Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Ethanol-based green RP-HPLC method for atogepant using analytical quality by design: validation and forced degradation profiling

Prabhu Dev H
JSS College of Pharmacy
Pattaluchetty P
JSS College of Pharmacy
Pavan Kumar M R
JSS College of Pharmacy
Rohit Chapiyavar
JSS College of Pharmacy
Manasa Chittipolu
JSS College of Pharmacy
R S Chandan
rschandan@jssuni.edu.in

JSS College of Pharmacy

Research Article

Keywords: Atogepant, Box–Behnken design, Analytical quality by design, Green analytical chemistry, Ethanol, RP-HPLC, Forced degradation

Posted Date: November 27th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11786156/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Ethanol-based green RP-HPLC method for atogepant using analytical quality by design: validation and forced degradation profiling

Abstract

Background: Atogepant is a calcitonin gene-related peptide (CGRP) receptor antagonist that helps to prevent migraines. In this work, a green RP-HPLC method is developed using ethanol-water as the mobile phase, employing an AQbD approach.

Methods: Water content, flow rate, and column temperature were optimised using a 15-run Box-Behnken (BBD) experimental design. Conditions used were a C18 column (250 × 4.6 mm, 5 µm), ethanol-water (65:35, v/v), 0.8 mL/min, 35 °C, and UV detection at 280 nm. Validation was per ICH Q2(R2). A blank injection demonstrated specificity, and forced degradation was performed under acidic, alkaline, oxidative, heat, and light conditions. Green analysis was performed with AGREE, GAPI, MoGAPI, ComplexGAPI, and HPLC-EAT.

Results: The ATO peak was at 4.8 min. Calibration was linear at 4-64 µg/mL (y = 18816x + 13413; R2 = 0.9998). LOD and LOQ were 1.006 and 3.047 µg/mL. Recovery was 98.00-100.13%. Intra- and inter-day %RSD were<0.9%, robustness %RSD was <2%. No peak was detected at the ATO retention time in the blank. Degradation led to a 10.32- 16.92% loss of ATO. BBD predicted only the peak area; the selected conditions were at the boundary of the design space. AGREE, and MoGAPI scores were 0.58 and 63%, respectively. GAPI displayed seven green, five yellow, and three red fields. HPLC-EAT was 43% lower than the reference using acetonitrile.

Conclusion: Ethanol substitution improved greenness, but offline operation and waste volume are issues. Specificity was shown against a blank only; peak purity and resolution were not determined. Not a stability-indicating method; use in dosage form would require validation.

Keywords: Atogepant; Box–Behnken design; Analytical quality by design; Green analytical chemistry; Ethanol; RP-HPLC; Forced degradation

Background

Migraine is a neurological disorder characterised by recurrent headache attacks that may be accompanied by nausea, photophobia, phonophobia and other neurological symptoms **(1) .** The calcitonin gene-related peptide (CGRP) pathway has emerged as an important therapeutic target in migraine management **(2).** Atogepant (ATO), an orally administered small-molecule CGRP receptor antagonist, is used for migraine prevention **(3).** Reliable analytical methods are therefore needed to quantitatively determine ATO in pharmaceutical research and quality assessment.

Published analytical work on gepants is limited. Reported methods include RP-HPLC for ubrogepant **(4)**, a stability-indicating method for ubrogepant impurities **(5) ,** a QbD-based method for rimegepant **(6) ,** and an LC-MS method for ubrogepant and ATO in plasma **(7) .** For ATO itself, RP-HPLC methods described as stability-indicating have been reported for bulk drug and tablets **(8)** and for dissolution studies **(9) .**

AQbD, described in ICH Q14 **(10) ,** builds quality into a method by first defining its purpose, identifying the parameters that matter through risk assessment, and mapping the region in which the method works using designed experiments. AQbD has been applied to HPLC methods for many drugs, and recent reviews describe the shift towards such strategies **(11)** . Many of these studies also assess environmental impact, combining green analytical tools with AQbD.

To the best of the authors' knowledge, no AQbD-based HPLC method that uses ethanol instead of acetonitrile and is judged with several greenness tools has been reported for ATO. This study therefore aimed to develop such a method, to optimise it with a Box–Behnken design, to validate it according to ICH Q2(R2) **(12)** , to study the degradation of ATO under forced degradation conditions, and to judge its greenness with five tools: AGREE, GAPI, MoGAPI, ComplexGAPI and HPLC-EAT.

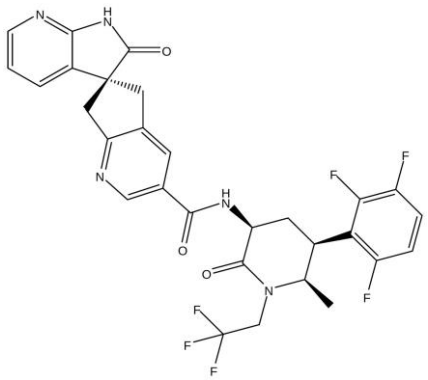


Fig. 1 Chemical structure of atogepant.

Methods

Chemicals and reagents

ATO reference standard (purity 98%) was a gift from Micro Labs Limited, Bengaluru, India. HPLC-grade ethanol was purchased from Merck Life Science Pvt. Ltd. Water was purified with a Milli-Q system (Millipore). Hydrochloric acid, sodium hydroxide and hydrogen peroxide were of analytical grade.

Instrumentation

Analysis was performed on a Shimadzu LC-2030C Plus HPLC system equipped with a UV–visible detector and controlled by LabSolutions software (Shimadzu Corporation, Japan). Separation was carried out on a C18 column (250 × 4.6 mm, 5 µm). UV spectra were recorded on a Shimadzu UV-1900i UV–visible spectrophotometer controlled by LabSolutions UV-Vis

software (version 1.14). Experimental design and statistical analysis were performed with Design-Expert software (version 13; Stat-Ease Inc., USA).

Standard solutions

ATO (10 mg) was dissolved in ethanol in a 10 mL volumetric flask to give a stock solution of 1000 µg/mL. 1mL of this solution was diluted to 10 mL with ethanol, yielding a 100 µg/mL working solution. Calibration standards of 4, 8, 16, 32 and 64 µg/mL were prepared from the working solution by serial dilution with ethanol.

Analytical quality by design

The method was developed in accordance with ICH Q14 **(10)** . The detection wavelength was selected by scanning a 10 µg/mL ATO solution in ethanol over the 200–400 nm range; the maximum absorbance was observed at 280 nm. A C18 column was chosen based on the physicochemical properties of the drug, and ethanol was used as the organic solvent and diluent because it completely dissolves ATO.

Analytical target profile and critical method attributes

The analytical target profile (ATP) was to determine ATO with accurate, precise and robust results in a short run using a green solvent. The critical method attributes (CMAs) and their acceptance criteria are given in Table 1.

Table 1 Critical method attributes (CMAs) and acceptance criteria

Critical method attribute	Acceptance criterion
Retention time	Consistent and reproducible
Peak area	Linear response
Tailing factor	< 2
Theoretical plates	> 2000
Specificity	No interference at the ATO retention time
Accuracy	98–102% recovery
Precision	< 2% RSD

Risk assessment

An Ishikawa (fishbone) diagram (Fig. 2) was used to list the variables that could affect performance: mobile phase, chromatographic system, sample preparation, detection, analyst and environment. Variables with the highest risk priority number (RPN) in the failure mode and effects analysis (Table 2) were taken as critical method parameters (CMPs): the percentage of water in the mobile phase, flow rate and column temperature (Table 3).

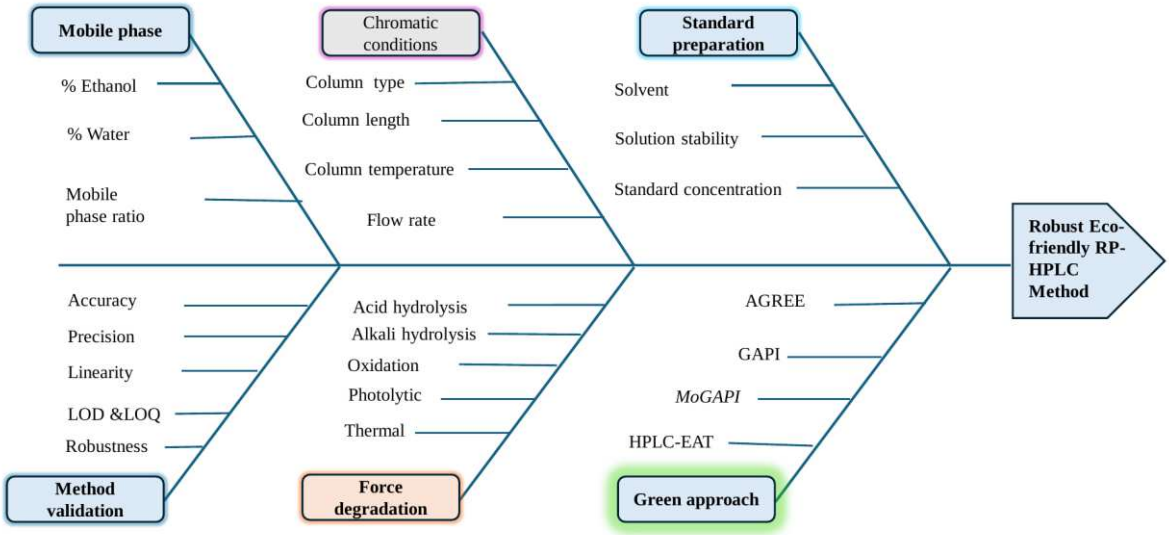


Fig. 2 Ishikawa (fishbone) diagram for the preliminary risk assessment. The diagram covers the AQbD-based RP-HPLC method.

