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Journal of Pharmaceutical Analysis

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ORIGINAL ARTICLE

Kinetic spectrophotometric method for determination of amlodipine besylate in its pharmaceutical tablets

Ashraf M. Mahmoud, Hanaa M. Abdel-Wadood, Niveen A. Mohamed*

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt

Abstract A simple and sensitive kinetic spectrophotometric method has been developed and validated for determination of amlodipine besylate (AML). The method was based on the condensation reaction of AML with 7-chloro-4-nitro-2,1,3-benzoxadiazole in an alkaline buffer (pH 8.6) producing a highly colored product. The color development was monitored spectro-phometrically at the maximum absorption λ_{max} 470 nm. The factors affecting the reaction were studied and the conditions were optimized. The stoichiometry of the reaction was determined, and the reaction pathway was postulated. Moreover, both the activation energy and the specific rate constant (at 70 °C) of the reaction were found to be 6.74 kcal mole^{-1} and 3.58 s^{-1}, respectively. The initial rate and fixed time methods were utilized for constructing the calibration graphs for the determination of AML concentration. Under the optimum reaction conditions, the limits of detection and quantification were 0.35 and 1.05 μg/mL, respectively. The precision of the method was satisfactory; the relative standard deviations were 0.85–1.76%. The proposed method was successfully applied to the analysis of AML in its pure form and tablets with good accuracy; the recovery percentages ranged from 99.55±1.69% to 100.65±1.48%. The results were compared with that of the reported method.

1. Introduction

Amlodipine besylate (AML), (4R, S)-3-ethyl-5-methyl 2-(2-amino-ethoxy-methyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl pyridine-3,5-dicarboxylate monobenzene sulphonate, is a potent long-acting calcium channel blocking agent [1]. It is widely used for the treatment of hypertension as well as the stable and variant angina [2]. It is more effective than β-blockers in the treatment of variant angina because it prevents and reverses the coronary spasms resulting in increased blood

*Corresponding author. Tel.: +20 882411255; fax: +20 882332776.
E-mail address: niveenam5606@yahoo.com (N.A. Mohamed).

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flow and myocardial oxygen supply [3]. Moreover, it inhibits selectively the arterial vascular smooth muscle cell proliferation resulting in prevention of the progressive narrowing of the arteries [4,5]. Amilodipine is official in BP, USP and EUP [6–8].

Because of the therapeutic importance of AML, many methods have been developed for its determination in pharmaceutical dosage forms and/or biological fluids. These methods include high performance liquid chromatography (HPLC) [6–13], high performance thin layer chromatography (HPTLC) [14,15], gas chromatography (GC) [16,17], capillary electrophoresis [18], flow injection analysis [19], differential-pulse square-wave anodic voltammetry [20], and enzyme immunoassay [21]. Spectrophotometry because of its inherent simplicity is considered one of the most convenient alternative techniques. Few spectrophotometric methods have been reported for the determination of AML [22–32]. However, some of these methods are expensive, somewhat time consuming and require sophisticated instruments that may be unavailable in many quality control laboratories. In comparison with HPLC technique the developed method is simple, cost effective and it is sensitive than other reported methods as HPLC [9], HPTLC [15] and more selective and sensitive than reported spectrophotometric method which its linear range is 5.0–30.0 μg/mL [22]. Therefore, the development of more simple, rapid, selective and sensitive method for the determination of AML is necessary. Kinetic spectrophotometric methods are becoming of great interest in pharmaceutical analysis [33]. Application of the kinetic methods offered some advantages such as improved selectivity, avoiding the interference of the colored and/or turbidity background of the samples, and possible avoiding of the interference of the other active compounds present in the commercial product if they are resisting the reaction conditions established for the proposed kinetic method. No attempts have yet been made to develop kinetic spectrophotometric method for determination of AML. For these reasons, the aim of the present study was directed to the development of kinetic spectrophotometric method for the determination of AML in tablets using 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) reagent which is selective for primary and secondary amines. The method was based on the condensation reaction of AML with (NBD-Cl) in an alkaline buffered medium producing a highly colored product exhibiting maximum absorption peak at 470 nm. The rate of color development was monitored spectrophometrically, and employed in the development of the proposed method.

