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Research Article

A sensitive bioanalytical method for the simultaneous determination of amlodipine and glibenclamide

Febrina Amelia Saputri[1,2*], Aliya Nur Hasanah[1], Mutakin[1], Taofik Rusdiana[3], Ingrid Suryanti Surono[4], Rizky Abdulah[5,6]

[1] Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, Jalan Raya Bandung-Sumedang KM 21, Jatinangor, Indonesia
[2] Faculty of Pharmacy, Universitas Indonesia, Jalan Margonda Raya, Depok, Indonesia
[3] Department of Pharmaceutical and Formulation Technology, Faculty of Pharmacy, Universitas Padjadjaran, Jalan Raya Bandung-Sumedang KM 21, Jatinangor, Indonesia
[4] Food Technology Department, Faculty of Engineering, Bina Nusantara University, Jakarta, Indonesia
[5] Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Jalan Raya Bandung-Sumedang KM 21, Jatinangor, Indonesia
[6] Center of Excellence in Higher Education for Pharmaceutical Care Innovation, Universitas Padjadjaran, Jalan Raya Bandung-Sumedang KM 21, Jatinangor, Indonesia

ABSTRACT

Type 2 diabetes mellitus triggers hypertension as a complication. The use of amlodipine and glibenclamide drugs simultaneously results in a synergistic and effective lowering of blood sugar and blood pressure. In the testing of bioavailability and bioequivalence, as well as the monitoring of drug concentrations in the blood, a sensitive bioanalytical approach that meets existing reference requirements, such as the European Medicines Agency (EMA) recommendation, is required. Presently, there is no simultaneous bioanalytical method of amlodipine and glibenclamide that meets EMA requirements. This study aimed to develop a sensitive bioanalytical method that fulfills EMA requirements for determining the levels of amlodipine and glibenclamide simultaneously. Amlodipine and glibenclamide in plasma were extracted with acetonitrile at 10°C. The derivatization was conducted using 0.08% 4-chloro-7-nitrobenzofurazan at pH 8.6 with Teorell and Stenhagen buffer for 20 min at 70°C, followed by the addition of 0.1 N sulfuric acid. High-performance liquid chromatography analysis used a LiChrospher RP 18 column with a size of 125×40 mm ID; mobile phase, acetonitrile: 0.01% phosphoric acid (52:48); flow rate of 1 mL/min; and emission and excitation wavelength for glibenclamide and amlodipine at 346 and 300 nm and 535 and 480 nm, respectively. The concentration ranges were 0.1-20 ng/mL for amlodipine and 1-200 ng/mL for glibenclamide. The average ranges of percentage coefficient of variation and percentage difference were 1.76%-14.62% and 4.48%-11.18% for amlodipine and 0.56%-11.92% and 2.92%-12.75% for glibenclamide. This sensitive and simultaneous bioanalytical method for amlodipine and glibenclamide fulfills the EMA requirements.

Keywords:
Amlodipine, Glibenclamide, Fluorescence, Sensitive, Simultaneous

1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by increased blood sugar levels or hyperglycemia. The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014, 90% of whom have type 2 diabetes mellitus (T2DM), and it is expected to increase to 578 million by 2030[1-3]. In T2DM, patients experience insulin resistance, which causes an increase in tissue inflammation and an increase in the production of reactive oxygen species. It causes an increase in renin-angiotensin-aldosterone system activity, which affects the increase in aldosterone secretion. The increase in aldosterone secretion causes an increase in sympathetic nerve activity and an increase in salt retention, resulting in an increase in blood volume, which

***Corresponding author:**
*Febrina Amelia Saputri Email: febrina.amelia@farmasi.ui.ac.id

triggers hypertension (HT) as a complication of diabetes mellitus[4-5].

In the treatment of T2DM with HT, the simultaneous use of drugs is needed to produce an effect on both. Simultaneous use of amlodipine and glibenclamide for the treatment of T2DM patients with HT has resulted in good treatment, marked by a significant decrease in blood sugar levels, total cholesterol, urine creatinine, and creatinine clearance[6]. The interaction of both drugs provides lower blood glucose levels compared with the combination of glibenclamide and enalapril drugs[7].

A sensitive bioanalytical method that meets existing reference requirements, such as the European Medicines Agency (EMA) guideline, is required in the study of bioavailability and bioequivalence, as well as the monitoring of drug concentration in the blood. EMA requires that a bioanalysis method can be used for pharmacokinetic tests if the lower limit of quantification (LLOQ) is not more than 5% of the maximal concentration in the blood (Cmax)[8]. The Cmax of amlodipine and glibenclamide are 10.6 and 156 ng/mL, respectively[9-11]. This allows the LLOQ method for bioanalysis of amlodipine and glibenclamide that still meets EMA requirements to have a maximum of 0.53 and 7.8 ng/mL, respectively. Presently, there is no simultaneous bioanalytical method of amlodipine and glibenclamide that meets EMA requirements. Previous concurrent methods using high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector yielded LLOQ of 25 ng/mL for amlodipine and 50 ng/mL for glibenclamide,[12]. whereas nonderivatization HPLC fluorescence obtained LLOQ of 166 ng/mL for amlodipine and 316 ng/mL for glibenclamide[13].