Table 2. Failure mode and effects analysis of the candidate parameters

Parameter	Severity	Occurrence	Detection	RPN
Water (%)	9	7	3	189
Flow rate	8	5	3	120
Oven temperature	8	4	3	96

RPN, risk priority number (severity × occurrence × detection).

Table 3 Critical method parameters (CMPs) and the reason for their selection

Parameter	Reason
Water in mobile phase (%)	Controls retention time and elution of ATO
Flow rate	Controls retention time, efficiency and back-pressure
Column temperature	Changes mobile-phase viscosity, retention and efficiency

Box–Behnken design

A three-factor, three-level BBD with 15 runs (12 edge-midpoint runs and three centre points) was generated in Design-Expert. The factors were water in the mobile phase (A: 35, 50 and 65%, equal to 65, 50 and 35% ethanol), flow rate (B: 0.8, 1.0 and 1.2 mL/min) and column temperature (C: 25, 35 and 45 °C) (Table 4). A 50 µg/mL ATO solution was injected for each design run, and the detection wavelength was maintained at 280 nm. The responses were theoretical plates (NTP), peak area (PA), tailing factor (TF) and retention time (RT). Quadratic models were fitted for each response and assessed using ANOVA, adjusted and predicted R^2, and adequate precision.

Table 4 Factors and levels of the Box–Behnken design

Critical method parameter	Low (−1)	Middle (0)	High (+1)
A: Water in mobile phase (%)	35	50	65
B: Flow rate (mL/min)	0.8	1.0	1.2
C: Column temperature (°C)	25	35	45

Method operable design region

The method operable design region (MODR) was defined as the part of the design space in which the CMA criteria in Table 1 are met for retention time, peak area, tailing factor and theoretical plates.

Chromatographic conditions

The final conditions were: C18 column (250 × 4.6 mm, 5 µm); mobile phase, ethanol–water (65:35, v/v); isocratic flow rate, 0.8 mL/min; column temperature, 35 °C; detection at 280 nm; injection volume, 10 µL; run time, 10 min. Standards were prepared in ethanol and injected directly.

Method validation

The method was validated in accordance with ICH Q2(R2) (12) .

System suitability. Six injections of an 8 µg/mL standard solution were made, and theoretical plates, retention time, peak area and tailing factor were recorded.

Specificity. The diluent (ethanol) was injected as a blank under the final chromatographic conditions, and the chromatogram was inspected for any peak at the retention time of ATO.

Linearity. Five calibration standards (4, 8, 16, 32 and 64 µg/mL) were analysed, and peak area was regressed on concentration by least squares. The regression equation, coefficient of determination and residuals were calculated from these five levels.

Sensitivity. LOD and LOQ were calculated from the final calibration curve as $3.3\sigma/S$ and $10\sigma/S$, where σ is the residual standard deviation of the regression line, and S is the slope.

Accuracy. Standard addition at three levels (50, 100 and 150%) was performed by spiking 8, 16 and 24 µg/mL of ATO into a 10 µg/mL solution, with three replicates per level. Recovery was calculated from the peak area of the added amount (total area minus initial area) using the calibration equation ($y = 18816x + 13413$).

Precision. Intra-day precision was measured at 8, 16 and 24 µg/mL ($n = 3$), and inter-day precision at the same levels on three days ($n = 9$).

Robustness. Triplicate injections of an 8 µg/mL standard solution were made after deliberate changes in mobile-phase composition (67:33 and 63:37 ethanol–water), flow rate (0.7 and 0.9 mL/min) and column temperature (33 and 37 °C), and %RSD was calculated for each condition.

Solution stability. An 8 µg/mL standard solution was injected after 0, 1, 2, 6, 12 and 24 h, and the peak area was compared with the initial peak area.

Forced degradation

Forced degradation followed the general approach of ICH Q1A(R2) (13) and was carried out to study how ATO degrades under stress, not to establish specificity. A 100 µg/mL ATO solution was stressed under the following conditions, and each solution was neutralised where needed and diluted with ethanol. Acidic: 0.1 N HCl at 60 °C for 2 h, neutralised with 0.1 N NaOH. Alkaline: 0.1 N NaOH at 60 °C for 1 h, neutralised with 0.1 N HCl. Oxidative: 3% (v/v) hydrogen peroxide at room temperature for 12 h. Thermal: 60 °C for 2 h. Photolytic: UV light in a UV chamber for 2 h. The light dose was not measured, so the photolytic test is not a full photostability study according to ICH Q1B **(14)**. Stressed samples were diluted to a nominal concentration of 32 µg/mL, and degradation was calculated against the unstressed control at the same nominal concentration.

Greenness assessment

The environmental impact was evaluated with five metrics: the Analytical GREEnness calculator (AGREE) **(15),** the Green Analytical Procedure Index (GAPI) **(16)**, its modified version (MoGAPI) **(17),** the complementary GAPI (ComplexGAPI) **(18)** and the HPLC Environmental Assessment Tool (HPLC-EAT) **(19).** The results were interpreted in light of the 12 principles of green analytical chemistry **(20)**. AGREE scores each of the 12 principles from 0 to 1 and gives an overall score; equal weights were used. GAPI and MoGAPI rate 15 stages of the procedure, from sample collection to waste treatment, with a green–yellow–red scheme, and MoGAPI converts the pictogram into a percentage score. ComplexGAPI adds a zone for pre-analytical steps, such as reagent and material preparation. HPLC-EAT multiplies the mass of each solvent used per analysis by its solvent-specific safety, health, and environmental factors, then sums the impacts; lower values indicate a greener result. All assessments follow the routine procedure for a single sample (standard preparation and one injection) under the

final conditions; solvents used only in forced degradation were excluded. The input data are in Table 16.

Results

Method development

ATO was separated on a C18 column using ethanol–water as the mobile phase, with detection at 280 nm. The UV spectrum of a 10 µg/mL ATO solution in ethanol (Fig. 3) showed a maximum at 280 nm (absorbance 0.438) and a minimum at 259 nm. Absorbance rises steeply below about 220 nm, where the solvent also absorbs, so the 280 nm maximum was used. A mobile phase of 65:35 ethanol–water at 0.8 mL/min gave a sharp, symmetrical ATO peak at about 4.8 min (Fig. 4). Based on the risk assessment (Fig. 2, Tables 2 and 3), water content, flow rate and column temperature were the CMPs and were studied by BBD.

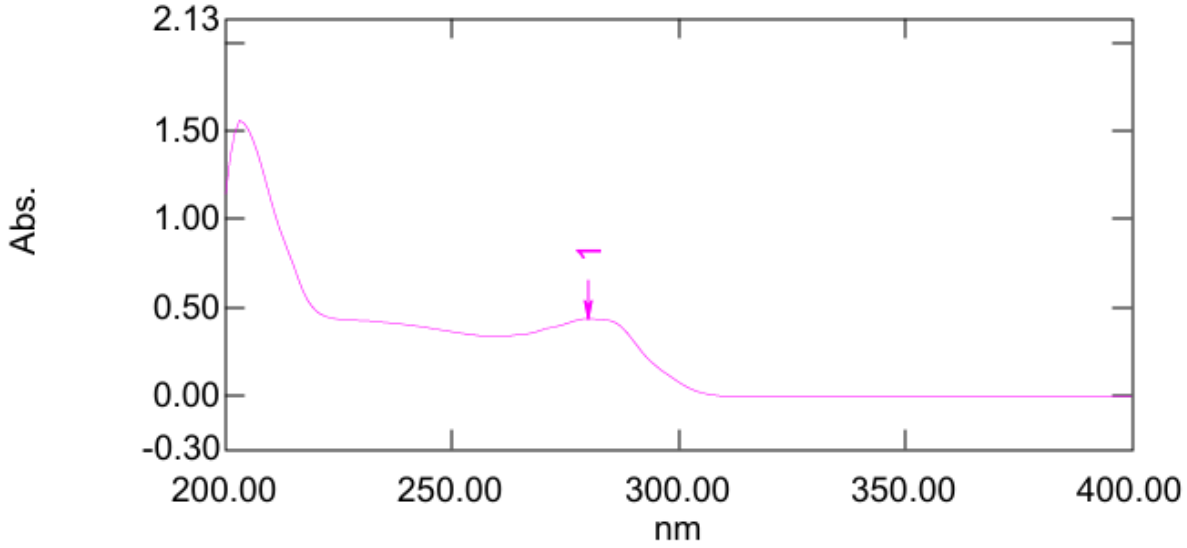


Fig. 3. UV spectrum of ATO (10 µg/mL) in ethanol. Recorded from 200 to 400 nm on a Shimadzu UV-1900i spectrophotometer; the maximum is at 280 nm.

