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Spectrochimica Acta Part A 70 (2008) 564–570
SPECTROCHIMICA ACTA PART A
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# Validated spectrofluorometric methods for determination of amlodipine besylate in tablets

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Received 3 June 2007; received in revised form 30 July 2007; accepted 31 July 2007

**Abstract**

Two simple and sensitive spectrofluorometric methods have been developed and validated for determination of amlodipine besylate (AML) in tablets. The first method was based on the condensation reaction of AML with ninhydrin and phenylacetaldehyde in buffered medium (pH 7.0) resulting in formation of a green fluorescent product, which exhibits excitation and emission maxima at 375 and 480 nm, respectively. The second method was based on the reaction of AML with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) in a buffered medium (pH 8.6) resulting in formation of a highly fluorescent product, which was measured fluorometrically at 535 nm ($\lambda_{ex}$, 480 nm). The factors affecting the reactions were studied and optimized. Under the optimum reaction conditions, linear relationships with good correlation coefficients (0.9949–0.9997) were found between the fluorescence intensity and the concentrations of AML in the concentration range of 0.35–1.8 and 0.55–3.0 $\mu g\,ml^{-1}$ for ninhydrin and NBD-Cl methods, respectively. The limits of assays detection were 0.09 and 0.16 $\mu g\,ml^{-1}$ for the first and second method, respectively. The precisions of the methods were satisfactory; the relative standard deviations were ranged from 1.69 to 1.98%. The proposed methods were successfully applied to the analysis of AML in pure and pharmaceutical dosage forms with good accuracy; the recovery percentages ranged from 100.4–100.8 ± 1.70–2.32%. The results were compared favorably with those of the reported method.


*Keywords:* Amlodipine; Spectrofluorometry; NBD-Cl; Ninhydrin

## 1. Introduction

Amlodipine besylate (AML), (4*R*,*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 relatively new potent long-acting calcium channel blocking agent [1]. AML is widely used for the treatment of hypertension as well as stable and variant angina [2]. It, rather than β-blockers, is more effective for the variant angina because it prevents and reverses the coronary spasms resulting in increased blood 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]. Owing to the therapeutic importance of AML, numerous different analytical methods have been developed for its quantitative determination in pure, pharmaceutical dosage forms and/or biological fluids. These methods include high-performance liquid chromatography [6–13], high-performance thin layer chromatography [14,15], gas chromatography [16,17], capillary electrophoresis [18], flow injection analysis [19], differential-pulse voltammetry [20], enzyme-linked immunosorbent assay [21], and spectrophotometry [22–30]. These methods, except spectrophotometric methods, offered the required sensitivity and selectivity for the analysis of AML in biological fluids, however their sophisticated instrumentation and high-analysis cost limited their use in quality control laboratories for analysis of AML in its pharmaceutical dosage forms. Moreover, these instruments are not available in most quality control laboratories specially, third world countries. In general, spectrofluorometry is considered one of the most convenient analytical techniques, because of its inherent simplicity, low cost, and wide availability in most quality control laboratories. To the best of our knowledge, there were no reported spectrofluorometric methods on AML in spectrofluorometric analysis. For these reasons, the present study describes two simple, sensitive and economical spectrofluorometric methods

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doi:10.1016/j.saa.2007.07.055

for the analysis of amlodipine in its pure and pharmaceutical dosage forms. The first method was based on its condensation reaction with ninhydrin and phenylacetaldehyde in neutral buffered medium. The second method was based on its reaction with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) reagent in slightly basic buffered medium.

## 2. Experimental

### 2.1. Apparatus

RF-5301 PC spectrofluorimeter (Shimadzu, Japan), with 1-cm matched quartz cells, were used for all measurement. The spectrofluorimeter was set at excitation and emission slit width of 3 nm. Super-mixer (Lab-line Instruments, Inc., USA). Thermostatically controlled water bath (Schetzort, Germany).

