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Hantzsch pre-column derivatization for simultaneous determination of alendronate sodium and its pharmacopoeial related impurity: Comparative study with synchronous fluorometry using fluorescamine

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ABSTRACT

High performance liquid chromatographic (HPLC) method with a pre-column derivatization based on Hantzsch condensation reaction was applied for simultaneous determination of alendronate sodium (ALN) and its main related impurity, 4-Aminobutanoic acid (ABA) at its pharmacopeial limit. The separation of colored condensation products of ALN and ABA were achieved on Agilent Zobrax Eclipse SB-C18 analytical column (250 × 4.6 mm, 5 μm) using a mobile phase composed of acetonitrile–0.1 M acetate buffer, pH 5.0 (15:85, v/v). The flow rate was 1 mL min^{-1}. The detection was carried out at 340 nm using photo-diode array detector. Peak areas were used for the linear regression line in the range of 10–500 and 0.2–40 μg mL^{-1} for ALN and ABA, respectively. Different conditions for the optimization of the derivatization reactions as well as for the HPLC measurement were studied. The proposed method was validated for linearity, precision, accuracy, specificity and robustness. This method was used to check the purity of ALN in the presence of ABA (related impurity) at the pharmacopeial limit (0.5%). For comparison purpose, another method was proposed which involves synchronous fluorescence measurement after ALN reaction with *fluorescamine*. In this method, the third derivative synchronous spectra were estimated as peak to peak measurement from 339 to 370 nm for ALN determination with LOD and LOQ of 24 and 73 ng mL^{-1}, respectively, showing very high sensitivity. Both methods have been applied for determination of the alendronate sodium (ALN) in bulk and pharmaceutical preparations without interference of additives in tablets or oral solution.

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1. Introduction

Alendronate sodium (ALN) is an amino bisphosphonate compound which is used for the treatment of a variety of bone diseases including osteoporesis, Paget's disease and hypocalcemia associated with malignancy [1].

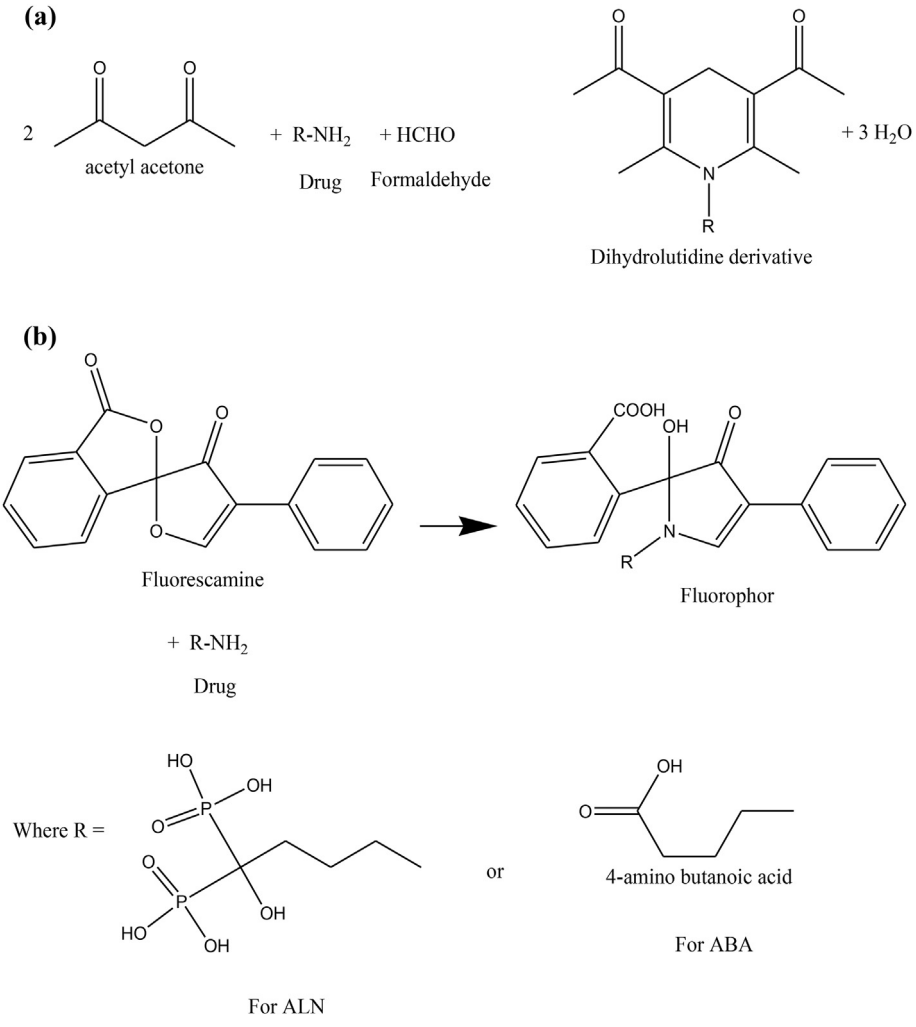
ALN is designated chemically as (4-amino-1-hydroxybutylidene) biphosphonic acid monosodium salt trihydrate [2] (Scheme 1). ALN was prepared by reacting 4-aminobutanoic acid with phosphorous acid and a halophosphorous compound, in the presence of diphenyl ether to obtain alendronic acid then alendronic acid was treated with sodium hydroxide to obtain alendronate sodium [3]. So, 4-aminobutanoic acid (ABA, Scheme 1) is expected to be a possible impurity in ALN raw material and pharmaceutical preparations which has a specified pharmacopeial limit.

