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Research article

Bio-analytical study of Efonidipine Hydrochloride Ethanolate and Telmisartan in human plasma by RP-HPLC

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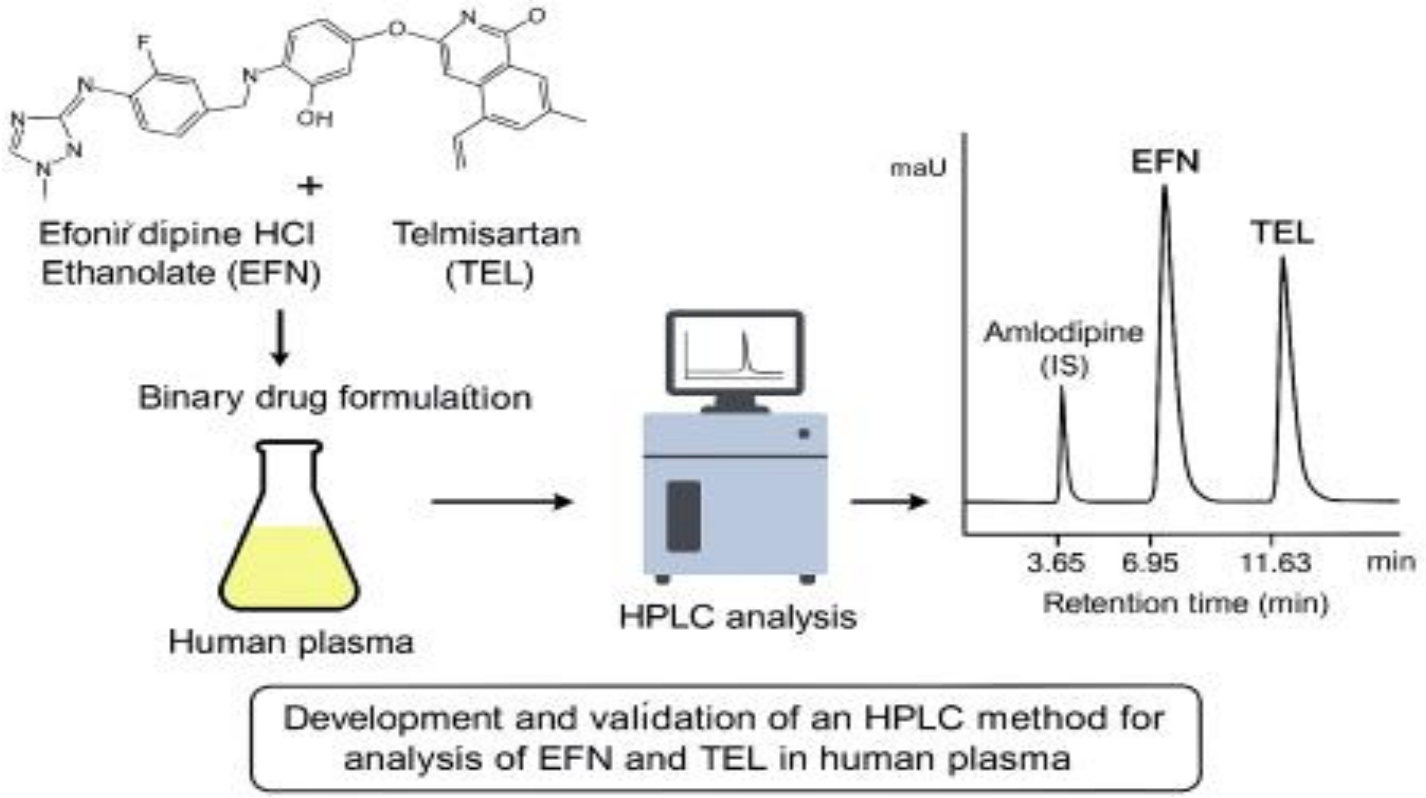
ABSTRACT

Hypertension is now commonly treated with two or three-drug combinations, often using two drugs. So, a binary drug combination was selected for this analytical study. The formulation comprises Efonidipine HCl Ethanolate (EFN) and Telmisartan (TEL), which treat cardiovascular conditions such as hypertension. Amlodipine was used as the Internal Standard (IS) to develop and validate a new and reliable RP-HPLC analytical technique for the simultaneous analysis of EFN and TEL in human plasma. Method novelty, sensitivity, rapidity, precision, and accuracy were evaluated.



This process involved spiking 50µL of EFN, 50µL of TEL, and 50µL of Amlodipine (IS) to a 350µL sample of human plasma, which was then extracted using solid phase extraction (SPE). Utilizing an Agilent RP-HPLC system featuring a Shimadzu column C18 (250mm×4.6mm ID,5µ), compound separation was successfully achieved at a temperature of 30°C.

