

# Validation of HPLC for identification of captopril, enalapril, and lisinopril in traditional Indonesian herbal medicine

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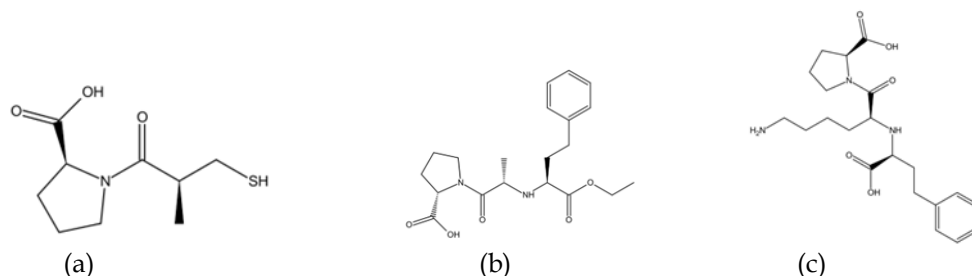
**ABSTRACT:** Traditional Indonesian herbal medicine (Jamu) is widely used for health maintenance and disease prevention. The addition of medicinal chemicals to Jamu is not in accordance with quality and safety requirements. The purpose of this study was to develop a valid HPLC method for the identification of captopril, lisinopril, and enalapril in Jamu for hypertension. In this study, they were identified using high-performance liquid chromatography with a photodiode array detector. The stationary phase was a C18 YMC column (length 250 mm, diameter 4.6 mm, particle size 5 µm), methanol - 0.1 % phosphoric acid (45:55) as the mobile phase with isocratic elution, a flow rate of 1.0 mL/min, a column temperature of 40 °C, an injection volume of 20 µL, and detection at 210 nm. The results showed that this method fulfilled the specificity requirement, with detection limits of captopril, enalapril, and lisinopril of 0.715, 0.795, and 1.403 ppm, respectively. This HPLC method can be used for the identification of captopril, enalapril, and lisinopril in commercial Jamu.

**KEYWORDS:** HPL; hypertension; identification; Jamu; medicinal chemistry.

## INTRODUCTION

The World Health Organization (WHO) estimates that approximately 1.28 billion adults aged 30-79 years worldwide suffer from hypertension. Most of them live in low- and middle-income countries. One of the global goals for non-communicable diseases is to reduce the prevalence of hypertension by 33% between 2010 and 2030 [1]. According to the 2023 Indonesian Health Survey, the prevalence of hypertension in Indonesia was 33.2% [2].

Captopril (C<sub>9</sub>H<sub>15</sub>NO<sub>3</sub>S), enalapril (C<sub>20</sub>H<sub>28</sub>N<sub>2</sub>O<sub>5</sub>) and lisinopril (C<sub>21</sub>H<sub>31</sub>N<sub>3</sub>O<sub>5</sub>) that their structure are shown in Figure 1 are included in the class of antihypertensive drugs, namely the Angiotensin-Converting Enzyme Inhibitor (ACE-Inhibitor). ACE inhibitors are the most commonly indicated medications for the treatment of cardiovascular diseases, including heart failure, acute coronary syndrome, and hypertension [3]. This class of drugs works by inhibiting the conversion of angiotensin I to angiotensin II, which has side effects such as cough, kidney failure, angioneurotic edema, hypotension, and hyperkalemia with long-term use [4]. These drugs also play a vital role in protecting the heart, brain, and kidneys in hypertensive patients with comorbidities, such as diabetes [5]. Captopril, in addition to amlodipine, is the drug of choice to overcome hypertension [6]. Both of these have the same effectiveness [7].



**Figure 1.** Figure 1. The structure of captopril (a)[9]; enalapril (b)[10]; lisinopril (c)[11]

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All of these structures contain chromophore groups. Captopril contains two carbonyl (C=O) groups, whereas enalapril and lisinopril contain three carbonyl groups and an aromatic ring. Therefore, they can absorb ultraviolet radiation.

