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Validation, measurement uncertainty estimation and evaluation of UHPLC greenness for simultaneous determination of metoprolol tartrate and hydrochlorothiazide in binary tablet

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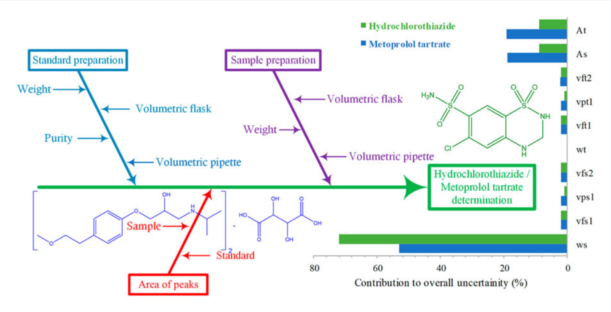
ABSTRACT

A novel green ultra high performance liquid chromatography (UHPLC) method was validated and estimated the measurement uncertainty for simultaneous determination of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) in binary tablet. A Zorbax® SB-C18 column with isocratic elution (flow rate 0.9 mL min^{-1}) at 25°C was used. Analytes and HCT degradation product were separated in approximately 1 min using acetonitrile:water:triethylamine (17:83:0.2, v/v) as mobile phase. Analytical curves were linear with ($R^2 > 0.99$ for MET and HCT) with detection limits of 2.42 and 1.05 µg mL^{-1} for MET and HCT, respectively. Precision measurements showed RSD% from 0.39 to 1.2% and method accuracy by recovery tests was 100 ± 2%. Measurement uncertainties using Eurachem procedure were 100.1 ± 2.8% and 100.3 ± 2.4% for MET and HCT, respectively. This UHPLC method was accurate, precise, linear and selective, making it suitable for pharmaceutical quality control. It also proved eco-friendly compared to other reported methods.



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1. Introduction

Among cardiovascular diseases, hypertension is one of the most serious medical conditions characterized by an increase in systolic blood pressure higher than 130 mm Hg [1] with an estimated 1.28 billion people worldwide [2]. The challenge of diagnosing and treating hypertension is immense because most hypertensive patients are asymptomatic and the symptoms are usually associated with vital organs such as brain, heart and kidney [3]. However, all organs suffer from the consequences of high-pressure levels, as all vessels can be reached [4].

WHO and Eighth Joint National Committee recommend thiazide diuretics as the first-line therapy for primary hypertensive condition due to their effectiveness and reduced cost [5]. Among the most commonly prescribed drugs include hydrochlorothiazide, chlorthalidone, indapamide and metolazone [6]. Hydrochlorothiazide (Log $P = -0.07$; $pKa1 = 7.9$ and $pKa2 = 9.2$) is chemically 2*H*-1,2,4-benzothiadiazine-7-sulfonamide, 6-chloro-3,4-dihydro-1,1-dioxide (Figure 1(A)) and among the pioneer diuretics [7] prescribed in combination with other anti-hypertensive drugs, such as metoprolol

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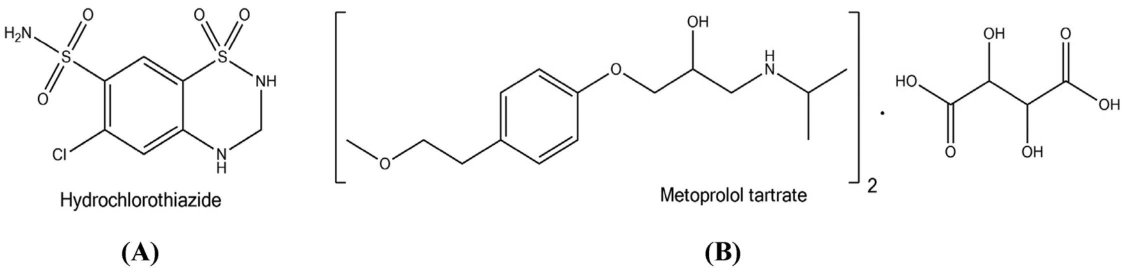


Figure 1. Chemical structure of hydrochlorothiazide and metoprolol tartrate.

tartrate (cardioselective beta-adrenergic antagonist) in hypertrophic cardiomyopathy, myocardial infarction, vascular headache and migraine which proved to be more effective than any other drug used by itself [8]. Metoprolol tartrate (Log $P = 1.76$; pKa1 $= 9.67$ and pKa2 $= 14.09$) is chemically 1,1-dioxide 1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]-2-propanol, 2*R*,3*R*-dihydroxybutanedioate (Figure 1(B)).

Several methods are available to determine the metoprolol tartrate and hydrochlorothiazide in their single formulation or in combined dosage forms including supercritical fluid chromatography [9], liquid chromatography-mass spectrometry [10–16], gas chromatography-mass spectrometry [17], ultra performance liquid chromatography-mass spectrometry [18], high performance liquid chromatography (HPLC) [19–23], ultra performance liquid chromatography [24,25], voltammetry [26], spectrophotometry [27–29], potentiometry [30] and capillary electrophoresis [31].

The literature reveals few HPLC methods to determine simultaneously both active pharmaceutical ingredients in tablets, but longer retention time, high amount of carbon-based solvents in the mobile phase, and buffer solution have been used [20,21,23,32–36]. *Żuromska-Witek B. et al.* [32] identified both drugs in approximately 18 min using mobile phase consisting of acetonitrile and phosphate buffer (pH 2.5) with gradient elution. *Wahajuddin et al.* [33] determined metoprolol tartrate, hydrochlorothiazide and other seven substances in pemeability samples (study in rats) by using methanol and 10 mM phosphate buffer, pH 2.5 as mobile phase with gradient elution. Hydrochlorothiazide and metoprolol were identified in approximately 12 min. *Ramadan et al.* [34] determined metoprolol tartrate and hydrochlorothiazide in the salamide (hydrochlorothiazide impurity) presence using acetonitrile and potassium dihydrogen phosphate (20:80 v/v, pH 3) as mobile phase, the substances were separated in approximately 7 min. The hydrochlorothiazide was not submitted to stress degradation, salamide impurity standard was added to the solution containing metoprolol tatrate and hydrochlorothiazide. *Ravisankar et al.* [35] separated metoprolol succinate, hydrochlorothiazide, atenolol, amlodipine and nebivolol hydrochloride in less than 6 min. The separation was performed by using phosphate buffer (pH 3 adjusted with triethylamine) and acetonitrile (50:50, v/v) as mobile phase. The authors only applied HPLC method to determine metoprolol succinate in tablets. *Patil et al.* [36] separated metoprolol succinate and hydrochlorothiazide impurities and its degradation products using buffer solution and methanol as mobile phase in gradient elution. The substances were separated in approximately 40 min. *Garg et al.* [20] identified the analytes in 8 min using high quantity of methanol (95%), while *Rawool et al.* [21] determined the analytes in 11 min using methanol and phosphate buffer (40:60) as mobile phase. Though, the latter did not provide sufficient information for system suitability. Another author separates the metoprolol tartrate, hydrochlorothiazide and relevant impurities in 13 min using acetonitrile and sodium phosphate buffer as mobile phase in gradient elution mode [23].