Glibenclamide has a molecular weight of 494.004 g/mol, logP of 4.7, and pKa of 4.32[14]. Amlodipine besylate has a molecular weight of 567.1 g/mol, pKa of 9.1, and logP of 2.96[15]. Glibenclamide has a secondary amine group, and amlodipine has a primary amine group, causing these two compounds to be derivatized by 4-chloro-7-nitrobenzofurazan (NBD-Cl) to produce fluorescent compounds[9,16]. As an internal standard, nortriptyline was used because this compound has a secondary amine group that can also be derivatized by NBD-Cl[17]. The use of derivatization fluorescence detectors against UV detectors has been conducted and provides a more sensitive detection[16]. The analysis using HPLC with a fluorescence detector can produce a low detection limit value, such as 20-37 pg/mL[18-20].

This study aimed to develop and validate the optimum condition of a sensitive bioanalytical method for simultaneous analysis of amlodipine and glibenclamide in human plasma using an HPLC fluorescence detector with derivatization. NBD-Cl, which reacts with primary and secondary amines, was used as a derivatized agent or fluorotag. As an extraction procedure, the protein precipitation method using base and low temperature was used.

2. MATERIALS AND METHODS

2.1. Materials

Amlodipine besylate, glibenclamide, nortriptyline hydrochloride (internal standard), and NBD-Cl were purchased from Sigma Aldrich (Merck Groups, United States). Methanol, acetonitrile, ethanol, hydrochloric acid, sodium hydroxide, boric acid, sulfuric acid, and phosphoric acid were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade except acetonitrile, which was HPLC grade. Double distilled water was purchased from Ikapharmindo (Indonesia). Plasma samples were obtained from the Indonesian Red Cross in Bandung (Indonesia).

2.2. Solutions

Weighing amlodipine besylate, which was equivalent to 10 mg of amlodipine free base, was then dissolved in 2 mL of ethanol and diluted to 100 mL with water. It was diluted again with water to obtain a concentration of 1 µg/mL[9]. By dissolving glibenclamide in methanol, a standard stock solution of 1 µg/mL of glibenclamide was created[11,21]. Subsequent serial dilution of the plasma calibration sample was made to obtain concentrations of 0.1, 0.2, 1, 5, 10, and 20 ng/mL for amlodipine and 1, 2, 10, 50, 100, and 200 ng/mL for glibenclamide.

A nortriptyline stock solution of 5 µg/mL as an internal standard (IS) was made by dissolving nortriptyline hydrochloric acid with water[9]. Then, it was diluted to obtain a working solution of 500 ng/mL. An NBD-Cl solution with a concentration of 0.08% was prepared freshly in methanol[22].

A Teorell and Stenhagen buffer with a pH range of 7-9 was prepared. In a 50 mL volumetric flask, 0.445 g disodium phosphate, 0.35 g citric acid crystals, 0.177 g boric acid crystals, and 12.15 mL sodium hydroxide 1 N were mixed, and then, CO_2-free water was added until the boundary mark was reached. The buffer solution was made by inserting 10 mL of buffer stock solution into a 50 mL volumetric flask and adding 0.1 N hydrochloric acid as much as 16.76, 15.68, 14,73, 14.10, 13.42, and 12.56 mL for pH of 7.0, 7.4, 7.8, 8.2, 8.6, and 9, respectively, and then, CO_2-free water was added until the boundary mark[23].

2.3. Laboratory instrument

The HPLC analysis was conducted using Waters e2695 with a fluorescence detector Waters 2475 FLR at an excitation wavelength and emission wavelength of 300 and 346 nm for glibenclamide and 480 and 535 nm for amlodipine, respectively. Chromatographic separa-

tion was achieved isocratically on a LiChrospher RP 18 (125 mm×4 mm, I.D) and a guard column LiChrospher RP 18 (4 mm×4 mm, I.D) with a particle size of 5 µm. The mobile phase, acetonitrile: 0.01% phosphoric acid (52:48), was used at a flow rate of 1 mL/min at ambient temperature.

2.4. Sample preparation

A 0.5 mL plasma sample was extracted using protein precipitation. One milliliter of acetonitrile and 100 µL of 0.1 N NaOH were added to the plasma sample and then placed at 9°C-10°C for 30 min. The solution was centrifugated for 15 min at 4500 rpm at 10°C. The supernatant was taken, and extraction was conducted twice. The extracted solution was evaporated under nitrogen.

The dried extracts were reconstituted for derivatization by adding 200 µL of acetonitrile. Then, 100 µL of 0.1 N NaOH, 100 µL of buffer at pH of 8.6, and 100 µL of 0.08% NBD-Cl solution were added. The reaction was performed at 70°C for 20 min. To stop the reaction, the reaction product was cooled and mixed with 100 µL of 0.1N sulfuric acid. The chromatographic apparatus was injected with the solution.

