Analyzing different cardiovascular medication combinations with vincamine: a simple, simultaneous, and environmentally friendly evaluation

1K.SARASIRISHA,2D CHAITANYA DIXIT,3P.T.NAGARAJU,4B Akhila,5E Nagadeepthi

Department Of Pharmaceutical Analysis

Abstract

It is crucial for sustainable pharmaceutical analysis to develop two environmentally friendly analytical methods that can simultaneously determine eight cardiovascular drugs: hydrochlorothiazide (HCT), captopril (CPL), lisinopril (LSP), valsartan (VAL), atorvastatin (ATR), bisoprolol (BSL), amlodipine (AML), and carvedilol (CVL). Additionally, the nutraceutical vincamine (VIC) should be tested. Micellar Electro Kinetic Chromatography (MEKC) and High-Performance Liquid Chromatography (HPLC) are investigated in this work as potential tools for this objective. The MEKC technique separates substances by using fused silica capillaries with an internal diameter of 41.5 cm and a background electrolyte that includes 50 mM borate buffer with a pH of 9, 50 mM sodium lauryl sulfate (SLS), and 10% Acetonitrile. A ZORBAX Extend-C18 column, measuring 4.6×250 mm and having a particle size of 5 μm, was used for separation in the HPLC technique. The mobile phase, which consisted of methanol and 50 mM phosphate buffer pH 3, was used in the gradient. With minimal values of LOD and LOQ, both techniques achieved high linearity (r > 0.9996). Both approaches were effective in identifying the examined medications' co-administered single, binary, and ternary dose forms. As an added bonus, different combinations of co-administered

dose forms in rat plasma, proving the techniques' viability in various matrices. In line with green chemistry principles, micellar solutions are used in MEKC to increase separation efficiency and decrease organic solvent use. To minimize toxicity and waste, HPLC procedures were fine-tuned utilizing solvents that are safe for the environment. To make sure the approaches were completely sustainable, we looked at their ecological effect, safety, and practical efficiency using green, white, and blue criteria. The techniques were perfect for frequent usage in pharmaceutical analysis since they were cost-effective, time-saving, and attained excellent efficiency, sensitivity, and repeatability.

Keywords Cardiovascular drugs, Vincamine, MEKC, HPLC, Greenness, Blueness, Whiteness

Introduction

Cardiovascular disorders (CVDs) continue to be the pri- mary global cause of illness and mortality, accounting for 17.9 million deaths yearly, according to the World Health Organization (WHO) [1]. These disorders encom- pass a range of conditions affecting the heart and blood vessels, such as coronary artery disease, hypertension, heart failure, and arrhythmias. Effective management of these disorders is crucial for reducing the associated health burden and improving patient outcomes. Pharma- cotherapy plays a pivotal role in the treatment of CVDs, with a diverse array of cardiovascular drugs employed to address the underlying pathophysiology, alleviate symp- toms, and prevent complications

Cardiovascular drugs, including antihypertensives, antianginals, anticoagulants, antiarrhythmics, lipid-low- ering agents, and vasodilators, form the cornerstone of CVD management. These medications function through various mechanisms to help avoid blood clots, lower cho- lesterol, or lower blood pressure, regulate heart rhythms, and enhance cardiac output. Their targeted actions not only mitigate the immediate risks associated with cardio- vascular events but also contribute to long-term cardio- vascular health [3, 4].

In this research, eight cardiovascular drugs from differ- ent classes were studied including, hydrochlorothiazide (HCT, Fig. 1a), which is the most common thiazide diu- retic recommended for the treatment of hypertension and edema. captopril (CPL, Fig. 1b) and lisinopril (LSP, Fig. 1c) are angiotensin converting enzyme inhibitors (ACEIs) used to treat hypertension combined with beta blockers or thiazide diuretics. They are the only ACEIs that are not prodrugs. Valsartan (VAL, Fig. 1d) is one of the angiotensin II receptor blockers (ARBs) that prefer- entially attach to the angiotensin receptor 1 (AT1) and inhibit the binding of the angiotensin II protein, resulting in lowered blood pressure, decreased aldosterone levels, decreased cardiac activity, and elevated salt excretion. Atorvastatin (ATR, Fig. 1e) functions by competitively inhibiting the enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, which is responsible for cata- lyzing the conversion of HMG-CoA to mevalonic acid. This results in lipid lowering effect for those patients with high risk of CVDs. Amlodipine (AML, Fig. 1f) is a calcium channel blocker used to treat hypertension and angina. Bisoprolol (BSL, Fig. 1g) and carvedilol (CVL, Fig. 1h) are non-selective and β-1 adrenergic antagonists, respectively. Both are used to treat hypertension and myocardial infarction [5].

In addition to conventional cardiovascular drugs, there is growing interest in the potential therapeutic benefits of naturally derived compounds, such as vin- camine. Vincamine (VIC, Fig. 1i), an alkaloid extracted from the Vinca minor plant, is utilized as a nutraceutical for its potential benefits in managing cardiovascular dis- eases. Known for its vasodilatory properties, vincamine enhances blood flow, particularly in cerebral vessels, which can help improve cognitive function and reduce symptoms related to poor circulation [6]. It also exhib- its antioxidant and anti-inflammatory effects [7], which contribute to cardiovascular health by protecting the heart and blood vessels from oxidative stress and inflam- mation, common factors in cardiovascular diseases. As a supplement, vincamine is promoted for its ability to support overall cardiovascular wellness. Recent stud- ies suggest that vincamine may offer valuable adjunctive benefits in the treatment of cardiovascular disorders [8].

Many analytical techniques for the separation and quantification of such drugs, either alone or in conjunc- tion with other cardiovascular drugs, in various matrices, bulk, dose forms, and biological fluids, were reported in the literature.

Among the recent reported HPLC and CE methods for the studied drugs, the determination of a mixture of HCT/CPL was performed using HPLC [9–11] and CZE [12]. Also, the determination of a mixture of HCT/ LSP was performed by HPLC [13–19], CZE [12] and MEKC [20] methods. For

HCT/VAL mixture, the recent reported methods includes HPLC [9, 11, 21–25] and CE [12]. For HCT/VAL/AML mixture, HPLC [26–35] and CZE [36] methods were applied. In addition to previ- ous mixtures, HCT/BSL mixture has been reported to be determined by HPLC [37–43]. Also, for the analysis of HCT/CVL mixture, HPLC [11, 44–47] and MEKC [48] methods are reported. A mixture of AML/VAL has been separated and quantified using HPLC [49–56] and CE [57–59]. A mixture of AML/ATR has been analyzed using HPLC [60–71] and CE [72]. A reported HPLC [73] method showed the simultaneous analysis of AML/ VAL/ATR together. For VIC, a nutraceutical of interest, various HPLC [74–76] methods were reported for its determination.

