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Analytical approach for conversion of conventional to green RP-HPLC methods for analysis of lipophilic neutral and basic active pharmaceutical ingredients

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ABSTRACT

Conventional RP-HPLC methods in pharmaceutical industry still dominantly rely on hazardous solvents like methanol (MeOH) and acetonitrile (ACN), presenting significant environmental and safety challenges. Although ethanol (EtOH) is recognized as greener alternative, isoeluotropic relationships between EtOH and these conventional solvents will broaden its implementation in routine HPLC analyses. To address this issue, the study presents an empirical approach using volumetric fraction (φ) equivalence to establish initial chromatographic conditions, enabling predictable substitution of MeOH and ACN with EtOH in the LC mobile phase without altering other chromatographic parameters. The approach was established using adsorption (log-log) retention model across multiple C_{18} stationary phases, applicable for lipophilic neutral and basic active pharmaceutical ingredients (APIs). For neutral APIs, φ MeOH ≈ 1.6 φ EtOH and φ ACN ≈ 1.1 φ EtOH, while for basic APIs φ MeOH ≈ 1.5 φ EtOH and φ ACN ≈ 1.0 φ EtOH. The general applicability was confirmed under isocratic conditions by successfully converting conventional RP-HPLC methods for an independent set of APIs into green, EtOH-based alternatives. Notably, this work extends previous research focused on lipophilic acidic to neutral and basic APIs, broadening its utility for the most widely used APIs in the pharmaceutical industry. The proposed framework supports sustainable analytical practices and improves laboratory safety without extensive method redevelopment.

Keywords: green RP-HPLC transformation, EtOH-based mobile phase, retention model, lipophilic neutral APIs, lipophilic basic APIs, isoeluotropic series

INTRODUCTION

Reverse-phase high-performance liquid chromatography (RP-HPLC) is an essential tool in the pharmaceutical analysis, but its dependence on hazard organic solvents poses significant environmental and safety concerns. The adoption of green analytical chemis-

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try (GAC) principles, particularly solvent substitution as an effective approach for greening HPLC methods, offers a direct route to reducing hazardous waste, lowering occupational exposure, and improving overall sustainability (1–11). Ethanol (EtOH) has been identified as one of the most widely used green organic solvents in RP-HPLC (1, 6, 10, 11) due to its low toxicity, biodegradability, and reduced environmental impact compared to methanol (MeOH) and acetonitrile (ACN), yet its broader implementation has been limited by the lack of simple conversion strategies. Additionally, the "greenness" of EtOH is also literately confirmed through the ChlorTox base (12) and the CHEMS-1 model (13).

Although the chromatographic behaviour of EtOH has been studied (6), the available data are neither as extensive nor as systematically developed as those reported for acetonitrile (ACN) or methanol (MeOH) (11, 14, 15). Moreover, reported applications of EtOH are typically limited to specific case examples involving selected analytes (11) rather than being presented as a general approach that could be applicable to a broad range of active pharmaceutical ingredients (APIs).

Developing isoeluotropic relationships between EtOH and conventional solvents can greatly facilitate this transition by enabling straightforward replacement without extensive method redevelopment, thereby minimizing time, resource use and solvent waste. Retention models such as the log-linear (LSS), quadratic (Q), and adsorption (log-log, ADS) have broad applications in determining solvent elution strength, optimizing and transferring methods and characterizing stationary phases. These models describe the relationship between the retention factor (k) of an analyte and the volume fraction (φ) of the organic solvent in the mobile phase (16, 17).

Building on previous work that established these relationships for acidic APIs (15), this study aims to extend the approach to lipophilic neutral and basic APIs using a practical, elution strength-based framework. The validity of this analytical approach was demonstrated through the successful and simplified transformation of several conventional RP-HPLC methods to green EtOH-based methods under isocratic conditions, encouraging the routine use of EtOH.

EXPERIMENTAL

Selected APIs and reagents

The APIs used for the research were secondary reference standards of APIs (produced by and kindly donated by Alkaloid AD, N. Macedonia). The lipophilic neutral and basic APIs were divided into an establishment set used for building the approach and an independent verification set used to evaluate its applicability. The establishment set contained five neutral (clindamycin hydrochloride – CLD, prazosin hydrochloride – PRZ, chloramphenicol – CHL, phenobarbital – PHN, nimesulide – NMS), and seven basic APIs (lamotrigine – LMT, sitagliptin phosphate monohydrate – SPM, zolpidem tartrate – ZOT, fluoxetine hydrochloride – FLH, naphazoline hydrochloride – NPH, nifuroxazide – NFX and risperidone – RSP). Verification set consisted of one neutral API (dapagliflozin propylene glycol monohydrate – DPM) and two basic APIs (bisacodyl – BSC and diazepam – DAZ). Chemical structures of APIs are presented in Fig. 1.

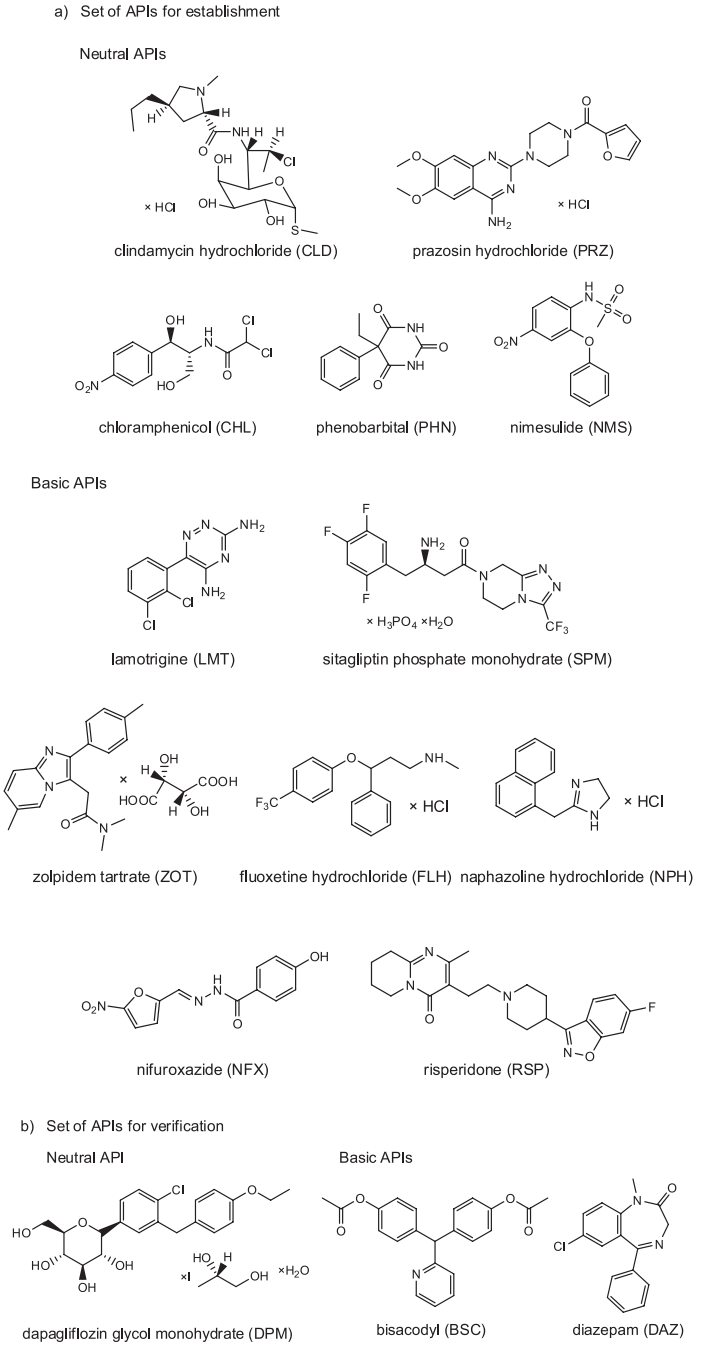

Fig. 1. Selected active pharmaceutical ingredients (APIs) for: a) establishment set; b) verification set.

