

Ethanol-based, stability-indicating RP-HPLC of the hydrolytically labile prodrug azilsartan medoxomil on a phenyl stationary phase: response-surface development and greenness assessment

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Abstract

Azilsartan medoxomil is an angiotensin II receptor blocker formulated as a hydrolytically labile ester prodrug, so its quantitative determination requires chromatographic conditions that resolve the intact ester from its hydrolysis product and limit degradation in solution during analysis. An eco-friendly, stability-indicating reversed-phase high-performance liquid chromatographic method was developed and validated for the assay of azilsartan medoxomil in tablets. Azilsartan medoxomil and its hydrolysis product azilsartan were separated on a phenyl stationary phase with a mobile phase of aqueous ammonium acetate and ethanol as the sole organic modifier. Response surface methodology was applied to model the resolution between the two compounds as a function of mobile-phase pH and ethanol content, and the apparent chromatographic acid dissociation constant of the analyte was determined to support the choice of working pH. Hydrolysis of the prodrug in the diluents examined was quantified and controlled through a low-temperature sample-handling regime, ensuring that the intact ester rather than its degradation product was measured. Validation followed ICH Q2(R2) and covered specificity, linearity, accuracy, precision, and the limits of detection and quantitation. Forced degradation under acidic, basic, oxidative, thermal and photolytic stress confirmed the stability-indicating capability, with the parent peak resolved from every degradation product detected under conditions that left a measurable parent peak (minimum resolution 2.01), and the limits of detection and quantitation were 0.065 and 0.198 mg/mL, respectively. Application to a commercial tablet formulation gave results consistent with the label claim. The environmental profile was assessed with four complementary metrics, yielding an Analytical Eco-Scale score of 88, a MoGAPI index of 77 and a CaFRI score of 77, together with an AGREE index of 0.56; the residual penalties arose from solvent throughput and the off-line, single-analyte format rather than from reagent hazard. The method provides a greener alternative for routine quality control and stability evaluation of azilsartan medoxomil in solid dosage forms.

Introduction

Arterial hypertension is among the most prevalent modifiable risk factors for cardiovascular, cerebrovascular, and renal morbidity, and pharmacological control of blood pressure remains a cornerstone of its management[1, 2]. Within the therapeutic armamentarium, angiotensin II receptor blockers (ARBs, "sartans") occupy a central position through their selective antagonism of the angiotensin II type 1 (AT₁) receptor and their favorable tolerability profile [3, 4]. Azilsartan medoxomil (AZM), the medoxomil ester prodrug of azilsartan, is one of the more recently introduced members of this class and has demonstrated competitive blood pressure-lowering efficacy relative to earlier ARBs in comparative clinical studies[5–7], sparked interest in reliable analytical procedures for its quality control. This interest is driven by the molecule's structure, which has direct and largely underappreciated consequences for method design: AZM combines a biphenyl scaffold, a tetrazole ring, and a 1,2,4-oxadiazol-5(4H)-one moiety so that its chromatographic retention is governed jointly by hydrophobic partitioning and by the ionization state of the acidic tetrazole, whose pK_a lies in the weakly acidic region characteristic of sartans[8], whereas the pronounced aromatic and heteroaromatic character of the analyte suggests that aryl-bonded stationary phases, through supplementary π – π interactions, may

afford a selectivity distinct from that of conventional C₁₈ surfaces[9]. Superimposed on these retention considerations is the hydrolytic lability of the medoxomil ester, the very feature that confers oral bioavailability: in aqueous and hydro-organic media, the prodrug is readily converted to azilsartan[10], so that any quantitative procedure intended for routine control or stability monitoring must not only resolve the parent compound from this principal hydrolysis product but also be designed – in its choice of diluent, mobile phase, and thermal regime – to suppress on-solution and on-column hydrolysis throughout the analytical sequence, a constraint that is seldom addressed explicitly in published assays.

Reported chromatographic procedures for AZM have predominantly relied on acetonitrile- or methanol-based mobile phases combined with nonvolatile phosphate buffers[10]; however, while such systems provide adequate performance, they depend on petrochemically derived, hazardous organic modifiers and buffers that are incompatible with mass spectrometric detection and prone to precipitation at elevated organic contents. More recent reports have begun to introduce green chemistry and quality-by-design considerations into AZM analysis[11, 12], a development that is consistent with the broader reframing of solvent choice as a determinant of overall method sustainability rather than a purely chromatographic variable, in which successive solvent selection guides consistently rank low-molecular-weight, renewable alcohols – mainly ethanol – well above acetonitrile and methanol on the basis of combined environmental, health, and safety criteria[13–15]. The higher viscosity and somewhat lower elutropic strength of ethanol have historically constrained its use as a reversed-phase modifier; however, these limitations are largely manageable through modest adjustments of mobile-phase composition and column temperature[16], and the replacement of phosphate with a volatile ammonium-based buffer further lowers the operational and environmental burden while retaining compatibility with hyphenated detection. Multivariate, model-based development that integrates quality-by-design with green analytical chemistry – coupling risk assessment, design of experiments, and design-space definition so that method robustness is secured while environmental burden and analyst exposure are minimised – is established practice for stability-indicating assays required to resolve an active ingredient from its hydrolytic degradation products[17]. Despite these advantages, methods that simultaneously employ a bioderived organic modifier, a volatile and MS-compatible buffer, an aryl-bonded stationary phase selected on a mechanistic basis, and an explicit strategy for stabilizing the labile prodrug remain scarce; furthermore, no systematic, model-based development of an ethanol-based, stability-indicating assay for AZM on an aryl-bonded phase has been reported. Design-of-experiments approaches have been applied to AZM before, but within acetonitrile/phosphate systems and on alkyl-bonded phases[18].

The present study develops an ethanol-based, stability-indicating assay for azilsartan medoxomil on a phenyl phase in which the hydrolytic lability of the prodrug is treated as a primary constraint on method design rather than an incidental complication. Using response surface methodology, the joint dependence of retention and AZM-azilsartan resolution on the mobile-phase pH and ethanol content was mapped on a phenyl stationary phase, and the apparent chromatographic pK_a of the analyte was characterized to rationalize the selection of the working pH. A common but rarely examined weakness of previously reported procedures is that the medoxomil ester hydrolyzes to azilsartan under the same conditions used to dissolve and dilute the sample. Methods that do not measure or control this

conversion during sample preparation may therefore report too little of the intact prodrug and too much of its main degradation product, treating hydrolysis caused during preparation as if it were the true content of the tablet. Accordingly, the hydrolytic instability of AZM in candidate diluents was quantified explicitly and suppressed through a low-temperature handling regime so that the intact ester rather than its hydrolysis product is what the procedure actually measures. The method was then validated for specificity, linearity, accuracy, precision, and sensitivity in accordance with ICH Q2 (R2)[19], with the objective of providing a reliable and environmentally responsible alternative to established acetonitrile/phosphate procedures for the routine quantification and stability monitoring of azilsartan medoxomil in tablet dosage form.

Methods

Materials and methods

All reagents and solvents were of analytical or HPLC grade.

Reference material: azilsartan medoxomil potassium, reference standard manufactured by the State Enterprise “Scientific Pharmacopoeial Center for Quality of Medicines” (Kharkiv, Ukraine). (Assay: 98%).

Analyzed sample: Edarbi[®], tablets 40 mg

Reagents: ethanol (96% (v/v), LUX grade) was obtained from the State Enterprise Ukrspyrtr (Ukraine). Ammonium acetate, glacial acetic acid, 2-propanol, acetone, dimethyl sulfoxide (DMSO), hydrochloric acid, sodium hydroxide and hydrogen peroxide (all ACS reagent grade) were obtained from Sigma-Aldrich.

Equipment and chromatography conditions

Glassware: volumetric glassware - volumetric flasks and pipettes, class A.

Analytical balance: Mettler Toledo XS204 (maximum capacity 220 g, readability 0.1 mg).

Ultrasonic bath: Daihan, WucA010H, Republic of Korea.

UV-spectrophotometer: UV 1800, Shimadzu (Japan)

Method development and validation were performed on a Nexera LC-30 system (Shimadzu, Kyoto, Japan) and on an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with a G1315A diode-array detector. Spectral data used for peak-purity evaluation were acquired and processed with Agilent ChemStation rev. A.08.03.

Stationary phases: XBridge Phenyl 250×4.6 mm, 5 µm; XBridge C18 250×4.6 mm, 5 µm.

Chromatography conditions for azilsartan medoxomil determination in tablets:

Column: XBridge Phenyl 250×4.6 mm, 5 µm.

The mobile phase was a mixture of 0.02 mol/L ammonium acetate solution (pH 3.5, adjusted with acetic acid) and ethanol (96%) at a ratio of 35:65 (v/v).

The column thermostat temperature was 25 °C.

Autosampler temperature: 5 °C.

Wavelength: 250 nm.

The flow rate of the mobile phase was 1.0 mL/min.

Injection volume: 2 µL.

Solution preparation

Unless otherwise specified, all standard and sample solutions were prepared via precooled 96% ethanol (5 °C).

Preparation of the solution for testing the suitability of the chromatographic system:

System suitability was verified with respect to column efficiency (N), the repeatability of the response expressed as the relative standard deviation (RSD) of the peak areas, peak symmetry (As), and resolution (Rs) between azilsartan medoxomil and its closest-eluting degradation product. For efficiency, RSD, and symmetry, no dedicated solution is needed: these parameters were obtained from six consecutive injections of the *standard solution* (0.8 mg/mL) described below, which serves directly as the system suitability solution. Resolution, which requires the degradation product to be present, was measured on a thermally stressed solution prepared as described below under thermally forced degradation.

Standard solution (for quantitative determination and linearity): A total of 20.0 mg of the azilsartan medoxomil potassium reference standard was placed into a 25 mL volumetric flask. The standard was dissolved and diluted to volume with the diluent. This solution has a nominal concentration of 0.8 mg/mL. Concentrations and results are expressed as azilsartan medoxomil (M = 568.53 g/mol). The reference standard was the potassium salt (azilsartan medoxomil potassium, M = 606.62 g/mol; assay 98.0%), and weighings were corrected both for the certified assay value and for the salt-to-base factor of 0.9372. The label claim of the drug product (40 mg per tablet) is expressed on the same basis. The solution was freshly prepared before use.

Assay sample preparation (working solution): Tablets were weighed and finely powdered, and a portion of powder equivalent to 40 mg of azilsartan medoxomil (180.0 mg) was transferred to a 50 mL volumetric flask, dispersed in the diluent and made up to volume. The flask was then placed in an ice-water ultrasonic bath (5 °C) for 5 minutes to ensure complete extraction. The resulting solution was filtered through a 0.45 µm syringe filter, and the first 3 mL was discarded.