An isocratic mobile phase (pH 3.0) of a 30:70% v/v mixture of acetonitrile and 0.1M phosphate buffer was used at a flow rate of 0.8 ml/min for optimal performance. The compounds were detected using UV detection at a 254 nm wavelength and a precise injection volume of 20 µL. The analytical run time was 20 minutes, with retention times of 11.63 minutes, 6.95 minutes, and 3.65 minutes for EFN, TEL, and Amlodipine (IS), respectively. Following ICH M10 requirements, the RP-HPLC technique was validated for linearity, precision, accuracy, specificity, and sensitivity. The method's linearity was observed in human plasma over a 5.0-200 ng/ml concentration series. EFN and TEL exhibited correlation coefficients (r^2) of 0.9997 and 0.9995, respectively.

Keywords: Efonidipine HCl Ethanolate, Telmisartan, Bioanalytical method, Validation, RP-HPLC.

INTRODUCTION

Analytical and bio-analytical technique is needed to estimate the quality and purity of the drug in the bulk as well as in a formulation. Analytical and bio-analytical support is also necessary to comply with the regulatory requirements. Compliance with regulatory requirements is a prerequisite to selling the drug across the globe.

There are single-component and multicomponent analytical methods available to do quality and purity evaluation of drugs [1]. Single-component analytical methods are quite easy than multi-component analytical methods. Multicomponent analysis is challenging and needs many systematic experimental trials to prove its rational [2]. The analytical method of a drug is required before a bio-analytical study. The analytical method development and its validation is needed to validate the claim [3]. Bio-analytical techniques involve the collection, selection, and storage of biological matrix, and it is challenging to carry out the sample preparation by using different extraction techniques for bio-analysis [4, 5]. Due to their complexity, biological fluids such as human plasma and whole blood are often not able to be directly injected into an analytical instrument for drug substance detection. The proposed study is a multicomponent bio-analytical study, which is important to estimate Efonidipine Hydrochloride ethanolate (EFN) and Telmisartan (TEL) in a combined formulation. So, it is a significant bio-analytical approach for EFN and TEL in the combined formulation.

Chemical name for EFN is 2-(*N*-benzylanilino) ethyl 5-(5, 5-dimethyl-2-oxo-1, 3, 2λ5-dioxaphosphinan-2-yl)-2, 6-dimethyl-4-(3-nitrophenyl)-1, 4 dihydropyridine – 3 -carboxylate; ethanol; hydrochloride shown in Figure 1[6]. EFN is mainly used to treat angina pectoris and hypertension. It is a member of the 1,4-dihydropyridine derivative family. With its capability to inhibit L-type as well as T-type calcium channels, EFN functions as a calcium channel blocker. Introduced in Japan as an antihypertensive medication in 1994, it is characterized by a slow rate of action and extended half-life [7].

Chemical name for TEL is 2-[4-[[4-methyl-6-(1-methylbenzimidazol-2-yl)-2-propylbenzimidazol-1-yl] methyl] phenyl] benzoic acid, which belongs to the benzimidazole derivative class and functions as a non-peptide angiotensin II type 1 receptor antagonist, shown in Figure 2[7]. It is widely prescribed to manage hypertension and heart failure. By exhibiting selectivity and reversibility, TEL specifically binds to the adrenal gland and vascular smooth muscle. This binding disrupts the interaction between angiotensin II and the angiotensin II AT1 receptor. This action results in arteriole vasodilation and inhibition of the renin-angiotensin system (RAS) stimulation [8].

The combined administration of EFN and TEL is a widely employed approach for hypertension treatment, as it leverages their synergistic mechanism to effectively regulate blood pressure.

There are various analytical and bio-analytical methods available to determine EFN and TEL individually and in combination, such as LC-MS/MS [9], RP-HPLC [10,11], HPTLC [12], and UV spectroscopy methods[13].There is no known bio-analytical method for the simultaneous evaluation of TEL and EFN in human plasma. The current work consists of a multi-component analysis in which an initial analytical method was developed and then, it was used to develop a bio-analytical method and which was validated for the analysis of EFN and TEL. Consequently, this research aims to establish a simple, accurate, specific, and precise bio-analytical method utilizing RP-HPLC to simultaneously quantify EFN and TEL in human plasma. The proposed approach was validated as per the International Conference for Harmonisation (ICH) M10 guidelines [14, 15].