This paper aims to develop and validate robust analyti- cal methods, Micellar Electro Kinetic Chromatography (MEKC) and High-Performance Liquid Chromatogra- phy (HPLC), for the simultaneous separation and quan- tification of multiple cardiovascular drugs, HCT, CPL, LSP, VAL, ATR, BSL, AML and CVL, together with vin- camine, dietary supplement, in a single run. This study seeks to establish a comprehensive, reliable, and efficient analytical methods capable of supporting the simultane- ous analysis of multiple dosage forms of cardiovascular drugs, including emerging natural compounds like vin- camine in a green, blue and white perspectives.

Experimental
Instrumentation
Agilent CE equipment for data manipulation (Agilent Technologies, Waldbronn, Germany, series 7100) with DAD and a PC equipped with Agilent Chem Station soft- ware. The capillary that was utilized was acquired from Agilent Technologies, Inc. A deactivated fused silica cap- illary with an i.d. of 50 µm and a total length of 50 cm and an effective length of 41.5 cm was used.

Agilent 1200 series HPLC–DAD (vacuum degasser, auto- injector, quaternary pump, diode array, and mul- tiple wavelength detectors G1315 C/D and G1365 C/D) was utilized. It was linked to a PC running Agilent Chem- Station Software (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was achieved using a ZORBAX Extend-C18 (4.6 × 250 mm, 5 µm) col- umn (Agilent Technologies, Santa Clara, CA, USA).

A 4-digit German analytical balance, the Kern AEJ 220–4 M balance, was utilized to weigh drugs and dos- age forms precisely, while a 3-digit Sartorius BL 310 bal- ance was utilized for accurate weighing of buffer and surfactant. Jenway model 3505 (Germany) pH meter was utilized for pH adjustment throughout the study. Christ rotational vacuum concentrator, Germany was used in plasma sample extraction.

Materials and reagents
HCT (purity > 99.5%, Pharco Pharmaceuticals Co., Alex- andria, Egypt), CPL (purity > 99.5%, EIPICO pharma, Egypt), VAL (purity 99.7%, Novartis Pharma S.A.E., Cairo, Egypt), ATR (purity 99.8%, EIPICO pharma, Egypt), VIC (purity 99%, October pharma, Egypt), BSL (purity > 99%, Global Napi Pharmaceuticals, 6th October, Egypt), CVL (purity > 99.8%, Chemipharm Pharmaceuti- cal Industries, Egypt), LSP (purity > 99.85%, AstraZen- eca, Egypt) and AML (purity 99.8%, Pfizer Egypt S.A.E., Cairo, Egypt) were used in this study.

All dosage forms utilized in the study were purchased from the local market. Capoten 50® (50 mg CPL/tablet), Zestril 10® (10 mg LSP/tablet) Tareg 80® (80 mg VAL/ tablet), Ator 10® (10 mg ATR/tablet), Concor 10® (10 mg BSL/tablet), Norvasc 10® (10 mg AML/tablet), Dilatrol 25® (25 mg CVL/tablet), and Brain Ox® (30 mg VIC/cap- sule. Co-tareg® (80 mg VAL and 12.5 mg HCT/tablet), Exforge ® (10 mg AML and 160 mg VAL/tablet), Exforge HCT® (10 mg AML,160 mg VAL and 12.5 mg HCT/ tablet), Capozide® (50 mg CPL and 25 mg HCT/tablet), Zestoretic® (20 mg LSP and 12.5 mg HCT),

Concor plus® (10 mg BSL and 25 mg HCT/tablet), Caduet® (5 mg AML and 10 mg ATR/tablet) and Co-dilatrol® (25 mg CVL and 12.5 mg HCT). In addition, HCT laboratory made tablets labeled to contain 25 mg HCT due to the lack of its com- mercial dosage form were used.

Boric acid (Oxford Lab Chem, Mumbai, India), HPLC grade ethyl acetate and orthophosphoric acid (LAB- SCAN Analytical Sciences, Poland), HPLC grade ace- tonitrile and methanol (Sigma-aldrich Chemie GmbH, Buchs, Switzerland), sodium hydroxide and sodium lau- ryl sulphate (SLS) and phosphate monobasic (El-Nasr Chemical Industry company, Egypt) and deionized water were used.

Animals

The study utilized adult male Wistar rats weighing 200– 250 g from the Faculty of Pharmacy animal facility at Alexandria University in Alexandria, Egypt. The Insti- tutional Animal Care and Use Committee at Alexandria University in Egypt granted approval for all experimental

procedures and animal manipulations (Approval No. AU/06.2023.4.12.2.147).

After the rats were given isoflurane anesthesia, about 2 mL of blood samples were taken from their orbital sinus (retro-orbital plexus). Blood was collected in pre- coated tubes coated with ethylene diamine tetra-acetic acid after the retro-orbital venous plexus was punctured using un-heparinized glass capillary tubes. The tubes were then centrifuged at 1200 rpm for 15 min. For addi- tional examination, the supernatant plasma layer was col- lected into Eppendorf tubes and kept at – 80 °C. Lastly, rats were euthanized by overdose of thiopental (100 mg/ kg).

Experimental steps and calibration graphs construction
MEKC running buffer and HPLC mobile phase preparation
In MEKC, deionized water was used to prepare 50 mM borate buffer, which was then adjusted to pH 9 with

0.5 M NaOH in a 100 mL volumetric flask. The previ- ously made buffer was used to prepare a 50 mM SLS. The finally used back ground electrolyte (BGE) consists of 50 mM borate buffer (pH 9) containing 50 mM SLS and HPLC-grade acetonitrile (90:10, v/v), respectively.

In HPLC, 50 mM phosphate buffer pH 3 and metha- nol were used in a gradient elution starting with the ratio 70:30 by volume. This ratio was changed at 5, 7, 8 min, where methanol was increased to be 60, 80 and 90%, respectively. The methanol ratio was restored to 30% before subsequent injections.

