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Simple, Accurate and Multianalyte Determination of Thirteen Active Pharmaceutical Ingredients in Polypills by HPLC-DAD

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Abstract: A new universal HPLC-DAD method has been developed for the separation and simultaneous determination of thirteen active pharmaceutical ingredients (APIs): ramipril, lisinopril, enalapril; atenolol, metoprolol; losartan, candesartan; rosuvastatin, atorvastatin, simvastatin; amlodipine; hydrochlorothiazide, acetylsalicylic acid in polypills used in the treatment of hypertension. The chromatographic analysis of the APIs was performed on an ACE-5 C18-PFP column (250 mm × 4.6 mm, 5 µm) with 0.01 M phosphate buffer (pH = 2.50) and acetonitrile in gradient elution as the mobile phase at a flow rate 1.0 mL min^{-1}. UV detection was performed at 230 nm. The analysis time was 35 min. The elaborated method meets the acceptance criteria for specificity, linearity, sensitivity, accuracy, and precision for all examined substances. The linearity range was observed in a wide concentration range, whereas the determination coefficients (R^2) for the linear model were greater than 0.990. The sensitivity of the method was good with the LOD and LOQ values ranged from 0.0009 to 0.0923 mg mL^{-1} and from 0.0027 to 0.2794 mg mL^{-1}, respectively. The proposed method showed good precision with RSD less than 1.91% and the accuracy expressed as percent recovery was from 95.20% to 104.62%. The proposed HPLC-DAD method was successfully applied to determine APIs in prepared model mixtures corresponding to the commercially available polypill tablets. The obtained results of the measured contents were with good accuracy (95.84–103.92%) and high precision (RSD < 0.95%) indicating the applicability of the proposed method for the simultaneous determination of the polypill components. Therefore, the method can be an effective tool in the quality control of polypills.

Keywords: polypills; active pharmaceutical ingredients; quantitative analysis; HPLC-DAD

Citation: Żuromska-Witek, B.; Stolarczyk, M.; Szlósarczyk, M.; Kielar, S.; Hubicka, U. Simple, Accurate and Multianalyte Determination of Thirteen Active Pharmaceutical Ingredients in Polypills by HPLC-DAD. *Chemosensors* **2023**, *11*, 25. https://doi.org/10.3390/chemosensors11010025

Academic Editors: Eugenia Fagadar-Cosma and Dana Vlascici

Received: 21 November 2022
Revised: 20 December 2022
Accepted: 23 December 2022
Published: 28 December 2022

1. Introduction

According to data from the World Health Organization (WHO), cardiovascular diseases are the main cause of death in the world, in addition to cancer and infectious diseases. Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels being major causes of health loss worldwide cause death of 17.9 million people every year, 31% of the population worldwide [1]. Moreover, current trends suggest that the perspective of reducing premature mortality due to CVD is difficult especially in low-income countries [2]. The failure of CVD prevention is caused by many factors, regardless of the existence of effective pharmacological and non-pharmacological interventions [3]. Patients with CVDs or who are at high risk of cardiovascular events are included in the treatment of 2, 3, and sometimes 4 different pharmaceutical preparations, whose synergistic effect improves the condition of the circulatory system. The pharmacological groups used most often in the form of poly-therapy include anticoagulants, β-blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, statins, diuretics, and calcium channel blockers. It is estimated that about 50% of patients stop treatment after a year, and an additional 35%—2 years after starting treatment. It is influenced by many factors,

but it should be emphasized that the patient's adherence with medical recommendations worsens as the number of tablets taken during the day increases [4]. In 2003, Wald and Law introduced the concept of a so-called polypill, with the hope of reducing cardiovascular events by more than 80% through an acceptable, and cost-saving approach [5]. Researchers have proposed a fixed-dose combination (FDC) drug containing statin (atorvastatin 10 mg or simvastatin 40 mg), three drugs that reduce blood pressure (a thiazide, a β-blocker, and an ACEI), each at half the standard dose; folic acid (0.8 mg); and aspirin (75 mg). Since then, the polypill concept has evidently evolved adopting even 3D-printing method of tablet (printlet) production, but the basic character of its composition has been preserved [6]. Several randomized trials were conducted in many countries to determine the effect of the use of this type of drug in a specific group of patients compared to a placebo or in the case of polytherapy with one or two component formulations [7–16]. The polypill strategy based on a single daily pill containing three or more specific APIs has been shown to be one of the useful tools to improve patient adherence and reduce the risk of death, myocardial infarction, or stroke. Such extensive research on pharmaceutical preparations in the form of polypills caused the appearance on the pharmaceutical market of a whole range of different medicines containing anticoagulant, statin, sartan, angiotensin converting enzyme inhibitor, and β-blocker in various qualitative and quantitative configurations (Table 1).

Table 1. Fixed-dose combination drugs used in the treatment of hypertension [17].

Polypill	Antiplatelet Drug	Thiazide	β-Blocker	Ca-Channel Blocker	ACE-Inhibitor or Sartan	Statin
Zycad-4 [a]	ACES 75 mg	No	MET (succ.) 50 mg	No	RAM 5 mg	ATO 10 mg
Starpill [b]	ACES 75 mg	No	ATE 50 mg	No	LOS 50 mg	ATO 10 mg
Deplatt-CV [c]	ACES 75 mg *	No	No	No	No	ATO 20 mg
CV-Pill Kit [c]	ACES 75 mg	No	MET (succ.) 50 mg	No	RAM 5 mg	ATO 10 mg
Polycap [d]	ACES 100 mg	HCT 12.5 mg	ATE 50 mg	No	RAM 5 mg	SIM 20 mg
Polytorva [e]	ACES 75 mg	No	No	No	RAM 5 mg	ATO 10 mg
Modlip Cad [c]	ACES 75 mg	No	No	No	RAM 2.5 mg	ATO 10 mg
Exforge HCT [f]	No	HCT 25 mg	No	AML 10 mg	VAL 160 mg	No
Trinomia, Sincronium, Iltria [g]	ACES 100 mg	No	No	No	RAM 2.5–10 mg	SIM 40 mg
Polypill-E [h]	ACES 81 mg	HCT 12.5 mg	No	No	ENA 5 mg	ATO 20 mg
Polypill-V [h]	ACES 81 mg	HCT 12.5 mg	No	No	VAL 40 mg	ATO 20 mg

[a] Zydus Cadila, Mumbai, India; [b] Cipla Ltd., Mumbai, India; [c] Torrent Pharmaceuticals Ltd., Gujarat, India; [d] Cadila Pharmaceuticals Ltd. Mumbai, India; [e] US Vitamins Ltd., Mumbai, India; [f] Novartis Pharma, Basel, Switzerland; [g] Ferrer, Barcelona, Spain (different brand names); [h] Iran Alborz Darou Pharmaceutical, Teheran, Iran; * combined with Clopidogrel 75 mg.

