A Rapid HPLC Method for the Concurrent Determination of Several Antihypertensive Drugs from Binary and Ternary Formulations

Mahesh Attimarad [1,*] , Katharigatta N. Venugopala [1,2] , Nagaraja Sreeharsha [1,3] , Muhammad S. Chohan [4], Sheeba Shafi [5], Anroop B. Nair [1] and Shinu Pottathil [4]

1 Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa 31982, Saudi Arabia; kvenugopala@kfu.edu.sa (K.N.V.); sharsha@kfu.edu.sa (N.S.); anair@kfu.edu.sa (A.B.N.)
2 Department of Biotechnology and Food Technology, Durban University of Technology, Durban 4001, South Africa
3 Department of Pharmaceutics, Vidya Siri College of Pharmacy, Off Sarjapura Road, Bangalore 560035, India
4 Department of Biomedical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa 31982, Saudi Arabia; mshwhan@kfu.edu.sa (M.S.C.); spottathail@kfu.edu.sa (S.P.)
5 Department of Nursing, College of Applied Medical Sciences, King Faisal University, Al-Ahsa 31982, Saudi Arabia; sheeba@kfu.edu.sa
* Correspondence: mattimarad@kfu.edu.sa; Tel.: +966-553269799

Abstract: A rapid, synchronized liquid chromatographic method was established for the estimation of hydrochlorothiazide (HCZ), amlodipine (AMD), olmesartan (OLM), telmisartan (TEL), and irbesartan (IRB) in binary and ternary coformulations using the same chromatographic conditions. Five analytes were separated on a Zorbax C18 column using isocratic elution with a mobile phase consisting of acetonitrile, methanol, and 20 mM phosphate buffer (pH 3.5) in a ratio of 45:20:35% v/v. The analytes were detected at a wavelength of 230 nm at ambient temperature. Furthermore, the proposed liquid chromatographic procedure was validated for linearity, precision, accuracy, stability, and robustness using an experimental design. Analytes were separated with good resolution within 3.5 min. Analytes showed good linearity in a concentration satisfactory to analyze the different ratios of these analytes in the formulations. Pareto charts showed that the flow rate and mobile phase composition have a significant effect on the peak area of analytes and hence need to be carefully controlled, however, the method is robust. Finally, the different formulations consisting of HCZ, AMD, OLM, TEL, and IRB in different ratios were analyzed with high accuracy using an optimized HPLC method and compared with reported methods. Furthermore, the reported HPLC procedure is simple, rapid, and accurate and therefore can used for regular quality control of binary and ternary formulations using the same stationary and mobile phase.

Keywords: HPLC; determination; antihypertensive drugs; experimental design; sartans; amlodipine; hydrochlorothiazide; formulations

1. Introduction

Hypertension is a leading cause of mortality in elderly patients; however, due to the stresses of modern life, even younger people are now suffering from hypertension [1,2]. Globally, about 25% of the population are suffering from hypertension, and this is expected to increase to 30% by 2025 [3]. Different classes of antihypertensive agents are available to control hypertension. However, the most commonly used class of drugs includes calcium channel blockers, angiotensin receptor blockers, and diuretics [4]. A combination therapy showed better control of hypertension than monotherapy due to the different mechanisms of action of a combination of drugs [5–7]. Calcium channel blockers reduce blood pressure by acting on the heart as a beta-blocker and due to a peripheral vasodilation effect on the

arteries and veins. Amlodipine besylate (AMD) is the most extensively used, long-acting calcium channel blocker used for the treatment of hypertension, heart attack, and angina. Sartans, angiotensin II receptor antagonists, reduce blood pressure by inhibiting the vaso-constriction effects of angiotensin on the blood vessels. Telmisartan (TEL), olmesartan (OLM), and irbesartan (IRB) are used for the management of hypertension and cardiac failure, either alone or in combination with other antihypertensive drugs and diuretics such as hydrochlorothiazide (HCZ) [4,8–10]. Diuretics reduce blood pressure by reducing blood volume through increasing the excretion of sodium and water in the urine. Telmisartan, olmesartan, and irbesartan are available on the market in combination with amlodipine and hydrochlorothiazide (Figure 1), manufactured by the same or different pharmaceutical industries [4]. Different analytical methods such as spectrophotometric [11–18], derivative spectrofluorimetric [19], HPLC [20–27], electrophoresis [28], and HPTLC [29] were reported for the concurrent determination of sartans, amlodipine, or hydrochlorothiazide from formulations and biological fluids. The literature has also reported a few analytical methods for the concurrent determination of ternary mixtures of sartans, amlodipine, and hydrochlorothiazide [30–34]. Furthermore, the estimation of several angiotensin II receptor antagonists with other cardiovascular drugs has been reported in the literature [35–39].
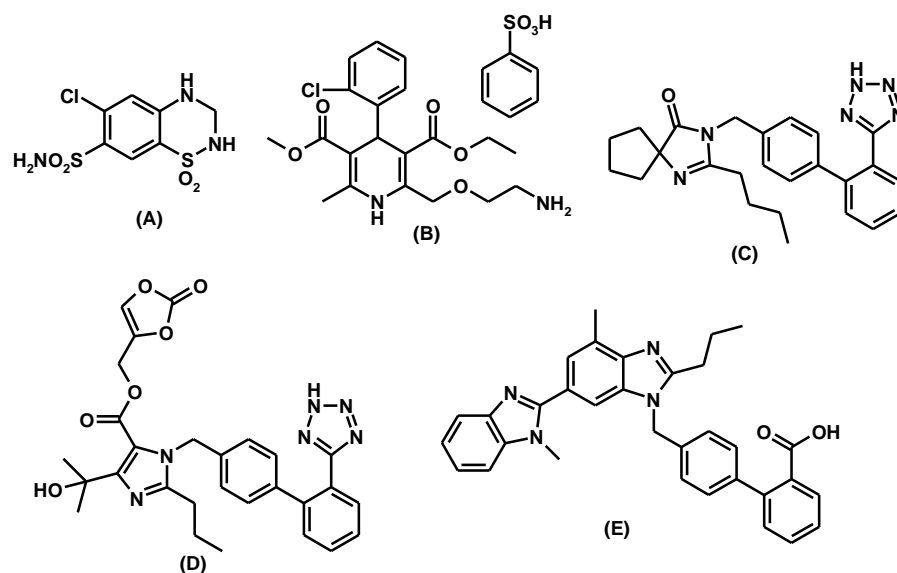

Figure 1. Chemical structures of hydrochlorothiazde (**A**), amlodipine besylate (**B**), irbesartan (**C**), olmesartan medoxomil (**D**), and telmisartan (**E**).