MEKC procedure
The daily conditioning of the capillary was set that the capillary was flushed by 0.5 M NaOH for 10 min, then water for another 10 min. Afterwards, 0.1 M NaOH for 5 min, waiting 2.5 min to ensure full activation of the inner wall of the capillary, then washed with water for 5 min. Lastly, it was allowed to equilibrate with BGE for 10 min.

To maintain proper repeatability of run-to-run injec- tions, buffer vials were replenished after every 5 con- secutive runs. Between successive runs, the capillary was washed for 2 min with the BGE.

Using the hydrodynamic mode, injections were made at the anodic side for 10 s at a pressure of 50 mbar. The applied voltage was constant at 30 kV. The analysis was performed at wavelengths 210 and 220 nm.

Calibration graphs: stock and working solution preparation
Standard stock solutions of 2000 μg/mL of HCT, CPL, LSP, VAL, ATR, VIC, BSL, AML and CVL were prepared in HPLC-grade methanol. For MEKC, working solutions were prepared using proper aliquots of the stock solu- tions to cover the concentration ranges of 5–50 μg/mL for HCT, ATR, BSL and AML, 10–100 μg/mL for CPL and VIC and of 20–200 μg/mL for VAL. Similarly, in HPLC method, the concentration ranges were 0.5–50 μg/ mL for HCT, VIC, CVL and ATR, 1–50 μg/mL for LSP, BSL and AML and of 2–200 μg/mL for VAL (Table 2).

The final dilution was performed with distilled water or methanol in MEKC or HPLC, respectively. For every solution, three injections were made.

Analysis of pharmaceutical preparations

Assay of single dosage forms Ten tablets of each single dosage forms (Capoten 50®, Tareg 80®, Ator 10®, Con- cor 10®, Zestril 10®, Dilatrol 10® and Norvasc 10®) were weighed and finely grounded powder. A set of 25 mL volu- metric flasks were filled with precisely weighed quantities of each powdered tablet, and the mixture was sonicated for 15 min in 15 mL methanol. After the flasks were com- pleted to final volume, stock solutions containing 2 mg/ mL were obtained by filtration (Whatman filter paper, Grade 1, 110 mm). Similar procedure was performed for HCT in its laboratory made tablets (containing 25 mg HCT/tablet as in the brand Hydrex®, together with stand- ard tablet excipients). For BrainOX® capsules, the con- tent of 10 capsules were taken and an accurately weighed portion of VIC was treated similarly. Serial dilutions of each extracted single dosage forms were prepared to the required concentrations (Table S3). For every solution, three injections were made.

Assay of binary and ternary dosage forms Ten tab- lets of each combined dosage form were weighed and finely powdered. An accurately weighed portions of Co-tareg® (80 mg VAL/12.5 mg HCT) or of Exforge® (10 mg AML/160 mg VAL) or of Exforge HCT® (10 mg AML/160 mg VAL/12.5 mg HCT) or of Capozide® (50 mg CPL/25 mg HCT) or of Concor plus® (10 mg BSL/ 25 mg HCT) or of Caduet® (5 mg AML/10 mg ATR) or of Zestoretic® (20 mg LSP and 12.5 mg HCT) or of Co- dilatrol® (25 mg CVL and 12.5 mg HCT) were put into a series of 25 mL volumetric flasks and sonicated in 15 mL of methanol for 15 min. After

the flasks were completed to final volume, stock solutions containing 2 mg/mL were obtained by filtration (Whatman filter paper, Grade 1, 110 mm). Serial dilutions of each extracted dosage form were prepared to the required concentrations (Table 3). For every solution, three injections were made.

Plasma sample preparation

Using the protein precipitation method, the studied drugs were recovered from spiked rat plasma. A centri- fuge tube was filled with an aliquot of 100 μL of blank rat plasma that had been spiked with various aliquots of the drugs under study. Next, 2 mL of ethyl acetate was added.

The tubes were then centrifuged for 15 min at 6000 rpm after being vortexed for 5 min. In a vacuum concentra- tor set at 40 °C, the supernatant was separated and evap- orated until it was completely dry and the residue was reconstituted with 1 mL water or 100 μL methanol to be injected after filtration (using 0.22 μm Millipore filter) in the MEKC or HPLC system, respectively.

Calibration in plasma and quality control standards

Spiking 100 μL of blank rat plasma with different ali- quots of the prepared working standard solutions for the studied drugs followed by extraction, evaporation and reconstitution as illustrated in Sect. "Plasma sample prep- aration". to get final concentration ranges of 50–1000 μg/ mL for HCT, ATR, BSL and AML, 100–1000 μg/mL for CPL, 10–1000 μg/mL for VIC and 50–2000 μg/mL for VAL for MEKC system. Similarly, in HPLC system, the final concentration ranges reached were 1–100 μg/mL for HCT, LSP and AML, 2–100 μg/mL for VIC, 5–100 μg/ mL for BSL, CVL and ATR and 2–200 for VAL (Table 4). As shown in tables S6 & S7, the four reported qual- ity control (QC) samples were prepared similarly to the calibration standards in order to ensure precision and accuracy. They were then handled to obtain the final con- centrations for the LLOQ (lower limit of quantitation), LQC (low quality control),

MQC (medium quality con- trol), and HQC (high quality control) for each analyte.

Determination of different co-administered dosage forms of the studied drugs with vincamine in spiked plasma samples

Cardiovascular diseases require multiple therapy that results in co-administration of different dosage forms. Brain OX$^{®}$ (VIC) is a nutraceutical that is commonly administered in most of cardiovascular diseases. Thus, the determination of various co-administered cardio- vascular drugs together with VIC in rat plasma was per- formed using MEKC and HPLC methods.

Volumes of 100 μL blank rat plasma were spiked with different aliquots of drugs in the ratio of the co-adminis- tered dosage forms in different combinations as in illus- trated in Table 5. The samples were treated as previously mentioned regarding the extraction, evaporation, recon- stitution and injection.

Results and discussion

Two simple, rapid and selective methods, MEKC and HPLC, were suggested for the separation and simul- taneous determination of various drugs commonly co-administered in different combinations for the treat- ment of cardiovascular diseases. Method I, involves the use of MEKC for the simultaneous separation of HCT, CPL, VAL, ATR, VIC, BSL and AML, whereas method II presents an HPLC method for the separation of HCT, LSP, VIC, BSL, CVL, AML, VAL and ATR. The multi-

ple wavelength detector in both methods was efficiently applied for the quantification of each analyte at its opti- mum wavelength.

