Original Article

Development and validation of simple simultaneous analysis for amlodipine and glibenclamide by nonderivatization high-performance liquid chromatography-fluorescence

Febrina Amelia Saputri, Anisahtul Alawiyah, Ayu Brilliany Firsty, Sandra Megantara, Arif Satria Wira Kusuma[1], Taofik Rusdiana[2], Aliya Nur Hasanah, Mutakin Mutakin, Ingrid S. Surono[3], Rizky Abdulah[4]

Departments of Pharmaceutical Analysis and Medicinal Chemistry, [1]Biological Pharmacy, [2]Pharmaceutical and Formulation Technology and [4]Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, [3]Department of Food Technology, Faculty of Engineering, Bina Nusantara University, Indonesia

J. Adv. Pharm. Technol. Res.

Abstract

Studies have shown that about 65% of diabetics have hypertension. Treatment for diabetic patients with hypertension is usually given a combination of drugs such as amlodipine (AML) and glibenclamide (GLI). The aim of this study was to develop and validate the simple simultaneous analysis method for separation of AML and GLI using high-performance liquid chromatography (HPLC) with fluorescence detector without derivatization. The arrangement of isocratic and gradient methods, mobile phase compositions, and flow rates to develop and validate the simple simultaneous analysis method for separation of AML and GLI by nonderivatization HPLC fluorescence was done. Optimum condition was obtained using an RP 18 (125 mm × 4 mm, i.d., 5 μm) and guard column RP 18 (4 mm × 4 mm, i.d., 5 μm) with mobile phase composition containing acetonitrile and phosphate buffer pH 3.0 using a 20:80 gradient condition at flow rate 1.0 ml/min measured at 361 nm for λ excitation and 442 nm for λ emission for AML and 235 nm for λ excitation and 354 nm for λ emission for GLI. The analysis of AML and GLI demonstrated a valid result with r^2 value 0.999, recoveries were 100.04% and 99.14% relative standard deviations were 0.508% and 0,797%, respectively, detection limits were 0.055 and 0.104 μg/ml, and quantification limits were 0.166 and 0.316 μg/ml, respectively. An accurate method of separation for AML and GLI using HPLC with fluorescence detector without derivatization has been validated.

Key words: Amlodipine, glibenclamide, high-performance liquid chromatography-fluorescence, nonderivatization, simultaneous

INTRODUCTION

Diabetes mellitus is a disorder characterized by elevated blood sugar levels or chronic hyperglycemia. Some of the body's metabolism can trigger the occurrence of diabetes if there is interference metabolism of fats, carbohydrates, and proteins and can also be exacerbated by vascular disorders.[1] Diabetes is usually accompanied by various complications, such as increased kidney disorders, heart attacks, and stroke. Research has shown that about 65% of diabetic patients are usually accompanied by hypertension.

Address for correspondence:
Mrs. Febrina Amelia Saputri,
Jl. Raya Bandung Sumedang, Km. 21, Jatinangor,
Sumedang 45363, Indonesia.
E-mail: febrina@unpad.ac.id

Access this article online	
Quick Response Code:	Website: www.japtr.org
	DOI: 10.4103/japtr.JAPTR_315_18

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Saputri FA, Alawiyah A, Firsty AB, Megantara S, Wira Kusuma AS, Rusdiana T, *et al*. Development and validation of simple simultaneous analysis for amlodipine and glibenclamide by nonderivatization high-performance liquid chromatography-fluorescence. J Adv Pharm Technol Res 2018;9:124-9.

