

ANALYTICAL METHOD OPTIMIZATION AND VALIDATION OF GLIBENCLAMIDE AND METFORMIN HYDROCHLORIDE IN DIABETIC HERBS PRODUCT BY THIN-LAYER CHROMATOGRAPHY-DENSITOMETRY

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Received: 02 October 2019, Revised and Accepted: 24 December 2019

ABSTRACT

Objective: This study aimed to develop and validate an analytical method using thin-layer chromatography (TLC)-densitometry for detecting the presence of glibenclamide and metformin hydrochloride in herbal diabetic products.

Methods: The *jamu* samples were extracted using methanol, followed by analysis using TLC-densitometry with methanol-aquades-glacial acetic acid (9:1:0.25) as the mobile phase at a wavelength of 237 nm.

Results: From the validation results, the correlation coefficients for glibenclamide and metformin hydrochloride were determined to be 0.9998 and 0.9981, respectively. The limits of detection and quantitation for glibenclamide were 49.97 µg/mL and 66.55 µg/mL, respectively, while those of metformin hydrochloride were 74.75 µg/mL and 249.25 µg/mL, respectively. The method developed in this study met the criteria of selectivity, accuracy, and precision. From seven *jamu* samples tested, four tested positive for glibenclamide, with sample 1=4.9522%, sample 2=4.1495%, sample 3=4.2578%, and sample 4=4.9412%.

Conclusion: None of the *jamu* samples contained metformin hydrochloride.

Keywords: Glibenclamide, Metformin hydrochloride, Optimization, Thin-layer chromatography densitometry, Validation.

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INTRODUCTION

Traditional medicine has been widely used by Indonesian people since ancient times. It can be used in the treatment of diseases, as well as part of an overall strategy for disease prevention and health optimization. Traditional medicines are substances or formulas of substances in the form of herbal mixes, animal compounds, minerals, essence preparations (galenic), or a mixture thereof, and have been used from generation to generation for treatments based on experience [1]. The use of traditional medicine is generally preferred over that of synthetic drugs, also known as modern medicine. The side effects of traditional medicines are considered fewer than in those of modern medicine such that traditional medicine is said to be safer than modern medicine [2]. However, in Indonesia, many traditional medicines have been found to contain medicinal chemicals that are harmful to human health.

In November 2015, the BPOM announced 50 traditional medicines and food supplements that were considered harmful because they contained medicinal chemicals. The medicinal chemicals that have been found in traditional medicines and food supplements include paracetamol, caffeine, tadalafil, and sildenafil citrate [1]. However, according to the Republic of Indonesia Indonesian Minister of Health Regulation No. 007 (2012) concerning the Registration of Traditional Medicines, traditional medicines are prohibited to contain medicinal chemicals, which are the result of isolation or synthetic preparation and have medicinal properties [3].

One example of a traditional medicine commonly consumed by Indonesian people is *jamu*. Several studies have reported the discovery of medicinal chemicals in herbal medicines, including the addition of corticosteroid hormones in *jamu* for aching pain [4]. In addition, using thin-layer chromatography (TLC)-densitometry, mefenamic acid, paracetamol, and ibuprofen have been found to be present in *jamu* formulations used for gout [5]. Accordingly, many herbal formulations

that have not been studied remain, including *jamu* for diabetes. While a medicinal chemical analysis was performed on *jamu* for diabetes by Jayanti *et al.* in 2015, the method was only performed qualitatively [6].

Therefore, the development of an analytical method for determining the presence of chemical drugs in *jamu* for diabetes by TLC-densitometry, both qualitatively and quantitatively, is needed. In addition, it is important to determine the limits of detection (LOD) of chemical drugs in *jamu* samples. It is expected that the LOD of analytes in *jamu* samples may give better results than those found in the previous studies. This study was also conducted to determine if there are dangerous levels of medicinal chemicals present in *jamu* samples. The compounds we analyzed for were glibenclamide and metformin hydrochloride, which are common drugs used in the treatment of diabetes. TLC-densitometry was chosen as the best method because it uses simple equipment, the mobile phase is relatively inexpensive, and it can quickly analyze for compounds in *jamu*.