### 2.2. Chemicals and reagents

Amlodipine besylate (Hetero Drugs Ltd., Hyderabad, India) was used as received (the purity was 99.2–100.2%). Ninhydrin (WINLAB, Leicestershire, UK) was 0.1% (w/v) in water, freshly prepared daily. Phenylacetaldehyde (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was 0.02% (v/v) in ethanol and the solution was stable for at least 1 week at 4 °C. 7-Chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl; Merck, Darmstadt, Germany) was 0.08% (w/v) in methanol and it was freshly prepared daily. Ascorbic acid (Prolabo, ADWIC, Egypt) was 0.2% (w/v) dissolved firstly in 1 ml water and diluted to 100 ml with dimethylformamide. All solvents and other chemicals used throughout this study were of analytical grade. Water was doubly distilled.

#### 2.2.1. Tablets

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 amlodipine besylate per tablet.

### 2.3. Preparation of solutions

#### 2.3.1. Stock standard solution

An accurately weighed amount (25 mg) of AML was transferred into a 50-ml volumetric flask. The powder was dissolved in 10-ml distilled water for ninhydrin method or dimethylformamide for NBD-Cl method. The solution was then diluted to the mark with the same solvent to obtain a working standard solution of 0.5 mg $ml^{-1}$ of AML. Further dilutions were made to obtain the suitable drug concentrations. The solutions were found to be stable for at least 1 weak when kept in the refrigerator.

#### 2.3.2. Tablets solution

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 into a 50-ml calibrated flask, dissolved in 25 ml water for ninhydrin method or dimethylformamide for NBD-Cl method, swirled and sonicated for 5 min, completed to volume with the same solvent, shaken well for 10 min, and filtered. The procedure was completed as described for preparation of stock standard solution.

#### 2.3.3. Buffer solutions

Teorell and Stenhagen buffer solution [31] of the pH range 5.72–9.61 were prepared in freshly boiled and cooled distilled water. The buffer composed of phosphoric acid, citric acid, 1 M sodium hydroxide, adjusted to the required pH with 0.1 M hydrochloric acid.

### 2.4. General analytical procedures

#### 2.4.1. Ninhydrin method

One milliliter of Torell and Stenhagen buffer solution (pH 7.0) was transferred into test tube, and then 1.0 ml of ninhydrin reagent (0.1%, w/v in water), 1.0 ml of the standard or sample solution (3.5–18 μg $ml^{-1}$), and 1.0 ml of phenylacetaldehyde reagent (0.02%, v/v in ethanol) were added, respectively. The reaction was allowed to proceed at 90 °C in a water bath for 10 min. Then, the test tubes were cooled in ice bath and the contents were quantitatively transferred into 10.0-ml calibrated flasks and the flasks were diluted to the mark with ethanol. The fluorescence intensity of the resulting solutions was measured at 480 nm ($\lambda_{ex}$, 375 nm) against reagent blanks treated similarly.

#### 2.4.2. NBD-Cl method

One milliliter of the standard or sample solution (5.5–30 μg $ml^{-1}$) was transferred into test tube, then 1.0 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 20 min. Then, the test tubes were cooled in ice bath and 1.0 ml of 8 M sulphuric acid was added and mixed well. The contents were quantitatively transferred into 10.0-ml calibrated flasks and the flasks were diluted to the mark with dimethylformamide. The fluorescence intensity of the resulting solutions was measured at 535 nm ($\lambda_{ex}$, 480 nm) against reagent blanks treated similarly.

## 3. Results and discussion

### 3.1. Involved reaction, design and strategy for assays development

Ninhydrin reacts with primary amines and gives a colored and/or fluorescent derivative [31–33]. This derivatization reaction has been used for determination of primary amino groups-containing compounds and has become a classical method because of its reproducibility, accuracy, and low cost of analysis. NBD-Cl reagent is an activated halide derivative that has been used as fluorogenic reagent for determination of secondary and primary amines [31,34].