Quality control laboratories of pharmaceutical industries and regulatory agencies prefer to use common analytical techniques for the analysis of diverse pharmaceutical formulations. The development of a common HPLC method saves time and solvents and increases the rate of routine analysis of different batches of formulations. The use of the same HPLC column and solvents reduces the time required to attain equilibrium between the stationary and mobile phases [40,41]. Hence, in the present work, a simple RP-HPLC method has been established and validated using the same reverse-phase high-performance liquid chromatography (RP-HPLC) column and mobile phase for the analysis of amlodipine and/or hydrochlorothiazide with different sartans. The major advantage of the proposed method is that the sartans, along with amlodipine and/or hydrochlorothiazide, can be determined as binary and ternary combinations using the same chromatographic conditions.

2. Materials and Methods

2.1. Materials

Standards AMD (99.56%) and HCZ (99.6%) were purchased from Sigma Aldrich (Buchs, Switzerland). Standards TEL (assay 99.4%) and OLM (assay 99.8%) were purchased

from Biokemics (Hyderabad, India), and IRB (Assay 99.4%) was provided by Apotex Advancing Generics (Bangalore, India) as a gift sample. HPLC grade acetonitrile and methanol procured from Sigma Aldrich (St. Louis, MO, USA). Analytical grade potassium dihydrogen phosphate and orthophosphoric acid were acquired from Scharlau (Sentmenata, Spain). Various formulations (Table 1) consisting of these analytes were purchased from the market. Ultra-pure water prepared using Milli-Q (Millipore, Burlington, MA, USA) was used for preparing the mobile phase.

Table 1. List of formulations.

Drug Products	Composition
Amlokind-H	Hydrochlorothiazide (12.5 mg) + Amlodipine (5 mg)
Lisart Plus (40 mg + 12.5 mg)	Telmisartan (40 mg) + Hydrochlorothiazide (12.5 mg)
Lisart Plus (80 mg + 12.5 mg)	Telmisartan (80 mg) + Hydrochlorothiazide (12.5 mg)
Cresar plus	Telmisartan (40 mg) + Hydrochlorothiazide (12.5 mg) + Amlodipine (5 mg)
Olsar Plus (40 mg/12.5 mg)	Olmesartan Medoxomil (40 mg) + Hydrochlorothiazide (12.5 mg)
Olsar Plus (20 mg/12.5 mg)	Olmesartan Medoxomil (20 mg) + Hydrochlorothiazide (12.5 mg)
Sevikar HCZ (20 mg/12.5 mg/5 mg)	Olmesartan Medoxomil (20 mg) + Hydrochlorothiazide (12.5 mg) + Amlodipine 5 mg
Arena Plus (150 mg/12.5 mg)	Irbesartan (150 mg) + Hydrochlorothiazide (12.5 mg)
Arena Plus 300/25	Irbesartan (300 mg) + Hydrochlorothiazide (25 mg)
Aprovasc 300 mg/5 mg	Irbesartan 300 mg + Amlodipine (5 mg)
Aprovasc 150 mg/10 mg	Irbesartan 150 mg + Amlodipine (10 mg)

2.2. Instrumentation

The HPLC system used for the development of the liquid chromatographic method was an Agilent LC system (1200 series, Agilent, Waldbronn, Germany) equipped with a degasser, auto sampler, quaternary pump, and PDA detector. The chromatogram was monitored using Agilent Chemstation software (Version B.2.4.1). A benchtop pH meter (Mettler-Toledo, Bekasi, Indonesia) was used for adjusting the pH of the mobile phase.

2.3. Chromatographic Conditions

All five antihypertensive drugs, AMD, HCZ, TEL, OLM, and IRB, were chromatographically separated using an Agilent chromatographic system. The column used was a Zorbax Agilent HPLC analytical C18 column (150 mm × 4.6 mm, 5 μM), the mobile phase was potassium dihydrogen phosphate (20 mM, pH adjusted to 3.5 by orthophosphoric acid), and acetonitrile and methanol were at a ratio of 35:45:20% *v/v*. The mobile phase was pumped at a flow rate of 1.5 mL/min at ambient temperature. The chromatogram of eluting was recorded by maintaining the wavelength of PDA at 230 nm. Then, 20 μL of analyte solutions were injected into the HPLC system for the analysis.

2.4. Preparation of Standard Solutions

Standards AMD, HCZ, OLM, and IRB (100 mg each) were weighed into 100 mL measuring flasks separately consisting of 50 mL of methanol, whereas TEL was dissolved in ethanol. The flasks were mixed to solubilize the drugs, and the final volume was adjusted to the 100 mL mark with methanol to get a 1 mg/mL concentration of analytes. Furthermore, working standard solutions for the calibration curve, accuracy, and precision were arranged by adding the required amount of mobile phase to the standard solutions.

2.5. Preparation of Sample Solutions

Twenty tablets consisting of different combinations of AMD, HCZ, TEL, OLM, and IRB (Table 1) were weighed and separately crushed into powder. Tablet powders consisting of different ratios of analytes were weighed and transferred to graduated flasks containing a sufficient amount of solvent. Formulations consisting of TEL were dissolved in ethanol, whereas other formulations were dissolved in methanol. The flasks were sonicated for

15 min to dissolve the analytes, then filtered into other volumetric flasks, and the final volume was adjusted with methanol. Moreover, the solutions were diluted with mobile phase to make the concentration of analytes in the range of the calibration curve. Then, 20 μL of the sample was introduced into the HPLC system for analysis. The concentration of analytes was calculated using corresponding regression equations.