Figure 1: EFN structure

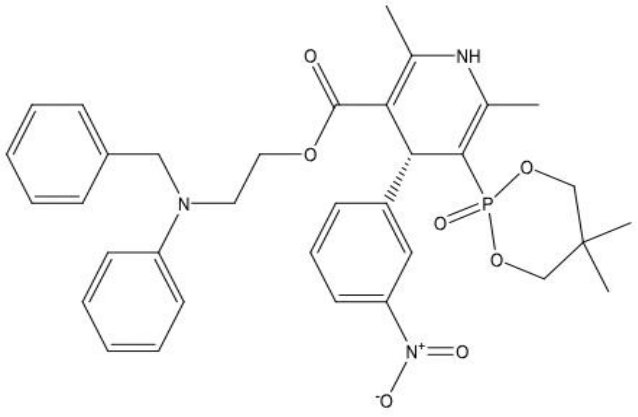
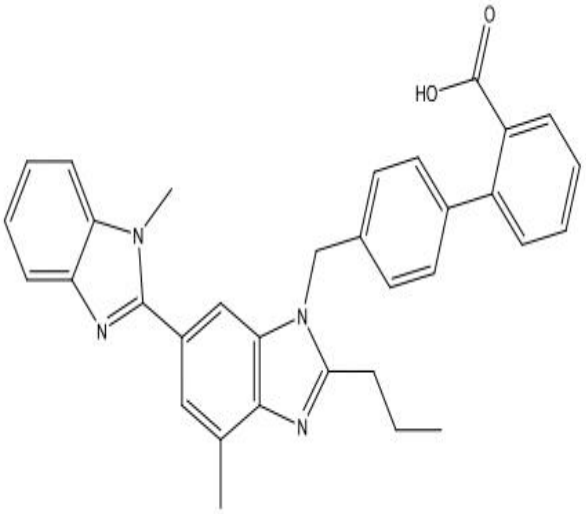


Figure 2: TEL Structure



MATERIALS AND METHODS

Equipment and Materials

The RP-HPLC system used for chromatographic development was an Agilent Technologies (Model no: Infinity 1260) with a UV Detector and quaternary pump. The injection volume capacity was 100µl, and the data acquisition was done on the Control Panel Open Lab software. All weighing was done on a Sartorius analytical and semi-micro weighing balance (Capacity-Max. 200 gm./Min. 25 mg and Capacity-Max. 10 gm./Min. 5mg respectively).

Zuventus Healthcare Ltd., located in Mumbai, India, generously provided the EFN gift sample. Atra Pharmaceuticals Limited, located in Aurangabad, India, generously provided the TEL gift sample. Glochem Industries, based in Hyderabad, India, gifted the Internal Standard (IS), Amlodipine. The human plasma sample, used in the study, was procured from Dr. Vasantrao Pawar Medical College Hospital and Research Centre, Nashik, India, and was stored at a temperature of -20 °C. HPLC grade solvents as well as analytical reagent grade chemicals were used. Acetonitrile and methanol were acquired from Merck, Mumbai (India), and the SPE cartridge (Strata-X) was bought from Phenomenex.

Preparation of Mobile Phase

To prepare the buffer, 13.6 g KH_2PO_4 was added to a litre reagent container and dissolved using 1 litre of Milli-Q water. By using diluted orthophosphoric acid, the buffer pH was accurately adjusted to 3.0 (±0.5) and filtered through a 0.45-micron filter after being sonicated for 10 minutes. For the preparation of the mobile phase, 0.1M phosphate buffer and HPLC-grade acetonitrile were mixed in a 30:70 v/v ratio and then sonicated for 5 minutes. A 70:30 v/v mixture of acetonitrile and water was used as the diluent to prepare working solutions, IS, and sample solutions [16].

Preparation of a Working Solution of Analytes

10 mg of TEL and EFN were weighed individually and transferred to 10 ml volumetric flasks. The weighted content was dissolved by adding 5 ml of diluent, and the volume was made to 10 ml with diluent. After further diluting these stock solutions with the diluent, working solutions of the analytes were prepared, ensuring appropriate concentrations within the desired range.

Preparation of Amlodipine (IS)

10 mg of amlodipine was weighed and transferred into a 10 mL volumetric flask. It was dissolved with 10 ml of diluent, resulting in a final concentration of 1000 µg/ml. After that, the stock solution was diluted with the diluent to produce the Amlodipine (IS) working solution, which had a concentration of 100 µg/ml.

Preparation of Calibration Curve Standards and Quality Control (QC) Samples

Working stock solutions of each of the analytes were added to the plasma blank for preparing calibration standards and QC samples. This resulted in a concentration range of 5 ng/ml to 200 ng/ml for EFN and TEL, respectively. Furthermore, samples for quality were prepared from the working solutions, comprising the lowest LOQ QC of 5.04 ng/ml, low QC of 12.7 ng/ml, medium QC of 100.8 ng/ml, high QC of 173.38 ng/ml, and upper LOQ QC of 201.6 ng/ml concentrations within the range of the calibration curves. To ensure light protection, all samples were carefully kept at a temperature of -20 °C.