Jamu has also been used for hypertension treatment. Jamu is a traditional Indonesian herbal medicine made from natural plant-based ingredients and prepared according to ancestral practices. It must meet the criteria of being safe in accordance with the requirements, claims of efficacy can be proven based on empirical data, and meet the quality requirements [8]. According to the National Agency for Drug and Food Control (BPOM), there are four groups of natural medicines: Jamu, standardized herbal medicines, phytopharmaceuticals, and other natural medicines. Jamu is formulated using plant materials and is traditionally served in the form of brewed powders, pills, powders, or liquids. It has been used for decades or even hundreds of years. It must meet the following criteria: safe in accordance with the requirements, claims of efficacy can be proven based on empirical data, and meet applicable quality requirements [8].

To provide a rapid effect of medicinal plants or herbs, there is a potential for the addition of medicinal chemicals in Jamu for high blood pressure treatment [12]. Medicinal chemicals are chemical substances used as the main ingredients of chemical medicines, which are usually added to traditional medicines to strengthen their indications. According to Decree No. 007 of the Minister of Health of the Republic of Indonesia, the traditional medicine industry is prohibited from producing traditional medicines that contain medicinal chemicals from isolation or synthesis, narcotics and psychotropics, and other materials that are in accordance with health considerations and based on research can cause harm to health [13].

Currently, the National Agency for Drug and Food Control (BPOM) still finds several Jamu containing medicinal chemicals. According to the 2023 Guidelines for Sampling and Testing of Traditional Medicines, Quasi-Medicines, Health Supplements, and Cosmetics, the identification of captopril and enalapril is a parameter for testing traditional herbal medicine samples with hypertension claims [14]. The BPOM used the densitometry analysis to identify captopril and enalapril. In addition to captopril and enalapril, lisinopril is a similar drug used to treat hypertension. Because TLC densitometry has limitations in terms of sensitivity, it is necessary to look for more sensitive methods. In the analysis of ACE inhibitors, mobile phases such as methanol-water (50:50) with phosphoric acid at pH 3.1 [15], captopril in tablet dosage form using 550 mL of methanol and 450 mL of water containing 0.50 mL of phosphoric acid [16], methanol-water-phosphate buffer pH 3.0 (60:40) [17]; acetic buffer-methanol-acetonitrile (60:20:20) [18] and methanol-acetonitrile-water (40:50:10) have been used [19]. The structures of captopril, enalapril, and lisinopril show that they have some chromophore groups and can absorb ultraviolet radiation, so PDA can detect them simultaneously. There is no HPLC method for the simultaneous identification of captopril, enalapril, and lisinopril using a photodiode array (PDA) detector. Photodiode array (PDA) detectors have advantages over other detectors in that they can provide a collection of chromatograms simultaneously at different wavelengths in a single run, check peak purity, and provide a lot of sample composition information compared to UV-Vis detectors [20]. The data elements required for the validation of identification are specificity and detection limit [21], [22]. This study aimed to determine the optimum HPLC system, evaluate its specificity and sensitivity, and apply it for the identification of captopril, enalapril, and lisinopril in commercial Jamu products.

## ▪ MATERIALS AND METHODS

### Materials

Captopril Indonesian Pharmacopeia reference standard (IPRS), enalapril maleate IPRS, and lisinopril IPRS were obtained from the Indonesian Food & Drug Authority. Methanol for HPLC and 85% phosphoric acid (Merck, Darmstadt, Germany) were purchased from PT Triandar Jastekta (Jakarta, Indonesia), and demineralized water (Milli-Q) was obtained as a gift from the Indonesian Food & Drug Authority Regional Office, Jakarta. A commercial for hypertension was purchased from an herbal medicine store in Jakarta.

## Instrumentation and equipment

Analysis was performed using HPLC Shimadzu LC 20AD with UV spectrophotometer and PDA as detector (Shimadzu, Kyoto, Japan), column of YMC C18 (250 mm × 4.6 mm with particle size of 5 µm (YMC-Triart, Japan) with equipment of ultrasonicator (Branson M8800-E, New York, USA), centrifuge (Rotina 380R, Germany), membrane filter 0.45µm (Millipore, Merck, Darmstadt, Germany).

## Preparation of solution

### Reference standard solution

A 25-mL volumetric flask was used to add 0.5 mL each of 20 µg/mL captopril, 20 µg/mL enalapril maleate, and 20 µg/mL lisinopril IPRS, diluted with mobile phase to volume, shaken until homogeneous, and filtered with a 0.45 µm membrane filter [21].

