Chapter

Green Strategies toward Eco-Friendly HPLC Methods in Pharma Analysis

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Abstract

The global need for changing the processes in order to meet the green analytical chemistry (GAC) criteria is a great challenge for the pharmaceutical industry. High-performance liquid chromatography (HPLC), as one of the most frequently used techniques in various stages in the pharmaceutical industry, generates huge amounts of organic toxic waste. Therefore, the implementation of the GAC principles in pharma analysis is highly required. Although the number of published papers concerning green chromatography approaches is constantly increasing, the use of eco-friendly HPLC methods in the pharma industry has not been widely implemented. The reasons for this mainly include the need for adaptation of the conventional HPLC instruments, lack of time, lack of experience, or uncertainty of the analysts regarding fulfillment of the method criteria. In this chapter, an overview of green strategies that can be easily applied to conventional instruments for liquid chromatography (LC) in developing eco-friendly HPLC methods in pharma analysis is given. The aim is to emphasize that the green method development in pharma analysis can be easily accomplished and to encourage the analytical community in the pharmaceutical industry not only to develop but also to transfer the already established conventional HPLC methods into green ones.

Keywords: green analytical chemistry, reversed-phase liquid chromatography, pharmaceutical analysis, green solvents, micellar liquid chromatography, per aqueous liquid chromatography, ethanol, propylene carbonate, glycerol, surfactants

1. Introduction

The concept of "green chemistry" (GC) refers to the design of chemical processes and products that enable elimination (or reduction) of the use or creation of substances that are harmful to humans and the environment. The GC concept is based on 12 principles set out by Paul Anastas and John Warner in 1998 [1]. An extensive overview of these principles could be found on the American Chemical Society (ACS) webpage [2]. The term "green analytical chemistry" (GAC) was introduced in 2001 [3]. The GAC refers to the development of new, effective analytical methodologies

that will enable minimization and/or elimination of hazardous chemicals and chemical waste while enabling faster and more energy-efficient analysis. Therefore, the above-mentioned 12 principles of green chemistry were modified to formulate the main features determining the green character of the analytical chemistry and place it in the function of GAC. The GAC principles could be summarized in four main topics: (1) elimination (or reduction) of the consumption of reagents from analytical procedures; (2) minimization of energy consumption; (3) proper management of analytical waste; and (4) increased safety for the operator [2–4]. Scientists, researchers, and analysts across the world recognized the need for implementation of the GAC principles in analytical methods, which resulted in constant increase of the number of published papers in different fields of GAC (fundamentals, spectroscopy, electrochemistry, and separation methods) [5].

The pharma industry is not immune to the global need of changing the processes to meet GAC criteria. The study conducted in 2019 by researchers from McMaster University in Canada showed that the pharmaceutical industry globally emitted a higher amount of greenhouse gases compared to the automotive production sector [6]. Pharma analysis is a fundamental part of the pharmaceutical industry, whereas high-performance liquid chromatography (HPLC) is one of the most commonly used techniques. HPLC finds application in various stages of the lifecycle of medicines, starting with pharmacokinetic, pharmacodynamics, and bioequivalent studies; quality control of active pharmaceutical ingredients (APIs) and excipients; control of the manufacturing process; quality control of the finished product; stability studies; and so on.

During the development and validation of the chromatographic methods, focus is placed on the chromatographic parameters such as accuracy, precision, and robustness, as well as the analysis runtime. However, other aspects regarding the impact of the chromatographic method on the analyst's safety and the environment are still insufficiently taken into account.

The following text summarizes the green strategies (along with their strengths and drawbacks) that are available for analysts in the development of eco-friendly chromatographic methods that can be immediately applied to conventional HPLC instruments. This summary will contribute toward the easy acceptance of the need for transformation of the already-established conventional HPLC methods in the pharma industry into "green" chromatographic methods.

2. Tools for evaluation of the greenness of chromatographic methods

The implementation of the GAP principles in the processes of green chromatographic method development, as well as the transfer of the conventional HPLC methods into eco-friendly solutions, is an essential part of a development strategy of the pharma industry. Alongside the green method development, it is also equally important to consider the available approaches aimed at evaluating the greenness of the developed methods. The analyst should take into consideration the greenness of the proposed method and compare it with the existing ones. This kind of evaluation provides quantitative data regarding how well each of the different segments of the method conforms to the GAC principles. In this way, the analyst can have additional information for further method improvement in terms of critical method parameters.

At the beginning of the implementation of the GAC principles in chromatographic methods, the analysts did not address this issue enough. Over the years, more and

more emphasis has been placed on the greenness assessment of the published methods in the field of pharma analysis. Several tools are used for evaluation of the different aspects of the methods and their compliance with the GAC principles, providing general assessment (e.g., National Environment Methods Index) or quantitative estimation (e.g., analytical eco-scale). In this section, a brief description of the tools that are most commonly used for evaluation of the greenness of the chromatographic method is presented.

The National Environment Methods Index (NEMI) is one of the oldest available tools for the assessment of the greenness of methods [7]. This approach evaluates the method in four different fields: persistent, bio-accumulative and toxic (PBT); hazard; corrosive; and waste. The greenness profile of the method is presented as a simple pictogram, divided into four sections. The color of each section can be green (if the term meets the particular criteria) or black (if the term does not meet the green criteria). In this manner, a general assessment is obtained, which is very easily visualized. However, the NEMI index does not take into consideration energy consumption, as one of the GAC principles. Another disadvantage of this assessment tool is that it cannot be considered a qualitative tool, and in cases where non-typical chemicals are used, the process of preparation of the symbols is more time-consuming.