METHODS

The materials used were the drugs glibenclamide (PT. Indofarma Tbk, Indonesia) and metformin hydrochloride (PT. Dexa Medica Tbk, Indonesia); chemicals in the form of aquades (Merck, Germany), methanol p.a (Merck), toluene p.a (Merck), glacial acetic acid p.a (Merck), ammonia 25% p.a (Merck), and ethyl acetate p.a (Merck). The samples analyzed were seven samples of *jamu* for diabetes, consisting of three samples of branded *jamu* (samples MX, CR, and NE) and four samples of concoction *jamu* (samples BI, SU, SP, and LN), which were obtained from the stores in Tangerang, Indonesia.

The spots obtained after TLC were analyzed using densitometer (Camag TLC Scanner 3), a computer connected to a scanner and equipped with "Wincats" applications, chamber chromatography, sonicators, TLC Silica gel 60 F₂₅₄ plates, UV Cabinet, volume pipettes, capillary pipes, Whatman® grade 41 filter paper, and glassware.

Preparation of standard solution

To prepare the glibenclamide standard solution, a total of 10 mg of glibenclamide was weighed and placed into a 10 mL volumetric flask; methanol was then added to the volume limit to obtain a standard solution concentration of 1 mg/mL (1000 µg/mL). Similarly, to prepare the metformin hydrochloride standard solution, a total of 10 mg metformin hydrochloride was weighed and placed into a 10 mL volumetric flask; methanol was then added to the volume limit to obtain a standard solution concentration of 1 mg/mL (1000 µg/mL).

Preparation of sample solution

A single dose sample of each herbal formulation was weighed and placed into a mortar and crushed until homogeneous. A total of 350 mg (or equal to 1/20 of the *jamu* weight) was weighed carefully and placed into a 10 mL volumetric flask; methanol was then added to represent half of the solvent. The mixture was then shaken until homogeneous, sonicated until dissolved, and methanol added to the volume limit.

Preparation of sample solutions that adduct with drug compounds

A single dose sample for each herbal formulation was weighed and placed into a mortar and crushed until homogeneous. A total of 350 mg (or equal to 1/20 of the *jamu* weight) was weighed carefully and placed into a 10 mL volumetric flask; methanol was then added to represent half of the solvent. The mixture was then shaken until homogeneous and sonicated until dissolved. Two milliliters each of the standard solutions of glibenclamide (1000 µg/mL) and metformin hydrochloride (1000 µg/mL) were then added to sufficient volume.

Mobile phase selection

This experiment was conducted using 2 µL glibenclamide and metformin hydrochloride standard solutions on the TLC plate, followed by elution with the mobile phase that had been used in the previous studies with an elution limit of up to 9 cm. A mobile phase that could separate the four compounds well and had a retention factor (Rf) range of 0.2–0.8 (Table 1) was chosen.

Optimum wavelength optimization

From the solution made for mobile phase optimization, repeated analysis using densitometry at the wavelength of each drug compound based on the literature was performed [7,8]. The optimal wavelength that could analyze each compound by providing the largest area (Table 2) was selected.

Extraction system optimization

A total of 350 mg (or equal to 1/20 of the *jamu kosong* weight) was weighed carefully and placed into a 10 mL volumetric flask; approximately 2.0 mL of glibenclamide solution was added to sufficient volume. The same procedure was performed with the metformin hydrochloride solution. To separate the insoluble particles, filtration and precipitation were performed for 5 min. Then, the filtrate, supernatant, and standard solution were dropped on one TLC plate and

eluted with the selected mobile phase. A separation method that would provide a larger area of the analysis results and closest to the standard area was chosen.

Method validation

Specificity and selectivity

Specificity and selectivity tests were performed by comparing the results of the analyses of the *jamu* solutions that did not contain medicinal chemicals, the *jamu* solution that drug compounds were added, and standard solutions. The method was considered selective, if there was no spotting for the *jamu* solution that did not contain the medicinal chemicals at the Rf for each standard solution and the adducted *jamu* solution.