### Spike sample

An accurately weighed quantity of approximately 100 mg of Jamu powder was placed into a 15 mL centrifuge tube, and approximately 10 mg of captopril IPRS, enalapril maleate IPRS, lisinopril IPRS, and 10.0 mL of the mobile phase were added. The mixture was sonicated for 15 min and centrifuged at 3500 rpm for 10 min. Approximately 0.5 mL of supernatant was taken and placed into a 25 mL measuring flask and diluted with solvent, and the solution was filtered with a 0.45 µm membrane filter.

### Test solution sample

Homogenized Jamu (approximately 100 mg) was accurately weighed and placed in a 15 mL centrifuge tube. The mobile phase (10.0 mL) was added, and the mixture was sonicated for 15 min and centrifuged at 3500 rpm for 10 min. A large amount of 0.5 mL supernatant was placed into a 25-mL volumetric flask and diluted with the mobile phase, and the solution was filtered using a 0.45 µm membrane filter.

## Optimization of HPLC system

Optimization was conducted using of methanol-0.1 % phosphoric acid (55:45) isocratic elution [16, 23]; methanol-0.1% phosphoric acid (50:50) [24] isocratic elution, methanol- 0.1% phosphoric acid (45:55) and methanol-0.1% phosphoric acid gradient elution. Each condition was performed at 25 °C and 40 °C temperatures.

## System suitability test

A mixture of 20 µg/mL captopril, enalapril maleate, and lisinopril IPRS solution was filtered with a 0.45 µm membrane injected into an optimum HPLC system with six times replication [22].

## Specificity and sensitivity test

The mixed reference standard solution, test sample, and sample spike solution were injected sequentially into the optimum HPLC system. The chromatograms were observed and analyzed for specificity [22].

The detection limit, as a sensitivity parameter, was calculated from the linear regression curve. Accurately weighed quantity 100 mg of the homogenized Jamu sample was accurately weighed, placed into a 15 mL centrifuge tube, and then approximately 10 mg of captopril, 10 mg of enalapril maleate, and 10 mg of lisinopril, 10.0 mL of solvent, sonicated for 15 min, and centrifuged at 3500 rpm for 10 min. The supernatant (50, 75, 100, 200, and 300 µL) was added to five 25 mL volumetric flasks and diluted to volume. The solution was filtered through a 0.45 µm membrane filter and injected into the optimum HPLC system.

Validation for qualitative analysis refers to the checklist in the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH Harmonized Guideline) Validation of Analytical Procedures (Q2(R2)). The data element required for qualitative method validation is specificity [22]. Detection limit tests were performed to determine the sensitivity of the method.

## Identification of captopril, enalapril, and lisinopril in commercial Jamu

An accurately weighed quantity of 100 mg of homogenized hypertension Jamu was placed into a 15 mL centrifuge tube, 10.0 mL of solvent was added, sonicated for 15 min, and centrifuged at 3500 rpm for 10 min.

Approximately 0.5 mL of the filtrate was placed into a 25 mL flask and diluted until the mark, the solution was filtered with a 0.45  $\mu\text{m}$  membrane filter, placed into a vial, and analyzed under optimum conditions.

### Data analysis

Selectivity was evaluated when there were no chromatographic peaks in the placebo and solvent test solutions with the same retention time as the spiked chromatogram of the sample and reference standard. The chromatogram peaks of the spiked solution of the sample provided the same retention time and spectrum as the captopril, enalapril, and lisinopril reference standard solutions. The reference standard peak was significantly separated from the other compounds if the resolution ( $R_s$ ) was  $\geq 1.5$  [23].

Sensitivity was expressed as the detection limit.

$$\text{Detection limit (DL)} = \frac{3.3}{S} \quad [22]$$

$\sigma$  = The standard deviation of the response,

S = the slope of the calibration curve

## RESULTS

### Optimization of HPLC system

Based on the optimization results, the composition of the methanol 0.1% phosphoric acid (45:55) was selected as the mobile phase and further optimized using a column at temperatures of 25 °C (Figure 1) and 40 °C (Figure 2). The results of the calculation of the resolution and tailing factor are shown in Table 1 for a temperature of 25 °C and in Table 2 for a temperature of 40 °C.