2.5. Validation of the method

2.5.1. Selectivity

A total of six plasmas from different sources were prepared to obtain the LLOQ of amlodipine and glibenclamide, which were 0.1 and 1 ng/mL, respectively. The response of compounds to their retention times was analyzed. The interference response must be <20% LLOQ for the test compounds and <5% for internal standards[8].

2.5.2. Carryover

The blank sample was injected after the injection of the sample at high concentrations or at the upper limit of quantification (ULOQ), which was 20 ng/mL for amlodipine and 200 ng/mL for glibenclamide. The response of an analyte should not exceed 20% of LLOQ and may not exceed 5% of internal standards[8].

2.5.3. LLOQ

Plasma solutions were added with standard solutions to obtain the concentrations of amlodipine at 0.05, 0.1, and 0.2 ng/mL; glibenclamide at 0.5, 1, and 2 ng/mL; and nortriptyline at 500 ng/mL. The plasmas were prepared and then injected into the HPLC system for five replications. The values of percentage difference (%diff) and percentage coefficient of variation (%CV) from the measurement were calculated[8].

2.5.4. Accuracy and precision

The within-run and between-run accuracy and precision were determined by analyzing plasma samples spiked with amlodipine at concentrations of 0.1, 0.3, 8, and 16 ng/mL and glibenclamide at concentrations of 1, 3, 80, and 160 ng/mL with five replicates on the same day, as well as on a separate day[8].

2.5.5. Stability

Plasma samples were spiked at concentrations of quality control low and quality control high, which were 0.3 and 16 ng/mL for amlodipine and 3 and 160 ng/mL for glibenclamide, respectively. The samples were tested in the following[8]:

1. Short-term stability: the samples were stored in the preparation area, and then the samples were tested at 0 and 24 h after storage.
2. Long-term stability: the samples were stored at -80°C, and then the samples were tested at 0 and 28 days after storage.
3. Freeze-thaw stability: the samples were stored at -80°C and then thawed and frozen for up to three cycles. The samples were then tested at 0 and 3 cycles.

3. RESULTS AND DISCUSSION

Amlodipine, glibenclamide, and nortriptyline as is were derivatized using NBD-Cl because it can react with both a primary amine and secondary amine[16,24-25]. The reaction between amlodipine and NBD-Cl occurs in primary amines because of the absence of steric hindrance. The reaction between glibenclamide and NBD-Cl is predicted to occur in the secondary amine flanked by a sulfone and a carbonyl group. Because the sulfone and carbonyl groups attract electrons, the electrons in the secondary amine flank are more positively charged. Consequently, the nitrogen-hydrogen bond becomes unstable and susceptible to breaking. This unstable bonding between nitrogen and hydrogen causes the hydrogen from the secondary amine to easily react with the chlorine from NBD-Cl. The nitrogen from glibenclamide that loses hydrogen will tend to bind to one of the carbon atoms of NBD-Cl, which is positively charged and unstable because it has lost chlorine, causing the bond between glibenclamide and NBD-Cl. Nortriptyline has only one amine group, which is the secondary amine that reacts with NBD-Cl.

Therefore, there is no steric hindrance in this secondary amine, so it makes the binding between nortriptyline and NBD-Cl easier. This is one of the reasons for using nortriptyline as an IS. Figure 1a depicts the reaction between amlodipine and NBD-Cl, and Figure 1b and Figure 1c depict the prediction of the reaction