In 2012, a quantitative tool named analytical eco-scale index had been presented [8]. According to this index, a perfectly green method is assigned with 100 points (theoretically ideal green method). A lower number of points for a given method indicate that the method has a larger deviation from the GAC principles and lower greenness. The analytical eco-scale points can be calculated by subtracting the number of penalty points from 100. The penalty points are calculated on various grounds. For example, the number of penalty points given for the reagents depends on the type and the volume of reagents used for the procedure. The penalty points assigned for energy consumption are determined by the type of instrumentation used. For example, titration is a technique that consumes the least amount of energy, while liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are the most energy-consuming techniques, leading to higher penalty points. After summarizing the total number of penalty points for the different segments of the method, the analytical eco-scale points are obtained. A score above 75 points represents an excellent green method; a score ranging from 75 to 50 indicates an acceptable green method, while a score below 50 designates inadequate green analysis. The analytical eco-scale index, as a quantitative tool, facilitates the process of comparison of the greenness of the methods. In addition, the calculation of penalty points is simple, and it allows analysts to assess which part of the procedure has the major contribution toward nonconformity with GAC principles. The analytical eco-scale approach has been widely accepted by scientists.

The green analytical procedure index (GAPI) is a tool that enables evaluation of the greenness of the whole analytical procedure, starting from the sample collection to the final determination [9]. This tool takes into account the advantages of the previously discussed tools, such as NEMI and eco-scale index. The GAPI tool visually is presented in terms of five pentagrams, which represent the different segments of the analytical procedure, and they are further subdivided into 15 different segments. These pentagrams can be colored green, yellow, or red, depending on their environmental impact. The first pentagram refers to the sample, and it is subdivided into four parts, representing the collection (1), preservation (2), transportation (3), and storage (4) of the sample. The second pentagram is positioned in the middle, and it refers to the type of the method (5), whether the method is direct or indirect. The third

pentagram refers to the sample preparation, and it includes the scale of extraction (6), used solvents (7), and additional treatments (8). The fourth pentagram refers to the reagents, and it is subdivided into three parts: amount (9), health hazard (10), and safety hazard (11). The last pentagram refers to the instrumentation, and it contains five parts: energy (12), occupation hazard (13), waste (14), and waste treatment (15). The GAPI index is very comprehensive; thus, it can be considered as a qualitative and a quantitative tool for the method of greenness assessment.

The analytical method greenness score (AMGS) metric is an online, freely accessible calculator, which can be found on the ACS webpage [10]. This web-based calculator is only applicable for LC methods (normal-phase LC, ultra-pressure LC, LC-MS, and preparative LC-MS) and supercritical fluid chromatography (SFC) methods such as preparative SFC and SFC-MS. The authors [11] introduced this tool for the assessment of the environmental, health, and safety impact (EHS) score of the solvents, the solvent energy demand, and the instrument energy consumption. The AMGS score is automatically calculated when the users enter several pieces of information about the method, that is: the type of technique used, number of compounds of interest, number of injections, flow rate, rut time of the analysis, solvents used for the mobile phase, type and volume of the sample solvent, and so on. A lower AMGS score implies a greener method. In addition, the users get quantitative information about the impact of the three main categories (instrument energy, solvent energy, and solvent safety) on the score. These three main categories can be colored green, yellow, or red, depending on their contribution to the AMGS value. The yellow and red colors indicate the area where the method can be improved. The current limitations of the AMGS calculator are that this tool is not applicable in cases where more than three components are used in the mobile phase and in cases where techniques different from LC and SFC are used. However, taking into account that LC has the widest application in the pharma industry and that the process of gathering information about the greenness score of the method is very simple, AMGS calculator is considered as a very useful tool.

In 2020, the analytical greenness (AGREE) metrics approach and software were published [12]. This metric system takes into account the 12 principles of GAC, so the method is assessed for each principle separately. The 12 GAC principles are converted into a numerical value (score) ranging from 0 to 1. The overall AGREE score is a product of the individual results for each GAC principle. In addition to the quantitative result, the AGREE score is presented in the form of a pictogram, enclosing the final result in the middle of the pictogram. The performance of the method regarding the 12 variables is presented as 12 segments in the outer part of the pictogram. The segments are in a color scale (green-yellow-red), and the width of each corresponding segment in the pictogram reflects the contribution of the variables to the AGREE score. The AGREE approach is a very comprehensive tool for the greenness method assessment because it takes into account each of the 12 principles, but at the same time, it is a very simple tool, allowing easy interpretation of results. The same group of authors published a tutorial for the use of the AGREE metrics for sample preparation [13].

3. Green solvents as a mobile phase

The reverse phase high performance liquid chromatography (RP-HPLC) as a technique requires a large number of organic solvents as eluents in the mobile phase.

It is well known that methanol (MeOH) and acetonitrile (ACN) are the most consumed organic solvents in the RP-HPLC mobile phase. These solvents are hazardous, and both have acute and chronic toxic effects. Methanol is a toxic alcohol that can cause retina damage and serious acidosis [14, 15]. Acetonitrile toxicity is manifested through inhalation of vapors or contact with skin and eyes. Acetonitrile in vivo is metabolized to cyanide, leading to cytotoxic anoxia [16]. Another issue with the ACN is its price on the market. This reagent is a byproduct of acrylonitrile, and the decreased demand for acrylonitrile in 2008 led to reduced production and a shortage of ACN [17]. Therefore, the price of acetonitrile on the market increased many times after the ACN crisis. It becomes evident that the use of these solvents in the mobile phase raises issues related to the health of the analysts as well as brings increased expenses for the analytical labs. Additionally, the negative ecological impact of these solvents cannot be neglected because of the huge amount of chemical waste. The chemical waste generated per year by HPLC instruments worldwide is approximately 34 million liters [18].