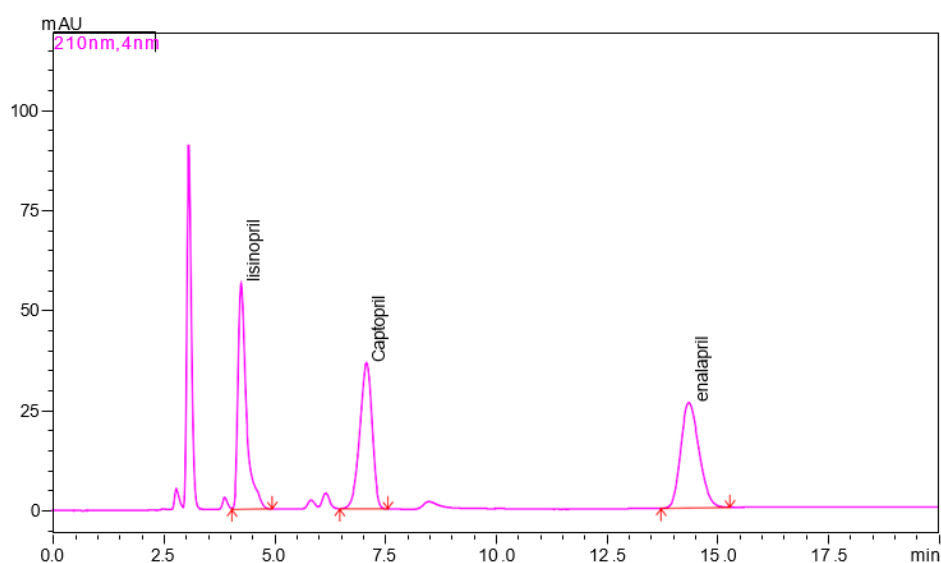
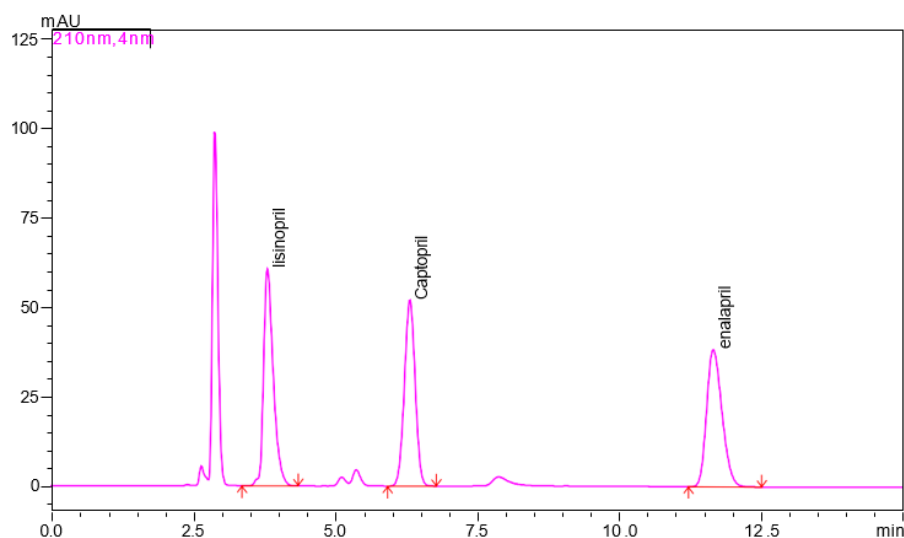


Figure 1. Chromatogram of mixed reference standard solution at room temperature (25 °C)



**Figure 2.** Chromatogram of mixed reference standard solution at temperature 40 °C.

**Table 1.** Results of resolution and tailing factor analyses at temperature of 25 °C.

Analyte	Retention Time (minutes)	Area	Resolution	Tailing factor
Lisinopril	4.265	736219	0.000	1.975
Captopril	7.098	713501	6.738	0.886
Enalapril	14.374	754542	11.265	1.206

**Table 2.** Results of resolution and tailing factor analyses at 40 °C.

Analyte	Retention Time (minutes)	Area	Resolution	Tailing factor
Lisinopril	3.811	721985	0.000	1.461
Captopril	6.322	689899	7.416	0.996
Enalapril	11.671	725303	12.271	1.242

### System suitability test

The system suitability test results for the analysis of captopril, enalapril, and lisinopril are shown in Table 3.