And it has been proven that cardiovascular disorders can lead to morbidity and mortality in diabetic patients.[2]

Amlodipine (AML) is a dihydropyridine derivative with a working mechanism inhibiting calcium ion channels that can be used for the treatment of antihypertensive and angina pectoris. It inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle, thereby reducing blood pressure and increasing blood flow to the heart muscle.[3] The molecular weight of AML is 408.879 g/mol. AML has two hydrogen bonds donor and seven hydrogen bonds acceptor. It has logP of 3 and 75.3 mg/L solubility in water.[4]

Glibenclamide (GLI), 1-(4-[2-(5-chloro-2-methoxybenzamido) ethyl] benzenesulfonyl)-3-cyclohexylurea, is a second-generation sulfonylurea hypoglycemic agent that appears to lower blood glucose by stimulating the release of insulin from the pancreas.[5] The logP of GLI is 4.7, with the solubility in water being 4 mg/L. The molecular weight of GLI is 494.003 g/mol. It has three hydrogen bonds donor and five hydrogen bonds acceptor.[6]

Simultaneous use of AML and GLI for the treatment of diabetics accompanied by hypertension resulted in good treatment outcomes characterized by decreased blood glucose, total cholesterol, creatinine urine, and creatinine clearance significantly.[2] Interaction of the use of both drugs provides a higher percentage of inhibition small compared to the percentage of drug combination inhibition between GLI and enalapril.[7]

AML is a fluorescent compound as well as fluorescence GLI that can be analyzed using high-performance liquid chromatography (HPLC) fluorescence.[8,9] The advantages of fluorescence detector are the time required to be rapid and at low cost and can increase the detection and selectivity limits of a compound in the analysis.[10,11]

The use of a fluorescence detector against a spectrophotometric instrument has been performed and provides a more sensitive detection limit when compared to the use of an ultraviolet (UV) detector of 0.05 µg/ml.[8] The use of an HPLC instrument with a UV detector to analyze the compound has been carried out by a more sensitive detection limit value than the use of the spectroscopy instrument of 2 ng/ml, but the lack of this method is the presence of peak interference that may interfere with the results of the analysis.[12] The analytical method using HPLC without derivatization is also capable of producing a low detection limit value of 50 pg/ml, 0.18 ng/ml, and 0.4 ng/ml.[13-15]

The purpose of this study was to validate the optimum conditions for the simultaneous separation of AML and GLI using HPLC without derivatization process so that this method of analysis could be used as a simple and validated alternative method for the determination of AML and GLI.

MATERIALS AND METHODS

AML besylate and GLI standards were purchased from Sigma Aldrich, NaH_2PO_4 and Na_2HPO_4 pro analysis were purchased from Merck, acetonitrile and methanol HPLC grade were purchased from Merck, and aqua bidestillata was purchased from IPHA Laboratories.

Experimental

An HPLC System (Waters e2695 Separation Module from Waters Associates, Milford, Massachusetts, USA) with the fluorescence detector (Waters Separation 2475 FLR Detector from Waters Associates) was used. The data were recorded using Empower Software from Waters Associates. The mobile phase used the percentage of acetonitrile with buffer phosphate. Stationary phase used was LiChrospher RP 18 (Merck, Darmstadt, Germany) (125 mm × 4 mm, i.d., 5 µm) and guard column Lichrospher RP 18 (Merck) (4 mm × 4 mm, i.d., 5 µm).

Preparation of standard stock solution

A total of 5 mg AML besylate and GLI were separately weighed and dissolved in 50 ml methanol HPLC grade. The standard solution concentration of 100 µg/ml was obtained. The standard stock solution of each solution was prepared to be a mixture of AML and GLI solutions, resulting in a mixed concentration of 0.1, 0.5, 1, 5, and 10 µg/ml, sonicated for 5 min and cooled to room temperature, mixed well, and filtered through 0.45 µm Millipore filter. We injected separately 10 µl of the standard solution into the equilibrated HPLC system in five replicates and measured the response of the major peak due to AML besylate and GLI.

System suitability testing of high-performance liquid chromatography

The system suitability of HPLC was carried out by gradient elution method and isocratic elution method using the mixed solution of 10 µg/ml which then calculated some parameters such as resolution, capacity factor, selectivity factor, tailing factor, theoretical plate, and height equivalent to a theoretical plate. The conditions of HPLC are shown in Table 1.

Validation of the method

Validation of analytical method was done using mixed AML and GLI solution, and values of some parameters such as accuracy, precision, linearity, specificity, detection limit, quantification limit, and robustness were validated.