2.6. Validation of HPLC Method

The optimized chromatographic method was validated for different validation parameters such as linearity, system suitability, LOD, LOQ, accuracy, precision, and robustness, per the ICH guidelines [42].

2.6.1. System Suitability Test

A low concentration of all analytes (2 μg/mL of HCZ, 1 μg/mL of AMD, 5 μg/mL of OLM and TEL, and 10 μg/mL of IRB) and six injection replicates were used for the system suitability tests. The different parameters calculated were peak area, retention time, resolution, and peak symmetry.

2.6.2. Linearity

The linearity range was determined by injecting standard solutions of all analytes in a series of 1 to 15 μg/mL of AMD, 2 to 30 μg/mL of HCZ, 5 to 160 μg/mL of OLM and TEL, and 10 to 300 μg/mL of IRB in triplicate. The average peak area was calculated for corresponding chromatograms and a linearity curve was prepared compared to respective concentrations. The regression coefficient (R^2), along with a regression equation consisting of slope and intercept generated, were used for further calculation of the analyte concentrations in samples.

2.6.3. Sensitivity

The limit of detection (LOD) and quantification (LOQ) indicate the sensitivity of the analytical method. The signal-to-noise ratio (S/N) method has been used to determine the LOD and LOQ. The chromatogram was generated by injecting a very low concentration of analytes, and signal-to-noise ratios of $3.3 \times$ S/N and $10 \times$ S/N were used to determine the LOD and LOQ, respectively.

2.6.4. Precision

The overall precision of the proposed method was assessed in the form of intraday and interday precision and expressed as the percent relative standard deviation. Intraday precision was assessed by analyzing three different concentrations (low, medium, and high), covering the entire calibration range. The analysis was performed in triplicate. Interday precision was assessed by analyzing the above solution on three consecutive days.

2.6.5. Accuracy

Accuracy was calculated by determining the percent assay and percent relative error using the standard addition technique. To the previously analyzed laboratory-prepared mixture (HCZ 10 mg + AMD 5 mg + TEL 40 mg + OLM 20 mg + IRB 100 mg), a known amount of standard drug was added (50%, 100%, or 150%) and the total analyte amount was determined using the optimized HPLC method. Furthermore, the accuracy of the procedure was confirmed by computing the percent recovery of the added amount of drugs and the percent relative error.

2.6.6. Robustness

According to the ICH guidelines, robustness has to be assessed for the newly proposed analytical method to understand the effect of slight changes on the experimental conditions. The multivariate approach provides complete information regarding the effect of simultaneous variation in the different independent variables. Hence, the full factorial 2^n factorial

approach, where *n* indicates the number of independent variables, was performed using Design Expert version 12 software (Minneapolis, MN, USA). The variables considered were flow rate (1.4, 1.5, or 1.6 mL/min), the pH of the mobile phase (3.0, 3.5, or 4.0), and the mobile phase composition (buffer: 30%, 35% or 40%; the volume of acetonitrile and methanol were changed equally to make the total volume 100%) on the percent assay. The final assay depends on the peak area; hence, the peak area of analytes was selected in response to studying the effect of independent variables. Thirteen runs were performed per the Plackett-Burman design, including five center points. Pareto charts were generated to show the effect of the independent variables on the peak area.

Further, a robustness study was performed to study the effect of wavelength (230 ± 2 nm), injection volume (20 ± 2 μL), and pH (3.5 ± 0.2) of the mobile phase on the peak area. One parameter was varied at a time by keeping all the other parameters at the optimized level. Analysis was performed in triplicate and percent relative standard deviation of peak area was calculated.