A variety of formulations in the form of polytablets, in terms of quality and quantity, in the case of their analysis require the use of efficient analytical techniques. In this case, multianalyte procedures seem to be the method of choice and allow the analysis of several compounds with a single sample pretreatment, saving time and resources following trends of "green chemistry" [18]. There are many available publications describing the simultaneous determination of substances mentioned above as APIs in FDC drugs; a laboratory mixture based on such a composition or in some cases adulterants in herbal-based products [19,20]. Different analytical techniques were used for the determination of the mixture of several substances, such as thin-layer chromatography [21,22], or liquid chromatography using different procedures [20]. However, the separation techniques were selected in the majority of the applications including liquid chromatography with reverse phase. The development of multi-analyte chromatographic methods with "broad spectrum" characteristics for the separation and simultaneous determination of several structurally or pharmacologically related drugs is one of the modern trends in drug analysis [23]. Pawar et al. proposed RP-HPLC methods for the analysis of atorvastatin, aspirin, enalapril, and metoprolol succinate in bulk and a polypill [24] and aspirin, ramipril, and simvastatin [25]. The substances included in the Starpill and their possible degradation products were separated using the LC method [26]. Another RP-HPLC method was used for the

quantitative analysis of atorvastatin alone and in combination with fenofibrate, ezetimibe, atenolol, losartan potassium, telmisartan, metformin hydrochloride, glimepiride, aspirin and clopidogrel bisulfate [27]. The mixture of ramipril, atorvastatin, and aspirin with a slightly different quantitative composition corresponding to the Polytorva and Modlip Cad preparation was determined using the isocratic RP-HPLC and HPTLC method [22,28]. The HPLC method was also used in the quantitative analysis of Exforge HCT. The proposed chromatographic conditions were also directed towards the study of the stress degradation of the three antihypertensive drugs amlodipine, valsartan, and hydrochlorothiazide; as well as the simultaneous determination of these drugs in their combined formulation [29]. Ibrahim et al. developed the HPLC method for the simultaneous estimation of losartan, hydrochlorothiazide, and atorvastatin in laboratory prepared pharmaceutical tablets [30]. Kumar et al. described RP-HPLC methods for the determination of the possible components of a polypill, i.e., lisinopril, aspirin, and one each among atenolol/hydrochlorothiazide and atorvastatin/simvastatin/pravastatin, in the presence of their main degradation products [31]. Similar RP-HPLC studies were carried out for the determination of atenolol, hydrochlorothiazide, acetylsalicylic acid, ramipril and simvastatin [32]. The RP-HPLC method was also proposed for the simultaneous determination of atenolol, lisinopril, hydrochlorothiazide, enalapril maleate, amlodipine besylate, losartan potassium, valsartan and atorvastatin calcium [33].

Recently, multianalyte analysis is a primary goal of analytical laboratories, which creates new challenges in terms of rapid analytical response as well as problems with matrix effect and interferences between analytes [18]. Such a large number of active substances and the possibility of their occurrence in various quantitative and qualitative configurations in polypill preparations [34] prompted the authors to develop and validate a chromatographic method that would allow the simultaneous determination of the active substances found in polypills regardless of their quantitative and qualitative relationship. In this paper, a new HPLC-DAD method was developed for the separation and simultaneous determination of thirteen compounds (ACE inhibitors: ramipril, lisinopril, enalapril; β-blockers: atenolol, metoprolol; sartans: losartan, candesartan; statins: rosuvastatin, atorvastatin, simvastatin; calcium channel blocker: amlodipine; diuretic: hydrochlorothiazide, anticoagulant: acetylsalicylic acid), which can occur in polypills used in the treatment of CVDs. The usefulness of this work was assessed by validation and measurements in prepared mixtures according to available pharmaceutical formulations.

2. Materials and Methods

2.1. Reagents

Methanol of HPLC grade was purchased from WITKO LTd (Łódź, Poland). Acetonitrile and 85% orthophosphoric acid of HPLC grade were purchased from Merck (Darmstadt, Germany). Dipotassium phosphate of analytical grade was purchased from Sigma-Aldrich (Darmstadt, Germany). HPLC-grade water was obtained from an HLP 5 (HYDROLAB Poland).

The 0.01 M phosphate buffer with pH = 2.50 was prepared as follows: 1.7 g of dipotassium hydrogen phosphate was weighed and dissolved in approximately 900 mL of HPLC-grade water, then the solution was adjusted to pH 2.50 with 85% orthophosphoric acid and made up to 1000.0 mL with HPLC-grade water.

2.2. Standard Solutions and Substances

The following standard substances according to European Pharmacopoeia requirements, were used: HCT CAS No. 58-93-5 Sigma-Aldrich, ROS calcium salt CAS No. 147098-20-2 Sigma-Aldrich, CAN cilexetil CAS No. 145040-37-5 Sigma-Aldrich, ATO calcium salt CAS No. 344423-98-9 Sigma-Aldrich, MET tartrate CAS No. 56392-17-7 Sigma-Aldrich, ACES CAS No. 50-78-2 Sigma-Aldrich, ATE CAS No. 29122-68-7 Sigma-Aldrich, LOS monopotassium salt CAS No. 124750-99-8 Sigma-Aldrich, SIM CAS No. 79902-63-9 Sigma-Aldrich, RAM

CAS No. 87333-19-5 Sigma-Aldrich, LIS CAS No. 83915-83-7 Sigma-Aldrich, ENA maleate salt CAS No. 76095-16-4 Sigma-Aldrich, AML besylate CAS No. 111470-99-6 Sig-ma-Aldrich.

