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Research Article

Analytical Method Validation of Sildenafil Citrate and Caffeine in Herbal Medicine for Increasing Stamina using Thin Layer Chromatography – Densitometry

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Abstract: Traditional medicine has been trusted for generations as an effective remedy for various health conditions and is widely recognized for its safety when compared to synthetic pharmaceuticals. One of the main reasons individuals choose herbal medicines is their relative affordability and the perception that they are free from harmful chemical additives. However, recent public warnings issued between 2020 and 2025 have revealed the presence of hazardous medicinal chemicals (Bahan Kimia Obat/BKO) such as sildenafil citrate and caffeine in some stamina-enhancing herbal products. This study aims to develop and validate an analytical method using Thin Layer Chromatography (TLC) coupled with densitometry for the detection of sildenafil citrate and caffeine in herbal medicines. The method was chosen due to its simplicity, low operational cost, and ability to effectively separate these two compounds. A category II validation was performed to determine the presence of impurities, with a focus on parameters including selectivity and the Limit of Detection (LOD). The mobile phase employed was a combination of chloroform and ethanol (9.5:0.5 v/v), and detection was carried out at 254 nm using a densitometer. The TLC results showed that sildenafil citrate and caffeine had Rf values of 0.32 and 0.52, respectively, with a resolution (Rs) of 2, indicating a good separation. The LOD for sildenafil citrate was 8.5418 µl/ml (0.0854 mg per 600 mg sample), while the LOD for caffeine was 4.7987 µl/ml (0.0959 mg per 600 mg sample). These findings highlight the importance of routine quality control testing to ensure the safety and authenticity of herbal medicines in the market.

Keywords: Adulteration; Caffeine; Herbal drug; Sildenafil citrate; TLC

1. Introduction

Traditional medicine has long been trusted by the Indonesian people as an alternative to modern medicine, and it has been proven to be safe and beneficial for certain health purposes. Jamu, which is a form of traditional medicine, is made from ingredients or concoctions derived from plants, animals, or minerals. These preparations, based on societal norms, have been used for generations to treat various ailments (KRITERIA DAN TATA LAKSANA REGISTRASI OBAT BAHAN ALAM, 2023). Jamu comes in various forms, including powdered infusions, pills, and liquid extracts from whole or part of the plant ingredients. One common example of jamu is the formulation designed to enhance male stamina, often referred to as "jamu kuat pria" (male strong medicine). This type of herbal remedy is used to address erectile dysfunction.

Many people prefer herbal medicine because it is generally cheaper and has fewer side effects compared to modern pharmaceuticals (Fuad Alkindi et al., 2021). Currently, the use of herbal medicine is on the rise, prompting many industries to produce these remedies. This

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increase in production has led to heightened competition among producers, which sometimes triggers unethical practices, including the addition of chemical drugs (BKO) to herbal products. Some irresponsible manufacturers exploit the public's lack of awareness regarding the dangers of using chemical drugs in herbal medicine. The inclusion of these substances aims to achieve quicker and more noticeable effects (Asra et al., 2018).

According to the Regulation of the Minister of Health of the Republic of Indonesia Number 007 of 2012, traditional medicines are prohibited from containing isolated or synthetic chemical drugs (KRITERIA DAN TATA LAKSANA REGISTRASI OBAT BAHAN ALAM, 2023). Despite these regulations, many herbal products still contain these substances. For instance, sildenafil citrate and caffeine are commonly found in stamina-enhancing herbal medicines. A press release from the Food and Drug Supervisory Agency highlights ongoing concerns about chemical drugs in herbal medicine. Public warning attachment No. HM.01.1.2.07.20.18 dated July 1, 2020, indicated that 40 herbal medicines still contained chemical drugs, including 13 stamina-enhancing herbal medicines with sildenafil citrate and one containing caffeine. Another warning dated October 4, 2022, revealed that 41 herbal medicines had chemical drugs, with 16 of those being stamina enhancers containing sildenafil citrate. A third warning from October 13, 2021, listed 53 herbal medicines with chemical drugs, including 12 stamina enhancers containing sildenafil citrate and one containing caffeine. Some of these products lack the necessary distribution permits from Indonesian Food and Drug Agency (BPOM) (BPOM, 2021).