Preparation of solutions for linearity and accuracy: Nine solutions were prepared by spiking a placebo mixture. Approximately 140.0 mg of the placebo (excipient mixture) was placed into a series of 25 mL volumetric flasks. Azilsartan medoxomil potassium as a reference standard, ranging from 16.0 mg to 24.0 mg (in 1 mg increments), was added to each flask. The contents were diluted to volume with the diluent, sonicated in an ice-water bath for 5 minutes, and filtered through a 0.45 µm syringe filter, discarding the first 5 mL. This produced solutions in the concentration range of 0.64-0.96 mg/mL (80–120% of the nominal concentration).

Preparation of solutions for specificity (forced degradation)

Forced degradation studies were performed on crushed tablet powder.

(1) *Acid degradation:* 90 mg of powder was placed in a 25 mL volumetric flask, and 5 mL of 0.1 M HCl and 5 mL of 96% ethanol were added.

(2) *Base degradation:* 90 mg of powder was placed in a 25 mL volumetric flask, and 5 mL of 0.1 M NaOH and 5 mL of 96% ethanol were added.

(3) *Oxidative degradation under alkaline conditions:* 90 mg of powder was placed in a 25 mL volumetric flask, and 5 mL of 0.1 M NaOH, 5 mL of 6% H₂O₂ and 5 mL of 96% ethanol were added.

(4) *Oxidative degradation under neutral conditions:* 90 mg of powder was placed in a 25 mL volumetric flask, and 5 mL of 6% H₂O₂ and 5 mL of 96% ethanol were added.

Solutions (1) – (4) were held for 2 h at 25 °C. The reactions were then quenched by adding 5 mL of 0.1 M NaOH to flask (1) and 5 mL of 0.1 M HCl to flasks (2) and (3); flask (4) required no neutralization. All four flasks were then diluted to volume with 96% ethanol.

(5) *Thermal degradation:* 90 mg of powder was heated on a hot plate at 170 °C for 10 min. The cooled powder was transferred to a 25 mL volumetric flask and diluted to volume with 96% ethanol.

(6) *Photolytic degradation:* 90 mg of powder was exposed to an 8 W UV lamp for 2 h. The exposed powder was transferred to a 25 mL volumetric flask and diluted to volume with 96% ethanol. Photolytic exposure was applied as a screening stress test and not as a formal photostability study in the sense of ICH Q1B[20].

All final degradation solutions (1) – (6) were filtered through a 0.45 µm syringe filter before injection.

Results and discussion

Organic solvent selection.

Conventional reversed-phase HPLC methods applied to the analysis of angiotensin II receptor blockers, including azilsartan medoxomil, predominantly rely on mobile phases composed of acetonitrile or methanol combined with inorganic buffer systems, most commonly phosphate buffers. While these solvent systems provide satisfactory chromatographic performance, they raise substantial concerns from both environmental and operational perspectives. Acetonitrile and methanol are classified as hazardous organic solvents because of their toxicity, flammability, and unfavorable environmental profiles, whereas phosphate buffers are nonvolatile and prone to precipitation, particularly at elevated organic modifier concentrations, which can compromise column integrity and system robustness.

In recent years, the principles of green analytical chemistry have increasingly influenced chromatographic method development, promoting the reduction or substitution of solvents associated with high environmental, health, and safety (EHS) risks. Within this framework, the concept of a “green solvent” extends beyond simple toxicity considerations and encompasses a comprehensive assessment of the solvent life cycle, including renewability, biodegradability, atmospheric emissions, energy demand for production, and waste management[13]. Consequently, solvent selection is now recognized as a critical determinant of the overall sustainability of analytical methods rather than a purely chromatographic optimization parameter.

Several solvent selection guides have been developed to support environmentally responsible decision-making in both industrial and analytical chemistry. These guides systematically rank solvents according to multidimensional criteria that integrate human toxicity, environmental persistence, ecological impact, and safety hazards such as flammability and explosiveness[14, 15]. A consistent conclusion emerging from these comparative assessments is that acetonitrile and methanol occupy an unfavorable position owing to their petrochemical origin, toxicity, and disposal challenges, whereas low-molecular-weight alcohols derived from renewable sources, particularly ethanol, are repeatedly classified among the most environmentally acceptable organic solvents[14, 15].

Ethanol has therefore attracted increasing attention as a viable green alternative to traditional organic modifiers in reversed-phase HPLC. From an environmental standpoint, ethanol offers several advantages: it can be produced from renewable biomass, is readily biodegradable, has low ecotoxicity, and poses significantly reduced risks to laboratory personnel compared with acetonitrile and methanol[13]. Importantly, modern solvent selection frameworks emphasize that ethanol’s favorable classification is not merely qualitative but is supported by quantitative life cycle assessment metrics, in which ethanol consistently demonstrates lower cumulative energy demand and reduced greenhouse gas emissions[13, 14].

From a chromatographic perspective, the use of ethanol as an organic modifier has historically been limited by concerns regarding higher viscosity and reduced elution strength relative to methanol.

However, multiple studies and practical evaluations have demonstrated that high-purity ethanol, when used under optimized conditions, provides chromatographic performance comparable to that of methanol in reversed-phase systems, particularly for moderately lipophilic analytes[15, 21]. Although ethanol has slightly different eluotropic strengths than acetonitrile[16] does, this effect can be compensated for by modest adjustments in the organic phase proportion without compromising resolution or peak symmetry. Importantly, the physicochemical properties of ethanol allow it to maintain sufficient solvating power while offering superior safety and environmental characteristics.

Overall, the developed eluent system, which is based on ethanol and a volatile ammonium acetate buffer, exemplifies the practical implementation of green solvent selection principles in pharmaceutical analysis. By replacing acetonitrile or methanol with ethanol, this method substantially reduces the environmental burden and occupational hazards while maintaining chromatographic efficiency suitable for routine quality control of azilsartan medoxomil. This approach is consistent with contemporary solvent selection guides and demonstrates that environmentally friendly solvents can be successfully integrated into reliable HPLC methods.

Wavelength selection

Ethanol as a component of the mobile phase has sufficient optical transparency for wavelength selection in the UV range from 210 to 400 nm[22]. Wavelength selection was performed in our previous study, where it was demonstrated that azilsartan medoxomil exhibited a distinct absorption band in the range of 230–260 nm regardless of the pH of the medium [23]. Based on this data, a detection wavelength of 250 nm was selected for the present study.

Salt modifier selection

The replacement of nonvolatile phosphate buffers with volatile buffer systems aligns both with chromatographic requirements and with green analytical chemistry principles. Ammonium salts have been applied successfully to the chromatography of sartans, including an ammonium acetate-based mobile phase used for the simultaneous determination of valsartan and atenolol in a fixed-dose combination [24]. Ammonium acetate was therefore selected as the buffering agent: it is volatile, it is compatible with both ultraviolet and mass spectrometric detection, and it carries no risk of the precipitation that limits the use of phosphate buffers at the high organic content required here.

Investigation of the retention factor at different pH values

Therefore, an initial study was conducted on the behavior of azilsartan medoxomil in reversed-phase chromatography (RPC) mode using ethanol as both the mobile phase component and the sample diluent.

The retention factor (k) expresses the ratio of the time an analyte spends in the stationary phase to the time it spends in the mobile phase, $k = (t_R - t_0)/t_0$, where t_0 is the column hold-up time ($t_0 = 2.02$ min

under the working conditions), obtained from the first baseline disturbance after injection of the diluent. According to the fundamental principles of liquid chromatography, values of k between approximately 1 and 10 are generally preferred for effective method development[25].

Maintaining the k value within this range is critical for several reasons. A retention factor that is too low ($k < 1$) often results in the analyte eluting too close to the "solvent front" or the t_0 -disturbance, which is frequently caused by refractive index changes between the sample solvent and the mobile phase. Conversely, a k value that is excessively high leads to unnecessarily long analysis times and broader, less intense peaks, which can compromise the sensitivity of the quantitative determination. For the purpose of developing a rapid and efficient routine control method for azilsartan medoxomil, a target k range of 2–5 was prioritized

To investigate the RPC behavior of azilsartan medoxomil, chromatographic conditions were evaluated at three different pH values (7.0, 4.0, and 2.0) and four ethanol concentrations (50%, 60%, 70%, and 80% v/v). The combined results of this study are presented in Fig. 1.

The data in Fig. 1 illustrate two critical trends:

1. Effect of ethanol: At all three pH values, the retention factor (k) decreases as the concentration of ethanol increases (from 50% to 80%). This is consistent with a standard reversed-phase retention mechanism in the ethanol-based mobile phase.
2. Effect of pH: the retention of azilsartan medoxomil is strongly pH dependent. At any given ethanol concentration, the k values at pH values of 4.0 and 2.0 are nearly identical and significantly higher than the k value at pH 7.0. At pH 7.0, the retention is minimal (e.g., $k \approx 0.5$ at 60% EtOH), which is insufficient for a reliable assay.

The dependence of azilsartan medoxomil retention on the mobile phase pH exhibited a characteristic sigmoidal profile, which was consistent with an ionization-controlled retention mechanism in reversed-phase chromatography. As shown in Fig. 2(A), the transition from the strongly retained neutral form to the weakly retained ionized form occurred within a relatively narrow pH range, and the inflection point of the fitted sigmoid, corresponding to the apparent chromatographic pK_a , was located at $pK_{a, app} = 4.729$, with a standard error of 0.109 and a 95% confidence interval of 4.25–5.03 ($t = 2.776$, 4 df; $R^2 = 0.991$). To corroborate the nonlinear fit, the first analytical derivative of the model, dtR/dpH , was computed and is presented alongside the experimental numerical differences in Fig. 2(B). The minimum of the derivative curve, which corresponds to the steepest change in retention and thus the inflection point of the sigmoid, coincided with the fitted $pK_{a, app}$ value, providing an independent graphical confirmation of the result.

Although the experimental design was designed primarily for chromatographic method optimization rather than formal physicochemical characterization, the obtained pK_a value of 4.73 is in good agreement with the reported aqueous pK_a range of 4.5–5.0 for the tetrazole ionization of azilsartan and

structurally related angiotensin II receptor blockers[8]. The minor systematic offset between the chromatographically determined and literature aqueous values is consistent with the expected medium effect introduced by the high proportion of organic modifier in the mobile phase, which reduces the effective dielectric constant of the eluent and is known to shift apparent pKa values relative to the fully aqueous reference state[26].

