression equation of spiked human plasma calibration curve. The results as shown in Fig.4 (a, b, c and d) and **Table 7.** Furthermore, NEB is primarily metabolized by CYP2D6 liver enzyme by hydroxylation, oxidation, glucuronidation and n-dealkylation to active metabolites. The time of peak drug concentration (T_{max}) and maximum concentration (C_{max}) of NEB are 1.50 – 4.0 hours and 8.02 ± 3.47 ng/mL.The route of eliminations of NEB are 38% in urine and 44% in feces[16]. Valsartan undergoes minimal liver metabolism. Valeryl-4-hydroxy valsartan metabolite was formed by using liver CYP2C9 isoenzyme. The time of peak drug concentration (T_{max}) and maximum concentration (C_{max}) of VAL are 4.0 – 6.0 hours, 1.64mg/L and 3.25 mg/L. The route of eliminations of VAL are 13% in urine and 83% in feces[17]. HCZ is not metabolized. The time of peak drug concentration (T_{max}) and maximum concentration (C_{max}) of HCZ are 1.0 – 5.0 hours and 70-490 ng/mL. The route of elimination of HCZ is eliminated unchanged in urine[18].

Table 6: Robustness results of the proposed method.

Parameters	Modification	AUC			% Recovery		
		NEB*	VAL*	HCZ*	NEB	VAL	HCZ
Flow rate (mL/min)	0.70 mL/min	54499	46020	189999	99.48	102.48	98.17
	1.3 mL/min	54999	46030	190955	100.26	102.50	98.73
Wavelength (nm)	282 nm	54449	46018	189952	99.40	102.46	98.14
	278 nm	54400	46000	189902	99.33	102.44	98.11

* Determined concentration was 10.0 µg/mL for each drug.

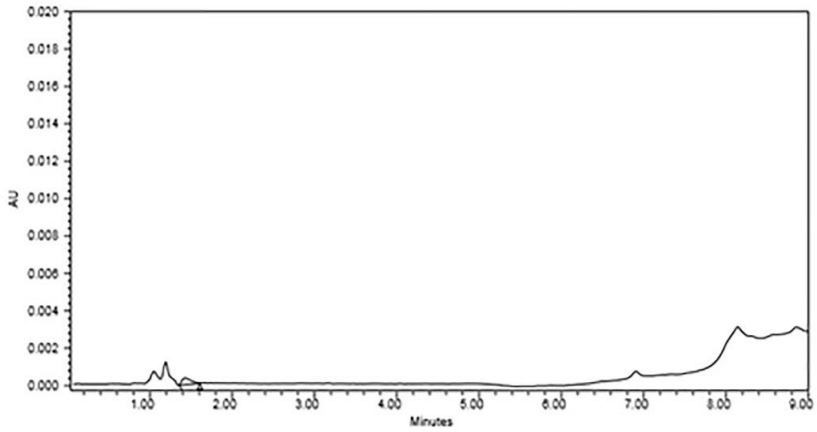


Fig.4: (a) Chromatogram showed blank human plasma.

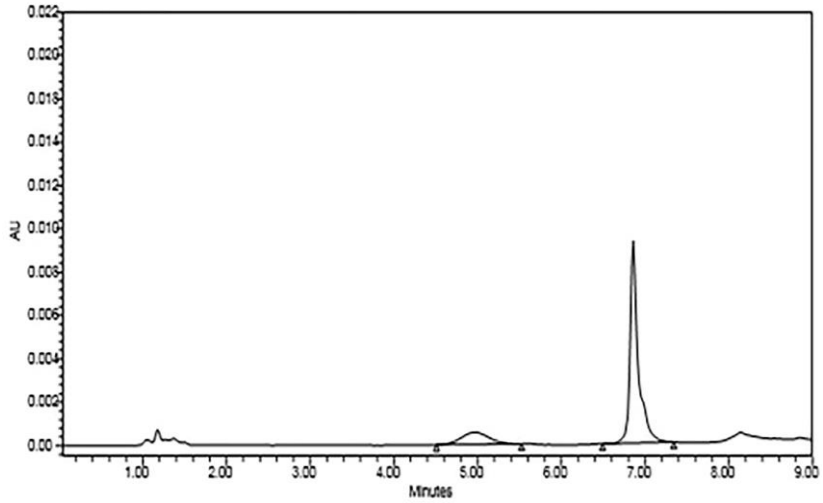


Fig.4: (b) Chromatogram shows human plasma spiked with combined dosage form of 5.0 µg/mL NEB with 7.0 mins retention time and 25.0 µg/mL HCZ with 5.0 mins retention time.