2. Literature Review

Sildenafil citrate is marketed under brand names such as Viagra and Revatio. It is commonly used to treat male erectile dysfunction and can also be prescribed for pulmonary arterial hypertension (Sumiati et al., 2017). Regulation classified sildenafil citrate and its derivatives to be used under medical supervision. Inappropriate use or lack of supervision can lead to serious side effects, such as headaches, flushing, vision problems (e.g., achromatopsia, cyanopsia), nasal congestion, digestive issues (e.g., dyspepsia), respiratory problems, strokes, heart attacks, and even death (Husna & Mita, 2020).

Caffeine, an isolate commonly derived from coffee (Coffea sp), tea (Camelia sinensis), and cocoa beans (Cacao sp), acts as a stimulant for the central nervous system (CNS), heart, and respiratory system. Its effects also include smooth muscle relaxation and increased diuresis. However, excessive caffeine intake can lead to negative side effects like increased heart rate, elevated blood pressure, and higher blood flow to muscles. In metabolism, caffeine boosts glucose production in the liver, thereby enhancing fat mobilization and glycogen utilization. Overuse of caffeine can result in symptoms such as restlessness, headaches, tremors, rapid breathing, and insomnia. Chronic excessive consumption can even lead to

addiction (Willson, 2018). Furthermore, excessive intake can lead to negative effects such as abnormal heart rhythms, anxiety, restlessness, impaired memory, insomnia, and digestive issues (Özpalas et al., 2017). Therefore, it is strongly advised to consume caffeine within recommended limits. The maximum recommended intake is 150 mg per day or 50 mg per serving.

The addition of chemical drugs to herbal medicine is prohibited by BPOM because it poses risks to public safety. To prevent adverse effects from the combination of sildenafil citrate and caffeine in herbal medicine products, a reliable method for identifying these chemical drugs is essential. This study utilize Thin Layer Chromatography (TLC) - Densitometry for analysis. TLC offers numerous advantages as a separation technique. It is versatile, applicable to nearly all compounds, cost-effective, and allows for quick separation using high-quality adsorbents and pure solvents, making it highly effective for separating unknown mixtures (Rosamah, 2019).

Thin Layer Chromatography is a simple, cost-effective, and easy-to-use planar chromatography technique that has been employed in general chemistry laboratories for decades to routinely separate chemical and biochemical compounds. Traditionally, chemical and optical methods are used to visualize the spots on a TLC plate. TLC involves a glass, metal, or rigid plastic piece coated with a thin layer of silica gel or alumina, where silica gel (or alumina) serves as the stationary phase. Often, the stationary phase includes a substance that fluoresces under UV light. The mobile phase consists of an appropriate liquid solvent or solvent mixture (Rosamah, 2019).

A densitometer is used to scan the developed TLC plate to determine the area related to the sample concentration, allowing for visualization of the spot spectrum. Given its separation principles, TLC is well-suited for this study, which aims to separate the herbal matrix containing sildenafil citrate and caffeine. The separation principle in TLC is similar to that of general chromatography, involving differences in affinity between the stationary phase, mobile phase, and analyte (Rosamah, 2019). The Densitometry method offers several advantages, including high specificity, reliable results, ease of use, and flexible mobile phase selection, making it feasible to optimize separation using various techniques (Savitri & Megantara, 2019).

Analytical method validation is essential to ensure that the procedure meets its intended purpose (Harmonised Tripartite Guideline. Q2 (R2): Validation of Analytical Procedure, 2024). The test parameters include specificity/selectivity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). In this research, sildenafile citrate and caffeine are categorized as impurities, and hereby the method validation focused on specificity and limit of detection (LOD) (Savitri & Megantara, 2019; USP, 2021).