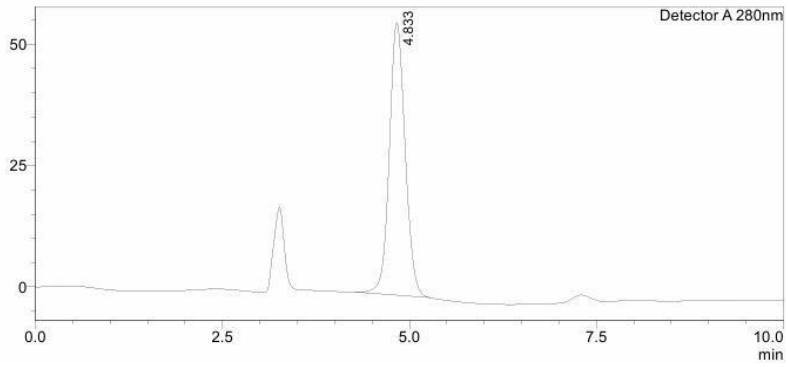


Fig. 4. Optimised chromatogram of ATO (50 µg/mL).

Box–Behnken design

The 15 runs and their responses are in Table 5. Five runs (runs 2, 7, 10, 13 and 14) gave no ATO peak within the run time: all four runs at 65% water and the run at 50% water, 0.8 mL/min and 25 °C. Because a design matrix needs a value for every run, zero was entered for all four responses in these runs. This has important consequences for the models, which are discussed below. The three centre-point runs (3, 8, and 12) gave identical responses, so pure-error variance could not be estimated, and the lack of fit was not tested.

Table 5. Box–Behnken design matrix and responses for ATO

Run	A: Water (%)	B: Flow (mL/min)	C: Temp (°C)	NTP	PA	TF	RT (min)
1	50	1.2	25	2269	558271	0.992	8.72
2	50	0.8	25	0	0	0	0
3	50	1.0	35	2132	693455	1.066	8.78
4	35	1.2	35	2009	552742	1.085	3.26
5	50	0.8	45	2184	789621	1.026	9.32
6	35	1.0	45	2619	651165	1.072	3.73
7	65	0.8	35	0	0	0	0
8	50	1.0	35	2132	693455	1.066	8.78
9	35	1.0	25	1860	662127	1.091	4.095
10	65	1.0	45	0	0	0	0
11	35	0.8	35	2450	829584	1.066	4.833
12	50	1.0	35	2132	693455	1.066	8.78
13	65	1.2	35	0	0	0	0
14	65	1.0	25	0	0	0	0
15	50	1.2	45	3533	556441	0.924	6.44

NTP, theoretical plates; PA, peak area; TF, tailing factor; RT, retention time. Zeros mark runs with no ATO peak.

Run 15 (50% water, 1.2 mL/min, 45 °C) gave the highest plate count (3533) and the lowest tailing factor (0.924) but a low peak area (556,441), similar to the other runs at 1.2 mL/min (runs 1 and 4: 558,271 and 552,742). It appears to deviate from the fitted values for plate count and tailing factor in Fig. 7.

Model fit and statistics

ANOVA results are in Table 6, the regression coefficients in Table 7, and the R^2 statistics in Table 8. The peak-area model was significant ($p < 0.0001$; $R^2 = 0.992$), with a predicted R^2 of 0.877 and an adequate precision of 20.5 (Table 8), indicating that it describes the data well, although a lack-of-fit test could not be performed. The tailing-factor model ($p = 0.0132$) and the retention-time model ($p = 0.0186$) were significant, but their predicted R^2 values were low (0.065 and −0.080), so they should not be used to predict new conditions. The theoretical-plate model was not significant ($p = 0.1054$), and its predicted R^2 was strongly negative (−1.354). Adequate precision was above 4 for all four models (5.5–20.5), but this only indicates signal-to-noise and does not make a model predictive. The coefficients of variation were high (12.4–49.5%), which is expected when five of 15 runs are zero.

Table 6. ANOVA summary of the quadratic models

Statistic	Theoretical plates	Peak area	Tailing factor	Retention time
R^2	0.853	0.992	0.941	0.932
Adjusted R^2	0.588	0.978	0.836	0.810
Coefficient of variation (%)	49.52	12.36	29.70	37.25
Standard deviation	769.85	50934.2	0.2070	1.66
Mean of response	1554.67	412000	0.696	4.45
F-value	3.22	71.88	8.96	7.67
p-value	0.1054	< 0.0001	0.0132	0.0186

Table 7. Regression coefficients of the quadratic models (coded factors)

Model term	Theoretical plates	Peak area	Tailing factor	Retention time
Intercept	2132.0	693500	1.07	8.78
A (water)	−1117.25	−337000	−0.539	−1.99
B (flow rate)	397.12	−56568.5	0.1136	0.533
C (temperature)	525.88	34504.0	0.1174	0.834

Model term	Theoretical plates	Peak area	Tailing factor	Retention time
AB	110.25	69210.5	−0.0047	0.3933
AC	−189.75	2740.5	0.0047	0.0912
BC	−230.0	−323100	−0.2735	−2.90
A^2	−947.00	−185200	−0.361	−5.46
B^2	−70.25	−162700	−0.1667	−1.30
C^2	−65.25	−179900	−0.1638	−1.36

Table 8 R^2, adjusted R^2, predicted R^2 and adequate precision of the models

Response	R^2	Adjusted R^2	Predicted R^2	Adequate precision
Theoretical plates	0.8529	0.588	−1.354	5.5085
Peak area	0.9923	0.978	0.877	20.539
Tailing factor	0.941	0.836	0.065	7.787
Retention time	0.932	0.8109	−0.0803	7.59

In Table 7, water content (A) or its quadratic term (A^2) has the largest coefficient for every response (A for plates, peak area and tailing factor; A^2 for retention time). Their negative signs mainly reflect the loss of the peak at 65% water, since those runs are treated as zero in the models.

Graphical interpretation

The effects of water content (A) and flow rate (B), with temperature at its centre level (35 °C), are shown as contour plots (Fig. 5) and three-dimensional response surfaces (Fig. 6). Retention time and peak area form closed, curved contours, in line with their significant quadratic models. In reversed-phase chromatography, retention is expected to rise steadily as water content increases, so the fitted maximum of retention time at mid-range water is a modelling effect of the zero entries and not a true chromatographic optimum. Theoretical plates and tailing factor show open, nearly flat surfaces, consistent with the non-significant plate-count model. In the predicted-versus-actual plots (Fig. 7), the peak-area points lie close to the 45° line, whereas the points for the other responses are more scattered. The no-peak runs (blue points) scatter around zero, especially for theoretical plates and retention time, which explains the negative predicted R^2 values of these models.

