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ORIGINAL STUDY

Green Flow-gradient Micellar High-performance Liquid Chromatograpic Method for Determination of a Hypertensive Combination; Azilsartan Medoxomil and Chlorthalidone: Application to Spiked Human Plasma and Urine

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Abstract

A green novel cost-effective flow-gradient micellar high-performance liquid chromatographic method was developed and validated for the simultaneous determination of two antihypertensive drugs, Azilsartan Medoxomil and chlorthalidone in plasma and urine. The eco-friendly method showed good linearity over the ranges of 15.00−80.00 μg/ml and 12.50−62.50 μg/ml with limits of detection of 0.19, 0.12 μg/ml and limits of quantification of 0.59, 0.36 μg/ml for Azilsartan Medoxomil and chlorthalidone, respectively. The proposed method was successfully applied for the simultaneous analysis of the studied drugs in their single tablets, laboratory mixed tablets in human plasma, and urine without prior extraction procedure. The method was verified on the Analytical Greenness metric approach (AGREE) and it was found to be an excellent green method.

Keywords: Azilsartan medoxomil, Chlorthalidone, Flow-gradient, Green micellar method, Micellar liquid chromatography, Validation

1. Introduction

Green chemistry is going towards eco-friendly safe methods that avoid chemicals having a harmful impact on the environment. One of the green chemistry branches is Micellar liquid chromatography (MLC). MLC technique has many advantages over other chromatographic techniques having small amount of organic modifiers used. MLC is a versatile well-known branch of high-performance liquid chromatography (HPLC) that has been known to be an attractive alternative to conventional reversed phase chromatography in which the mobile phases are aqueous solutions of a surfactant at a concentration above critical micelle concentration. Moreover, it combines the advantages of micellar media with the separation capability of liquid chromatography (Esteve-Romero et al., 2005). MLC has been extensively used for multiple compound determination in pharmaceutical formulations, biological samples, and environmental samples. MLC has a major advantage over other techniques, which is direct on-column injection of physiological fluids with unique separation selectivity.

Gradient elution is the steady changes in the mobile phase condition during the chromatographic run. When the analytes are of different polarities, they will retain differently on the column being either poorly retained or strongly retained. Those early eluting bands who will elute near the void volume will suffer from poor resolution. On the other hand, some late-eluting peaks experience too

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long separation times. Hydrophobic analytes will show very long retention times, being very broad with decreased sensitivity. The leading objective of gradient elution is to move strongly retained components of the mixture faster while having the least retained component well-resolved (Schellinger and Carr, 2006).

Flow gradient elution which is an increase in flow rate during separation, is used especially for late peaks that are too broad. The ability to use this approach is attributed to pressure tolerance of the system (Scott and Lawrence, 1969; Snyder, 1970; Aitzetmüller, 1990). A significant increase in front-end resolution from flow programming (relative to normal elution) is noticed along with a corresponding lowering of back-end resolution. It is worth mentioning that the previously mentioned nonisocratic techniques rise the ability of liquid chromatography to handle difficult samples and to cope with unusual situations or requirements.

Azilsartan medoxomil (AZL) (5-methyl-2-oxo-1,3-dioxol-4-yl) methyl 2-ethoxy-3-[[4-[2-(5-oxo-4*H*-1,2,4-oxadiazol-3 yl) phenyl] phenyl] methyl] benzimidazole-4-carboxylate, (Fig. S1a (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1)) is an anti-hypertensive drug used in the treatment of hypertension (Martindale, 2014). It is an angiotensin II receptor antagonist. It selectively blocks the binding of angiotensin II to the AT_1 receptors in the vascular smooth muscle and the adrenal gland promoting vasodilation and a decrease in the effects of aldosterone (Cheng, 2013).

Chlorthalidone (CLT) (Fig. S1b (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1)) 2-chloro-5-(1-hydroxy-3-oxo-2H-isoindol-1-yl) benzenesulfonamide is a diuretic drug belonging to thiazide diuretics, used in the treatment of hypertension. It reduces hypertension directly by its effect on the distal convoluted tubule of the nephron leading to a decrease in extracellular fluid volume, plasma volume, and total exchangeable sodium (Cheng, 2013).

Determination of a mixture of AZL and CLT has been described previously using derivative ultraviolet (UV)-spectrophotometric technique in bulk and pharmaceutical preparations (Ebeid et al., 2014). Different chromatographic techniques have been extensively described for the simultaneous determination of AZL and CLT in different matrices using different columns, elution systems, and detectors (Sravani et al., 2014; Ramakrishna et al., 2015; Sohni et al., 2016; Lavanya et al., 2017; Samanthula et al., 2018; Kumar et al., 2019; Jahangir et al., 2023).

The developed micellar HPLC method has the advantage over the previously reported HPLC methods of low cost and low toxicity of the mobile phase due to the low percentage of organic solvent used (only 12% n-propanol). Moreover, the solubilizing ability of micelles permits the direct injection of biological fluids eluting protein in the solvent front. To the best of our knowledge, nothing has been reported for micellar determination of both drugs. This motivated us to establish and validate a robust gradient direct injection micellar liquid chromatographic determination method for the simultaneous determination of AZL and CLT. Different chromatographic parameters were investigated to select the optimum conditions for the separation. It was also aimed to assess the ability of the proposed direct injection MLC method to determine AZL and CLT in commercial tablets, human plasma, and human urine.

2. Experimental

2.1. Apparatus

Gilson 1260 series HPLC instrument consisting of (a quaternary pump, auto sampler, vacuum degaser, diode array, and UV-VIS detector model (156) with auto-sampler injector) was used for chromatographic separation. Data acquisition was done using Unipoint software. The mobile phase was filtered with membrane filters (Millipore, Ireland) through Charles Austen pumps Ltd filter, and degassed with a vacuum membrane degasser built in the accella pump. The pH was measured with a Jenway pH meter, 3510, (Essex-UK). The ultrasonic bath used was Falc, (Treviglio-Italy).

2.2. Materials and reagents

All the chemicals used were of Analytical Reagent grade, and the solvents were of HPLC grade. High-purity water was obtained by Elga lab water, prima 7 (UK) and it was used throughout the study.