**Table 3.** System suitability test results.

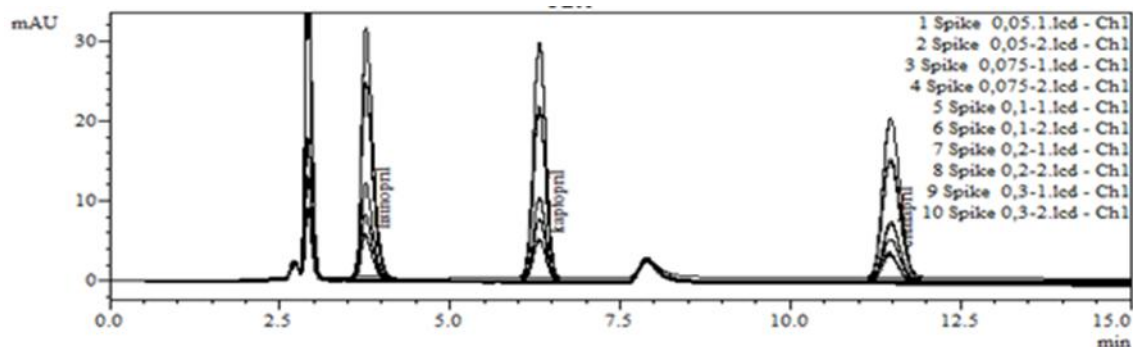
	Lisinopril	Captopril	Enalapril
Rt (minute)	3.724	6.314	11.228
Area $\pm$ SD	727645 $\pm$ 3054	689266 $\pm$ 1289	725175 $\pm$ 752
RSD (%)	0.419	0.187	0.103

### Specificity

The results of the specificity test showed that lisinopril, captopril, and enalapril were perfectly separated. Interpretation of the results of the specificity test, namely the absence of chromatogram peaks in the solvent and matrix of the test sample that had the same retention time as the reference standard and spiked samples analyzed.

## Sensitivity

The linearity chromatogram is shown in Figure 3.

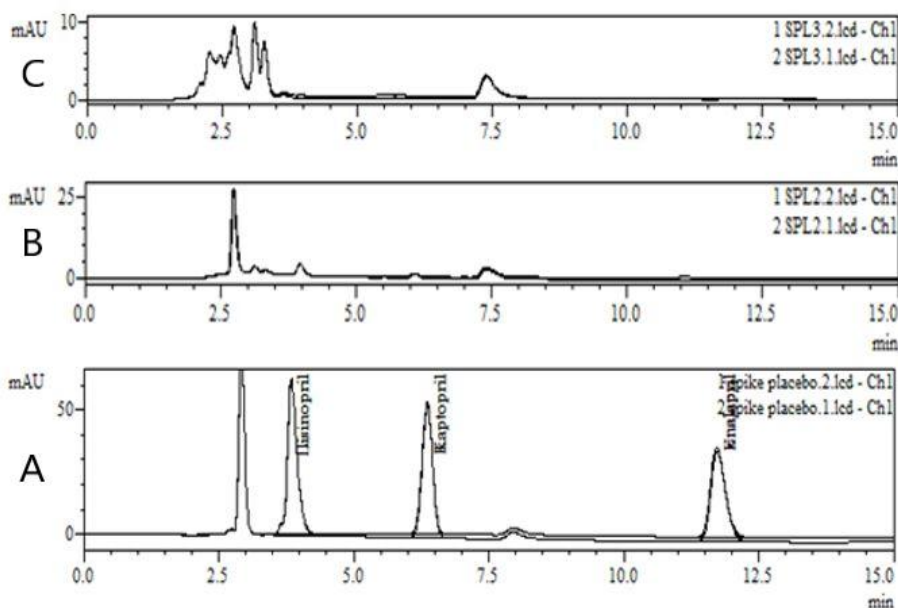


**Figure 3.** Chromatograms of various concentration of captopril, lisinopril and enalapril in the sample matrix.

The relationship between sample concentration and area showed that the equations of the calibration curves of lisinopril, captopril, and enalapril, were  $y = 32082x + 111174$  with a correlation coefficient ( $r$ ) = 0.995;  $y = 34645x + 5497.4$  with a value of 0.998; and  $y = 31670x + 6535.5$  with a value of = 0.998 respectively. In the detection limit test of the HPLC method for the identification, the limit values for captopril, enalapril, and lisinopril were 0.715; 0.795 and 1.403 ppm, respectively.