MeOH, ACN and EtOH, all LC grade and with purity of ≥ 99.9 % (Supelco-Merck, Germany) were used for the preparation of the mobile phases and diluents. KH_2PO_4 *pro analysis* (Merck) and *o*-H_3PO_4 *pro analysis* (Merck) were used for the preparation of the buffer solution for the mobile phase. Regenerated cellulose (RC) membrane filter 0.45 μm (Sartorius, Germany) was used for filtration of the solutions.

Chromatographic analysis

The chromatographic analysis for lipophilic neutral APIs was performed on Vanquish Duo UHPLC (ThermoFisher Scientific, USA) system, equipped with DAD detector, while for basic APIs the analysis was conducted on Dionex UltiMate 3000 Method Scouting UHPLC (ThermoFisher Scientific) system, also equipped with DAD detector. Both instruments were fully controlled by the Chromeleon Chromatography Data System, version 7.3.2.

For the experiments, four different octadecyl silica (C_{18}) columns (dimension 150 mm × 4.6 mm) without a pre-column were used: Agilent Zorbax Extend 5 μm, Agilent Zorbax StableBond 5 μm, ThermoFisher Scientific BDS Hypersil 3 μm and Waters XBridge 5 μm. The chromatographic separation was performed using different mobile phases composed of 20 mmol L^{-1} CH_3COONH_4 (pH 5.0) as a buffer solution for the analysis of lipophilic neutral APIs and 20 mmol L^{-1} KH_2PO_4 (pH 6.5) for lipophilic basic APIs and suitable organic eluent (MeOH, ACN or EtOH) in isocratic elution mode. The fraction φ of the organic eluent (MeOH, ACN or EtOH) in the corresponding mobile phase ranged from 30 to 70 %, *V*/*V* (with 5 % increment). The flow rate was 1 mL min^{-1}, the column temperature was set to 35 °C and the autosampler temperature was set to 25 °C. The injection volume for all tested model APIs was 20 μL. The selected APIs were monitored on their respective wavelengths for maximum absorption.

Preparation of test solutions

Test solutions of the model compounds, each in concentration of 0.2 mg mL^{-1}, were prepared by dissolving the APIs in the corresponding organic solvent (MeOH, ACN or EtOH). Dilution to a final concentration of 0.05 mg mL^{-1} was made with a mixture of corresponding buffer solution and suitable organic solvent (MeOH, ACN or EtOH) in a ratio of 1:1 (50 %, *V*/*V*). All solutions were filtered before injection through RC membrane filter 0.45 μm.

Retention model for determination of elution strength of EtOH

The adsorption (log-log, ADS) retention model was used to evaluate the elution strength of EtOH in comparison to MeOH and ACN, which was identified as the most suitable tool based on a statistical evaluation of the chromatographic data (15). The used ADS model comprises of logarithmical transformation of k values as a function of φ values. This model is also known as log-log or Snyder-Soczewiński model (16, 17) and is presented by the following equation:

$$\ln k = \ln k_0 - n \ln \varphi$$

where n is the slope (so-called solvation number) and it represents the ratio of surface areas occupied by adsorbed molecules of the organic eluent and the analyte, $\ln k_0$ (often denoted

as ln k_w), refers to the isocratic k of a solute when pure water is used as a mobile phase and ln φ is the logarithm of φ of the organic eluent (16, 17). When the model was used for the calculations presented in this manuscript, an inverse linear correlation between ln k and ln φ was obtained.

Statistical analysis was performed using regression analysis.

Verification of the applicability of the proposed analytical approach for conversion of conventional RP-HPLC methods into EtOH-based methods

The applicability of the proposed analytical approach was verified by conversion of conventional RP-HPLC methods, used for determination of APIs that are different from those used for the establishment of the approach. The verification set comprised DPM as a representative lipophilic neutral API, alongside BSC and DAZ as representative lipophilic basic APIs. Two conventional RP-HPLC methods used for assay of DPM were transformed into EtOH-based methods. The methods, available in literature, were isocratic conventional methods and used MeOH and water in ratio of 4:1 (80:20 %, *V*/*V*) (18) and ACN and water in ratio of 3:2 (60:40 %, *V*/*V*) (19).

For assay of BSC and for DAZ two conventional isocratic RP-HPLC methods were selected for verification of the proposed analytical approach. The method for assay of BSC, published in USP (20) specifies the use of ACN and ammonium formate (pH 5.0) in ratio of 4.5:5.5 (45:55 %, *V*/*V*). The method reported in the literature for the assay of DAZ is based on a mobile phase consisting of 50 mmol L^{-1} phosphate buffer (pH 4.5) and MeOH in a ratio of 2:3 (40:60 %, *V*/*V*) (21).

The chromatographic separation for all of the APIs used for the verification process was performed using XBridge BEH C_{18} column (150 mm × 4.6 mm, 5 μm).

The φ (%) of conventional eluent (MeOH or ACN) in the mobile phase and diluent was replaced with the corresponding φ (%) of EtOH. The required EtOH fraction to achieve the elution strength as the conventional mobile phases was determined using the proposed approach. In the EtOH-based methods, all the other chromatographic conditions remained the same as those in the reference conventional RP-HPLC methods, with the only change being the substitution of the organic eluent in the mobile phase and the solvent used for sample preparation.

RESULTS AND DISCUSSION

Selection of APIs used as model compounds for establishment and verification of the approach

For establishment of the approach five neutral and seven basic APIs were used. During the selection of the APIs as model compounds, attention was made to cover a wide range of log*P* and PSA values, so that the proposed transformational model would be applicable on a variety of APIs. Subsequently, an independent verification set comprising structurally diverse neutral and basic APIs was selected to challenge the approach under realistic laboratory scenarios. For this verification process DPM was selected as a neutral, lipophilic compound undergoing purely hydrophobic interactions during RP-HPLC analysis, alongside BSC and DAZ as lipophilic basic APIs capable of secondary silanol or

ion-exchange interactions on C_{18} stationary phases. All of the used APIs with their physico--chemical characteristics (22) are presented in Table I.