Considering these data, it becomes understandable that the removal of these toxic solvents from the RP-HPLC mobile phases and their replacement with greener alternatives have a key role in the development of eco-friendly HPLC methods. In addition, solvents used for the sample preparation steps for one HPLC method should also be taken into consideration. It is well known that the polarity of the solvents used for sample preparation should match the polarity of the mobile phase to obtain symmetrical chromatographic peaks. Therefore, the composition of the solvent for the sample preparation process should correspond to the composition of the mobile phase. Taking into account the abovementioned, it becomes evident that the waste generated from the whole HPLC procedure should be considered during the ecological assessment of the HPLC method.

Several organic solvents such as ethanol (EtOH), acetone, ethyl acetate, 2-propanol, glycerol, and propylene carbonate (PC) are used as green alternatives for conventional organic eluents in the mobile phase. However, not all of the mentioned green organic alternatives have the same advantages and allow easy method transfer from conventional to eco-friendly HPLC methods. Namely, the main disadvantage of acetone and ethyl acetate is that they have very high UV cutoffs (330 and 260 nm, respectively), so their use becomes incompatible with UV detector. Another issue is their high viscosity, leading to higher column back pressure [19–21].

In this chapter, green organic solvents that are easily affordable and that produce good chromatographic performance, such as ethanol, propylene carbonate, and glycerol, will be discussed.

3.1 Ethanol

Several review papers have highlighted the advantages of the use of ethanol as a green solvent in RP-HPLC mobile phases [21–24]. Although ethanol, according to Snyder's classification of organic solvents [25], belongs in the same group as methanol, this solvent can be used as a replacement for acetonitrile too. Ethanol has several advantages over these two solvents.

Regarding human health effects, ethanol has lower vapor pressure, so the toxic effects of inhalation of ethanol vapors are reduced. The toxic effect of ethanol is more related to long-term ingestion and not to its use as a reagent. Considering the environmental impact, ethanol is biodegradable and has less negative environmental impact compared to acetonitrile and methanol [21].

From an analytical point of view, ethanol has higher eluotropic strength, so lower quantities are needed to achieve comparable retention time to acetonitrile and methanol. The UV cutoff is acceptable, and it's around 210 nm [25]. The main drawback is the higher viscosity of the ethanol-water mobile phase, leading to higher column backpressure. Our experience shows that this column backpressure can be easily overcome with the use of higher column temperatures of 35 or 40°C or with lower flow rates. Considering the GAP principles for reducing reagent consumption, the lower flow rate will not be an issue but an added value to the green method.

From an economic point of view, the market price of ethanol is lower than those of acetonitrile and methanol, so the method cost is lower. In addition, since ethanol is less toxic, the waste disposal cost is lower. This goes in favor of the overall reduction of expenses in the pharmaceutical analysis.

All these features have contributed to ethanol being the most preferred green alternative for acetonitrile and methanol. There are numerous examples of the application of ethanol-based green mobile phase in RP-HPLC in pharma analysis. Yabré et al. gave an extensive review of the pharmaceutical applications regarding the use of ethanol-based mobile phase [21].

This chapter gives an overview of certain publications, from 2018 to date, concerning the use of ethanol-based HPLC mobile phase in pharma analysis. In 2022, a comparative study of two HPLC methods for the determination of four antipsychotics (quetiapine fumarate, aripiprazole, asenapine maleate, and chlorpromazine hydrochloride) was published [26]. The authors developed one green and one conventional RP-HPLC method for the determination of these antipsychotics in bulk and pharmaceutical formulations (Quitapex®, Asenapine®, Aripiprazole®, and Neurazine® tablets). The mobile phase for the green method consisted of ethanol and 20 mM sodium dihydrogen phosphate in a ratio of 35:65, *v/v* (pH 5.0), and the separation was achieved in isocratic mode within 11 minutes. For the conventional HPLC method, acetonitrile was used instead of ethanol, and the separation of the studied antipsychotics was achieved in gradient mode in 15 minutes. The flow rate for both methods was 1 mL/min. Both methods were validated following International Conference on Harmonization (ICH) Q2R1 Guideline [27]. It was observed that the green HPLC method showed better limits of detection (LOD) and limits of quantification (LOQ) for the analyzed antipsychotics. In addition, the evaluation of the obtained results using Student's t-test and F-ratio showed that there was no statistically significant difference between the accuracy and the precision of the green method and the conventional HPLC method. The greenness of the proposed methods was evaluated using the GAPI index. The green HPLC method for the determination of the four antipsychotics had nine green and six yellow pentagrams, while the conventional method had six green, six yellow, and three red pentagrams. This research demonstrates that the ethanol-based LC mobile phase not only gives benefits from an ecological point of view but also leads to better method performances such as shorter runtime and better LOD and LOQ.

Another group of authors applied the design of experiment (DoE) approach to optimize a green HPLC method for the determination of atorvastatin in tablets and in the presence of its degradation products. The optimized mobile phase consisted of ethanol and 0.5% aqueous acetic acid (*v/v*) in a ratio of 57.5–42.5, with a flow rate of 0.91 mL/min and a column temperature of 40°C. The method was validated, and the statistical evaluation of the assay results showed that there was no significant difference between the proposed and the reference methods. The greenness of the method was evaluated using four different approaches: GAPI, AMGS, analytical eco-scale,

and AGREE software-based tool. The applied approaches suggested that the method was more environmentally friendly compared to other published HPLC methods for the determination of atorvastatin [28].