3. Results and Discussion

The chromatographic conditions were optimized to get the highest resolution for all five analyte peaks through the selection of different stationary phases, mobile phase compositions, pH values of the mobile phase, and flow rates. Different stationary phases such as chromolith speed rod, Sepax BR-C18, and Zorbax C18 were also tried, and the Zorbax C18 column showed good separation of analytes with excellent resolution and shape of peaks. The mobile phase composition was elevated by observing the effect of the mobile phase on the peak shape, resolution, capacity factor, and peak symmetry. Potassium dihydrogen phosphate was selected to maintain the ionic strength. Different strengths from 10 mM to 50 mM were tried, with 10 mM tailing observed in AMD and TEL peaks. However, at 20 mM an acceptable peak shape was observed; hence, 20 mM was selected for further development of the HPLC method. The organic modifier acetonitrile was selected for its low viscosity and to reduce the column backpressure. However, with a phosphate buffer and acetonitrile, AMD, OLM, and IRB could not be separated. To separate all five analytes with good resolution, methanol was added to the mobile phase. Varied ratios of phosphate buffer, acetonitrile, and methanol were studied (Figure 2). With less than 35% of phosphate buffer, the HCZ peak was broad; furthermore, more than 35% of telmisartan was eluted after 5 min. In addition, with the increase in the amount of acetonitrile, the retention times of analytes were reduced; however, with more than 45% OLM the IRB resolution was less than 2, so 45% acetonitrile was selected. Furthermore, 20% methanol led to good resolution for all the analytes. The effect of mobile phase pH was evaluated in acidic pH values of 3, 3.5, and 4 (Figure 3). At pH 3, good resolution was observed between HCZ and AMD; however, the TEL and IRB peaks overlapped and a baseline separation was not observed. At pH 4, the AMD and TEL retention time increased and the AMD peak overlapped completely with the IRB peak. At pH 3.5, all five analytes were separated with good resolution and peak shape. The different flow rates of 1 mL/min, 1.2 mL/min, and 1.5 mL/min were evaluated. A flow rate of 1 or 1.2 mL/min took a long analysis time, whereas 1.5 mL/min gave good resolution with baseline separation of all analytes within 3.5 min. From the UV absorption spectra (16, 17, Figure S3) of all five analytes, a wavelength of 230 nm was selected for recording the chromatogram. Finally, the optimized mobile phase, consisting of 20 mM phosphate buffer, acetonitrile, and methanol in a ratio of 35%:45%:20% *v/v*, led to acceptable retention times of 1.01 min, 1.42 min, 1.8 min, 2.1 min, and 3.3 min for HCZ, AMD, OLM, IRB, and TEL, respectively (Figure 4).
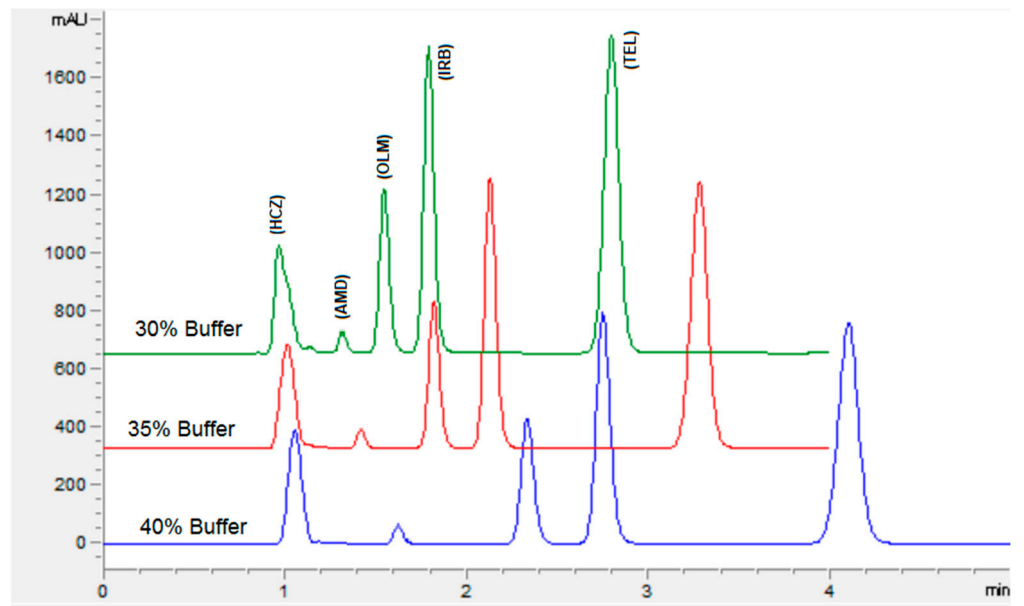
Figure 2. Chromatogram showing the effect of buffer concentration on the separation of analytes. Mobile phase: potassium dihydrogen phosphate (20 mM, pH adjusted to 3.5 via orthophosphoric acid) and acetonitrile and methanol. Flow rate 1.5 mL Min^{-1}, wavelength 230 nm.
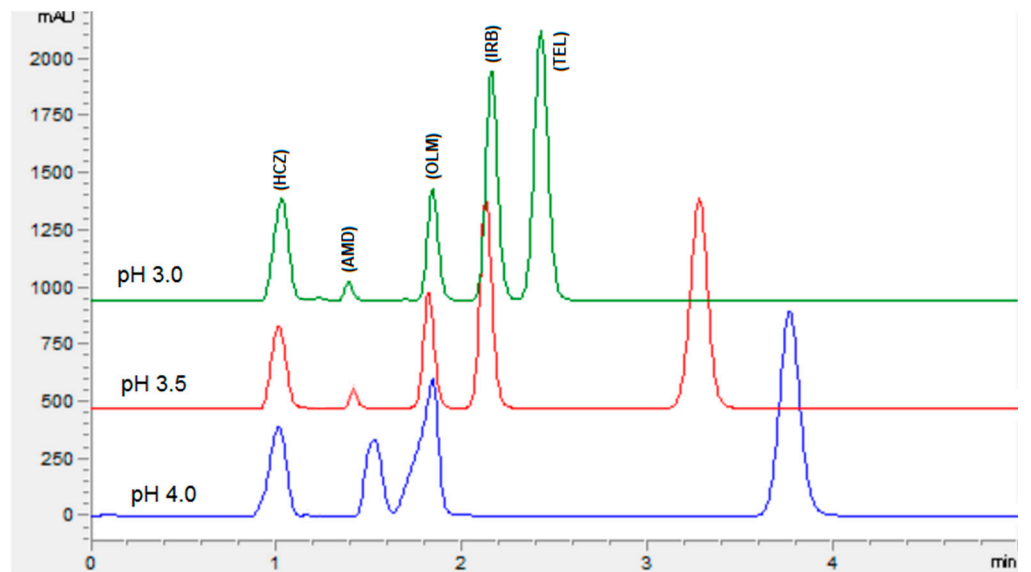

Figure 3. Chromatogram showing the effect of the pH of the mobile phase on the separation of analytes. Mobile phase: potassium dihydrogen phosphate (20 mM, pH adjusted via orthophosphoric acid) and acetonitrile and methanol were at a ratio of 35:45:20% *v/v*. Flow rate 1.5 mL Min^{-1}, wavelength 230 nm.
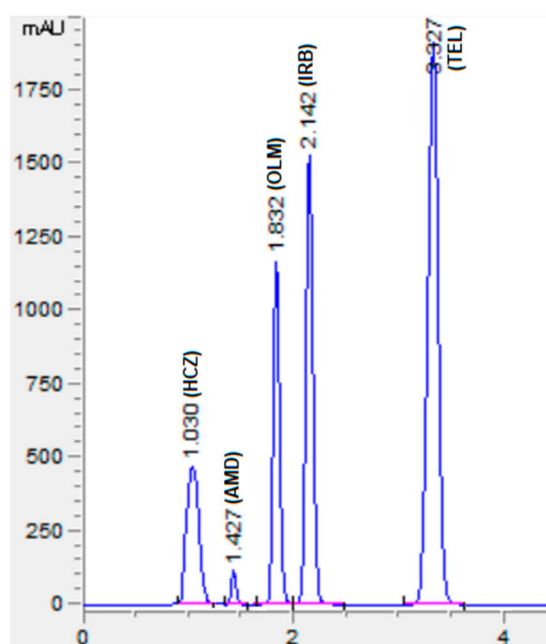
Figure 4. Representative chromatogram of standard solutions of HCZ (25 μg/mL), AMD (20 μg/mL), OLM (100 μg/mL), IRB (300 μg/mL), and TEL (100 μg/mL). Mobile phase: potassium dihydrogen phosphate (20 mM, pH adjusted to 3.5 via orthophosphoric acid) and acetonitrile and methanol were at a ratio of 35:45:20% *v/v*. Flow rate 1.5 mL Min^{-1}, wavelength 230 nm.