(a) Methanol, ethanol, n-propanol, 2-propanol, Acetonitrile (ACN) (HPLC grade), orthophosphoric acid (85% w/v), and sodium hydroxide were obtained from Sigma - Aldrich (Germany).
(b) Triethylamine (TEA) and sodium dodecyl sulphate (SDS, 99%) were obtained from Riedel-de Häen (Sleeze, Germany).
(c) Fresh human plasma samples were obtained from Vacsera (Giza, Egypt) and were kept frozen until use after gentle thawing, and urine sample was taken from a healthy person.

(d) Azilsartan (99.5%), was kindly supplied by EDA-CADAC (Cairo, Egypt). CLT hydrochloride (99.3%), was kindly supplied from Laboratories Limited (Giza, Egypt), and pharmaceutical preparations containing the drugs were purchased from the pharmacy.
(e) Edarby tablets (Takeda Pharmaceuticals America, USA), were labeled to contain 40 mg AZL/tablet, and Hygroton tablets (Novartis, USA) were labeled to contain 25 mg CLT/tablet.

2.3. Chromatographic conditions

An intersil BDS column (150 mm × 4.6 mm I.D., particle size 5 μm) (NACALAI TESQUE, Japan) was used. The chromatographic conditions was optimized and a mobile phase consisting of 0.12 M SDS, 12% n-propanol, 0.3% TEA, prepared in 0.02 M orthophosphoric acid for determining the cited drugs. The pH of the mobile phase was adjusted to pH 7.0 using sodium hydroxide. The mobile phase was filtered through a 0.2 μm Millipore micro filter and sonicated for a few mins before use. The initial flow rate was 0.90 ml/min. At 3.50 min, a 1.00 min flow gradient ramp was executed to a final flow rate of 1.60 ml/min. The column was operated at room temperature and the wavelength was monitored at 260 nm.

Additionally, the system was flushed with water, followed by a 20 min purge in a solution of water and MeOH (1:1) in order to remove the adsorbed surfactants from the stationary phase.

2.4. Standard solutions

Standard methanolic stock solutions of 400.00 μg/ml AZL and 250 μg/ml CLT, were prepared separately in 50 ml volumetric flasks. Then the solutions were sonicated with the aid of an ultrasonic bath. Working standard solutions were prepared by appropriate dilution of the stock solutions with the mobile phase to reach a concentration range of 15.00–80.00 and 12.50–62.50 μg/ml for AZL and CLT, respectively.

The solutions were sonicated for 5 min and filtered through a disposable syringe filter (0.45 μm) before column injection. 50 μL aliquots of each standard solution were injected in triplicate and eluted with the mobile phase under the previously described chromatographic conditions. The average peak areas of AZL and CLT were plotted versus the corresponding concentrations in μg/ml to obtain the calibration graphs. Alternatively, the corresponding regression equations were derived.

2.5. Procedures

2.5.1. Construction of the calibration graphs of pure bulk powder

Calibration standard samples for the two drugs in bulk powder were prepared by transferring different aliquots of the stock solutions to different volumetric flasks and completing the mark with the mobile phase, resulting in calibration standard solutions with concentrations of 15.00–80.00 and 12.50–62.50 μg/ml for AZL and CLT, respectively.

The solutions were sonicated for 5 min and the procedure described under 'Standard solutions' was then applied.

2.5.2. Construction of the calibration graphs of spiked human plasma and urine

New calibration graphs were constructed using spiked biological fluids. Aliquots of AZL or CLT working standard solution were transferred into a series of 10 ml volumetric flasks. The final concentration is in the range of 20.00–50.00 μg/ml and 12.50–35.00 μg/ml for AZL and CLT, respectively, in spiked plasma and 20.00–75.00 μg/ml and 15.00–50.00 μg/ml for AZL and CLT, respectively in spiked urine. The contents of the flasks were diluted to about 8 ml with the mobile phase (pH 7), to avoid precipitation of plasma proteins with methanol. 1 ml of human plasma or human urine was added to each flask, and the volumes were completed to the mark with the mobile phase and mixed well. The solutions were then filtered through a 0.45 μm membrane filter and directly injected. A blank experiment was carried out simultaneously. The experiment was carried out in triplicate. Each solution was injected in triplicate. The procedure described under 'Standard solutions' was then applied. The average peak areas of AZL and CLT were plotted versus the corresponding concentrations in μg/ml to obtain the calibration graphs. Alternatively, the corresponding regression equations were derived.

2.6. Applications

2.6.1. AZL and CLT in binary mixtures assay

Different aliquots of stock solutions of AZL and CLT standard solutions were transferred to a series of 10 ml volumetric flasks in their pharmaceutical ratio. The solutions were diluted to the mark with the mobile phase and mixed well to cover the concentration range of 15.00–80.00 and 12.50–62.50 μg/ml for AZL and CLT, respectively.

2.7. Analysis of AZL and CLT in their tablets

2.7.1. AZL and CLT in their single tablets

Ten tablets of Edarby tablet and Hygroton tablet were weighed separately and the average weight for each product was determined, homogenized in a mortar, then an accurately weighed amount of each powder corresponding to 20.00 mg AZL and 12.50 mg CLT declared active principle was transferred into 50 ml volumetric flasks. About 30 ml of methanol was added followed by sonication in an ultrasonic bath for 30 min. Afterwards, the solutions were completed to the volume with methanol, mixed well, and filtered using a disposable syringe filter (0.45 μm). Aliquots of the filtrates were transferred into 10 ml volumetric flasks, diluted to volume with the mobile phase, and mixed well.

2.7.2. AZL and CLT in their laboratory mixed tablets

Ten tablets of Edarby tablet and Hygroton tablet were weighed separately and the average weight for each product was determined, homogenized in a mortar, then an accurately weighed amount of the powder corresponding to 20.00 mg AZL and 12.50 mg CLT declared active principle were transferred into 50 ml volumetric flasks.