Determination of ALN represents a real analytical challenging example because its structure lacks any chromophore, and hence it cannot be determined by direct spectrophotometric or HPLC-UV methods. ALN is official in the BP 2013 [4] and USP 36 [5]. These official methods exhibit some difficulties and need sophisticated instruments. In USP method [5], the reagent used for pre-column derivatization of ALN is very toxic and carcinogenic. Furthermore, separation from excess reagent is needed by performing an extraction step using methylene chloride. The BP method [4] depends on assay of ALN using ion exchange chromatography, which consumed time as the equilibration of the column takes a long time.

Literature reviews have been reported some methods for ALN assay, most of them depend on derivatization steps. These methods include: Spectrophotometric methods [6–16], HPLC methods [8,17,18], voltammetric method [19], fluorometric methods [20,21] and capillary electrophoresis method [22].

Fluorometry is considered one of the most accessible analytical techniques, because of its low cost, wide availability in most quality control laboratories and high sensitivity. Synchronous fluorescence spectroscopy has several advantages [23], such as high selectivity, simple spectra, low interference, etc. In addition, derivative synchronous fluorometry is more advantageous than differentiation of the conventional direct fluorometry in terms of sensitivity [24].

However, a comprehensive literature survey except the pharmacopeial ones revealed the lack of a selective method



Scheme 1 – Schematic illustration of chemical reactions of (a) ALN and ABA with the reagents in Hantzsch condensation reaction or (b) ALN with fluorescamine.

for the simultaneous determination of ALN and its related impurity, ABA. Moreover, no synchronous fluorometric method has been applied previously for the assay of ALN in pharmaceutical preparations. Furthermore, only one spectrophotometric method has been reported for the assay of ALN in oral solution [16], however, it is tedious and time consuming because of the extraction steps requirement for condensed product after derivatization reaction.

The aim of this work is to develop two validated methods for determination of ALN in bulk and pharmaceutical preparations (tablets and oral solution). The first method (method I) depends on the application of a pre-column HPLC-DAD derivatization technique based on Hantzsch condensation reaction. This proposed method allowed the simultaneous determination of ALN and its main related impurity, ABA at the pharmacopeial limit of 0.5%. The second method (method II) is achieved by applying the third derivative synchronous fluorometry following the derivatization with fluorescamine. Both methods were validated in compliance with ICH guidelines.