The numerous benefits of the analytical quality by design (AQbD) approach contributed to the more extensive use of this methodology for the green method development. The Central Composite Design (CCD) model has been applied for the optimization of the chromatographic conditions for the stability-indicating RP-HPLC method for the determination of escitalopram and etizolam in tablets [29]. The ethanol-potassium dihydrogen phosphate buffer (60:40 v/v, respectively) mobile phase allows the separation of the APIs from their degradation products obtained under stress conditions (acidic and alkaline hydrolysis, oxidation, photodegradation, and dry heat). The greenness of the proposed method was confirmed by four different assessment methods: NEMI, GAPI, AMGS, and AGREE.

In another study, QbD methodology was also used for method development for the simultaneous determination of impurities of artesunate and amodiaquine as APIs [30]. The method development was complex mostly due to the differences in the polarity of the impurities of the analyzed APIs. Another challenge was the low UV absorption maximum (210 nm) for artesunate and its impurities, leading to poor detection with ethanol-based mobile phase driven by the UV cutoff of ethanol (210 nm). The QbD approach allowed the development of a simple and robust HPLC method using an ethanol-based mobile phase. The separation of nine impurities, including acidic, basic, and structurally related compounds, was achieved using ethanol and 10 mM acetic acid in gradient mode. The analytical eco-score of the proposed method was 95, confirming that the method complied with the GAP principles.

Another research that was published recently also demonstrated the applicability of the ethanol-water mobile phase for the simultaneous determination of three different APIs in tablets [31]. In this study, famotidine, paracetamol, thiocolchicoside, and caffeine (as internal standard) were separated using ethanol and 50 mM sodium dihydrogen phosphate (pH 4.6) in gradient mode. The method was validated following ICH guidelines [27]. The robustness testing showed that the method was robust. The proposed method was compared in terms of method performance and greenness with other published methods for the determination of the studied compounds. The evaluation showed that the proposed method was eco-friendly and had the same or better LOD/LOQ compared with previously published methods. Another paper [32] presents the use of an ethanol-water mobile phase (containing 0.05% triethanolamine at pH 4.5, in a ratio of 90:10, respectively) for the determination of two different moxifloxacin combinations (moxifloxacin/dexamethasone and moxifloxacin/prednisolone). The optimized and validated green method was successfully applied for the determination of the studied APIs in an eyedrop solution, ophthalmic and otic solution, as well as ophthalmic suspension. As for the previously mentioned papers, the authors of this paper also made a comparison between the results obtained from the proposed green method and the reference methods using the Student t-test and the variance ratio F-test. The results showed that there was no statistically significant difference between the proposed and the reference methods. The authors of this paper also evaluated the greenness of the proposed method using the three most-often-used approaches: NEMI, GAPI index, and analytical eco-scale index. In addition, there is a detailed tabular view for assigning and counting the eco-scale penalty points for the proposed and previously reported methods for the studied compounds. The eco-scale score was found to be 94, indicating excellent agreement with the GAC principles.

Ethanol can be used as a green alternative to acetonitrile even for chiral separations. An example of the use of ethanol-based chiral mobile phase is the stability-indicating RP-HPLC method for simultaneous determination of timolol and latanoprost in eye drops [33]. The mobile phase consists of β-cyclodextrin, sodium octane sulphonate, and ethanol in gradient mode. The optimized mobile phase enabled the separation of the two APIs in the presence of latanoprost C3-epimer, latanoprost acid, and timolol-related compounds C and E and from the degradation products obtained under stress conditions. Considering that the organic solvent influences the inclusion of the β-cyclodextrin, the successful replacement of acetonitrile with ethanol opens an opportunity for the use of this green solvent in chiral separations. The detection wavelength was set at 210 nm, showing that the detection near the UV cutoff of ethanol was applicable. The column temperature was 35°C, while the flow rate was 1.2 mL/min. The issue with the higher column backpressure was solved with the use of monolithic columns. The monolithic columns are packed with a single piece of porous material (called "silica rod"), which fills the column, so there are no interparticle voids. This type of design enables higher total porosity, higher separation efficiency, and lower pressure drop. A major advantage of the monolithic columns is that they can work under high flow rates (up to 10 mL/min) without generation of high column backpressure [34]. The author highlighted the applicability of the use of monolithic columns as an approach for dealing with the high column pressure generated from the ethanol-based mobile phase. The method validation showed that the method was robust. The green analysis assessment was made using the GAPI pictogram.

Table 1 summarizes some of the recently published articles regarding the use of ethanol as a green chromatographic eluent for pharma analysis.

3.2 Propylene carbonate

Propylene carbonate belongs to the group of cyclic carbonated solvents, and it is derived from carbon dioxide. This solvent, as a green aprotic solvent with high polarity, can be used as a replacement for the toxic aprotic polar solvents such as acetonitrile, dimethyl formamide, or dimethyl sulfoxide [42, 43]. PC was used for the first time as an organic modifier in the LC mobile phase in 2011 [44]. Since then, there are only a few articles regarding the use of this green solvent in the LC mobile phase [45–47]. The investigations showed that the problem with the limited miscibility of PC with water could be solved by using a mixture of PC with methanol or ethanol. Considering the GAC concept, ethanol should be considered as a third solvent in the PC/water mobile phase. The mixture of PC/EtOH/water has acceptable viscosity. In addition, the PC has an acceptable UV cutoff of 210 nm.