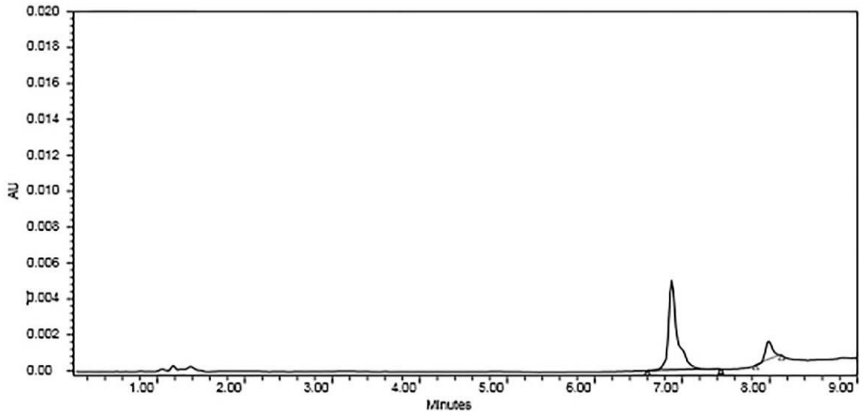


Fig.4: (c) Chromatogram shows human plasma spiked with combined dosage form of 5.0 µg/mL NEB with 7.0 mins retention time and 80.0µg/mL VAL with 8.0 mins retention time.

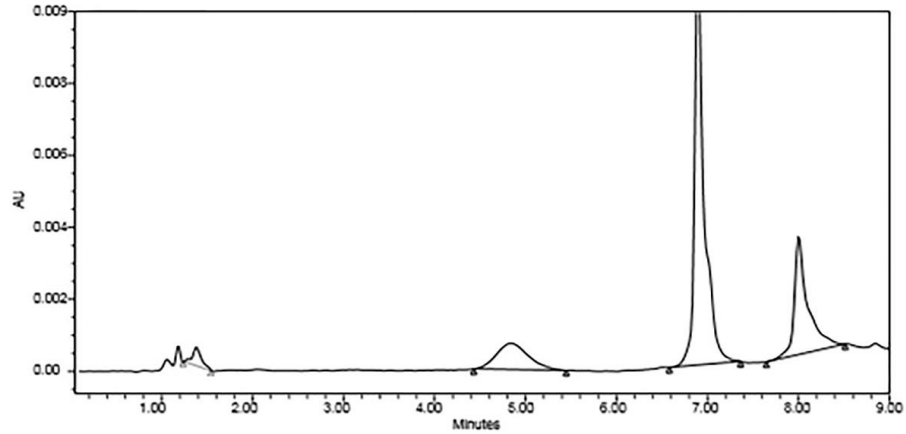


Fig.4: (d) Chromatogram obtained from human plasma spiked with the three drugs in their dosage forms, retention time of 25.0 µg/mL HCZ was at 5.0 mins, retention time of 5.0 µg/mL NEB was at 7.0 min and retention time of 80 µg/mL VAL was at 8.0 mins.

Table7: Validation parameters of NEB, HCZ and VAL in spiked human plasma using HPLC method.

Parameters	**NEB**	**HCZ**	**VAL**
Linearity range	5.0-30.0 µg/mL	8.0-40.0 µg/mL	14.0-80.0 µg/mL
Slope	163.02	1805.9	517.29
Intercept	19597	83094	9280
Correlation coefficient	0.9980	0.9970	0.9980
Accuracy% [a] (mean ± SD)	99.73 ± 1.41	100.26 ± 1.69	99.10 ± 0.87
LOD (µg/mL)	1.99	2.65	4.76
LOQ (µg/mL)	6.04	8.04	14.42
Precision Intraday %R.S.D[b]	1.04	0.72	0.84
Precision Interday %R.S.D[b]	1.33	1.0	1.07

[a] Data presented as mean recovery ± standard deviation.
[b] % Relative standard deviation

Statistical Analysis

Comparing the proposed method with two reported methods; it was found that this method could determine simultaneously the three drugs either in dosage forms or plasma with good linearity, acceptable accuracy and precision as presented in **Table 8. Table 9** represents the statistical comparison between the results of our proposed method and the reported methods. The calculated t and F values showed that there is no significant difference between the proposed method and the reported methods.

Table 8: Comparison of the chromatographic parameters between the proposed method and the reported methods.