2. Experimental

2.1. Material and reagents

Pharmaceutical grade of alendronate sodium (99.85%) was kindly supplied by Borg Pharmaceutical Industries, Alexandria, Egypt. All the reagents used are of analytical grade. 4-aminobutanoic acid (Merck KGaA, Darmstadt, Germany), fluorescamine (Sigma–Aldrich, Hamburg, Germany), boric acid, sodium hydroxide, sodium acetate trihydrate, glacial acetic acid (El-Nasr Chemical Ind. Co., Cairo, Egypt).

All pharmaceutical preparations are purchased from commercial market. Fosamax® tablets labeled to contain 10 mg ALN/tablet (MSD, Merck Sharp & Dohme Pharmaceutical Co., New Cairo, Cairo, Egypt). Borgalendro® Oral solution labeled to contain 91.35 mg ALN (equivalent to 70 mg alendronic acid) per 75 mL (BORG pharmaceutical Industries, Borg El Arab New City, Alexandria, Egypt).

2.2. Apparatus

The HPLC system (Agilent, Germany) consisted of Agilent 1200 Series Quaternary pump G1311A which comprises a solvent cabinet, an Agilent 1200 Series Vacuum Degasser G1322A and a four-channel gradient pump; Agilent 1200 Series Diode Array and Multiple Wavelength detector G1315D. The LC system is equipped with Agilent 1200 Series Thermostated Column Compartment G1316A and Agilent 1200 Series Manual Injector which uses a Rheodyne 7725i7-port sample injection valve and fitted with a 20 μL sample loop. LC separations were performed on Agilent Zobrax Eclipse SB-C18 analytical column (250 × 4.6 mm, 5 μm) column.

Fluorescence spectra and measurements were carried out on a Perkin–Elmer LS45 Luminescence Spectrometer, equipped with 1-cm quartz cell. The instrument is interfaced to a computer loaded with FL WinLab software and the computer is connected to a Hewlett–Packard DeskJet 640C printer.

2.3. Preparation of standard solutions

For method I, stock solutions were prepared by dissolving ALN and ABA in distilled water to obtain stock solutions having concentrations of 2 mg mL^{-1}. For method II, stock solution of 1 mg mL^{-1}ALN was prepared in water then further dilution with water was performed to obtain 50 μg mL^{-1} working solution. The solutions were kept in a refrigerator at 4 °C.

2.4. Preparation for reagents

2.4.1. Preparation of 0.1M acetate buffer, pH5

A weight of 13.6 g of sodium acetate trihydrate was dissolved in 800 mL distilled water. The pH was adjusted to 5 using glacial acetic acid. The volume was completed with distilled water to 1 L and the solution was mixed well.

2.4.2. Preparation of pre-column derivatizing reagent

The derivatizing reagent was freshly prepared by mixing 6 mL of distilled water, 1 mL of 0.1 M acetate buffer (pH 5), 1 mL of formaldehyde and 2 mL of acetyl acetone. Four drops of formaldehyde were added till the solution become clear.

2.4.3. Preparation of fluorescamine

Fluorescamine reagent was prepared as 0.6 mg mL^{-1} in acetonitrile.

2.4.4. Preparation of borate buffer, pH 9.8

Boric acid solution (0.1 M) was prepared by dissolving 0.6183 g in 80 mL distilled water and its pH was adjusted to 9.8 using 1M NaOH then the volume was completed to 100 mL with distilled water.

2.5. Chromatographic conditions

- Mobile phase: A mixture of acetonitrile: 0.1 M acetate buffer (pH 5) in the ratio of 15:85 (v/v), filtered using a 0.45 mm pore size membrane filter then degassed prior to use.
- Flow rate: 1 mL min^{-1}.
- Wavelength of detection: 340 nm using photo-diode array detector
- Temperature: ambient
- Injection volume: 20 μL.