3. Method

This study was designed as a laboratory experiment. The materials used in the experiment included pure forms of sildenafil citrate and caffeine as standards, along with a matrix component consisting of Myristica fragrans, Panax ginseng Radix, Zingiberis Rhizoma, Eurycoma longifolia Radix, Curcumae Rhizoma, Kaempferiae Rhizoma, Pyllanthi Herba, and Piper retrofracti Fructus. The mobile phase components comprised chloroform, methanol, and ethanol. Additionally, ten stamina-enhancing herbal medicines available on the market were purchased to be analyzed using the proposed method.

The tools used in this study included analytical scales, parchment paper, stirring rods, measuring cups, beakers, micropipettes, and silica gel plates F254. A chamber was utilized to elute the plates, and UV light at 254 nm was employed to observe the separation of stains from the two compounds. A TLC scanner / densitometer was used to analyze the area and spectral data of each solution.

3.1. Mobile Phase Selection

To achieve optimal separation, the mobile phase was selected from three different compositions. The following mobile phase solutions were prepared:

a. Chloroform: Ethanol (9.5:0.5 v/v)
b. Chloroform: Ethanol (9:1 v/v)
c. Chloroform: Methanol (1:4 v/v)

Twenty milliliters of each solution was stirred until homogeneous and then saturated in a chamber. The spotted plate was eluted in an optimized mobile phase. The plate was spotted with a 5-microliter solution of sildenafil citrate and caffeine working standard, along with the matrix solution. Sildenafil citrate and caffeine working solution was prepared with concentration 400 ppm in ethanol. A matrix solution was prepared by extracting 300 mg of the matrix in 5 mL of ethanol. The filtrate was collected for further use. The same preparation method was used for sample preparation.

The separation was analyzed based on the resulting retention factor and resolution. Mobile phase resulting Rf value parameters between 0.2–0.8 and the Rs value ≥ 1.5 will be selected.

3.2. Limit of Detection

Once the mobile phase and wavelength were selected, a detection limit test (LOD) was conducted. Solutions with concentrations of 30, 40, 50, 60, and 70 ppm were prepared. Each solution was spotted on a silica gel 254 plate and eluted in the chosen mobile phase. Observations were made using a TLC scanner to assess the area of each spot. Regression analysis was performed between concentration and area, followed by calculations: LOD = 3σ/S (Appendix F: Guideline for Standard Method Performace Requirements, in: AOAC Official Method of Analysis, 2016).

3.3. Sample Analysis

The study continued by observing samples of stamina-enhancing herbal medicine. Each sample (300 mg) was weighed, dissolved in 5 ml of ethanol, filtered, and the filtrate was taken. Each sample solution was then spotted on a silica gel 254 plate and eluted in the selected mobile phase. Further observations were made with a TLC scanner, and the spectrum of each sample suspected of being positive was compared with that of the comparative raw material.

4. Results and Discussion

The selection of the mobile phase was conducted by eluting the spots of sildenafil citrate, caffeine, and the matrix solution using a stationary phase in the form of a silica gel 60 F 254 plate. The mobile phase that offers optimal selectivity, evaluated through the Rf and Rs parameters, will be utilized in subsequent experiments.

Based on the experimental results, the most effective mobile phase for separating sildenafil citrate, caffeine, and the matrix in herbal medicine samples is a mixture of chloroform and ethanol in a 9.5:0.5 v/v ratio. This is evidenced by the Rf values for the sildenafil citrate and caffeine spots, which fall within the range of 0.2 to 0.8 (with Rf values of 0.32 for sildenafil citrate and 0.52 for caffeine). Additionally, there is good separation between the spots, as indicated by an Rs value of 2. Furthermore, with the chloroform:ethanol (9.5:0.5 v/v) mobile phase, the matrix spot is adequately separated from the two analytes without interfering with the analysis.