Five solutions of each substance in methanol were prepared at concentrations from 0.0125 mg mL^{-1} to 0.0374 mg mL^{-1} for HCT; from 0.0250 mg mL^{-1} to 0.0750 mg mL^{-1} for AML; from 0.0375 mg mL^{-1} to 0.1125 mg mL^{-1} for ACES; from 0.0200 mg mL^{-1} to 0.0600 mg mL^{-1} for ROS; from 0.0150 mg mL^{-1} to 0.0449 mg mL^{-1} for ATO; from 0.0150 mg mL^{-1} to 0.0449 mg mL^{-1} for SIM; from 0.0100 mg mL^{-1} to 0.0300 mg mL^{-1} for LOS; from 0.0160 mg mL^{-1} to 0.0480 mg mL^{-1} for CAN, from 0.2650 mg mL^{-1} to 0.7950 mg mL^{-1} for RAM, from 0.1885 mg mL^{-1} to 0.5655 mg mL^{-1} for LIS, from 0.1250 mg mL^{-1} to 0.3750 mg mL^{-1} for ENA, from 0.0250 mg mL^{-1} to 0.0749 mg mL^{-1} for ATE, from 0.0500 mg mL^{-1} to 0.1500 mg mL^{-1} for MET.

2.3. Pharmaceutical Preparations

The following preparations have been used to prepare combined polypills: Enarenal—film-coated tablets containing 20 mg ENA (Polpharma, Starogard Gdański, Poland), Carzap—film-coated tablets containing 16 mg CAN (Zenvita, Prague, Czech Republic); Suvardio—film-coated tablets containing 10 mg ROS (Sandoz, Austria); Amlonor—film-coated tablets containing 5 mg AML (Polfa, Pabianice, Poland); Normocard—film-coated tablets containing 50 mg ATE (Polfa, Warszawa, Poland); Ximve 20—film-coated tablets containing 20 mg SIM (Recordati, Warszawa, Poland); Bestpirin—film-coated tablets containing 75 mg ACES (Teva Pharmaceuticals Polska Ltd, Warszawa, Polska); Metocard—film-coated tablets containing 50 mg MET (Polfarma, Starogard Gdański, Poland); Tritace—film-coated tablets containing 2.5 mg RAM (Sanofi -Aventis, Warszawa, Poland); Presartan—film-coated tablets con-taining 50 mg LOS (Pharma Swiss, Praha Holesovice, Czech Republic); Hydrochlorothiazidum—film-coated tablets containing 25 mg HCT (Polpharma, Starogard Gdański, Poland); Lisi-Hennig—film-coated tablets containing 5 mg LIS (Hennig Arzneimittel, Flörsheim am Main, Germany); Atoris—film-coated tablets containing 20 mg ATO (KRKA, Nove Mesto, Slovenia).

2.4. Sample Preparation

The combined formulations containing the tested APIs were not available in the market and, therefore, the following model mixtures have been prepared using the preparations described in the section Pharmaceutical preparations: M-1 corresponding to the Red Heart Pill^{TM} 1 preparation (containing 75 mg ACES, 50 mg ATE, 10 mg LIS and 40 mg SIM); M-2 corresponding to the preparation Zycad-4 (containing 75 mg ACES, 10 mg ATO, 5 mg RAM, 50 mg MET); M-3 corresponding to the Atacand HCT preparation (containing 12.5 mg HCT, 16 mg CAN, spiked with 10 mg ROS); and M-4 (containing 25 mg LOS, 2.5 mg AML, 2.5 mg ENA).

Ten tablets of each preparation were weighed and finely powdered. The respective amount of each powder was accurately weighed and transferred into 25 mL volumetric flasks, extracted for 20 min with 15 mL of methanol in an ultrasonic bath and then the volume was completed with the same solvent to obtain polypill solution. The suspensions were centrifuged for 10 min at 1500 rpm. Appropriate volumes of the clear supernatant solutions were diluted with methanol in 10 mL volumetric flasks. Finally, all the solutions were filtered through a 0.45 µm Millipore nylon membrane filter before chromatographic analysis.

The following amounts of powdered formulations were weighed: for the preparation of M-1 26.2 mg ACES, 41.4 mg ATE, 49.6 mg LIS, 103, 2 mg SIM; for the M-2 compound 37.5 mg ATO, 26.3 mg ACES, 51.5 mg RAM, 38.6 mg MET; for making M-3 24.7 mg HTC, 40.2 mg CAN, 28.2 mg ROS; and for the preparation of M-4 78.4 mg LOS, 37.5 mg AML, 80.5 mg ENA.

2.5. Instrumentation and HPLC Conditions

The liquid chromatography system, HITACHI, High-Technologies Corporation (Tokyo Japan) equipped with a solvent delivery pump (L-2130), degasser, an autosampler (L-2200), a photodiode array detector (L-2455), and a column oven (L-2350) was used. The chromatographic analysis of 13 APIs was performed on the ACE-5 C18-PFP column (Advanced Chromatography Technologies, Aberdeen, Scotland) (250 mm × 4.6 mm, 5 µm particle size) coupled with a guard column. The column temperature was 25 °C. The chromatographic separation was achieved using a gradient elution of the mobile phase (Table 2) composed of 0.01 M phosphate buffer (pH = 2.50) and acetonitrile. The flow rate of the mobile phase was 1.0 mL min^{-1}, and the injection volume was 5 µL. The analysis time was 35 min. The samples were monitored at 230 nm.

Table 2. The percentage composition of the mobile phase.