2.6. Procedure

2.6.1. Method I (Pre-column derivatization)

2.6.1.1. Blank solution preparation. The blank solution was prepared by mixing 3 mL of distilled water with 1 mL of pre-column derivatizing reagent into a 10-mL screw capped test tubes. Each test tube was heated in water bath adjusted at 55 °C for 15 min. The solution was cooled, transferred quantitatively into a 10-mL volumetric flask and completed to volume with 0.1 M acetate buffer.

2.6.1.2. *Construction of calibration curves*. Different aliquots of each stock solution were transferred into 10 mL screw-capped test tubes and volumes were completed to 3 mL using distilled water. Then, 1 mL of pre-column derivatizing reagent was added to each test tube. The test tube was heated in a water bath (55 °C) for 15 min. The yellow coloured solutions produced were cooled, transferred quantitatively into 10 mL volumetric flasks and completed to volume with 0.1 M acetate buffer to obtain different working standard solutions of ALN and ABA in the ranges of 10.0–500 μg mL^{-1} and 0.20–40 μg mL^{-1}, respectively. Each concentration was injected in triplicate of 20 μL volumes and chromatographed using the optimized conditions. The peak areas were obtained and used for constructing the calibration plot for each compound against the corresponding concentration.

2.6.1.3. *Analysis of ALN and ABA mixtures*. Working standard mixtures' solutions of ALN and ABA were prepared by mixing different aliquots of their stock solutions to give final concentrations within the linearity range of each drug with ratios of 1:25, 1:50 and 1:200 of ABA:ALN, respectively. To these different aliquots, 1 mL of the pre-column derivatizing reagent was added and the procedure was continued as described under the section of construction of calibration curves.

2.6.2. *Method II (Derivative synchronous fluorometry)*
Accurately measured aliquots of ALN working solution (50 μg mL^{-1}) were transferred into separate 5-mL volumetric flasks to obtain a series of ALN solutions covering the working range of 0.1–1.5 μg mL^{-1} in the final solution. Addition of 2.5 mL borate buffer of pH 9.8 followed by 0.8 mL of fluorescamine solution (0.6 mg mL^{-1}) was done to each flask. Then the flasks were completed to volume with acetonitrile. A reagent blank was prepared similarly using water instead of the ALN working stock solution.

The synchronous spectra of the ALN solutions after the derivatization reaction were obtained by scanning both monochromators at $\Delta\lambda = 100$ nm. The scan rate was kept 600 nm min^{-1} and the excitation and emission slits were 10 nm. Derivative spectra were generated by Excel for Microsoft Windows ($\Delta\lambda = 20$ nm).

Calibration curve was obtained by plotting the concentrations versus the third derivative of synchronous fluorometric peak amplitudes from 339 to 370 nm.

2.7. Assay of ALN pharmaceutical preparations

2.7.1. *Analysis of ALN in tablets*
Ten Fosamax® tablets were weighed and finely powdered. A portion of the tablet powder equivalent to 50 mg ALN was accurately weighed and transferred into a 25-mL volumetric flask using 15 mL water. The sample solution was sonicated for 20 min. Dilution was made to volume in the flask with distilled water followed by filtration through Whatman No. 1 filter paper to give solution of 2 mg mL^{-1} ALN. Portions of 150 μL of this solution were transferred into 10 mL screw-capped test tubes (to give final concentration of 30 μg mL^{-1} ALN) and the procedure was then completed as mentioned under construction of calibration curves.

For method II, further dilution was done using distilled water to prepare 50 μg mL^{-1} working solution from which portions of 0.1 mL were transferred into 5-mL volumetric flasks (to give final concentration of 1 μg mL^{-1}) and the procedure was then completed as mentioned under construction of calibration curves.