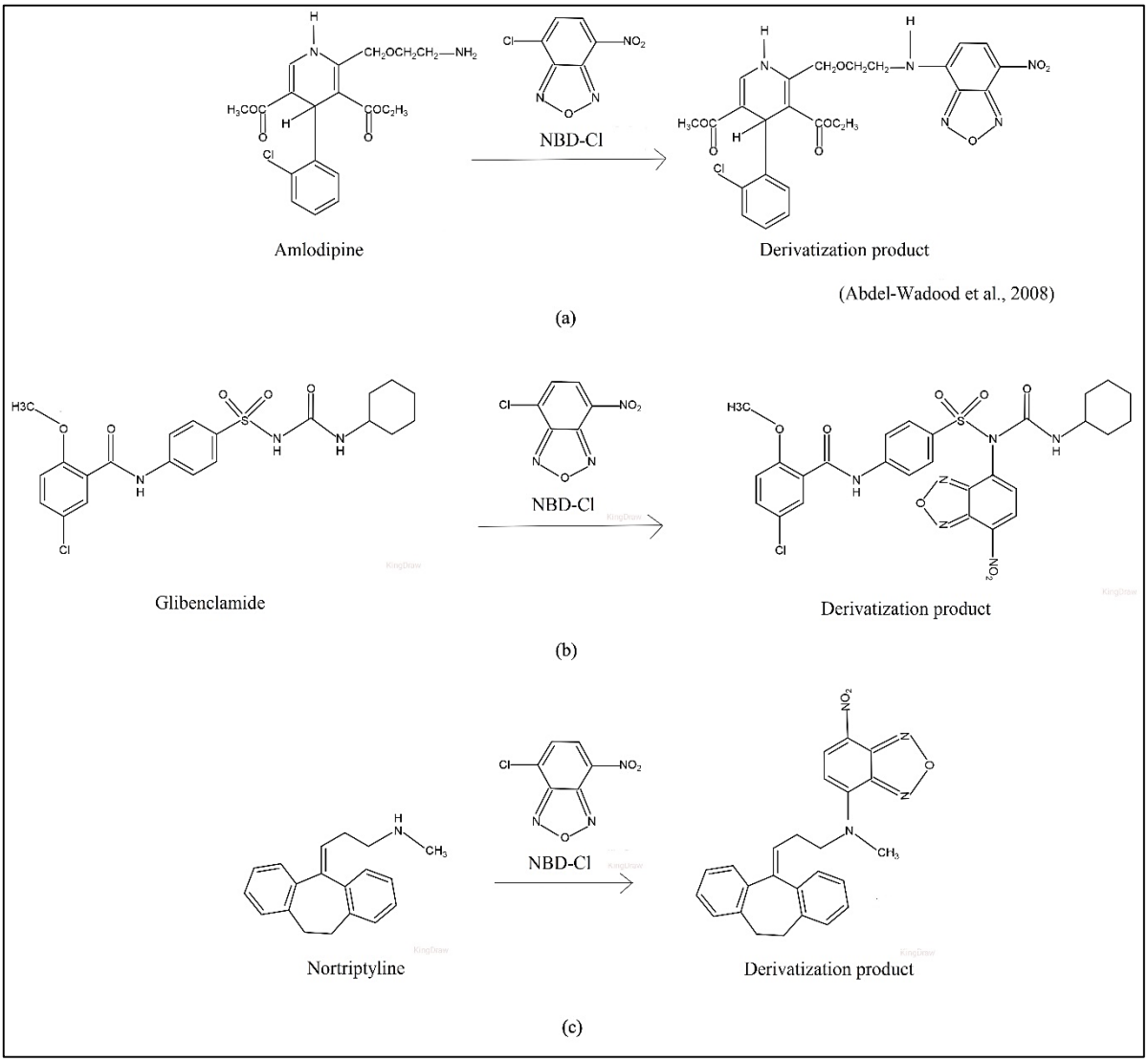

Figure 1. Reaction and prediction of the reaction between amlodipine, glibenclamide, and nortryptiline with NBD-Cl.

between glibenclamide-NBD-Cl and nortriptyline-NBD-Cl, respectively.

Screening at the maximum wavelength was conducted to give the most sensitive method. The screening was conducted on the standard solution of amlodipine and glibenclamide separately. The maximum wavelengths of excitation and emission are 480 and 535 nm for amlodipine (Figure 2a) and 300 and 346 nm for glibenclamide (Figure 2b).

pH was optimized for the reaction between amlodipine and NBD-Cl. The reaction was performed in a neutral to basic ratio. The base will react with hydrogen from amlodipine's main amine to allow the NBD-Cl to attach to it. The solution was cooled after the reaction, and acids were added to stop it. The added acids were sulfuric acid and hydrochloric acid. Figure 3 presents the results of pH optimization and acidification. As shown in this figure, the optimum pH for the reaction between amlodipine and NBD-Cl was 8.6, whereas the acidic solution that produces the best results is if 0.1 N sulfuric acid is used. This is because hydrochloric acid can cause quenching in fluorophores[26].

The extraction technique used was protein precipitation, because it is relatively simple and provides a quick sample cleanup[27]. In this study, we used acetonitrile and methanol as organic solvents. These solvents cause a decrease in the dielectric constant, which causes water to be displaced from the hydrophobic portion of the protein surface. It further causes hydrophobic connections between proteins in the plasma to be disrupted, leading to proteins precipitating out of the solution[27]. Acetonitrile extracts amlodipine better than methanol because the -C≡N group on acetonitrile is more reactive in breaking the protein-water hydrogen bonds that lead to a displacement of water, which causes the better protein precipitation. The temperature setting and addition of bases were conducted to optimize the extraction process. In this study, amlodipine and glibenclamide in plasma were extracted with acetonitrile, added with 0.1 N NaOH, mixed with a vortex, and then put in a refrigerator at 9°C-10°C for 30 min. Our study is in agreement with the research conducted by Ganesh et al[28]. It is known