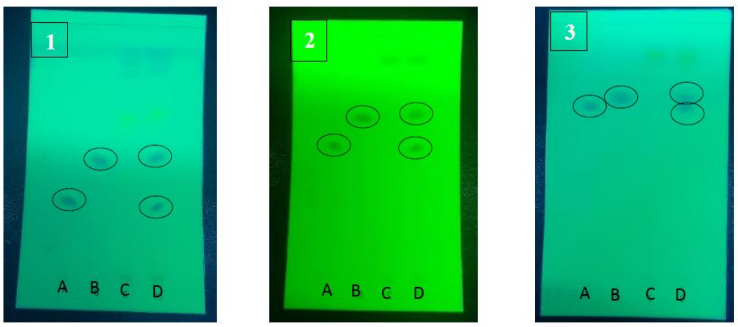

Figure 1. Eluation from three different mobile phase

a. Chloroform: Ethanol (9.5:0.5 v/v)
b. Chloroform: Ethanol (9:1 v/v)
c. Chloroform: Methanol (1:4 v/v)

In the second mobile phase, a mixture of chloroform and ethanol in a 9:1 ratio was used. This phase successfully separated the two analytes as well as the matrix, similar to the first mobile phase. The Rf values obtained were 0.61 for sildenafil citrate and 0.74 for caffeine. The resolution between the two analytes was measured at 1.7, which is considered good, although slightly lower than that of the first mobile phase.

In the third mobile phase, a combination of chloroform and methanol in a 1:4 ratio was tested. However, this phase did not achieve good separation, with a resolution (Rs) value of 0.4, which is below the optimal requirement of 1.5. The results of these experiments can be found in Figure 1 and Table 1, which detail the calculations.

Table 1. Selectivity of the Mobile Phase

No.	Mobile Phase	Rf		Rs
		Sildenafil Citrate	Caffeine	
1.	Chloroform: Ethanol (9,5:0,5 v/v)	0,32	0,52	2
2.	Chloroform: Ethanol (9:1 v/v)	0,61	0,74	1,73
3.	Chloroform: Methanol (1:4 v/v)	0,76	0,8	0,4

The detection limit refers to the smallest amount of analyte that can be reliably detected using a specific analysis method. In this study, the detection limit was determined using the regression curve method. This approach involves constructing a linear curve that illustrates the relationship between concentration and the area readings obtained with a densitometer (Harmonised Tripartite Guideline. Q14: Analytical Procedur Development., 2024). Standard solutions with concentrations of 30, 40, 50, 60, and 70 ppm were prepared, and 5 microliters of each solution was spotted and analyzed at a wavelength of 254 nm using the densitometer. The linear relationship between analyte concentrations and the device's response is illustrated in Figure 2.

The linear relationship between the concentration and response of sildenafil citrate and caffeine is indicated by their correlation coefficient values, which exceed the critical r table value of 0.878. Specifically, the correlation coefficient for sildenafil citrate is 0.988, while for caffeine, it is 0.996. To calculate the limit of detection (LOD), we determined the residual standard deviation and used it in the equation where LOD is defined as 3 times the residual standard deviation divided by the slope. The calculated LOD values for sildenafil citrate and caffeine are presented in Tables 2 and 3.

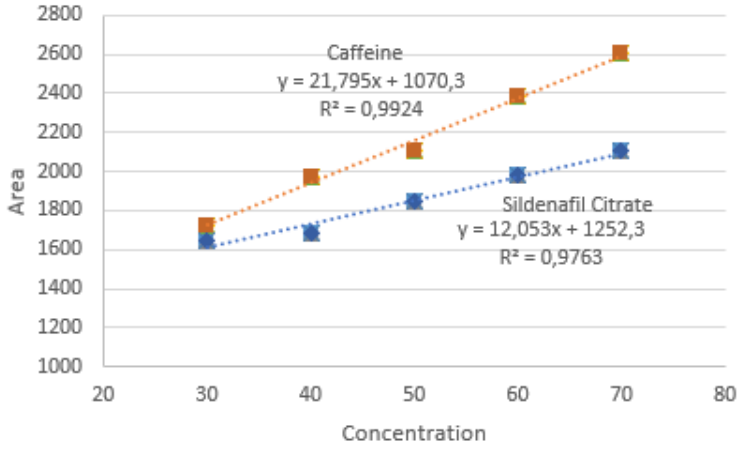

Figure 2. Linear relationship between the concentration and response of analyte

Table 2. Limit of Detection (Sildenafil Citrate)