The most recent research that demonstrates the applicability of PC as a replacement for ACN has focused on the separation of a mixture of 39 pharmaceutical compounds in two-dimensional liquid chromatography (2D LC) [48]. The authors revealed that the effective peak distribution and peak capacity obtained with PC as an organic modifier were comparable to those obtained using ACN and MeOH. Despite the significantly reduced runtime (32 min for methods with PC *vs* 52 min for methods with ACN and MeOH), an improvement in separation selectivity was achieved [48]. This research confirmed the role of propylene carbonate as an effective substitute for acetonitrile, providing greater separation power even in 2D LC. The results from this research open a possibility for wider applications of this solvent in the future.

API	Medicinal product	Mobile phase	Column	Reference
Antipsychotics	Tablets	EtOH: 20 mM NaH_2PO_4 pH 5.0 = 35:65	Thermo C18 250 × 4.6 mm, 5 μm	[26]
Atorvastatin calcium	Bulk, Tablets	0.5% Acetic acid: EtOH = 42.5:57.5	Zorbax Eclipse Plus C18 150 × 4.6 mm, 5 μm	[28]
Escitalopram and etizolam	Tablets	EtOH: KH_2PO_4 pH 2.5 = 60:40	C18	[29]
Artesunate and amodiaquine impurities	Tablets	Aqueous solution: ethanol, gradient elution	XBridge BEH C18 150 × 3 mm 5 μm	[30]
Famotidine, paracetamol and thiocolchicoside	Tablets	50 mM NaH_2PO_4 pH 4.6 and EtOH, gradient elution	C8 150 × 4.6 mm, 5 μm	[31]
Moxifloxacin/ dexamethasone and moxifloxacin/prednisone	Eye drop solution, Ophthalmic suspension, Ophthalmic and otic solution	EtOH: water = 90:10, containing 0.05% triethanolamine, pH 4.5	Hypersil C8 250 × 4.6 mm, 5 μm	[32]
Timolol and latanoprost	Eye drop solution	(β-cyclodextrin + sodium octane sulphonate): EtOH = 40:60, gradient elution	Kinetex XB-C18 150 × 4.6 mm	[33]
Ketoconazole and beclomethasone	Bulk, Cream formulation	EtOH: 0.1 M KH_2PO_4 pH 2.5 = 33:67	ODS 250 × 4.6 mm, 5 μm	[35]
Pyridoxine HCl and doxylamine succinate	Tablets	EtOH: 0.01 M phosphate buffer pH 5.0 = 10:90	Xterra C18 100 × 4.6 mm, 5 μm	[36]
Tafluprost	Bulk, Ophthalmic formulation	EtOH: 0.01 M phosphate buffer pH 4.5 = 60:40	Hyperclone C18 150 × 4.6 mm, 5 μm	[37]
Lamivudine, zidovudine and nevirapine	Tablets	EtOH and 0.1 M ammonium acetate pH 4.5, gradient mode	C18 250 × 3.0 mm, 5 μm	[38]
3,4-methylenedioxymetham phetamine (MDMA)	Tablets	H_2O with 0.1% formic acid, pH 5.0: EtOH = 85:15	ACE-5 Phenyl	[39]
Rosuvastatin	Tablets	EtOH: MeOH: EtAc = 6:3:1	Nucleodor C8 150 × 4.6 mm, 5 μm	[40]
Secnidazole	Tablets	(H_2O + 0.7% acetic acid): EtOH = 78:22	Luna CN 250 × 4.6 mm, 5 μm	[41]

Table 1.
Examples of HPLC methods using ethanol-water mobile phase in pharma analysis.

3.3 Glycerol

Glycerol is an organic solvent that is the most recently included in the development of green chromatographic methods. In 2021, glycerol was used for the separation of four antiviral medicines under reversed-phase chromatographic conditions [49]. The same group of authors used this solvent as a mobile phase modifier for the determination of ascorbic acid and glutathione in tablets [50].

Several features make this solvent suitable for the development of eco-friendly LC methods. First of all, glycerol is a nonvolatile safe solvent, so the risk to the analyst's health due to vapor inhalation is reduced. The advantage regarding lab safety is that under normal storage conditions, glycerol has low flammability and high stability. The benefit in terms of environmental protection is that it is biodegradable, and it is available from renewable, cheap sources [51, 52]. According to the CHEM21 selection guide of classical and less classical solvents, the health, safety, and environmental scores of ethanol are 3, 4, and 3, respectively, whereas for glycerol, these scores are 1, 1, and 7, respectively [43]. From an analytical point of view, glycerol has an advantage over ethanol and PC in terms of UV cutoff and miscibility with water. Namely, glycerol has a UV cutoff of 207 nm, which is slightly lower compared to ethanol, and it is completely miscible with water in comparison to PC, which has limited miscibility.

The higher viscosity of glycerol could be seen as a drawback from one side, but on the other hand, it could bring a potential benefit. Due to the higher viscosity, it is recommended for glycerol to be premixed with the water phase, followed by sonication. This step will reduce the pump load of the LC system and will facilitate the mixing process [49, 50]. In addition, the issue with higher viscosity could be overcome with the use of higher column temperature, because this parameter has a significant impact on the column back pressure. For instance, the increase of the column temperature of 10°C led to a 20% decrease in column back pressure. The research results regarding the influence of the glycerol viscosity on the terms from the Van Deemter equation showed that higher viscosity of the glycerol-based mobile phase led to a decrease of the eddy diffusion (A term) and a decrease of the longitudinal diffusion (B term) [49]. This could be the reason for the good chromatographic performance in terms of peak symmetry, column efficiency, and resolution. Therefore, in this case, the higher viscosity of the glycerol-based mobile phase could be seen as a potential benefit. Regarding the elution strength, glycerol is in the middle between water as a weak eluent and acetonitrile and methanol as strong eluents in RP-HPLC. This property allows analysts to use glycerol for better adjustments of the elution strength of the mobile phase and to obtain better selectivity when needed.