During development of an RP-HPLC method it is important to know whether the tested API is acidic, basic or neutral, as well as to know its pK_a. But, classifying APIs based solely on a single pK_a value can appear contradictory if the pH value of the mobile phase

Table I. Physico-chemical characteristics of the APIs used for the establishment and verification of the approach (22)

Set of neutral APIs for establishment	log*P*	PSA (Å^2)
Clindamycin hydrochloride (CLD)	Experimental: 2.16 Predicted: 1.76; 1.04	102.26
Prazosin hydrochloride (PRZ)	Experimental: 1.3 Predicted: 1.93; 1.65	106.95
Chloramphenicol (CHL)	Experimental: 1.14 Predicted: 1.15; 0.88	112.7
Phenobarbital (PHN)	Experimental: 1.47 Predicted: 1.4; 1.41	75.27
Nimesulide (NMS)	Experimental: 2.60 Predicted: 2.56; 1.79	98.54
Set of neutral APIs for verification	**log*P***	**PSA (Å^2)**
Dapagliflozin propylene glycol monohydrate (DPM)	Experimental: 2.7 Predicted: 2.11; 2.52	99.38
Set of basic APIs for establishment	**log*P***	**PSA (Å^2)**
Lamotrigine (LMT)	Experimental: 1.93 Predicted: 1.87; 1.93	90.71
Sitagliptin phosphate monohydrate (SPM)	Experimental: 1.5 Predicted: 1.95; 1.26	77.04
Zolpidem tartrate (ZOT)	Experimental: 3.02 Predicted: 3.15; 3.02	37.61
Fluoxetine hydrochloride (FLH)	Experimental: 4.05 Predicted: 4.09; 4.17	21.26
Naphazoline hydrochloride (NPH)	Experimental: 3.88 Predicted: 3.44; 2.19	24.39
Nifuroxazide (NFX)	Predicted: 2.59; 1.75	117.97
Risperidone (RSP)	Predicted: 3.27; 2.63	61.94
Set of basic APIs for verification	**log*P***	**PSA (Å^2)**
Bisacodyl (BSC)	Predicted: 3.61; 4.71	65.49
Diazepam (DAZ)	Experimental: 2.82 Predicted: 2.63; 3.08	32.67

is not fully considered. APIs frequently contain multiple functional groups with distinct pK_a values. Therefore, their retention mechanisms in RP-HPLC are governed not by static structural labels, but by their actual operational ionization state at the chosen mobile phase pH value. APIs classified as neutral at the used mobile phase either lack ionizable groups within the analytical window (*e.g.*, CHL, DPM) or are weak acids whose ionization is effectively suppressed (*e.g.*, PHN, NMS). According to the Henderson-Hasselbalch relationship, these compounds remain predominantly in their unionized forms, and their retention is governed primarily by hydrophobic partitioning with minimal contribution from ionic interactions. At pH 6.5, analytes containing basic functionalities with pK_a values substantially above the mobile-phase pH exist predominantly as protonated cations (*e.g.*, FLH, SPM, RSP, ZOT). Conversely, weak bases (*e.g.*, LMT, DAZ, BSC) are deprotonated and exist as uncharged free bases, behaving operationally as neutral species.

Determination of the elution strength of EtOH for lipophilic neutral and basic APIs

All selected APIs used as model compounds were analyzed on four octadecylsilyl (C_{18}) columns (Zorbax Extend 5 μm, Zorbax StableBond 5 μm, BDS Hypersil 3 μm and XBridge 5 μm), as C_{18} columns represent the most commonly used stationary phases in RPLC (23).

The used mobile phases were varied by the type and φ (%) of the organic solvent (MeOH, ACN or EtOH) in a fraction of 30, 35, 40, 45, 50, 55, 60, 65 and 70 %. The φ of 70 % was used as the highest φ in order to avoid retention time values lower than the retention time of the used hold-up marker, and the lowest φ of 30 % was used in order to avoid peaks that will show band broadening, unacceptable asymmetry and reduced height.

During determination of the elution strength of EtOH for lipophilic neutral and basic APIs three different retention models were used: non-logarithmic, log-linear (LSS) and ADS. Statistical analysis with ADS gave the most appropriate values of r, R^2, standard error (%) and p-values for φ (%), (Supplementary file: Appendices A and B, resp.). Following, ADS was found to be the most suitable retention model for evaluation of the elution strength of EtOH.

The determination of EtOH elution strength is illustrated using the Zorbax Extend column as a representative of all of the used C_{18} columns. The obtained plots with the ADS model for the model APIs analyzed on Zorbax Extend column are shown in Fig. 2 (for neutral APIs) and in Fig. 3 (for basic APIs). The values for R^2 for neutral APIs, obtained by the ADS model, ranged from 0.991 to 0.999 for MeOH; from 0.991 to 1.000 for ACN and from 0.993 to 1.000 for EtOH. In the case of basic APIs, the values for R^2 ranged from 0.995 to 1.000 for MeOH; from 0.990 to 0.998 for ACN and from 0.990 to 1.000 for EtOH. This indicates that 99.0–100.0 % of the variance in the logarithmically transformed k values for neutral and basic lipophilic APIs could be explained by changes in φ (%) (Supplementary file: Appendices A and B, resp.). The standard error of the slope of the regression lines obtained using the ADS model was relatively low for both tested groups of APIs, for neutral they ranged from 1.3 to 3.6 % for MeOH, 0.3 to 3.5 % for ACN, and 0.5 to 3.2 % for EtOH (Appendix A) and for basic from 0.7 to 3.6 % for MeOH, 1.5 to 3.9 % for ACN, and 0.25 to 4.1 % for EtOH (Appendix B), indicating good precision of the slope values. The p-values for φ (%) were below 0.05, indicating that the ADS model is statistically significant for both groups of APIs. Additionally, the 95 % confidence intervals for log φ (%) across all model APIs do not include zero, for both groups of APIs, confirming that the true value for the coefficient of φ (%) is a negative number, indicating inverse relationship and statistically

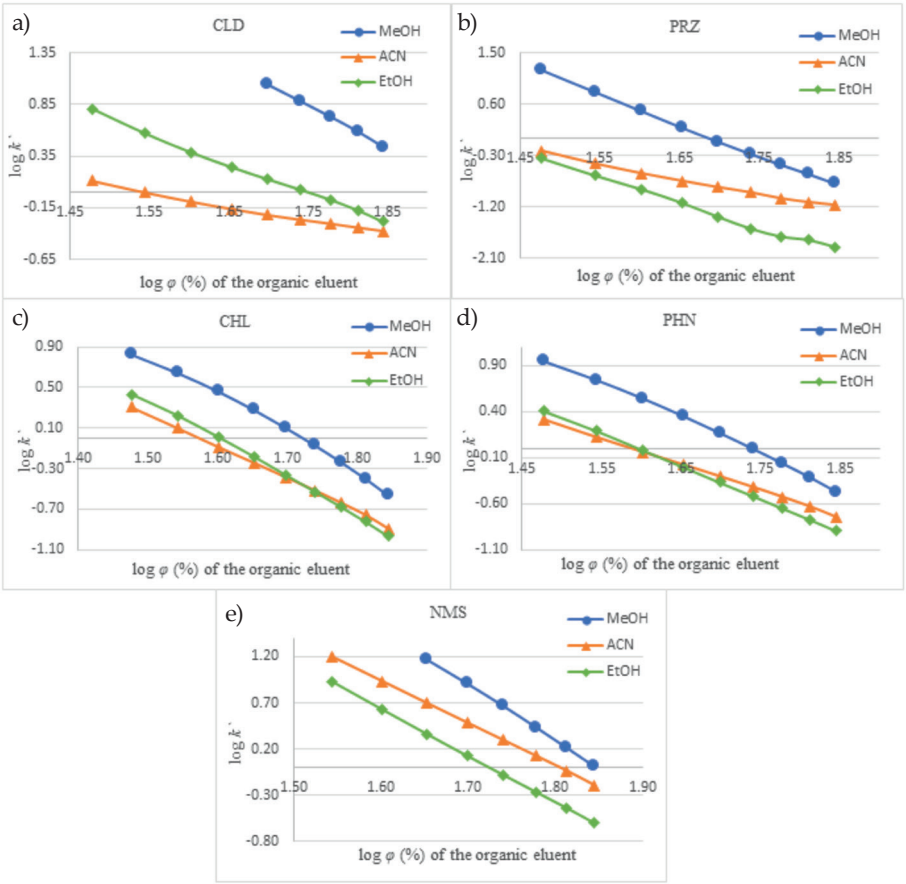

Fig. 2. Plots log k' values *vs.* log φ (%) of the organic eluent obtained with ADS regression model for: a) clindamycin hydrochloride (CLD); b) prazosin hydrochloride (PRZ); c) chloramphenicol (CHL); d) phenobarbital (PHN) and e) nimesulide (NMS).

significant in predicting the response variable k (24). The performance of the approach for neutral and basic lipophilic APIs, as supported by statistical analysis, aligns with the results previously obtained for acidic APIs using the ADS model (15).