## Identification results of captopril, enalapril, and lisinopril in commercial Jamu

The identification results for captopril, enalapril, and lisinopril are presented in Figure 4.



**Figure 4.** Chromatogram identification results in commercial Jamu which were supplemented with captopril, enalapril and lisinopril (spike sample) (A), commercial Jamu (B), and (C).

## DISCUSSION

The optimization of the HPLC system in this study was carried out by setting the composition of the mobile phase to isocratic or gradient. There are parameters to evaluate the quality of the HPLC system, namely the quality of separation of adjacent peaks (resolution) and analysis time. The chromatographic system had a good separation if it had a resolution value of  $\geq 1.5$  [23], so that a good separation of chromatogram peaks between substances was produced in a mixture of substances. The larger the number of theoretical plates ( $N$ ), the more efficient the column, resulting in a chromatogram with a sharp peak shape.

In Condition 1, with a methanol-0.1% phosphoric acid mobile phase of (55:45), lisinopril, captopril, and enalapril can be elucidated. The solvent peak was adjacent to the lisinopril peak. Condition 2 with methanol-0.1% phosphoric acid mobile phase (50:50) isocratically results that all analytes can be elucidated well, with a resolution of  $\geq 1.5$ , but the enalapril peak at minute 7 was very close to the solvent peak, which could cause poor separation quality. Condition 3 with a methanol-phosphoric acid mobile phase composition of 0.1% (45:55) was conducted based on the modification of the composition of conditions 1 and 2, and the results of the separation of lisinopril, captopril, and enalapril were obtained with a resolution of  $\geq 1.5$  [23]. Condition 4 was carried out using methanol-0.1% phosphoric acid gradient elution by adjusting the composition of the mobile phase at a certain time.

The gradient system used aimed to achieve good separation between the three analytes. The gradient system is a system in which the composition of the mobile phase can be adjusted or changed in such a way that the composition during the elution process is maintained from the beginning to the end of the elution. This aims to obtain the equilibrium point of the mobile phase so that there is an equilibrium of the mobile phase in the stationary phase, which will affect the analyte retention system in the test sample matrix, especially for multi-component analytes, so that there is no accumulation of retention time between analytes [25]. In this system, the lisinopril chromatogram that eluted in the first minute was adjacent to the solvent chromatogram in front of it; therefore, this gradient method could not be used for subsequent test conditions.

Based on the tailing factor and retention time from Tables 1 and 2, condition 3 with a column temperature of 40 °C was selected as the optimum system and used for the next step. The flow rate used was 1.0 mL/min, because if the flow rate was more than 1.0 mL/min, it would affect the pressure on the pump to be greater. Meanwhile, if the flow rate was less than 1.0 mL/min, the retention time would be longer and the peak shape would be wider, which can affect the tailing factors. The composition of the mobile phase affects the retention time of captopril, enalapril, and lisinopril, where the type of HPLC used was reverse-phase HPLC with a nonpolar C18 stationary phase, while the mobile phase was polar. The methanol-0.1% phosphoric acid (45:55) was the best mobile phase composition for separation using a C18 column. The optimization results showed that a column temperature of 40 °C was better than 25 °C for the identification of captopril, enalapril and lisinopril. This produced a chromatogram that separated the three active substances, with a sharp and symmetrical peak shape. Using a column temperature of 25 °C, the three analytes were separated well, but the shape of the lisinopril chromatogram was asymmetrical, and the enalapril chromatogram shape was widened. By using a column temperature of 40 °C, the analyte time was expanded, and the theoretical plate count was greater than that at 25 °C. Temperature is one of the factors that can affect the migration speed of a component band passing through a column [25]. A temperature of 40 °C is the optimum temperature for influencing the distribution of molecules between the stationary and mobile phases.

The general purpose of the HPLC optimization carried out in this study was to determine the effectiveness of separation. A system suitability test was performed to ensure the effectiveness of the operational system, both in the tools, methods, and conditions of the HPLC, so that it was able to provide good results [25].

Results of the system suitability test for lisinopril, captopril, and enalapril provided relative standard deviation (RSD) values of 0.419%, 0.187%, and 0.103%, respectively. The results of the system suitability test for each reference standard showed an RSD value of  $\leq 2\%$ . This value meets the requirements of the Indonesian Pharmacopoeia, edition VI [25]. In addition, the peak resolution of lisinopril, captopril, and enalapril met the requirement of  $\geq 1.5$ . From the above description, it can be concluded that the operational conditions in this analysis provide good results and can be used for the next step.