HPLC is a well-established separation technique that has been used in several areas, including chemistry, forensics, toxicology and clinical and environmental analysis, to solve many analytical problems. Recently many improvements have been incorporated into this technique, including the development of new stationary phases and chromatographic support [37]. Ultra high performance liquid chromatography (UHPLC) technique is an example of them that was developed with the introduction of porous particles in the stationary phase with less than 2 μm diameter, in response to the ongoing search for a faster and more efficient analysis [38]. This method is based on the same HPLC separation principle but uses chromatography columns from 5 to 10 cm in length, and internal diameters from 1 to 2.1 mm, filled with small-size ($\leq 2\,\mu m$) particles [39]. A higher mobile phase linear velocity increases the resolution and detectability while reducing the time analysis which ultimately leads to a significant increase in the chromatographic pressure. As a result, appropriate equipment, capable of operating at high pressure of up to 1000 bar ($\sim$15,000 psi) is used to achieve maximum chromatographic performance [40].

Green analytical chemistry, an offshoot of green chemistry, is defined as the exploitation of methodologies and techniques aiming to minimize the generation of hazardous chemical waste by selecting green reagents and solvents as well as by choosing energy-efficient instruments to the extent i.e. technically and economically feasible. To address and cover all the 12 principles of green chemistry [41,42], two highly recommended approaches i.e. Analytical GREEnness and Green Analytical Procedure Index were employed for comprehensive greenness evaluation of the proposed method.

Laboratories need to estimate measurement uncertainty for their quantitative methods to comply with ISO/IEC 17025 requirements. Measurement uncertainty is a component of uncertainty in all the individual steps of an analytical procedure [43]. Generally, a measurement result is only an approximation or estimation of the measured value and thus only complete when accompanied by a declaration of uncertainty of this estimate [44,45].

According to the authors' knowledge, there is no validation, measurement uncertainty estimation and greenness evaluation of UHPLC method reported for the simultaneous quantitation of hydrochlorothiazide and metoprolol tartrate in tablets. Therefore, the present research describes a fast, simple, validated and optimized UHPLC method for the simultaneous quantitation of hydrochlorothiazide and metoprolol tartrate in binary tablets. Both drugs were determined in less than 1 min using less quantity of organic solvent and without buffer solution as compared to the reported HPLC methods [20,21,23]. Moreover, measurement uncertainty calculation which is based on validation of the analytical procedure is described. Additionally, a comprehensive greenness assessment using two different green analytical chemistry metrics (Analytical GREEnness and Green Analytical Procedure Index) was performed for the proposed method and compared with previously reported HPLC methods.

2. Materials and methods

2.1. Chemical, reagent and sample

Metoprolol tartrate (98%) and hydrochlorothiazide (98%) were provided by Sigma-Aldrich (Sao Paulo, Brazil) and Foundation for Popular Medicine (Sao Paulo, Brazil). Selopress® coated tablets (hydrochlorothiazide 12.5 mg and metoprolol tartrate 100 mg) and placebo (monohydrate lactose, silicon dioxide, microcrystalline cellulose, magnesium stearate, povidone and sodium starch glycolate) were purchased from AstraZeneca (Sao Paulo, Brazil). Acetonitrile and methanol HPLC grade were obtained from J. T. Baker (Sao Paulo, Brazil). Hydrogen peroxide, sodium hydroxide, *o*-phosphoric acid, hydrochloric acid and triethylamine were obtained from Merck® (Sao Paulo, Brazil). The purified water was obtained using Milli-Q® system.

2.2. Instrumentation and conditions

An Agilent 1200 UHPLC system, equipped with a degasser, an autosampler, a quaternary pump and a photodiode array detector was used for drug analyses. A Zorbax® SB-C18 (50 mm × 2.1 mm i.d., 1.8 µm particle size) chromatographic column was used. The acetonitrile:water:triethylamine (17:83:0.2, v/v), pH 3.0 adjusted with *o*-phosphoric acid was used as mobile phase. The flow rate was 0.9 mL min^{-1}. An absorbance wavelength of 225 nm was selected, the column temperature was maintained at 25°C and the sample injection volume was 0.2 µL.

2.3. Standard stock solutions' preparation

Ten mg of metoprolol tartrate was accurately weighed and transferred into a 10 mL amber volumetric flask, 5 mL of methanol was added, and the mixture was stirred until completely dissolved. Milli-Q® purified water was used to complete the volume and the sonicatation was performed for 5 min. Metoprolol tartrate final concentration was 1000 µg mL^{-1}. The same procedure was used to prepare hydrochlorothiazide standard stock solution with final concentration of 1000 µg mL^{-1}.

2.4. Test-mixture standard preparation

Aliquots from hydrochlorothiazide and metoprolol tartrate stock standard solutions were transferred into amber volumetric flask (10 mL), methanol (5 mL) was added, and Milli-Q® purified water was used to complete the volume. Final concentrations were 12.5 µg mL^{-1} and 100 µg mL^{-1} for hydrochlorothiazide and metoprolol tartrate, respectively. The sonication of test mixture was done for 3 min, the solutions were filtered with 0.45 µm filter and injected into UHPLC instrument.

2.5. Sample stock and working solutions' preparation

Twenty tablets were weighed and crushed to obtain a fine powder (average tablet weight: 345 mg). Three quantities of powder (276, 345 and 414 mg) were accurately weighed and then transferred into three amber volumetric flasks (100 mL). After methanol (50 mL) was added, the flasks were shaken manually for a few minutes and then the volume was adjusted to the mark with Milli-Q® water; further, the flasks were sonicated for 10 min. Final concentrations of hydrochlorothiazide (100, 125 and 150 µg mL^{-1}) and metoprolol tartrate (800, 1000 and 1200 µg mL^{-1}) were obtained.

To prepare desired working sample solutions, aliquots from sample stock solutions were transferred into amber volumetric flasks (10 mL), methanol (5 mL) was added and finally, the volume was completed with Milli-Q® water. The solutions were sonicated for 5 min, filtered in a 0.45 µm filter and injected into UHPLC instrument.

2.6. Method validation

The method validation was performed according to the guidelines of United States Pharmacopoeia [46] and International Conference on Harmonization [47].

2.6.1. Specificity/selectivity

Method specificity was evaluated by confirming the separation of metoprolol tartrate and hydrochlorothiazide peaks from tablet excipients. Method selectivity was assessed by forced degradation conditions (photolytic, oxidative, neutral, alkaline and acidic hydrolysis). Neutral, acidic and alkaline degradations were carried out in ultrapure water, 0.1 M HCl and 0.1 M NaOH, respectively. After degradation, last two were neutralized with 0.1 M NaOH and 0.1 M HCl, respectively. 3% H_2O_2 solution was used for oxidative stress and photodegradation was performed by exposing the samples to a photostability UV light chamber for 60 h at 254 nm. Metoprolol tartrate (1000 µg mL^{-1}) and hydrochlorothiazide (125 µg mL^{-1}) sample solutions were prepared and refluxed for 2 and 24 h at 80°C except photolytic condition. Aliquots were taken and transferred to 10 mL amber volumetric flasks and diluted with Milli-Q® water to obtain final concentrations of 12.5 µg mL^{-1} and 100 µg mL^{-1} for hydrochlorothiazide and metoprolol tartrate, respectively and filtered before being injected. Peak purities were performed for selectivity assessment by using photodiode array detector.