4. Pure water as a mobile phase

Everyone would agree that there is no greener option for making the chromatographic analysis eco-friendlier than choosing pure water as LC mobile phase. This idea is present in the analytical community for more than 30 years [53], but its popularity has grown in the last decade.

Two approaches enable the use of pure water as a LC mobile phase. The first approach is to apply elevated temperatures along with stationary phases that are stable under these conditions. It should be considered that under the term "elevated temperature", a temperature above 100°C or water heated below its critical point conditions (374°C and 218 atm) should be considered [54]. In the literature studies,

this type of chromatography could be found under the name of superheated water chromatography (SHWC) or subcritical water chromatography (SCWC) [54, 55]. Subcritical water has different properties compared to water under ambient temperature conditions. Namely, the temperature increase leads to a decrease in the dielectric constant, viscosity, and surface tension of the water. The result is a decrease in the water polarity, so in the chromatographic system, it begins to behave more like the organic solvents. Therefore, the nonpolar compounds can be eluted under higher water temperatures, whereas lower temperatures are needed for the elution of polar compounds [54, 56]. Considering the limited temperature stability of the classical RP columns, the use of pure water under subcritical conditions requires the application of thermally stable stationary phases that could withstand the temperatures above 200°C. Some examples of stationary phases that could be applied for SCWC are polymer-based (such as polystyrene and divinylbenzene), zirconia-based, or carbon-based stationary phases [57].

The application of SHWC is related to the need for the adaptation of conventional HPLC instruments. One of the most crucial instrumental modifications is the requirement of special column ovens that can heat the column very fast, as well as detectors other than UV/Vis (e.g., amperometric detector, flame ionization detector, etc.) [54]. The need for instrument modification could be one of the reasons that this technique has not had wider application in pharma analysis yet.

The second approach is based on the use of pure water under ambient temperature conditions (below 60°C) [54, 58]. In such cases, there is no need for adaptation of the conventional HPLC instruments. In addition, the thermal stability of the stationary phases is not an issue anymore. The only requirement is to use columns that are stable under high water content surrounding. If silica-based stationary phases are used, then the term per aqueous liquid chromatography (PALC) should be used [59]. PALC should be distinguished from hydrophilic interaction liquid chromatography (HILIC) because although in PALC and HILIC the same type of stationary phases is used (silica-based columns), in HILIC, the water content in the mobile phase is very low, whereas in PALC, water is the dominant eluent in the mobile phase [53, 55, 57]. In PALC, the surface of the silica stationary phase becomes nonpolar in conditions where the water content in the mobile phase is very high. As explained in the literature [59], this effect is due to the higher participation of siloxane groups.

A variant of PALC is the water-only reversed-phase liquid chromatography (WRP-LC) [53]. The difference between PALC and WRP-LC is that the second one uses polar-embedded or polar-endcapped stationary phases instead of silica-based columns.

In cases like this, where the choice of the mobile phase composition is very limited (pure water or high water content), the selectivity is mostly controlled by the type of stationary phase. In the recent years, several different polar-embedded and polar-endcapped stationary phases have appeared on the market. The differences between the polar-embedded and polar-endcapped stationary phases are presented in comprehensive way [57]. The available polar-embedded and polar-endcapped stationary phases have different solvation properties, resulting in differences in selectivity. The importance of proper selection among the various types of polar-embedded stationary phases (Amino-P-C18, Diol-Ester C10, Diol-Ester C18, and Diol-Ester Phenyl) was presented by the separation of polar compounds (nucleic bases, nucleosides, and purine alkaloids) using pure water as a mobile phase [54]. Separation of four amino acids (hydroxyproline, proline, glycine, and alanine) using pure water as a mobile phase was achieved using a mixed-mode polar embedded column [60]. The stationary

phase used for this application has long alkyl chains and ion-exchange functional groups attached at the terminal end of this chain, thus providing the reverse phase and ion-exchange nature of this column [60]. This kind of stationary phase could increase the possibilities for the separation of components with different polarities, thus making WRP-LC as an approach for green chromatographic method development more attractive.

5. Surfactants as a mobile phase

The amphiphilic molecules have two different ends (hydrophilic, polar and hydrophobic, nonpolar) with an opposite affinity for the dispersion medium. These molecules can reduce surface tension, so they are called surface-active substances or surfactants. These molecules, when present in low concentrations in the dispersion medium, exist as individual molecules of a sub-colloidal size (monomers). By increasing the concentration of amphiphilic molecules, their aggregation occurs, and micelles are formed. The concentration of monomers necessary to form micelles is called the critical micellar concentration (CMC). Theoretically, micelles are formed at a certain concentration of monomers, but in practice, this happens in a narrow concentration range [61].

Surfactants have diverse applications in different fields of analytical chemistry [62]. Further text includes a brief overview of the application of surfactants as green eluents in the mobile phase. The chromatographic technique that uses surfactants as a mobile phase is called micellar liquid chromatography (MCL). This technique was introduced in 1980 [63], but it was "forgotten" during the past years. However, the increased awareness of the GAC principles contributed to the return of MLC in the focus of the analysts. Surfactants used in MLC are nontoxic, biodegradable, and have low environmental bioconcentration factors [64]; therefore, their use as eluents in the RP-HPLC mobile phase is considered as an effective strategy for greening the HPLC methods.