2.7.2. *Analysis of ALN in oral solution*
For method I, an accurate volume of Borgalendro® Oral solution equivalent to 0.3 mg was directly transferred into 10 mL screw-capped test tubes and volumes were completed to 3 mL using distilled water. Then, 1 mL of pre-column derivatizing reagent was added to each test tube. The test tube was heated in a water bath (55 °C) for 15 min. The yellow coloured solutions produced were cooled, transferred quantitatively into 10 mL volumetric flasks, the volumes were completed with the 0.1 M acetate buffer to obtain 30 μg mL^{-1} solution of ALN. Portions of 20 μL of this solution was chromatographed using the chromatographic conditions mentioned above.

For method II, an accurate volume of Borgalendro® Oral solution equivalent to 5 mg ALN was transferred into a 100-mL volumetric flask using 60 mL water, sonicated for 5 min (for mixing), completed to mark with distilled water giving final concentration of 50 μg mL^{-1}. Portions of 0.1 mL of this solution were transferred into 5-mL volumetric flasks (to give final concentration of 1 μg mL^{-1}) and the procedure was then completed as mentioned under construction of calibration curves.

3. Results and discussion

3.1. Method I (Pre-column derivatization)

Pre-column derivatization method based on Hantzsch condensation reaction was applied in which the 1^{ry} aliphatic amine group in both ALN and its related impurity, ABA, react with acetyl acetone and formaldehyde via Hantzsch condensation reaction to produce yellow coloured products (Scheme 1). However, both drugs exhibit maximum absorbance at 340 nm.

Therefore, the application of separation technique like HPLC following the pre-column derivatization reaction was adopted to permit the separation of condensation products of ALN and ABA and their simultaneous determination using diode array detector at 340 nm.

3.1.1. *Optimization of the pre-column derivatization reaction*
The influence of different reaction conditions, such as reaction temperature, buffer pH, buffer type, reaction time and reagent volume, on the derivatization reaction was studied to achieve maximum sensitivity and selectivity of the proposed method. This was done chromatographically, by checking the effect of parameters on area and sharpness of ALN condensation product peak and retention times for peaks of condensation product of ALN and blank.

3.1.1.1. *Effect of reaction temperature*. Different temperatures were studied (25, 40, 55, 80 and 95 °C) while keeping other parameters constant. Results indicate that at low temperatures, no reaction products were produced. While as the temperature increased, the peak area of ALN condensation

product increased, and thus increasing sensitivity, however the blank shows broader peak. The optimum reaction temperature was found to be 55 °C as sharp chromatographic peaks with suitable peak area that were well separated from the blank peak was obtained.

3.1.1.2. *Effect of buffer pH*. Various pH values of acetate buffer were tried. The optimum pH value of 5 was chosen which gave high peak area of ALN condensation product (Fig. 1a).

3.1.1.3. *Effect of buffer type*. Different buffer types (as acetate, phosphate and citrate) adjusted at pH 5 were tried. Both acetate and citrate buffer gave higher peak area than phosphate but acetate buffer gave the sharpest chromatographic peak.

3.1.1.4. *Effect of the reaction time*. Different reaction times (5, 10, 20, 30, 40 and 60 min) were tried. It was found that the best reaction time that gave maximum sensitivity and sharp peaks was 15 min. But, changing reaction time has no effect on the retention time of ALN peak (Fig. 1b).

3.1.1.5. *Effect of reagent volume*. Different volumes of the reagent were studied. The effects of reagent volumes on chromatographic peak were shown in (Fig. 1c). It was found that the reagent volume has great effect on peak area of ALN condensation product. On the other hand, the same retention times of both peaks were obtained for each volume used. It was found that using 1 mL of reagent gave highest chromatographic peak of ALN condensation product (Fig. 1c).