RESULTS

The system suitability test

Before performing the validation process, the system suitability test is necessary because the results of this test

Table 1: High-performance liquid chromatography conditions

Isocratic elution method			
n	Mobile phase composition		Flow rate (ml/min)
	Acetonitrile	Phosphate buffer pH 3	
1	45	55	0.8
			1.0
			1.2
2	40	60	0.8
			1.0
			1.2
3	38	62	0.8
			1.0
			1.2
4	35	65	0.8
			1.0
			1.2
Gradient elution method (flow rate 1 ml/min)			
n	Time (min)	Mobile phase composition	
		Acetonitrile	Phosphate buffer pH 3
1	0	30	70
	10	30	70
	30	70	30
	32	30	70
	40	30	70
2	0	20	80
	1	20	80
	20	80	20
	21	20	80
	25	20	80

will affect accuracy and precision. The system suitability test was performed using two separation conditions which were isocratic and gradient separation conditions. The results of system suitability test of some isocratic system elution and gradient elution system are shown in Tables 2 and 3.

The separation conditions from several different conditions can be concluded that the gradient separation conditions of 20 acetonitriles and 80 phosphate buffer of pH 3.0 are optimal conditions for performing simultaneous AML and GLI analysis processes because all conformity test parameters of the system meet the prescribed requirements [Table 4].

Validation of the method

Validation of the analytical method was carried out using gradient elution separation conditions using acetonitrile and phosphate buffer of pH 3.0 with a ratio of 20:80; the elution conditions are shown in Table 1. The excitation wavelengths used for the AML and GLI separation conditions were 361 nm and 235 nm, respectively. The emission wavelengths used for AML and GLI separation conditions are 442 nm and 354 nm, respectively.

Specificity

The specificity of the method is used to measure the ability of an analytical method to differentiate and measure the analyte in the presence of other components in the sample.[16] Specificity testing was performed by injecting standard mixed solutions with a concentration of 1 μg/ml and blanks injected into HPLC. For AML, specificity testing was performed at 361 nm excitation wavelength and 442 nm emission wavelength, while for GLI, specificity testing was performed at 235 nm excitation wavelength and 354 nm emission wavelength.

Linearity

Linearity testing was carried out using at least five different concentrations; the value of linearity is said to meet the requirements if the resulting correlation coefficient (r^2) is >0.98. Based on the research result, the correlation coefficient of AML besylate and GLI was obtained 0.9999 and 0.9999, respectively [Table 5].

Accuracy

Accuracy testing was performed using three different concentrations and performed in triplicate. Accuracy was expressed by the value of %recovery; %recovery is good if it is in the range of 98%–102%.[16] The average of %recoveries is 100.04% and 99.14% for AML and GLI, respectively. The accuracy data of the two compounds are shown in Table 6.

Precision

Precision testing was performed using three different concentrations and performed in triplicate. Precision is expressed by the value of %relative standard deviation (RSD); %RSD is declared good if not more than 2%.[16] The average of %RSD is 0,508% and 0,797% for AML and GLI, respectively. The precision data of the two compounds are shown in Table 7.

Robustness

Robustness testing was carried out by undergoing a change in test conditions; changes in conditions include changes in flow rate of ±0.2 ml/min and pH of the mobile phase (±0.2 units). Robustness testing was done and compared the %recovery value obtained; the %recovery value is declared to be good if it is in the range of 98%–102%.[16] Robustness testing data from both compounds are shown in Table 8.

DISCUSSION

The system suitability test conducted in this experiment was done using two methods, isocratic and gradient methods. The isocratic system is a system in which the composition of the same phase of motion during measurement, while the gradient system is a system in which the mobile phase composition changes during the measurement.[17] The variation in the comparison of the components of the mobile phase influences the peak retention time resulting from

Table 2: The suitability test of high-performance liquid chromatography system with isocratic elution system