Establishment of isoeluotropic series between EtOH, MeOH and ACN for lipophilic neutral and basic APIs

Isoeluotropic series between EtOH, MeOH and ACN for lipophilic neutral and basic APIs were established using the ADS retention model. The calculations were based on obtaining φ (%) of each organic eluent required to achieve identical k values for all tested model compounds, on the used C_{18} columns.

For example, when NMS was analyzed on Zorbax Extend column using the obtained regression equations for MeOH ($y = -6.0274x + 11.145$) and EtOH ($y = -5.0648x + 8.7432$) it

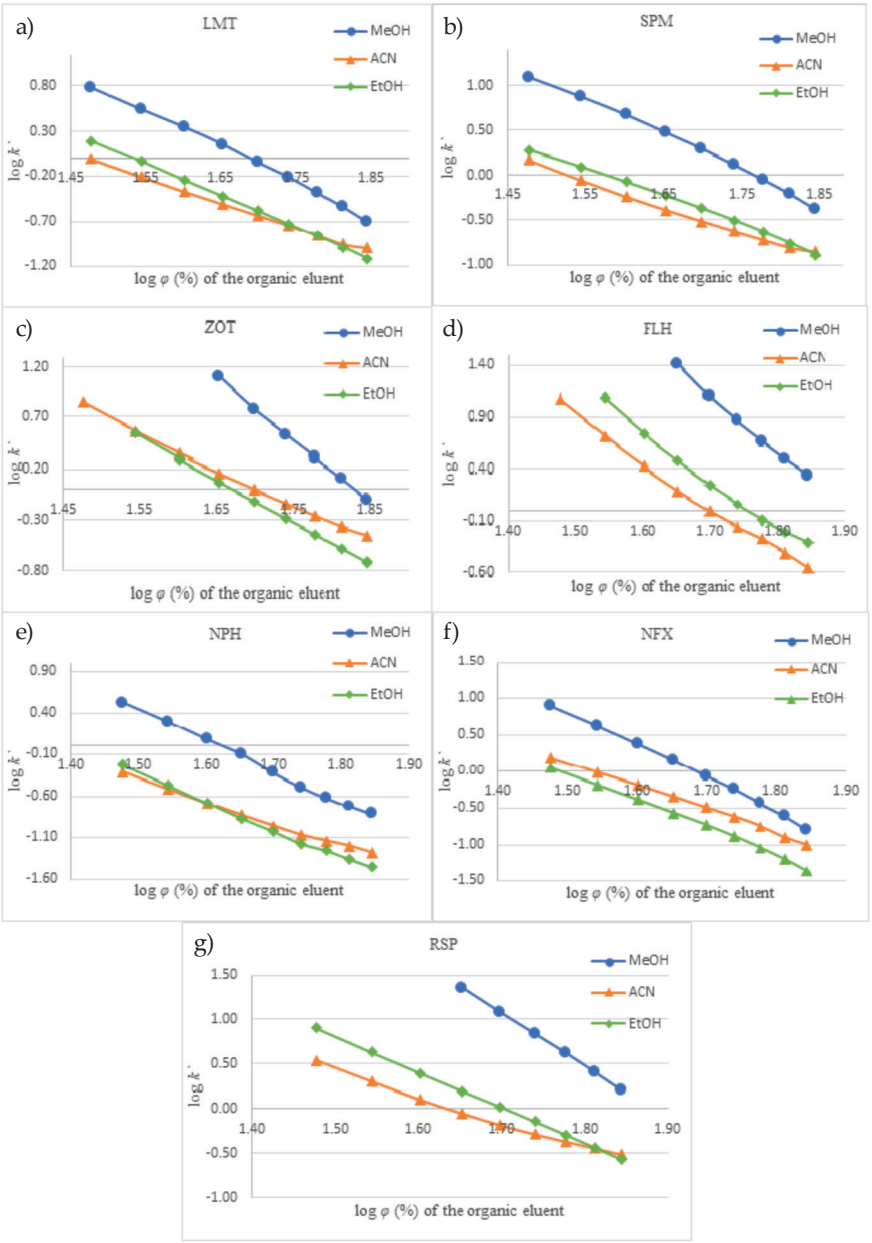

Fig. 3. Plots log k' values *vs.* log φ (%) of the organic eluent obtained with ADS regression model for: a) lamotrigine (LMT); b) sitagliptin phosphate monohydrate (SPM); c) zolpidem tartrate (ZOT); d) fluoxetine hydrochloride (FLH); e) naphazoline hydrochloride (NPH); f) nifuroxazide (NFX) and g) risperidone (RSP).

was calculated that the same k value (*e.g.*, 2.67) would be obtained if 60 % of MeOH in the mobile phase would be replaced with around 44 % of EtOH. In the case of ACN (y = –4.6133x + 8.3252) the same k value (*e.g.*, 1.32) would be obtained if 60 % of ACN in the mobile phase is replaced with around 50 % of EtOH. Accordingly, it can be concluded that less φ (%) of EtOH is needed to achieve the same elution power as the stated φ (%) of MeOH and ACN. The same calculation was performed for each of the analyzed APIs, on each of the four C_{18} columns, for the tested organic eluents (MeOH, ACN and EtOH) using φ in range from 30 to 70 %.

In order to assess the influence of the type of the C_{18} stationary phase on the determination of EtOH's elution strength relative to MeOH and to ACN, four different C_{18} stationary phases (Zorbax Extend, Zorbax StableBond, BDS Hypersil and XBridge) were used for both tested groups of APIs. The stationary phases varied in particle diameter, carbon load, endcapping, working pH range, pore size and particle type. The φ (%) of EtOH was determined for all four chromatographic columns for each of the tested APIs, yielding consistent results across columns in terms of the required φ (%) of EtOH. For example, on the Zorbax Extend column, 50 % MeOH is equivalent in elution strength with 31.8 % EtOH for neutral APIs – a value that remained consistent across all four C_{18} stationary phases, while 50 % ACN corresponds to 49.1 % EtOH on the Zorbax Extend column, compared to an average of 46.9 % EtOH on the other columns. For basic APIs, 50 % MeOH is equivalent to 31.6 % EtOH on Zorbax Extend *versus* an average of 32.7 % EtOH on the other stationary phases, while 50 % ACN corresponds to 50.5 % EtOH on Zorbax Extend *versus* an average of 47.4 % EtOH on other stationary phases. The variation between columns in obtained φ (%) of EtOH equivalent to ACN is observed in comparison to MeOH, probably due to a slightly increased sensitivity of ACN-based mobile phase to the stationary phase characteristics. However, the predicted elution strength of EtOH relative to MeOH and ACN remains consistent across different C_{18} stationary phases.