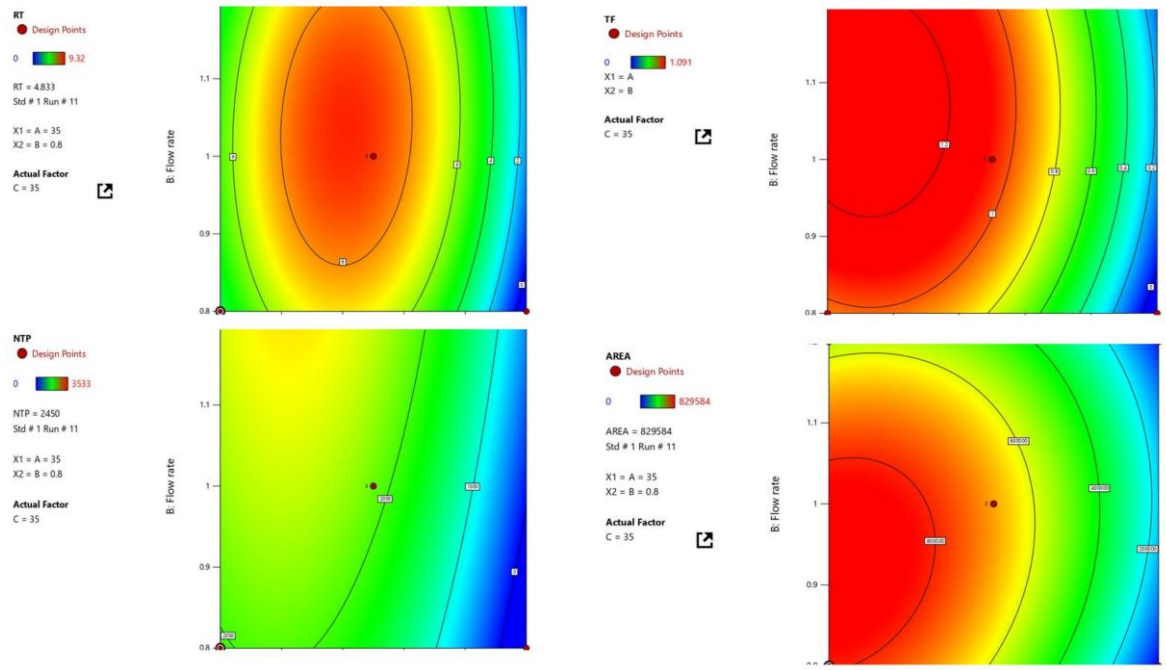


Fig. 5 Contour plots of the four responses against water content and flow rate. (a) Retention time, (b) tailing factor, (c) theoretical plates and (d) peak area against water content (A) and flow rate (B) at 35 °C.

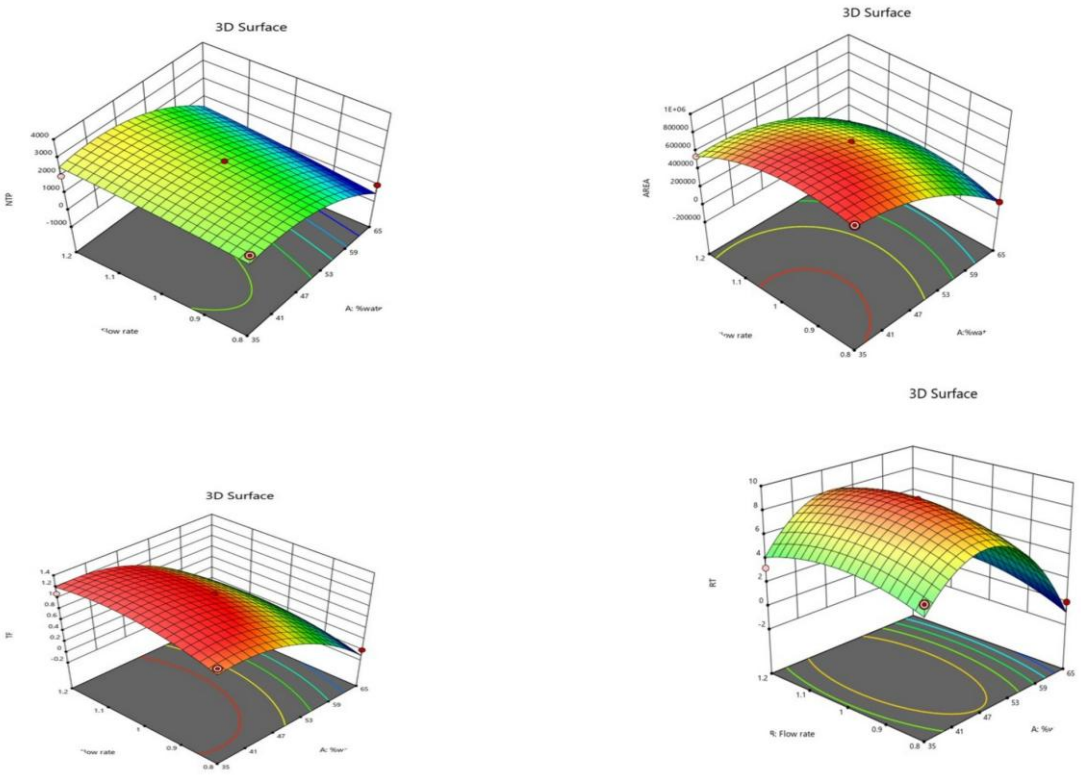


Fig. 6. Three-dimensional response surfaces of the four responses. (a) Theoretical plates, (b) peak area, (c) tailing factor and (d) retention time against water content (A) and flow rate (B) at 35 °C.

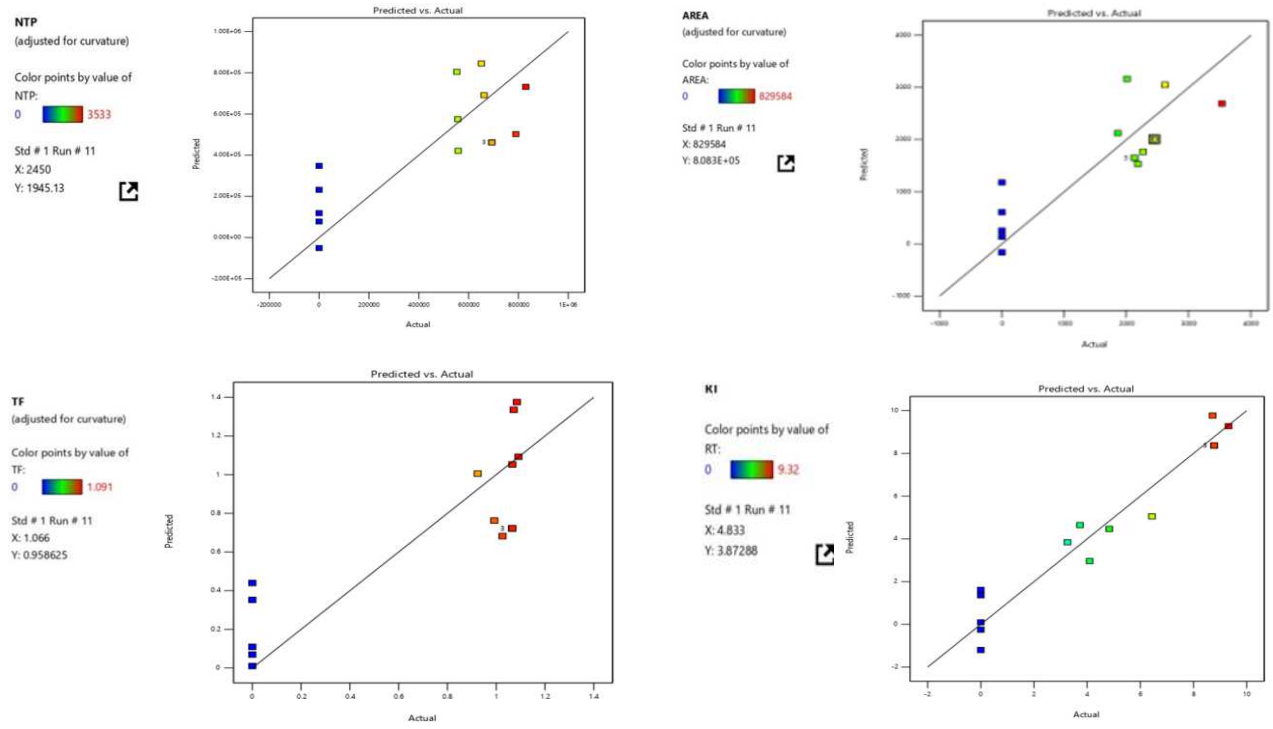


Fig. 7 Predicted versus actual plots for the four responses. (a) Theoretical plates, (b) peak area, (c) tailing factor and (d) retention time. Blue points are runs with no ATO peak.

Selected conditions and operable region

The working point (35% water, 0.8 mL/min, 35 °C; run 11) gave the highest peak area of all runs (829,584), theoretical plates above 2000 (2450), a tailing factor of 1.066 and a retention time of 4.83 min. Faster runs with similar plate counts (runs 4 and 6) had lower peak areas. The ethanol–water ratio of 65:35 at a flow rate of 0.8 mL/min was therefore chosen. Because this point lies at the lower limit of two factors (water and flow rate), the operable region is not bracketed on that side, and the design provides no support for operating below 35% water or 0.8 mL/min. The plate count at this point (2450) is only modestly above the ATP limit of 2000, and the plate-count model is not significant. For these reasons, the MODR is described here as the neighbourhood of the studied corner, as confirmed experimentally through validation, rather than as a region proven by the models.

Method validation

System suitability. Six injections of an 8 µg/mL standard solution gave a mean theoretical plate count of 2277.16, a retention time of 4.786 min and a tailing factor of 1.159 (Table 9). The plate count and tailing factor meet the criteria in Table 1.