Analysis conditions optimization
MEKC method

To determine the ideal conditions for the separation of the studied drugs in CE, several trials were conducted. Firstly, capillary zone electrophoresis (CZE) mode with various buffers were tried, as acetate buffer (10, 20, 50, and 100 mM) of pH 4.7, phosphate buffer (10, 20, 50, and 100 mM) of pH 7.4, and borate buffer (10, 20, 50, and 100 mM) of pH 9 were among the buffers used in the ini- tial mode trials. Additionally, 50 mM borate buffer was tested at various pH levels. All of the previously men- tioned CZE trials could not effectively separate the stud- ied drugs as some drugs appeared at the same migration time. Also, VIC was eluted with the electro-osmotic flow (EOF) and was not well separated.

Secondly, the MEKC mode was accessed by addition of SLS at a concentration greater than its critical micelle concentration (CMC). Phosphate buffer (20 or 50 mM) with (25 or 50 mM SLS), and borate buffer of 50 mM with (25 or 50 mM SLS) were tried. Borate buffer with 50 mM SLS was able to effectively separate all drugs and VIC was eluted away from EOF. Unfortunately, the peak shapes

Borate buffer at pH 9 was used in various concentra- tions (10, 20, and 50 mM) to investigate the impact of buffer concentration. The data showed that low con- centration buffer was uncapable of separating the seven drugs where some drug peaks were overlapped. The migration time of the analyzed drugs are influenced dramatically by increasing buffer concentration, result- ing in longer separation periods and well-resolved and separated peaks. Finally, 50 mM concentration of borate buffer was selected to achieve better analysis with good resolution and peak shape in a reasonable migration time.

SLS concentrations of 15, 25, and 50 mM were added to the BGE along with 10% acetonitrile to examine the impact of SLS concentration on the separation. Both migration times and resolution increase with increas- ing SLS concentration. However, 15 mM SLS gave sig- nificantly tailed peaks, while 25 mM of SLS showed few better separations. 50 mM of SLS was the optimum con- centration showing better peak shape in short analysis time.

The type and concentration of organic modifiers greatly influence how the drugs under study are separated. In the

absence of organic modifier, both AML and BIS peaks were not well-separated with poor resolution. VIC peak was forked. Upon using 10% Acetonitrile, BSL and AML separation was significantly altered, showing better res- olution. In addition, VIC was eluted with sharper peak. However, results were not repeatable when $\geq$ 20% (v/v) was utilized, as the high concentration of organic modi- fier can prevent micelle formation. Micelles are generally known to be unstable in water and organic solvent mixes that contain more than 20–30% organic solvent [77]. The peak shapes of both BIS and AML were deformed when methanol was used instead of acetonitrile. More- over, ethanol was tried showing no significant change in the shape of the peaks. Also, it failed to improve the separation of the peaks of drugs with poor resolution. By increasing the percentage of ethanol, destabilization of micelles occurred. Therefore 10% (v/v) of acetonitrile was selected.

Different trials for applied voltage (20, 25 and 30 kV) utilizing the improved BGE are shown in Fig. S1. As expected, migration times increased with lowering voltage because of a drop in EOF, as seen in Fig. S1. In addition to increasing migration times, resolution was affected for VIC, BSL and AML. Therefore, a voltage of 30 kV was selected.

The pressure values of 20, 30, and 50 mbar did not sig- nificantly alter the migration time of the studied drugs. Due to its better response, 50 mbar was determined to be the ideal pressure. Peak width and height in hydro- dynamic injection are influenced by injection time. Peak height and injection time are directly correlated under ideal circumstances. Sample solutions were injected at 50 mbar for 5 to 25 s, adjusting the injection time to find the ideal duration. Peak height increased with longer injection times; however, longer injection times also result in deformed peaks and a departure from linearity. Furthermore, altering the injection time did not result in any changes to the migration times of any of the analyzed drugs. Because of the good peak symmetry and linear relationship between peak height and injection volume, 10 s was determined to be the ideal injection time.

Fig. S4, shows UV spectra of the drugs to be studied in MEKC method. The proposed method permits sepa- ration of all drugs at 220 nm in ~ 7 min as shown in the obtained MEKC electropherogram (Fig. 1a). The quan- titative determination of CPL, VAL, ATR and AML was done at 210 nm, whereas HCT, VIC and BSL was done at 220 nm. Moreover, DAD confirms peak purity as the purity angles were below the threshold values as illus- trated in Fig. S4.

Within the FDA requirements, the suggested technique was found to have acceptable system suitability parame- ters: k' > 2, N > 2000, α > 1, Rs > 2, and T $\leq$ 2 (Table S4) [3]. The peaks showed good resolution, symmetry, sharpness, and a suitable migration time.

The impact of varying the operating temperature (20, 25, and 30 °C) on the separation of the drugs under study was investigated. BGE viscosity is decreased by high tem- peratures, resulting in a shorter run time. Even while 30 °C produced the best migration times for all drugs, the data lacked high repeatability. As a result, 25 °C was cho- sen for the analysis.

HPLC method

Achieving adequate resolution and satisfactory peak symmetry within a reasonable run time is the most cru- cial factor in the development of HPLC methods. Many tests were run to optimize the stationary and mobile phases in order to accomplish this goal.

Analytical columns tried in this study were Agilent Zorbax SB-C8 Stable Bond column (4.6 × 250 mm, 5 μm), Agilent ZORBAX Extend-C18 (4.6 × 250 mm, 5 μm) and Waters Symmetry C18 (3.9 × 150 mm, 5 μm). The Agilent ZORBAX Extend-C18 (4.6 × 250 mm, 5 μm) gave the best resolution between the tested drugs in a relatively short run time. Accordingly, it was chosen as the working col- umn for this study. The column was coupled with Agi- lent ZORBAX Extend-C18 guard column (4.6 × 12.5 mm, 5 μm).

Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers

adjusted at various pH values. The intended separation was not achieved by the isocratic mobile

phase. It was discovered that the optimal mobile phase combination for the assay of this complex mixture was methanol with phosphate buffer at pH 3. Ethanol was tried with phosphate buffer in different eluting modes. Unfortunately, ethanol was not able to separate as many drugs as desired and showed undesired tailed peaks of the eluted drugs. Also, acetonitrile and methanol were tried with phosphate buffer at pH 3 in different gradi- ent elution modes, but with acetonitrile, most drug peaks overlapped and were not well separated. Addition- ally, various gradient elution programs were tested with 50 mM phosphate buffer pH 3 and methanol. The best chromatograms and shortest run times were achieved with gradient elution starting with a ratio of 70:30 by vol- ume. At 5, 7, and 8 min, methanol was increased to 60, 80, and 90%, respectively. Prior to the next injection, the methanol ratio was brought back to 30%.