2.7.3. Analysis of AZL and CLT in spiked human plasma and urine

Different Aliquots of AZL or CLT standard solution were transferred into a series of 10 ml volumetric flasks to cover the concentration range of 20.00–50.00 μg/ml and 12.50–35.00 μg/ml for AZL and CLT, respectively, in spiked plasma and 20.00–75.00 μg/ml and 15.00–50.00 μg/ml for AZL and CLT, respectively, in spiked urine. The contents of the flasks were diluted to about 8 ml with the mobile phase (pH 7), to avoid precipitation of plasma proteins with methanol. 1 ml of human plasma or human urine was added to each flask, and the volumes were completed to the mark with the mobile phase and mixed well.

The procedure described under 'Standard solutions' was then applied. The nominal contents of the antihypertensive drugs in their binary mixture, single tablets, laboratory mixed tablets, and plasma and urine were calculated using the corresponding regression equations.

3. Results and discussions

The proposed MLC method represents a novel, rapid, direct-injection technique for the simultaneous determination of the newly prescribed antihypertensive drug combination, AZL and CLT via gradient elution. The two drugs were determined with good resolution in their pharmaceutical dosage forms (Edarby tablet, 40 mg AZL/tablet) and (Hygroton tablet, 25 mg CLT/tablet), in spiked human plasma and urine with no need for tedious sample pre-treatment steps.

Starting with the low condition; less flow rate, gradient elution will permit the separation of the least retained components. Strongly retained components will stay on the adsorbent surface on the top of the column moving very slowly (Snyder, 1970). Then, after increasing the conditions, the strongly retained analytes will move faster and faster and this is attributed to the steady increase of the competition for the adsorption sites. The initial gradient conditions are adjusted to retain and resolve those peaks eluted early (such as our case with CLT) or with the solvent peak. Then the elution strength is increased to elute the analytes which elute late (AZL) with optimum conditions. The final condition should guarantee all the analytes elution within a reasonable time.

The experimental parameters influencing the chromatograms of the studied drugs were accurately considered and optimized. The parameters giving the highest number of theoretical plates and the best peak shape were selected. Figure 1 shows a typical chromatogram for CLT and AZL under the described chromatographic conditions in pure forms at 260 nm. The separation was performed within short retention time Rt = 2.96 and 6.41 min for CLT and AZL in pure form, Rt = 2.90 and 6.37 min for CLT and AZL in spiked urine, and Rt = 2.90 and 7.06 min for CLT and AZL in spiked plasma, respectively.

3.1. Chromatographic performance optimization

We carried out careful optimization of the gradient elution system to develop the proposed separation method with the best resolution in a reasonable time. During this optimization, we considered that a suitable compromise between resolution, peak shape, and analysis time should be reached. Well-defined symmetrical peaks were developed to obtain the perfect parameters such as type of column, detection wavelength, type and concentration of the selected organic modifier, concentration of SDS, and pH of the mobile phase. The results of the optimization study can be presented as follows:

3.1.1. Choice of appropriate detection wavelength

For selection of the optimum detection wavelength, the studied drugs absorption spectra were

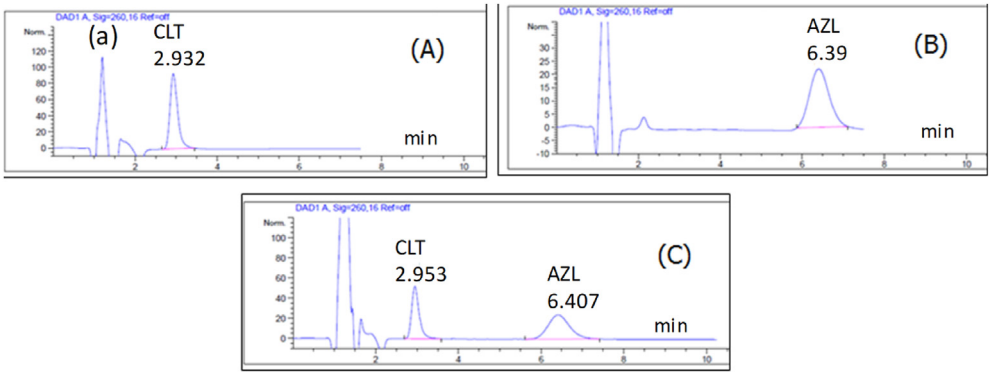


Fig. 1. Typical chromatograms using the proposed Micellar liquid chromatography method at 260 nm where; (A) (a) solvent front, 40.00 µg/ml chlorthalidone; (B) 40.00 µg/ml Azilsartan Medoxomil and (C) binary mixture of 25 µg/ml chlorthalidone and 40 µg/ml Azilsartan Medoxomil mixture.

scanned using UV spectrophotometer. It was found that CLT and AZL exhibit their maximum absorption at 242 and 266 nm, respectively. The most suitable wavelength for the determination of both drugs in the mobile phase was found to be 260 nm at which the sensitivity of the method was very high, and separation occurs with a good response. The absorption spectrum of the two antihypertensive drugs in the mobile phase is shown in Fig. S2 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1).

3.1.2. Choice of column

To investigate the chromatographic performance, different columns were tried. These include RP C_8, RP C_{18}, spherisorb cyano, and bonded phase phenyl columns. Experimental trials revealed that the intersil ODS column, waters Corporation, Ireland, was the most suitable one giving narrower symmetric peaks and the highest number of theoretical plates allowing the best separation of the two drugs within a reasonable analysis time as shown in Fig. 1.

3.1.3. Mobile phase composition

The mobile phase consisted of TEA, a quaternary amine compound, which is used in MLC as a mobile phase additive in a concentration of 0.3% to cause a reduction peak tailing (Rizk et al., 2014a; 2014b), decrease in retention factor and an increase in peak symmetry and efficiency.

To achieve the best chromatographic conditions, the mobile phase composition was optimized to provide high selectivity and sensitivity in a short analysis time.

The studied variables included; the pH of the mobile phase, concentration of SDS and concentration of the organic modifier. The results of the optimization study are presented in Table 1.