Table 9 System suitability results (mean of six injections)

Parameter	Result	%RSD
Theoretical plates	2277.16	0.865
Retention time (min)	4.786	0.062
Peak area	160206.8	1.231
Tailing factor	1.159	0.391

Specificity. The chromatogram of the blank (ethanol) showed no peak at the retention time of ATO (about 4.8 min) (Fig. 8). Specificity is therefore claimed based on the blank only. A peak

at about 3.3 min, present in the standard chromatograms, elutes well before ATO and does not overlap with it (Figs. 4 and 9).

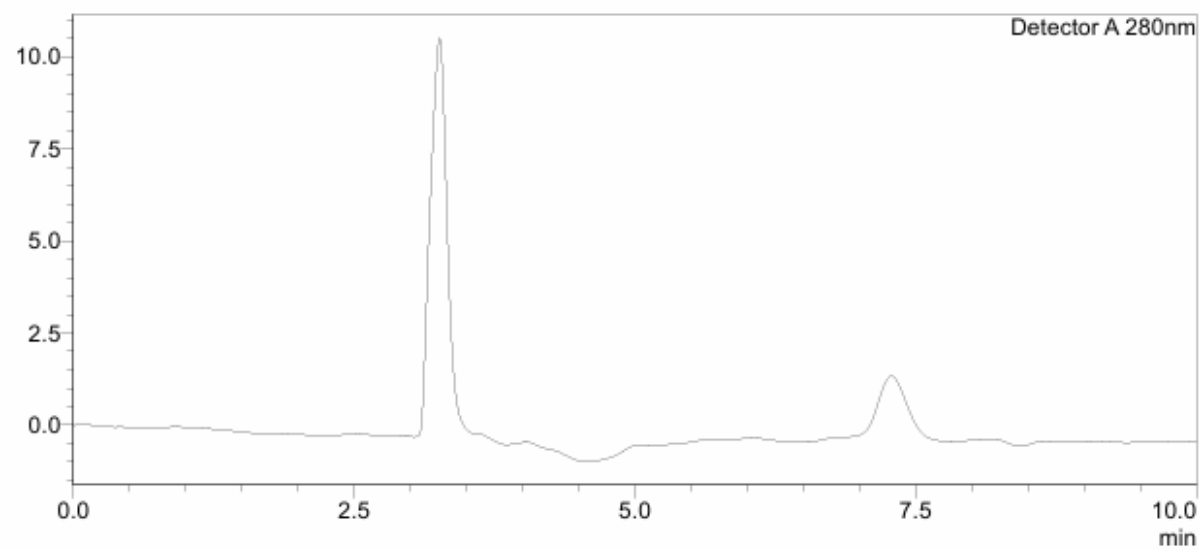


Fig. 8 Chromatogram of the blank (ethanol) under the final conditions.

Linearity, residuals and sensitivity. The LOD and LOQ calculated from the calibration curve were 1.006 and 3.047 µg/mL, respectively, so the lowest calibration level (4 µg/mL) is above the LOQ. The calibration was linear from 4 to 64 µg/mL (Fig. 9 and Fig. 10a), with the equation $y = 18816x + 13413$ (y, peak area; x, concentration in µg/mL) and a coefficient of determination (R^2) of 0.9998. Residuals were of both signs and did not increase with concentration (Table 10, Fig. 10b). The relative residual was within ±1.9% of the predicted area from 8 µg/mL upward, but it reached −4.9% at 4 µg/mL, the lowest level, which is close to the LOQ.

Table 10. Residual analysis of the calibration curve (4–64 µg/mL)

Concentration (µg/mL)	Observed area	Predicted area	Residual	Residual (% of predicted)
4	84350	88675.6	−4325.6	−4.88
8	160894	163938.5	−3044.5	−1.86
16	319687	314464.3	5222.7	1.66
32	621120	615515.8	5604.2	0.91
64	1214162	1217618.9	−3456.9	−0.28

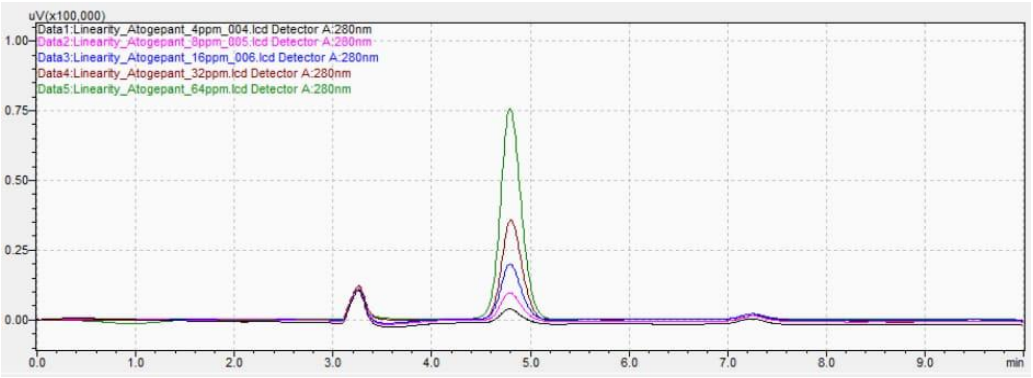


Fig. 9 Overlay of chromatograms of ATO calibration standards. 4, 8, 16, 32 and 64 µg/mL.

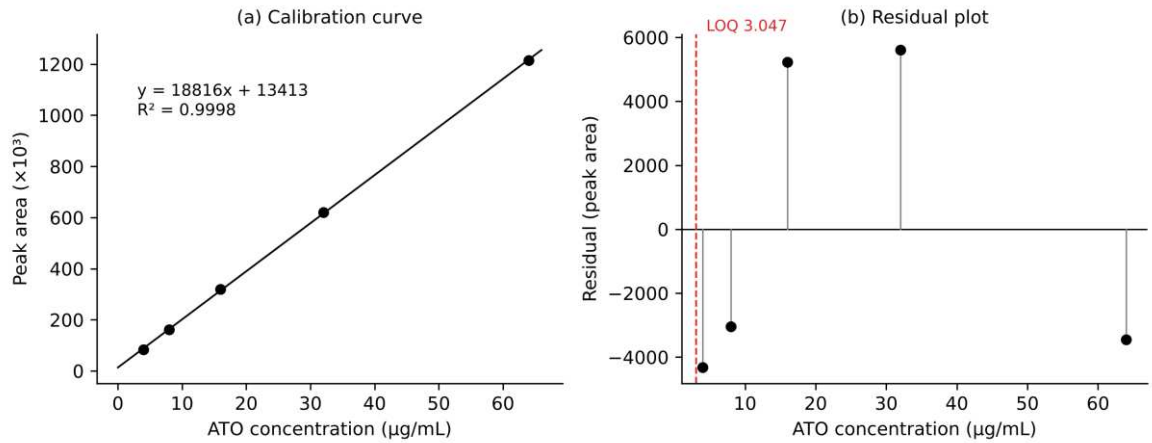


Fig. 10 Calibration curve and residual plot of ATO. (a) Calibration curve (y = 18816x + 13413, R^2 = 0.9998) and (b) residuals for the five levels from 4 to 64 µg/mL. The dashed line marks the LOQ (3.047 µg/mL).

Accuracy. Accuracy was tested at 50, 100 and 150% levels (Table 11). Recoveries ranged from 98.00 to 100.13%, with level means of 98.03, 99.51 and 99.80% and an overall mean of 99.11% (RSD 0.87%). All individual recoveries were within the 98–102% criterion, the lowest being 98.00% at the 50% level.

Table 11. Accuracy by standard addition to a 10 µg/mL ATO solution

Level (%)	Spiked (µg/mL)	Initial drug (µg/mL)	Initial area	Total area	Spiked area	Recovered (µg/mL)	Recovery (%)	Mean (%)
50	8	10	203124	364118	160994	7.843	98.04	98.03
	8	10	202113	363098	160985	7.843	98.04	
	8	10	203312	364248	160936	7.840	98.00	

Level (%)	Spiked (µg/mL)	Initial drug (µg/mL)	Initial area	Total area	Spiked area	Recovered (µg/mL)	Recovery (%)	Mean (%)
100	16	10	203100	515133	312033	15.871	99.19	99.51
	16	10	202409	515529	313120	15.928	99.55	
	16	10	203131	516964	313833	15.966	99.79	
150	24	10	203124	664964	461840	23.832	99.30	99.80
	24	10	202712	667541	464829	23.991	99.96	
	24	10	202251	667850	465599	24.032	100.13	

Spiked area = total area − initial area; recovered amount = (spiked area − 13413)/18816, where 13413 and 18816 are the intercept and slope of the calibration equation (y = 18816x + 13413; 4–64 µg/mL).

Precision. Intra-day %RSD was 0.355, 0.840 and 0.046% at 8, 16 and 24 µg/mL, and inter-day %RSD was 0.361, 0.346 and 0.043% (Table 12). All values were far below the 2% criterion.