2. Experimental

2.1. Apparatus

UV-1601 PC, Ultraviolet–visible spectrophotometer (Shimadzu, Japan), with 1-cm matched quartz cells, was used for all measurements. Super-mixer (Lab-line Instruments, Inc., USA). MLW type thermostatically controlled water bath (Memmert GmbH, Co. Schwa bach, Germany).

2.2. Chemicals and tablets

Amlodipine besylate (AML; Hetero Drugs Ltd., Hyderabad, India), was used as received (the purity was 99.2–100.2%).

NBD-Cl (Merck, Darmstadt, Germany) was 0.08% (w/v) in methanol and it was freshly prepared daily. The following available tablets were used in the present investigation: Regcor tablets (Egyptian International Pharmaceutical Industries Co., Cairo, Egypt) and Alkapress tablets (Alkan Pharma, Cairo, Egypt) are labeled to contain 5 mg of AML per tablet. All solvents and other chemicals used throughout this study were of analytical grade. Water was doubly distilled.

2.3. Preparation of solutions

2.3.1. Stock standard solution
An accurately weighed amount (25 mg) of amlodipine besylate was dissolved in 10 mL methanol in a 50-mL volumetric flask. The solution was then diluted to the mark with methanol to obtain a working standard solution of 0.5 mg/mL of AML. The solution was found to be stable for at least one weak when kept in the refrigerator.

2.3.2. Tablets sample solutions
Twenty tablets of each formulation were weighed and finely powdered. A quantity of the mixed powder equivalent to 25 mg of the active component was transferred to a 50-mL calibrated flask, dissolved in 25 mL methanol, swirled and sonicated for 5 min, completed to volume with methanol, shaken well for 10 min, and filtered. The procedure was completed as described for preparation of stock standard solution.

2.3.3. Buffer solution
Teorell and Stenhagen buffer solution [34] at pH 8.6 was prepared in freshly boiled and cooled distilled water. The buffer was composed of phosphoric acid, citric acid, and 1 M sodium hydroxide, adjusted to the required pH with 0.1 M hydrochloric acid.

2.4. General analytical procedures

One milliliter of the standard or sample solution (20–1000 μg/mL) was transferred to test tube, then 1 mL of Torell and Stenhagen buffer solution (pH 8.6) and 1 mL of NBD-Cl reagent (0.08%, w/v in methanol) were added, respectively. The reaction was allowed to proceed at 70 °C in a water bath for the specific time. Then, the test tubes were cooled in ice bath and 0.2 mL of concentrated sulphuric acid was added and mixed well. The contents were quantitatively transferred to 10-mL calibrated flasks and diluted to the mark with methanol. The absorbance of the resulting solutions was measured or monitored at 470 nm against reagent blanks treated similarly.

2.5. Determination of the molar ratio of the reaction

The Job's method of continuous variation [35] was employed. Master equimolar solutions of AML and NBD-Cl reagent were prepared. The concentrations of these solutions were 5×10^{-3} M in methanol. Series of 10-mL portions of the master solutions of AML with the reagent were made up of different complementary proportions (0:10, 1:9, ……, 9:1, 10:0, inclusive) in test tubes. Then, 1 mL of Torell and Stenhagen buffer solution (pH 8.6) was added to each tube. The reactions were allowed to proceed at 70 °C in a water bath for 35 min. Then, the test tubes were cooled in ice bath and 0.2 mL of concentrated sulphuric acid was added and mixed well. The contents were quantitatively transferred to 10.0-mL

calibrated flasks and diluted to the mark with methanol. The absorbance of the resulting solutions was measured at 470 nm against reagent blanks treated similarly.