3.1.4. Type of organic modifier

Different organic modifiers were tried to select the most suitable one for good chromatographic separation of both drugs under study. Methanol, absolute ethanol, ACN, n-propanol and 2-propanol were the organic modifiers tried. It was found that; using methanol, ACN, and 2- propanol made the separation takes longer time and produced broad peaks. Also, methanol and ACN showed low sensitivity for AZL and very low sensitivity for CLT. Absolute ethanol showed higher retention time with split peaks and low sensitivity for both drugs. Well-resolved and highly sensitive peaks within less retention time were obtained using n-propanol. Therefore, n-propanol was the organic modifier of choice giving good resolved and highly sensitive peaks within a reasonable time (less than 7.50 min).

3.1.5. Concentration of organic modifier

To study the influence of the concentration of n-propanol on the selectivity and retention times of CLT and AZL, it was varied over the range of 6.00–14.00%. As expected, the retention time of the studied drugs decreases as percentage of organic modifier increases. A concentration of 12.00% of n-propanol was chosen as the optimal concentration, regarding the resolution of the two drugs and the number of theoretical plates. Concentrations less than 6% resulted in a broad, less sensitive peaks and it was time consuming, whereas concentrations higher than 12% decreased number of theoretical plates for both drugs. Zero % propanol was tried and no peak appeared till 20 min.

3.1.6. Concentration of SDS

The effect of changing the concentration of SDS on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 0.05–0.15 M SDS at a

Table 1. Optimization of the chromatographic conditions for Azilsartan Medoxomil and chlorthalidone mixture using the proposed Micellar liquid chromatography method.

Parameters		Number of theoretical plates (N)		Retention Factor		Retention Time		Resolution (Rs)	Selectivity factor (α)	Tailing Factor	
		AZL	CLT	AZL	CLT	AZL	CLT			AZL	CLT
% concentration of organic modifier	6	265	772	9.09	2.19	18.58	5.90	10.38	4.15	0.981	1.336
	8	246	917	7.14	1.81	14.57	5.05	9.54	3.94	0.945	1.329
	10	248	661	5.76	1.43	12.5	4.5	9.07	4.03	0.95	1.8
	12	317	889	3.52	1.01	8.32	3.7	6.94	3.49	1.07	1.559
	14	282	1233	2.81	0.86	7.23	3.546	5.90	3.27	1.058	1.539
Concentration of SDS (M)	0.05	381	970	5.72	1.47	12.10	4.45	9.55	3.89	1.096	1.75
	0.1	317	889	3.52	1.01	8.32	3.7	6.94	3.49	1.07	1.559
	0.12	930	2100	3.27	0.92	8.12	3.65	6.27	3.55	0.96	1.524
	0.15	239	1032	3.27	0.85	6.78	3.518	5.2	3.85	1.032	1.979
pH of the mobile phase	4	307	965	4.03	1.11	8.80	3.69	12.10	3.63	1.07	1.5
	5	411	1231	4.84	1.06	10.33	3.64	11.11	4.57	1.02	1.35
	6	296	1208	4.10	0.91	9.69	3.63	8.47	4.51	0.98	1.51
	7	1051	1033	2.64	1.15	6.925	4.088	7.52	2.30	1.7	1.28
	8	857	1050	2.62	1.02	6.884	3.84	7.14	2.57	1.81	1.21
Flow rate (mL/min)	0.8	820	1055	2.38	0.99	7.45	4.385	10.75	2.40	1.99	1.34
	1	1051	1033	2.64	1.15	6.925	4.088	7.52	2.30	1.7	1.28
	1.2	1135	2343	2.63	0.41	6.171	2.39	7.29	6.41	1.58	0.94
	gradient	817	1424	3.57	1.07	6.4	2.9	9.54	3.34	0.83	0.74

Where: Selectivity Factor(α)=k_2/k_1. Retention Factor= (tr-t)/t. Resolution (Rs)=$2\Delta t/(w_1+w_2)$.

constant 12.00% concentration of n-propanol. 0.07 M SDS mobile phase resulted in too broad a peak. The retention time of the drugs decreased as the molar concentration of SDS increased. The study revealed that the optimum chromatographic performance was attained when using 0.12 M SDS regarding the resolution of the two drugs and the number of theoretical plates. Concentrations less than 0.05 M SDS resulted in a high increase in the retention time, while concentrations higher than 0.12 M SDS decreased number of theoretical plates.

3.1.7. pH of the mobile phase

To select the optimum pH value for the analysis of CLT and AZL, the pH of the mobile phase was studied over the range of 4.00–8.00 taking into account the drug nature (neutral or ionic) and drug pka values. The trials were made at different pH values with 0.12 M SDS and 12% n-propanol, to achieve the best resolution conditions. It was found that the retention times of CLT were not greatly affected by the change in pH. Decreasing the pH of the mobile phase below 7.00 resulted in an increase in the retention time of AZL accompanied by a decrease in a number of theoretical plates. Thus, pH 7.00 was selected as the optimum pH value for the mobile phase in this study which yields the highest number of theoretical plates, and lower retention time with the best resolution conditions.

3.1.8. Flow rate of the mobile phase

The effect of the flow rate of the mobile phase on the retention of CLT and AZL was investigated over the range of 0.80–2.00 ml/min. Flow rate 1.30 ml/min and above causes a complete overlap of CLT with the solvent peak due to its rapid elution. With a Flow rate below 1.30 ml/min, AZL was very broad and suffered a high tailing factor.

However, upon the application of an isocratic elution for 3.50 min, it was successful in increasing the retention time of CLT being separated from the solvent peak (Fig. 2) followed by a 3.00 min gradient elution, AZL eluted at 6.39 ± 0.10 min with better peak shape within a reasonable time.