Table 12 Intra-day and inter-day precision

Precision	Concentration (µg/mL)	Mean peak area ± SD	%RSD
Intra-day (n = 3)	8	161058.7 ± 571.1	0.355
	16	318633.7 ± 2676.2	0.840
	24	480839.7 ± 220.6	0.046
Inter-day (3 days, n = 9)	8	161325.3 ± 582.5	0.361
	16	320533.0 ± 1109.3	0.346
	24	480698.3 ± 206.1	0.043

Robustness. Table 13 shows triplicate results after deliberate changes. The %RSD was below 2% for all six changes: 67:33 (0.67%), 63:37 (0.99%), 0.7 mL/min (0.13%), 0.9 mL/min (0.43%), 33 °C (1.33%) and 37 °C (1.42%). The %RSD within a condition measures repeatability under that condition, not the shift from the nominal result. The mean peak areas differed between the two flow rates (165,990 and 135,043) and between the two temperatures (137,782 and 150,675), so flow rate and column temperature need to be closely controlled.

Table 13 Robustness of the method (peak area, n = 3)

Parameter	Change	Individual areas	Mean	SD	%RSD
Mobile phase (ethanol: water)	67:33	144693, 143078, 144793	144188.0	962.6	0.67
	63:37	146395, 147767, 144881	146347.7	1443.6	0.99
Flow rate	0.7 mL/min	165850, 166240, 165881	165990.3	216.8	0.13
	0.9 mL/min	135162, 135551, 134415	135042.7	577.3	0.43
Column temperature	33 °C	139770, 137399, 136176	137781.7	1827.3	1.33

Parameter	Change	Individual areas	Mean	SD	%RSD
	37 °C	153118, 149731, 149175	150674.7	2134.2	1.42

Solution stability. The peak area of the 8 µg/mL standard solution decreased slowly with storage time: −0.05% at 1 h, −0.21% at 2 h, −0.78% at 6 h, −1.42% at 12 h and −2.09% at 24 h relative to the initial area (Table 14). The overall %RSD for the six peak areas was 0.85%, and for the retention time it was 0.07%. The deviation remained within 2% up to 12 h but exceeded 2% at 24 h; therefore, the solution should be used within 12 h, and fresh solutions should be prepared for longer storage.

Table 14 Solution stability of ATO over 24 h

Time (h)	RT (min)	Peak area	Deviation from initial area (%)
0	4.826	160254	—
1	4.828	160169	−0.05
2	4.825	159919	−0.21
6	4.830	159010	−0.78
12	4.833	157983	−1.42
24	4.833	156899	−2.09

Forced degradation

Forced degradation resulted in a 10.32–16.92% loss of ATO (Table 15, Fig. 11). The order of degradation was acidic > photolytic > alkaline > oxidative > thermal.

Table 15. Forced degradation of ATO

Stress condition	Stress applied	ATO found (µg/mL)	Degradation (%)
Acidic	0.1 N HCl, 60 °C	26.58	16.92
Alkaline	0.1 N NaOH, 60 °C	27.08	15.37
Oxidative	3% H_2O_2	27.87	12.91
Thermal	60 °C, 2 h	28.70	10.32
Photolytic	UV light, 2 h	26.98	15.67

Degradation was calculated against the unstressed control.

Compared with the unstressed solution (Fig. 4), an extra peak at about 7.2 min (relative retention about 1.5) was larger in the acidic, alkaline, oxidative and photolytic samples (Fig. 11A, B, C and E) and stayed at the control level after thermal stress (Fig. 11D). The oxidative sample also showed a split peak at about 3.3 min. A peak at about 3.3 min was also present in the unstressed solution and in all calibration standards (Fig. 9), and its height varied between

the stressed samples; its origin was not investigated. The ATO peak (4.8 min) remained visible in all stressed samples, and the other peaks did not visibly overlap it. Resolution values were not calculated, and peak purity could not be assessed because a single-wavelength UV detector was used. The stress study is therefore used only to describe the extent of degradation; it is not used to claim specificity or stability-indicating ability.

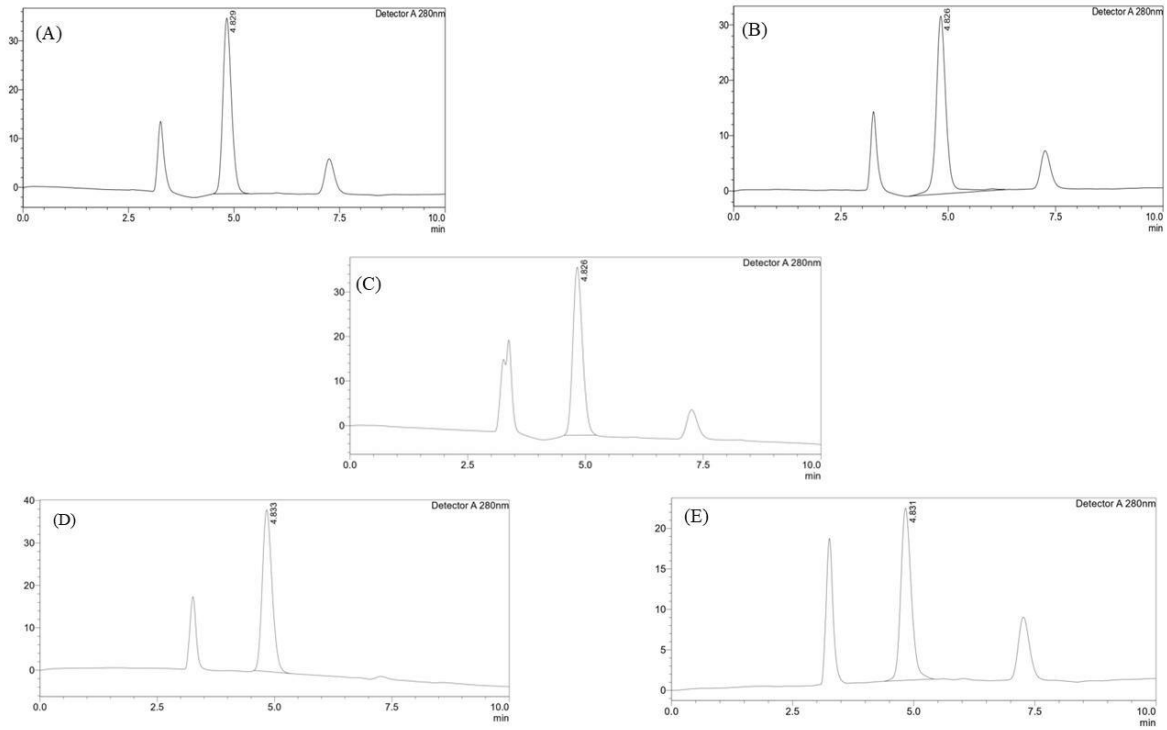


Fig. 11 Chromatograms of ATO after forced degradation. (A) Acidic, (B) alkaline, (C) oxidative, (D) thermal and (E) photolytic stress.

Greenness assessment

The greenness of the method was assessed with the five metrics described in the Methods; the inputs are in Table 16. The method scores in the mid-range of each scale: the solvent is its main strength, and offline sample handling and waste volume are its main weaknesses.

Table 16 Input data for the greenness assessment (routine procedure, one sample)

Parameter	Value	Basis
Mobile phase	Ethanol–water 65:35 (v/v), isocratic	Chromatographic conditions
Flow rate/run time	0.8 mL/min / 10 min	Chromatographic conditions
Mobile phase per run	8.0 mL (5.2 mL ethanol + 2.8 mL water)	Calculated

Parameter	Value	Basis
Sample preparation	Dissolution and dilution in ethanol; 10 mL ethanol per sample; no extraction or derivatisation	Standard solutions
Total solvent per sample	18.0 mL (14.8 g)	Calculated
Energy	< 0.1 kWh per sample	Estimated for HPLC-UV with column oven
Throughput	6 samples/h; one analyte	Calculated
Solvent hazards	Ethanol: flammable liquid (H225), eye irritant (H319); water: none	Safety data sheet
Waste handling	Collected as organic solvent waste; no on-site treatment	Assumed

AGREE. The overall AGREE score was 0.58 (Fig. 12), in the middle of the 0–1 scale. The highest scores were for no derivatisation (principle 6, 1.00) and no toxic reagents (principle 11, 1.00), followed by minimum sample size (principle 2, 0.90) and operator safety (principle 12, 0.75), which was limited only by ethanol's flammability. The lowest scores were for offline operation (principle 3, 0.00), sample treatment (principle 1, 0.30) and throughput (principle 8, 0.30) (Table 17).