3.1.2. *Optimization of HPLC conditions*

The main target of the proposed HPLC method was to get maximum separation of closely eluting peak of ALN condensation product from ABA condensation product peak. During method development, various experimental conditions were studied and optimized such as, using different mobile phase compositions and different flow rates as followed:

3.1.2.1. *Effect of mobile phase components ratio*. Different ratios of acetonitrile were tried in the mobile phase consisting of acetonitrile and acetate buffer pH 5. Fig. 1d shows the retention times of the blank and condensation products of ALN and ABA peaks as a function of acetonitrile %. It was found that, at lower concentrations of acetonitrile (<15%) separation occurred but with increased retention times for all peaks. At 20% of acetonitrile, the ALN condensation product peak is well separated from blank peak but partially overlapped with ABA condensation product peak. Increasing acetonitrile concentration (>20%) led to loss of resolution and overlapped peaks. It was found that, the optimum resolution within reasonable total running time was achieved using 15% acetonitrile.

3.1.2.2. *Effect of flow rate*. Different flow rate values of 0.5, 1, 1.5 and 2 mL min^{-1} were tried. At flow rate of 0.5 mL min^{-1}, very broad peaks were obtained. At flow rate of 1.5 mL min^{-1}, ALN condensation product peak is separated from blank peak but overlapped with ABA condensation product peak. At flow rate of 2 mL min^{-1}, all peaks are overlapped. So, the flow rate of 1 mL min^{-1} was chosen for optimum chromatographic measurement.

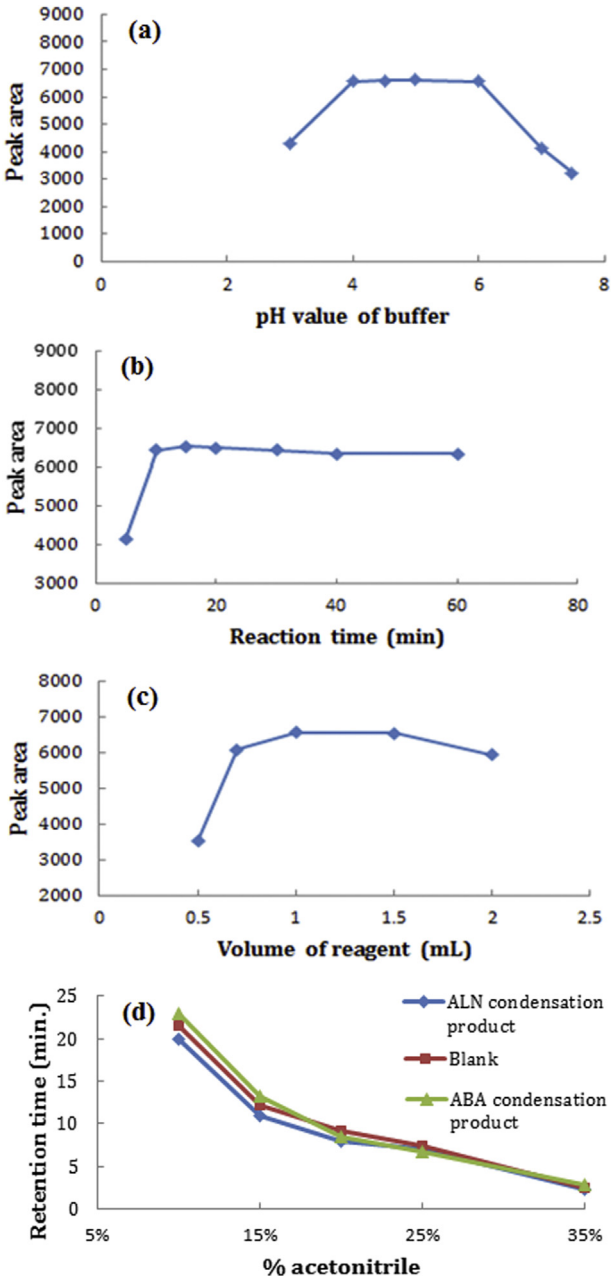


Fig. 1 – Effect of (a) the buffer pH values, (b) the reaction time and (c) the volume of reagent on the peak area of ALN condensation product obtained from the reaction of 500 μg mL^{-1} ALN with the pre-column derivatizing reagent and (d) effect of % acetonitrile in mobile phase on the retention times of ALN condensation product, ABA condensation product and blank obtained from the reaction of 500 μg mL^{-1}ALN and 2.5 μg mL^{-1}ABA with the pre-column derivatizing reagent.