Parameters	0.8 ml/min		1.0 ml/min		1.2 ml/min	
	AML	GLI	AML	GLI	AML	GLI
Percentage acetonitrile: percentage phosphate buffer (45:55)						
Retention time	2.36	13.009	1.880	10.346	1.579	8.591
Capacity factor	0.75	8.63	0.75	8.64	0.76	8.58
Number of theoretical plate	932.02	5137.30	433.21	2449.32	280.52	2708.48
High equivalent to a theoretical plate	0.01	0.002	0.03	0.005	0.04	0.004
Tailing factor	2.0	1.3	1.5	1.3	1.5	1.4
Selectivity factor	11.49		11.49		11.28	
Resolution	20.55		14.14		13.52	
Percentage acetonitrile: percentage phosphate buffer (40:60)						
Retention time	3.153	-	2.53	19.067	2.13	15.971
Capacity factor	1.35	-	1.36	16.77	1.37	16.79
Number of theoretical plate	257.47	-	179.48	7182.84	22.96	9267.69
High equivalent to a theoretical plate	0.049	-	0.07	0.002	0.54	0.001
Tailing factor	1.63	-	1.33	1.5	1.44	2.0
Selectivity factor	-		12.35		12.23	
Resolution	-		19.98		11.34	
Percentage acetonitrile: percentage phosphate buffer (38:62)						
Retention time	4.025	32.249	2.981	25.319	2.491	21.19
Capacity factor	3.93	38.47	1.78	22.60	1.79	22.70
Number of theoretical plate	249.73	2772.85	239.19	2207.99	90.65	2383.05
High equivalent to a theoretical plate	0.05	0.004	0.05	0.005	0.14	0.005
Tailing factor	2.0	1.7	1.67	2.0	1.33	1.4
Selectivity factor	9.79		12.70		12.70	
Resolution	16.27		15.26		13.43	
Percentage acetonitrile: percentage phosphate buffer (35:65)						
Retention time	5.221	23.883	4.1	-	3.44	34.635
Capacity factor	2.87	16.71	2.80	-	2.78	37.10
Number of theoretical plate	296.47	128.6212	261.95	-	423.80	2980.602
High equivalent to a theoretical plate	0.04	0.09	0.05	-	0.03	0.004
Tailing factor	0.67	1.33	1.00	-	1.00	1.7
Selectivity factor	5.818		-		13.325	
Resolution	3.873		-		19.46	

AML: Amlodipine, GLI: Glibenclamide

Table 3: The suitability test of high-performance liquid chromatography system with gradient elution system

Parameters	20 acetonitrile: 80 phosphate buffer		30 acetonitrile: 70 phosphate buffer	
	AML	GLI	AML	GLI
Retention time	9.069	14.6650	6.8	17.5
Capacity factor	12.95	21.56	20.45	54.26
Number of theoretical plate	5261.69	7046.579	639.26	7223.66
High equivalent to a theoretical plate	0.002	0.001	0.020	0.001
Tailing factor	1.50	0.11	1.33	0.11
Selectivity factor	1.664		2.65	
Resolution	9.335		11.28	

AML: Amlodipine, GLI: Glibenclamide

the compound and influences the polarity of the mobile phase.[18] The effect of variation in the flow rate will affect the retention time of the compound. The system suitability of HPLC showed that the greater the flow rate, the faster the peak generated from the compound and will affect the resolution value. The resolution is smaller with an increase in the flow rate of the mobile phase.[18] Changing the flow rate will affect the separation efficiency as it affects the widening of the peak.[18] From the robustness test, it can be seen that the %recovery is out of acceptable range. It means that the analysis must be strictly done at the optimum pH because pH affects the ionization of AML and GLI compounds.