2.6.2. Linearity

Aliquots of hydrochlorothiazide and metoprolol tartrate stock standard solutions were transferred to five amber volumetric flasks (10 mL) and ultrapure water was used to complete the final volume. Linearity was evaluated over the concentration range of 80–120% of the test concentration (100 and 12.5 µg mL^{-1} for metoprolol tartrae and hydrochlorothiazide, respectively), by preparing three individual replicates at each concentration level for metoprolol tartrate (80, 90, 100, 110 and 120 µg mL^{-1}) and hydrochlorothiazide (10, 11.25, 12.5, 13.75 and 15 µg mL^{-1}). Analytical curve was constructed by plotting metoprolol tartrate and hydrochlorothiazide concentrations against the mean response (peak areas) for each concentration. Least-square method was used to calculate the regression lines. Method's linearity (p-value $= 0.05$) was determined by using the analysis of variance.

2.6.3. Detection and quantitation limits

According to the International Conference on Harmonization guideline [47], detection and quantitation limits were calculated based on response standard deviation and analytical curve slope ($n = 3$). The respective equations (1 and 2) are mentioned below:

$$\text{Detection limit} = 3.3\,\sigma/S \tag{1}$$

$$\text{Quantitation limit} = 10\sigma/S \tag{2}$$

where σ – standard deviation of y-intercepts of regression lines, and S – slope estimated from analytical curves of metoprolol tartrate and hydrochlorothiazide.

2.6.4. Precision

Method precision (repeatability and intermediate precision) was studied. Repeatability was performed by determination of 10 independent samples in the median concentration (100%) of the analytical curve. Samples (12.5 µg mL^{-1} for hydrochlorothiazide and 100 µg mL^{-1} for metoprolol tartrate) were prepared and analysed on the same day under the same experimental conditions. Intermediate precision was analysed at three different concentration levels (10, 12.5 and 15 µg mL^{-1} for hydrochlorothiazide and 80, 100 and 120 µg mL^{-1} for metoprolol tartrate) in triplicate over three consecutive days. All results were calculated by average percent relative standard deviation.

2.6.5. Accuracy

Accuracy was determined as the percent recovery of standard added at known concentration to the sample. Recovery based on three concentration levels (80, 100 and 120% of the label claim) was assessed. Known quantities of hydrochlorothiazide (5, 6.25 and 7.5 µg mL^{-1}) and metoprolol tartrate (40, 50 and 60 µg mL^{-1}) standard solutions were added to commercial sample solutions. Accuracy was determined in triplicate.

2.7. Measurement uncertainty evaluation and risk assessment

Measurement uncertainty estimations were performed according to Eurachem/Citac guides, both using bottom-up and top-down approaches [44,45]. Combined uncertainties associated with the quantification of metoprolol tartrate and hydrochlorothiazide were obtained from individual uncertainties components through weighing and diluting (volumetric flasks and pipettes) associated with standard and sample solution preparations as well as repeatability from peak areas obtained from chromatograms. The general uncertainty propagation law equation is presented below:

$$u_y = \sqrt{\begin{array}{c}\sum_{i}^{n}\left(u_{x_i}{}^2\left(\frac{\partial y}{\partial x_i}\right)^2\right)\\ +\sum_{i}^{n}\sum_{j}^{n}\left(u_{x_i}u_{x_j}\left(\frac{\partial y}{\partial x_i}\right)\left(\frac{\partial y}{\partial x_j}\right)r_{ij}\right)\end{array}} \tag{3}$$

where y and u_y are measured value and respective combined standard uncertainty, u_{x_i} and u_{x_j} are the uncertainty values of x_i and x_j inputs, and r_{ij} is the correlation between the x_i and x_j input values.

Alternatively, measurement uncertainties associated with the quantification of metoprolol tartrate and hydrochlorothiazide were estimated based on the results obtained from in-house validation. In this case, the measurement uncertainties are associated with accuracy and precision used to calculate combined and expanded uncertainties [48]. The general model equation used to evaluate in-house validation uncertainty data is presented below:

$$u_y = \sqrt{(1-R)^2 + s_P{}^2} \tag{4}$$

where u_y – combined standard uncertainty, R – mean recovery value (accuracy), and s_P – standard deviation of the intermediate precision.

The false risk conformity decisions due to measurement uncertainties was estimated using a frequentist probability approach. Consumers' (probability to accept a lot that should be rejected) and producers' (probability to reject a lot that should be accepted) by Monte Carlo method. This method was applied using MS-Excel spreadsheet with 50,000 simulations. Monte Carlo method was employed using MS-Excel formula " = NORM.INV(RAN(), y, u_y)", where y and u_y are the measured values (e.g. metoprolol tartrate or hydrochlorothiazide assay value) and respective combined standard uncertainty. The consumers' and producers' risk values were calculated as $N_{OUT}/(N_{OUT}+N_{IN})$ and $N_{IN}/(N_{OUT}+N_{IN})$, where N_{IN} and N_{OUT} are the number of simulated values within and out of the specification limits (note: $N_{IN} + N_{OUT} = 50{,}000$). The MS-Excel spreadsheet used to calculate the consumers' and producers' risk values is available as *supplementary material* (*Risk Assessment.xlsx*).

3. Results and discussion

3.1. Method development and optimization

An UHPLC method was developed to separate metoprolol tartrare, hydrochlorothiazide and their degradation products. It is well known that mobile phase composition, pH and UV wavelength are critical parameters for chromatographic analysis. In this work, metoprolol tartrare and hydrochlorothiazide standard solutions were scanned separately in the wavelength range from 200 to 350 nm. Both analytes had UV absorbances at 225 and 275 nm, the wavelength at 225 nm was selected because the analytes had maximum UV absorption (Figure 2(A)).