In addition, the results obtained for both lipophilic neutral and basic APIs confirm that although MeOH and EtOH belong to the same solvent group according to Snyder's polarity triangle (25), EtOH exhibits a stronger elution strength than MeOH. This observation is also supported by existing literature findings (1, 2, 15, 23). Appendix C provides a detailed comparison of the φ (%) of EtOH required to replace specific φ (%) of MeOH for both neutral and basic lipophilic APIs. For each analyzed API, the reported results correspond to the average values obtained from the four tested C_{18} columns. Even though Snyder's classification (25) places ACN and EtOH in distinct solvent groups, EtOH exhibits an elution strength comparable to ACN (Appendix C).

Conversion of conventional RP-HPLC methods via MeOH/ACN substitution with EtOH

The proposed analytical approach presented in this study provides a guidance for the φ (%) of EtOH needed to replace MeOH or ACN as eluents in a conventional RP-HPLC method, while maintaining unchanged chromatographic conditions (Appendix D). This approach offers two options for replacement, one based on the exact percentage of the organic eluent used in the analytical methods, and the other based on the calculated ratios of MeOH and ACN in relation to EtOH. The first based on the exact percentage of the organic eluent used in the analytical methods allows the user to directly read the equivalent φ (%) of EtOH corresponding to the specific φ (%) of MeOH or ACN used in the con-

ventional method straight from the established approach without performing additional calculations. The second, based on the calculated ratios of MeOH and ACN in relation to EtOH, utilizes derived conversion factors where the user takes the original φ (%) of MeOH or ACN from the conventional method and divides it by the corresponding conversion factor (*e.g.*, dividing φ (%) of MeOH by 1.58 for neutral compounds or 1.54 for basic compounds) to determine φ (%) of EtOH.

According to literature data (23), the φ (%) of MeOH is approximately 1.27 times the φ (%) of ACN. In our study, the organic modifier relationship was established as φ MeOH = 1.58 φ EtOH and φ ACN = 1.06 φ EtOH for lipophilic neutral APIs, compared to φ MeOH = 1.54 φ EtOH and φ ACN = 1.05 φ EtOH for lipophilic basic APIs. Notably, these estimated conversion factors are nearly identical across both classes of APIs, demonstrating that the proposed green transformation analytical approach remains consistent across diverse molecular structures and functional groups. In summary, roughly, φ MeOH is almost equal to 1.5 φ EtOH, while φ ACN is almost equal to the φ EtOH. The margin of error (26) was also calculated for the presented conversion factors for MeOH and ACN (Appendix C).

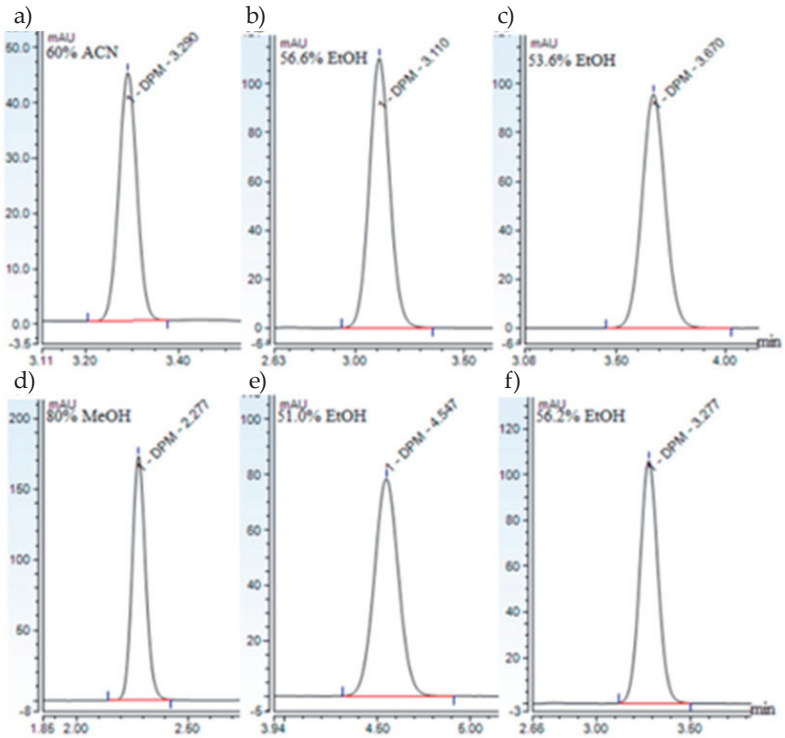

Fig. 4. Chromatograms obtained for dapagliflozin propylene glycol monohydrate (DPM): a) conventional RP-HPLC method using 60 % ACN; b) converted green method with prediction based on φ ACN = 1.06 φ EtOH (60 % ACN = 56.6 % EtOH); c) converted green method with prediction based on exact φ of ACN (60 % ACN = 53.6 % EtOH); d) conventional RP-HPLC method using 80 % MeOH; e) converted green method with prediction based on φ MeOH = 1.58 φ EtOH (80 % MeOH = 51.0 % EtOH) and f) converted green method with prediction based on exact φ of MeOH (80 % ACN = 56.2 % EtOH).

The analytical approach is intended for a guidance toward setting the starting (initial) chromatographic conditions of the green method. When the approach is used the sample preparation diluent should likewise be replaced appropriately. By changing the organic eluent in the mobile phase of the obtained green methods, in certain cases, there may be a need for further adjustment of the chromatographic conditions to ensure the converted RP-HPLC method meets system suitability criteria.

Verification of the applicability of the proposed analytical approach

To assess external validity and avoid overfitting, the applicability of the proposed analytical approach (Appendix D) was verified under isocratic conditions using an independent set of APIs distinct from those used during establishment of the approach. This was achieved by transforming conventional RP-HPLC methods into green, EtOH-based for DPM as a neutral API, alongside BSC and DAZ as basic APIs. The physico-chemical properties of these APIs are presented in Table I. Two selected RP-HPLC methods for assay determination of DPM, reported in the literature, based on isocratic elution using MeOH (18) and ACN (19) as organic eluents, were converted into EtOH-based RP-HPLC methods.

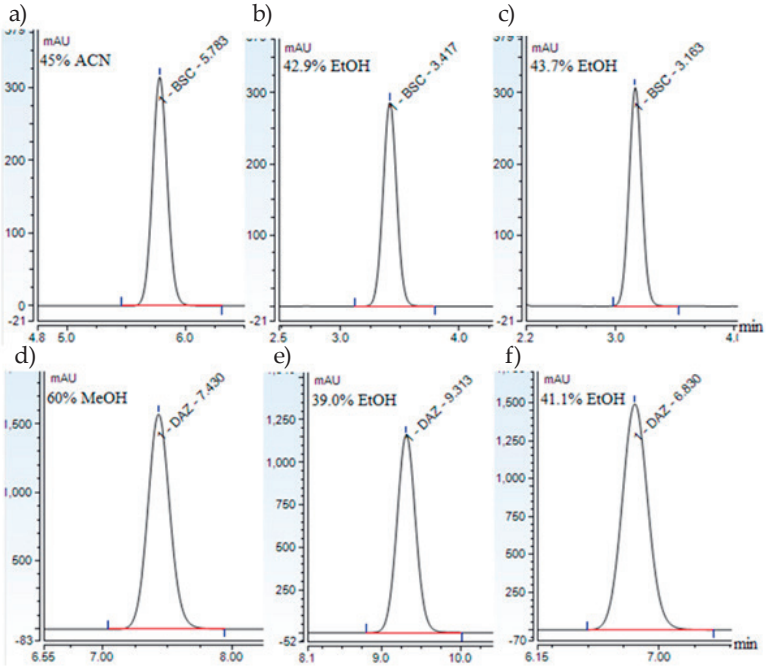

Fig. 5. Chromatograms obtained for: a) bisacodyl (BSC) with conventional RP-HPLC method using 45 % ACN; b) converted green method with prediction based on φ ACN = 1.05 φ EtOH (45 % ACN = 42.9 % EtOH); c) converted green method with prediction based on exact φ of ACN (45 % ACN = 43.7 % EtOH); d) diazepam (DAZ) with conventional RP-HPLC method using 60 % MeOH; e) converted green method with prediction based on φ MeOH = 1.54 φ EtOH (60 % MeOH = 39.0 % EtOH) and f) converted green method with prediction based on exact φ of MeOH (60 % MeOH = 41.1 % EtOH).