After optimization of these variables, we use a mobile phase consisting of 0.12 M SDS, 12% n-propanol, 0.3% TEA, prepared in 0.02 M orthophosphoric acid for determining the cited drugs at 260 nm. The pH of the mobile phase was adjusted to pH 7.0 with an initial flow rate 0.90 ml/min. At 3.50 min, a 1.00 min flow gradient ramp was executed to a final flow rate of 1.60 ml/min. The column was operated at room temperature. These conditions provide the best peak shape and

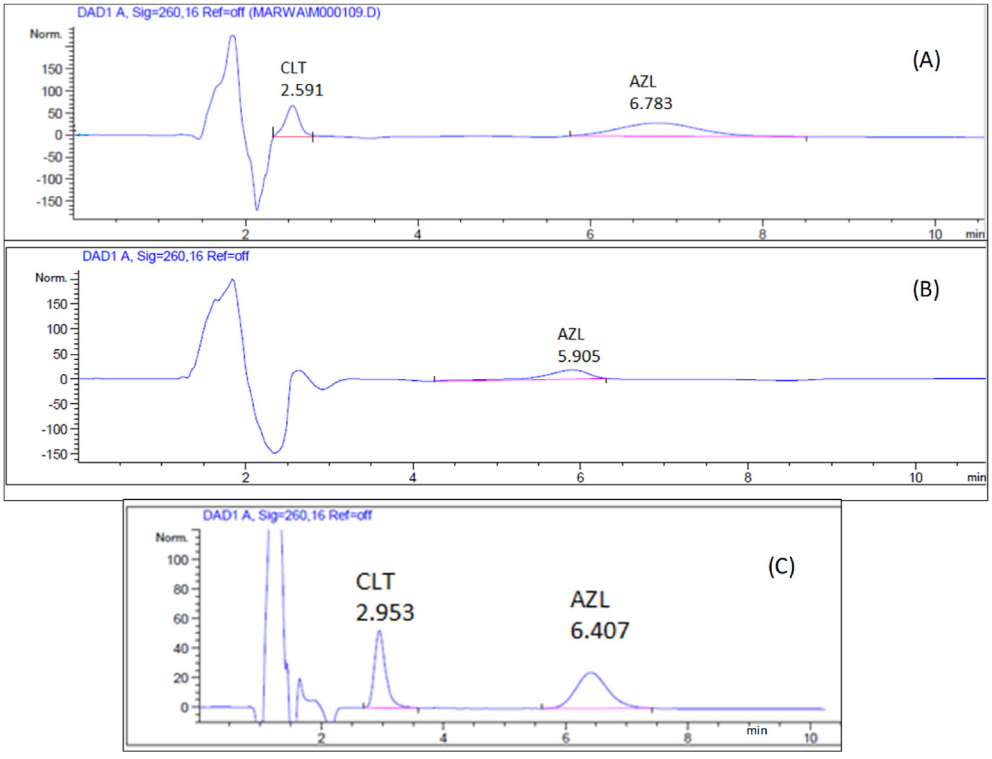


Fig. 2. Chromatogram of 25 µg/ml chlorthalidone and 40 µg/ml Azilsartan Medoxomil using 0.12 M sodium dodecyl sulphate, 12% n-propanol, 0.3% Triethanolamine, prepared in 0.02 M orthophosphoric acid (pH 7.00) using (A) isocratic elution with flow rate 1.00 ml/min, (B) isocratic elution with flow rate 1 and (C) Flow-Gradient elution.

lowest peak tailing with well-defined peaks and good sensitivity within a reasonable analytical run time.

Certain care should be performed to prolong the age of the stationary phase. It should be regularly cleaned with water and then for 15 min with a mixture of water: MeOH (1:1) mixture. This is essential to prevent surfactant precipitation and protect the column against salt crystallization. The stationary phase is then regenerated by washing with 100% methanol to remove the surfactant adsorbed.

Figure 2c represents a chromatogram indicating the good retention of CLT and AZL under the optimum chromatographic conditions. The results obtained are listed in Table 1 and represented in Fig. 3.

3.2. Method validation

The validity of the proposed method was assessed by studying these parameters: linearity, range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, selectivity, sample solution stability, mobile phase stability, system suitability, and robustness, according to ICH guidelines (Guideline IHT, 2005).

3.2.1. Linearity and range

Using the optimized experimental conditions, a linear relationship was established by plotting the average peak area against the drug concentration of each of the two antihypertensive drugs in pure drug, human urine, and human plasma in μg/ml as shown in Fig. S3 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1). The calibration graphs were found to be rectilinear over the concentration range of 15.00–80.00 and 12.50–62.50 μg/ml for AZL and CLT, respectively, in pure form, 20.00–50.00 μg/ml and 12.50–35.00 μg/ml for AZL and CLT, respectively in spiked plasma and 20.00–75.00 μg/ml and 15.00–50.00 μg/ml for AZL and CLT, respectively in spiked urine. Linear regression analysis of the data gave the following equations:

For AZL and CLT in pure form

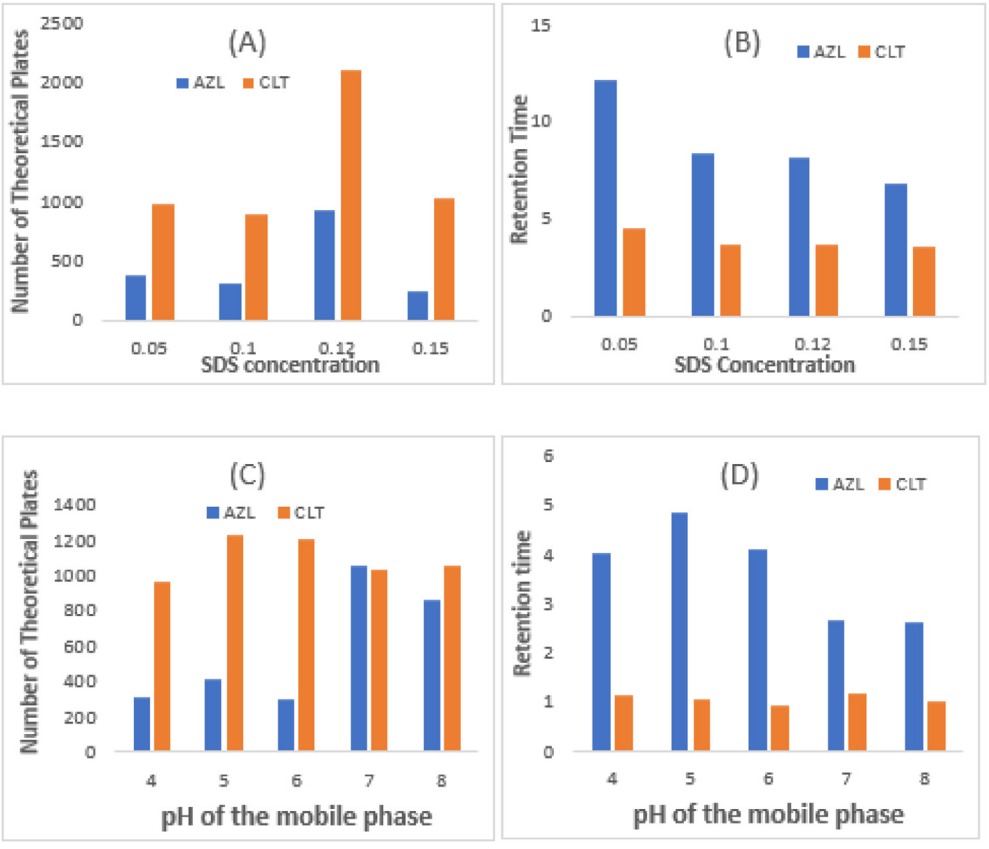


Fig. 3. Effect of changing (A) sodium dodecyl sulphate concentration on Number of theoretical plates of Azilsartan Medoxomil and chlorthalidone, (B) sodium dodecyl sulphate concentration on Retention time of Azilsartan Medoxomil and chlorthalidone, (C) pH of the mobile phase on Number of theoretical plates of Azilsartan Medoxomil and chlorthalidone, and (D) pH of the mobile phase on Retention time of Azilsartan Medoxomil and chlorthalidone.

$P = 20.495C + 71.182$ ($R^2 = 0.9995$) for AZL.
$P = 18.261C + 17.408$ ($R^2 = 0.9991$) for CLT.

For AZL and CLT in spiked human plasma

$P = 14.292C + 32.103$ ($R^2 = 0.9994$) for AZL.

$P = 10.692C + 88.192$ ($R^2 = 0.9992$) for CLT.

For AZL and CLT in spiked human urine

$P = 12.518C + 83.616$ ($R^2 = 0.9998$) for AZL.

$P = 13.646C + 57.666$ ($R^2 = 0.9996$) for CLT.

Where: P is the average peak area, C is the concentration of the drug in μg/ml and R^2 is the determination coefficient.

Statistical analysis of the data obtained by the proposed method gave a high value of the determination coefficient (R^2) of the regression equation, accepted values of the standard deviation of residuals ($S_{y/x}$), standard deviation of intercept (S_a), standard deviation of slope (S_b), accepted value of the % relative standard deviation and the % relative error as shown in Table 2. These data proved the linearity of the calibration curves and less scattering of the points around the calibration curves.

3.2.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD was determined by establishing the minimum level at which the analyte can be detected. The LOQ was determined by establishing the lowest concentration that can be measured according to ICH recommendations (Lavanya et al., 2017) below which the calibration graph is non-linear. The results of LOD and LOQ are summarized in Table 2.

The values of LOD and LOQ were calculated according to the following equations:

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where σ = the residual standard deviation of the response.

And S = slope of the calibration curve.

Table 2. Analytical performance data for the determination of (A) Azilsartan Medoxomil; and (B) chlorthalidone their pure form, spiked human plasma, and spiked human urine by the proposed Micellar liquid chromatography method.

Parameters	Bulk	Plasma	Urine
(A) AZL in pure form, spiked human plasma and spiked human urine			
Concentration range (μg/ml)	15.00–80.00	20.00–50.00	20.00–75.00
LOD (μg/ml)	0.19	0.34	0.33
LOQ (μg/ml)	0.59	1.02	1.00
Determinationcoefficient (R^2)	0.9995	0.9994	0.9998
Slope	20.495	14.292	12.518
Intercept	71.182	32.103	83.616
Standard deviation of Residuals ($S_{y\ /\ x}$)	1.21	1.46	1.25
S.D. of intercept (S_a)	9.40	6.81	5.38
S.D. of slope (S_b)	0.20	0.20	0.1
% RSD	1.50	1.22	30.94
SE	0.61	0.55	0.42
(B) CLT in pure form, spiked human plasma, and spiked human urine			
Concentration range (μg/ml)	12.50–62.50	12.50–35.00	15.00–50.00
LOD (μg/ml)	0.12	0.54	0.23
LOQ (μg/ml)	0.36	1.63	0.70
Determination coefficient (R^2)	0.9991	0.9992	0.9996
Slope	18.26	10.692	13.646
Intercept	17.41	88.192	57.666
Standard deviation of Residuals ($S_{y\ /\ x}$)	0.66	1.74	0.95
SD of intercept (S_a)	9.46	4.42	5.70
SD of slope (S_b)	0.25	0.17	0.17
% RSD	1.88	1.49	1.25
SE	0.71	0.67	0.60

Where RSD is the relative standard deviation.
R^2 is the Determination coefficient.
S_a is the Standard deviation of intercept.
S_b is the Standard deviation of slope.
$S_{y/x}$ is the Standard deviation of residuals.
SD is the Standard deviation and.
SE is the Standard error.

3.2.3. Accuracy

The accuracy of the proposed method was checked by comparing the results of the assay of the studied drugs with those obtained using the reported method (Sravani et al., 2014), where Student's *t*-test and variance ratio F-test showed no significant difference in the two methods performance regarding the accuracy and precision, respectively, as shown in Table 3 indicating high accuracy and precision of the proposed method. The reported method (Sravani et al., 2014) depends on using reversed-phase HPLC for determination of AZL and CLT in pure form and dosage form with UV detection at 230 nm, using a mobile phase consisting of 0.1% orthophosphoric acid buffer and ACN in the ratio of (30:70, v/v). The proposed procedure offers more advantages over the reported one in that the proposed method could be extended to the analysis of AZL and CLT in spiked human plasma and urine with direct injection technique.

3.2.4. Precision

The intra- and inter-day precision was assessed by assaying freshly prepared solutions of pure form, dosage form and spiked human urine in triplicate at three concentration levels on the same day and on three different days, respectively using the proposed method. The results are summarized in Table S1 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1).