The preliminary chromatographic method for simultaneous determination of hydrochlorothiazide and metoprolol tartrate was developed using Zorbax® SB-C18 column (50 mm × 2.1 mm i.d., 1.8 µm particle size), different mixtures of acetonitrile and water, pH 3.0 adjusted with *o*-phosphoric acid were performed as mobile phase. The flow rate was set at 0.9 mL min^{-1}, the column temperature at 25°C, and the detection wavelength was fixed at 225 nm. Figure 2(B,C) shows the separation of analytes when using acetonitrile:water (30:70, v/v) and (20:80, v/v), respectively, however, the peaks are deformed and presenting enlargement at baseline

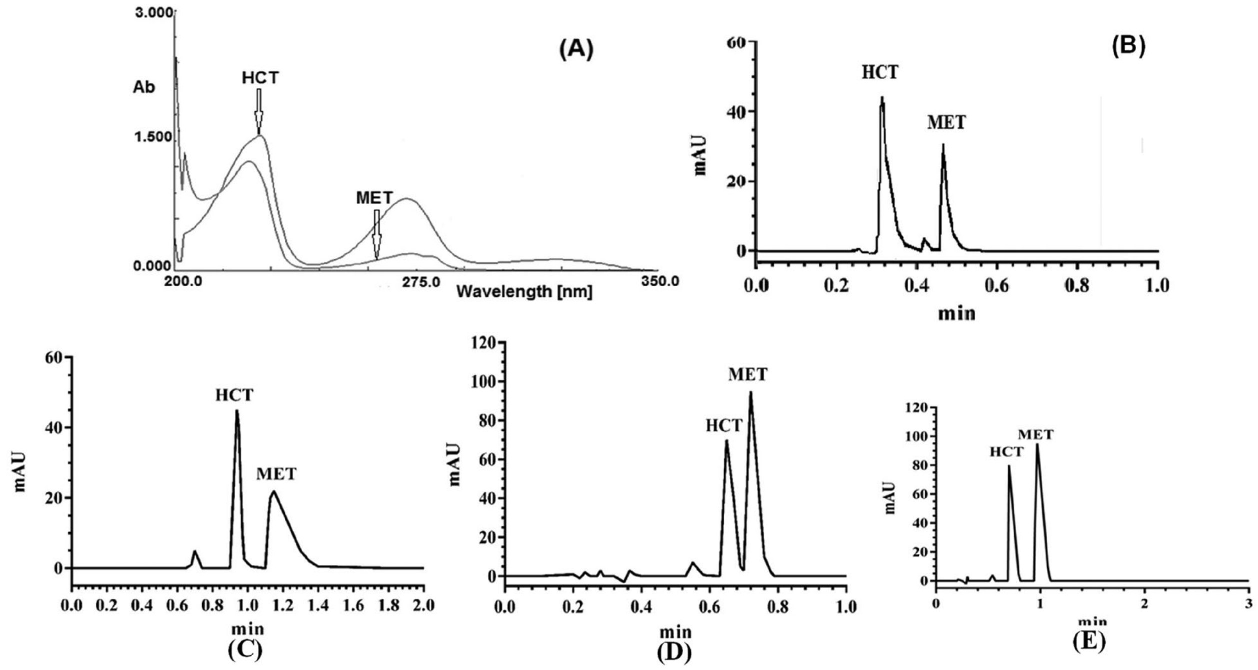


Figure 2. (A) UV absorption spectra of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) solutions using methanol:water (50:50, v/v) as blank. Chromatograms of MET and HCT, conditions: Zorbax® SB-C18 column (50 mm × 2.1 mm i.d., 1.8 µm particle size), pH 3.0 adjusted with *o*-phosphoric acid, column temperature 25°C, flow rate 0.9 mL min^{-1} and detection at 225 nm. Mobile phase: (B) acetonitrile:water (30:70, v/v); (C) acetonitrile:water (20:80, v/v); (D) acetonitrile:water:triethylamine (20:80:0.2, v/v) and (E) acetonitrile:water:triethylamine (17:83:0.2, v/v).

Table 1. Central composite design for hydrochlorothiazide (Y1) and metoprolol tartrate (Y2) resolutions as function of pH (X1) and acetonitrile (% ACN) proportion (X2).

		Resolution	
X1 (pH)	X2 (% ACN)	Y1 (HCT)	Y2 (MET)
2.3	17	4.82	7.14
2.5	12	1.6	14.19
2.5	22	0	1.6
3.0	10	0	20.9
3.0	17	4.85	6.68
3.0	24	0.86	1.08
3.5	12	0	16.32
3.5	22	1.42	0.92
3.7	17	4.75	7.6

(peak symmetry > 2). The metoprolol tartrate asymmetry is due to the interaction of amine functionality with the residual silanols of the stationary phase. To improve peak symmetry, 0.2% triethylamine was added to the mobile phase which will cover residual silanols, hence avoiding the peaks enlargement but resolution was a little bit compromised ($Rs = 1.2$) (Figure 2(D)).

To determine the most appropriate conditions for the determination of metoprolol tartrate and hydrochlorothiazide by UHPLC, the method was optimized in order to verify under which conditions it would be possible to obtain better resolution. In this sense, Central Composite Design with Doehlert was used to adjust quadratic models [49,50]. The tested models are expressed in Table 1, while their statistical significance assessed by analysis of variance are expressed in Table 2.

The regression equations and determination coefficients, evaluated through analysis of variance, for hydrochlorothiazide (Y1) and metoprolol tartrate (Y2) resolutions as function of pH (X1) and acetonitrile proportion (X2) are expressed in equations (5 and 6), respectively.

$$\mathrm{Y1} = -18.40 + 2.528 * X2 \; - \; 0.0736 * X2^2 \tag{5}$$

$$\begin{aligned}\mathrm{Y2} = 36.64 + 5.31 * X1 \; - \; 3.129 * \mathrm{X2} + 0.0754 * \mathrm{X2}^2 \\ - 0.281 * \mathrm{X1} * \mathrm{X2}\end{aligned} \tag{6}$$

Figure 3(A) represents 3D response surface plot for hydrochlorothiazide (Y1) resolution and Figure 3(B) for metoprolol tartrate (Y2) resolution as function of pH (X1) and acetonitrile proportion (X2). Hydrochlothiazide resolution ($\mathrm{Rs}_{(\text{hydrochlorothiazide degradation product –hydrochlorothiazide})}$) and metoprolol tartrate resolution ($\mathrm{Rs}_{(\text{hydrochlorothiazide –metoprolol tartrate})}$) suffer more interference with variation in acetonitrile concentration and there was almost no change with pH variation.

Figure 3(C) shows the global graph of UHPLC method optimization, in which we can observe that the resolution was maximized, when 17% of acetonitrile and pH 3 were used. Under these conditions, resolutions of 4.85 for hydrochlorothiazide degradation product – hydrochlorothiazide and 6.68 for hydrochlorothiazide degradation product – metoprolol tartrate were obtained.

The proposed UHPLC method for determining both drugs simultaneously was validated using the following conditions: Zorbax® SB-C18 column (50 mm × 2.1 mm i.d., 1.8 µm particle size), and acetonitrile:water:triethylamine (17:83:0.2, v/v) pH 3.0 adjusted with *o*-phosphoric acid. The injection volume was 0.2 µL, the column temperature was set at 25°C and the eluent was monitored by the detection wavelength of 225 nm.

Figure 2(E) shows metoprolol tartrate and hydrochlorothiazide chromatogram obtained at optimized chromatographic conditions.

3.2. *Method validation*

The assessed parameters were system suitability, specificity/selectivity, linearity, detection and quantification limits, precision and accuracy [46,47]. Robustness was assessed by Central Composite Design response from method development and optimization.