3.1. Validation of HPLC Method

3.1.1. System Suitability Studies

The different system suitability parameters such as peak area, retention time, resolution, and peak symmetry were calculated by injecting analytes in triplicate. The results are tabulated in Table 2. All the system suitability parameters were well within the acceptable range. (SM S1)

Table 2. System suitability and regression analysis results.

Parameters	HCZ	AMD	OLM	IRB	TEL
System Suitability Results					
$t_R \pm$ SD	1.01 ± 0.02	1.42 ± 0.021	1.83 ± 0.014	2.14 ± 0.022	3.32 ± 0.012
Peak area ± SD	1162.46 ± 6.27 [a]	92.08 ± 1.16 [b]	515.24 ± 2.34 [c]	4695.45 ± 68.42 [d]	3160.15 ± 57.83 [e]
Resolution ± SD	–	2.43 ± 0.015	2.89 ± 0.012	2.21 ± 0.013	5.56 ± 0.031
Tailing factor ± SD	0.98 ± 0.03	1.08 ± 0.01	1.06 ± 0.01	1.02 ± 0.02	0.99 ± 0.01
Linearity					
Linearity range (μg/mL)	5–30	1–15	5–160	10–300	5–160
Slope	143.41	18.94	30.12	46.56	81.202
Intercept	−628.48	−3.18	−80.49	23.95	−100.2
Regression coefficient (r^2)	0.9993	0.9998	0.9999	0.9999	0.9999
Sensitivity					
LOD (μg/mL)	1.42	0.22	1.34	2.33	1.36
LOQ (μg/mL)	4.31	0.68	4.07	7.06	4.14

t_R: retention time, [a] 12.5 μg/mL, [b] 5 μg/mL, [c] 20 μg/mL, [d] 100 μg/mL, [e] 40 μg/mL.

3.1.2. Linearity

Linearity was established by plotting a graph of the concentrations of the analyte against the corresponding peak areas. The regression equation and regression coefficient (Microsoft Excel 10) were computed from the calibration curve (Table 2). Linearity with a good regression coefficient was observed in the concentration of 2–30 μg/mL for HCZ, 1–15 μg/mL for AMD, 5–160 μg/mL for OLM and TEL, and 10–300 μg/mL for IRB (Figure S1).

3.1.3. Limit of Detection and Quantification

LOD and LOQ were determined by the signal-to-noise ratio and the results are tabulated in Table 2. The low LOD and LOQ values confirm the sensitivity of the method.

3.1.4. Precision

Intraday and interday precision were assessed at three different levels for all analytes. The % RSD for both intraday and interday precision (Table 3) was found to be 1.03 to 1.49 for HCZ, 1.42 to 1.98 for AMD, 1.38 to 1.70 for OLM, 1.22 to 1.30 for TEL, and 0.84 to 1.32 for IRB. The low % RSD confirms the precision of the method. The percent relative error (% RE) was in the acceptable range ($\pm 2\%$) for all analytes, confirming the precision of the optimized HPLC method.

Table 3. Precision results for analytes using the optimized HPLC method.

Drug	Amount of Drug (μg/mL)	Interday			Intraday		
		Amount Found Mean (n = 3) $\pm$ SD	%RSD	%RE	Amount Found Mean (n = 9) $\pm$ SD	%RSD	%RE
AMD	1	0.98 ± 0.01	1.02	−2.00	1.01 ± 0.02	1.98	1.00
	7.5	7.61 ± 0.12	1.58	1.47	7.45 ± 0.14	1.88	−0.67
	15	14.87 ± 0.12	0.81	−0.87	14.75 ± 0.21	1.42	−1.67
HCZ	5	4.97 ± 0.03	1.61	−0.60	5.01 ± 0.07	1.40	0.20
	15	15.02 ± 0.11	0.73	0.13	14.83 ± 0.18	1.21	−1.13
	30	29.68 ± 0.31	1.04	−1.07	30.12 ± 0.31	1.03	0.40
OLM	5	4.94 ± 0.06	1.21	−1.20	5.06 ± 0.07	1.38	1.20
	80	79.42 ± 1.28	1.61	−0.72	79.26 ± 1.35	1.70	−0.92
	160	158.14 ± 1.73	1.09	−1.16	158.54 ± 2.57	1.62	−0.91
IRB	10	9.85 ± 0.12	1.22	−1.50	9.86 ± 0.13	1.32	−1.40
	150	147.89 ± 1.54	1.04	−1.41	148.55 ± 1.25	0.84	−0.97
	300	297.54 ± 3.24	1.09	−0.82	294.87 ± 2.55	0.86	−1.71
TEL	5	5.04 ± 0.1	1.98	0.80	4.93 ± 0.06	1.22	−1.40
	80	79.05 ± 1.22	1.54	−1.19	78.98 ± 0.98	1.24	−1.28
	160	158.27 ± 1.24	0.78	−1.08	157.71 ± 2.05	1.30	−1.43

3.1.5. Accuracy

The standard addition method with the determination of percentage recovery was adopted for the confirmation of the accuracy of the optimized HPLC method. The mean of percent recovery was found to be 99.61% for HCZ, 99.42% for AMD, 98.71% for OLM, 98.83% for TEL, and 99.48% for IRB. Furthermore, the low percent relative error confirmed the accuracy of the method (Table 4).

Table 4. Recovery study results for the accuracy of the optimized HPLC method.