AML contains a primary aliphatic amino group that was found to react with both ninhydrin in presence of phenylacetaldehyde and NBD-Cl. These condensation reactions have not been

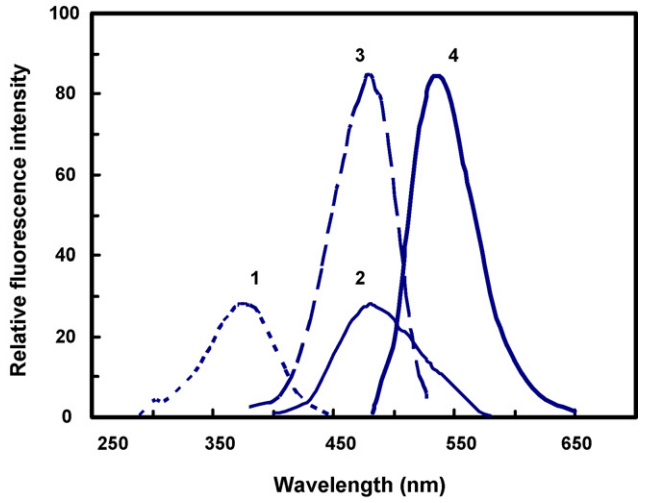


Fig. 1. Excitation and emission spectra of the reaction products of amlodipine with ninhydrin/phenylacetaldehyde (1 and 2) and NBD-Cl (3 and 4) reagents. The concentrations of amlodipine were 1.0 and 1.5 μg ml$^{-1}$ for ninhydrin/phenylacetaldehyde and NBD-Cl, respectively.

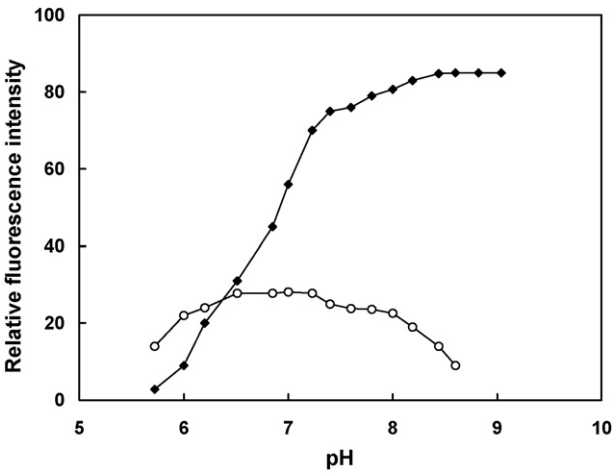


Fig. 2. Effect of pH on the reaction of amlodipine with ninhydrin/phenylacetaldehyde (◯) and NBD-Cl (♦). The concentrations of amlodipine were 1.0 and 1.5 μg ml$^{-1}$ for ninhydrin/phenyl-acetaldehyde and NBD-Cl, respectively.

reported yet for AML. Therefore, we investigated these reactions in the present study. Under the recommended conditions, the derivatized AML was found to be fluorescent product exhibiting highest fluorescence intensity at $\lambda_{ex}$ 375 nm and $\lambda_{em}$ 480 nm (with ninhydrin–phenylacetaldehyde) and at $\lambda_{ex}$ 480 nm and $\lambda_{em}$ 535 nm (with NBD-Cl). Fig. 1 shows the spectral characteristics of the derivatized AML with both reagents.

Preliminary experiments in the present work indicated that these two reactions are nearly identical for both AML base and its corresponding besylate salt. Therefore, the reactions involved in the present study were carried out on the besylate salt rather than the free base of AML.

The following sections describe the optimization of different factors affecting the chemical reactions, and the use of the optimized conditions in the development of the assay procedures.

### 3.2. Optimization of reaction conditions

#### 3.2.1. Ninhydrin method

The factors affecting the reaction conditions (pH, the concentrations of phenylacetaldehyde and ninhydrin reagents, reaction time and temperature, and the diluting solvent) were performed by altering each variable in turn while keeping the others constant.

For investigating the effect of pH, the reaction was performed at different pH values (5.72–9.61). The results indicated that the fluorescence intensity was pH dependent (Fig. 2). The optimum pH was found to be 7.0.

The fluorescence forming reaction was also studied as a function of phenylacetaldehyde and ninhydrin reagents concentrations. The results revealed that the fluorescence intensity was dependent on both reagents (Fig. 3). The higher fluorescence intensity was attained when the concentration of phenylacetaldehyde in the final measured solution was between 0.0015 and 0.0025%, v/v; a concentration of 0.002%, v/v was selected for further experiments. While, the optimum concentration of ninhydrin reagent in the final measured solution was 0.01%, w/v and it was selected for further experiments.