Sample Preparation

To prepare samples, a variety of extraction methods are available, including protein precipitation, liquid-liquid extraction, and solid-phase extraction. However, we opted for the SPE approach due to its advantages, including high recovery rates, minimal use of organic solvents, and prevention of cross-contamination. This extraction method offers excellent selectivity and reproducibility. 375µL of plasma was spiked with 50µL of each analyte (EFN and TEL), 50µL of Amlodipine (IS), and 500µL of buffer (0.1 M phosphate buffer), to accomplish the extraction. The spiked plasma mixture was thoroughly vortexed for 1 minute before being put into a glass tube. After that, the sample was added to a Strata-X cartridge that had already been pre-conditioned. This cartridge had previously been treated with one ml of methanol and equilibrated with one ml of water. Carefully loading the sample onto the cartridge allowed it to travel through at the ideal rate. The cartridge was thereafter dried and given a two mL water wash. In the end, the cartridge's analytes were eluted.

Chromatographic Conditions

Separation of the compounds was accomplished using an Agilent HPLC system. The column used was a Shimadzu C18 Column (250mm x 4.6mm ID, Particle size: 5µ) operating at a temperature of 30 °C. Acetonitrile and 0.1 M potassium hydrogen solution in a 30:70 v/v ratio served as the mobile phase [17]. The separation was carried out at a flow rate of 0.8 ml/min with an injection volume of 10µl and UV detection at a wavelength of 254 nm. The total run time was 20 minutes. The retention time for EFN and TEL was determined to be 11.6 minutes and 6.9 minutes, respectively.

Method validation

To develop a precise and reliable RP-HPLC technique to determine EFN and TEL in plasma, various stationary and mobile phases, and sample preparation procedures were attempted. The final method was validated as per ICH M10 bio-analytical method validation and study sample analysis. The critical parameters, including system suitability, selectivity, sensitivity, linearity, precision, accuracy, recovery, and stability, were carefully evaluated.

System Suitability

A system suitability analysis was carried out prior to the analysis of every batch of samples to guarantee the chromatographic system's repeatability. Five samples of diluted drug and Amlodipine (IS) were injected into the HPLC system's linear section of the calibration curve for the analysis, and the %CV (coefficient of variation) was calculated. This was done to assess the system's consistency and guarantee the validity of the analysis's findings.

Selectivity

Six batches of blank human plasma, together with two haemolysed and two lipemic plasma blanks, were analysed to confirm the method's specificity. This was done to make sure that the Amlodipine (IS) and analytes in the plasma were not being hampered by any endogenous compounds.

Linearity

For EFN and TEL, a standard calibration curve was produced with eight concentrations that range from 5-200 ng/ml. For the calibration graph, each concentration's peak area was determined and plotted against that concentration. To reach the necessary concentration range, working standards were made using different concentrations of EFN and TEL spiked with a fixed concentration of Amlodipine (IS) solutions in plasma. Following sample extraction, an RP-HPLC system was used to examine the data. When compared to drug concentration, the plots were made of the drug/ Amlodipine (IS) peak area ratio and presented as % Relative Standard Deviation (RSD).

Sensitivity (LLOQ)

The sensitivity of the procedure was evaluated over six replicates using plasma samples spiked with LLOQ-QC concentrations of EFN and TEL.

Accuracy and Precision

The accuracy and precision of the developed methods were determined by evaluating the QC sample at three separate concentrations, which are low, medium, and high ranges of the calibration curve. Six samples were injected for each concentration on a similar day to analyze intra-day variance. The procedure's accuracy is the percentage of observed concentrations against theoretical concentrations (observed concentrations*100 / theoretical concentrations), whilst precision was given as a % RSD.

Recovery

Known concentrations of these substances were spiked into drug-free plasma to assess the recovery of the Amlodipine (IS), TEL, and EFN. The calibration curves' low, middle, and high ranges were represented as three separate concentrations. Using the peak regions of recovered standards with the same concentrations as the peaks of the extracted QC samples, the recovery rates were calculated. The method accuracy may be evaluated against how effectively analytes, as well as Amlodipine (IS), are recovered through the plasma matrix.

Stability

Two distinct methods were used to examine the analytes' short-term stability. After the initial injection cycle, the first technique entailed keeping human plasma samples overnight on the bench at normal temperature (15-20 °C). The following day, more injections of the samples were made. The second procedure involved freezing human plasma samples at -20 °C for a whole night before letting them thaw naturally at ambient temperature.

RESULTS AND DISCUSSION

Solid-phase extraction, or SPE, has yielded superior results than other extraction processes such as precipitation of proteins and liquid-liquid extraction, throughout the development of the method. SPE demonstrated excellent analyte extraction performance regarding separation, recovery, and efficiency.

System suitability

Area ratios, retention times, tailing factors, and theoretical plates were chosen as validation parameters to evaluate the proposed approach. Table 1 displays the corresponding values. The method's % RSD was estimated to be less than 2%, suggesting the suitability of the optimized chromatographic conditions and verifying the method's overall validity. The system suitability parameters are within the acceptable range, as demonstrated in Figure 3. Therefore, the system is appropriate for the method employed.