Specificity is the ability of an analytical method to measure the intended analyte precisely and specifically in the presence of other components in the sample matrix, such as impurities, degradation products, and matrix components. Specificity is expressed as the resolution value. A good separation has a resolution value of  $\geq 1.5$ . Many compounds in Jamu can absorb ultraviolet radiation. In this development method, the interference of similar compounds was tested through standard addition to the sample matrix (placebo). The specificity test was carried out by comparing the solution containing the sample matrix and the solvent with the comparative standards of lisinopril, captopril, and enalapril in the optimum HPLC system. The retention times in the sample and solvent were not the same as those of the reference standard. A confirmation test was

then performed using a PDA detector. In the PDA detector, the spectrum produced was compared with the reference standard spectra and the sample solution.

The results were qualified if the chromatogram peaks of each compound were well separated with a resolution value of  $\geq 1.5$  [23]. In addition, the peak chromatogram of the sample should not show the same retention time response as the standard retention time of captopril, enalapril, lisinopril, and spiked samples. This specificity test was performed to determine the ability of the analysis method to distinguish the analyte from other components in the sample matrix. This shows that the method used meets the requirements, namely, the ability to distinguish analytes precisely and specifically in the presence of other components in the sample matrix.

The detection limit was defined as the lowest concentration of analytes in the sample that could still be detected. In this study, the detection limit was determined based on the calibration curve of the spiked sample at the lowest concentration [26]. The chromatogram peaks of the detected captopril, enalapril, and lisinopril areas were calculated using the standard deviation, and the detection limit was obtained by comparing the slope value of the linearity curve.

Linearity is the ability of a method to obtain test results that are directly proportional to the concentration of analytes in the sample. Linearity connected the response ratio ( $y$ ) with concentration ( $x$ ), and the slope, intercept, and correlation coefficient ( $r$ ) were determined. The linear relationship was indicated by the correlation coefficient parameter ( $r$ ) for the regression analysis  $y = a + bx$ . Linearity data were acceptable when  $r \geq 0.990$  [26]. The results showed a linear relationship because they provided comparable or directly proportional results between the concentrations of the three analytes in the sample and the peak area obtained, and met the criteria for accepting the value of  $r \geq 0.990$  [26].

The detection limits for captopril and enalapril were much lower than those obtained by TLC densitometry, which were 52.11 ppm and 12.31 ppm (internal data), respectively. Therefore, the HPLC method was more sensitive than TLC densitometry in detecting captopril and enalapril in Jamu for hypertension. However, the limit of detection of reverse-phase HPLC to determine the level of captopril in tablets was 1.75 ng/mL [17] and enalapril of 0.04 ppm [19]. In the tablet dosage form, HPLC methods were more sensitive than in Jamu. Jamu was rich in natural compounds so the matrix in Jamu for hypertension is more complex than tablet dosage form.

This HPLC system was used to identify captopril, enalapril, and lisinopril in hypertension Jamu. This method was tested on three commercial Jamu samples, namely sample A, a simulated sample made using the spiked method, and samples B and C, routine samples of hypertension Jamu. The samples tested were Jamu, which was suspected to contain chemicals such as captopril, enalapril, and lisinopril. The results of the medicinal chemical identification test on the samples were compared. Captopril, lisinopril, and enalapril were not detected in samples B and C. The identification results met the safety and quality requirements of the regulation of the Head of the Indonesian Food and Drug Authority No. 32 of 2019, which states that traditional medicine, including Jamu, must not contain medicinal chemicals [27].

## CONCLUSION

The HPLC method with a C18 stationary phase (250 mm  $\times$  4.6 mm, particle size 5  $\mu$ m), PDA detector at an optimum wavelength of 210 nm, isocratic elution with a mobile phase of methanol-0.1% phosphoric acid (45:55) at a flow rate of 1.0 mL/min and a column temperature of 40  $^{\circ}$ C had resolution values that met the requirements with detection limits for captopril, enalapril, and lisinopril of 0.715 ppm, 0.795 ppm, and 1.403 ppm, respectively. This method can be applied to identify the three analytes in commercial Jamu.

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**Conflict of interest statement:** The authors declare no conflicts of interest.

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