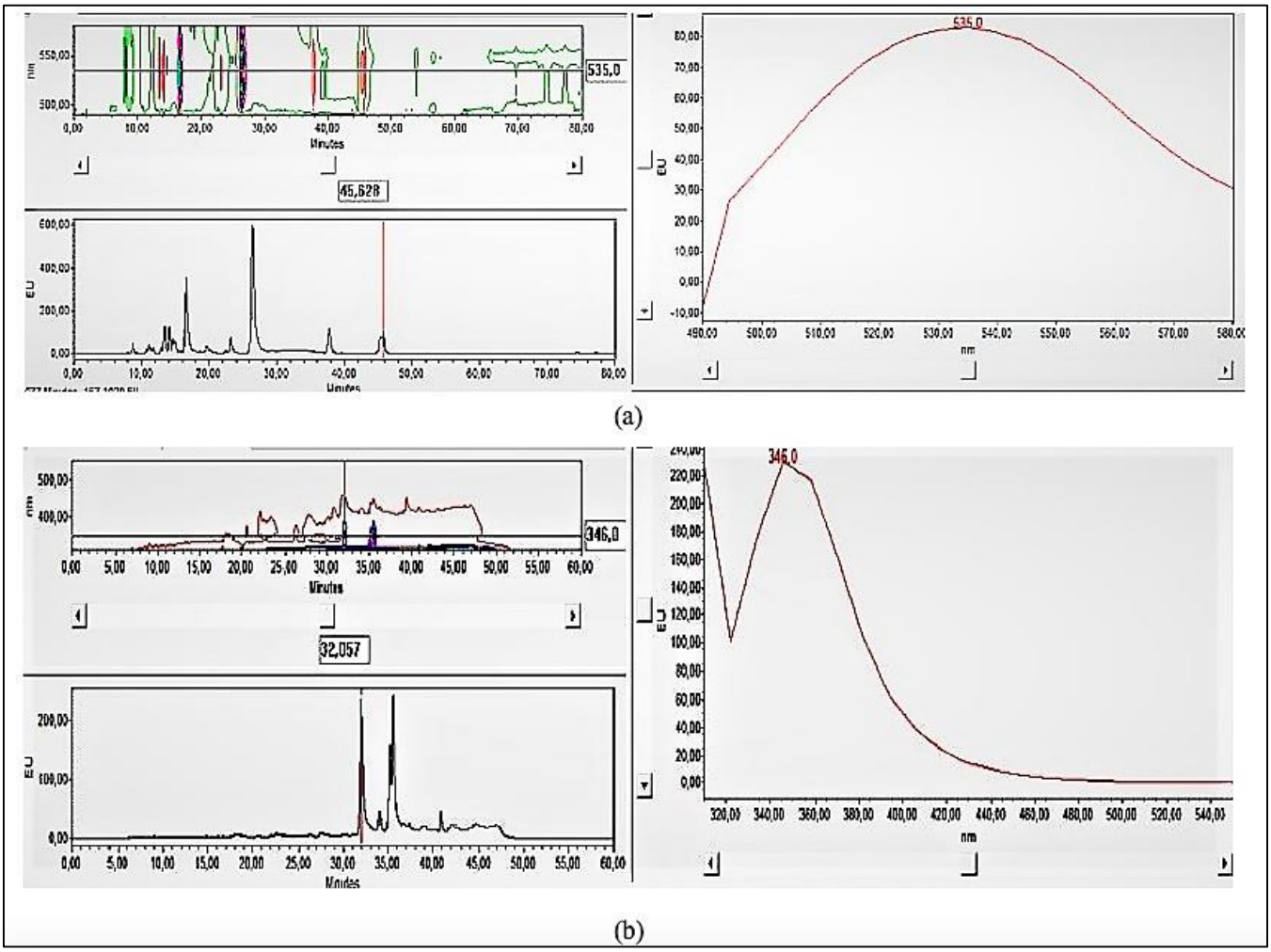

Figure 2. The maximum wavelength of excitation and emission of amlodipine and glibenclamide.