The effect of temperature on the reaction time was also studied by performing the reaction at different temperatures (30, 40, 60, 70, 80, 90, and 100 °C) and the results indicated that maximum readings were obtained at 80 °C (Fig. 4). For more precise readings further experiments were carried out at 90 °C. Moreover, the results obtained from optimizing the reaction time indicated that the maximum fluorescence intensity was attained after 10 min (Fig. 5). Longer reaction time decreased the fluorescence intensity. Therefore, further experiments were carried out at 90 °C for 10 min.

In order to select the most appropriate solvent for dilution, different solvents were tested: water, methanol, ethanol, ace-

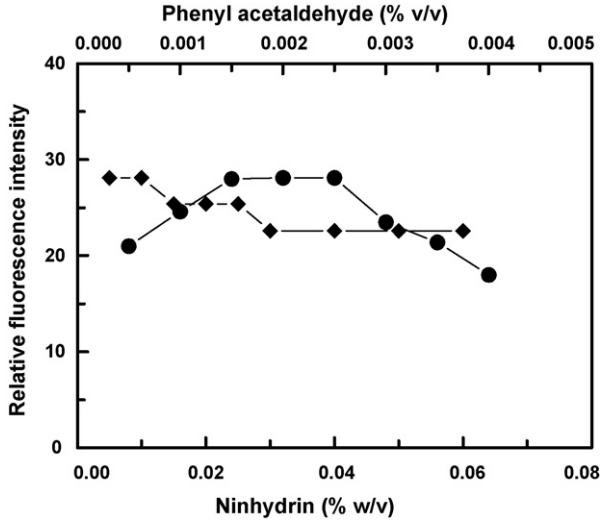


Fig. 3. Effect of ninhydrin (♦), and phenylacetaldehyde (●) reagent concentrations on their reaction with amlodipine (1.0 μg ml$^{-1}$).

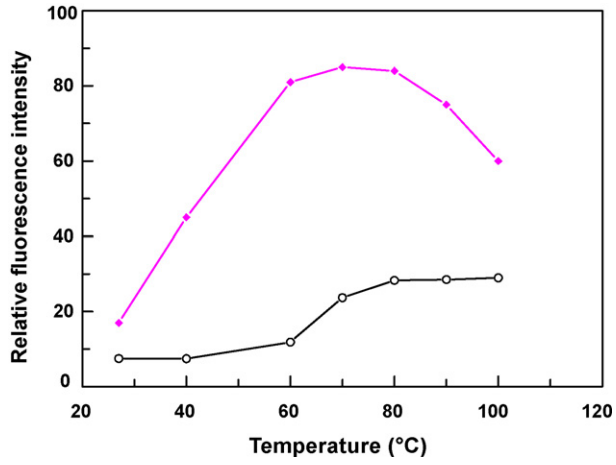


Fig. 4. Effect of temperature on the reaction of amlodipine (1.0 μg ml$^{-1}$) with ninhydrin/phenylacetaldehyde (◯) and NBD-Cl (♦) reagents.

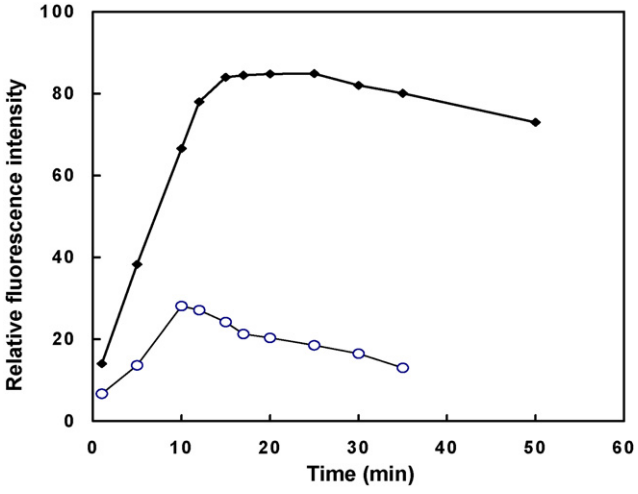


Fig. 5. Effect of time on the reaction of amlodipine with ninhydrin/phenylacetaldehyde (◯) and NBD-Cl (♦). The concentrations of amlodipine were 1.0 and 1.5 μg ml$^{-1}$ for ninhydrin/phenylacetaldehyde and NBD-Cl, respectively.

tonitrile, dimethylformamide, dimethylsulphoxide, and acetone. Ethanol was found to be an ideal diluting solvent (Table 1) as it afforded maximum sensitivity, and therefore it was selected for further investigations.