2.6. Activation energy of the kinetic reaction of AML with NBD-Cl

The reaction time of AML with NBD-Cl was performed at different temperatures; 25, 40, 60, 70, and 80 °C using 20 μg/mL of AML and 1 mL of 0.08% (w/v) of NBD-Cl reagent at pH 8.6. The absorption-time curves at these temperatures were constructed to determine the initial rates, then plotting $1/T$ against $\log k$ to determine the slope of the line (activation energy of the reaction).

2.7. Data acquisition and processing

The kinetic data recorded for the proposed method were transformed to the Slide Write Plus software, version 5.011 (Advanced Graphics Software, Inc., CA, USA) for curve fitting, regression analysis, and statistical calculations. The initial rate (V) of the reaction at different concentrations was obtained from the slope of the tangent to the absorbance-time curve. The calibration curve was constructed by plotting the logarithm of the initial rate ($\log V$) of reaction versus logarithm of the concentration ($\log C$) of AML. Alternatively, the calibration curve was constructed by plotting the absorbance measured after a fixed time of 30 min.

The limits of detection (LOD) and limits of quantitation (LOQ) were determined [36] using the formula: LOD or LOQ $= \kappa SD_a/b$, where $\kappa = 3.3$ for LOD and 10 for LOQ, SD_a is the standard deviation of the intercept, and b is the slope.

3. Results and discussion

3.1. Optimum conditions for the reaction of AML with NBD-Cl

NBD-Cl is an activated halide derivative, which was first introduced as a fluorigenic reagent for the determination of some amines [30,34]. In further, it was used as a chromogenic reagent for the colorimetric determination of many pharmaceutical amines: e.g. β-blokers [37] and H$_2$-receptor antagonists [38]. In the present study, the reaction of AML with NBD-Cl produced a highly colored product exhibiting maximum absorption peak at 470 nm (Fig. 1). It was found that the formation of the colored product increased with time, therefore it was deemed useful to elaborate a kinetically based method for the determination of AML. The following paragraphs describe the conditions under which the reaction of AML with NBD-Cl fulfills the requirements necessary for its spectrophotometric analysis.

Owing to the presence of labile chloride in the chemical structure of NBD-Cl, a daily fresh solution is recommended. The effect of NBD-Cl concentration on the reaction was studied at room temperature (25 ± 5 °C) and away from direct sun or artificial daylight. As shown in Fig. 2, the reaction of AML with NBD-Cl was dependent on the concentration of NBD-Cl reagent. The highest absorbance intensity was attained when the concentration of NBD-Cl reagent in the final solution was
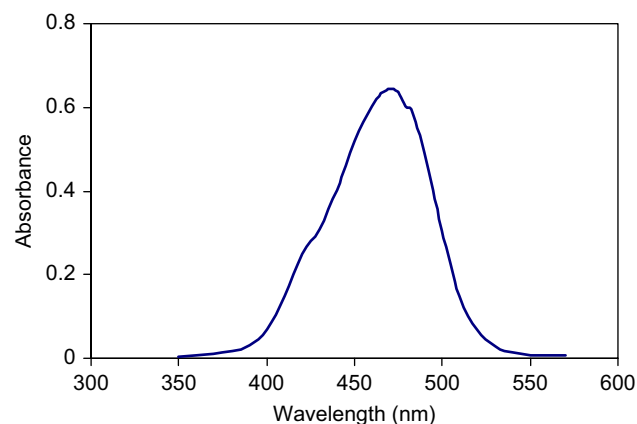

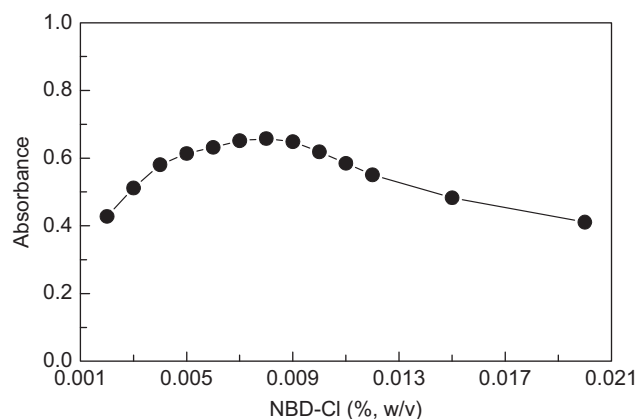**Fig. 1** Absorption spectrum of the reaction product of AML (20 μg/mL) with NBD-Cl (0.08% w/v).