Importantly, only minor differences in retention factor were observed between pH 2.0 and 4.0, indicating that azilsartan medoxomil remained sufficiently retained across the acidic region of the investigated chromatographic space. This finding suggested that the use of strongly acidic conditions was not analytically mandatory to maintain acceptable retention. Considering the greater stability of the ethylene-bridged hybrid (BEH) stationary phase[27] within the recommended working pH range of the chromatographic column, acidic pH levels should be prioritized as optimal for sustainable analysis[28].

Column Type Selection

Screening of several stationary phases is a routine step in the chromatography of sartans, where a number of column chemistries commonly have to be compared before acceptable selectivity and efficiency are obtained[29]. At this stage the mobile-phase composition was held constant at 0.02 mol L⁻¹ ammonium acetate and 96 % (/v) ethanol (40:60, v/v), a mid-range value of the interval subsequently examined, so that both columns were compared under identical conditions. XBridge C18 and XBridge Phenyl columns (250 × 4.6 mm, 5 µm) were compared, the pH of the buffer being varied from 1.5 to 7.5 to establish its effect on retention and peak shape. The results are presented in Fig. 3.

Under the investigated conditions, the XBridge Phenyl stationary phase demonstrated consistently longer retention for azilsartan medoxomil than the C18 phase across the entire pH range (e.g., 7.78 min vs. 4.39 min at pH 3.5). This observed behavior is consistent with the enhanced aromatic selectivity often associated with aryl-bonded phases[9, 30]. The improved retention performance is likely attributable to a dual-mode mechanism: hydrophobic partitioning supplemented by potential π - π interactions between the analyte's biphenyl scaffold and the phenyl ligands of the stationary phase. While these specific interactions appear to contribute to the greater selectivity and retention stability observed in this study, such effects are highly dependent on the mobile phase environment and the aromatic character of the analyte [31].

pH 3.5 lies approximately 1.2 units below the experimentally determined pKa, app of 4.73, ensuring that azilsartan medoxomil exists predominantly in its neutral, nonionized form under these operating conditions. According to the Henderson–Hasselbalch equation, this pH offset corresponds to approximately 94% of the analyte remaining in the neutral state, which is consistent with the well-established chromatographic practice of operating below the pKa to maximize hydrophobic retention and ensure reproducible peak shape. This interpretation is further supported by the ionization behavior of structurally analogous sartans: the tetrazole pKa values of losartan (pKa₂ ≈ 4.84) and valsartan (pKa₂ ≈ 4.82), determined by pH-spectrophotometric titration[8], confirm that pH 3.5 places azilsartan medoxomil well within the protonation domain. In addition, pH 3.5 remains within the documented

stability range of XBridge columns, supporting the long-term durability of the stationary phase under routine operating conditions.

At pH 3.5, the XBridge Phenyl column produced 4483 theoretical plates, whereas 2135 was obtained with the C18 column under identical chromatographic conditions, corresponding to a 2.1-fold increase in efficiency. While reports in the literature frequently describe the improved performance of phenyl phases for aromatic analytes due to supplementary π - π interactions, direct quantitative comparisons are highly method dependent, and the magnitude of the efficiency gain observed in the present study reflects system-specific selectivity effects rather than the universal superiority of phenyl over C18 phases. Given the highly aromatic and heteroaromatic architecture of azilsartan medoxomil, comprising a biphenyl scaffold, a tetrazole ring, and a 1,2,4-oxadiazole moiety, enhanced π -electron interactions with the phenyl-functionalized stationary phase likely contributed to the observed improvement in chromatographic efficiency.

Response surface modeling and selection of the working mobile phase composition

The experimental results obtained across the 3×4 screening grid during the investigation of the dependence of the retention factor on the mobile phase pH and ethanol concentration were further processed via response surface modeling. All the statistical computations, ANOVA, model fitting, and graphical outputs were performed via Design-Expert® software (version 13; Stat-Ease Inc., Minneapolis, MN, USA).

For this purpose, chromatographic data obtained at three pH values (2.0, 4.0, and 7.0) and four ethanol (96%) concentrations (50, 60, 70, and 80% v/v) were modeled via response surface methodology (RSM) on the XBridge Phenyl stationary phase. The critical response selected for optimization was the chromatographic resolution (R_s) for separation between azilsartan medoxomil and its primary process impurity and degradation product, azilsartan. This pair was selected as the critical response because, in the intended quantitative assay of the finished product, azilsartan medoxomil and its hydrolytic counterpart azilsartan are the only two species expected to be present and detected; the additional degradants observed under forced stress are not anticipated under normal handling and were therefore not used as optimization targets. Ensuring adequate resolution of this pair across the experimental domain was accordingly taken as the governing requirement for a stability-indicating quantitative method.

The results of the 3×4 factorial design were subjected to analysis of variance (ANOVA) to assess the statistical significance of the linear, quadratic, and interaction terms in the quadratic polynomial model. The ANOVA table for the $\ln(R_s)$ model (Table 1) provides a detailed breakdown of the variance components, allowing for the identification of the dominant factors governing the separation. All model terms, with the exception of the quadratic ethanol term (B^2 , $p = 0.958$), were found to be statistically significant ($p < 0.001$), confirming the adequacy of the fitted model. The overall model F value of 217.67 ($p < 0.001$) indicates that the model is highly significant relative to the residual variance, with excellent

goodness-of-fit ($R^2 = 0.9945$, Adj. $R^2 = 0.9899$). Notably, the 3×4 grid did not include replicate runs or center points; consequently, the error term used in the analysis corresponds to the residual variance of the fitted model rather than to an independent estimate of pure experimental error, and a formal lack-of-fit test could not be performed. The reliability of the model was therefore additionally assessed via leave-one-out cross-validation (Q^2), and the agreement between the predicted and experimentally obtained values at the selected working point was used to confirm its practical adequacy.

Table 1
ANOVA Table for the $\ln(R_s)$ Quadratic Model

Source	Sum of Square	Df	Mean Square	F value	p value	
Model	5.9882	5	1.1976	217.67	< 0.001	significant
A-pH (linear)	0.8222	1	0.8222	149.44	< 0.001	significant
B-%EtOH (linear)	4.1662	1	4.1662	757.19	< 0.001	significant
A ² (pH ²)	0.7522	1	0.7522	136.71	< 0.001	significant
B ² (%EtOH ²)	0.0000	1	0.0000	0.003	0.958	
AB (interaction)	0.1394	1	0.1394	25.33	0.002	significant
Residual	0.0330	6	0.005502	-	-	
Total	6.0213	11	-	-	-	

The final predictive model was expressed as a second-order polynomial equation in terms of actual factors. The regression equation for the natural logarithm of the critical resolution was:

$$\ln(R_s) = 3.354 + 0.979 \times pH - 0.0302 \times (\%EtOH) - 0.0891 \times pH^2 - 1.19 \times 10^{-5} \times (\%EtOH)^2 - 4.69 \times 10^{-3} \times pH \times (\%EtOH) \quad (1)$$

where pH refers to the aqueous buffer component and %EtOH denotes the volume fraction of 96% (v/v) ethanol in the mobile phase. Eq. (1) serves as the basis for the response surface plots and for the identification of the optimal mobile phase composition for the AZM assay.

The two factors enter the model in qualitatively different ways. Ethanol content acts almost purely linearly: its quadratic coefficient is vanishingly small (-1.19×10^{-5}), consistent with the non-significant B² term in the analysis of variance ($p = 0.958$), so that resolution declines steadily as eluent strength rises, without an interior optimum. Mobile-phase pH, by contrast, carries a large positive linear coefficient (+ 0.979) together with a negative quadratic term (-0.0891), which produces a concave surface with a genuine maximum in the acidic region. Setting the partial derivative of Eq. (1) with respect to pH to zero locates that maximum at pH 3.78 for 65% (v/v) ethanol, in the region where azilsartan medoxomil is predominantly neutral and its hydrophobic affinity for the phenyl surface is greatest. The negative

interaction coefficient (-4.69×10^{-3}) shifts the position of the optimum with eluent strength: the stationary point moves from pH 4.18 at 50% ethanol to pH 3.39 at 80%, so that the pH-dependent gain in resolution is progressively eroded as the organic fraction increases. This is the quantitative expression of the significant AB interaction ($p = 0.002$) and the reason why the two factors cannot be optimized independently. The working point of pH 3.5 lies marginally below the modelled optimum; it was preferred because it places the analyte further onto the plateau of the retention–pH sigmoid, and hence provides a margin against small pH deviations, at a negligible cost in predicted resolution. The coupled effects of both factors are visualized in Fig. 4 as a 3D response surface and in Fig. 5 as a 2D contour plot, both generated in Design-Expert® 13.

The response surface revealed two distinct roles for the two factors. Ethanol content was the dominant source of variance in $\ln(R_s)$ ($SS = 4.17$; $F = 757.2$), reflecting the expected loss of retention, and hence of separation margin, as eluent strength increases. Mobile-phase pH accounted for considerably less of the total variance ($SS = 0.82$; $F = 149.4$) but was the only factor to show significant curvature (A_2 , $p < 0.001$; B_2 , $p = 0.958$) and therefore governed the location of the optimum rather than the overall level of the response, the highest resolution values being located in the acidic region. This trend is consistent with the ionization behavior of azilsartan medoxomil: at pH values below the pK_a , app (pH 2.0 and 4.0), the molecule exists predominantly in its neutral, nonionized form, maximizing the hydrophobic affinity for the phenyl-modified stationary phase and thereby enhancing both retention and resolution. Conversely, at pH 7.0 ($> pK_a$, app), deprotonation of the acidic moiety increases molecular polarity, leading to rapid elution and a significant reduction in separation efficiency. The response surface further revealed that the effect of ethanol content could not be interpreted independently of pH, as the selectivity of the system changed in a coupled manner across the experimental domain – an effect captured by the statistically significant AB interaction term ($p = 0.002$).

On the basis of the generated response surface, a suitable operating point was identified at pH 3.5 and 65% (v/v) ethanol (96%), representing an interpolated optimum within the experimental grid rather than an extrapolated estimate. This composition was selected as the final mobile phase because it provided the best overall compromise between selectivity and practical assay performance. At lower ethanol contents, the model predicted further increases in resolution, but at the cost of excessive retention and unnecessarily prolonged analysis time; at higher ethanol fractions, the retention factor and separation margin progressively decreased.

The model-based predictions for the selected operating points are summarized in Table 2.

Table 2
Working point prediction for the selected mobile phase composition (pH 3.5/65% (v/v) EtOH).