Table 17 AGREE scores for the 12 principles of green analytical chemistry

No.	Principle	Score	Basis
1	Direct analysis, avoid sample treatment	0.30	External preparation: dissolution and dilution
2	Minimum sample size and number of samples	0.90	10 mg of the sample
3	In situ measurement	0.00	Offline analysis
4	Integration of operations, fewer steps	0.50	Few steps, not integrated
5	Automation and miniaturisation	0.50	Automated injection; 4.6 mm i.d. column
6	Avoid derivatisation	1.00	No derivatisation
7	Minimise waste	0.50	18.0 mL per sample
8	Multi-analyte determination, high throughput	0.30	One analyte; 6 samples/h
9	Minimise energy use	0.70	< 0.1 kWh per sample
10	Renewable reagents	0.50	Ethanol (can be bio-based) and water
11	Avoid toxic reagents	1.00	No toxic reagents
12	Operator safety	0.75	Flammable solvent only
	Overall score (equal weights)	0.58	

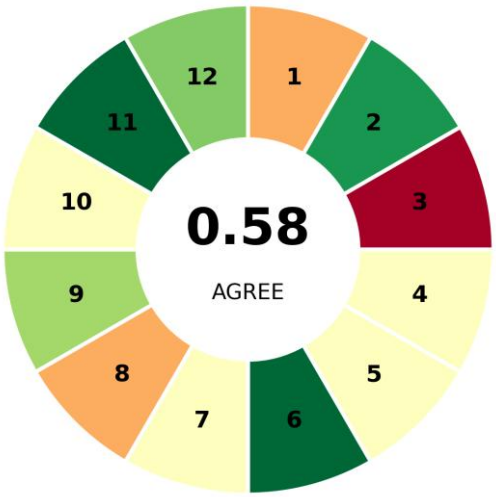


Fig. 12 AGREE pictogram of the proposed method (overall score 0.58). Segments 1–12 are the 12 principles; colour runs from red (0) to green (1).

GAPI, MoGAPI and ComplexGAPI. The GAPI pictogram (Fig. 13a) has seven green, five yellow and three red fields (Table 18). The red fields are offline sample collection (field 1), waste volume (field 14) and no on-site waste treatment (field 15). The green fields cover no preservation, transport, storage, extraction or extra treatment, together with energy use below 0.1 kWh per sample and a closed chromatographic system. The five yellow fields reflect the simple dilution step, the green solvent, a solvent volume of 10–100 mL, and the moderate health and flammability ratings of ethanol. The MoGAPI score was 63% (Fig. 13b), which falls in the acceptable band (50–74%). In ComplexGAPI (Fig. 13c), the pre-analytical zone was not scored because the ATO reference standard was supplied as a gift and no reagents or materials were prepared in-house; the main pictogram is therefore the same as GAPI.

Table 18 Field-by-field GAPI and MoGAPI assessment

Field	Criterion	Assessment	Colour
1	Sample collection	Offline	Red
2	Preservation	None	Green
3	Transport	None	Green
4	Storage	None; analysed immediately	Green
5	Type of method	Simple dilution, no extraction	Yellow
6	Scale of extraction	No extraction	Green
7	Solvents/reagents used	Green solvent (ethanol)	Yellow

Field	Criterion	Assessment	Colour
8	Additional treatments	None	Green
9	Amount of solvent/reagent	18.0 mL (10–100 mL)	Yellow
10	Health hazard	Ethanol, moderate rating	Yellow
11	Safety hazard	Flammable (ethanol)	Yellow
12	Energy	< 0.1 kWh per sample	Green
13	Occupational hazard	Closed HPLC system	Green
14	Waste	> 10 mL	Red
15	Waste treatment	No on-site treatment	Red

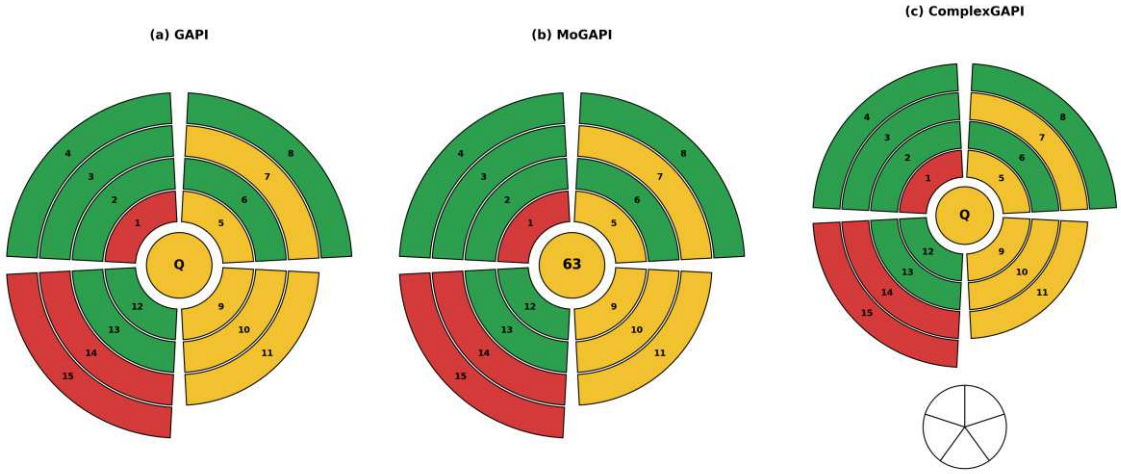


Fig. 13 GAPI, MoGAPI and ComplexGAPI pictograms of the proposed method. (a) GAPI, (b) MoGAPI (score 63%) and (c) ComplexGAPI. Numbers are the field numbers in Table 18.

HPLC-EAT. The HPLC-EAT score was 30.7 per analysis: safety 22.5, health 2.4 and environment 5.8 (Table 19, Fig. 14). safety accounted for about 73% of the total, mainly because ethanol is flammable. For comparison, the same calculation for a hypothetical conventional method (acetonitrile–water 50:50 v/v, 1.0 mL/min, 10 min run, acetonitrile as diluent) gave 53.7. The proposed method was therefore about 43% lower overall, with the health component about 80% lower, the environmental component about 36% lower, and the safety component about 30% lower.

Table 19 HPLC-EAT impacts per analysis

Method	Solvent	Mass (g)	Safety	Health	Environment	Total
Proposed	Ethanol	11.99	22.5	2.4	5.8	30.7
	Water	2.80	0	0	0	
Reference[a]	Acetonitrile	11.79	32.1	12.5	9.1	53.7

Method	Solvent	Mass (g)	Safety	Health	Environment	Total
	Water	5.00	0	0	0	

A hypothetical conventional method used only for comparison (acetonitrile–water 50:50 v/v, 1.0 mL/min, 10 min, acetonitrile as diluent). Solvent factors per gram (safety/health/environment): ethanol 1.872/0.204/0.485; acetonitrile 2.724/1.056/0.772 **(19)** .

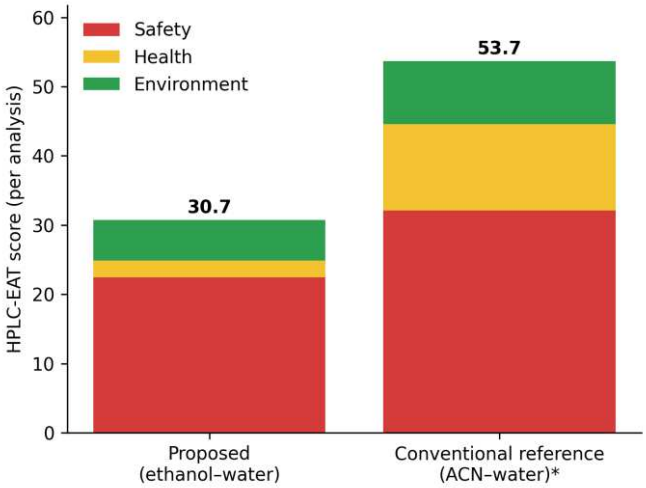


Fig. 14 HPLC-EAT scores per analysis for the proposed and reference methods. The proposed ethanol–water method is compared with the hypothetical acetonitrile–water reference.

Table 20. Summary of the greenness assessment

Tool	Result	Interpretation
AGREE	0.58	Moderate; weakest principles 3, 1 and 8
GAPI	7 green / 5 yellow / 3 red fields	Red: collection, waste volume, waste treatment
MoGAPI	63%	Acceptable greenness (50–74%)
ComplexGAPI	Pre-analytical zone not applicable	The main pictogram is the same as GAPI
HPLC-EAT	30.7 vs 53.7 (reference)	About 43% lower than the reference

Discussion

Greenness of the method

The five tools give the same overall picture. The method is greener than a conventional acetonitrile-based RP-HPLC method, but it is not fully green. It is better described as an improvement on usual practice than as an ideal green method.