Evaluation of the intra-day precision of the proposed method in spiked human plasma was achieved by three replicate determination of human plasma spiked at three concentration levels, 20.00 and 25.00 μg/ml for CLT and AZL, respectively as Quality Control Low (QCL), 25.00 and 30.00 μg/ml for CLT and AZL, respectively as Quality control medium (QCM), and 30.00 and 40.00 for CLT and AZL, respectively as Quality control high (QCH) on the same day for evaluation of the intra-day precision and on three different days for inter-day evaluation, and the results are shown in Table S2 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1).

The relative standard deviations were found to be very small indicating good repeatability and intermediate precision of the proposed method.

3.2.5. Selectivity

The interference from common excipients in pharmaceutical formulations was observed through the analysis and it was proved from the analysis results that these additives did not affect the results of the proposed method. Moreover, there was not any interference faced from human spiked plasma and urine matrix. The high % recovery and high accuracy with low SD indicated that excipients and plasma matrix did not affect the results of the proposed method and this was indicated by three dimensional detection as shown in Fig. S4 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1) and peak purity calculation as illustrated in Fig. S5 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1).

Furthermore, to evaluate the specificity of the proposed method for the determination of the cited drugs in human plasma, blank human plasma and urine were diluted with the mobile phase and directly injected using the recommended chromatographic conditions. No endogenous interference was observed at the retention times of the cited drugs, proving the specificity of the method as shown in Fig. S6 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1).

3.2.6. Sample solution stability and mobile phase stability

Evaluation of the stability of the cited drugs in its dosage forms solution was achieved by quantification of the cited drugs on three successive days and comparison to freshly prepared dosage forms solution. Similarly, the stability of the mobile phase was checked. No significant changes were observed in standard solution or mobile phase responses. The results obtained in both cases proved that the sample solution and mobile phase used during the assay were stable for up to 3 days in the refrigerator.

3.2.7. System suitability test (SST)

Evaluation of the system suitability test (SST) parameters was performed during the development and optimization of the method. Moreover, to ascertain the effectiveness of the final operating system, it was subjected to suitability testing. The test was performed by injecting the standard sample in triplicate and the parameters were calculated as reported by USP (USP, 2017). SST parameters include capacity factor (k⍀), selectivity factor (α), Resolution factor (*R*s), and column efficiency (number of theoretical plates, N). The final SST parameters under the optimum chromatographic conditions are abridged in Table 4. Examining the separated peaks under the optimum conditions

Table 3. Accuracy of the proposed Micellar liquid chromatography method for the determination of the antihypertensive drugs in pure forms.

		Method	% found						Mean ± S.D	Student *t*-test	Variance ratio F-test
Pure form	AZL	Proposed Method (μg/ml)	15.00	30.00	40.00	50.00	60.00	80.00	99.98 ± 1.04	1.60 (2.26)*	1.26 (6.26)*
			99.05	101.3	98.69	99.53	100.44	100.84			
		Reported Method (μg/ml)	20.00	30.00	40.00	50.00	60.00		100.94 ± 0.93		
			102.00	100.80	101.70	99.70	100.50				
	CLT	Proposed Method (μg/ml)	12.50	20.00	25.00	30.00	40.00	62.50	100.55 ± 1.91	0.28 (2.26)*	1.82 (6.26)*
			103.21	101.56	99.58	97.58	100.29	101.08			
		Reported Method* (μg/ml) (Sravani et al., 2014)	15.00	20.00	25.00	30.00	35.00		100.84 ± 1.41		
			99.21	99.75	102.75	101.54	100.95				
Dosage form *Edarby® tablet (40 mg AZL/tab) and Hygroton® tablet (25 mg CLT/tab)*	AZL	Proposed Method (μg/ml)	20.00		30.00	40.00			102.53 ± 0.83	0.15 (2.78)*	2.78 (19)*
			103.07		101.57	102.94					
			25.00		30.00	40.00			102.61 ± 0.50		
			102.54		103.14	102.15					
	CLT	Reported Method (μg/ml) (Sravani et al., 2014)	20.00		25.00	40.00			101.10 ± 0.84	1.53 (2.78)*	3.16 (19)*
			101.67		100.14	101.49					
			20.00		25.00	40.00			101.95 ± 0.47		
			102.17		101.41	102.27					

Each result is the average of three separate determinations.
N.B. *Figures in parentheses are the tabulated t and F values, respectively, at $P = 0.05$ (Miller and Miller, 2018).

Table 4. Chromatographic characteristics of system suitability test using the proposed Micellar liquid chromatography method at 260 nm.

Parameters	AZL	CLT
R_t ±%RSD	2.90 ± 0.14	6.39 ± 0.20
Capacity factor (K\)	3.57	1.07
Symmetry	0.74	0.83
Theoretical plates (N)	817	1421
Resolution	9.54	
Selectivity factor	3.34	

*Each result is the average of three different separate determinations.
Where RSD is the relative standard deviation and SD is the standard deviation. (Whitley, 2002) and (Synek & Kříženecká (2023)).

reveals the system suitability and validity of the method. The calculated resolution for CLT and AZL was 9.54 (>2), which ensures complete separation. The tailing factor was 0.74 and 0.83 (<2) for CLT and AZL, respectively, which shows the symmetry of the produced peaks within the stated Pharmacopeia's range used for quantitative analysis. The number of theoretical plates (N) and retention time (R_t) were 1421, 817, and 2.90, 6.39 for CLT and AZL, respectively, which were adequate for the separation of the two antihypertensive drugs.

3.2.8. Robustness of the method

To assess the robustness of the proposed MLC method, chromatographic conditions were deliberately altered. The tested variables included; concentration of n-propanol (12% ± 0.5% v/v), strength of ortho-phosphoric acid (0.02 M ± 0.005), and pH of the mobile phase (±0.2). In all the varied chromatographic conditions, the efficiency of the separation of AZL and CLT was not affected indicating the reliability of the proposed method.