Regarding the lipophilic basic APIs, USP method for assay of BSC based on ACN as eluent in the mobile phase (20) and literature-reported assay method for DAZ using MeOH as organic eluent (21) were selected to verify the applicability of the proposed analytical approach. The obtained chromatograms are presented in Figs. 4 and 5.

System suitability parameters including retention time (t_R), symmetry factor (*As*) and theoretical plates number (*N*) were monitored and compared between the conventional and the converted EtOH-based methods (Table II). Regarding the system suitability

Table II. Comparison of the chromatographic parameters for dapagliflozin propylene glycol monohydrate (DPM), diazepam (DAZ), and bisacodyl (BSC) using conventional vs. converted EtOH-based HPLC methods

Dapagliflozin propylene glycol monohydrate (DPM)					
		φ (%) of the organic eluent	t_R (min)/RSD (%) (n = 6)	As	N
Conventional reference method (18)		80 % MeOH	2.27/0.2	1.1	6929
Transformed green methods	Prediction based on exact φ	56.2 % EtOH	3.26/0.7	1.1	5379
	Prediction based on φ ACN = 1.03 φ EtOH	51.1 % EtOH	4.55/0.0	1.0	5750
Conventional reference method (19)		60 % ACN	3.29/0.1	1.0	33318
Transformed green methods	Prediction based on exact φ	53.6 % EtOH	3.68/0.1	1.1	5525
	Prediction based on φ ACN = 1.03 φ EtOH	56.6 % EtOH	3.11/0.0	1.1	5270
Diazepam (DAZ)					
		φ (%) of the organic eluent	t_R (min)/ RSD (%) (n = 6)	As	N
Conventional reference method (21)		60 % MeOH	7.50/0.8	1.1	8894
Transformed green methods	Prediction based on exact φ	41.1 % EtOH	6.83/0.1	1.0	6544
	Prediction based on φ MeOH = 1.54 φ EtOH	39.0 % EtOH	9.27/0.3	1.0	7114
Bisacodyl (BSC)					
		φ (%) of the organic eluent	t_R (min)/RSD (%) (n = 6)	As	N
Conventional reference method (20)		45 % ACN	5.78/0.2	1.1	9988
Transformed green methods	Prediction based on exact φ	43.7 % EtOH	3.16/0.1	1.0	3890
	Prediction based on φ ACN = 1.05 φ EtOH	42.9 % EtOH	3.41/0.1	1.0	4017

requirements of the transformed green methods, system precision was fully satisfied. The RSD for retention time obtained from six consecutive injections of the tested APIs were below 2.0 % (Table II). The peak area precision was also found to be below 2.0 % for all tested methods. Furthermore, peak symmetry remained in strict compliance with *Ph. Eur.* requirements, falling well within the designated 0.8 to 1.8 range. In marketing authorization holder analytical procedures for drug quality control, a column efficiency threshold of $N \geq 2000$ is typically required. While the substitution of MeOH or ACN with EtOH resulted in a decrease in N values, an effect most pronounced when replacing ACN, the N values consistently remained well above this required 2000 threshold. This reduction in column efficiency is primarily attributed to the higher viscosity of EtOH compared to ACN and MeOH. Higher viscosity slows down the mass transfer of the analyte between the mobile phase and the stationary phase, leading to wider peaks and lower N. Crucially, this drop in N did not compromise the other critical chromatographic performance characteristics, demonstrating that the green mobile phase transition completely fulfils the standard system suitability criteria. Additionally, the relevant guidelines for validation of analytical methods (27) does not state a requirement for the value of N, it is to the analyst to decide on the relevance of the obtained value.

Greenness assessment of the conventional and converted green EtOH-based methods

Both the conventional and transformed methods were evaluated using the Analytical Eco-Scale (28) and the AGREE metric (29). Given that the mobile phase elution power and all other chromatographic conditions remained identical, the organic modifier was the sole variable, allowing a direct comparison of the solvents' environmental and safety attributes. Under the Analytical Eco-Scale framework, replacing ACN and MeOH with EtOH consistently increased the greenness index from 80 for MeOH-based and 83 for ACN--based methods to 88 for EtOH-based methods (Table III). This improvement is primarily driven by the superior hazard profile of EtOH, which carries fewer GHS pictograms and lower toxicity, thereby reducing penalty points and the chemical risk footprint during analysis.

Similarly, the AGREE evaluation showed a substantial score elevation, rising from approximately 0.5 for conventional ACN and MeOH methods to around 0.7 for the green EtOH-based methods (Table III). This substitution exerts its most profound impact on GAC principle 11 (toxic reagents) and principle 12 (operator safety). For principle 11, the score of this segment of the pictogram transitions from red/orange to light green. While ACN and MeOH present dual threats as highly flammable and aquatic toxins, the aquatic toxicity threat is entirely eliminated with EtOH. For principle 12, the metric shifts from yellow to green. ACN carries acute toxicity warnings due to its systemic metabolism into cyanide, and MeOH carries a GHS health hazard pictogram for irreversible target organ toxicity to the eyes and central nervous system (1). Conversely, EtOH lacks severe systemic toxicity, drastically lowering the risk profile for the analyst. Furthermore, utilizing agricultural bioethanol satisfies principle 10 (renewable resources) and avoids the petroleum-depletion penalties associated with fossil-derived ACN and MeOH. Finally, transitioning to EtOH streamlines laboratory waste management, indirectly improving principle 7 (waste management) because EtOH waste is readily biodegradable or clean-burning, whereas ACN and MeOH waste streams require highly regulated, cost-intensive disposal.