The gain comes almost entirely from the solvent. Ethanol is renewable and has low toxicity, and it is rated as recommended in the CHEM21 solvent guide, whereas acetonitrile is rated as problematic **(21)** . In HPLC-EAT, this change lowered the health impact by about 80% and the environmental impact by 36%. The safety impact fell by 30% even though ethanol is flammable. Flammability remains the main residual hazard and explains the yellow fields 10 and 11 in GAPI and the score of 0.75 for principle 12 in AGREE.

The points that were lost come from how the procedure is run, not from the solvent: offline analysis (principle 3, field 1), 18.0 mL of solvent and waste per sample (principle 7, field 14), no waste treatment (field 15), one analyte per run (principle 8) and a conventional 4.6 mm column (principle 5). A green solvent therefore does not make a green method by itself. Part of the waste can be avoided. In one analysis, most of the ethanol is the sample diluent rather than the mobile phase; in this model, sample preparation accounts for about 66% of the ethanol volume per analysis. Halving the diluent volume would lower the HPLC-EAT score from 30.7 to approximately 20.6 (a 33% reduction). Collecting and redistilling ethanol waste would move field 15 away from red, and a shorter, narrower column would further reduce mobile-phase use.

Ethanol–water mixtures are more viscous than acetonitrile–water mixtures, which raises column back-pressure. This is a reason to use a moderate flow rate (0.8 mL/min) and a column temperature of 35 °C.

The assessment has limits. First, the scores depend on stated assumptions (diluent volume, energy use and waste handling). Second, the tools weigh criteria differently, and some inputs need judgement, so scores from different tools should not be compared numerically. Third, the comparator in HPLC-EAT is a hypothetical conventional method rather than a published ATO method. Finally, these metrics describe environmental and operator impact, not analytical performance; a greener method is useful only if it also meets the validation criteria in the ATP.

Limitations of the study

Several limitations should be considered. The BBD models are reliable only for peak area, because five runs without a peak were entered as zero and the three centre points were identical. The selected conditions lie at the lower limit of water content and flow rate, so the operable region was not fully bracketed. Specificity was shown with a blank injection only. The forced degradation results were not used to claim specificity because peak purity and resolution were not assessed, and the nature of the peak at approximately 3.3 min was not established. The method is therefore not claimed to be stability-indicating; that claim would require peak-purity evidence (for example, from a photodiode-array or mass-spectrometric detector) and resolution data for the degradation products. Accuracy and precision were tested on the bulk drug at 8–24 µg/mL; use in finished dosage forms would therefore require separate validation in the presence of excipients. Finally, peak area changed noticeably with flow rate and column temperature in the robustness study, so these two parameters must be tightly controlled.

Conclusions

A green RP-HPLC method for atogepant was developed with an AQbD approach and a Box–Behnken design. The final conditions were a C18 column (250 × 4.6 mm, 5 µm), ethanol–water (65:35, v/v), 0.8 mL/min, 35 °C and detection at 280 nm, with ATO eluting at about 4.8 min. The method was linear from 4 to 64 µg/mL (R^2 = 0.9998), with LOD and LOQ of 1.006 and 3.047 µg/mL, recoveries of 98.00–100.13% and precision below 0.9% RSD. The blank showed no interference at the ATO retention time. Forced degradation resulted in a 10.32–16.92% loss of ATO; however, because peak purity and resolution were not assessed, the stress study is not used to support specificity, and the method is not claimed to be stability-indicating. Repeatability remained below 2% RSD across six deliberate changes in conditions. The BBD models were useful only for peak area, and the chosen conditions lie at the edge of the studied

design space. Replacing acetonitrile with ethanol made the method greener: AGREE 0.58, MoGAPI 63%, GAPI with seven green, five yellow and three red fields, and an HPLC-EAT score about 43% lower than that of a conventional acetonitrile-based reference. The remaining weak points are offline operation, waste volume and the lack of waste treatment, and the largest practical gain would come from smaller sample-preparation volumes and solvent recovery.

References

1. Zhang Y jie, Li X yu, Guo Z lin. Temporal trends of migraine and tension-type headache burden across the BRICS: implications from the Global Burden of Disease study 2019. Front Neurol. 2023 Dec 22;14. doi:10.3389/fneur.2023.1307413
2. Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies — successful translation from bench to clinic. Nat Rev Neurol. 2018 Jun 24;14(6):338–50. doi:10.1038/s41582-018-0003-1
3. Ailani J, Lipton RB, Goadsby PJ, Guo H, Miceli R, Severt L, et al. Atogepant for the Preventive Treatment of Migraine. New England Journal of Medicine. 2021 Aug 19;385(8):695–706. doi:10.1056/NEJMoa2035908
4. A.Venkata Suresh Babu HKS. Method Development And Validation Of Ubrogepant In Bulk And Pharmaceutical Dosage Form By Using Rp-Hplc. J Pharm Negat Results. 2022 Dec 31;7690–6. doi:10.47750/pnr.2022.13.S07.929
5. Nagababu U, Sujatha D, Jyothi U, Vissa MR, Srinivasa Kumar B. Validated Stability Indicating HPLC Method for the Quantification of Process Related Impurities of Ubrogepant in Pharmaceutical Formulations. EURASIAN JOURNAL OF CHEMISTRY. 2023 Mar 27;28(1 (109)). doi:10.31489/2959-0663/1-23-7
6. Goraksh TA, Gitaram VD. QbD-Based RP-HPLC Method Development and Validation for Quantitation of Rimegepant in Standard and Pharmaceutical Formulations. International Journal of Pharmaceutical Research and Applications. 2025 Feb;10(2):942–5. doi:10.35629/4494-1002942945
7. Phaneendra Rao PG, Rao BS. Development and Validation of Novel Analytical LC-MS Method for Simultaneous Quantification of Calcitonin Gene-Related Peptide Receptor Antagonists Ubrogepant and Atogepant in Human Plasma. Asian Journal of Chemistry. 2022 Oct 19;34(11):2865–71. doi:10.14233/ajchem.2022.23913

8. Kumar HMS, Chandrasekhar KB. Determination of Atogepant Using RP-HPLC in Bulk and Table ts. Asian Journal of Pharmaceutical Research and Health Care. 2022 Jul;14(3):161–7. doi:10.4103/ajprhc.ajprhc_4_22
9. Rizwana I, Anam SU. Stability Indicating RP-HPLC Method for Development and Validation of Atogepant and its Application in Dissolution Studies. Int J Pharm Sci Rev Res. 2022 Nov 15;160–4. doi:10.47583/ijpsrr.2022.v77i01.026
10. International Council for Harmonisation. ICH. 2023. ICH Q14: Analytical procedure development.
11. Deshmukh DS, Bhalekar S, Gosavi OR, Aher KN, Lamkhade G, Ramteke K. STABILITY-INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION: CONTEMPORARY ANALYTICAL STRATEGIES, REGULATORY EVOLUTION, AND RECENT ADVANCES. EPRA International Journal of Multidisciplinary Research (IJMR). 2026 May 27;817. doi:10.36713/epra27370
12. International Council for Harmonisation. ICH Q2(R2): Validation of analytical procedures. 2023.
13. International Council for Harmonisation. ICH Q1A(R2): Stability testing of new drug substances and products. 2003.
14. International Council for Harmonisation. ICH Q1B: Photostability testing of new drug substances and products. 1996.
15. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE─Analytical GREEnness Metric Approach and Software. Anal Chem. 2020 Jul 21;92(14):10076–82. doi:10.1021/acs.analchem.0c01887
16. Płotka-Wasylka J. A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index. Talanta. 2018 May;181:204–9. doi:10.1016/j.talanta.2018.01.013
17. Mansour FR, Płotka-Wasylka J, Locatelli M. Modified GAPI (MoGAPI) Tool and Software for the Assessment of Method Greenness: Case Studies and Applications. Analytica. 2024 Sep 6;5(3):451–7. doi:10.3390/analytica5030030
18. Płotka-Wasylka J, Wojnowski W. Complementary green analytical procedure index (ComplexGAPI) and software. Green Chemistry. 2021;23(21):8657–65. doi:10.1039/D1GC02318G
19. Gaber Y, Törnvall U, Kumar MA, Ali Amin M, Hatti-Kaul R. HPLC-EAT (Environmental Assessment Tool): A tool for profiling safety, health and environmental

impacts of liquid chromatography methods. Green Chemistry. 2011;13(8):2021. doi:10.1039/c0gc00667j

20. Gałuszka A, Migaszewski Z, Namieśnik J. The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. TrAC Trends in Analytical Chemistry. 2013 Oct;50:78–84. doi:10.1016/j.trac.2013.04.010
21. Prat D, Wells A, Hayler J, Sneddon H, McElroy CR, Abou-Shehada S, et al. CHEM21 selection guide of classical- and less classical-solvents. Green Chemistry. 2016;18(1):288–96. doi:10.1039/C5GC01008J