Parameter	Value
Status	Interpolation (65% EtOH within 50–80% measured range)
Predicted Rs (EP)	13.54 [95% PI: 10.98–16.69]
Predicted k (API)	2.78 [95% PI: 1.80–4.29]
R ² (Rs model)	0.9945
Adj. R ²	0.9899
Q ² (LOO-CV)	0.9658

The experimental resolution of 11.70 shows good agreement with the model-predicted value of 13.54, which falls well within the 95% prediction interval (10.98–16.69). The experimental resolution remained within the model-predicted 95% prediction interval, supporting the practical adequacy of the selected operating point. The retention factor k was predicted with high accuracy (2.5% error), and the obtained experimental retention factor was 2.85, validating the linear relationship between the solvent strength and retention in the ethanol system.

Stability of Azilsartan Medoxomil

To evaluate stability, several “green” organic solvents, including 2-propanol, acetone, DMSO, 96% ethanol, and aqueous mixtures (DMSO-buffer pH 4.0 and ethanol-buffer pH 4.0), were used. Azilsartan medoxomil (2 mg/mL) was insoluble in 2-propanol and acetone, rendering them unsuitable as diluents. The stability in the remaining solvents was monitored by high-performance liquid chromatography (HPLC) over 12 hours at ambient (20–25°C) temperatures. The results in Table 3 show the percentage of degradation over time.

Table 3
Stability of azilsartan medoxomil in organic solvents at ambient temperature (% degradation).

Solvent	1 hour	2 hours	6 hours	12 hours
2-propanol	2 mg/ml insoluble			
acetone	2 mg/ml insoluble			
DMSO	0.0%	0.6%	3.1%	3.2%
Ethanol 96%	0.8%	1.9%	6.7%	15.6%
DMSO/buffer 4.0 (1:1)	14.9%	25.8%	56.8%	79.5%
Ethanol/buffer 4.0 (1:1)	10.7%	19.8%	45.1%	73.8%

As shown in Table 3, azilsartan medoxomil is significantly more stable in pure organic solvents than in mixtures with aqueous buffers. The presence of water clearly accelerated hydrolysis, with degradation in the buffer mixtures reaching ~ 45–57% in just 6 hours.

Notably, DMSO provided the most favorable stability profile at ambient temperature, with degradation levels remaining below 3.2% over 12 hours. However, despite being categorized as a "green" or "recommended" solvent in some previously mentioned guidelines because of its low vapor pressure and high flash point, DMSO is classified as "Problematic" according to more recent and comprehensive frameworks such as the CHEM21 selection guide[32]. A primary drawback in terms of green analytical chemistry is its exceptionally high boiling point (189°C). This high boiling point significantly hinders solvent recovery through distillation, as the process becomes excessively energy intensive and often impractical in a standard laboratory setting.

In addition to environmental concerns, DMSO poses specific safety risks to laboratory personnel; it is a potent skin penetration enhancer that can facilitate the rapid transdermal transport of both the solvent and dissolved pharmaceutical ingredients into the bloodstream. Given these combined environmental, health, and safety concerns, ethanol was prioritized as a safer, more manageable, and truly sustainable alternative for routine quality control.

However, even in 96% ethanol at ambient temperature, the degradation reached 15.6% after 12 h, which is unacceptable for a reliable quantitative method. Given this rapid hydrolysis, lowering the storage temperature was investigated as a primary strategy to increase the stability of the analytical solutions.

To maintain a comprehensive stabilization environment, the sample compartment was kept at 5°C. This allows the analytical solutions to remain chilled prior to injection, mirroring the precooled 96% ethanol used during sample preparation and effectively suppressing hydrolysis throughout the entire analytical sequence. In this study, the stability of the analyte was evaluated in both 96% ethanol and 100% ethanol, with the solutions maintained at a constant temperature of 5°C. The comparative results of this investigation are presented in Table 4.

Table 4
Stability of azilsartan medoxomil in 96% and 100% ethanol at 5°C (% degradation).

Ethanol 96%	1 hour	2 hours	6 hours	9 hours
	0.8%	0.9%	1.9%	3.2%
Ethanol 100%	0.0%	0.2%	1.1%	1.3%

The experimental data demonstrate that 96% ethanol at 5°C provides an acceptable stability profile for at least 6 hours, which is sufficient to cover the duration of a standard analytical sequence from sample preparation to final chromatographic injection. While absolute ethanol offered slightly greater protection

against degradation, the stability result for 96% ethanol, which resulted in only approximately 2% degradation after 6 h, was deemed entirely acceptable for routine quality control purposes.

Consequently, 96% ethanol was used as the primary solvent for both standard and sample preparation in this method. This selection ensures the chemical integrity of the prodrug throughout the analysis while further aligning with the principles of Green Analytical Chemistry by utilizing a readily available, bioderived organic modifier.

Column thermostat temperature selection

The optimization of the column temperature in ethanol-based HPLC requires a systematic evaluation of both the physical properties of the mobile phase and the chemical stability of the analyte. Ethanol possesses a notably higher viscosity than traditional modifiers such as acetonitrile, which typically results in elevated system back pressure. As emphasized in a recent comprehensive review[33], this relatively high viscosity remains a significant challenge in green HPLC, often acting as a primary barrier to its adoption in routine analysis. However, this can be effectively mitigated under the principles of GAC by increasing the column temperature to reduce the eluent's dynamic viscosity and enhance mass transfer[34]. However, in the case of azilsartan medoxomil, the applicability of this approach is limited by its pronounced susceptibility to hydrolysis, which may be further accelerated under elevated temperatures in aqueous-ethanolic media.

To identify the optimal operating conditions, the influence of temperature on chromatographic parameters and system pressure was investigated across a range of 25°C to 40°C. The experimental findings are summarized in Table 5.

Table 5
Effect of column temperature on the chromatographic parameters and system pressure of azilsartan medoxomil

Column Temperature (°C)	Retention Time (min)	Theoretical Plates (N)	System Pressure, bar (psi)
25	9.05	3499	235 (~ 3408)
30	8.42	3605	214 (~ 3103)
35	7.90	3596	196 (~ 2843)
40	7.71	3662	190 (~ 2756)

As observed in Table 5, increasing the temperature led to a significant reduction in system pressure, from 235 bar at 25°C to 190 bar at 40°C. While higher temperatures successfully reduced the retention time from 9.05 min to 7.71 min, they also increased the risk of on-column hydrolysis. Nevertheless, column efficiency was preserved throughout the investigated column temperature range (3499–3662 theoretical plates), indicating stable stationary phase performance under the applied thermal conditions.

A column temperature of 25°C was selected as an optimal compromise between system backpressure and analyte stability. This temperature provides sufficient resolution from the solvent front while minimizing the risk of thermally induced degradation of the ester prodrug. Under these conditions, a retention time of approximately 9 min was achieved, representing a suitable baseline for routine analysis.

Validation of the azilsartan medoxomil assay procedure

The developed procedure was validated in accordance with the ICH Q2(R2) guideline to confirm its suitability for the intended quantification and stability monitoring of azilsartan medoxomil in the tablet dosage form. The validation encompassed the assessment of system suitability, specificity and selectivity, linearity, accuracy, repeatability and intermediate precision, and the limits of detection and quantitation, with each characteristic evaluated against predefined acceptance criteria. The results obtained for each of these parameters are presented in the following sections.

Chromatographic system suitability

The system suitability met all the predefined acceptance criteria (Table 6), confirming that the chromatographic system was suitable for the intended analysis.

Table 6
System suitability requirements and results.

Parameter*	Requirement	Result
RSD, % of peak areas	≤ 1.0%	0.44%
Efficiency (N)	≥ 2000	4600
Resolution (Rs) (with degradation impurity)	≥ 1.5	2.01
Symmetry (As)	0.8–1.8	1.22
* for 6 consecutive injections (n = 6) of the standard solution.		

Specificity and selectivity

Interference from Placebo

To evaluate the potential impact of the tablet matrix on the determination of azilsartan medoxomil, a comparative study was conducted using three distinct solutions prepared according to the finalized protocol

1. Standard solution: a reference solution of azilsartan medoxomil potassium at the target concentration (0.8 mg/mL).
2. Spiked solution (standard + placebo): a sample prepared by adding a known quantity of the reference standard to the placebo matrix to monitor potential peak distortion or recovery issues.

3. 3. Placebo solution: A placebo obtained from the manufacturer and prepared from all formulation excipients - mannitol, fumaric acid, sodium hydroxide, hydroxypropyl cellulose, croscarmellose sodium, microcrystalline cellulose and magnesium stearate – in their relative proportions, without the active pharmaceutical ingredient (API). The resulting chromatograms are presented in Fig. 6.

Analysis of the chromatograms revealed that the placebo matrix (3) exhibited no detectable peaks or baseline disturbances at the characteristic retention time of azilsartan medoxomil (approximately 8.8 min). The chromatographic profile of the spiked sample (2) was identical to that of the standard solution (1) in terms of peak shape, symmetry, and retention time. Furthermore, the absence of any coeluting peaks at the main peak position was confirmed by the peak purity factor (acceptance threshold 990 on the 0-1000 scale of the software), indicating that the analyte signal is spectrally pure and free from matrix interference. **Figure SEQ Figure * ARABIC 6.** Overlay of chromatograms for specificity testing: Standard (1), Standard + Placebo (2), and Placebo only (3)

Selectivity (forced degradation)

the fundamental requirement for a stability-indicating method is its ability to monitor the active pharmaceutical ingredient without interference from its degradation products. For azilsartan medoxomil, a prodrug characterized by a highly labile ester moiety, the primary challenge lies in resolving the parent molecule from various hydrolytic and oxidative degradants that exhibit similar structural features. To prove the stability-indicating nature of the method, forced degradation studies were conducted on the drug products (crushed tablets) under various stress conditions (acidic, basic, oxidative, thermal, and photolytic).

The results of the stress studies are summarized in Table 7.

Table 7
Summary of forced degradation results for azilsartan medoxomil tablets.

Stress Condition	% Degradation	Peak Purity Factor
0.1 M HCl (120 min)	48.6%	999
6% H ₂ O ₂ (120 min)	80.3%	999
Thermal (170°C, 10 min)	60.1%	999
UV Light (120 min)	34.7%	999
0.1 M NaOH (120 min)	100%	N/A
H ₂ O ₂ 6% + 0.1 M NaOH	100%	N/A
NA = Not Applicable, as the main peak was 100% degraded.		