Table 1
Effect of solvents on the fluorescence intensity of the reaction product of amlodipine with ninhydrin/phenylacetaldehyde and NBD-Cl reagents

| Solvent | RFI[a] | |
|---|---|---|
| | Ninhydrin method | NBD-Cl method |
| Water | 15.6 | 15.0 |
| Methanol | 26.4 | 20.0 |
| Ethanol | 28.11 | 32.0 |
| Acetonitrile | 12.7 | 28.1 |
| Dimethylformamide | 8.8 | 84.8 |
| Dimethylsulphoxide | 8.9 | 78.0 |
| 1,4-Dioxane | – | 40.0 |
| Acetone | 9.4 | 44.1 |

[a]Values for all solvents are mean of three determinations; the R.S.D. for the readings were <2%.

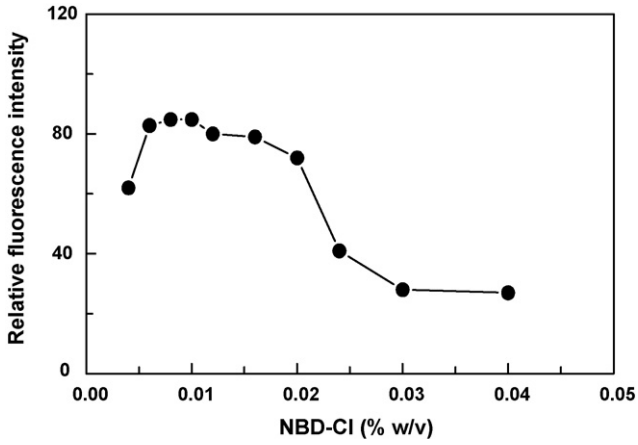


Fig. 6. Effect of the concentration of NBD-Cl reagent on its reaction with amlodipine (1.5 μg ml$^{-1}$).

### 3.2.2. *NBD-Cl method*

The factors affecting the reaction between AML and NBD-Cl reagent were also studied.

Investigating the effect of pH indicated that the fluorescence intensity was pH dependent and the optimum pH was found to be 8.6 (Fig. 2). Studying the effect of different NBD-Cl reagent concentrations on the produced fluorescence intensity revealed that highest fluorescence intensity was attained when the concentration of NBD-Cl reagent in the final solution was between 0.006 and 0.010%, w/v (Fig. 6). Therefore, a concentration of 0.008%, w/v was selected for further experiments.

With respect to the influence of temperature on the reaction between AML and NBD-Cl reagent, the results showed that increasing the temperature to 70 °C accelerated the reaction. Besides the effect of temperature also the influence of time was studied in order to optimize the assay conditions. The results revealed that the maximum intensity was attained after 20 min (Fig. 5). Longer reaction time decreased the fluorescence intensity. Therefore, further experiments were carried out at 70 °C for 20 min. Dimethylformamide was found to be the best diluting solvent (Table 1), and therefore it was selected for further investigations.

NBD-Cl is hydrolyzed in alkaline medium to give NBD-OH which has excitation and emission maxima at 462 and 532 nm, respectively. Therefore, it was necessary to acidify the reac-

Table 2
Effect of acids on the fluorescence intensity of the reaction product of amlodipine with NBD-Cl

| Acid | RFI[a] |
|---|---|
| Hydrochloric | 61.3 |
| Sulphuric | 84.8 |
| Nitric | 62.3 |
| Perchloric | 55.0 |
| Phosphoric | 20 |
| Acetic | 8.5 |

[a]Values for all solvents are mean of three determinations; the R.S.D. for the readings were <2%.

tion mixture to pH 2.0 before carrying out the measurements to quench the emission of the reagent blank [35]. In order to select the most appropriate acid for the acidification of the reaction mixture, different acids were studied (Table 2). The results indicated that sulphuric acid was the most suitable acid as it gave the highest fluorescence intensity.