Table III. Greenness assessment of conventional and converted EtOH-based HPLC methods for dapagliflozin propylene glycol monohydrate (DPM), bisacodyl (BSC) and diazepam (DAZ) using analytical eco-scale and AGREE tools

Dapagliflozin propylene glycol monohydrate (DPM)				
Analytical eco-scale			AGREE pictogram	
Methods	Conventional MeOH-based (18)	Proposed EtOH-baseds	Conventional MeOH-based (18)	Proposed EtOH-baseds
Reagent hazard related PP*	15 (3 * 5)	9 (3 * 3)		
Energy consumption PP (≤ 1.5 kW per sample)	1	1		
Occupation hazard PP	1	1		
Chemical waste PP	3	1		
Total PP	20	12		
Eco-scale index	80	88		
Analytical eco-scale			AGREE pictogram	
Methods	Conventional ACN-based (19)	Proposed EtOH-based	Conventional ACN-based (19)	Proposed EtOH-baseds
Reagent hazard related PP*	12 (3 * 4)	9 (3 * 3)		
Energy consumption PP (≤ 1.5 kW per sample)	1	1		
Occupation hazard PP	1	1		
Chemical waste PP	3	1		
Total PP	17	12		
Eco-scale index	83	88		
Bisacodyl (BSC)				
Analytical eco-scale			AGREE pictogram	
Methods	Conventional ACN-based (20)	Proposed EtOH-based	Conventional ACN-based (20)	Proposed EtOH-baseds
Eco-scale index**	83	88		
Diazepam (DAZ)				
Analytical Eco-Scale			AGREE pictogram	
Methods	Conventional MeOH-based (21)	Proposed EtOH-based	Conventional MeOH-based (21)	Proposed EtOH-based
Eco-scale index**	80	88		

* Reagent hazard related penalty points (PP) = Amount PP * Hazard PP; ** Same principle for calculation of PP as for DPM.

CONCLUSIONS

This study presents a simple and practical analytical approach for conversion of conventional to green RP-HPLC methods for analysis of lipophilic neutral and basic APIs, based on the isoeluotropic series between EtOH (as green solvent) against MeOH and ACN (as the most commonly used conventional eluents). The estimated ratio of the organic eluent fractions (φ MeOH ≈ 1.5 φ EtOH; φ ACN ≈ φ EtOH), considering the verification results, can be used for green conversion of the RP-HPLC methods without compromising the chromatographic performance. The approach was successfully applied for establishment of the initial chromatographic conditions of four different conventional methods into green methods, under isocratic conditions, bypassing the resource-intensive "trial and error" phase. Demonstrating predictable method conversion across both neutral and basic lipophilic drugs confirms the robustness of the EtOH-based approach regardless of compound charge state or specific solute-stationary phase mechanisms. It is to the analyst for a final decision whether with the obtained green method a satisfactory chromatographic separation is achieved or there may be a need for an additional adjustment of the chromatographic conditions. The converted methods require final verification and formal validation before routine implementation.

The authors acknowledge the need for further investigation in the area of gradient methods toward achieving a wider application of this analytical approach. Additionally, the findings of this study will be used as a foundation for the development of a machine learning-based model, which is expected to provide substantial benefits, including enhanced predictive performance and improved robustness in the process of greening the RP-HPLC methods.

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Appendix A

Regression data for log k vs. log φ (%) plots of neutral APIs on Zorbax Extend C18 column.

***Regression statistics* (*MeOH*)**										
API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
CLD	0.99933	0.99867	0.010	8.025	-4.106	0.086	2,09	2.06E-05	-4.381	-3.831
PRZ	0.99940	0.99880	0.025	9.115	-5.381	0.070	1,30	1.73E-11	-5.548	-5.215
CHL	0.99543	0.99089	0.049	6.499	-3.791	0.137	3,61	2.11E-08	-4.116	-3.466
PHN	0.99791	0.99582	0.034	6.760	-3.898	0.095	2,44	1.37E-09	-4.124	-3.672
NMS	0.99968	0.99936	0.012	11.145	-6.027	0.076	1,26	1.51E-07	-6.238	-5.816
***Regression statistics* (ACN)**										
API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
CLD	0.99557	0.99115	0.016	2.004	-1.300	0.046	3,54	1.90E-08	-1.410	-1.190
PRZ	0.99623	0.99247	0.030	3.552	-2.584	0.085	3,29	1.08E-08	-2.785	-2.383
CHL	0.99948	0.99895	0.014	5.069	-3.216	0.039	1,21	1.09E-11	-3.309	-3.123
PHN	0.99966	0.99931	0.010	4.485	-2.824	0.028	0,99	2.45E-12	-2.891	-2.758
NMS	0.99998	0.99996	0.003	8.325	-4.613	0.013	0,28	2.78E-14	-4.644	-4.583
***Regression statistics* (*EtOH*)**										
API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
CLD	0.99825	0.99651	0.023	4.994	-2.858	0.064	2,24	7.34E-10	-3.009	-2.707
PRZ	0.99647	0.99295	0.049	6.080	-4.363	0.139	3,19	8.60E-09	-4.691	-4.034
CHL	0.99906	0.99813	0.022	6.109	-3.820	0.062	1,62	8.23E-11	-3.967	-3.672
PHN	0.99990	0.99980	0.007	5.654	-3.545	0.019	0,54	3.40E-14	-3.590	-3.500
NMS	0.99993	0.99986	0.007	8.743	-5.065	0.025	0,49	8.75E-13	-5.125	-5.005

Appendix B

Regression data for log k vs. log φ (%) plots of basic APIs on Zorbax Extend C_{18} column.

***Regression statistics* (*MeOH*)**

API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
LMT	0.99795	0.99591	0.03433	6.7445	-4.0118	0.10	2,49	1.27E-09	-4.24	-3.78
SPM	0.99748	0.99497	0.03783	7.0232	-3.9821	0.11	2,76	2.64E-09	-4.24	-3.73
ZOT	0.99938	0.99877	0.01741	11.2881	-6.1747	0.11	1,78	5.72E-07	-6.48	-5.87
FLH	0.99751	0.99503	0.03157	10.5840	-5.5679	0.20	3,59	9.29E-06	-6.11	-5.02
NPH	0.99889	0.99779	0.02368	6.0934	-3.7633	0.07	1,86	1.49E-10	-3.92	-3.60
NFX	0.99887	0.99774	0.02913	7.7008	-4.5820	0.08	1,75	1.60E-10	-4.78	-4.39
RSP	0.99989	0.99978	0.00721	11.2688	-5.9950	0.04	0,67	1.90E-08	-6.12	-5.87

***Regression statistics* (*ACN*)**

API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
LMT	0.99919	0.99838	0.01469	4.0112	-2.7312	0.04	1,46	4.97E-11	-2.83	-2.63
SPM	0.99720	0.99441	0.02813	4.2766	-2.8073	0.08	2,85	3.82E-09	-3.00	-2.62
ZOT	0.99825	0.99650	0.02787	6.0052	-3.5213	0.08	2,27	7.38E-10	-3.71	-3.33
FLH	0.99640	0.99281	0.04917	7.3967	-4.3263	0.14	3,24	9.20E-09	-4.66	-4.00
NPH	0.99677	0.99355	0.02832	3.5392	-2.6312	0.08	3,04	6.30E-09	-2.82	-2.44
NFX	0.99888	0.99777	0.02020	4.9269	-3.1993	0.06	1,88	1.53E-10	-3.33	-3.06
RSP	0.99508	0.99018	0.03759	4.6583	-2.8259	0.11	3,89	2.74E-08	-3.08	-2.57

***Regression statistics* (*EtOH*)**

API	r	R^2	Standard error of the regression	Intercept	Slope	*For the slope*				
						Standard error	*Standard error (%)*	*P-value*	*Lower 95% confidence interval*	*Upper 95% confidence interval*
LMT	0.99992	0.99984	0.00605	5.4280	-3.5393	0.02	0,57	1.63E-14	-3.58	-3.50
SPM	0.99859	0.99718	0.02243	4.9774	-3.1593	0.06	1,90	3.46E-10	-3.31	-3.01
ZOT	0.99985	0.99970	0.00819	7.0075	-4.1909	0.03	0,72	8.28E-12	-4.26	-4.12
FLH	0.99514	0.99030	0.05195	8.1744	-4.6350	0.19	4,10	2.86E-07	-5.09	-4.18
NPH	0.99815	0.99631	0.02710	4.6614	-3.3313	0.08	2,40	8.94E-10	-3.51	-3.15
NFX	0.99808	0.99617	0.03123	5.6420	-3.7697	0.09	2,39	1.02E-09	-3.98	-3.56