Under all conditions applied, degradation exceeded the 5–20% range commonly targeted in forced-degradation studies. Twelve chromatographically distinct degradation products were formed and

resolved, compared with the two or three reported in earlier studies of this prodrug, one of which is the hydrolysis product azilsartan [10, 35]. Selectivity was therefore assessed against a wider set of potential interferences than a less severe stress design would provide. Under alkaline and alkaline-oxidative stress no parent peak remained; these conditions establish the base lability of the ester but do not contribute evidence of chromatographic resolution, and at this extent of degradation the mass balance cannot be verified. Figure 7 shows the chromatograms of the most informative stress conditions, demonstrating the formation of multiple degradation products that were well resolved from the AZM peak. The minimum chromatographic resolution observed between AZM and the nearest degradation product was $R_s = 2.01$ under thermal degradation conditions (3).

Most degradation products eluted earlier than azilsartan medoxomil did, which is consistent with the formation of more polar species; under thermal stress, one later-eluting component suggested the formation of a less polar degradation product.

The results of the forced degradation, categorized by the RRT of the observed impurities, are summarized in Table 8. The forced degradation profiles revealed the presence of both common and unique degradation products under different stress conditions, with degradation product 2 (azilsartan) being detected under all the stress conditions.

Table 8
Summary of RRTs for azilsartan medoxomil and its degradation products
under various stress conditions.

Impurity ID	RRT*	Acidic	Oxidative	Thermal	Photolytic
AZM (API)	1.000	+	+	+	+
Deg. Prod. 5	0.322	–	+	–	–
Deg. Prod. 1	0.377	+	–	+	+
Deg. Prod. 6	0.439	–	–	+	+
Deg. Prod. 2	0.499	+	+	+	+
Deg. Prod. 10	0.522	–	–	–	+
Deg. Prod. 3	0.582	+	–	+	–
Deg. Prod. 11	0.614	–	–	–	+
Deg. Prod. 4	0.678	+	–	+	–
Deg. Prod. 12	0.731	–	–	–	+
Deg. Prod. 7	0.791	–	–	+	–
Deg. Prod. 8	0.861	–	–	+	–
Deg. Prod. 9	1.647	–	–	+	–
*RRT calculated relative to the azilsartan medoxomil peak ($t_R \approx 8.8$ min)					

The absence of coelution was confirmed by peak purity analysis via ChemStation software. In all cases of partial degradation, the peak purity factor exceeded 999, which is well above the conservative acceptance threshold of 990, confirming the spectral purity of the analyte peak. Overall, these analytical findings confirm that the proposed method is selective and can be used for assays and stability monitoring throughout its lifecycle.

Linearity

The linearity of the method was examined at concentrations ranging from 0.646 to 0.969 mg/ml. Measurements were performed for nine solutions of different concentrations. As shown in the data in Table 9, the proposed method satisfies all the criteria; the method is linear in the studied range of 0.646–0.969 mg/ml azilsartan medoxomil. The intercept was not significantly different from zero ($t = 70.9/64.39 = 1.10$, against a critical value of 2.365 for seven degrees of freedom) and amounted to 2.8% of the response at the nominal concentration. The range of the procedure was accordingly established as 0.646–0.969 mg/mL, corresponding to 80–120% of the nominal assay concentration.

Table 9
Linearity results.

Parameter	Parameter value	Requirement	Result
y-intercept	-70.9	$\leq 2*SD$	Pass
SD of y-intercept	64.39	–	N/A
Slope	3250	–	N/A
SD of slope	79.09	–	N/A
Coefficient of regression r^2	0.998	≥ 0.998	Pass

Repeatability and intermediate precision

To verify the precision of the method, two series of measurements were performed by two analysts on different days. Each series comprised six independent test solutions ($n = 6$). Repeatability was expressed as the relative standard deviation within a single series performed by one analyst on one day, whereas intermediate precision was evaluated from the comparison of the two series obtained by different analysts on different days. The difference between the mean results of the two series was 0.5%, and the within-series relative standard deviations did not exceed the acceptance limit, confirming that both the repeatability and intermediate precision complied with the requirements.

Table 10
Results of repeatability and intermediate precision for AZM

Result, mg/tab	Analyst 1	Analyst 2	Requirement	Result
	40.32	40.11	Value difference being no more than 2.0%	Pass
RSD, %	0.77	0.44	No more than 2.0%	Pass

Accuracy

Sample-preparation recovery was assessed by residue reextraction to verify the completeness of the sample treatment procedure. The residual amount of azilsartan medoxomil recovered from the tablet matrix after the primary extraction step did not exceed 1.7% of the total analyte content ($n = 9$).

Analytical accuracy was assessed on nine independently prepared placebo-spiked samples covering 80–120% of the nominal assay concentration in nominal increments of 5%, as summarized in Table 11. Recovery ranged from 98.32% to 101.04%, with an overall mean of 100.00% (SD 0.77%, RSD 0.77%, $n = 9$). The 95% confidence interval of the mean recovery, 99.41–100.59%, includes 100%, indicating the absence of a statistically significant systematic error over the range studied. Grouped into the lower (80–90%), central (97–105%) and upper (110–121%) thirds of the range, mean recoveries were 99.88%, 100.36% and 99.76%, with RSD values of 0.18%, 0.39% and 1.37%, respectively, showing no concentration-dependent bias. These data confirm the suitability of the proposed procedure for the quantitative analysis of azilsartan medoxomil in tablets.

Table 11
Results of accuracy for azilsartan medoxomil tablets.

Sample Nº	Added, mg	Found, mg	% Recovery
AZM1	16.14	16.10	99.70
AZM2	17.42	17.43	100.06
AZM3	18.00	17.98	99.89
AZM4	19.39	19.55	100.78
AZM5	19.98	19.96	100.02
AZM6	21.00	21.05	100.27
AZM7	22.12	22.10	99.91
AZM8	23.06	22.68	98.32
AZM9	24.22	24.46	101.04
Mean	N/A	N/A	100.00

Limit of detection (LOD) and the limit of quantitation (LOQ)

The sensitivity of the developed HPLC method was assessed through the determination of the LOD and LOQ, which define the lowest concentrations of the analyte that can be reliably detected and quantified with acceptable accuracy and precision.

In accordance with the recommendations of the ICH Q2(R2), the LOD and LOQ values were calculated on the basis of the standard deviation of the response and the slope of the calibration curve. The LOQ value of 0.198 mg/mL was significantly lower than the lowest concentration of the validated linear range (0.6456 mg/mL) and remained well below the target assay concentration (0.8 mg/mL). The obtained LOD value of 0.065 mg/mL demonstrated that the method possesses sufficient sensitivity for the intended analytical procedure. These findings confirm that the developed method is suitable for routine quality control and stability studies of azilsartan medoxomil in pharmaceutical dosage forms. Moreover, the relatively low detection limits indicate that the method is capable of monitoring low-level changes associated with potential degradation processes, supporting its applicability as a stability-indicating analytical procedure.

Quantitative determination of azilsartan medoxomil in solid dosage form

The developed method was further applied to commercial azilsartan medoxomil tablets (Edarbi®). Taking the two precision series together (n = 12 independent tablet preparations), the determined content was 40.22 ± 0.46 mg/tablet (mean $\pm$ SD; RSD 1.14%), corresponding to a mean assay value of

100.6% of the label claim expressed as azilsartan medoxomil. These results indicate satisfactory agreement with the declared content and support the suitability of the proposed method for routine tablet analysis.

Greenness assessment

The environmental profile of the developed procedure was evaluated using four complementary metric tools, applied in order of increasing scope: the Analytical Eco-Scale, which resolves the contribution of each individual reagent[36]; the Analytical GREENness (AGREE) index, which scores the procedure against the twelve principles of green analytical chemistry[37]; the Modified Green Analytical Procedure Index (MoGAPI), which covers the entire life cycle of the procedure from sample collection to waste management[38]; and the Carbon Footprint Reduction Index (CaFRI), which estimates the associated greenhouse-gas burden[39]. The complete sets of input parameters and penalty points are given in Supplementary Tables S1-S5, and the resulting pictograms are presented in Fig. 8.

The Analytical Eco-Scale assigns penalty points to those features of a procedure that depart from an ideal green analysis – the amount and hazard of each reagent, classified according to the Globally Harmonized System of Classification and Labelling of Chemicals, together with energy demand, occupational exposure, and the volume and fate of the waste – and subtracts them from a maximum of 100, scores above 75 denoting excellent green analysis, 50–75 acceptable and below 50 inadequate[36]. The procedure accumulated 12 penalty points and attained a score of 88. As the Analytical Eco-Scale does not provide a standardized pictogram, the distribution of the penalty points and the position of the resulting score relative to the classification thresholds are presented in Fig. 9 as a graphical representation. The largest single contribution arose from the waste volume and the second largest from the amount of ethanol consumed, whereas hermetic containment of the chromatographic process, recycling of the organic modifier and the absence of any hazard classification for ammonium acetate incurred no penalty at all. The reagent-resolved format makes explicit that the residual penalties of the method are volumetric rather than toxicological in origin.

MoGAPI was applied as a complementary tool, since it covers the entire life cycle of the procedure – from sample collection and storage through reagent hazards to waste management – and represents the outcome as a colour-coded pictogram supported by a numerical index[38]. The procedure attained a MoGAPI score of 77 (Fig. 8A), with green fields assigned to the use of green solvents, the absence of any additional sample treatment, hermetic containment of the analytical process and the quantitative nature of the determination. The remaining penalties were concentrated in off-line collection and in the reagent volumes and waste generated by regular bore liquid chromatography, whereas the physical stabilization of the analyte at 5°C was accounted for as a preservation requirement imposed by the hydrolytic lability of the medoxomil ester. In contrast to the AGREE index, MoGAPI weights the nature of the reagents and the fate of the waste rather than their volume alone, which explains the more favorable outcome and highlights that the volatile ammonium acetate-ethanol eluate is amenable to distillative recovery.

The AGREE metric converts the twelve principles of green analytical chemistry into a normalized 0–1 index, with equal weights assigned to all criteria in the present evaluation[37]. The procedure scored 0.56 (Fig. 8B), the highest contributions arising from the absence of derivatization, the elimination of toxic reagents and the limited number of sample-preparation operations, and the principal penalties from off-line measurement, single-analyte determination and the waste volume generated by a 250 × 4.6 mm column operated at 1.0 mL/min. The latter criteria are inherent to the assay of a single-component solid dosage form rather than to the solvent system employed, and restrict the achievable index for this class of procedure regardless of the mobile phase.