3.2.1. *System suitability*

In chromatographic methods, system suitability assessment is very important to confirm the acceptable performance of the instrument used for analytical measurements [51]. This process generates the production of accurate and replicable data which can be submitted with confidence to regulatory agencies. Typically, the system suitability parameters include tailing factor, resolution, separation factor, retention time and number of theoretical plates [46,52]. From Table 3, we can observe good column efficiency, separation factor and short retention time (less than 1 min), the *Rs* was above 2, tailing factors were less than 2 for both drugs. The obtained results are in accordance with United States Pharmacopoeia and FDA guidelines [46,52].

3.2.2. *Specificity/selectivity*

Method specificity was evaluated through a solution containing a mixture of tablet excipients. No interferences were detected at retention times of metoprolol tartrate and hydrochlorothiazide in the sample solution. Peak purities higher than 99.8% were obtained for metoprolol tartrate and hydrochlorothiazide by using photodiode array detector, which shows the excipients did not coelute with main peaks (Figure 4(J)). Thereby, the presented method is applicable for the determination of both active pharmaceutical ingredients.

Method selectivity was assessed by performing metoprolol tartrate and hydrochlorothiazide under forced degradation conditions (photolytic, oxidative, neutral, basic and acidic hydrolysis) to demonstrate whether their degradation products either interfere or not for the determination of hydrochlorothiazide and

Table 2. Analysis of variance results for hydrochlorothiazide (Y1) and metoprolol tartrate (Y2) resolutions as function of pH (X1) and acetonitrile proportion (X2).

	Y1				Y2			
Source	Coef	DF	SS	*p* value	Coef	DF	SS	*p* value
Regression		2	16.0811	0.078		4	411.549	0.000
R^2	0.57				0.995			
Constant	−18.40				36.64			
Linear		1	0.1324	0.806		2	392.817	0.000
X1					5.31	1	0.554	0.350
X2	2.528	1	0.1324	0.806	−3.129	1	392.263	0.000
Quadratic		1	15.9487	0.030		1	16.758	0.004
X1								
X2	−0.0736	1	15.9487	0.030	0.0754	1	16.758	0.004
Interaction						1	1.974	0.117
X1 $*$ X2					−0.281	1	1.974	0.117
Error		6	12.0012			4	1.981	
Total		8	28.0823			8	413.529	

Note: R^2 = Determination Coefficient; Coef = Coefficient; DF = Degrees of Freedom; SS = Sum of Squares.

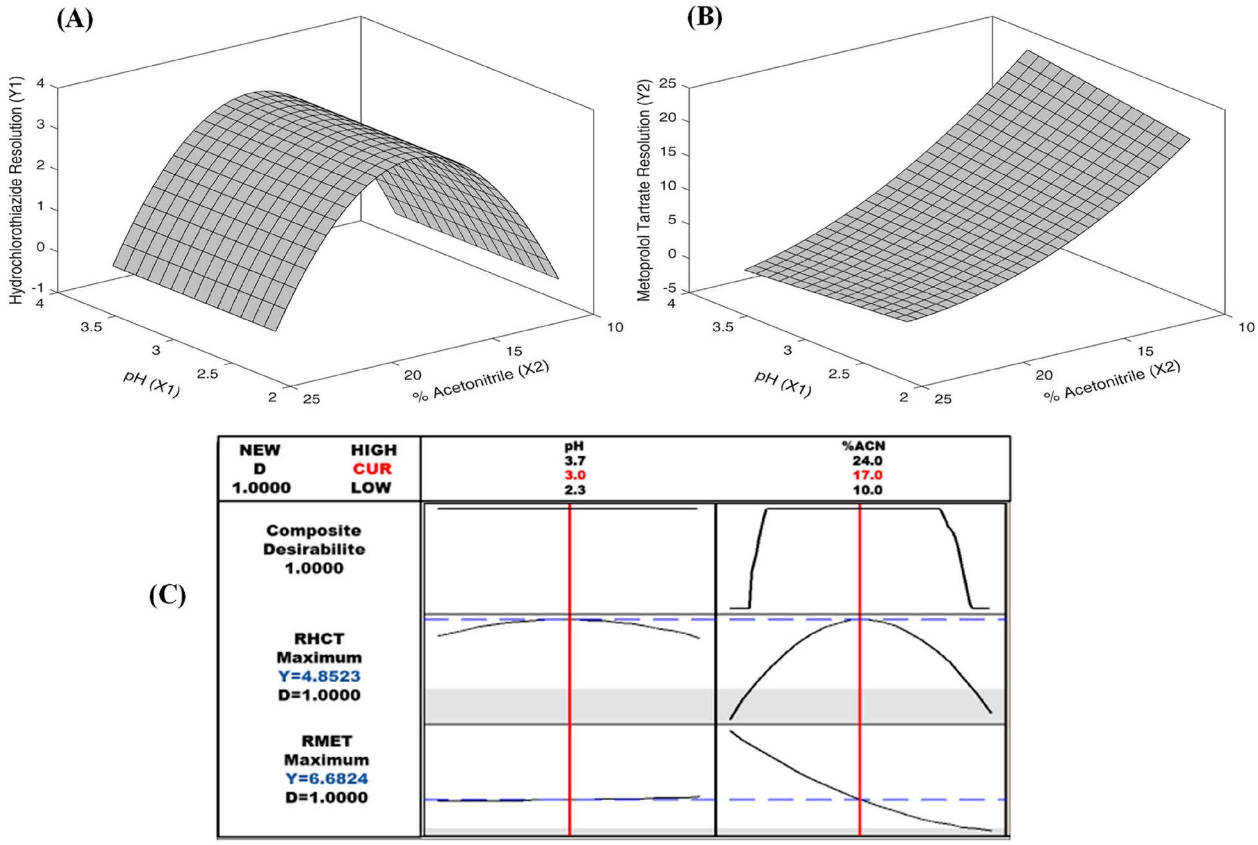


Figure 3. Response surface. (A) hydrochlorothiazide (Y1) and (B) metoprolol tartrate (Y2) resolutions as function of pH (X1) and acetonitrile proportion (X2); and (C) Global graph of UHPLC method optimization results.

metoprolol tartrate. One hydrochlorothiazide degradation product was observed after 2 and 24 h for oxidative and neutral hydrolysis (0.56 min), acidic and basic hydrolysis (0.55 min), while no degradation product was observed with metoprolol tartrate (Figure 4). Peak purities were performed and purity factor higher than 999 (metoprolol tartrate 999.95 and hydrochlorothiazide 999.96) were obtained by using photodiode array detector. There was no interference from the obtained degradation products with the quantification of analytes under study.

The results are in agreement with the acceptance criteria established in United States Pharmacopoeia, International Conference on Harmonization and FDA guidelines [46,47,52] i.e. the excipient compounds, impurities or degradation products must not interfere with the analysis of the targeted analyte.