4. Applications

4.1. Determination of AZL and CLT in single pharmaceutical tablets

The proposed method was successfully used to quantify AZL in Edarby tablets and CLT in Hygroton tablet, their single pharmaceutical dosage forms as shown in Fig. S7 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1). The standard addition technique was used to assess the matrix effect of the tablet additives and its contribution in the deviation of the results obtained by the proposed method as shown in Table S3 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1). The obtained results revealed no significant matrix effect.

4.2. Determination of AZL and CLT in laboratory mixed tablets

The proposed method was successfully used to simultaneously quantify AZL in their laboratory mixed tablets, as shown in Fig. S8 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1) and Table S4 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1). It was concluded that good recoveries were achieved for the studied drugs in their laboratory-prepared mixed tablets.

4.3. Determination of AZL and CLT in spiked human plasma and urine

The proposed method as discussed before under section *III.4.6.3.3* was successfully used to determine AZL and CLT in spiked human plasma and urine as shown in Fig. 4 and Fig. S9 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&typex=additional&preview_mode=1), respectively without interfering with the plasma peak.

Table S5 (https://tast.researchcommons.org/cgi/viewcontent.cgi?filename=0&article=1016&context=journal&type=additional&preview_mode=1) shows the results obtained from simultaneous determination of AZL and CLT using the proposed MLC direct injection method in spiked human plasma and urine. It was noticed that the retention time of AZL in spiked human plasma was changed from 6.40 to 6.93 and this may be attributed to the change in the pH of the media.

5. Assessing the greenness of the proposed method

AZL and CLT were analyzed using several HPLC methods but all reported methods were using mobile phases consisting of organic solvents in different proportions. The extent of organic solvent usage is a crucial factor in determining the environmental impact of an analytical method. Hence, reducing the organic solvent concentration in the mobile phase enhances the method's greenness as the percentage of organic solvent in any analytical method is regarded as a key principle in assessing the method's greenness. The method's greenness was assessed using the Analytical Greenness Metric

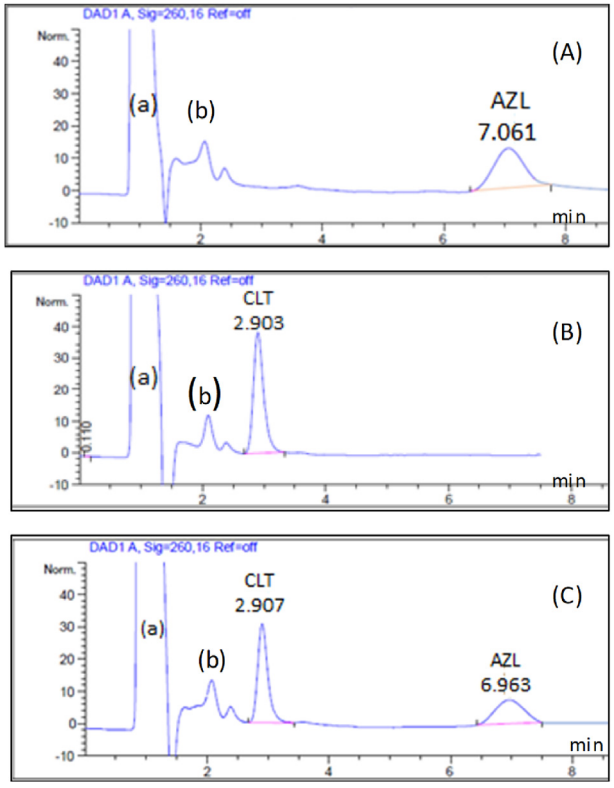


Fig. 4. Typical chromatograms where (a) solvent front; (b) plasma peak; using the proposed Micellar liquid chromatography method for the determination of (A) 48.00 µg/ml Azilsartan Medoxomil; (B) 15.00 µg/ml chlorthalidone; and (C) 48.00 µg/ml Azilsartan Medoxomil and 15.00 µg/ml chlorthalidone mixture, in spiked plasma at 260 nm.

(AGREE) (Pena-Pereira et al., 2020), which was founded on the 12 principles of green analytical chemistry (GAC). (AGREE) was used to evaluate the greenness of the proposed and the reported methods. Analytical Greenness Metric As shown in Table 5, the proposed method has seven green, four yellow, and only one red part so it has a lower impact on the environment compared with the reported method which contains six green, four yellow, and two red parts. So, the reported method is superior in greenness regarding to the reported method.

6. Conclusion

Green analytical chemistry is concerned with creating environmentally benign analytical procedures which are more safer to the environment. The proposed MLC method represents a green direct injection, novel, simple, accurate, rapid, less hazardous micellar liquid chromatographic method that could be used for the simultaneous determination of the antihypertensive combination AZL and CLT determination in bulk drug and dosage form. Moreover, the proposed method was successfully applied for the determination of AZL and CLT in spiked human plasma and spiked human urine with no need for any tedious sample pretreatment steps. The good validation criteria of the proposed method allow its use in quality control laboratories. Furthermore, the results are in good adherence with the reported methods.

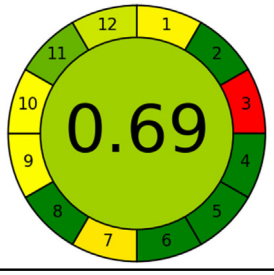
Authors contribution

Marwa Mohamed Azab carried out the experiment and wrote the manuscript. Dina Abd-Elfattah Mohamed made the green analysis calculations and wrote this part. Maha Mahmoud Abou El-Alamin, Maha Abd-Elrahman Sultan supervised the project.

Ethical statement

This work involved human plasma in its research. Approval of all ethical and experimental procedures

Table 5. Comparison between the proposed Micellar liquid chromatography and the reported high-performance liquid chromatography method for determination of drugs under study.

Method	Proposed method	Reported method (13)
Technique	MLC- C_{18} HPLC-UV	RP-HPLC-UV
Organic modifier	0.12 M SDS, 12% n-propanol, 0.3% Triethanolamine (TEA)	0.1% Ortho phosphoric acid buffer and acetonitrile in the ratio of (30:70)
Analytes	Azilsartan Medoxomil and Chlorthalidone	Azilsartan Medoxomil and Chlorthalidone
AGREE assessment		

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Conflicts of interest

There are no conflicts of interest.

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