### 3.3. Stoichiometry of the reactions

The stoichiometry of the reactions was studied adopting the limiting logarithmic method [36]. With respect to ninhydrin method, two straight lines were obtained. The first line was obtained by increasing the concentration of ninhydrin while keeping the concentration of AML constant. The second line was obtained by increasing the concentration of AML while keeping the concentration of ninhydrin constant. The values of the slopes of the two lines were 0.9127 and 0.8849 (Fig. 7; lines 1 and 2), indicating that the molar ratio of ninhydrin:AML was 1:1. Accordingly, the reaction pathway was proposed as described in Fig. 8. With respect to NBD-Cl method, values of the slopes of the two lines were 0.5863 and 0.5593 (Fig. 7, lines 3 and 4) proving that the molar ratio of NBD-Cl: AML was also 1:1, and the reaction pathway was proposed as in Fig. 8.

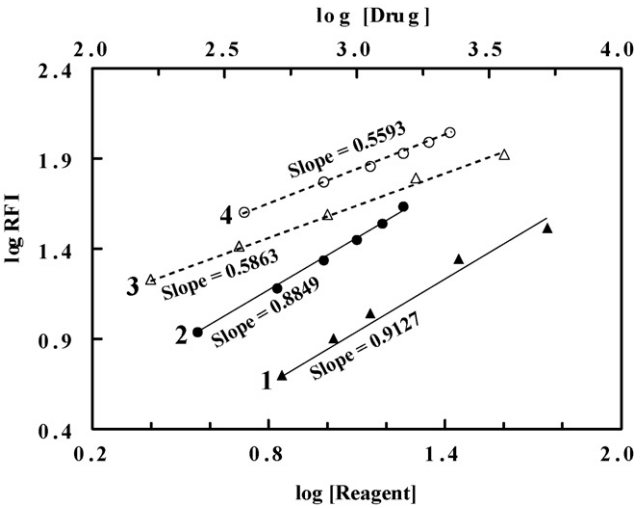


Fig. 7. Limiting logarithmic plots for the molar reactivity of amlodipine with both ninhydrin and NBD-Cl reagents: (1, ▲) log[fluorescence intensity] vs. log[ninhydrin] with [amlodipine] kept at $1.76 \times 10^{-5}$ M; (2, ●) log[fluorescence intensity] vs. log[amlodipine] with [ninhydrin] kept at $2.81 \times 10^{-3}$ M; (3, △) log[fluorescence intensity] vs. log[NBD-Cl] with [amlodipine] kept at $2.64 \times 10^{-5}$ M; (4, ○) log[fluorescence intensity] vs. log[amlodipine] with [NBD-Cl] kept at $4 \times 10^{-3}$ M.