Linearity and calibration curves

A total of 250 mg standard glibenclamide was weighed and placed into a 25 mL volumetric flask; a sufficient volume of methanol was then added to mark the boundary so that a standard solution concentration of 10,000 µg/mL was obtained. Aliquots of the solution were pipetted sequentially at 0.5, 1, 1.5, 2, 2.5, and 3 mL into a 10 mL volumetric flask; a sufficient volume of methanol was then added to mark the boundary so that concentrations of 500 µg/mL, 1000 µg/mL, 1500 µg/mL, 2000 µg/mL, 2500 µg/mL, and 3000 µg/mL were obtained.

A total of 100 mg standard metformin hydrochloride was weighed and placed into a 50 mL volumetric flask; a sufficient volume of methanol was then added to mark the boundary so that a standard solution concentration of 2000 µg/mL was obtained. Aliquots of the solution were pipetted sequentially at 3, 4, 5, 6, 7, and 8 mL into a 10 mL volumetric flask; a sufficient volume of methanol was then added to mark the boundary so that concentrations of 600 µg/mL, 800 µg/mL, 1000 µg/mL, 1200 µg/mL, 1400 µg/mL, and 1600 µg/mL were obtained.

Each solution was then dropped as much as 2 µL onto the TLC plate and analyzed.

LOD and limit of quantitation (LOQ)

From the calibration curve data, the LOD and LOQ were calculated. LOD and LOQ values were calculated by measuring the replicates of the blank sample and calculating the mean result and the standard deviation.

Accuracy and precision

Accuracy and precision tests were performed by diluting the standard solution of glibenclamide (10,000 µg/mL) and metformin hydrochloride (2000 µg/mL) to obtain solutions of three different concentrations.

Accuracy and precision tests for the standard solution of glibenclamide were performed at concentrations of 3500 µg/mL, 2000 µg/mL, and 500 µg/mL. For the standard solution of metformin hydrochloride, accuracy and precision tests were performed at concentrations of 1600 µg/mL, 1200 µg/mL, and 600 µg/mL. Each solution was put into a total of 350 mg (or equal to 1/20 the dosage) of the blank matrix that did not contain any medicinal ingredients and then dropped onto the TLC plate as much as six drops. The mixture was then eluted with the selected mobile phase. We calculated the percent recovery by entering the area data obtained from the TLC-densitometry analysis. Standard deviation values and the coefficient of variation were calculated from data obtained from the accuracy analysis.

Qualitative analysis of medicinal compounds in *jamu*

A total of 350 mg of the *jamu* sample were weighed and placed into a 10 mL volumetric flask and then dissolved in 5 mL of methanol with sufficient volume to mark the boundary. Two microliters of this solution were then dropped onto the TLC plate using capillary pipettes. The standard solution and the adducted *jamu* solution were also dropped as much as 2 µL onto the same TLC plate and eluted with the selected mobile phase, followed by TLC-densitometry analysis. From the positive results of the qualitative analysis of the *jamu* solution containing the chemical compounds, quantitative analysis was performed.

Table 1: Mobile phase selection

No.	Mobile phase
1.	Ethyl acetate-methanol-ammonia 25% (80:15:5)
2.	Methanol-ammonia 25% (100:1.5)
3.	Toluene-ethyl acetate-methanol (7.5:0.5:2)
4.	Aquades-methanol-glacial acetic acid (6:4:0.25)
5.	Aquades-methanol-glacial acetic acid (7:3:0.25)
6.	Aquades-methanol-glacial acetic acid (8:2:0.25)
7.	Aquades-methanol-glacial acetic acid (9:1:0.25)

Table 2: Wavelengths of each substance based on the literature

Medicinal compound	Wavelength
Glibenclamide	257 nm, 300 nm
Metformin hydrochloride	237 nm, 256 nm

Quantitative analysis of medicinal compounds in *jamu*

From the samples that had a positive result in qualitative testing, quantitative tests were performed by weighing 350 mg of the *jamu* sample that was detected to contain added medicinal chemicals qualitatively 2 times; this was then placed into a 10 mL volumetric flask with a sufficient volume of methanol to mark the boundary.