Fig. 2 Effect of NBD-Cl concentration on its reaction with AML (20 μg/mL).

between 0.006 and 0.010% (w/v). Therefore, a concentration of 0.008% (w/v) (1 mL of 0.08% (w/v) working reagent solution) was selected for further experiments.

To generate the nucleophile from AML, different buffers at different pH values (7.0–10.0) were investigated. Best results were obtained in the case of Teorell and Stenhagen buffer at pH 8.6 (Fig. 3). At higher pH values, precipitation of white colloid occurred upon addition of NBD-Cl, high blank readings, non reproducible results, and/or weak sensitivity were observed.

The effect of temperature on the reaction time was studied by performing the reaction at different temperatures (30, 40, 60, 70, 80, and 90 °C) and the results indicated that maximum readings were obtained at 70 °C (Fig. 4). At higher temperature, dramatic decrease in the absorbance values was observed. This was attributed probably to the instability of AML-NBD product at high temperature. NBD-Cl is hydrolyzed in alkaline medium to give NBD-OH which has absorption intensity at 470 nm. Therefore, it was necessary to acidify the reaction mixture to pH 2.0 before carrying out the measurements to decrease the absorbance of the reagent blank [39]. In order to select the most appropriate acid for the acidification of the reaction mixture, different acids were tested including sulphuric, hydrochloric, perchloric, nitric, phosphoric and acetic acids. The results indicated that sulphuric acid was the most suitable acid as it yielded the
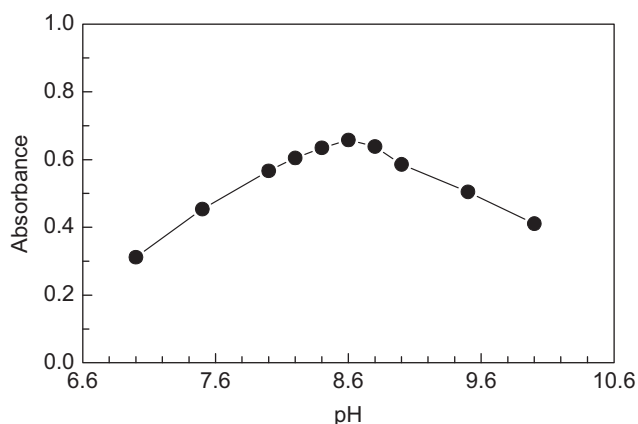
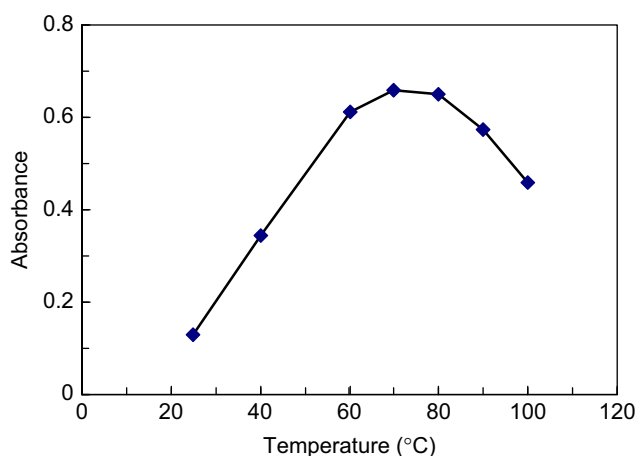**Fig. 3** Effect of pH on the reaction of AML (20 µg/mL) with NBD-Cl.