Parameters	Proposed method for three drugs	Reported method [19] (NEB and VAL)	Reported method [20] (NEB and HCZ)
Column used	Xterra C_{18} column (100 mm × 4.60 mm, 5.0 µm)	Hypersil ODS 3V (150mm 4.60mm, 5.0 µm)	LiChrosorb^R C_{18} column, with 250mm × 4.60 mm, 5.0µm)
Mobile phase	Acidified water of pH = 3.0 adjusted using 0.1% orthophosphoric acid: acetonitrile. Gradient mobile phase was started from 0-3.0 mins with ratios of (90:10, v/v) , then 4.0-8.0 mins with ratios of (20:80, v/v) and 9.0-13.0 mins (90:10, *v/v*) ratios	Isocratic mobile phase of ammonium Acetate Buffer with adjusted pH to 5.20 : Acetonitrile (60:40 v/v)	Isocratic mobile phase of methanol and water (80:20 v/v) with an apparent pH adjusted to 7.0 using 1.0 N potassium hydroxide solution
Flow rate	1.0 mL/min	1.0 mL/min	1.60 mL/min
Detector	Photodiode array detector adjusted at 280 nm	UV detector at 280 nm	UV detection at 286 nm
Retention time	• HCZ: 5.0 min • NEB: 7.0 min • VAL: 8.0 min	• NEB: 2.70 min • VAL: 4.70 min	• HCZ: 1.64 min • NEB: 3.67 min
Linearity and range	• HCZ: 8.0–60.0 µg/mL • NEB: 5.0–70.0 µg/mL • VAL: 5.0–80.0 µg/mL	• NEB: 2.50-15.0 µg/mL • VAL: 40.0-240.0 µg/mL	• HCZ: 2.0-70.0 µg/mL • NEB: 2.0-40.0 µg/mL

Table 9: RP-HPLC statistical results obtained by the proposed methods and the reported methods for determination of (NEB and VAL) and (NEB and HCZ) in pharmaceutical dosage forms.

Item	NEB		HCZ		NEB		VAL	
	Proposed	Reported[20]	Proposed	Reported[20]	Proposed	Reported[19]	Proposed	Reported[19]
Mean ± SD	99.84% ± 0.59	99.58% ± 0.75	100.1% ± 0.26	100.04% ± 0.37	100.95 % ± 0.44	100.25 ± 0.95	98.81% ± 1.0	99.48 ± 1.16
n	2	9	2	9	2	6	2	6
Student's t-test [a]	1.35		0.64		2.35		-1.77	
F-value [a]	1.61		2.02		4.73		1.34	
	(P=0.05) t tabulated =2.262				(P=0.05) t tabulated =2.447			
	(P=0.05) F tabulated =238.883				(P=0.05) F tabulated =230.162			

[a]Values in the parenthesis are the corresponding theoretical values of t and F at P = 0.05 [21].

Assessment of Greenness of the Method: The Analytical Eco-Scale

The analytical Eco-Scale is used to determine the greenness of the analytical method by giving penalty points to any variable in the analytical method that dispute with the green analysis. The results are evaluated on the scale, with more than 75 representing excellent green analysis, more than 50 representing acceptable green analysis and less than 50 representing inadequate green analysis. The evaluation is based on four parameters: quantity of reagents, hazardousness, energy consumption and waste generation[22]. Our method is rated as an acceptable green analysis as shown in **Table 10**.

Green analytical procedure index (GAPI)

It is a new tool for visual assessment, which used to evaluate the greenness of analytical method. The five pentagrams as shown in Fig.5 represented sample collection, sample preparation, reagents and solvents, instrumentation and type of method. Each step can be given one of three colors: green, yellow and red to represent low, medium, high environmental impact [23]. The two red regions of sample collection and transport in the first pentagram were mandatory as there is a distance between the production and quality control department in the pharmaceutical industry. The other red region in fifth pentagram that corresponds to waste which was more than 10 mL. The method showed 6 green regions, 6 yellow regions and 3 red regions.

Table 10: The penalty points used to calculate the eco-scale.