3.2.3. Linearity

Metoprolol tartrate (concentration range: 80–120 µg mL^{-1}) and hydrochlorothiazide (concentration range: 10–15 µg mL^{-1}) were used to construct the analytical curves. Good linearities with determination coefficients > 0.99 for both active pharmaceutical ingredients were obtained. To check the method good fitting, an analysis of variance was performed and

Table 3. System suitability, linearity, limits of detection and quantitation, precision and accuracy of the proposed method.

Analytical feature	Metoprolol tartrate	Hydrochlorothiazide
Retention time (min)[a]	0.96 ± 0.01	0.70 ± 0.01
Column efficiency (*N*)[a]	2931.50 ± 0.81	2094.22 ± 0.47
Separation factor (α)	1.51	
Tailing factor[a]	1.36 ± 0.03	1.32 ± 0.03
Resolution (*Rs*)[a]	4.03 ± 0.05	
Determination coefficient (R^2)	0.9991	0.9903
Concentration range (µg mL^{-1})[b]	80.0–120.0	10.0–15.0
Intercept	2.893	−13.794
Slope (*S*)	2.187	10.381
Critical	2.48	2.48
P	0.6147	0.2840
Detection limit (µg mL^{-1})	2.42	1.05
Quatitation limit (µg mL^{-1})	7.34	3.19
Intra-day precision (% RSD)[c]	1.20 ± 2.70	0.72 ± 0.87
Inter-day precision (% RSD)[d]	0.62 ± 0.30	0.39 ± 0.22
Accuracy (% Recovery) (Mean ± SD)[e]	101.13 ± 0.64	100.60 ± 0.26

a: Mean ± SD of six determinations.
b: Five concentrations, each concentration level in triplicate.
c: Mean ± SD of 10 determinations.
d: Mean ± SD of nine determinations.
e: Average of nine determinations.
% RSD = percent relative standard deviation.

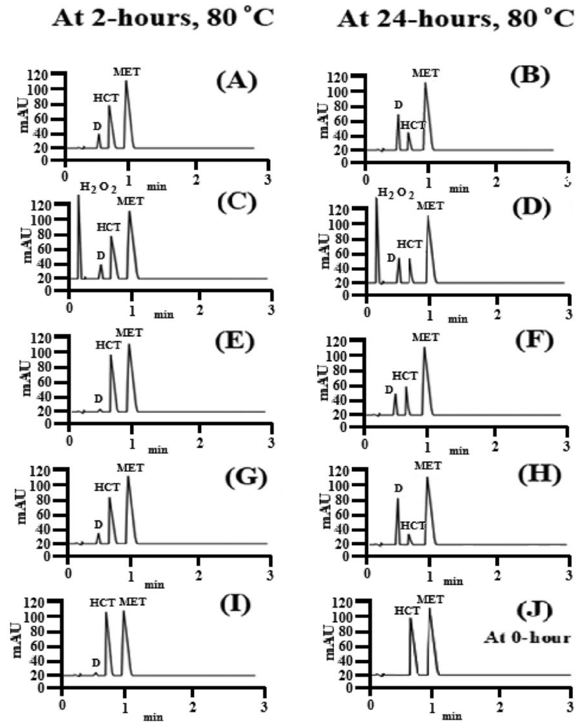


Figure 4. Chromatograms of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) after forced degradation conditions at 2 h – 80°C and 24 h – 80°C. (A) and (B) neutral hydrolysis; (C) and (D) oxidative; (E) and (F) basic hydrolysis; (G) and (H) acidic hydrolysis; (I) photolytic and (J) 0-hour without stress. Conditions: Zorbax® SB-C18 column (50.0 mm × 2.1 mm i.d., 1.8 µm particle size); mobile phase: acetonitrile:water:triethylamine (17:83:0.2, v/v) pH 3.0 adjusted with *o*-phosphoric acid, sample injection 0.2 µL, flow rate 0.9 mL min^{-1}, column temperature 25°C and UV detection at 225 nm. D = degradation product.

there was significant regression, $F_{\text{calculated}} = 152.85 > F_{\text{critical}} = 2.48$ for hydrochlorothiazide and $F_{\text{calculated}} = 1934.34 > F_{\text{critical}} = 2.48$ for metoprolol tartrate, $\alpha = 0.05$. It demonstrates that linear model is suitable for the curves, since critical *F* values were higher than *p*-values, indicating *Y* (response variances) is independent of *X* (concentration levels). Moreover, *F* values observed from the regression analysis were greater than critical *F*. Therefore, Table 3 shows the existence of a linear relationship between *X* and *Y* variables. The results are in agreement with the acceptance criteria established in United States Pharmacopoeia, International Conference on Harmonization and FDA guidelines [46,47,52] i.e. for the assay of a drug substance or a finished product, the correlation coefficient of minimum five concentration levels will be ≥ 0.999 for the range of 80–120% of the test concentration.

3.2.4. Detection and quantitation limits

Detection and quantitation limits for metoprolol tartrate were 2.42 and 7.34 µg mL^{-1} and for hydrochlorothiazide were 1.05 and 3.19 µg mL^{-1}, respectively. Detection and quantitation limits were calculated according to the equations mentioned in section 2.6.3.

3.2.5. Precision

The intra-day (repeatability) and inter-day (intermediate) precisions were calculated as percent relative standard deviation for sample solutions containing metoprolol tartrate and hydrochlorothiazide to prove its applicability. The intra-day precision was estimated to be 0.72 ± 0.87 and 1.20 ± 2.70% for hydrochlorothiazide and metoprolol tartrate, respectively; and the inter-day precision was estimated to be 0.39 ± 0.22% for hydrochlorothiazide and 0.62 ± 0.30% for metoprolol tartrate. The results confirmed that the proposed method was highly precise [53] (Table 3) and in accordance with FDA acceptance criteria i.e. RSD should be ≤ 2% for both bulk drugs and finished products [52].

3.2.6. Accuracy

Recovery tests were carried out at three concentration levels (low, middle and high) from the analytical curve to obtain the method of accuracy. Recovery was obtained by spiking known quantities of hydrochlorothiazide and metoprolol tartrate standards to known sample quantities in triplicate. The average percentage recoveries for hydrochlorothiazide and metoprolol tartrate demonstrated that the presented method was highly accurate [53] (Table 3) based on pharmaceutical industry acceptance criteria i.e. 100 ± 2% for an assay of an active ingredient in a drug product over the range of 80–120% of the test concentration [46,47].

3.3. Uncertainty measurement

To indicate the test reliability, the measurement uncertainties were estimated according to Eurachem/Citac

Table 4. Quantification of uncertainties and conversion to standard deviation of the uncertainty of each component which is used to estimate the combined uncertainties associated with the quantification of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) in pharmaceutical binary tablet.