Upon investigating the pKa of the studied drugs (Table 1), the effect of mobile phase pH was investigated within the range 3–6 at 1.0 pH unit interval and the best chromatogram was obtained at pH 3. The peaks of AML, VAL, and ATR were overlapped at pH 5, however, the VIC showed a forked peak at pH 6. However, because buffer pH 4 required a longer run time to achieve the same level of peak separation, pH 3 was used for this investigation.

After studying the impact of flow rate, it was discov- ered that 1 mL/min produced the best results in terms of runtime, peak asymmetry, and column pressure. Lastly, during the chromatographic run, the column tempera- ture was maintained at 25 ºC.

The separation of the studied drugs was performed at 220 nm (Fig. 1d). The quantitative determination for LSP, VAL, ATR and AML was done at 210 nm, whereas for HCT, VIC, BSL and CVL was done at 220 nm.

Within an appropriate run time, the chromatographic

conditions previously stated showed almost symmet- ric peaks and high resolution between the eight drugs. According to the FDA's requirements, system suitabil- ity criteria were determined for the analyzed drugs and found to be acceptable (Table S4): $N > 2000$, $\alpha > 1$, $Rs > 2$, $k' > 2$, and $T \leq 2$ [3]. Also, DAD confirms peak purity as the purity angles were below the threshold values as illus- trated in Fig. S5.

Validation of the proposed methods
Validation of the proposed methods was assessed as per the International Conference on Harmonization (ICH) guidelines [78].

Linearity and concentration ranges
In MEKC method, the linearity was performed in the concentration ranges of 5–50 µg/mL for HCT, ATR, BSL

and AML, 10–100 µg/mL for CPL and VIC, 20–200 µg/ mL for VAL. The linearity parameters are collected and summarized in Table 2.

In HPLC method, the linearity was verified in the range of 0.5–50 µg/mL for four drugs HCT, VIC, CVL and ATR, 1–50 µg/mL LSP, BSL and AML, 2–200 µg/mL for VAL.

Table 2, demonstrates the obtained linearity param- eters. Regression calculations for both methods showed good linearity as proved by the correlation coefficient val- ues that are not less than 0.9996. Moreover, the methods showed high F-values providing a steeper regression line and low significance F resulting in minimal scattering of experimental points around the regression line [79].

Many attempts were made to optimize both methods in order to separate the same drugs. Regretfully, CVL was co-eluted with AML and LSP was co-eluted with HCT in the MEKC method. As a result, BSL proved enough as a β-blocker, and CVL was removed. Consequently, AML was retained as a typical case for calcium channel block- ers. Furthermore, as both CPL and LSP are non-prod- rugs and members of the

same class (ACEIs), they were substituted for one another keeping HCT as the most commonly used diuretic found in most combined phar- maceutical formulations with other cardiovascular drugs. Nevertheless, CPL and VIC peaks were overlapped dur- ing HPLC optimization, despite numerous attempts to separate the drugs. Ultimately, in the HPLC method it was better to switch out CPL with LSP, a similar drug that was successfully maintained isolated from VIC and the other drugs undergoing research.

Detection and quantitation limits

In MEKC method, the LOD and LOQ were in the range of 1.28–4.74 and 3.88–14.36 µg/mL, respectively as illustrated in Table 2. Meanwhile, in HPLC method, the LOD and LOQ were in the range of 0.11–0.57 and 0.34–
1.72 µg/mL, respectively as illustrated in Table 2.

proposed methods showed good percentage recover- ies indicating good specificity of the method (Tables S1 & S2). Furthermore, the application of the proposed methods for the determination of the tested drugs in their single and combined dosage forms, without the interference of the excipients, demonstrated the speci- ficity of the method (Table 3 and S3).

The method specificity was demonstrated by simi- lar electropherograms (MEKC method) and chroma- tograms (HPLC method) and essentially unchanged migration/retention durations of the drugs under study obtained from the standard solutions when compared to that of dosage form solutions (Figs. 1a, d, 2, 3, S2 & S3).

In addition, the peak purity of all drugs was checked by using a G1315 C/D and G1365 C/D photo diode array detector (DAD) (Fig. S4 & S5). The purity angle in every sample was found to be under the purity threshold limit, indicating that no extra peaks were co-eluting with any of the analytes and demonstrating the proposed methods' capability to evaluate the target analyte even in the pres- ence of possible interferences.

Robustness

Deliberate adjustments were made to the methods' parameters, the robustness was assessed by comput- ing the SD and RSD of both peak area ratios and migra- tion or retention times. Triplicate injections were used for analysis, and each time, the parameters under study underwent a single modification. The proposed methods were robust since the examined modifications had no sig- nificant impact on the studied drugs' peak area ratios or migration periods, as indicated by RSD% values that were less than or equal to ± 2, as indicated in Table S5.

Stability of solutions

The stability of the nine medicines in their working solutions was examined throughout the analysis duration. The normal working solutions were prepared and left at room temperature for two, four, and six hours. We tested the answers using the suggested methods at different intervals. Analysis revealed that the medications were unchanged and stable under these settings. There were no notable changes seen in the peak area values and migration or retention periods of the medicines that were studied when the %RSD values were below 2%. The stability of stock solutions was also determined to be at least two weeks when kept at 4 °C. The discrepancy was found to be less than 2% when the suggested procedures were applied to the computed concentrations of both newly prepared solutions and those that had been held for two weeks.

Assay of tablets dosage forms

The test of the nine medications that were investigated was conducted using the suggested procedures. The pharmaceuticals were accessible in Egypt in single, binary, and ternary dose forms. A final concentration within the required linearity range was achieved by following the procedures outlined in Section "Analy- sis of pharmaceutical preparations" during sample preparation and subsequently diluting aliquots prior to injection. The migration or retention durations for each medication were precisely measured and

recorded. There were no interference peaks seen in the dosage form matrix or any of the inactive components (Figs. 2, 3, S2 & S3). Tables S3 and 3 provide the computed and illustrated recoveries and %RSD, which represent the acceptable values.