Selectivity

A chromatogram obtained from blank plasma has been verified to demonstrate the absence of any significant interference from endogenous components (Figure 4).

Linearity

The calibration curves of EFN and TEL in human plasma exhibited better linearity within the concentration ranges of 5-200 ng/ml. The method displayed a strong linear relationship in this range. Figure 5 illustrates typical calibration curves of spiked plasma samples, along with the regression equation and corresponding correlation coefficient (r^2) values for EFN and TEL. The correlation coefficient for EFN was determined to be 0.9997, while for TEL it was found to be 0.9995, as shown in Tables 2 and 3.

Figure 3: Amlodipine (IS) and system suitability samples chromatogram

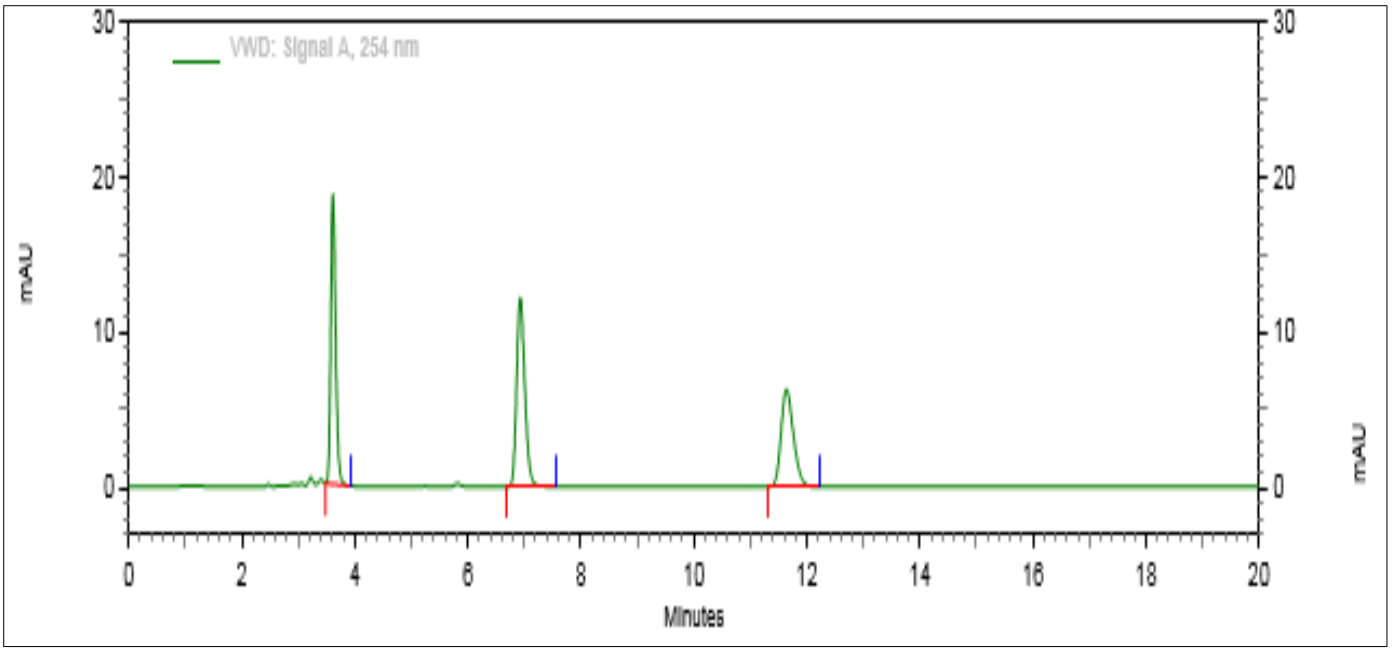


Table 1: System suitability of EFN and TEL

Sr. No.	TEL				Amlodipine (IS)			EFN				
	RT	AREA	TP	TF	RT	AREA	TP	TF	RT	AREA	TP	TF
1	6.93	2069862	10820	1.24	3.61	1875793	8527	1.25	11.64	1607340	13004	1.24
2	6.92	2065683	10915	1.22	3.6	1872900	8638	1.23	11.63	1603592	13094	1.22
3	6.93	2067462	10861	1.23	3.61	1875805	8572	1.24	11.64	1606993	12960	1.23
4	6.92	2069648	10908	1.22	3.62	1878481	8644	1.23	11.63	1609503	12879	1.22
5	6.91	2073610	10990	1.23	3.61	1880195	8526	1.24	11.62	1613260	12947	1.23
AVG	**6.92**	**2069253**	**10899**	**1.23**	**3.61**	**1876635**	**8581**	**1.24**	**11.63**	**1608138**	**12977**	**1.23**
SD	**0.008**	**2977.5**	**63.8**	**0.008**	**0.007**	**2803.1**	**57.5**	**0.008**	**0.008**	**3560.6**	**79.4**	**0.008**
% CV	**0.12**	**0.14**	**0.59**	**0.65**	**0.19**	**0.15**	**0.67**	**0.65**	**0.07**	**0.22**	**0.61**	**0.65**

Where TP- Theoretical plates; TF- Tailing factor; RT- retention time.