Validation of the method of analysis was performed before the testing process, and this study was the development

Table 4: Optimum condition for suitability system test of high-performance liquid chromatography

Parameters	20 acetonitrile: 80 phosphate buffer		Terms of ICH[16]	Suitable/unsuitable
	AML	GLI		
Retention time	9.069	14.6650	-	-
Capacity factor	12.95	21.56	2<K' <20	Suitable
Number of theoretical plate	5261.69	7046.579	N >2000	Suitable
High equivalent to a theoretical plate	0.002	0.001	-	Suitable
Tailing factor	1.50	0.11	Tf<2	Suitable
Selectivity factor	1.664		α >1	Suitable
Resolution	9.335		Rs >2	Suitable

AML: Amlodipine, GLI: Glibenclamide, ICH: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use

Table 5: The linearity of AML and GLI by the proposed HPLC method

n	Concentration (μg/ml)	Area of AML	Area of GLI	Statistical analysis of AML	Statistical analysis of GLI
1	0,1	276,699.3	429,637	Slope: 1,708,295	Slope: 1,461,498
2	0,5	957,809	955,786	Intercept: 104,354	Intercept: 230,620
3	1	1,802,012	1,664,516	Correlation coefficient: 0.9999	Correlation coefficient: 0.9999
4	5	8,576,042	7,493,725		
5	10	17,159,304	14,870,306		

AML: Amlodipine, GLI: Glibenclamide

Table 6: The accuracy data of AML and GLI by the proposed HPLC method

AML				GLI			
Area	Amount found (μg/ml)	Amount added (μg/ml)	Recovery (%)	Area	Amount found (μg/ml)	Amount added (μg/ml)	Recovery (%)
958,877	0.504	0.5	100.84	958374	0.491	0.5	98.23893
958,193	0.503	0.5	100.76	961823	0.494	0.5	98.71128
956,357	0.502	0.5	100.55	967162	0.497	0.5	99.44247
1,814,141	1.006	1	100.62	1699320	0.999	1	99.85654
1,789,932	0.991	1	99.2	1695957	0.996	1	99.62626
1,801,963	0.999	1	99.9	1678272	0.984	1	98.41526
8,637,022	5.01	5	100.21	7402050	4.904	5	98.07143
8,513,662	4.938	5	98.76	7488067	4.962	5	99.24945
8,576,042	4.974	5	99.49	7591060	5.033	5	100.66

GLI: Glibenclamide, AML: Amlodipine

Table 7: The precision data of AML and GLI by the proposed HPLC method

Concentration	Area			Average	SD	RSD (%)
	I	II	III			
AML						
0.5	958,877	958,193	956,357	957,809	1303.147	0.136
1	1,814,141	1,789,932	1,801,963	1,802,012	12,104.57	0.671
5	8,637,022	8,513,662	8,577,442	8,576,042	61,691.92	0.719
GLI						
0.5	958,374	961,823	967,162	962,453	4427.74	0.46
1	1,699,320	1,695,957	1,678,272	1,691,183	11,306.98	0.67
5	7,402,050	7,488,067	7,591,060	7,493,725	94,631.97	1.26

RSD: Relative standard deviation, SD: Standard deviation, GLI: Glibenclamide, AML: Amlodipine

of the analytical method for the AML analysis using fluorescence HPLC.[16] The purpose of the validation of the analysis method was to confirm the inspection evidence and to have a method that in accordance with the testing objectives.[16]

CONCLUSION

The optimum condition for simultaneous analysis of AML and GLI simultaneously using HPLC fluorescence without derivatization was obtained using an RP

Table 8: Robustness of amlodipine and glibenclamide

n	Validation parameter	Average peak area		Amount found (μg/ml)		Amount added (μg/ml)		Recovery (%)	
		AML	GLI	AML	GLI	AML	GLI	AML	GLI
1	Flow rate (0.8 ml/min)	14,865,627	5,055,904.14	8.66	3.47	10	10	86.6	34.67
2	Flow rate (1.2 ml/min)	9,721,776	19,970,821.4	5.64	13.43	10	10	56.4	134.3
3	pH mobile phase (2.8)	13,350,544.33	7,182,357.94	7.78	4.84	10	10	77.8	48.3
4	pH mobile phase (3.2)	14,455,967.33	6,349,620.79	8.43	4.27	10	10	84.3	42.7

AML: Amlodipine, GLI: Glibenclamide

18 (125 mm × 4 mm, i.d.) and guard column RP 18 (4 mm × 4 mm, i.d.) with mobile phase composition containing acetonitrile and phosphate buffer of pH 3.0 using a 20:80 gradient condition at flow rate 1.0 ml/min measured at λ excitation and λ emission at 361 nm and 442 nm for AML and λ excitation and λ emission at 235 nm and 354 nm for GLI. All parameters have been validated.