The carbon footprint and resource intensity of the procedure were further evaluated using CaFRI, which extends the assessment beyond reagent hazards to energy consumption, emission factor, sample storage and transport, personnel demand, waste management and recycling[39]. The procedure attained a score of 77 out of 100 (Fig. 8C). Favorable contributions arose from the moderate power demand of the chromatographic system, the absence of energy-intensive non-analytical equipment, the elimination of sample transport and specialised storage, the limited number of hazard pictograms associated with the reagent inventory and the recycling of the organic modifier within the same procedure. The principal penalties were associated with the volume of organic solvent consumed and the waste generated per sample, which reflect the conventional-bore chromatographic format rather than the composition of the mobile phase. Taken together, the four assessments indicate that the environmental burden of the proposed method is governed primarily by solvent throughput, whereas every criterion relating to reagent hazard, renewability and waste fate favours the ethanol-ammonium acetate system over conventional acetonitrile-phosphate procedures.

Conclusions

An eco-friendly RP-HPLC method was developed for the quantitative determination of azilsartan medoxomil in tablet dosage forms. The proposed method uses an ethanol-ammonium acetate mobile phase and a phenyl stationary phase, providing adequate selectivity and efficient separation of the parent compound from its major degradation products. Replacement of conventional organic solvents with ethanol significantly reduces the environmental impact of the analysis while maintaining satisfactory chromatographic performance.

The validation results confirmed that the method meets the requirements for pharmaceutical analysis in terms of linearity, precision, and accuracy. Forced-degradation studies demonstrated the susceptibility of azilsartan medoxomil to alkaline and oxidative conditions and confirmed the stability-indicating capability of the developed procedure. Crucially, the hydrolytic lability of the medoxomil ester was quantified in the candidate diluents and suppressed by a low-temperature handling regime, so that the procedure measures the intact prodrug rather than its hydrolysis product. The environmental performance of the procedure was quantified using four complementary metric tools, giving an Analytical Eco-Scale score of 88, MoGAPI and CaFRI indices of 77, and an AGREE index of 0.56. The residual burden is governed by solvent throughput and the off-line, single-analyte format, whereas every

criterion relating to reagent hazard, renewability and waste fate favours the ethanol-ammonium acetate system. Therefore, the proposed approach can be considered a reliable and environmentally more responsible alternative for routine quality control of azilsartan medoxomil in pharmaceutical preparations.

Abbreviations

AZM

azilsartan medoxomil

ARB

angiotensin II receptor blocker

AT₁

angiotensin II type 1 receptor

HPLC

high-performance liquid chromatography

RP-HPLC

reversed-phase high-performance liquid chromatography

RPC

reversed-phase chromatography

MS

mass spectrometry

API

active pharmaceutical ingredient

EHS

environmental, health and safety

GAC

green analytical chemistry

DMSO

dimethyl sulfoxide

EtOH

ethanol

RSM

response surface methodology

ANOVA

analysis of variance

LOO-CV

leave-one-out cross-validation

k

retention factor

t_r

retention time

t_0

dead (void) time

R_s

chromatographic resolution

N

column efficiency (number of theoretical plates)

A_s

peak asymmetry (symmetry) factor

RSD

relative standard deviation

pKa, app

apparent chromatographic pKa

RRT

relative retention time

SE

standard error

CI

confidence interval

PI

prediction interval

LOD

limit of detection

LOQ

limit of quantitation

ICH

International Council for Harmonisation

UV

ultraviolet

AGREE

analytical GREEnness metric approach

MoGAPI

modified green analytical procedure index

CaFRI

carbon footprint reduction index

Declarations

Ethics approval and consent to participate

Not applicable.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article. Further data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Hlib Hikal: Performing the experimental work, mathematical modeling, validation, writing – original draft.

Oleksandr Syrotchuk: Conceptualization, methodology, method development, supervision, writing – review & editing.

Olga Glushachenko: reviewing and publishing editing, supervision

Roman Markin: translating, reviewing and publishing editing

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Figures

retention factor(k) dependance on pH level and content of EtOH

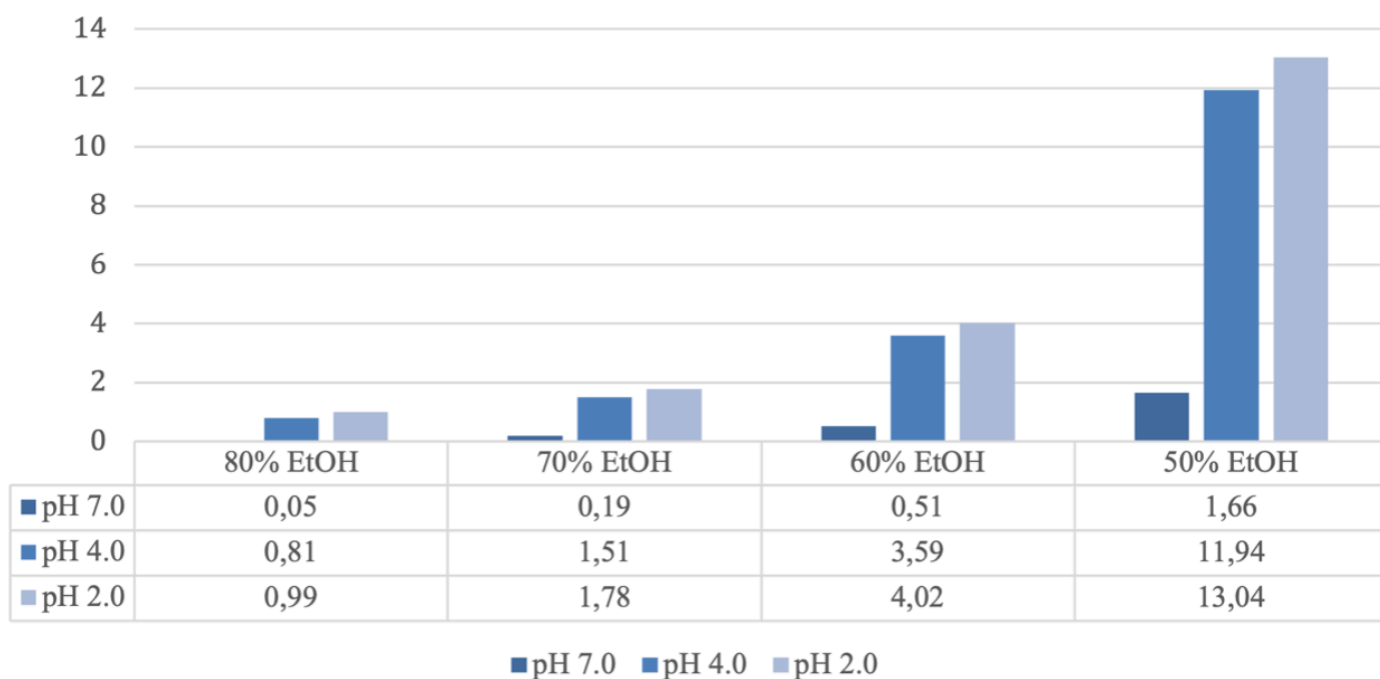


Figure 1

Dependence of the retention factor (k) on the mobile phase pH and ethanol (EtOH) content.

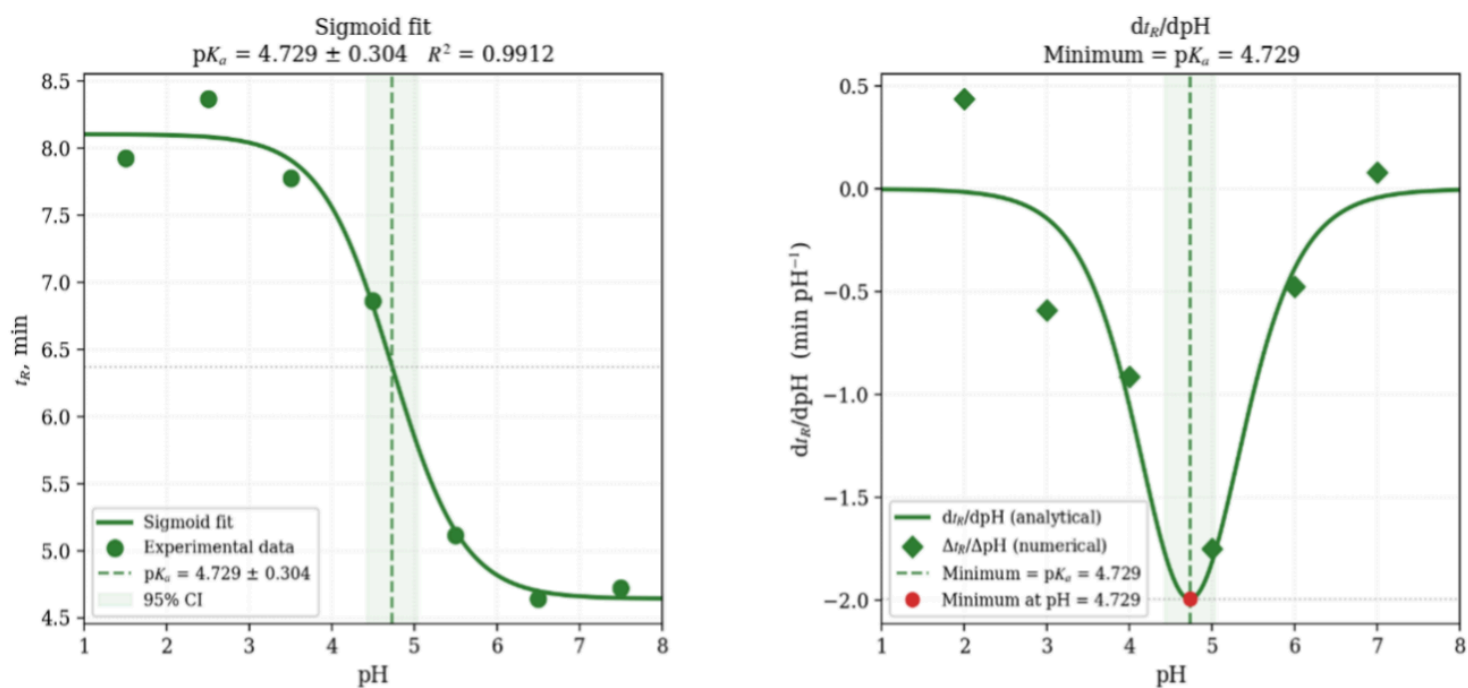


Figure 2

(A) Sigmoidal fit of the retention time (t_R) as a function of mobile phase pH via a Henderson-Hasselbalch-type model; the dashed vertical line indicates the inflection point of the fitted curve corresponding to $pK_a, app = 4.729 \pm 0.304$ (95% CI: 4.425-5.033; $R^2 = 0.991$). **(B)** First analytical derivative of the fitted sigmoid (dt_R/dpH) as a function of pH; numerical differences ($\Delta t_R/\Delta pH$) calculated from experimental data points are shown as filled diamonds. The minimum of the derivative curve (red circle) coincides with the inflection point of the sigmoid, providing a graphical confirmation of the $pK_a, app = 4.729$.