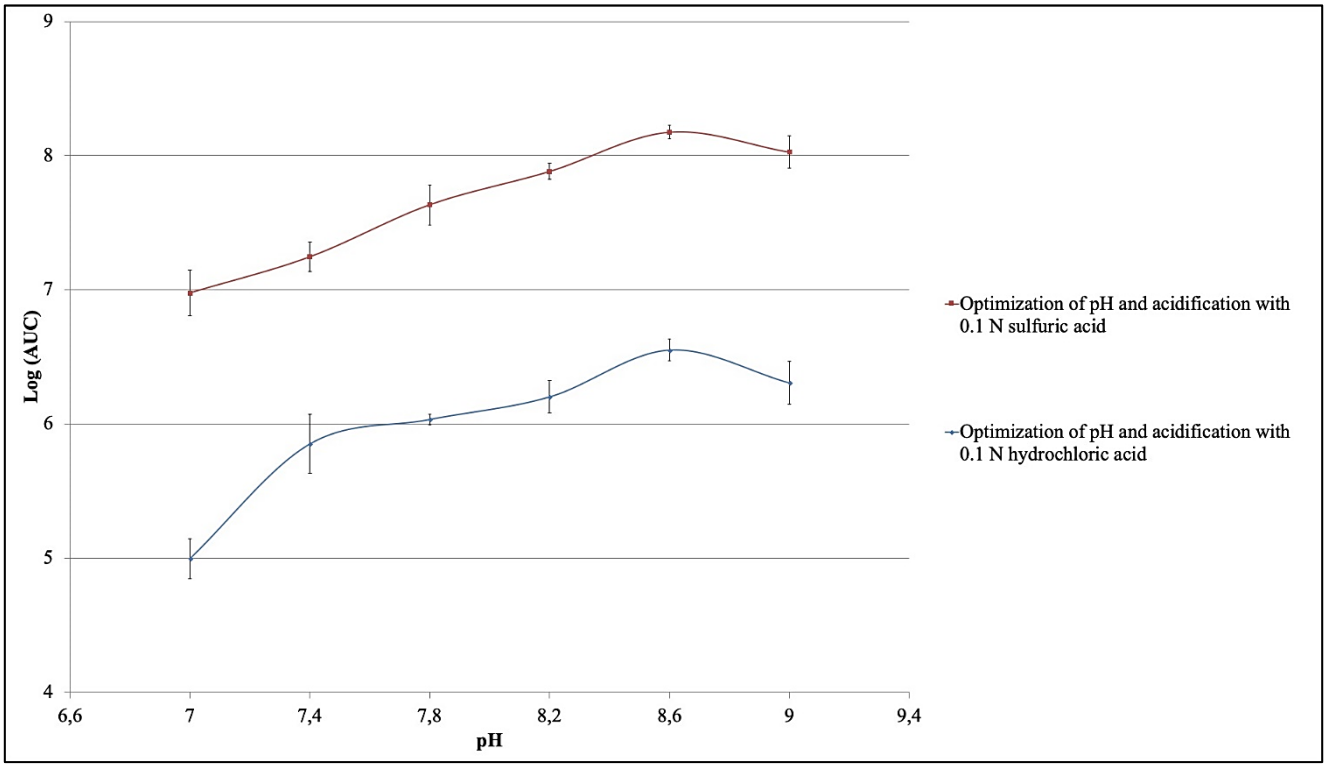

Figure 3. pH optimization and acidification of amlodipine and NBD-Cl reaction.

that the average optimum temperature for maximum stability of some globular proteins unfolding is 9.42°C[28]. The study by Saputri et al.[29] also showed that the detection limit of amlodipine is worse when the extraction of amlodipine with acetonitrile was conducted at room temperature, which is 2.5 ng/mL[29]. Amlodipine is bound to albumin, and 98% of glibenclamide is also bound to albumin[30-31]. Albumin is a weak acid protein[32]. The nitrile group of acetonitrile has high electronegativity and will break the hydrogen bonds between proteins, whereas the use of low temperatures is conducted to stabilize the unfolding albumin as a globular protein.

3.1. Validation of the method

3.1.1. Selectivity

Selectivity was tested on plasma blanks from six different sources. The plasmas were prepared to obtain the concentration of analytes in LLOQ, which was 0.1 and 1 ng/mL for amlodipine and glibenclamide, respectively. Consequently, the interference response has the retention time of test compounds of <20% LLOQ, which was 11.724% for amlodipine and 8.445% for glibenclamide, and the internal standard retention times of <5%, which was 0.037% for nortriptyline, which meets the validation requirements[8].

3.1.2. Carryover

Carryover was tested by injecting sample blanks after injection of samples at high concentrations or at ULOQ, which were 20 ng/mL for amlodipine and 200 ng/mL for glibenclamide. The result was that the blank response after ULOQ injection at amlodipine retention time was 10.959%, at glibenclamide retention time was 19.865%, and at nortriptyline retention time was 0.065%. These results meet the validation requirements because the response in blanks after ULOQ injection for analytes was <20% and that for internal standards was <5%[8].

3.1.3. LLOQ

The LLOQ method was determined by using signal to noise (S/N) and %CV data and then confirmed by experiment. During LLOQ experiments, amlodipine concentrations of 0.05, 0.1, and 0.2 ng/mL and glibenclamide concentrations of 0.5, 1, and 2 ng/mL were used. Nortriptyline was used as an IS at a concentration of 500 ng/mL. Amlodipine and glibenclamide LLOQ from this method, which has %diff and %CV of <20%, were 0.1 and 1 ng/mL. Table 1 lists the LLOQ test results.

Table 1. LLOQ of amlodipine and glibenclamide.

Analyte	C (ng/mL)	C NOR (ng/mL)	%CV (average±SD)	%*diff* (average±SD)
Amlodipine	0.05	500	129.90 ± 0.15	132.02 ± 9.15
	0.1		6.77 ± 0.02	11.23 ± 3.16
	0.2		5.43 ± 0.19	4.76 ± 0.48
Glibenclamide	0.5	500	122.06 ± 0.33	188.71 ± 9.59
	1		16.73 ± 0.15	10.83 ± 5.95
	2		6.40 ± 0.12	7.26 ± 2.97

C: Concentration, NOR: Nortryptiline, %CV: %Coefficient of Variation, %diff: %Difference, SD: Standard Deviation