The MLC is a type of reversed-phase chromatography; hence, conventional nonpolar stationary phases (C18, C8, etc.) are used. The mobile phase consists of an aqueous solution of surfactants in a concentration above their CMC. As this mobile phase travels through the column, it forms two different constituents: the micelles (also called micellar pseudophase) and the surfactant monomers that are present in the aqueous environment. In addition, surfactants modify the surface of the stationary phase: the hydrophobic end of the surfactants binds to the nonpolar stationary phase, whereas the hydrophilic end is directed toward the mobile phase. The surfactants' orientation on the surface of the stationary phase forms a kind of an open micelle structure. Therefore, the polarity and the charge of the surface of the stationary phase depend on the nature of the surfactant used (anionic, cationic, or nonionic). The analytes are separated based on their different partitioning between the modified stationary phase, the pseudophase, and the bulk solvent. The unique characteristics of the surfactants, as well as their position and effects on the stationary phase, enable the existence of different kinds of retention mechanisms (hydrophobic, ionic, and steric), allowing separation of analytes with different polarities [65, 66].

The most commonly used surfactants as eluents in MCL are the anionic sodium dodecyl sulfate (SDS), the cationic cetyl trimethyl ammonium bromide (CTAB), and the nonionic polyoxyethylene-23-lauryl ether (Brij-35). These surfactants have low CMC (e.g., 8.2 mM for SDS and 0.09 mM for Brij-35) [65], providing acceptable

viscosity of the mobile phase. The most important chromatographic parameters that should be considered during the method development are the column temperature, the type and concentration of the surfactant used, and the type of co-eluents, if needed. The working column temperature should be above the Kraft point (the temperature at which the solubility of the amphiphilic molecules equals the CMC). Considering that the Kraft point for the commonly used surfactants is on ambient temperature (e.g., for SDS, the Kraft point is on 15°C) [65] and that the viscosity of the mobile phase is temperature dependent, the chromatographic separation is usually performed between 25 and 60°C. The increased temperature contributes to faster mass transfer kinetics, which often improves the separation efficiency [67]. Generally, the increase in the column temperature leads to a shorter retention time of the analytes [68, 69]. However, in some cases, it was observed that higher column temperature contributed to the increased value of the tailing factor [69].

The surfactant concentration usually has an inverse influence on the retention time, due to the stronger association of the analytes with the micelles [70]. An exception to this dependence is the chromatographic behavior of phenolic compounds, where the concentration of Brij-35 has a positive correlation with the retention time. This could be explained by a hydrogen bond formation between the phenolic compounds and the hydroxyl groups of Brij-35; thus, increase in the retention time is observed [71, 72].

Co-eluents in the surfactant-based mobile phase are usually needed to decrease the retention of the nonpolar compounds and to improve the mass transfer to the stationary phase. Short-chain alcohols such as methanol, ethanol, n-propanol, n-butanol, n-pentanol, or acetonitrile in low concentrations (3–15%) are frequently used to enhance the elution strength of the surfactant-based mobile phase [73]. These alcohols act by reducing the ability of the monomers to form micelles; thus, the ability of the analyte to bind the micelles is decreased. This effect is more pronounced when long-chain alcohols are used [62, 66]. This finding was also confirmed during the determination of two ternary mixtures (phenylephrine hydrochloride, ibuprofen, and chlorpheniramine maleate as mixture 1 and pseudoephedrine hydrochloride, ibuprofen, and chlorpheniramine maleate as mixture 2) utilized for cold treatment [74]. In this case, use of methanol and acetonitrile in the mobile phase provided longer retention times of the analytes, but asymmetrical peaks were obtained, which was not the case when n-propanol was used.

In recent years, in order to fulfill the GAC criteria, the organic alcohols have been replaced with the addition of more polar surfactants (e.g., Brij-35) in the mobile phases. The combination of two types of surfactants in the LC mobile phase (usually SDS and Brij-35) modifies the MLC. This modified MLC is called mixed-mode micellar liquid chromatography (mixed MLC). The mixed MCL allows the separation of neutral and charged analytes. Brij-35, as a more polar surfactant compared to SDS, decreases the polarity of the stationary phase, leading to reduced retention time of the polar compounds. The SDS, as an anionic surfactant, provides a negative charge to the surface of the stationary phase; thus, polar compounds with a positive charge bind more strongly, and consequently, their retention time is longer. Brij-35, as a nonpolar surfactant, reduces this negative charge of the modified stationary phase; thus, the column keeps its neutral character [72]. The mixed micellar mobile phase was used to separate 10 commonly used antihypertensive medicines (hydrochlorothiazide, chlorthalidone, atenolol, losartan, amiloride, valsartan, spironolactone, olmesartan, bisoprolol, and irbesartan) using C18 core-shell column [75]. An interesting finding is that the increase in the concentration of the surfactants did not have the same effect

on the retention of all target compounds. The expected behavior is that the increase in the concentration of Brij-35 will decrease the retention time of the analytes (higher elution power of the mobile phase). However, the authors noticed that longer retention time of hydrochlorothiazide was observed with the increase of concentration of Brij-35 from 0.01 M to 0.04 M. On the other hand, valsartan first showed a decrease in the retention time, and afterward, the retention time increased. Similar behavior but in the opposite direction has been observed for spironolactone. For this compound, the increase of the concentration of Brij-35 from 0.01 M to 0.03 M led to a longer retention time, and when the concentration of Brij-35 was 0.04 M, the retention time of this compound decreased. This finding shows that in mixed MLC, it is very hard to predict the retention behavior of the analytes; thus, the method development becomes a more challenging process. Considering that various factors affect the retention behavior of the analytes and that their effect could not be easily predicted, the use of the DoE approach during the method development process is highly recommended.