Time [min]	0.01 M Phosphate Buffer pH = 2.50 [%]	Acetonitrile [%]
0	95	5
10	80	20
30	0	100
35	95	5

2.6. Method Validation

The HPLC method was validated for specificity, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, precision, and robustness according to the International Conference on Harmonization (ICH) guidelines [35]. The specificity of the method was assessed by comparing chromatograms of the pure standard substances, chromatograms of the drug preparation solutions, blank chromatograms, and chromatograms obtained for methanol used to dissolve tested substances. In all obtained chromatograms, the retention time (t_R), resolution factor (Rs), and asymmetry factor (As) values of the analysed substances, the peak areas, and the purity of the peaks were taken into account. The system suitability was checked by five replicate injections of standard solutions of ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS, and SIM. The system suitability parameters were defined with respect to retention times, asymmetry factors, resolution factors, and a number of theoretical plates of the examined drug peaks. The system was considered to be suitable for the analysis when the number of the theoretical plates was greater than 2000, the asymmetry factor was less than 1.5, and the resolution factor was greater than 1.5 for five replicate injections.

The calibration plots for ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS and SIM were constructed by the analysis of five separately prepared solutions for each tested substance covering a range of concentrations 50–150% as described in section Standard solutions and substances. The further analytical procedure was as described in the Instrumentation and HPLC conditions. Linearity was assessed in triplicate based on the relationship between peak areas and concentration, in milligrams per millilitre. The slope of the regression lines, y-intercept, the standard deviation of slope and intercept, the correlation coefficient, the R^2 value, and the standard error of residuals of the calibration curves were calculated using the program Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Then, to determine whether the residuals have a normal distribution, the Shapiro–Wilk statistical test was used. The limit of detection (*LOD*) and the limit of quantitation (*LOQ*) for the examined drugs were estimated based on the residual standard deviation of a regression line (*Se*) and the slope (*a*) of the calibration plots, following the formulas:

$$LOD = \frac{3.3 \cdot Se}{a} \quad (1)$$

$$LOQ = \frac{10 \cdot Se}{a} \quad (2)$$

The detection and quantification limits were validated by the analysis of the solutions at the concentrations prepared at the detection and quantification limits.

The repeatability of the method was determined by the analysis of three replicates of standard solutions of the tested substances from individual weighing. The study was performed for three concentration levels: 50%, 100%, and 150%. The intermediate precision was obtained for the same concentration of freshly prepared solutions by different analysts who performed the analysis over a period of 1 week. The results were expressed as the relative standard deviation (RSD).

The accuracy of the method was determined by the quantitative analysis of polypills solutions of the tested substances prepared as described in the section Pharmaceutical preparations and solutions. The study was carried out for a concentration level of 100%. Recovery was evaluated as the percentage relative error between the determined content of tested substances calculated from the regression equation and the weighed amount.

The robustness was evaluated by intentional minor modifications of the proposed method parameters. The impact of small changes in the pH of the phosphate buffer (±5% from the initial pH) and the flow rate (±0.1 mL min^{-1}) on the separation of the drugs studied was checked.

3. Results

3.1. Optimization of Chromatographic Conditions

The main target of our work was to develop and validate the universal HPLC-DAD method for the identification and determination of APIs present in polypills commonly used in the therapy of cardiovascular diseases. We decided to analyse the following substances: ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS and SIM. In the literature survey, there are methods reported for the simultaneous determination of a few but not all APIs selected by us.

Three chromatographic columns were tested as stationary phases: ACE-5 C18 (250 mm × 4.60 mm, particle size 5 µm), ACE-5 C18-PFP (250 mm × 4.60 mm, particle size 5 µm) and Kinetex 5u XB-C18 100A (250 mm × 4.60 mm, particle size 5 µm core-shell type) Phenomenex. All columns selected for analysis can be used in the reverse phase, which is the main mode for pharmaceutical analysis. The ACE-5 C18 and Kinetex 5u XB-C18 columns contain an octadecyl silica (C18) stationary phase that is most commonly used in reverse phase chromatography. The advantage of the Kinetex column over the ACE-5 is that the stationary phase particles are superficially porous, resulting in significantly reduced plate height and higher separation efficiency. While the column containing the stationary phase C-18-PFP combines the hydrophobic characteristics of a C18 phase with the increased selectivity based on multiple retention mechanisms offered by a pentafluorophenyl (PFP) phase.

The ACE-5 C18 column was used as the first stationary phase, while the mobile phase was a mixture of 0.01 M phosphate buffer at pH = 3.00—acetonitrile-methanol at a ratio of 30:20:50 (*v*/*v*/*v*). The column temperature was set at 25 °C, the flow rate of 1.0 mL min^{-1} and the detection at λ = 230 nm. Under the applied conditions, the retention times of many test compounds coincided or were very similar. Other mobile phases with the following proportions of the ingredients listed above; 20:30:50 and 20:20:60 (*v*/*v*/*v*) were checked but no satisfactory separation of the tested substances was obtained.

The stationary phase was then replaced with the Kinetex 5u XB-C18 column. The analyses were repeated, but under the applied conditions, no satisfactory separation of the tested substances was achieved.

The separation on the octadecyl silica (C18) phase is mainly based on hydrophobic interactions and, to a much lesser extent, on shape selectivity. In contrast, the PFP stationary phase shows many mechanisms increasing the retention of analytes. Mainly these are π–π interactions, dipole–dipole, hydrogen bond formation, shape selectivity, and to a much lesser extent, hydrophobic interactions. The C18-PFP phase exhibits all retention mechanisms of the PFP phase, which may exploit in order to resolve mixtures that are difficult to

separate on the traditional C-18 phase while maintaining hydrophobicity, stability, and low bleed characteristics of C-18 phases. Therefore, it was decided to use the ACE-5 C18-PFP column as the stationary phase. The mobile phases composed of methanol-water (95: 5 v/v) and 0.01 M phosphate buffer pH = 2.50—acetonitrile-methanol (20: 30: 50 $v/v/v$) were tested. In the checked mobile phases using isocratic elution, we have not obtained a good separation of all compounds.

Accordingly, it was found that, due to the very different hydrophobicity of the 13 tested compounds (log Kow from −1.22 to 6.36), to obtain better resolution and symmetry of the peaks gradient elution should be used. Finally, a linear gradient based on a binary mixture of phosphate buffer (pH = 2.50) and acetonitrile were used (Table 2). In the initial conditions of the gradient, a mobile phase with a lower elution strength was used, consisting of 95% phosphate buffer and 5% acetonitrile, then the amount of acetonitrile was gradually increased at the expense of the buffer to reach 100% of its content after 30 min, which resulted in a linear increase in the elution strength of the mobile phase.