	Concentration (ppm) (x)	Area (y)	Yi	y-yi	$(y - yi)^2$	LOD
C1	30	1649,4	1613,92	35,48	1258,83	8,5418 ppm (0,0854 mg In 600 mg preparation)
C2	40	1689,1	1734,45	-45,35	2056,62	
C3	50	1847,2	1854,98	-7,78	60,5284	
C4	60	1985,2	1975,51	9,69	93,8961	
C5	70	2104	2096,04	7,96	63,3616	
				Σ	3533,24	

Table 3. Limit of Detection (Caffeine)

	Concentration (ppm) (x)	Area (y)	Yi	y-yi	$(y - yi)^2$	LOD
C1	30	1725,5	1724,18	1,32	1,7424	4,7987ppm (0,0479 mg In 600 mg preparation)
C2	40	1971,9	1942,13	29,77	886,2529	
C3	50	2109,6	2160,08	-50,48	2548,2304	
C4	60	2384,4	2378,03	6,37	40,5769	
C5	70	2609	2595,98	13,02	169,5204	
				Σ	3646,323	

Testing of herbal medicine samples available in the market yielded the results displayed in Table 4. The presence of chemical drug impurities can be identified not only by the RF value but also by comparing it to the standard analyte spectrum. The test results indicated that two out of ten samples tested positive for Sildenafil, both showing RF values and UV spectra similar to standard Sildenafil. This evidence suggests that preparations containing chemical drugs are still readily found, highlighting the need for increased supervision and decisive action from authorities to regulate their distribution.

Table 4. Result of Sample Analysis

Sampel	Rf		UV Spectra	
	Sildenafil Sitrat	Kafein	Sildenafil Sitrat	Kafein
A	+	-	+	-
B	+	-	+	-
C	-	-	-	-
D	-	-	-	-
E	-	-	-	-
F	-	-	-	-
G	-	-	-	-
H	-	-	-	-
I	+	+	-	-
J	-	-	-	-

5. Conclusions

Traditional drug adulteration can negatively impact health (Ekar & Kreft, 2019; Foroughi et al., 2017; Izzo & Ernst, 2009; Xu et al., 2019). As of now, there has been no research investigating the simultaneous identification of sildenafil citrate and caffeine in stamina-enhancing herbal medicines. This study aims to develop a reliable method for identifying both sildenafil citrate and caffeine in stamina-enhancing herbal products available on the market. The goal is to create a simple and reliable method that can be applied in daily analyses. The findings regarding adulteration are expected to inform both consumers and authorities.

Analytical methods using thin-layer chromatography (TLC) densitometry can detect the presence of banned substances such as chemical drug in traditional medicine preparations with simple processing and at relatively low costs compared to other chromatography methods, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Haneef et al., 2013; Pyka, 2014). In the context of BKO analysis in traditional medicinal preparations, TLC has the advantage of allowing multiple samples to be tested simultaneously during one elution. Given that the purpose of the method is product screening, the number of samples to be analyzed is typically quite large. Furthermore, the solvent used for spotting samples will be evaporated before elution. This allows for the volume of the sample to be adjusted as needed, thereby increasing the amount of analyte and enabling the detection of small concentrations.

The identification of impurities in finished products falls under Category 2 of analytical method validation, which includes quantity and limit tests (Harmonised Tripartite Guideline. Q2 (R2): Validation of Analytical Procedure, 2024). This study focuses on the limit test section of Category 2, and therefore, the validation parameters will include selectivity and the limit of detection (LOD) test. This study demonstrated that sildenafil citrate and caffeine can be detected simultaneously using TLC with silica plates, employing a chloroform:methanol (9.5:0.5) eluting solvent, along with observations using UV light and densitometry. This method allows for the detection of these two ingredients at concentrations of less than 10

μg/mL in the test solution, making it sufficiently sensitive to identify their presence in traditional medicine preparations.

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