We compared the medications using stated reference techniques in both their single- and combined-dosage forms. Tables S3 and 3 show that the assay's accuracy and precision were adequate, as shown by the values of % recovery, SD, and % RSD. We used Student's t-tests for accuracy and variance ratio F-tests for precision to compare the recovery data from the suggested approaches to the published methods. At the 95% confidence level, the computed values in both tests were less than the theoretical ones (Table S3 & 3).

Bioanalytical validation of the proposed methods
The suggested techniques underwent validation in terms of linearity, LLOQ, accuracy, precision, recovery, stability, and selectivity in accordance with the FDA Bioanalytical Method Validation [80]. This validation was done so that the methods could be used for the analysis of different biological fluids.

Calibration curve
In order to generate calibration curves for the concentration ranges indicated in Table 4, 100 µL of rat plasma was spiked with different portions of HCT, CPL, LSP, VAL, ATR, VIC, BSL, AML, and CVL. The internal standard (IS) approach was used to generate the calibration curves. These curves were plotted against the respective concentrations of the researched medications and the ratio of their peak areas to the IS peak areas. All other medications were found to have ATR concentrations of 100 and 20 µg/mL as their IS, while ATR in MEKC and HPLC had concentrations of 100 and 20 µg/mL of HCT as their IS, respectively (Table 4).

Limit of detection (LOD) and lower limit of quantitation (LLOQ)
Five replicates were used to analyze the LOD and LLOQ, and the analyte's finding was compared to the blank response. (Table 4). LOD and LLOQ were calcu- lated using signal-to-noise (S/N) ratio of 3:1 and 5:1, respectively, to that of the blank response. LODs as low

Accuracy and precision
The accuracy and precision of the proposed methods were validated. For intra-day and inter-day assay vali- dation, six replicates of each of the four quality con- trol points, LLOQ, LOQ, MQC, and HQC, within the calibration range of each drug were determined on the same day and three separate days later.

A newly developed calibration curve was used every day to determine the concentration of the drugs under study. The precision was measured in terms of percent- age coefficient of variation (%RSD), while the accuracy was measured in terms of mean percentage recoveries and percentage error of the mean (%Er) (Tables S6 & S7).

Stability
A recovery of 85–115% of the original concentrations of the studied drugs in plasma confirms their stability. Achieving satisfactory recovery and RSD values for all the stability trials (Tables S8 & S9) of the drugs under study in rat plasma indicates their stability under various settings.

Application of different co-administered dosage forms of the studied drugs with vincamine in spiked plasma samples
The suggested methods were effectively used to deter- mine the administered cardiovascular drugs in rat plasma in combination with vincamine. In this work,
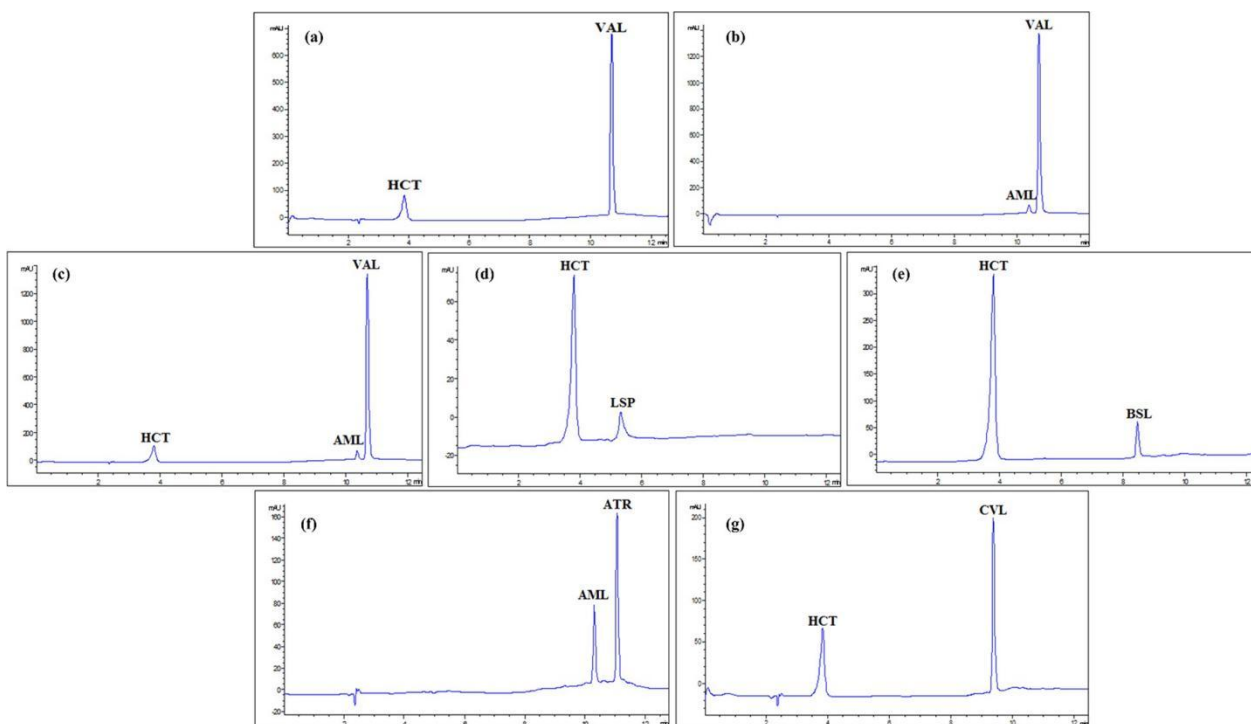
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Fig. 3 Chromatograms of HCT, LSP, VIC, BSL, CVL, AML, VAL and ATR prepared from their combined dosage forms; **a** Co-tareg®, **b** Exforge®, **c** Exforge HCT®, **d** Zestoretic®, **e** Concor plus®, **f** Caduet® and Co-dilatrol®, respectively, measured at 220 nm

the analysis of different possible combinations of co- administered dosage forms indicated for treating various CVDs together with VIC was performed and demonstrated good recovery rates with relative stand- ard deviations within acceptable limits, ensuring pre- cise and accurate quantification (Table 5, Fig. 4 and 5). The analyzed samples were prepared using the drugs' concentrations within the ratio of the co-administered dosage forms. The prepared ratios of the co- adminis- tered drugs in their dosage forms were, combination 1; 1, 16, 1.25, 1, 3 for AML, VAL, HCT, ATR, VIC, com-

The analysis was completed in a reasonable time frame, 7 and 11.5 min, for MEKC and HPLC, respec- tively, showcasing the efficiency of the techniques for simultaneous drug determination in biological matri- ces (Fig. 1c and f).