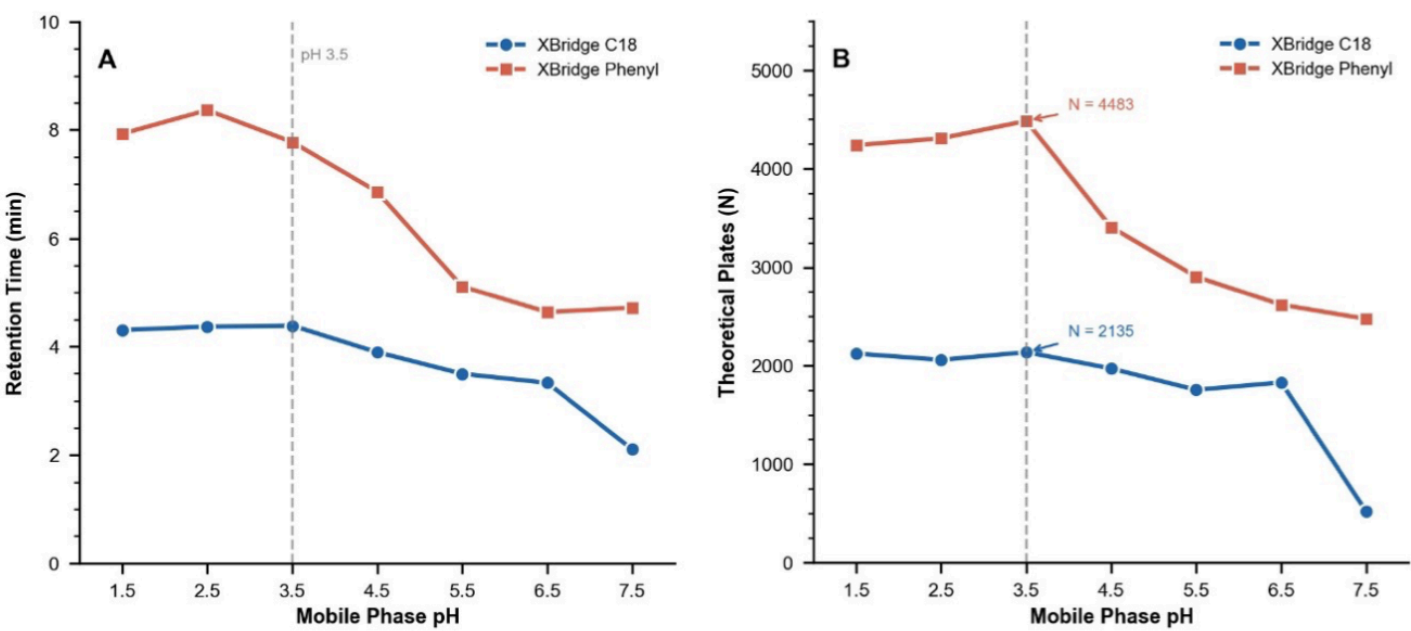


Figure 3

Effect of mobile phase pH on the retention time (A) and column efficiency (B) of azilsartan medoxomil on XBridge C18 and XBridge Phenyl columns.

Factor Coding: Actual

Rs

Design Points:

● Above Surface

● Below Surface

2.11 27.495

X1 = A

X2 = B

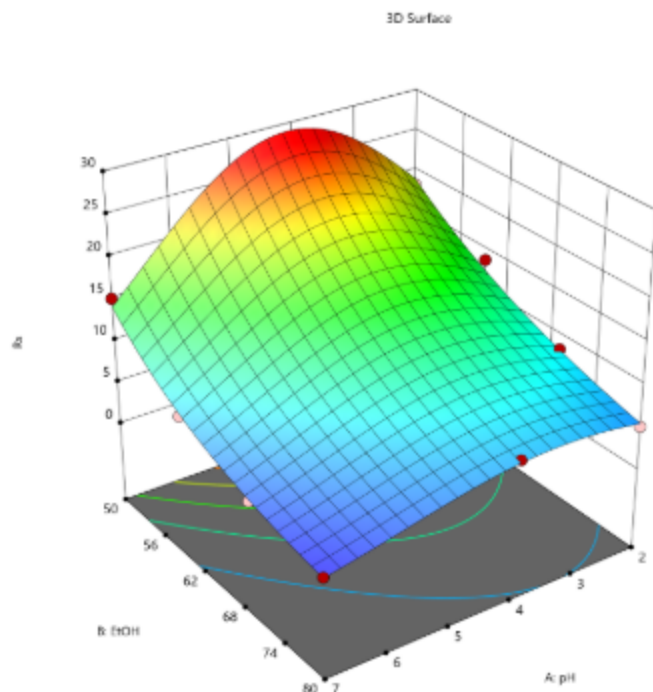


Figure 4

3D response surface plot of the resolution (Rs) as a function of the mobile phase pH and ethanol content. The three-dimensional surface illustrates the quadratic effect of pH (pH^2) on analyte separation on the Waters XBridge Phenyl stationary phase.

Factor Coding: Actual

Rs

● Design Points

2.11 27.495

X1 = A

X2 = B

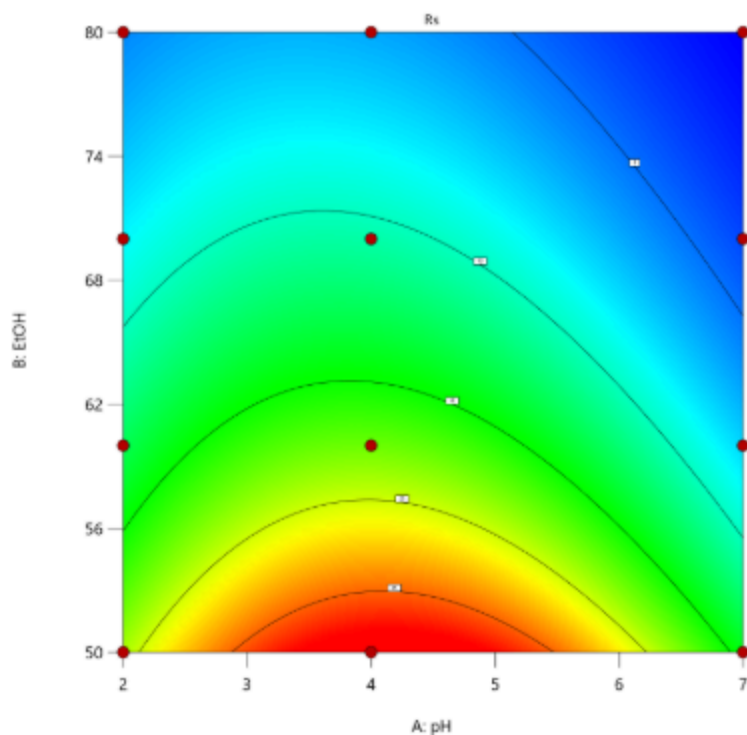


Figure 5

2D response surface contour plot for the optimization of azilsartan medoxomil resolution (Rs). The model is based on natural log transformation (ln(Rs)) as a function of the mobile phase pH (Factor A) and ethanol content, % v/v (Factor B). The design points (n = 12) are overlaid on the contour lines; the color gradient represents the resolution values ranging from the minimum (blue) to the maximum (red).

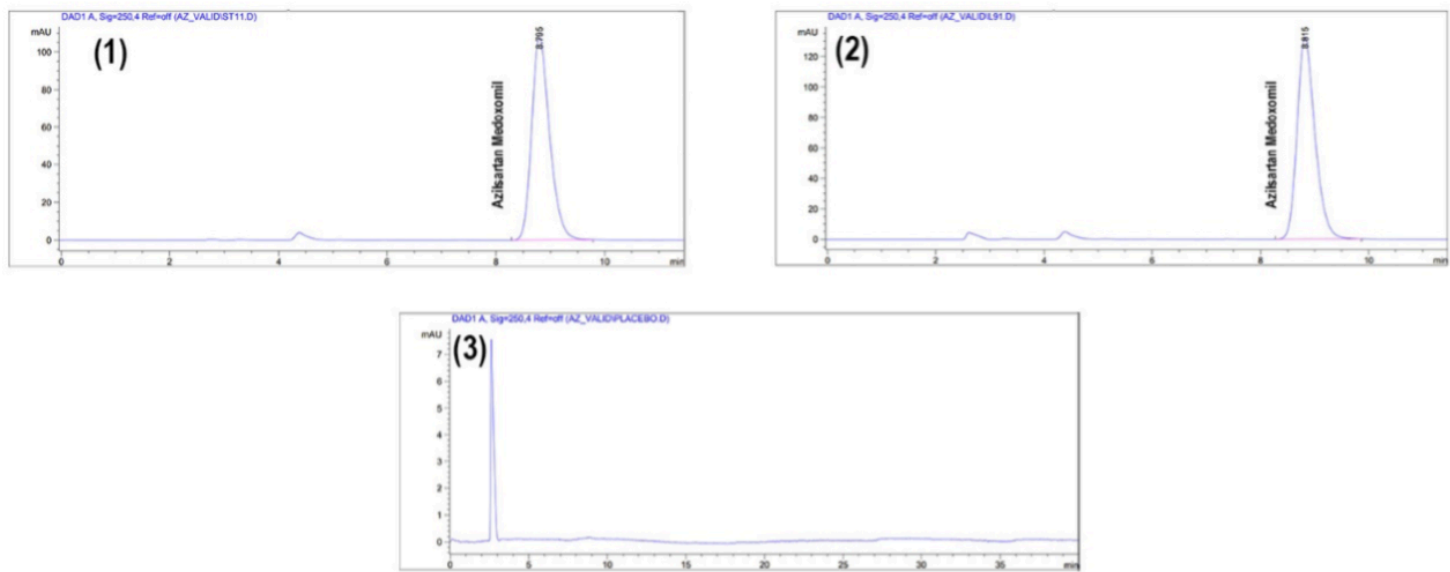


Figure 6

Chromatograms obtained during method validation for azilsartan medoxomil. (1) Standard solution of azilsartan medoxomil potassium at the target concentration (0.8 mg/mL); (2) placebo sample spiked with the reference. (3) Placebo solution.