Uncertainties sources	Standard uncertainty (x)	Relative standard uncertainty (u_x)
Preparation of standard solution		
Weight of MET RS (ws)	10.0 mg	0.1 mg
Weight of HCT RS (ws)	10.0 mg	0.1 mL
Volumetric flask ($vfs1$)	10.000 mL	0.017 mL
Volumetric pipette ($vps1$)	1.0000 mL	0.0017 mL
Volumetric flask ($vfs2$)	10.00 mL	0.017 mL
Preparation of sample solution		
Weight of sample (wt)	345.0 mg	0.1 mg
Volumetric flask ($vft1$)	100.00 mL	0.17 mL
Volumetric pipette ($vpt1$)	1.0000 mL	0.0017 mL
Volumetric flask ($vft2$)	10.000 mL	0.017 mL
Repeatability of peak area		
Area of MET in Standard (As)	221.73	1.33
Area of MET in Sample (At)	221.86	1.33
Area of HCT in Standard (As)	120.83	0.43
Area of HCT in Sample (At)	121.20	0.44
Expanded final uncertainty		
Quantification of MET (%)	100.1%	2.8%*
Quantification of HCT (%)	100.3%	2.4%*

Note: RS = Reference Standard.

*Expanded uncertainty using a coverage factor $k = 2.0$ with 95% confidence level.

guidelines [54]. The uncertainty estimation result of any test depends on the knowledge of critical points of the analytical procedure and it is essential to know the factors that can influence the final result. To avoid the double uncertainty contribution, which could lead to overestimation of the combined uncertainty, the diagram of cause and effect was constructed, also known as Ishikawa or Fishbone diagram (***graphical abstract***).

Standard uncertainties of metoprolol tartrate and hydrochlorothiazide were estimated based on the validation method, experimental studies, certificate information and calibration results (Table 4).

For each source of uncertainty, variables of entry were determined, and uncertainties associated with each source were quantified. Correlations between variables were assumed to be negligible. Combined and expanded uncertainties were calculated using the following equation:

$$u_{C_{\%}} = C_{\%}\sqrt{\begin{array}{c}\left(\frac{u_{ws}}{ws}\right)^2 + \left(\frac{u_{vfs1}}{vfs1}\right)^2 + \left(\frac{u_{vps1}}{vps1}\right)^2 \\ + \left(\frac{u_{vfs2}}{vfs2}\right)^2 + \left(\frac{u_{wt}}{wt}\right)^2 + \left(\frac{u_{vft1}}{vft1}\right)^2 \\ + \left(\frac{u_{vpt1}}{vpt1}\right)^2 + \left(\frac{u_{vft2}}{vft2}\right)^2 + \left(\frac{u_{As}}{As}\right)^2 \\ + \left(\frac{u_{At}}{At}\right)^2\end{array}} \quad (7)$$

where ws and u_{ws} are weight of reference standard and its respective uncertainty, $vfs1$ and u_{vfs1} are volume of volumetric flask used to prepare standard stock solution and its respective uncertainty, $vps1$ and u_{vps1} are volume of volumetric pipette used to prepare standard diluted solution and its respective uncertainty, $vfs2$ and u_{vfs2} are volume of volumetric flask used to prepare standard diluted solution and its respective uncertainty, wt and u_{wt} are weight of test sample and its respective uncertainty, $vft1$ and u_{vft1} are volume of volumetric flask used to prepare test stock solution and its respective uncertainty, $vpt1$ and u_{vpt1} are volume of volumetric pipette used to prepare test diluted solution and its respective uncertainty, $vft2$ and u_{vft2} are volume of volumetric flask used to prepare test diluted solution and its respective uncertainty, As and u_{As} are peak area obtained for reference standard diluted solution and its respective uncertainty, and At and u_{At} are peak area obtained for test diluted solution and its respective uncertainty.

The main uncertainties identified for the test were associated with the weight of metoprolol tartrate and hydrochlorothiazide reference standards, contributing to 53 and 72% of overall uncertainties. It can be explained due to the small amount of reference standards used. Moreover, the variability of peak areas for metoprolol tartrate and hydrochlorothiazide in sample and standard solutions contributed with 9 and 19% of overall uncertainty, respectively. A contribution summary of each component is shown in Figure 5(A).

The metoprolol tartrate and hydrochlorothiazide content results and their uncertainty measurement obtained using Eurachem procedure were 100.1 ± 2.8 and 100.3 ± 2.4%, respectively. These results were closely to ones found with Monte Carlo simulation (100.1 ± 2.74% for hydrochlorothiazide and 100.3 ± 2.37% for metoprolol tartrate) and using in-house validation data (100.0 ± 2.99% for hydrochlorothiazide and 100.3 ± 2.58% for metoprolol tartrate). Measurement uncertainty values obtained from validation data were calculated using the accuracy and precision results at 12.5 and 100 μg mL^{-1} for hydrochlorothiazide and metoprolol tartrate, respectively. 50,000 assay results were simulated and calculated by random raw data using Monte Carlo method.

It is worth to be mentioned that the uncertainty value for metoprolol tartrate ($U_{MET} = 2.8\%$) was found to be higher than the target uncertainty value ($U^t = 2.5\%$). On the contrary, the measurement uncertainty value obtained for hydrochlorothiazide ($U_{HCT} = 2.4\%$) was lower than the target uncertainty ($U^t = 2.5\%$). The uncertainty associated with weight of the reference standard may be improved in order to obtain an uncertainty value for hydrochlorothiazide below the target uncertainty value.

Measurement uncertainty information should be taken into account to reduce false risk of conformity/non-conformity decisions. Thus, considering the measurement uncertainty values obtained for metoprolol tartrate and hydrochlorothiazide, the false risk conformity decisions were estimated using Monte Carlo