RESULTS AND DISCUSSION

Mobile phase selection

To determine the optimal conditions for analysis, the selection of the best mobile phase for the analysis of glibenclamide and metformin hydrochloride was performed. From the experimental results, it was found that the mobile phase used to optimally separate glibenclamide and metformin hydrochloride was methanol-aquades-glacial acetic acid (9:1:0.25) with an Rf of 0.82 for glibenclamide and an Rf of 0.49 for metformin hydrochloride (Fig. 1).

Optimum wavelength determination

From the experimental results, the optimum wavelength that was used for the glibenclamide and metformin hydrochloride analysis was 237 nm, which obtained the largest area of the two compounds (Table 3).

Extraction system optimization

Extraction optimization was performed by comparing the area of the *jamu* adducts solution with the glibenclamide, and metformin hydrochloride filtered with filter paper and precipitated for 5 min. The best extraction optimization used consisted of precipitation for 5 min because an area larger than the filtered area was obtained (Table 4).

Method validation

Selectivity

For testing the selectivity, the standard solution, *jamu kosong* solution, and adducted *jamu* solution were spotted. The results showed that

this method was selective because there was no interference from the matrix in the Rf of glibenclamide and the Rf of metformin hydrochloride.

Linearity and calibration curves

From the results of spotting the standard glibenclamide solution at concentrations of 500 µg/mL, 1000 µg/mL, 1500 µg/mL, 2000 µg/mL, 2500 µg/mL, and 3500 µg/mL and the standard metformin hydrochloride solution at concentrations of 600 µg/mL, 800 µg/mL, 1000 µg/mL, 1200 µg/mL, 1400 µg/mL, and 1600 µg/mL, the method revealed a linear relationship. The glibenclamide calibration curve resulted in a correlation coefficient value of 0.9998, and the metformin hydrochloride calibration curve resulted in a correlation coefficient value of 0.9981 (Figs. 2 and 3).

The r-value for glibenclamide meets the specified linearity criteria of being >0.999 [9]. However, the r-value for metformin hydrochloride did not meet this standard. This may be due to errors in standard dilution,

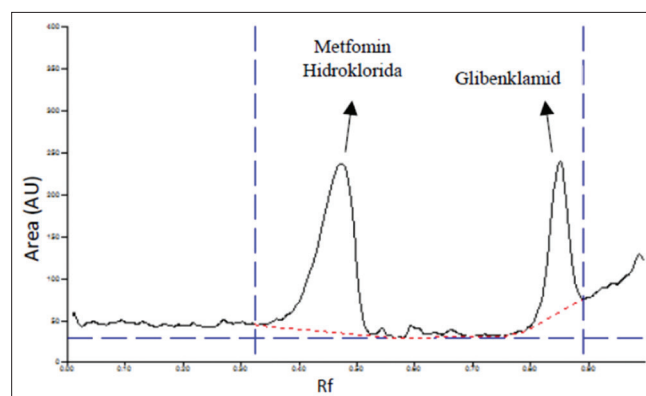


Fig. 1: Chromatogram of the glibenclamide and metformin hydrochloride compounds

Table 3: Optimum wavelength determination results

Wavelength (nm)	Area (AU)	
	Glibenclamide	Metformin hydrochloride
237	10921.0	19757.0
254	8424.8	15300.9
256	7584.6	13244.4
257	7032.1	10501.9
300	-	-

Table 4: Results of the extraction system selection

Extraction	Area (AU)	
	Glibenclamide	Metformin hydrochloride
Standard	7909.5	8845.5
Filtration	5472.0	5794.1
Precipitation	8931.8	6091.6

Table 5: Accuracy and precision test results

Medicinal compounds	Concentration (µg)	Recovery (%)	% CV
Glibenclamide	1.006	98.46	1.059
	4.024	99.15	1.506
	7.042	100.18	2.087
Metformin hydrochloride	1.2168	99.89	1.163
	2.4336	100.71	2.187
	3.2248	100.81	1.204