RSP	0.99995	0.99991	0.00506	6.7743	-3.9778	0.01	0,25	2.08E-15	-4.01	-3.94

Appendix C

Isoeluotropic EtOH composition (φ) relative to MeOH and ACN across four different C18 columns

Neutral APIs								
				φ* (%) *EtOH				
φ* (%) *MeOH	*CLD*	*PRZ*	*CHL*	*PHN*	*NMS*	**average**	*RSD*	*φ MeOH / φ EtOH*
70	41.07	40.17	53.29	51.97	52.23	**47.8**	13.7	1.47
65	37.01	35.75	49.56	47.95	47.94	**43.6**	15.3	1.49
60	33.07	31.53	45.82	43.96	43.69	**39.6**	17.0	1.51
55	29.27	27.53	42.07	40.00	39.51	**35.7**	18.9	1.54
50	25.60	23.74	38.32	36.07	35.38	**31.8**	20.9	1.57
45	22.08	20.17	34.56	32.17	31.32	**28.1**	23.1	1.60
40	18.72	16.83	30.79	28.31	27.33	**24.4**	25.5	1.64
35	15.52	13.72	27.02	24.49	23.42	**20.8**	28.1	1.68
30	12.50	10.86	23.23	20.72	19.59	**17.4**	31.1	1.73
								Average ± margin of error = **1.58** ± 0.058
φ* (%) *ACN				***φ* (%) *EtOH***		**average**	*RSD*	*φ ACN / φ EtOH*
70	73.62	44.61	63.87	61.05	56.81	**60.0**	17.6	1.17
65	70.72	42.52	60.09	57.63	53.17	**56.8**	18.1	1.14
60	67.71	40.38	56.26	54.15	49.50	**53.6**	18.6	1.12
55	64.59	38.17	52.37	50.60	45.79	**50.3**	19.3	1.09
50	61.35	35.88	48.42	46.98	42.05	**46.9**	20.1	1.07
45	57.95	33.52	44.40	43.28	38.27	**43.5**	21.1	1.03
40	54.37	31.06	40.30	39.49	34.45	**39.9**	22.3	1.00
35	50.59	28.49	36.11	35.60	30.57	**36.3**	23.8	0.96
30	46.56	25.79	31.81	31.57	26.64	**32.5**	25.7	0.92
								Average ± margin of error = **1.06** ± 0.054

Basic APIs										
					φ* (%) *EtOH					
φ* (%) *MeOH	*LMT*	*SPM*	*ZOT*	*FLH*	*NPH*	*NFX*	*RSP*	**average**	*RSD*	*φ MeOH / φ EtOH*
70	52.71	48.59	49.98	52.02	50.31	49.01	45.92	**49.8**	4.56	1.41
65	48.63	44.48	45.28	47.53	45.55	45.00	41.28	**45.4**	5.17	1.43
60	44.57	40.43	40.71	43.10	40.93	41.04	36.79	**41.1**	5.90	1.46
55	40.55	36.45	36.26	38.76	36.45	37.12	32.46	**36.9**	6.76	1.49
50	36.55	32.53	31.94	34.51	32.12	33.26	28.31	**32.8**	7.75	1.53
45	32.60	28.69	27.77	30.35	27.94	29.46	24.33	**28.7**	8.87	1.57

40	28.68	24.94	23.75	26.29	23.92	25.73	20.54	**24.8**	10.15	1.61
35	24.80	21.27	19.90	22.35	20.08	22.06	16.96	**21.1**	11.60	1.66
30	20.97	17.70	16.23	18.52	16.42	18.47	13.59	**17.4**	13.29	1.72
										Average ± margin of error = **1.54** ± 0.070
φ* (%) *ACN	***φ* (%) *EtOH***							**average**	*RSD*	*φ ACN / φ EtOH*
70	64.19	65.46	60.71	69.14	55.39	54.17	63.69	**61.8**	8.79	1.13
65	60.73	61.40	57.17	64.89	52.41	51.06	60.52	**58.3**	8.64	1.11
60	57.19	57.29	53.57	60.60	49.38	47.91	57.27	**54.7**	8.50	1.10
55	53.59	53.13	49.92	56.25	46.29	44.70	53.94	**51.1**	8.39	1.08
50	49.90	48.93	46.20	51.85	43.13	41.43	50.51	**47.4**	8.32	1.05
45	46.11	44.66	42.42	47.39	39.89	38.10	46.98	**43.7**	8.30	1.03
40	42.22	40.34	38.55	42.86	36.55	34.69	43.32	**39.8**	8.34	1.01
35	38.20	35.94	34.59	38.26	33.11	31.19	39.52	**35.8**	8.49	0.98
30	34.04	31.45	30.52	33.55	29.55	27.59	35.54	**31.8**	8.78	0.94
										Average ± margin of error = **1.05** ± 0.042

Appendix D

Equivalent φ of EtOH for conversion of conventional to green RP-HPLC method for lipophilic neutral and basic APIs

Neutral APIs		
Based on exact *φ* (%)		Based on calculated ratios
30% MeOH ↔ 17.4% EtOH	30% ACN ↔ 32.5% EtOH	
35% MeOH ↔ 20.8% EtOH	35% ACN ↔ 36.3% EtOH	
40% MeOH ↔ 24.4% EtOH	40% ACN ↔ 39.9% EtOH	*φ* (%) MeOH = 1.58 *φ* (%) EtOH
45% MeOH ↔ 28.1% EtOH	45% ACN ↔ 43.5% EtOH	
50% MeOH ↔ 31.8% EtOH	50% ACN ↔ 46.9% EtOH	
55% MeOH ↔ 35.7% EtOH	55% ACN ↔ 50.3% EtOH	
60% MeOH ↔ 39.6% EtOH	60% ACN ↔ 53.6% EtOH	*φ* (%) ACN = 1.06 *φ* (%) EtOH
65% MeOH ↔ 43.6% EtOH	65% ACN ↔ 56.8% EtOH	
70% MeOH ↔ 47.8% EtOH	70% ACN ↔ 60.0% EtOH	
Basic APIs		
Based on exact *φ* (%)		Based on calculated ratios
30% MeOH ↔ 17.4% EtOH	30% ACN ↔ 31.8% EtOH	
35% MeOH ↔ 21.1% EtOH	35% ACN ↔ 35.8% EtOH	
40% MeOH ↔ 24.8% EtOH	40% ACN ↔ 39.8% EtOH	*φ* (%) MeOH = 1.54 *φ* (%) EtOH
45% MeOH ↔ 28.7% EtOH	45% ACN ↔ 43.7% EtOH	
50% MeOH ↔ 32.8% EtOH	50% ACN ↔ 47.4% EtOH	
55% MeOH ↔ 36.9% EtOH	55% ACN ↔ 51.1% EtOH	
60% MeOH ↔ 41.1% EtOH	60% ACN ↔ 54.7% EtOH	*φ* (%) ACN = 1.05 *φ* (%) EtOH
65% MeOH ↔ 45.4% EtOH	65% ACN ↔ 58.3% EtOH	
70% MeOH ↔ 49.8% EtOH	70% ACN ↔ 61.8% EtOH	