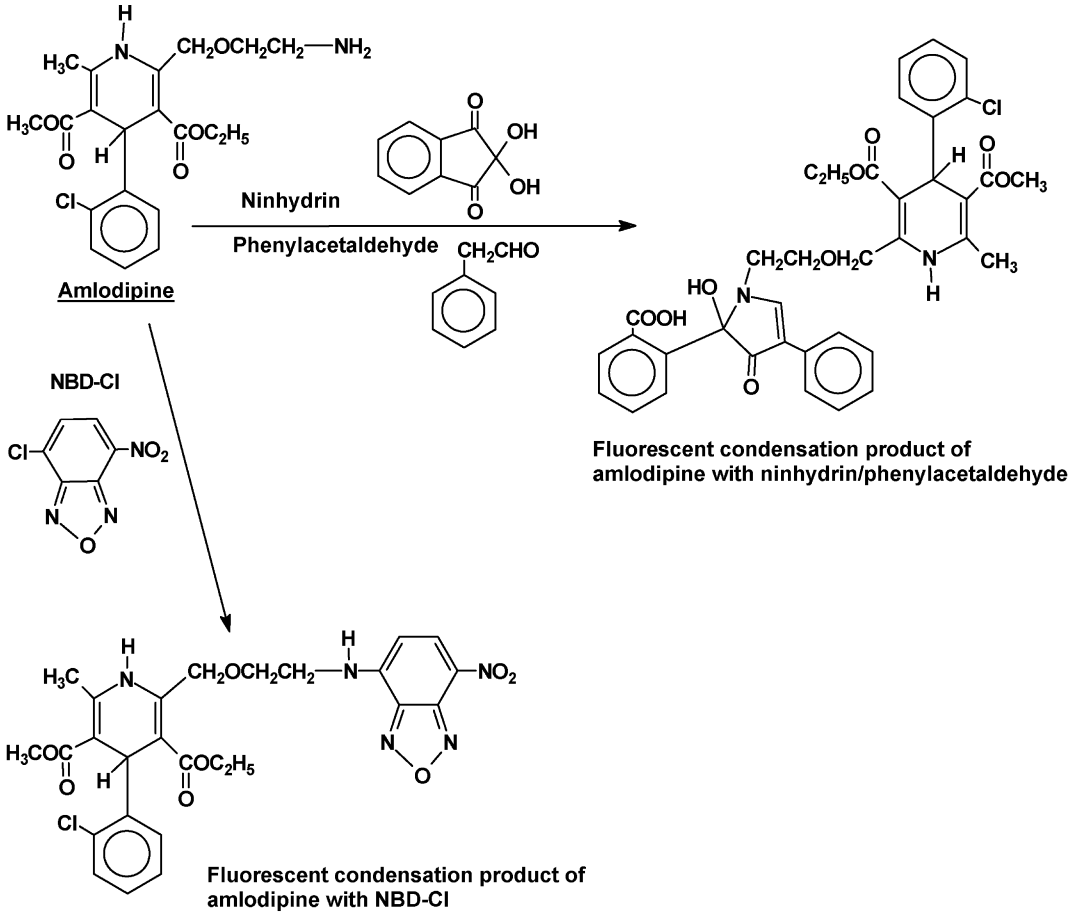


Fig. 8. The reaction pathway of amlodipine with each of ninhydrin/phenylacetaldehyde and NBD-Cl reagents.

Table 3
Quantitative parameters for the proposed spectrofluorometric methods for analysis of amlodipine by reaction with ninhydrin/phenylacetaldehyde and NBD-Cl reagents

| Method | Range (μg ml$^{-1}$) | Intercept ± S.D. | Slope ± S.D. | Correlation coefficient | LOD (μg ml$^{-1}$) | LOQ (μg ml$^{-1}$) |
|---|---|---|---|---|---|---|
| Ninhydrin | 0.35–1.8 | 2.1701 ± 0.7871 | 0.0259 ± 0.0007 | 0.9985 | 0.091 | 0.304 |
| NBD-Cl | 0.55–3.0 | 33.00 ± 1.8710 | 0.0345 ± 0.0008 | 0.9997 | 0.163 | 0.542 |

### 3.4. Validation of the proposed methods

#### 3.4.1. Linearity and sensitivity

Under the specified optimum reaction conditions, the calibration curves for AML with the different analytical reagents employed in the present work were constructed by measuring a series of eight concentrations of the standard solutions of AML. All measurements were carried out using six replicate measurements ($n = 6$). The assays were carried out according to the general procedures previously established for both ninhydrin and NBD-Cl methods. In all cases, standard curves were linear with acceptable intercepts and very good correlation coefficients in the general concentration range of 0.35–1.80 and 0.55–3.00 μg ml$^{-1}$ for ninhydrin and NBD-Cl methods, respectively (Table 3).

The LOD was 0.09 and 0.16 μg ml$^{-1}$, while the LOQ was 0.30 and 0.54 μg ml$^{-1}$ for ninhydrin and NBD-Cl methods, respectively. Limits of detection (LOD) and limits of quantitation (LOQ) were determined using the formula: LOD or LOQ = $\kappa$SDa/$b$, where, $\kappa = 3$ for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and $b$ is the slope [37]. Comparing the LOD and LOQ values for both methods showed that the sensitivity of ninhydrin method is relatively higher than NBD-Cl methods.

#### 3.4.2. Precision

The precisions of the two methods were estimated by measuring six replicate samples of AML. The assays gave satisfactory results; the relative standard deviations (R.S.D.) were less than 2. This level of precision of the proposed methods was adequate for the quality control analysis of AML in its pharmaceutical dosage forms.