The gradient elution described above was also applied on the Kinetex 5u XB-C18 column but a satisfactory separation for HCT and LIS was not obtained.

3.2. Selectivity and System Suitability

The developed method was specific to the APIs studied. On recorded chromatograms, there are no peaks for the mobile phase, and the mixture of solvents used to dissolve the tested substances where the components studied occur (Figure 1).

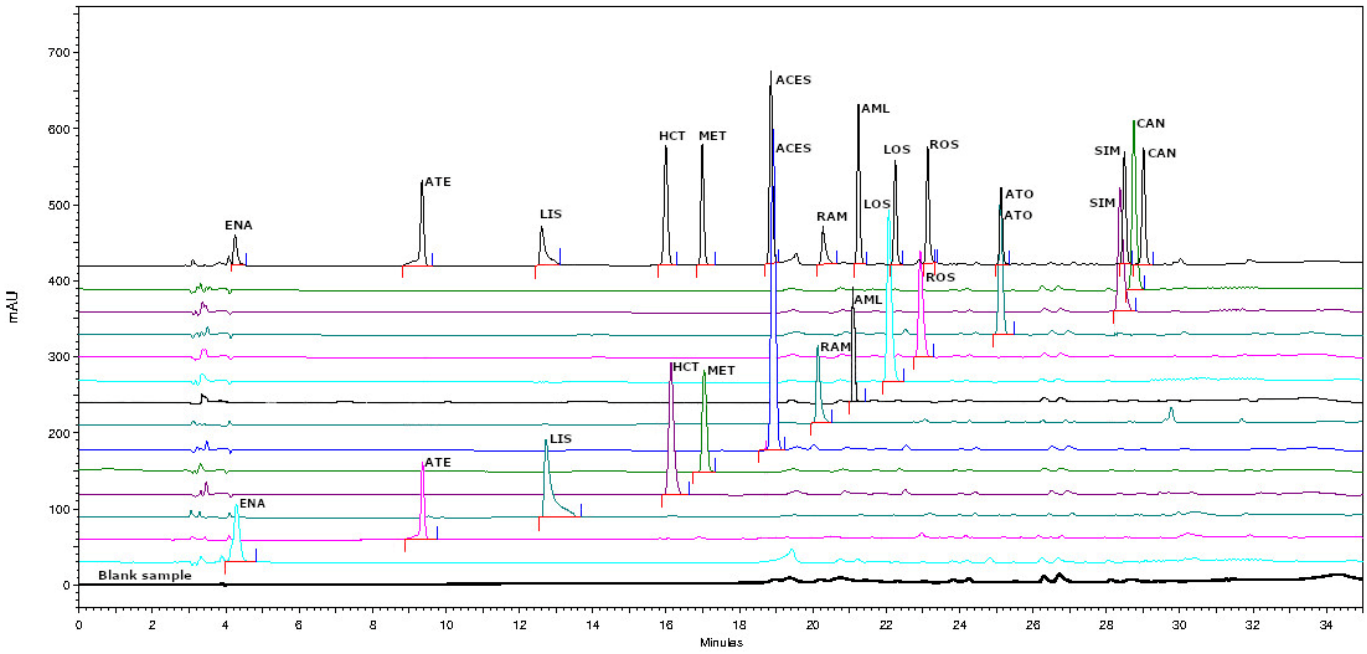

Figure 1. Overlay chromatogram of system specificity obtained from chromatograms registered for a blank sample, standards of 13 APIs, and their mixture.

The method also guaranteed obtaining well-shaped and pure peaks. Criteria for assessing the suitability of the system are described in the European Pharmacopoeias chapter 2.2.46 Chromatographic separation techniques. The asymmetry factor of the principal peaks should fall between 0.8 and 1.5, with a value of 1.0 indicating a perfectly symmetrical peak. Values greater than 2 are unacceptable. The asymmetry factors achieved for all studied substances meet the acceptance criteria. The As values obtained were greater than 0.8 and less than 1.38. Peaks of tested substances were also well resolved. The resolution factors obtained for all the compounds tested were greater than 1.50 which corresponds to the baseline separation. The highest Rs values were obtained for ATE and LIS. The number of theoretical plates for all APIs studied was greater than 2000 (Table 3).

An example of a chromatogram of a separated mixture containing all tested APIs is shown in Figure 2.

Table 3. System suitability parameters (n = 5).

Compound	t_R (min)	N	A_s	R_s [a]
ENA	4.21 SD = 0.104 RSD = 2.48%	12,064.0 SD = 344.8 RSD = 2.80%	1.07	-
ATE	9.32 SD = 0.06 RSD = 1.05%	49,535.0 SD = 1415.8 RSD = 2.86%	0.82	31.37
LIS	12.57 SD = 0.104 RSD = 0.50%	36,314.2 SD = 1150.9 RSD = 3.17%	1.37	14.89
HCT	15.93 SD = 0.06 RSD = 0.38%	121,376.2 SD = 342.60 RSD = 2.82%	0.99	14.37
MET	16.93 SD = 0.15 RSD = 0.88%	225,934.4 SD = 2888.4 RSD = 1.28%	1.22	5.83
ACES	18.81 SD = 0.08 RSD = 0.43%	239,086.6 SD = 5721.0 RSD = 2.39%	1.12	12.46
RAM	20.13 SD = 0.09 RSD = 0.46%	198,583.8 SD = 5743.1 RSD = 2.89%	1.27	5.84
AML	21.10 SD = 0.09 RSD = 0.42%	425,173.8 SD = 3890.8 RSD = 0.92%	1.18	5.50
LOS	22.09 SD = 0.09 RSD = 0.42%	406,365.8 SD = 7515.6 RSD = 1.84%	1.08	7.38
ROS	22.96 SD = 0.10 RSD = 0.43%	450,504.4 SD = 4158.7 RSD = 0.92%	1.16	6.31
ATO	24.94 SD = 0.12 RSD = 0.46%	499,586.6 SD = 4304.0 RSD = 0.86%	1.09	14.20
SIM	28.27 SD = 0.13 RSD = 0.47%	502,694.6 SD = 11958.5 RSD = 2.38%	1.08	22.30
CAN	28.77 SD = 0.14 RSD = 0.49%	516,520.0 SD = 9117.7 RSD = 1.80%	1.08	3.16

[a] Resolutions were calculated between two adjacent peaks. t_R—retention time; Rs—resolution; N—number of theoretical plates; As—asymmetry factor.