Fig. 4 Effect of temperature on the reaction of AML (20 µg/mL) with NBD-Cl.

highest absorption intensity, and the optimum volume of concentrated sulphuric acid was 0.2 mL.

In order to select the most appropriate solvent for dilution, different solvents of varying polarities were carefully tested: water, methanol, ethanol, acetonitrile, and acetone. Methanol was found to be an ideal diluting solvent as it afforded maximum absorption intensity, and therefore it was selected for further investigations. A marked hypsochromic shift with hypochromic effect was observed in the absorption spectrum of the colored product with increasing solvent polarity. This finding was in agreement with the fact that polar solvents cause stabilization of the ground state of $n \rightarrow \pi^*$ transition peaks through a hydrogen bonding [40], and was also coincident with previous results [41].

3.2. Stoichiometry and reaction mechanisms

The stoichiometry of the reaction of AML with NBD-Cl was determined by the Job's method [35]. The ratio of AML:NBD-Cl was found to be 1:1, although there are two amino groups available for reacting with NBD-Cl reagent. Based on the differential higher reactivity of the primary amino group than that of the secondary one, the reaction between AML and NBD-Cl was proposed to proceed according to the pathway given in Fig. 5.
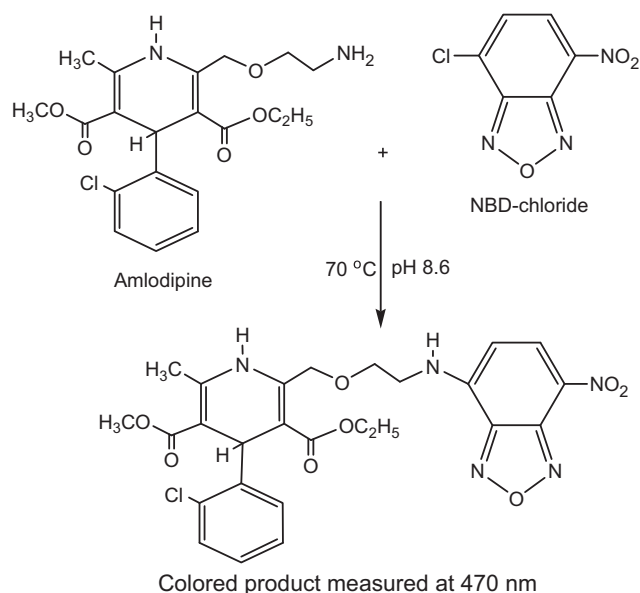

Fig. 5 Pathway for the reaction of AML with NBD-Cl.

3.3. Kinetics of the reaction

Under the above described optimum conditions (Table 1), the absorbance-time curves for the reactions of AML with NBD-Cl were generated (Fig. 6). The initial rates of the reaction were determined from the slopes tangents of the absorption-time curves. The order of the reaction with respect to the analytical reagent was determined by studying the reaction at different concentrations of the reagent with fixed concentration of AML. The plot of the initial rate, dA/dt, against the initial absorbance was linear passing through the origin, indicating that the initial order of the reaction with respect to the analytical reagent was one. The order with respect to AML was evaluated from the measurement of the rates of the reaction at several concentrations of AML at a fixed concentration of the reagent, which were found to be one. However under the optimized experimental conditions, the concentration of AML was determined using relative excess amount of NBD-Cl and the other conditional reagents. Therefore, pseudo-first order conditions were obtained with respect to their concentrations

3.4. Activation energy of the reaction

The activation energy, the minimum kinetic energy that a molecule must possess in order to undergo reaction, can be determined from Arrhenius equation:

$$V = k = Ae^{-Ea/RT}$$

where V (k) is the reaction rate, A is a constant known as frequency factor, Ea is the activation energy, T is the absolute temperature, and R is the gas constant; 1.987 cal/degree mole. The logarithmic form of the above equation is written as follow:

$$\mathrm{Log}\,k = \log A - Ea/2.303RT$$

The activation energy of the kinetic reaction of AML with NBD-Cl was determined by studying the reaction at different temperatures (25, 40, 60, 70, and 80 °C) using fixed concentrations of AML and the reagent. The absorption-time curves

Table 1 Analytical parameters for the initial rate method of the proposed kinetic spectrophotometric method for determination of AML.