Figure 4: Blank human plasma chromatogram

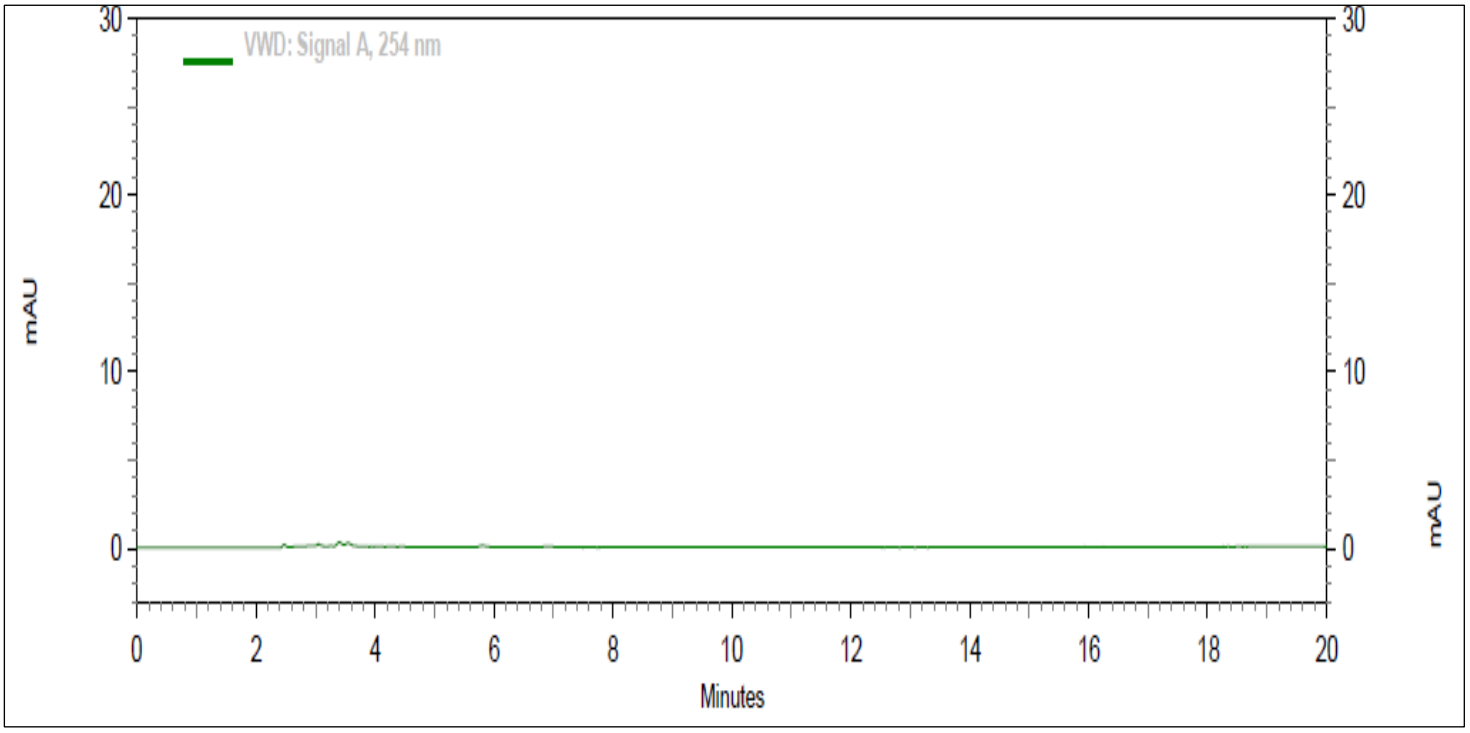


Figure 5: Calibration curve of (a) EFN and (b) TE

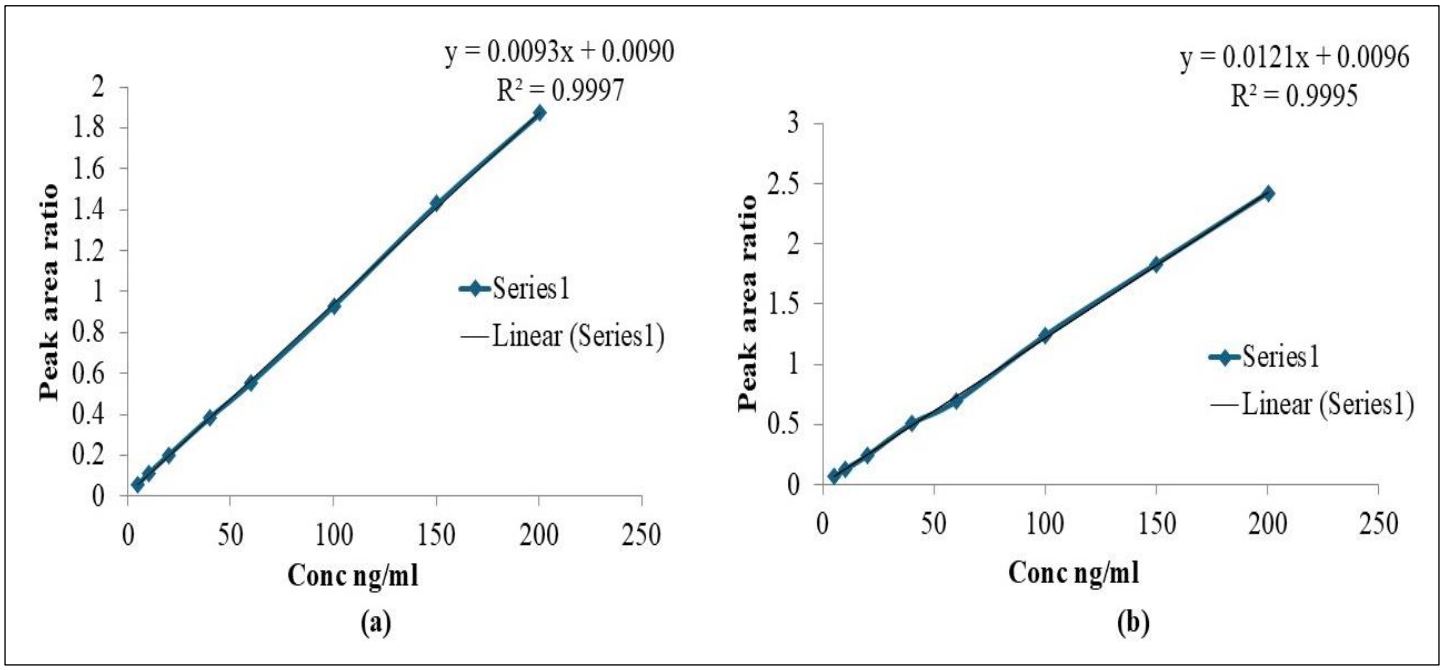


Table 2: Results for linearity of EFN

STD	EFN area	Amlodipine area	Conc. (ng/ml)	Area ratio	Actual Conc.	Slope	Intercept	Correlation coefficient (r^2)
a.	105879	1867238	5.02	0.0567	5.13	0.0093	0.009	0.9997
b.	201170	1858847	10.03	0.1082	10.67			
c.	377628	1876902	20.06	0.2012	20.67			
d.	714957	1862769	40.12	0.3838	40.3			
e.	1053581	1898149	60.18	0.5551	58.72			
f.	1832986	1969494	100.3	0.9307	99.11			
g.	2673953	1871353	150.44	1.4289	152.68			
h.	3578136	1909968	200.59	1.8734	200.47			

Table 3: Results for linearity of TEL

STD	TEL area	Amlodipine area	Conc. (ng/ml)	Area ratio	Actual conc.	Slope	Intercept	Correlation Coefficient (r^2)
a.	133088	1867238	5.01	0.0713	5.10	0.0121	0.0096	0.9995
b.	246903	1858847	10.01	0.1328	10.18			
c.	464020	1876902	20.02	0.2472	19.64			
d.	953434	1862769	40.04	0.5118	41.5			
e.	1324175	1898149	60.06	0.6976	56.86			
f.	2444482	1969494	100.1	1.2412	101.79			
g.	3431996	1871353	150.15	1.834	150.78			
h.	4622617	1909968	200.2	2.4203	199.23			

Sensitivity (LLOQ)

The experimental determination of the LLOQ involved diluting known concentrations of EFN and TEL in the plasma and conducting six replicate determinations. LLOQ of 5.0ng/ml for EFN and TEL in plasma has been achieved with the current assay method. This method demonstrated an intra-day precision with RSD of 4.42% and 4.35%, and accuracy values of 101.65% and 97.85% for EFN and TEL, respectively, as per Tables 4 and 5.

Precision and accuracy

LLOQ, LQC, MQC, HQC, and ULOQ samples for QC of the drug were all used in the estimation of the % RSD to evaluate the method's accuracy. For EFN, the intraday precision% RSD values ranged between 3.28 and 6.42, while for TEL, the values ranged between 1.75 and 4.90. Furthermore, the method's accuracy was assessed, and the results showed that it ranged from 96.88% to 101.65% for EFN and from 96.39% to 99.27% for TEL. Table 6 presents the results that determine the method's precision and accuracy to be satisfactory.