Greenness assessment of the proposed methods

The greenness of analytical methods is increasingly assessed using tools such as EcoScale, the Green Ana- lytical Procedure Index (GAPI), and the Analytical GREEnness (AGREE) metric. EcoScale evaluates the environmental impact of analytical methods based on factors like reagent toxicity and energy consumption, assigning a score to reflect sustainability criteria such as sample preparation, reagents, and instrumentation [81]. Unfortunately, ecoscale does not provide infor- mation regarding the risks' structures or the reasons for the analytical procedure's environmental impact, like the usage of solvents or other reagents, occupa- tional hazards, or waste generation. AGREE synthe- sizes multiple green chemistry principles into a single, user-friendly score [82]. It is characterized by automa- tion and highlighting the weak points that need fur- ther optimization in the suggested analytical method. GAPI provides a comprehensive, visual representation

Table 4 Characteristic parameters for the regression equations of the proposed MEKC and HPLC methods for the determination of HCT, CPL, LSP, VAL, ATR, VIC, BSL, AML and CVL in rat plasma

Parameters		HCT	CPL	LSP	VAL	ATR	VIC	BSL
Detection Wavelength (nm)	MEKC	220	210	210	210	210	220	220
	HPLC							
Linearity range (µg/mL)	MEKC	50–1000	100–1000		50–2000	50–1000	10–1000	50–1000
	HPLC	1–100	—	1–100	2–200	5–100	2–100	5–100
LLOQ (µg/mL)	MEKC	50	100	—	50	50	50	50
	HPLC	1	—	1	2	5	2	5
LOD (µg/mL)	MEKC	25	50	—	20	30	5	30
	HPLC	0.40	—	0.57	1.46	2.75	1.29	1.23
Intercept	MEKC	-2.03×10^{-1}	-1.05×10^{-1}	—	1.50×10^{-1}	4.35×10^{-2}	5.76×10^{-2}	-4.76×10^{-2}
	HPLC	1.83×10^{-2}	—	8.31×10^{-3}	2.81×10^{-2}	-1.35×10^{-2}	4.50×10^{-2}	7.06×10^{-4}
Slope	MEKC	1.55×10^{-2}	7.35×10^{-3}	—	1.10×10^{-2}	7.35×10^{-3}	2.05×10^{-2}	9.79×10^{-3}
	HPLC	1.89×10^{-1}	—	4.63×10^{-2}	4.79×10^{-2}	1.04×10^{-1}	1.10×10^{-1}	3.97×10^{-2}
Correlation coefficient	MEKC	0.9996	0.9997	—	0.9999	0.9998	0.9998	0.9999
	HPLC	0.9999	—	0.9999	0.9999	0.9995	0.9998	0.9999
^aS_a	MEKC	9.40×10^{-2}	4.49×10^{-2}	–	4.53×10^{-2}	2.31×10^{-2}	6.82×10^{-2}	2.93×10^{-2}
	HPLC	2.51×10^{-2}	–	8.78×10^{-3}	2.33×10^{-2}	9.56×10^{-2}	4.74×10^{-2}	1.63×10^{-2}
	MEKC	2.06×10^{-4}	9.13×10^{-5}	–	5.49×10^{-5}	5.07×10^{-5}	1.60×10^{-4}	6.43×10^{-5}
	HPLC	5.82×10^{-4}	–	2.03×10^{-4}	2.68×10^{-4}	1.87×10^{-3}	1.02×10^{-3}	3.20×10^{-4}
$^cS_{y/x}$	MEKC	1.64×10^{-1}	6.66×10^{-2}	–	9.57×10^{-2}	4.03×10^{-2}	1.37×10^{-1}	5.11×10^{-2}
	HPLC	5.20×10^{-2}	–	1.82×10^{-2}	4.76×10^{-2}	1.47×10^{-1}	8.62×10^{-2}	2.51×10^{-2}

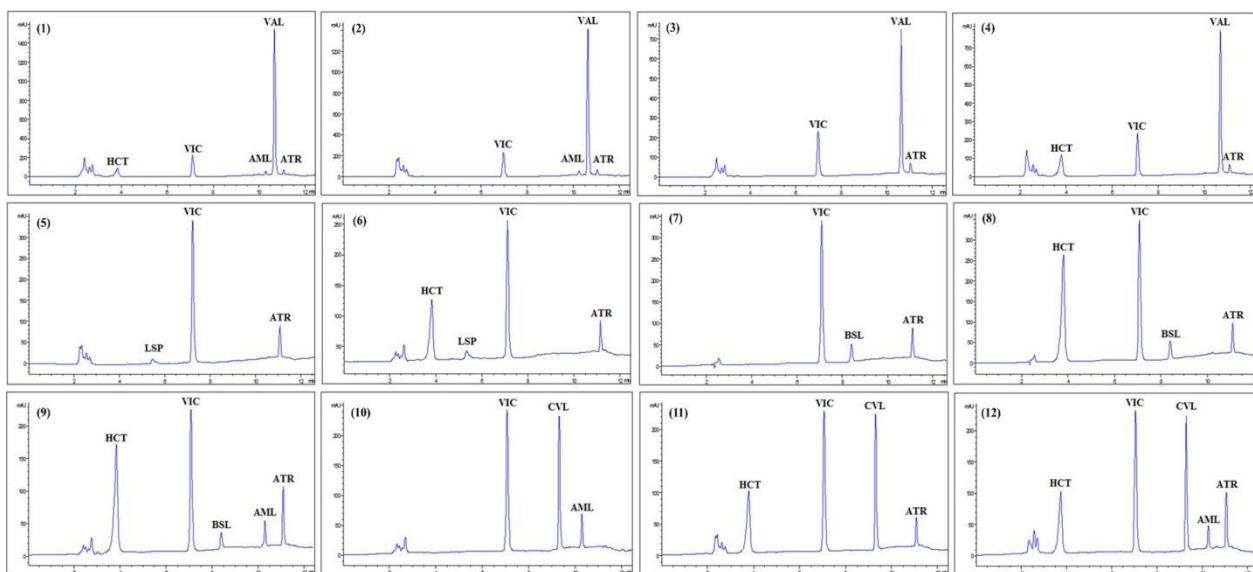**Fig. 5** Chromatograms of HCT, LSP, VIC, BSL, CVL, AML, VAL and ATR in their possible co-administered combined dosage forms in rat plasma; **1** Exforge HCT®/Ator 10®/Brain-OX®, **2** Exforge®/Ator 10®/Brain-OX®, **3** Tareg 80®/Ator 10®/Brain-OX®, **4** Co-tareg ®/Ator 10®/Brain-OX®, **5** Zestril 10®/Ator 10®/Brain-OX®, **6** Zestoretic®/Ator 10®/Brain-OX®, **7** Concor 10®/Ator 10®/Brain-OX®, **8** Concor plus®/Ator 10®/Brain-OX®, **9** Caduet®/ Concor plus®/Brain-OX®, **10** Norvasc 10®/Dilatrol 25®/Brain-OX®, **11** Co-dilatrol ®/Ator 10®/Brain-OX® and **12** Co-dilatrol ®/Caduet®/Brain-OX® measured at 220 nm