Drug	Amount of Drug (µg/mL)	Amount Found Mean (n = 3) ± SD	%RE	% Recovery
AMD	2.50	2.46 ± 0.04	−1.60	98.40
	5.00	5.04 ± 0.06	0.80	100.80
	7.50	7.43 ± 0.1	−0.93	99.07
		Across Mean		99.42
		% RSD		1.24
HCZ	5.00	4.96 ± 0.04	−0.80	99.20
	10.00	10.05 ± 0.09	0.50	100.50
	15.00	14.87 ± 0.26	−0.87	99.13
		Across Mean		99.61
		% RSD		0.77
OLM	10.00	9.88 ± 0.03	−1.20	98.80
	20.00	19.79 ± 0.15	−1.05	98.95
	30.00	29.51 ± 0.35	−1.63	98.37
		Across Mean		98.71
		% RSD		0.30
IRB	50.00	50.68 ± 0.18	1.36	101.36
	100.00	98.39 ± 1.09	−1.61	98.39
	150.00	148.05 ± 2.48	−1.30	98.70
		Across Mean		99.48
		% RSD		1.63
TEL	20.00	19.81 ± 0.13	−0.95	99.05
	40.00	39.59 ± 0.34	−1.02	98.98
	60.00	59.08 ± 0.62	−1.53	98.47
		Across Mean		98.83
		% RSD		0.32

3.1.6. Robustness

The robustness of the analytical procedure was assessed to identify the effect of variations in the experimental settings of the analysis. The univariate method was based on changing one factor at a time; it does not provide information about the combined effect of different independent variables. Hence, in accordance with the Plackett-Burman design, a factorial design was selected. The Pareto chart in Figure 5 illustrates the individual and combined effects of independent variables on the response. The effects of variables above the Bonferroni limit were highly significant, whereas the effects above the *t*-value limit were significant. The 13 runs carried out as per the software suggestion, in random order, showed that the buffer volume and flow rate had a significant effect on the peak area of HCZ and TEL, respectively. However, the combined variation of all these factors and pH × buffer volume had a highly significant effect on the peak area of OLM and IRB, respectively. In addition, the combined effect of flow rate × pH and flow rate × buffer volume had a significant effect on the peak area of TEL and OLM, respectively. Hence, these variables need to be controlled carefully during the analysis. Furthermore, a slight variation in individual factors did not affect the peak area of AMD, OLM, and IRB.
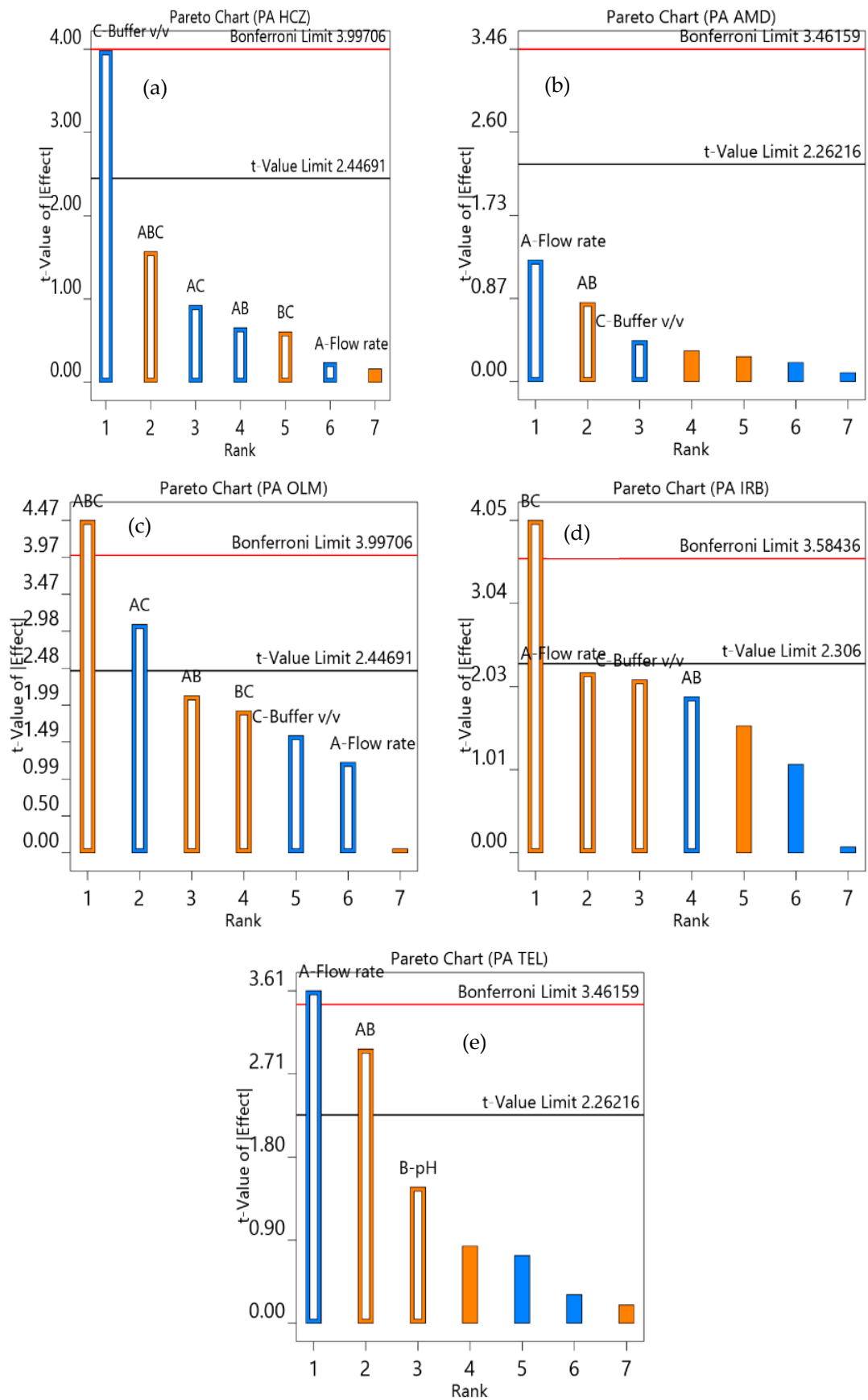
Figure 5. Pareto chart showing the effect of flow rate (A); pH (B); and percentage of buffer (C) on the peak area (PA) of HCZ (**a**), AMD (**b**), OLM (**c**), IRB (**d**), and TEL (**e**).