CV: Coefficient of variation

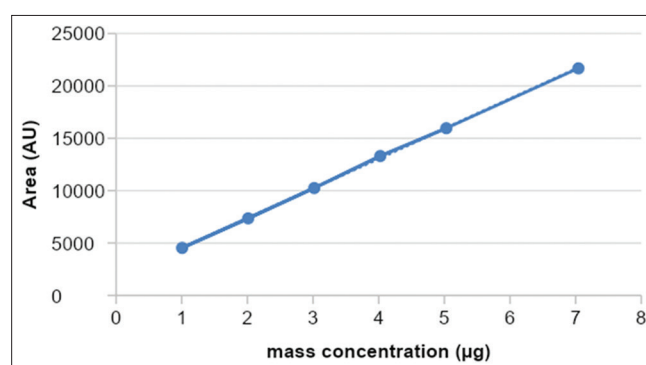


Fig. 2: Glibenclamide calibration curve

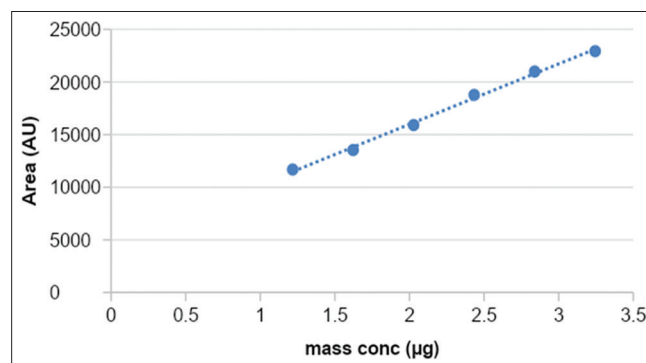
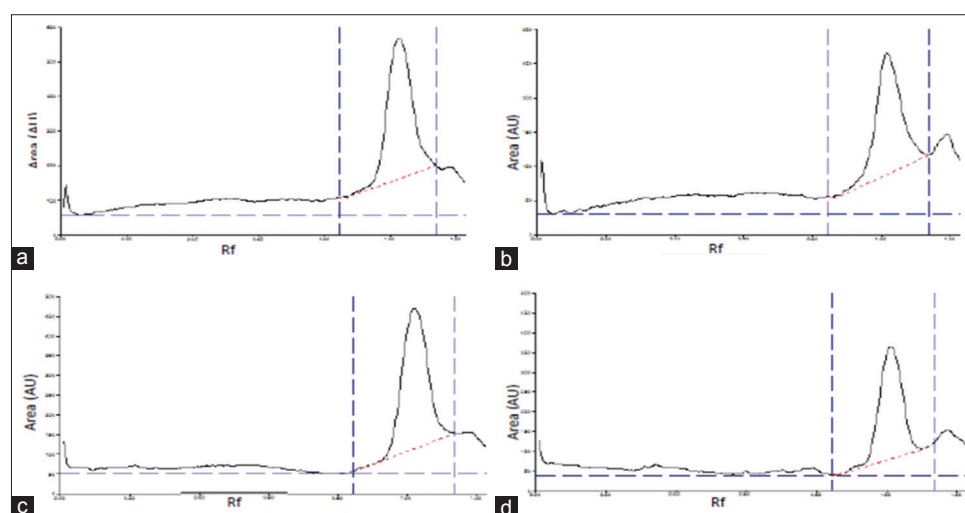


Fig. 3: Metformin hydrochloride calibration curve

Table 6: Quantitative sample test results

Jamu for diabetes	Levels ($\mu\text{g/mL}$)	Content (%)	Average content (%)	Weight of drug in sample packs (mg)
BI sample	1668.19	4.7602	4.9522	17.3327
	1801.62	5.1442		
SU sample	1454.11	4.1389	4.1495	14.5233
	1461.56	4.1601		
SP sample	1503.08	4.2699	4.2587	14.9055
	1493.85	4.2475		
LN sample	1765.03	5.0028	4.9412	17.2942
	1722.87	4.8796		

Fig. 4: Chromatograms of the *jamu* samples analyzed. (a) B1 sample; (b) SU sample; (c) SP sample; and (d) LN sample

errors when spotting the sample, and/or the influence of the particular instruments used. Nonetheless, the results are still considered acceptable in terms of linearity. According to Krausse (2003), the r -value of the linearity requirement is $r \geq 0.98$. As such, to obtain better linearity results, an experiment can be repeated [10].