Type of reagent	**Penalty points**
Methanol	(more than 100 mL)=18
Acetonitrile	(more than 100 mL)= 12
Phosphoric acid	(<10 mL)= 2
Hazardousness	(None)=0
Energy consumption	(less than or equal to 0.1k W h per sample)=0
Waste production	(>10 mL)= 5
Total penalty points	37
Analytical Eco-Scale total score	63
	Acceptable green analysis

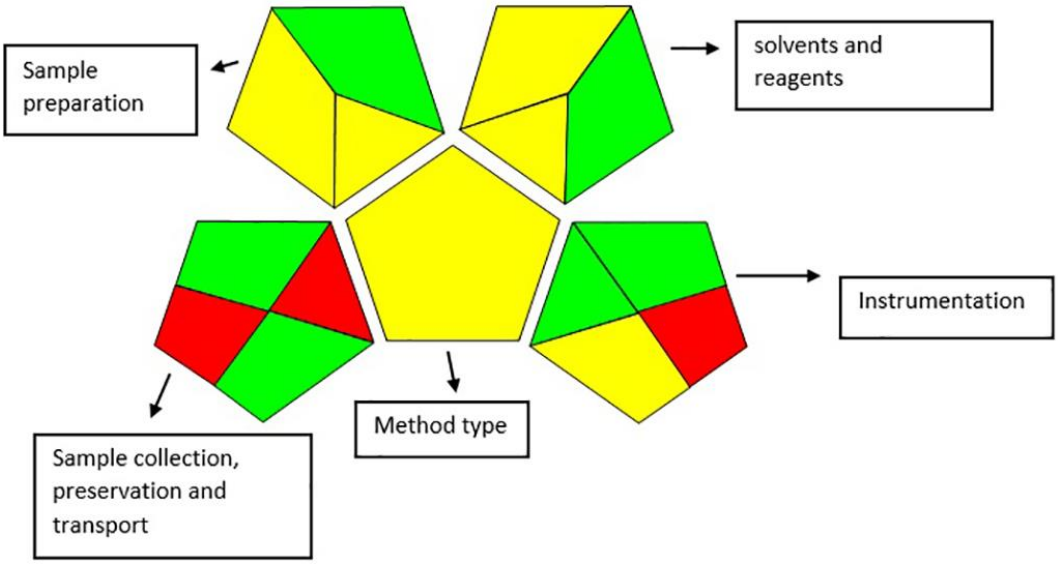


Fig.5: GAPI assessment profile of our proposed analytical method of HPLC-PDA.

.**Conclusion**

A simple, accurate and precise RP-HPLC–PDA method was developed for the simultaneous determination of two binary mixtures (Nebivolol with Valsartan) and (Nebivolol with hydrochlorothiazide) in pharmaceutical dosage forms. Moreover, the developed method was applied for the challenging separation of the three drugs. ICH guidelines were followed for confirming method validation. The greenness of the method has been evaluated by two scales: the analytical Eco-Scale and green analytical procedure index. Quality control laboratories can apply this method with advantages of cost effectiveness, simplicity and short run time.

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Bull. Pharm. Sci., Assiut University, Vol. 46, Issue 1, 2023, pp. 157-172.

نشرة العلوم الصيدلية
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تطوير طريقة فصل باستخدام كروماتوغرافيا السائل ذات الأداء العالي لتحديد متزامن لـــنيبفولول ، هيدروكلوروثيازيد و فالسارتان في مستحضرات الأدوية الخافضة للضغط التي يتم تناولها معاً و كذلك في البلازما البشرية

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تم تطوير طريقة باستخدام الكروماتوغرافيا السائلة عالية الأداء جنبًا إلى جنب مع استخدام الطيف (HPLC-PDA) من أجل التحديد المتزامن لاثنين من التركيبات الثنائية الخافضة للضغط ؛ إما نيبفولول مع هيدروكلوروثيازيد أو مع فالسارتان في المستحضرات الصيدلانية والبلازما البشرية. تم تطبيق الطريقة بنجاح لفصل الثلاثة أدوية تحت نفس الظروف الكروماتوجرافية المستخدمة في مستحضرين الأدوية ، وتم الفصل على عمود Xterra C18 باستخدام طور متحرك متدرج يتكون من ماء محمض بدرجة حموضة = ٣,٠ (تم ضبطه باستخدام ٠,١٪ orthophosphoric حمض) وأسيتونتريل بمعدل تدفق ١,٠ مل / دقيقة. تم تحديد الأدوية عند ٢٨٠ نانومتر. تم عرض نطاق تركيز كل تحليل خطي عند ٥,٠–٧٠,٠ ميكروغرام / مل لـــنيبفولول ، ٨,٠–٦٠,٠ ميكروغرام / مل لـــهيدروكلوروثيازيد و ٥,٠–٨٠,٠ ميكروغرام / مل لفالسارتان. تم عرض نتائج التحقق من صحة الطريقة وفقًا لإرشادات ICH، وقد تم إجراء التقييم الأخضر للمقترح باستخدام مقاييس مختلفين.