The results of deliberate changes in the experimental condition for the robustness determination included wavelength (230 ± 2 nm), injection volume (20 ± 2 μL), and pH (3.5 ± 0.2). The percentage RSD of the peak area of the analytes was found to be in the range of 0.67–1.73%, 0.78–1.16%, 1.23–1.58%, 1.55–1.81%, and 1.23–1.56% for HCZ, AMD, OLM, IRB, and TEL, respectively. In all the cases, the percentage RSD was less than 2%, indicating the robustness of the optimized HPLC method (Table 5).

Table 5. Robustness results of optimized HPLC method.

Chromatographic Condition		Peak Area				
		HCZ	**AMD**	**OLM**	**IRB**	**TEL**
Mobile phase pH	3.3 (Low)	1147.92	92.88	2385.09	6869.23	6389.15
	3.5 (Normal)	1162.46	91.86	2362.85	7070.25	6446.99
	3.7 (High)	1159.68	91.49	2315.24	7103.48	6586.2
	Across mean	1156.69	92.08	2354.39	7014.32	6474.11
	% RSD	0.67	0.78	1.52	1.81	1.56
Injection volume (μL)	18 (Low)	1177.92	93.48	2318.27	6922.3	6293.98
	20 (Normal)	1162.46	91.86	2362.85	7070.25	6446.99
	22 (High)	1189.68	91.58	2392.45	7134.95	6401.32
	Across mean	1176.69	92.31	2357.86	7042.50	6380.76
	% RSD	1.16	1.11	1.58	1.55	1.23
Wavelength (nm)	228 (Low)	1191.21	94.01	2328.34	6934.12	6318.75
	230 (Normal)	1162.46	91.86	2362.85	7070.25	6446.99
	232 (High)	1152.26	92.82	2386.19	7189.24	6503.42
	Across mean	1168.64	92.90	2359.13	7064.54	6423.05
	% RSD	1.73	1.16	1.23	1.81	1.47

3.2. Application to Formulations and Comparison with Reported Methods

The optimized HPLC method was applied for concurrent quantification of HCZ, AMD, OLM, IRB, and TEL in binary and ternary combination formulations (Figure 6). The assay results (Table 6) were in agreement with the label claim, and no interfering peaks were witnessed at the retention time of analytes (Figure 6 and S2). The percentage assay was found to be in the range of 98% to 102% for all the analytes (HCZ, AMD, OLM, IRB, and TEL). Furthermore, the present method was compared with the reported methods [27,28]. Student's *t*-test and *F* test were performed to compare the assay results. The calculated *t* and *F* values were found to be less than the critical values (Table 7), which indicated no significant difference in the assay outcomes between the present and reported methods.

Table 6. Analysis results of drugs from formulations using the optimized HPLC method.

Pharmaceutical Preparation	Drug	Label Claim (mg/Tab)	Amount Found (mg)	% Label Claim	% RSD
Amlokind-H	AMD	5	4.97	99.40	0.60
	HCZ	12.5	12.31	98.48	1.62
Lisart Plus (40 + 12.5)	TEL	40	39.78	99.45	1.03
	HCZ	12.5	12.52	100.16	0.88
Lisart Plus (80 + 12.5)	TEL	80	79.59	99.49	1.65
	HCZ	12.5	12.53	100.24	1.92
Cresar plus	TEL	40	40.32	100.80	1.54
	HCZ	12.5	12.46	99.68	1.04
	AMD	5	4.96	99.20	1.81

Table 6. *Cont.*

Pharmaceutical Preparation	Drug	Label Claim (mg/Tab)	Amount Found (mg)	% Label Claim	% RSD
Olsar Plus (40/12.5)	OLM	40	39.78	99.45	1.46
	HCZ	12.5	12.48	99.84	1.36
Olsar Plus (20/12.5)	OLM	20	20.03	100.15	0.80
	HCZ	12.5	12.35	99.80	1.54
Sevikar HCZ (20/12.5/5)	OLM	20	19.63	98.15	1.68
	HCZ	12.5	12.38	99.04	1.13
	AMD	5	5.02	100.40	0.80
Arena Plus (150/12.5)	IRB	150	148.54	99.03	1.73
	HCZ	12.5	12.35	98.80	1.70
Arena Plus (300/25)	IRB	300	296.65	98.88	1.36
	HCZ	25	12.40	99.20	1.77
Aprovasc (300/5)	IRB	300	294.79	98.26	1.30
	AMD	5	4.93	98.60	1.62
Aprovasc (150/10)	IRB	150	151.89	101.26	1.29
	AMD	10	9.87	98.7	1.52

Table 7. Assay and statistical comparison of the results of the optimized HPLC method with the reported methods.

Drug	Method	Mean (% Assay)	% RSD	*n*	Student's *t*-test (2.228) [a]	*F* test (5.050) [b]
AMD	Present method	99.20	1.81	6	0.758	1.276
	Ref method [28]	99.98	0.97			
HCZ	Present method	99.68	1.04	6	0..037	1.395
	Ref method [28]	100.27	1.46			
TEL	Present method	100.80	1.54	6	0.710	1.128
	Ref method [27]	98.82	1.39			
OLM	Present method	99.45	1.46	6	0.282	1.099
	Ref method [28]	99.58	0.86			
IRB	Present method	99.03	1.73	6	0.129	1.241
	Ref method [27]	100.87	1.29			