#### 3.4.3. Ruggedness and robustness

The ruggedness of the proposed methods was assessed by applying the procedures by different analysts at different elapsed time. Results obtained from analyst-to-analyst and day-to-day variations were found to be reproducible as R.S.D. did not exceed 3%.

Table 4
Influence of small variation in the assay conditions on the analytical performance of the ninhydrin and NBD-Cl methods for analysis of amlodipine

| Variation in | %Recovery ± S.D.[a] |
|---|---|
| Ninhydrin method | |
| Optimum conditions[b] | 101.7 ± 1.63 |
| pH | |
| 6.85 | 100.6 ± 1.60 |
| 7.23 | 100.7 ± 1.58 |
| Ninhydrin concentration (%, w/v) | |
| 0.005 | 101.5 ± 1.43 |
| 0.015 | 102.8 ± 1.84 |
| Phenylacetaldehyde concentration (%, v/v) | |
| 0.0015 | 101.3 ± 1.73 |
| 0.0025 | 101.7 ± 1.60 |
| Reaction time (min) | |
| 8 | 98.2 ± 1.59 |
| 12 | 99.8 ± 1.56 |
| NBD-Cl method | |
| Optimum conditions[c] | 100.76 ± 2.31 |
| pH | |
| 8.44 | 98.62 ± 2.21 |
| 8.82 | 100.8 ± 2.15 |
| NBD-Cl concentration (%, w/v) | |
| 0.006 | 99.70 ± 1.38 |
| 0.010 | 99.76 ± 1.13 |
| Reaction time (min) | |
| 15 | 100.16 ± 1.91 |
| 25 | 100.78 ± 2.00 |

[a] Each value is the mean of three determinations.
[b] The conditions were pH 7.0, ninhydrin reagent of 0.01%, w/v, phenylacetaldehyde reagent of 0.002% (v/v) and reaction time of 10 min at 90 °C.
[c] The conditions were pH 8.6, NBD-Cl reagent of 0.008%, w/v, and reaction time of 20 min at 70 °C.

Robustness of the procedures was assessed by evaluating the influence of small variation in experimental variables: concentrations of phenylacetaldehyde, ninhydrin and NBD-Cl reagents, reaction time, and pH on the analytical performance of the methods. In these experiments, one experimental

Table 5
Analysis of amlodipine in tablets

| Tablets | Recovery (% ± S.D.)[a] | | |
|---|---|---|---|
| | Ninhydrin method | NBD-Cl method | Reported method [23] |
| Alkapress | 101.77 ± 2.15 (0.84, 1.27)[b] | 100.77 ± 2.32 (0.08, 1.10) | 100.65 ± 2.43 |
| Regcor | 100.58 ± 1.70 (0.85, 1.08) | 100.43 ± 2.25 (0.85, 1.90) | 101.39 ± 1.64 |

[a] Values are mean of six determinations.
[b] Values in parenthesis are the calculated values of $t$ and $F$; the tabulated values at 95% confidence limit are 2.228 and 5.051, respectively.

parameter was changed while the other parameters were kept unchanged, and the recovery percentage was calculated each time (Table 4). The small variations in any of the variables did not significantly affect the results. This gave an indication for the reliability of the proposed methods during routine work.

### *3.4.4. Analysis of pharmaceutical dosage forms and accuracy testing*

AML tablets were subjected to the analysis by the proposed, as well as the reported methods [23] and the obtained results were statistically compared with each other. The mean percentage recoveries, relative to the labelled amounts, obtained by the proposed methods ranged from 100.43–101.77 ± 1.70–2.32% (Table 5). With respect to *t*- and *F*-tests, no significant differences were found between the calculated and theoretical values of both the proposed and the reported methods at 95% confidence level. This indicated similar accuracy and precision in the analysis of AML in tablets.

## 4. Conclusion

The present study described two validated spectrofluorometric methods for the analysis of AML in its tablets. These methods were simple, rapid, accurate, and reliable for the determination of AML in tablets without interference from the common excipients. The proposed methods are of great value in quality control analysis of AML owing to their improved simplicity, sensitivity, low-cost, and their independence on expensive instruments, or critical analytical reagents.

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