Linear range (M)	Least square equation (Log $V = \log K' + n \log C$)[a]		Correlation coefficient (r)	LOD (M)	LOQ (M)
	Intercept ($\log K$)	Slope (n)			
0.55×10^{-5}–8.22×10^{-5} $(5$–$30)$[b]	2.3322	0.9628	0.9998	0.09×10^{-5} (0.35)[b]	0.27×10^{-5} (1.05)[b]

[a] V is the reaction rate, K' is the conditional rate constant, n is the order of reaction, and C is the molar concentration of AML.
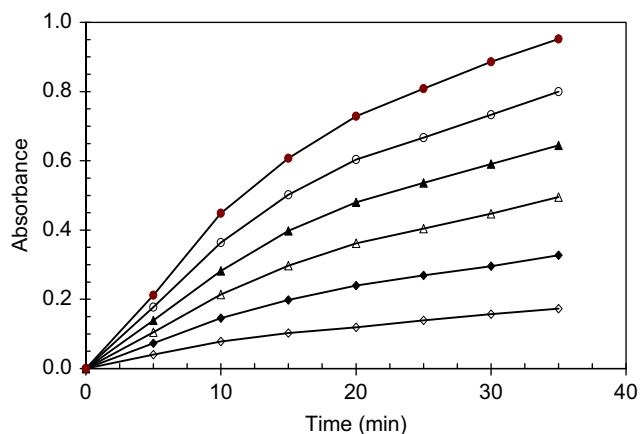[b] Figures in parenthesis are the linear range in µg/mL.

Fig. 6 Absorbance-time curves for the reaction of varying concentrations of AML (0.55×10^{-5}–8.22×10^{-5} M) with NBD-Cl.

Fig. 7 Arrhenious plot for kinetic reaction of AML with NBD-Cl.

at these temperatures were constructed to determine the initial rates, then plotting $1/T$ against $\log k$ to determine the slope of the line; $-Ea/2.303R$ (Fig. 7). The activation energy was found to be 6.74 kcal mole^{-1}. This low activation energy explained that the nucleophilic substitution reaction between AML and NBD-Cl could easily take place under mild conditions, and NBD-Cl could be used as a useful reagent in the spectrophotometric determination of AML.

3.5. Quantitation methods

3.5.1. Initial rate method
The initial rates of AML reaction with NBD-Cl would follow a pseudo-first order, and were found to obey the following equation:

$$V = \Delta A / \Delta t = K' C^n$$

where V is the reaction rate, A is the absorbance, t is the measuring time, K' is the pseudo-first order rate constant, C is the molar concentration of AML, and n is the order of the reaction. The logarithmic form of the above equation is written as follow:

$$\text{Log } V = \log \Delta A / \Delta t = \log K' + n \log C$$

Regression analysis using the method of least square was performed to evaluate the slope, intercept and correlation coefficient. The analytical parameters and results of regression analysis are given in Table 1. Specific rate constant of the kinetic reaction of AML with NBD-Cl (k, at 70 °C) was
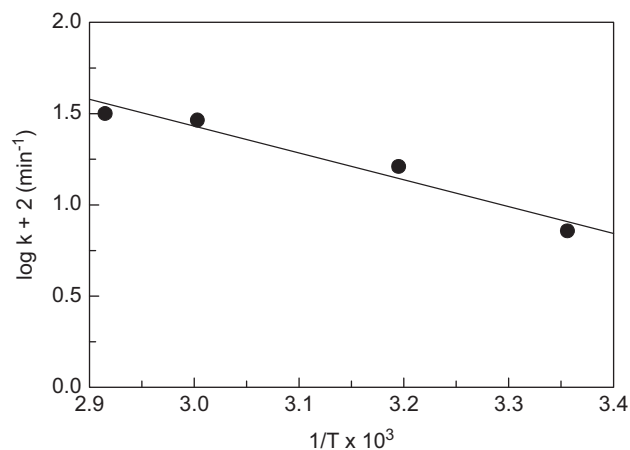
determined and found to be 214.88 min^{-1} or 3.58 s^{-1}. The value of n in the regression equation was 0.9623 (≈ 1), confirming that the reaction of AML with NBD-Cl was first order with respect to the AML concentration. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.35 and 1.05 µg/mL, respectively. These low values confirmed the good sensitivity of the initial rate method and consequently its capability to determine low amounts of AML.

3.5.2. Fixed time method
In this method, the absorbance of the reaction solutions containing varying amounts of AML was measured at a pre-selected fixed time. Calibration plots of absorbance versus the concentrations of AML were established at fixed periods of time for the reaction (Table 2). The regression equations, correlation coefficients, and the LOD and LOQ are given in Table 2. The lowest limits of detection and quantification were obtained with fixed times of 25, 30, and 35 min. However, the fixed time of 5 min showed wider concentration range for quantification. According to the ICH guidelines for validation of analytical procedures [42], the detection limit is not required to be part of the validation. Therefore, on the basis of wider concentration range and less time of analysis, the fixed time of 5 min was recommended for the determination of AML.

3.5.3. Validation of the proposed methods

3.5.3.1. Accuracy and precision. The accuracy and precision of the proposed kinetic spectrophotometric method were

Table 2 Analytical parameters for the fixed-time method of the proposed kinetic spectrophotometric method for the determination of AML.

Reaction time (min)	Linear range (μg/mL)	Correlation coefficient (r)	Slope (b)	SD_b	Intercept (a)	SD_a	LOD (μg ml^{-1})	LOQ (μg ml^{-1})
1	10.0–100.0	0.9995	0.0044	0.0001	0.0021	0.0014	1.06	3.21
5	5.0–75.0	0.9997	0.0067	0.0001	−0.0001	0.0016	0.81	2.47
10	5.0–50.0	0.9986	0.0147	0.0004	−0.0022	0.0031	0.69	2.09
15	4.0–45.0	0.9998	0.0202	0.0002	−0.0025	0.0036	0.59	1.78
20	3.0–35.0	0.9999	0.0243	0.0001	−0.0037	0.0030	0.40	1.22
25	3.0–35.0	0.9994	0.0267	0.0001	0.0034	0.0028	0.35	1.05
30	2.0–30.0	0.9993	0.0291	0.0002	0.0083	0.0032	0.36	1.08
35	2.0–30.0	0.9998	0.0314	0.0003	0.0176	0.0032	0.34	1.02

Table 3 Evaluation of the accuracy and precision of the initial rate and fixed time methods of the proposed kinetic spectrophotometric method for determination of AML.

Amount taken (μg/mL)	Recovery (% ± RSD)[a]	
	Initial rate method	Fixed time method
5.0	100.4 ± 1.76	99.7 ± 1.13
15.0	99.5 ± 1.22	100.6 ± 0.85
30.0	101.1 ± 1.04	100.9 ± 1.42

[a]Recovery was calculated as the amount found/amount taken × 100. Values are mean ± RSD for five determinations.

Table 4 Standard addition method for the determination of AML by the initial rate and fixed time methods of the proposed kinetic spectrophotometric method.