3.3. Linearity and LOD and LOQ

Regression analysis results obtained for examined compounds are listed in Table 3. The correlation coefficients (R) and determination coefficients (R^2) obtained for the linear model for all examined substances were greater than 0.990. The y-intercepts of the linear equation for ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS, and SIM were statistically insignificant. The distribution of the residuals can well be approximated with a normal distribution as it is shown by p-values ($p > 0.05$) of the Shapiro–Wilk normality test.

Based on the regression analysis, it was assumed that the calibration data fitted well to the linear model (Table 4). The linearity range was observed in a wide concentration range.

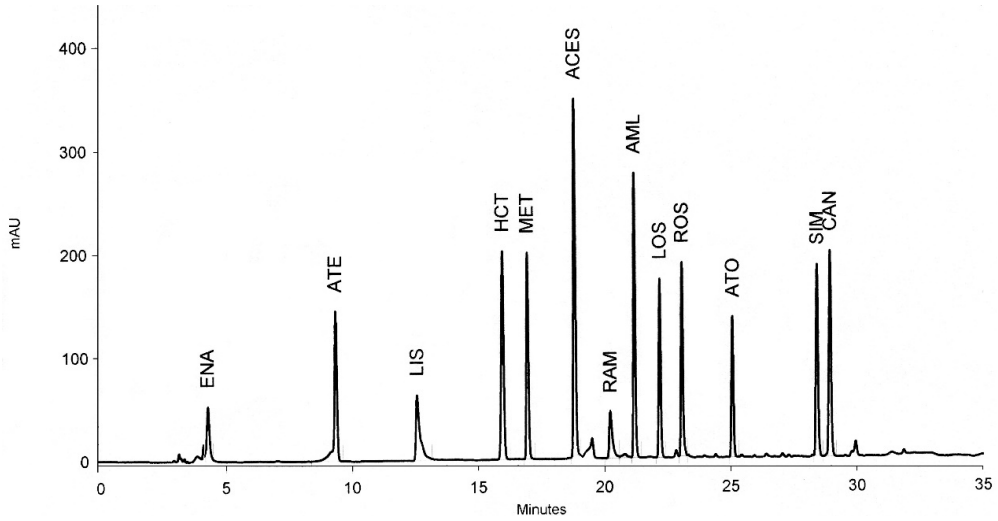

Figure 2. HPLC-DAD chromatogram of system suitability of 13 examined drugs obtained under the developed conditions.

Table 4. Calibration curves equations and statistical tests used for linearity assessment and LOD and LOQ of the method.

API	LOD [mg mL^{-1}]	LOQ [mg mL^{-1}]	Linearity Range [mg mL^{-1}]	Regression Coefficients $P = ac + b \pm S_e$ [a] (n = 15)	S_a [b] S_b	R^2	Normality of Residuals [c] (SW Test)	
							p	W
ENA	0.0416	0.1261	0.1250–0.3750	$a = 5642 \times 10^3$ $b = 11{,}501 \pm 71{,}163$	207,879 55,122.2	0.9827	0.5581	0.9521
ATE	0.0035	0.0106	0.0250–0.0749	$a = 7215 \times 10^4$ $b = -117 \times 10^3 \pm 76{,}684$	1,121,837 59,399	0.9969	0.9993	0.9897
LIS	0.0358	0.1085	0.1885–0.5655	$a = 7890 \times 10^3$ $b = -117 \times 10^3 \pm 85{,}636$	165,886.9 66,336.1	0.9943	0.1524	0.9134
HCT	0.0024	0.0071	0.0125–0.0374	$a = 1815 \times 10^5$ $b = -194{\cdot}10^3 \pm 1294 \times 10^2$	3,786,642 10,0248	0.9944	0.6269	0.9562
MET	0.0092	0.0279	0.0500–0.1500	$a = 3612 \times 10^4$ $b = -154 \times 10^3 \pm 1008 \times 10^2$	735,779.4 78,041.2	0.9946	0.0189	0.8526
ACES	0.0116	0.0350	0.0375–0.1125	$a = 1020 \times 10^5$ $b = -72 \times 10^3 \pm 3573 \times 10^2$	3,484,547 276,751	0.9850	0.8939	0.9726
RAM	0.0923	0.2794	0.2650–0.7950	$a = 7881 \times 10^3$ $b = 2458 \times 10^2 \pm 2205 \times 10^2$	303,884.8 170,828.8	0.9810	0.6950	0.9602
AML	0.0054	0.0163	0.0250–0.0750	$a = 9058 \times 10^4$ $b = -184 \times 10^3 \pm 1473 \times 10^2$	2,152,175 114,136	0.9927	0.9113	0.9739
LOS	0.0009	0.0027	0.0100–0.0300	$a = 1481 \times 10^5$ $b = -473 \times 10^2 \pm 40{,}683$	1,485,532 31,513	0.9987	0.7750	0.9648
ROS	0.0027	0.0081	0.0200–0.0600	$a = 8216 \times 10^4$ $b = -933 \times 10^2 \pm 66{,}954$	1,222,399 51,862	0.9971	0.9127	0.9740
ATO	0.0022	0.0067	0.0150–0.0449	$a = 7903 \times 10^4$ $b = -755 \times 10^2 \pm 5323 \times 10^1$	1,299,252 41,232	0.9965	0.8834	0.9718
SIM	0.0030	0.0092	0.0150–0.0449	$a = 1208 \times 10^5$ $b = -102 \times 10^3 \pm 1108 \times 10^2$	2,705,490 85,859	0.9935	0.4461	0.9448
CAN	0.0037	0. 0113	0.0160–0.0480	$a = 1167 \times 10^5$ $b = 70{,}580 \pm 1318 \times 10^2$	3,007,377 102,074	0.9914	0.9464	0.9772

[a] (P—peak area; c—concentration; a, b—regression coefficients) S_e—standard error of the estimate; [b] S_a, S_b—standard deviation of the regression coefficients a and b, respectively; [c] normal distribution of residuals if $p > 0.05$; SW—Shapiro-Wilk test.