of method greenness, considering the assessment and comparison of eco-friendly analytical procedures [83]. It can be used as a semi-quantitative tool for laboratory practice. Both AGREE and GAPI are represented as colored pictograms that are easily interpreted.

In Table S10, the proposed methods were compared to eight different reported methods [9, 31, 76, 84–88] to compare their greenness. The proposed MEKC method showed excellent green analysis by having an analytical Eco-scale of 89 (above 75) and the highest AGREE score (0.89). GAPI pictogram for both proposed methods were comparable to the reference methods, where proposed MEKC surpassed all the methods in the waste amount. This indicates the superior greenness of MEKC method.

Blueness assessment of the proposed methods
The proposed analytical methods, designed for the sep- aration of various cardiovascular drugs alongside vin- camine, emphasizes environmental sustainability by incorporating the Blue Applicability Grade Index (BAGI) [89]. This index evaluates the method's water and energy usage, toxicity, and waste production, ensuring a minimal environmental footprint. By achieving a high Blue Index rating, the method not only proves to be efficient and reliable for drug analysis but also aligns with eco-friendly practices. This approach underscores the importance of green chemistry principles in modern analytical tech- niques. In Fig. S6, the proposed methods showed dark

blue color showing good compliance of the analytical methods to the criteria. Both proposed methods scored 80 revealing excellent performance of the method. In comparison with the reported reference methods, our proposed methods showed comparable score in BAGI to two reported reference HPLC methods [9, 31]. This score outperformed other reference methods [84–88], whereas another HPLC reported method [76] showed the least score of 75 as illustrated in Fig. S6.

Whiteness assessment of the proposed methods
Analytical (red) and practical (blue) factors

are two more important factors that White Analytical Chemistry (WAC) considers when evaluating the method's quality. The coherence and synergy of the analytical, ecological, and practical elements are demonstrated by a white ana- lytical approach in connection to the RGB color model, which implies that the combination of red, green, and blue light beams produces the appearance of whiteness.

The proposed methods along with other reported methods [9, 31, 76, 84–88] were evaluated for their whiteness using the multicriteria RGB 12 model [90]. The resulted data was illustrated in Fig. S7. The proposed MEKC and HPLC methods showed superior perfor- mance over the reference methods.

The greenness, blueness, and whiteness approaches demonstrated the superiority of both the MEKC and HPLC procedures. Both techniques separated the drugs

in a comparatively short amount of time during analysis by using less hazardous reagents in modest amounts. The suggested methods received good score in these evalua- tions because of their capacity to separate multicompo- nent samples with various chemical classes. Additionally, the suggested approaches outperformed previously pub- lished reference methods in every area of WAC due to their reduced power consumption and straightforward sample preparation.

To summarize the evaluation of greenness, blueness, and whiteness, our suggested MEKC method is a superior white and green method compared to other documented chromatographic and spectrophotometric methods. In addition, this suggested method has unique advantages in terms of high separation power with quick analysis times (7 min), small amounts of toxic reagents with minimum energy and waste consumption that have little to no det- rimental effects on the environment or public health. On the other hand, the multianalyte analysis of eight medi- cations using our suggested HPLC was completed in less than 12 min and received good marks for greenness, blueness, and whiteness.

The proposed MEKC method showed high capabil- ity of simultaneous analysis of seven

drugs in relatively short time (7 min) with high resolution (Fig. 1). It is char- acterized by high green, blue and white profile with high throughput analysis per hour using minimal amount of solvents and waste as well (Table S10, Fig. S6 & S7). On the other side, proposed HPLC method showed higher sensitivity as per lower detection and quantitation limits (Tables 2 and 4). Moreover, eight drugs were simultane- ously determined using the HPLC method in less than 12 min (Fig. 1) with high selectivity and reproducibility. Additionally, the HPLC method showed high score in WAC approaches (Table S10, Fig. S6 &S7).

Our proposed methods, MEKC and HPLC, showed their efficacy for simultaneous analysis of various car- diovascular drugs with the nutraceutical, vincamine, in single and combined pharmaceutical formulations in rea- sonable time frame with good WAC profile. In addition to their applicability in biological matrices; plasma. This highlights the methods' suitability for possible routine use in pharmaceutical analysis.

Conclusion
Ultimately, the suggested procedure incorporates the deterrence of many cardiovascular medicines, each of which represents a distinct class of pharmaceuticals, in addition to the nutritional vincamine. Thanks to this all-encompassing technology, many drug classes may be analyzed at the same time. Regular use of the suggested analytical methodologies to the study of pharmaceutical formulations, whether for single or multiple medication treatment in combination or co-administration, has shown promising results. Reliable and precise quantification of various medicines within a short analysis time is ensured by these methods, which are cost-effective and efficient. Their environmentally conscious approach is in line with the principles of green chemistry, which makes these approaches a significant tool for improving the efficiency and sustainability of contemporary pharmaceutical analysis.

The possible health advantages of nutraceuticals are making them an increasingly significant class of medications that are often given in conjunction with other drugs. Because of this, analytical techniques to investigate these combinations are required. To understand if nutraceuticals and pharmaceuticals have synergistic or antagonistic effects, it is essential to find ways to evaluate them both at the same time.

Optimizing treatment effects and ensuring safety in combination use should be the focus of future research on these interactions. Since no prior approaches have been able to effectively separate vin-camine when other cardiovascular medications are present, the suggested methods meet a substantial need.

To ensure safe and successful therapy regimens, this study opens the door to further investigations into their possible interactions and combined usage in clinical settings.

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