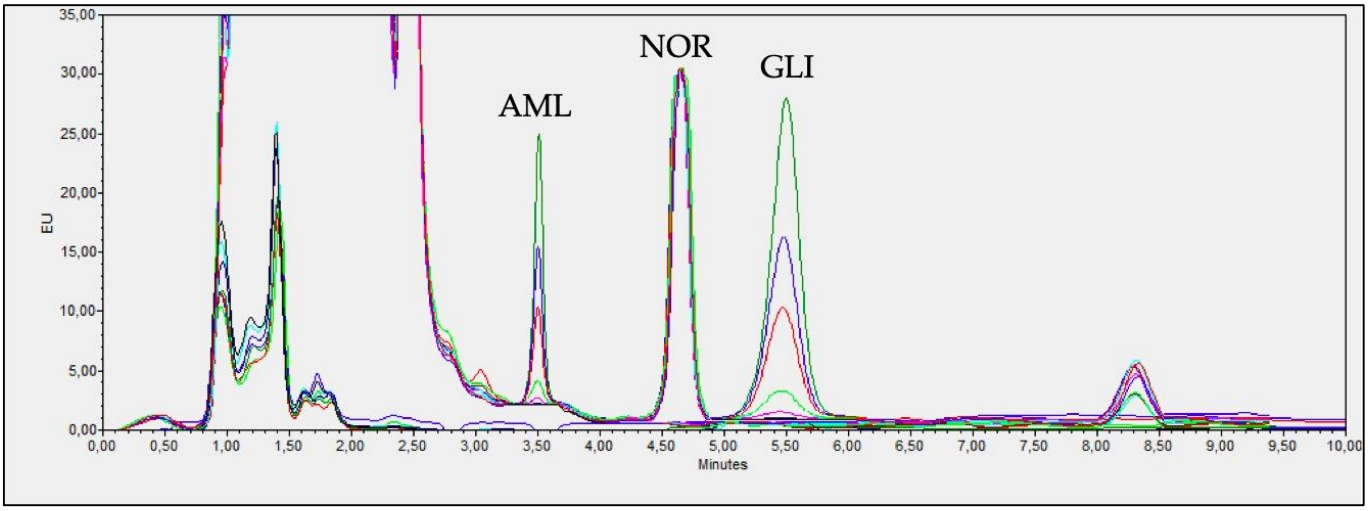

Figure 4. Chromatogram of amlodipine, glibenclamide, and nortriptyline.

3.1.4. Calibration curve

Calibration curves were made at concentrations of 0.1, 0.2, 1, 5, 10, and 20 ng/mL for amlodipine and 1, 2, 10, 50, 100, and 200 ng/mL for glibenclamide. The IS of nortriptyline was used at a concentration of 500 ng/mL. The linear regression equation for amlodipine was y = 0.0149x+0.0006, whereas the linear regression equation for glibenclamide was y = 0.0045x+0.0024, with r^2 >0.99 for both. Figure 4 shows the linear chromatograms.

3.1.5. Accuracy and precision

Accuracy and precision were measured through within-run and between-run testing of four different concentrations, namely, on LLOQ, quality control-low, medium, and high-which were 0.1, 0.3, 8, and 16 ng/mL for amlodipine and 1, 3, 80, and 160 ng/mL for glibenclamide. Accuracy and precision meet the requirements because the values of %diff and %CV were <20% for LLOQ and <15% for quality control samples[8]. Table 2 lists the results of accuracy and precision.

3.1.6. Stability

Stability was tested on QCL and QCH samples. Short-term stability was tested at room temperature, long-term stability was tested at -80°C, and freeze-thaw stability was tested for three freezing cycles. If the %diff is less than 20%, the sample is said to be stable[8]. Based on the results of the stability tests listed in Table 3, amlodipine and glibenclamide were stable during the test time.

The bioanalytical method of simultaneous analysis of amlodipine and glibenclamide meets the validation requirements according to the EMA, especially for the LLOQ parameter when these two compounds are quantified simultaneously. Table 4 summarizes the results of the bioanalysis validation compared with the validation requirements according to the EMA. This bioanalytical method can be used for the study of bioavailability and bioequivalence, as well as the monitoring of amlodipine and glibenclamide concentrations in the blood. The bioanalytical method obtained produces better sensitivity than the simultaneous methods previously obtained by Porwal and Talele[12] and Saputri et al.[13] Table 5 shows the comparison of the sensitivity of the current method with the previous methods. The result is in agreement with the theory that the use of a fluorescence detector with derivatization against a UV detector has been conducted and provides a more sensitive detection[16]. The sensitivity and selectivity of the fluorescence detector are due to its mechanism of action, which detects the light emitted by compounds at specific wavelengths. Only compounds that have a fluorophore, which fluoresces, can be detected[33]. Furthermore, the extraction method also affects the sensitivity of the method. Porwal and Talele extracted amlodipine and glibenclamide from plasma using the protein precipitation method, using cold aqueous 10% trichloroacetic acid and acetonitrile. In this experiment, extraction was also conducted through protein precipitation using acetonitrile but with temperature modification and the addition of NaOH. Additionally, in the simultaneous analysis of amlodipine and glibenclamide conducted by Porwal and Talele, there was no evaporation of the extracted solution, whereas in this experiment, evaporation and reconstitution were conducted in small volumes so that the concentration of samples was concentrated[12].

Our study has limitations. In the present study, we did not compare with other methods such as high-performance thin-layer chromatography and mass spectrometry. Despite this limitation, our study provides a sensitive bioanalytical method for the simultaneous determination of amlodipine and glibenclamide, which fulfills the EMA requirement.