The sensitivity of the method was good. The LOD and LOQ values were found to be from 0.0009 to 0.0923 mg mL^{-1} and from 0.0027 to 0.2794 mg mL^{-1}, respectively (Table 4).

3.4. Precision, Accuracy and Robustness

Good precision and intermediate precision at three concentration levels with percent RSD less than 1.80% and 1.91% respectively were observed. The accuracy of the method

expressed as percent recovery was from 95.20% to 104.62%. Detailed results are listed in Table 5.

Table 5. Precision and accuracy of the method.

API	Precision, RSD [%] * (*n* = 3)			Indirect Precision, RSD [%] (*n* = 3)			Recovery [%] (*n* = 5)	
	50%	100%	150%	50%	100%	150%	Mean	RSD%
ENA	0.99	0.99	1.50	1.21	1.62	1.65	101.60	1.24
ATE	1.02	1.11	0.70	1.50	1.43	1.35	95.20	1.74
LIS	1.80	0.22	0.31	1.86	1.20	1.35	99.40	1.15
HCT	0.99	0.99	1.50	1.30	0.85	0.77	97.60	0.82
MET	0.24	1.05	0.24	1.30	1.24	0.78	98.08	0.37
ACES	0.8	0.36	1.40	1.29	1.64	1.91	103.07	1.12
RAM	0.93	1.29	0.53	1.12	1.55	1.07	100.40	1.67
AML	0.84	0.98	0.47	1.56	1.24	0.75	97.04	0.85
LOS	0.86	0.59	0.55	1.38	0.60	0.64	96.80	1.27
ROS	0.89	0.67	0.59	0.94	0.76	1.35	104.62	1.85
ATO	0.36	0.71	0.41	1.70	1.15	0.70	103.80	0.81
SIM	1.02	1.11	0.70	1.50	1.43	1.35	97.62	1.56
CAN	0.43	0.72	0.70	1.11	0.78	1.18	95.20	1.74

* RSD = relative standard deviation.

In all the deliberately varied chromatographic conditions (flow rate and pH of phosphate buffer in the mobile phase), examined drugs were adequately resolved, the order of elution remained unchanged and peak areas showed no significant changes.

Application to the Analysis of Pharmaceutical Formulations

The proposed HPLC-DAD method was successfully applied for the determination of ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS, and SIM in our laboratory-prepared model mixtures corresponding to the commercially available polypill tablets. The determining contents of APIs were good and RSD values were 0.30–0.95% (Table 6). The good content values and low RSD values indicated the applicability of the proposed method for the accurate and precise simultaneous determination of the polypill components. Moreover, excipients, which were present in the samples, do not interfere. All the peaks were well separated in the formulation sample and no impurities were detected at the analyzed concentration level.

Table 6. Application of the proposed HPLC-DAD method for the determination of APIs in laboratory-prepared mixtures of polypill tablets.

Analysed Mixtures	Nominal Content	Determined Content [mg ± RSD] (*n* = 5)
M-1	ACES (75 mg)	73.64 ± 0.95
	ATE (50 mg)	51.96 ± 0.80
	LIS (10 mg)	10.16 ± 0.88
	SIM (40 mg)	38.56 ± 0.30
M-2	ACES (75 mg)	71.88 ± 0.59
	ATO (10 mg)	10.36 ± 0.53
	RAM (5 mg)	4.92 ± 0.91
	MET (50 mg)	49.10 ± 0.50
M-3	HCT (12.5 mg)	12.18 ± 0.69
	CAN (16 mg)	15.64 ± 0.86
	ROS (10 mg)	10.26 ± 0.53
M-4	LOS (25 mg)	25.70 ± 0.48
	AML (2.5 mg)	2.42 ± 0.79
	ENA (12.5 mg)	12.32 ± 0.68

4. Discussion

Many dosage forms of drugs are commercially available to enable effective therapy of patients with CVDs. High hopes for an effective fight against CVDs are associated with the introduction of polypills into therapy, containing three or more APIs in one tablet. There are many articles in the available literature on the development of chromatographic methods for the quantitative or stability analysis of drugs used in the treatment of hypertension in the form of FCDs.