Fig. 2 shows the HPLC chromatogram of the standard mixture of 20 μg mL^{-1}ALN and 10 μg mL^{-1}ABA after pre-column derivatization using Hantzsch condensation reaction. After pre-column derivatization using the optimized parameters mentioned above, the condensation products of ALN and ABA were separated with sharp well-resolved peaks at 10.9 and 13 min, respectively that are well separated from the blank peak.

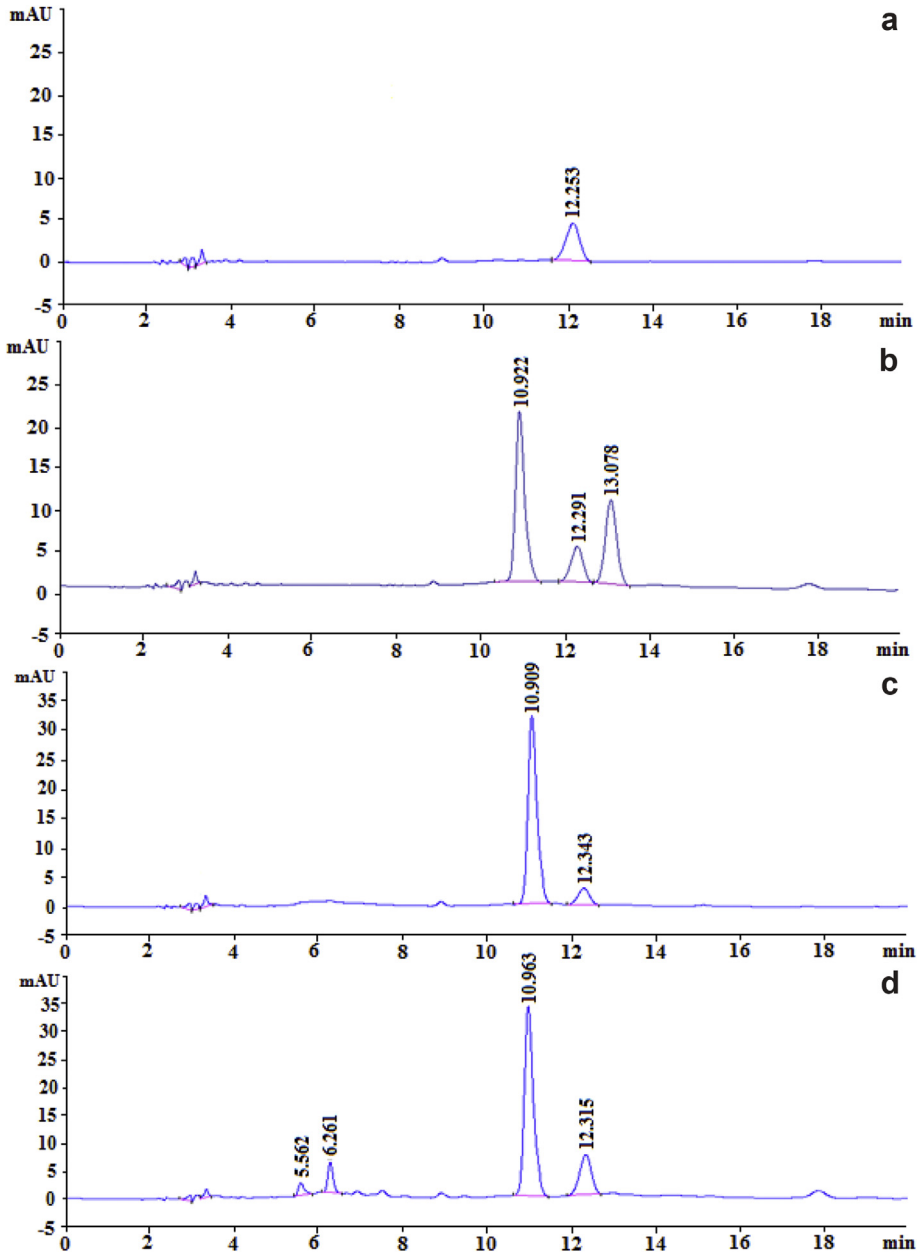


Fig. 2 – HPLC chromatogram of(a) reagent blank, (b) standard mixture of 20 μg mL^{-1}ALN and 10 μg mL^{-1}ABA (c) ALN tablet solution (30 μg mL^{-1}) and (d) ALN oral solution (30 μg mL^{-1}) after pre-column derivatization based on Hantzsch condensation reaction.

3.1.3. *System suitability*. Using the optimized chromatographic system, different system suitability parameters have been tested since they are important for the validation of the analytical method and they confirm the suitability and efficiency of the operating system [25]. System suitability parameters of the proposed method were as followed: retention time (R_t) (for ALN, 10.93; for ABA, 13.08), retention factor (k') (for ALN, 3.37; for ABA, 4.23). While, asymmetry factor (A_f) which used to evaluate the degree of peak asymmetry were 0.82 and 0.91 for ALN and ABA, respectively. A_f for both drugs did not exceed 1.2, indicating acceptable degree of peak asymmetry. In addition, the large number of theoretical plates which are 9592 and 21428 for ALN and ABA, respectively indicating high column efficiency. The obtained system suitability parameters indicated that the proposed HPLC–DAD method permitted adequate resolution of the ALN from ABA [good resolution (R_s) (3.29) and selectivity values (α) (1.26)] within reasonable runtime and high column efficiency.

3.2. *Method II (Derivative synchronous fluorometry)*

3.2.1. *Excitation and emission spectra*