LOD and LOQ

From the results of the LOD and LOQ tests, the LOD and LOQ values for glibenclamide were $49.97 \mu\text{g/mL}$ and $166.55 \mu\text{g/mL}$, respectively. The LOD and LOQ values for metformin hydrochloride were $74.75 \mu\text{g/mL}$ and $249.25 \mu\text{g/mL}$, respectively.

Accuracy and precision

From the results of the three concentrations (low, medium, and high) with six spottings, the average recovery value (Table 5) was obtained. The method we used met the accuracy requirements in terms of the recovery values being in the range of 98–102%. In addition, the method showed good precision, with correlation coefficient values below 2% [9] for glibenclamide compounds at low and moderate concentrations and for metformin hydrochloride compounds at low and high concentrations. Poor precision, as indicated by correlation coefficient values of more than 2%, maybe caused by errors when spotting the sample so that inaccurate analysis results are obtained. Moreover, there may also be an influence on the equipment or laboratory conditions. According to Riyanto (2014), variations in precision results can be due to random errors, such as the instability of instruments, variations in temperature or reagents used, and the diversity of techniques and different operators. To reduce these errors, an experiment can be repeated [11–13].

Qualitative analysis of samples

From the seven analyzed samples, four samples were positive for containing glibenclamide, namely, the BI, SU, SP, and LN samples; but there was no metformin hydrochloride found in any of the tested samples. This can be seen from the R_f value and its absorption compared

with the standard and adducted samples. The resulting R_f value is close to the same as the R_f standard of glibenclamide (Fig. 4).

Quantitative analysis of samples

From the results obtained, glibenclamide levels in the samples exceeded the LOQ. At these levels, the added glibenclamide would be expected to provide an increased antidiabetic effect within the *jamu* formulation. In addition, the amount of glibenclamide found in the *jamu* BI and *jamu* LN samples was equivalent to 3.5 times the usual dosage, while that found in the *jamu* SU and *jamu* SP samples was equivalent to 2.9 times the usual dose, where the value of the usual dose of glibenclamide is 5 mg. It can be concluded that these *jamu* formulations are very dangerous for the treatment of diabetes because they contain medicinal chemicals in the form of glibenclamide greater than the standard dosage (Table 6).

CONCLUSION

The selected mobile phase used for this study was methanol-aquades-glacial acetic acid (9:1:0.25) with an R_f value of 0.82 for glibenclamide and an R_f value of 0.49 for metformin hydrochloride. The best extraction system was obtained by precipitating the *jamu* for 5 min. The optimum wavelength for the analysis was 237 nm. Our validation results indicated that this method meets the requirements of linearity, selectivity, accuracy, and precision based on predetermined criteria.

From the seven *jamu* samples for diabetes, four contained glibenclamide, and none contained metformin hydrochloride. The content of glibenclamide in the samples was 4.9522% in the BI sample, 4.1495% in the SU sample, 4.2587% in the SP sample, and 4.9412% in the LN sample.

Action needs to be taken by the government to monitor the misuse of the addition of BKO to the herbal medicines available in the market. This is a matter of concern for health practitioners; the government should educate consumers and sellers about the dangers of products

in circulation. Stricter supervision of traditional medicine distribution needs to be done to tackle the circulation of dangerous products in the community.

ACKNOWLEDGMENTS

The authors would like to thank head, staff, and lecturers of the Faculty of Pharmacy, Universitas Indonesia, for their support during this research.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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