[a] critical value of Student's *t*-test and [b] critical value of *F* test at *p* = 0.05 [27]. Agilent C18 Zorbax column (250 mm × 4.6), 35% acetonitrile: 65% Phosphate buffer (10 mM, pH 6), 1 mL/min, 225 nm [28]: MEKC method, 55-cm capillary tube with background electrolyte (40 mM phosphate buffer, 20 mM sodium dodecyl sulphate (pH 6.0), and acetonitrile (90%:10% *v/v*), 220 nm.
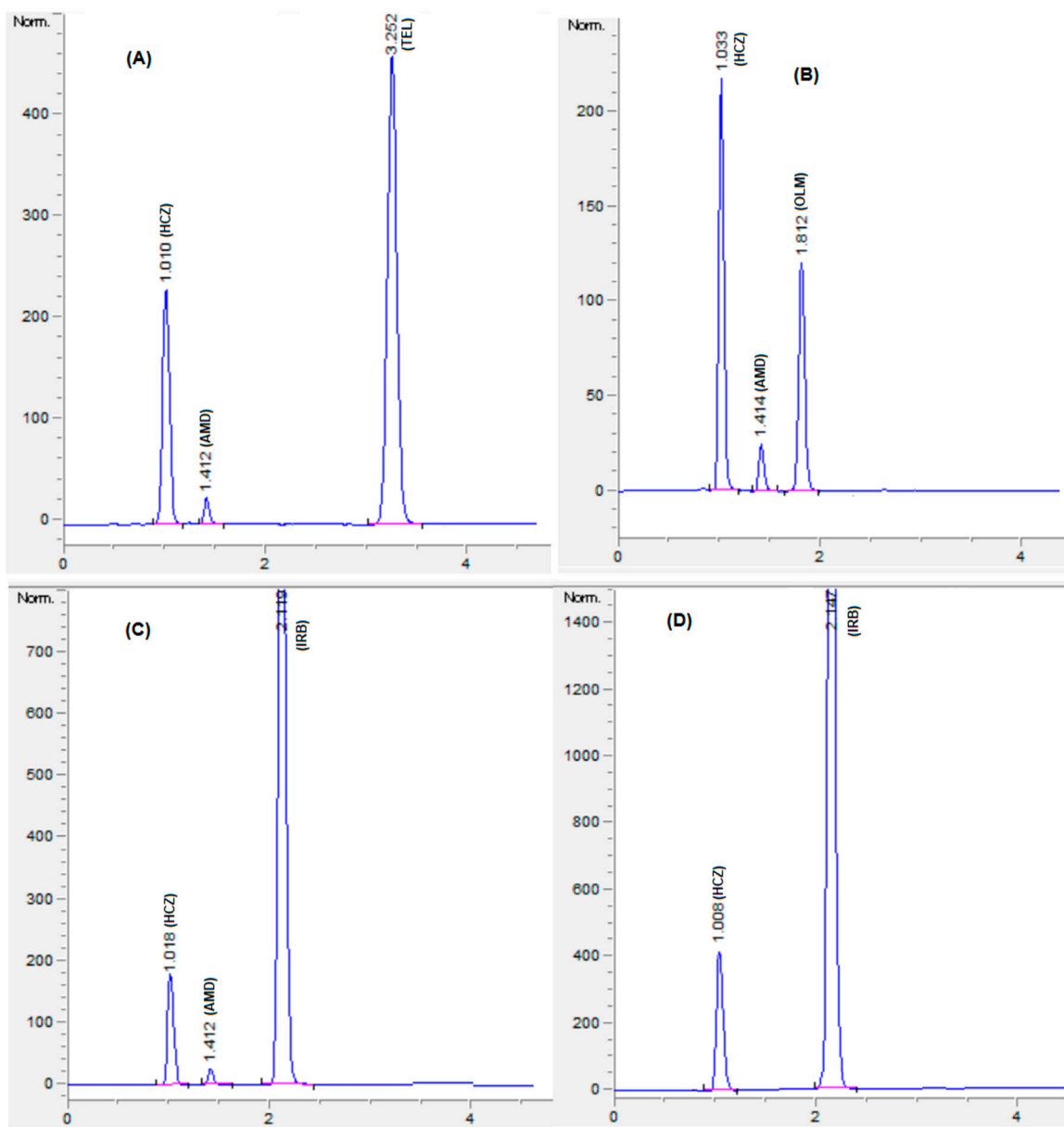
Figure 6. Chromatograms of formulations, HCZ:AMD:TEL:12.5:5:40 mg (**A**); HCZ:AMD:OLM:12.5:5:20 mg (**B**); HCZ:AMD:IRB:12.5:5:150 mg (**C**); HCZ:IRB:25:300 mg (**D**). Mobile phase: potassium dihydrogen phosphate (20 mM, pH 3.5 adjusted by orthophosphoric acid) and acetonitrile and methanol were at a ratio of 35:45:20% v/v. Flow rate 1.5 mL Min^{-1}, wavelength 230 nm.

4. Conclusions

A simple HPLC procedure was established for the concurrent determination of HCZ, AMD, OLM, IRB, and TEL from binary and ternary formulations, using similar experimental conditions. The developed HPLC method is economical and timesaving for analyzing several binary and ternary mixture formulations because it uses the same stationary and mobile phase. The formulation excipients did not interfere with the analysis and the method was rapid (<3.5 min). The robustness study showed the effect of different variable parameters on the analysis. A statistical comparison of the present method with the reported method showed no significant difference in the assay results.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/separations8060086/s1, Figure S1: Calibration curves of analytes from the optimized HPLC method. Figure S2: Chromatograms of formulations; HCZ AMD:12.5:5 μg/mL (A); HCZ:TEL:12.5:40 μg/mL (B); HCZ:TEL: 12.5:80 μg/mL (C); HCZ:AMD:TEL:12.5:5:40 μg/mL (D); HCZ:IRB:12.5:150 μg/mL (E); HCZ:IRB:12.5:300 μg/mL (F); AMD:IRB:10:150 μg/mL (G); HCZ:AMD:OLM:12.5:5:20 μg/mL (H); HCZ:TEL:12.5:40 μg/mL (I). Figure S3: UV absorption spectra of AMD, HCZ, and TEL. SM S1: Equations used for calculations and preparation of standard solutions for validation.

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