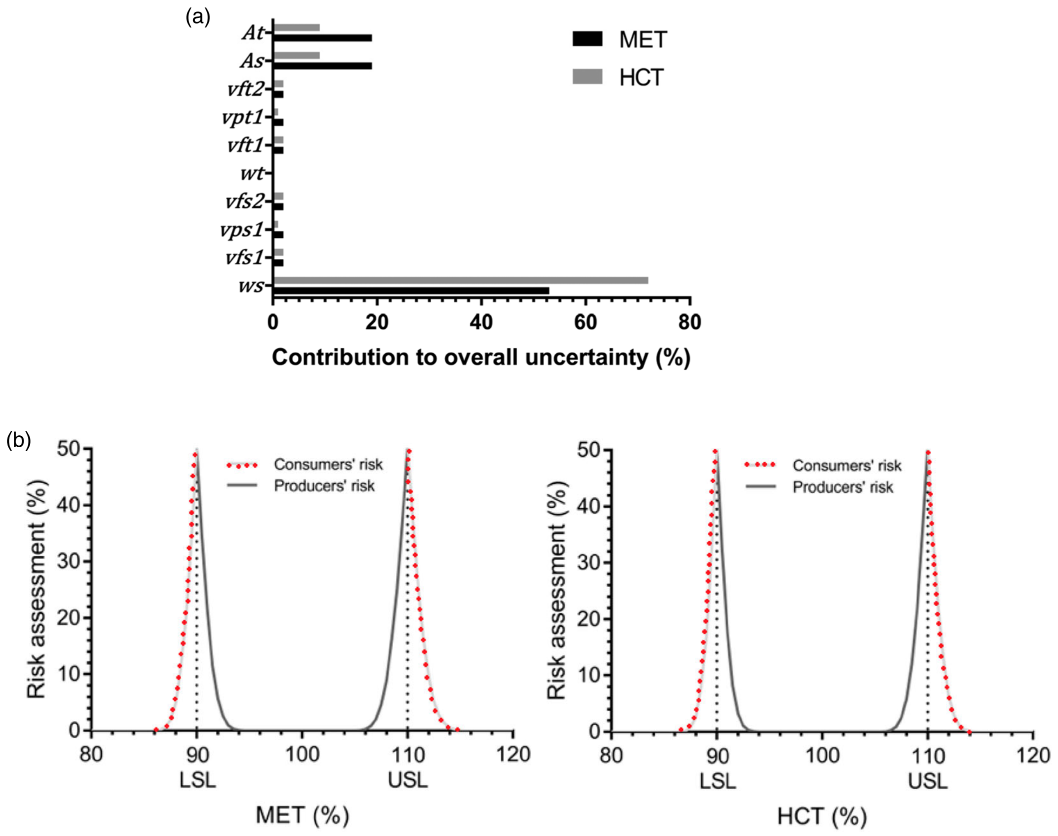


Figure 5. (A). Contribution of each source of uncertainty to the overall uncertainty associated with the quantification of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) by UHPLC. *ws* = weight of MET reference standard; *ws* = weight of HCT reference standard; *vfs1* = volumetric flask; *vps1* = volumetric pipette; *vfs2* = volumetric flask; *wt* = weight of sample; *vft1* = volumetric flask; *vpt1* = volumetric pipette; *vft2* = volumetric flask; *As* = area of MET in standard; *At* = area of MET in sample; *As* = area of HCT in standard; and *At* = area of HCT in sample. (B). Consumers' and producers' risks due to measurement uncertainties estimated as a function of metoprolol tartrate (MET) and hydrochlorothiazide (HCT) quantifications. LSL = Lower Specification Limit; USL = Upper Specification Limit.

simulations. Consumers' and producers' particular risks as a function of metoprolol tartrate and hydrochlorothiazide quantification are presented in Figure 5(B).

Alternatively, guard bands (*g*) may be used to define acceptance (or rejection) region which ensures a reduced risk of false acceptance (or rejection). Generally, by multiplying the uncertainty value (*u*) by an adequate coverage factor (*k*, usually 1.64 for a 5% risk of false decision), it is possible to obtain *g*. In order to provide a lower risk of false acceptance (consumer's risk), *g* is subtracted and added to the upper and lower specification limits, respectively. Considering measurement uncertainty values obtained for metoprolol tartrate and hydrochlorothiazide and specification interval from 90 to 110%, the acceptance limits are from 92 to 108% and from 92.3 to 107.7% for metoprolol tartrate and hydrochlorothiazide, respectively. On the other, *g* is added and subtracted to the upper and lower specification limits to provide a lower risk of false rejection (producer's risk). Thus, the lower and upper rejection limits will be 88 and 102% for metoprolol tartrate and 87.7 and 102.3% for hydrochlorothiazide, respectively.

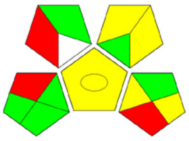
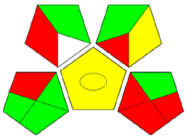
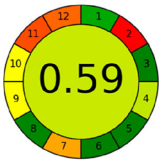
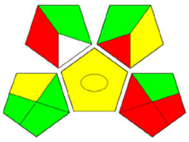
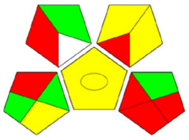
3.4. Application of the proposed method

Both active pharmaceutical ingredients (hydrochlorothiazide and metoprolol tartrate) were analysed using the validated UHPLC method in binary Selopress® coated tablets. The assay of hydrochlorothiazide and metoprolol tartrate in the pharmaceutical formulation indicated a mean value of 12.94 ± 0.53 mg/tab (103.52%, %RSD = 0.53) and 99.94 ± 0.08 mg/tab (99.94%, %RSD = 0.08), respectively that found to be in good compliance with label claim amount 90–110% [46].

3.5. Evaluation of proposed method greenness and comparison with three reported methods

Based on green analytical chemistry approaches (Analytical GREEnness and Green Analytical Procedure Index), the developed UHPLC method were evaluated. Green Analytical Procedure Index shows green, yellow, or red colours which represent the low, medium, or high environmental concerns. Results showed that 6 regions are shaded green, 5 yellow, 3 red and 1 white indicating the proposed method was greener compared

Table 5. Evaluation of proposed method and comparison with literature.

Method	Mobile phase	Run time (min)	Flow rate (mL min^{-1})	Waste (mL/run)	Green Analytical Procedure Index	Analytical GREEnness
UHPLC Proposed Method	acetonitrile:water: triethylamine (17:83:0.2, v/v)	3	0.9	2.7	 (6 Green, 5 Yellow, 3 Red)	
HPLC [20]	methanol:water (95:5, v/v)	13	1.2	15.6	 (6 Green, 2 Yellow, 6 Red)	
HPLC [21]	phosphate buffer:methanol (60:40, v/v)	14	1	14	 (6 Green, 3 Yellow, 5 Red)	
HPLC [23]	(A) sodium phosphate buffer 34 mM; (B) acetonitrile at a gradient elution: 15% B; 15%–90% B; 90%–15% B; 15% B	13	1	13	 (4 Green, 4 Yellow, 6 Red)	

to three reported high performance liquid chromatographic methods (Table 5).

Analytical GREEnness also reveals the greenness profile by specific pictogram colour ranging from red to dark green with a score from 0.0 to 1.0. The proposed UHPLC method was more eco-friendly with a final score of 0.79 as compared to three reported HPLC methods (Table 5). Therefore, both are highly recommended and widely used approaches that produce an informative and easily interpretable result for the assessment of greenness.

4. Concluding remarks

A fast, linear, precise, accurate and robust UHPLC method was developed, optimized and validated by using Central Composite Design for simultaneous quantitation of hydrochlorothiazide and metoprolol tartrate in pharmaceutical binary tablet and in presence of their degradation products. No interference was detected at retention times of both active pharmaceutical ingredients from matrix sample. The forced degradation conditions (photolytic, oxidative, neutral, acidic and alkaline hydrolysis) were also performed. No metoprolol tartrate degradation product was observed, while one hydrochlorothiazide degradation product was observed when the sample was submitted to photolytic, oxidative, neutral, acidic and alkaline hydrolysis.

The novelty of the described UHPLC method is the validation and measurement uncertainty estimation. The producer's and consumer's risks were estimated for compliance assessment using uncertainty measurement and Monte Carlo simulations to obtain a reliable analytical procedure. Moreover, the proposed method greenness is also evaluated and compared with the published HPLC methods. Therefore, the proposed method can be effectively implemented for analytical quality control in the pharmaceutical industries.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study is available from the corresponding author upon reasonable request.

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