The DoE approach was used for the development of a robust mixed MLC method for the determination of five antidiabetic medicines (metformin, glipizide, glimepiride, pioglitazone, and repaglinide) using Symmetry C18 column [64]. The use of CCD design allowed fast method optimization, and at the same time, the requirements for the method parameters were fulfilled. The separation of the medicines was accomplished in 10 minutes. Another method uses the same experimental design for the optimization of a mixed MLC method for the determination of six medicines (paracetamol, guaifenesin, pseudoephedrine, ibuprofen, chlorpheniramine, and dextromethorphan) for the treatment of common cold [72]. The response surface methodology proved to be successful in this case as well, allowing the separation of six analytes with adequate resolution in a short analysis time. The application of the DoE approach during the mixed MLC method development facilitates the optimization of the critical chromatographic parameters such as concentration of SDS and Brij-35 and allows timely perception of their interaction. Polysorbates are another type of nonionic surfactant that can be used for MLC, including polysorbate 20 (Tween 20) or polysorbate 40 (Tween 40) [68, 73]. The comparison of the influence of Tween 20 and Tween 40 as eluents in the MLC mobile phase on the retention of hydroxycinnamic acid compounds showed that shorter retention times and better peak shapes are observed using Tween 20 [68].

The gradient elution in MLC is possible by changing the concentration of either the surfactant or the organic solvent (if present). In both cases, the time needed for re-equilibration of the column is shorter, and analysis time is faster when compared to the gradient mode using conventional mobile phases. The faster re-equilibration time is due to the constant amount of surfactant being absorbed on the surface of the stationary phase. During the gradient elution, the increased monomer concentration leads to greater micelle formation, so the concentration of the monomer does not change, and it is kept around the CMC value [66, 73]. The gradient elution mode in MLC is not very suitable for nonpolar hydrophobic compounds, because they are more strongly retained. This could be overcome by the inclusion of an organic solvent or more polar surfactant as a co-eluent or with the use of more polar reversed-phase stationary phases.

It is very important that the analysts who are starting to use MLC on their HPLC systems for the first time provide information about the column conditioning, column care, and special considerations regarding surfactant mobile phase flushing. Detailed instructions for the care of the chromatographic system in MLC can

be found in literature studies [65, 76]. In brief, the reversed-phase column should be first flushed with 100% water (at least 30 column volumes) to remove any present organic solvent. Afterward, the surfactant-based mobile phase can be introduced in the system. The column equilibration with this kind of mobile phase takes a longer time. Another consideration that should be taken into account is that the mobile phase should be continuously flushed through the column. If the flushing stops, there is a great possibility of precipitation of the surfactant and consequently crystal formation around the seals of the pump or in the column. To prevent system blockage or column clogging, the pumps should work continuously overnight. To minimize solvent consumption, the flow rate should be set to a minimum (e.g., 0.1 mL/min) [65, 76]. After finishing the analysis, the HPLC system as well as the chromatographic column should be gradually washed up to 100% water to remove the surfactant. As a further step, the column should be restored according to the instructions given by the manufacturer for the column conditioning.

6. Conclusion

As presented in this chapter, the green strategies needed for the development of eco-friendly HPLC methods in pharma analysis could be easily accomplished and applied to conventional LC instruments.

Ethanol, as the most extensively used green organic solvent, provides better method performances (shorter runtime, better LOD, better LOQ, etc.) compared to conventional organic solvents (acetonitrile and methanol). Many of the published papers presented that there was no statistically significant difference between the results (regarding the method accuracy and precision) obtained with the ethanol-based methods and the reference methods based on the conventional LC mobile phase. Propylene carbonate is still not an extensively used green alternative, but recent publications have shown that analysts could take into account this solvent as an effective substitute for acetonitrile, even for 2D LC. Glycerol, as the most recently used green alternative for conventional organic solvents, has an elution strength that is in between the elution strength of water and methanol/acetonitrile. Even though the number of published papers concerning the use of glycerol is still scarce, analysts should consider this solvent as an option for green method development.

If we speak about the greenest solvent for the LC mobile phase, then pure water is the solution. The increased availability of modern materials that are used for the production of polar-embedded and polar-endcapped stationary phases resulted in increased applicability of PALC and WPR-LC as valuable techniques for eco-friendly HPLC method development.

Many papers demonstrate that MLC methods, based on surfactant mobile phase, fully meet the GAC criteria, allowing separation of compounds with different polarity. The main challenge in this type of green chromatography is the method development process. The use of the DoE approach facilitates the optimization of the critical chromatographic parameters and leads to faster and easily accomplished MLC method development. Regardless of the type of the mobile phase (ethanol, pure water, or surfactants), it is recommended to use the DoE methodology for the method optimization process, because this approach reduces the number of experiments, and it follows the GAP principles.

Different tools (such as NEMI, GAPI, Eco-scale index, AMGS, and AGREE) used for the evaluation of the greenness of the HPLC methods unambiguously demonstrate

that eco-friendly methods have advantages over the conventional methods in terms of ecological impact, operator's safety, and energy consumption. Along with ecological and economic benefits, the eco-friendly methods provide better method performances, being an additional motivation for implementation of the GAC concept in the R&D departments and quality control labs in the pharma industry. The implementation of the green strategies in the pharma analysis will provide benefits for the analysts (healthier working environment), the pharma industry itself (lower method cost and lower waste disposal costs), and the whole microcommunity (reduced negative environmental impact).

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Conflict of interest

The authors declare no conflict of interest.

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