5. CONCLUSION

In this study, we optimized the bioanalytical method for determining the concentration of amlodipine and glibenclamide simultaneously. The development of the method involved the use of acetonitrile for drug extraction from plasma, NBD-Cl as fluorotag, Teorell and

Table 2. Within run and between run accuracy and precision.

Analyte	C (ng/mL)	Within run		Between run	
		%CV (average±SD)	%*diff* (average±SD)	%CV (average±SD)	%*diff* (average±SD)
AML	0.1	14.619 ± 0.015	11.185 ± 7.651	17.309 ± 0.017	14.015 ± 9.488
	0.3	6.079 ± 0.019	6.969 ± 4.187	11.394 ± 0.035	9.213 ± 6.295
	8	3.059 ± 0.255	4.484 ± 3.196	6.104 ± 0.498	5.299 ± 3.505
	16	1.758 ± 0.299	6.609 ± 1.874	4.176 ± 0.718	7.494 ± 4.489
GLI	1	11.920 ± 0.134	12.748 ± 13.439	14.101 ± 0.147	14.613 ± 10.801
	3	4.999 ± 0.165	9.568 ± 1.077	7.226 ± 0.234	10.182 ± 4.452
	80	5.172 ± 4.424	7.129 ± 5.233	7.721 ± 6.490	7.937 ± 4.940
	160	0.561 ± 0.871	2.919 ± 0.544	4.001 ± 6.099	4.725 ± 3.811

C: Concentration, %CV: %Coefficient of Variation, %diff: %Difference, SD: Standard Deviation

Table 3. The results of stability testing.

Concentration	Stability	Time	%*diff* AML (average±SD)	%*diff* GLI (average±SD)
QCL	Short term	0 hour	0.851 ± 9.161	-1.063 ± 13.427
		24 hours	-1.942 ± 3.254	-6.152 ± 4.957
	Long term	0 day	1.445 ± 4.205	4.415 ± 4.124
		28 days	-7.651 ± 2.447	-5.744 ± 3.508
	Freeze thaw	0 cycle	2.405 ± 5.734	1.305 ± 3.795
		3 cycles	-5.538 ± 7.623	-7.836 ± 3.700
QCH	Short term	0 hour	5.093 ± 1.335	6.027 ± 2.585
		24 hours	2.419 ± 1.586	1.703 ± 3.420
	Long term	0 day	8.223 ± 4.934	3.191 ± 4.026
		28 days	4.265 ± 2.713	-3.134 ± 7.615
	Freeze thaw	0 cycle	7.058 ± 2.129	4.629 ± 1.908
		3 cycles	3.284 ± 3.049	-2.740 ± 2.321

QCL: Quality Control Low, QCH: Quality Control High, AML: Amlodipine, GLI: Glibenclamide, SD: Standard Deviation

Table 4. The results of validation compared to EMA requirements.

Parameters	EMA requirements (EMA, 2011)	The results of validation
Selectivity	Detector response <20% for LLOQ of analyte, <5% for IS	AML = 11.72%, GLI = 8.45%, IS = 0.04%
Carry over	Detector response <20% for LLOQ of analyte, <5% for IS	AML = 10.96%, GLI = 19.87%, IS = 0.07%
LLOQ	LLOQ <5% Cmax, %*diff* <20%, %CV <20%	AML= 0.1 ng/mL, %CV = 6.77±0.02, %*diff* = 11.23±3.16
		GLI = 1 ng/mL, %CV = 16.73±0.15, %*diff* = 10.83±5.95
Calibration curve	LLOQ = %*diff* <20%,	AML = 1.56-11.23%
	Others = %*diff* <15%	GLI = 0.54-10.83%
Accuracy	LLOQ = %*diff* <20%	AML = 4.48-11.18%
	QC = %*diff* <15%	GLI = 2.92-12.75%
Precision	LLOQ = %CV <20%	AML = 1.76-14.62%
	QC = %CV <15%	GlI = 0.56-11.92%
Stability	%*diff* <15%	AML = 1.94-7.65%
		GLI = 1.31-7.84%

IS: Internal Standard, AML: Amlodipine, GLI: Glibenclamide,, QC: Quality Control, LLOQ: Lower Limit of Quantification

Table 5. Comparison of the sensitivity.

Method	Instrumentation	LLOQ (ng/mL)		Reference
		AML	GLI	
Nonderivatization	HPLC-UV	25	50	12
Nonderivatization	HPLC-Fluorescence	166	316	13
Derivatization with NBD-Cl	HPLC-Fluorescence	0:01	1	Current method

AML: Amlodipine, GLI: Glibenclamide,, QC: Quality Control, LLOQ: Lower Limit of Quantification

Stenhagen buffers at pH 8.6, and modification of the time and temperature of the reaction. The solution was then analyzed using HPLC with a fluorescence detector. This method was sensitive and met the EMA's validation requirement.

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Author contribution

Conceptualization, FAS, ANH, MT, and RA; methodology, FAS, ANH, and MT; experiment, FAS; data analysis, FAS, ANH, MT, and TR; funding acquisition, RA; writing the manuscript, FAS; review and editing, MT, ANH, RA, and ISS. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare no conflicts of interest.

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Ethics approval
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