Recovery

To analyze the recovery rate of EFN, TEL, along Amlodipine (IS), predetermined quantities of these compounds were introduced to free from drugs in human plasma at three distinct concentrations, representing the low, middle, as well as high ranges that comprise the calibration curve. By correlating the peaks of the extracted QC samples and the peak area of the recovery standard with similar concentrations, the recovery rates were determined. The absolute recoveries of the extraction procedure for EFN were 95%, 96.5%, and 97.4% at the low, middle, as well as high concentrations, respectively. For TEL, the recoveries were 99.8%, 97.6%, and 99.7% at the corresponding concentration levels. The recovery of Amlodipine (IS) was determined to be 91.6%. The extraction recovery results demonstrated consistency, precision, and reproducibility for EFN and TEL, according to Table 7.

Table 4: Results for Sensitivity of EFN

LLOQ QC	EFN area	Amlodipine area	Area ratio	Actual conc.	% Accuracy	Limit (%)	Precision %(RSD)	Limit (%)
01	108002	1946742	0.0555	5.00	99.21	80-120	4.42	<20
02	113739	1939350	0.0586	5.33	105.75			
03	116371	1947368	0.0598	5.46	108.33			
04	107516	1936239	0.0555	5.00	99.21			
05	104728	1931881	0.0542	4.86	96.43			
06	109404	1942503	0.0563	5.09	100.99			

Table 5: Results for Sensitivity of TEL

LLOQ QC	TEL area	Amlodipine area	Area ratio	Actual conc.	% Accuracy	Limit (%)	Precision %(RSD)	Limit (%)
01	128202	1946742	0.0659	4.65	92.26	80-120	4.35	<20
02	136742	1939350	0.0705	5.03	99.8			
03	142617	1947368	0.0732	5.26	104.37			
04	136013	1936239	0.0702	5.01	99.4			
05	130382	1931881	0.0675	4.79	95.04			
06	132738	1942503	0.0683	4.85	96.23			

Table 6: The intraday precision results (% RSD) and accuracy values (%) for EFN and TEL

Conc. Nominal (ng/ml)	Drug Conc. (ng/ml)	Mean peak area		Accuracy (%)		% RSD	
		EFN	TEL	EFN	TEL	EFN	TEL
5(LLOQ QC)	5.04	0.0567	0.0693	101.65	97.85	4.42	4.35
12 (LQC)	12.7	0.1234	0.1605	96.88	98.18	6.42	2.77
100 (MQC)	100.8	0.9274	1.1999	98.77	98.25	3.28	4.9
170 (HQC)	173.38	1.6017	2.0922	98.77	99.27	3.64	1.75
200 (ULOQQC)	201	1.8612	2.3609	98.79	96.39	3.47	2.92

Table 7: Recovery of EFN and TEL

Samples	Peak area						
	EFN			TEL			Amlodipine (IS)
	LQC	MQC	HQC	LQC	MQC	HQC	Mean peak area
Unextracted	249120.83	1847734.5	3172043.33	308238.17	2364199.8	4047465.2	2099264
Extracted	236728.67	1782848.17	3089501.5	307751.5	2306696	4036841.17	1923466
% Recovery	95	96.5	97.4	99.8	97.6	99.7	91.6

Table 8: Stability study results

	Bench Top Stability				Freeze-Thaw Stability			
Particular	EFN		TEL		EFN		TEL	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
% Nominal	97.77	97.26	99.93	98.83	98.19	96.54	99.31	99.07
% RSD	3.95	2.37	4.43	3.39	5.18	3.5	4.13	4.21

Stability

The stability studies conducted on plasma samples containing EFN and TEL demonstrated that at room temperature, the samples can be handled securely (benchtop) for 4 hours. Additionally, they can undergo up to three cycles of freezing and thawing without experiencing significant degradation. When kept at 4 °C for 24 hrs or at -20 °C, the samples also maintained their stability without any notable degradation. The stability results meet the required acceptance requirements, as per Table 8.

CONCLUSION

The method used for EFN and TEL demonstrated its simplicity, novelty, sensitivity, rapidity, accuracy, and precision. SPE was used for the extraction of drugs from a plasma sample that had been spiked with them and an Amlodipine (IS). With correlation coefficients showing significant correlations between the analytes, the technique demonstrated good linearity across a concentration range ranging from 5 -200 ng/ml. The method validation was carried out as per ICH M10, and the results obtained are satisfactory. This validated RP-HPLC method provides a practical and efficient approach for the simultaneous evaluation of EFN and TEL in human plasma. It can be applied in various research and clinical settings to monitor drug levels, assess pharmacokinetics, and evaluate the effectiveness of combination therapy in treating cardiovascular conditions. The method's reliability and accuracy contribute to advancing the understanding and application of this drug combination for hypertension management and related cardiovascular disorders.

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Conflict of interest

The authors declare that there is no conflict of interest.

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