Tablet	Amount added (μg/mL)	Recovery (% ± RSD) [a]	
		Initial rate method	Fixed time method
Alkapress	20	99.2 ± 1.33	98.6 ± 1.11
Regcor	20	98.8 ± 0.79	99.4 ± 0.92

[a]Mean ± RSD for five determinations.

determined at three concentration levels of AML [36] by analyzing five replicate samples of each concentration by both the initial rate and fixed time methods. The relative standard deviations (RSD) for the results ranged from 1.04% to 1.76% and 0.85% to 1.42% for the initial rate and fixed time methods of the proposed kinetic spectrophotometric method, respectively (Table 3), proving the high reproducibility of the results and the precision of the method. This good level of precision was suitable for quality control analysis of AML in its pharmaceutical tablets.

3.5.4. *Analytical recovery and interference liabilities*
The accuracy of the proposed kinetic method was also tested by carrying out the recovery experiments using the standard addition method [43]. Known amounts of pure AML were added to the pre-analyzed AML-containing tablets, and then determined by the recommended kinetic procedures. The obtained recoveries and relative standard deviations were in the range of 98.6–99.4% and 0.79–1.33%, respectively (Table 4). These results prove the accuracy of the proposed methods and absence of interferences from the common excipients. It is worth noting that the proposed kinetic spectrophotometric method was performed in the visible region away from the UV-absorption region of the UV-absorbing interfering excipient materials that might be co-extracted from the AML-containing tablets.

3.5.5. *Robustness and ruggedness*
Robustness was tested by evaluating the influence of small variation in the experimental parameters on the analytical

performance of the method [44]. In these experiments, one parameter was changed while the others were kept constant, and the recovery percentage was calculated each time. It was found that none of these variables significantly affected the performance of the method; the recovery values were between 98.90 ± 1.46% and 101.30 ± 1.92%. This provides an indication of the reliability of the proposed method during the routine application of the proposed method. Ruggedness was also tested by applying the proposed method to the assay of AML using the same operational conditions but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab and day-to-day variations were reproducible, as the relative standard deviations (RSD) did not exceed 2.32%.

3.6. *Application of the proposed methods*

The initial rate and fixed time methods of the proposed kinetic spectrophotometric method for determining AML have been performed on commercial pharmaceutical tablets. The concentration of AML was computed from its corresponding regression equations. The results of the proposed methods (initial rate or fixed time) were statistically compared with those of the reported method [27], in respect to the accuracy and precision. The mean recovery values of the claimed amounts were between 99.55 ± 1.69% and 100.65 ± 1.48% (Table 5). The results of *t*- and *F*-tests indicated no significant differences between both the proposed and the reported methods at 95% confidence level. This indicated similar precision and accuracy in the analysis of AML in tablets.

Table 5 Analysis of AML-containing tablets by the reported and the initial rate and fixed time methods of the proposed kinetic spectrophotometric method.

Tablet	Label claim (% ± RSD)[a]		
	Initial rate method	Fixed time method	Reported method[b]
Alkapress	99.55 ± 1.69 (0.83, 2.07)[c]	100.05 ± 2.01 (0.43, 1.46)	100.65 ± 2.43
Regcor	99.84 ± 1.58 (1.52, 1.08)	100.65 ± 1.48 (0.68, 1.23)	101.39 ± 1.64

[a]Values are mean of five determinations.
[b]Ref. [19].
[c]Figures in parenthesis are the calculated values of t and F at 95% confidence limit, respectively. The tabulated t- and F-values are 2.78 and 6.39, respectively.

4. Conclusion

A simple and sensitive kinetic spectrophotometric method for the determination of AML has been successfully developed and validated. The method was based on the coupling reaction of AML with NBD-Cl. The initial rate and fixed time methods for the proposed kinetic spectrophotometric method can be easily applied to the determination of AML in its pure form and tablets. Also, the activation energy of the proposed kinetic reaction was determined. The proposed method is sensitive enough to enable determination of lower amounts of the drug, and compared with the previously reported methods in terms of accuracy and precision. The proposed method, because it involves measurements in the visible region, is more selective than the reported spectrophotometric methods that involve measurement in ultraviolet region. These advantages encourage the application of the proposed methods in routine analysis of AML in quality control laboratories, as alternatives for the existing methods.

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