Pawar et al. proposed the RP-HPLC method for the analysis of enalapril maleate, hydrochlorothiazide, atorvastatin, and aspirin in pure and simulated dosage forms. The separation was achieved using a C18 column. The mobile phase was a 50:25:25 ($v/v/v$) mixture of acetonitrile, methanol, and triethylammonium phosphate buffer (pH 2.5) [23]. The second method developed by Pawar et al. allows the simultaneous estimation of aspirin, ramipril, and simvastatin in bulk and simulated formulations. The separation and determination were carried out on a Lichrosphere 100 RP-18 column with the mobile phase consisting of acetonitrile and triethylammonium phosphate buffer pH 2.5 (70:30 v/v) [25]. The APIs contained in the Starpill and their possible degradation products were separated on the Inertsil ODS C18 column (150 × 4.6 mm, particle size 5 µm). The mobile phase was a combination of 0.1% orthophosphoric acid adjusted to pH 2.9 with triethylamine and acetonitrile, delivered in gradient mode [27]. The mixture of ramipril, atorvastatin, and aspirin with a slightly different quantitative composition corresponding to the Polytorva and Modlip Cad preparations was determined using the isocratic RP-HPLC method [28]. HPLC analysis was performed on a C18 column with a mixture of acetonitrile-methanol (65:35 v/v) (phase A) and 10 mM sodium dihydrogen phosphate buffer adjusted to pH 3.0 (phase B), and a mixture of A:B (60:40 v/v) was used as a mobile phase [28]. The HPLC method was also used in the quantitative analysis of Exforge HCT. Effective chromatographic separation was achieved using the Zorbax SB-C8 column with gradient elution of the mobile phase composed of 0.025 M phosphoric acid and acetonitrile. The proposed chromatographic conditions were also directed toward the stress degradation study of the three antihypertensive drugs amlodipine, valsartan, and hydrochlorothiazide; as well as to the simultaneous determination of these drugs in their combined formulation [29]. Ibrahim et al. developed the HPLC method for the simultaneous estimation of losartan, hydrochlorothiazide and atorvastatin in laboratory prepared pharmaceutical tablets. The analysis was carried out on a BDS Hypersil C18 column with acetonitrile and 0.02 mM sodium dihydrogen phosphate buffer (pH = 3) 50:50 (v/v) as mobile phase [30]. Kumar et al. described the RP-HPLC method with gradient conditions for the determination of four possible components of a polypill, i.e., lisinopril, aspirin, and one each among atenolol/hydrochlorothiazide and atorvastatin/simvastatin/pravastatin, in the presence of their major degradation products. The separation was achieved by three gradient elution modes for various drug combinations using acetonitrile and 10 mM potassium dihydrogen orthophosphate buffer pH 2.3 as the mobile phase. In this study, a C8 column was used [31]. Similar studies were carried out for the determination of atenolol, hydrochlorothiazide, acetylsalicylic acid, ramipril, and simvastatin. The individual drug components and their main degradation products were well separated using the RP-HPLC method using a C18 column (150 mm × 4.6 mm, particle size 5 µm) and a mobile phase containing acetonitrile—0.01 M potassium dihydrogen phosphate buffer (pH 2.3) in gradient mode [32]. Another RP-HPLC method was also proposed for the simultaneous determination of atenolol, lisinopril, hydrochlorothiazide, enalapril maleate, amlodipine besylate, losartan potassium, valsartan and atorvastatin calcium. Optimal separation conditions were obtained using a C-8 column (250 mm × 4.6 mm, particle size 5 µm) and gradient elution of the mobile phase consisting of acetonitrile and 10 mM dipotassium hydrogen phosphate buffer (pH 2.2) [33]. Furthermore, publications have been found that describe methods for the separation of enalapril and amlodipine on the C-18 phase (250 mm × 4.6 mm, particle size 5 µm) using a mixture of methanol-water (adjusted to pH 3.0 with phosphoric acid) in a proportion of 10: 90 (v/v) as a mobile phase [36] and also simvastatin and atorvastatin next to telmisartan and irbesartan on a C-18 column

(75 mm × 4.6 mm; 3.5 µm) using a mobile phase consisting of 10mM ammonium acetate buffer (pH 4.0) and acetonitrile in a ratio 40:60 (*v*/*v*) [37].

To sum up, in all of the cases, the C18 or rarely C8 HPLC columns were applied with a typical length of 250 mm (rarely 150 mm) and 4,6 mm inner diameter, filled with 5 µm particle size. As components of the mobile phases, acetonitrile, methanol, and a phosphate buffer at pH 2.3–3.0 were used. To obtain separation of the tested compounds, gradient elution was more often used than isocratic.

For our research, we have chosen ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS, and SIM which are commonly prescribed for the treatment of hypertension diseases. Based on the literature review, it was found that there were no reports of a method that would allow the simultaneous quantitative analysis of the thirteen drugs mentioned above. The pharmaceutical industry manufactures formulations of all mentioned APIs in the form of polypills or in a single dosage form. Our new simple validated and fast multianalyte HPLC-DAD method is advantageous because it allows the simultaneous quantification of all proposed thirteen APIs within a run time of 35 min without the need for the development of separate and distinct methods for each formulation. For the first time, we proposed using the C18-PFP phase for separation instead of the traditionally used C-18 or C-8 phase. The ACE C18-PFP combines a hydrophobicity of the C18 chain with PFP functionality, resulting in a phase that maintains the hydrophobic and stability characteristics of a leading C18 phase, whilst providing the multiple retention mechanisms of a PFP phase. Phase ACE C18-PFP is recommended for the separations of compounds that involve halogenated aromatic compounds, and position isomers. Due to better retention in the PFP phase, it shows an improvement in selectivity in the analysis of polar pharmaceutical compounds [38]. To date, the authors have developed methods for two to eight compounds that make up polypills [24–37]. The methods were also adapted to the capabilities of drug stability testing [26,29,31,32]. However, it was associated either with the analysis of a smaller amount of active substances or with a significant extension of the analysis time to an hour or more. The method we propose is universal and the most useful in the quality control of selected active substances occurring in polypills. The new method is less time-consuming and inexpensive, saving on the cost of replacing columns or different mobile phases use. Only one mobile phase and column can be used for the thirteen individual drugs and their combinations. The proposed method also meets criteria of a green analytical technique with a smaller amount of organic phase (acetonitrile).

5. Conclusions

Herein, we developed a universal HPLC-DAD method for the separation, identification, and determination of thirteen APIs used in fixed dose combinations. The developed and validated new chromatographic method is simple and allows the separation and quantitation of the following compounds: ACES, AML, ATE, ATO, CAN, ENA, HCT, LIS, LOS, MET, RAM, ROS and SIM in polypills of antihypertensive action. The elaborated method meets the acceptance criteria for specificity, linearity, sensitivity, accuracy, and precision. Therefore, the method can be an effective tool in the quality control of polypills.

Author Contributions: Conceptualization, U.H. and M.S. (Mariusz Stolarczyk); methodology, U.H. and B.Ż.-W.; Software, U.H., M.S. (Marek Szlósarczyk) and B.Ż.-W.; validation, B.Ż.-W.; formal analysis, B.Ż.-W. and S.K.; investigation, B.Ż.-W., M.S. (Mariusz Stolarczyk) and S.K.; writing—original draft, B.Ż.-W., M.S. (Mariusz Stolarczyk) and M.S. (Marek Szlósarczyk); writing—review and editing, M.S. (Marek Szlósarczyk) and U.H.; supervision, U.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CAN—candesartan, HCT—hydrochlorothiazide, ROS—rosuvastatin, ATO—atorvastatin, MET—metoprolol, ACES—acetylsalicylic acid, ATE—atenolol, LOS—losartan, SIM—simvastatin, RAM—ramipril, LIS—lisinopril, ENA—enalapril, AML—amlodipine, FDC—fixed dose combination, HPLC-DAD—high-performance liquid chromatography with a diode-array detector, ICH—International Conference on Harmonisation.

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