Financial support and sponsorship

The study was supported by the Indonesian Ministry of Research, Technology, and Higher Education (Grant-In-Aid for International Research Collaboration and Publication) for RA.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kaplan NM. Treatment of coexisting diabetes and hypertension. Curr Cardiol Rep 2001;3:498-503.
2. Owolabi OJ, Omogbai EK. Co-administration of glibenclamide and amlodipine induces resistance to hyperglycemic treatment in streptozotocin induced adapted/non adapted diabetic rats. J Clin Exp Pharmacol 2011;1:1-7.
3. Moussa BA, El-Zaher AA, Mahrouse MA, Ahmed MS. Simultaneous determination of amlodipine besylate and atorvastatin calcium in binary mixture by spectrofluorimetry and HPLC coupled with fluorescence detection. Anal Chem Insights 2013;8:107-15.
4. Website information. USA: National Center for Biotechnology Information. Available from: https://www.pubchem.ncbi.nlm.nih.gov/compound/amlodipine#section=Chemical-and-Physical-Properties. [Last accessed 2018 Sep 26].
5. Shweta H, Dhaneshwar SR. Determination of glibenclamide, metformin hydrochloride and rosiglitazone maleate by reversed phase liquid chromatographic technique in tablet dosage form. Chem Ind Chem Eng Q 2014;20:39-47.
6. Website Information USA: National Center for Biotechnology Information. Available from: https://www.pubchem.ncbi.nlm.nih.gov/compound/glyburide#section=Chemical-and-Physical-Properties. [Last accessed 2018 Sep 26].
7. Reddy K, Jeevan Kumar J, Reddy S, Goud R, Aparma N. Pharmacodynamic interactions of amlodipine and enalapril with glyburide in normal and diseased rats. Am J Pharm Tech Res 2016;6:503-31.
8. Kadioglu Y, Ozturk M. Spectrofluorimetric determination of amlodipine in human plasma without derivatization. Braz J Pharm Sci 2012;48:719-25.
9. Khalaf KD, Hassen PA. Spectrofluorimetric method for the determination of glibenclamide in pharmaceutical formulations. Baghdad Sci J 2012;9:296-301.
10. Angin BA. Rapid identification techniques for chromatographic separation using fluorescence detectors (In Bahasa). J Penelit MIPA 2008;2:28-32.
11. Karlida I, Saputri FA. Review article: Compound derivatization in HPLC with fluorescence detector (In Bahasa). Farmaka 2016;14:1-17.
12. Shah S, Asnani A, Kawade D, Dangre S, Arora S, Yende S, *et al*. Simultaneous quantitative analysis of olmesartan medoxomil and amlodipine besylate in plasma by high-performance liquid chromatography technique. J Young Pharm 2012;4:88-94.
13. Konda RK, Challa BR, Chandu BR, Chandrasekhar KB. Bioanalytical method development and validation of memantine in human plasma by high performance liquid chromatography with tandem mass spectrometry: Application to bioequivalence study. J Anal Methods Chem 2012;2012:101249.
14. Bhadoriya A, Rathnam S, Dasandi B, Parmar D, Sanyal M, Shrivastav PS. Sensitive and rapid determination of amantadine without derivatization in human plasma by LC-MS/MS for a bioequivalence study. J Pharm Anal 2018;8:202-7.
15. Bhandare P, Madhavan P, Rao B, Rao SN. Determination of amino acid without derivatization by using HPLC-HILIC column. J Chem Pharm Res 2012;2:372-80.
16. International Conference of Harmonisation. Validation of Analytical Procedure. USA: International Conference Harmonisation; 2005.
17. Kealey D, Haines PJ. Instant Notes: Analytical Chemistry. Oxford: BIOS Scientific Publishers Limited; 2002.
18. Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography. 3rd ed. London: John Wiley & Sons; 2010.