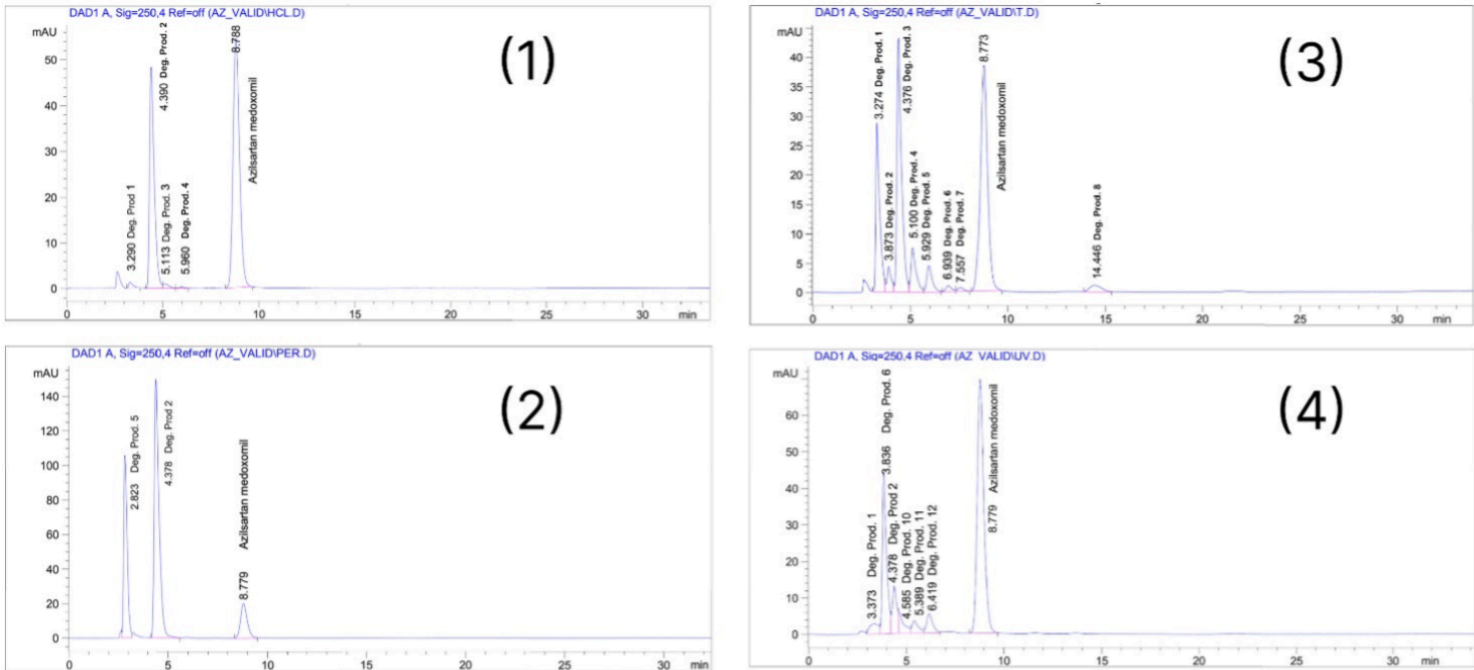


Figure 7

Composite of representative HPLC chromatograms for azilsartan medoxomil under various forced degradation conditions: (1) acidic hydrolysis, (2) oxidative stress, (3) thermal degradation, and (4) photolytic (UV) stress.

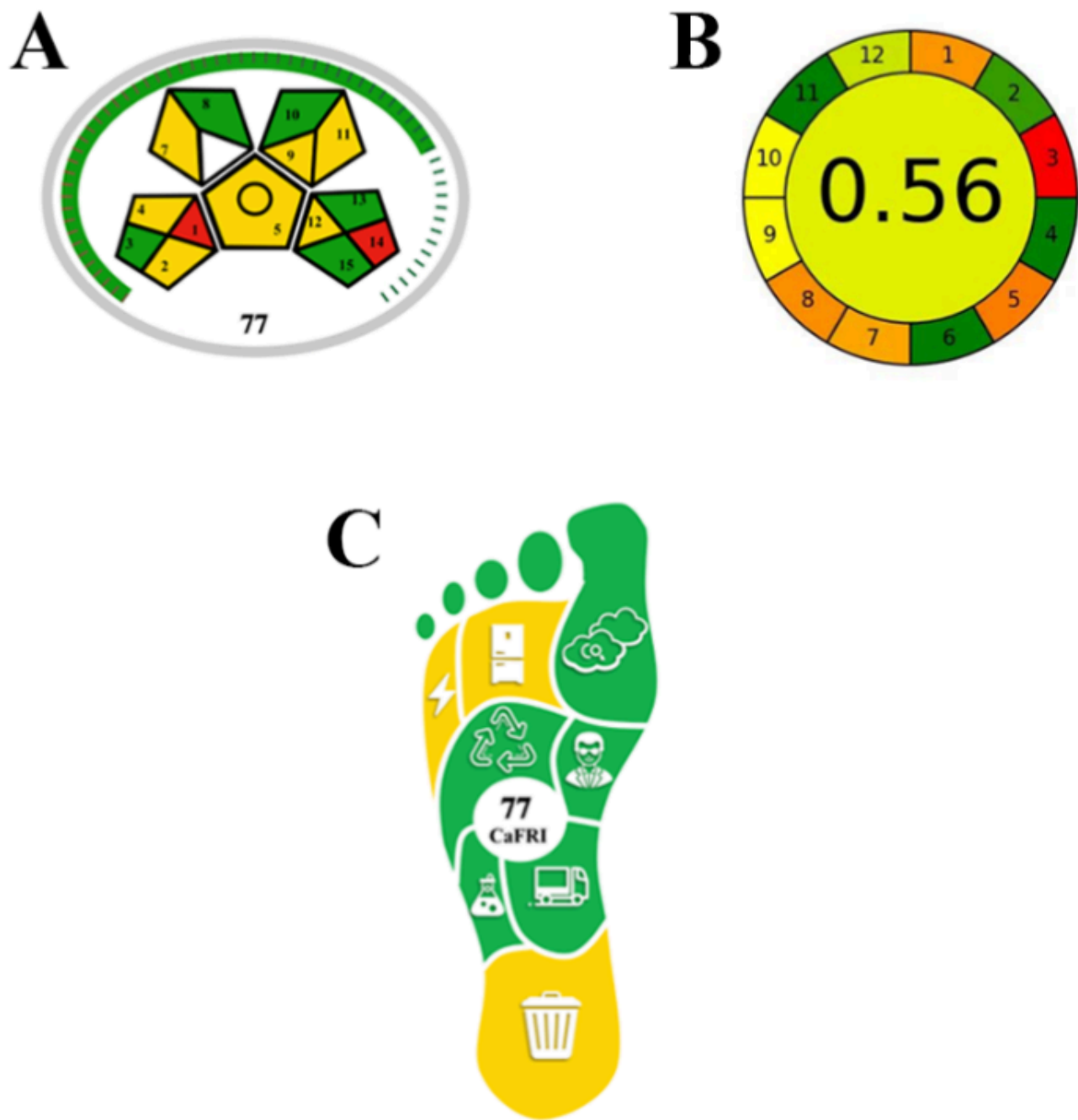


Figure 8

Greenness assessment of the proposed RP-HPLC procedure for the determination of azilsartan medoxomil: (A) MoGAPI pictogram (index 77); (B) AGREE pictogram (overall score 0.56); (C) CaFRI

pictogram (score 77). Color coding from green to red indicates decreasing conformity with the corresponding criterion.

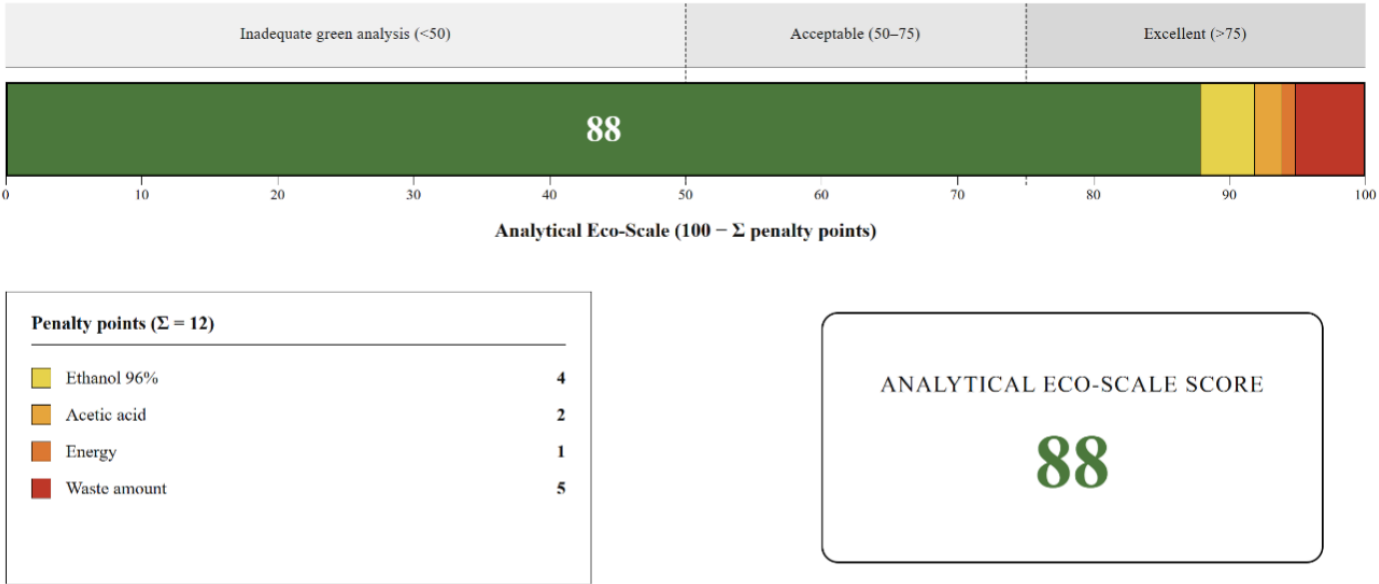


Figure 9

Graphical representation of the Analytical Eco-Scale assessment of the proposed RP-HPLC procedure. The stacked bar shows the distribution of the 12 penalty points among the individual reagents, energy demand and waste volume, and the position of the resulting score of 88 relative to the classification thresholds of 50 and 75. Penalty point assignments are given in Supplementary Table S2.

Supplementary Files

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- [Additionalfile1Supplementaryfiguresandtables.docx](#)