Because of the absence of native fluorescence of ALN, a derivatization step using fluorogenic reagent was requisite for

its fluorometric analysis. Fluorescamine was chosen as a derivatizing reagent because it forms highly fluorescent derivatives with primary amines under relatively mild reaction conditions. It was found that ALN reacts with fluorescamine to give a fluorescent derivative with maximum fluorescence intensity (λ_{em}) at 489 nm after excitation at wavelength (λ_{ex}) of 386 nm. Obviously, the emission spectra of ALN and the reagent blank are strongly overlapped with high blank emission reading. Therefore, ALN can be determined directly by normal fluorometry but with low sensitivity because of interference of the reagent blank fluorescence.

3.2.2. *Synchronous fluorescence spectra and third derivative spectra*

The synchronous fluorescence spectral peaks of ALN and the reagent blank are at 280 and 240 nm, respectively (Fig. 3a) Although a synchronous fluorescence technique was used, the synchronous fluorescence spectra of ALN and the reagent blank cannot be separated entirely. To resolve the problem of spectral overlap, derivative technique was used. The synchronous fluorescence spectrum of the mixture solution (ALN and the reagent blank) was derived. Obviously, peaks of ALN and the reagent blank were separated on the third derivative spectrum. ALN can be more conveniently and accurately determined by measuring the third derivative of synchronous spectrum from peak-to-peak at (339–370 nm) which correspond to the absence of any interference from reagent blank (Fig. 3b). The ratios of third derivative synchronous spectral peak amplitudes at these wavelengths were calculated for different concentrations of ALN standard solutions and pharmaceutical formulations. These ratios were used for the detection of the presence of any interferences [26].

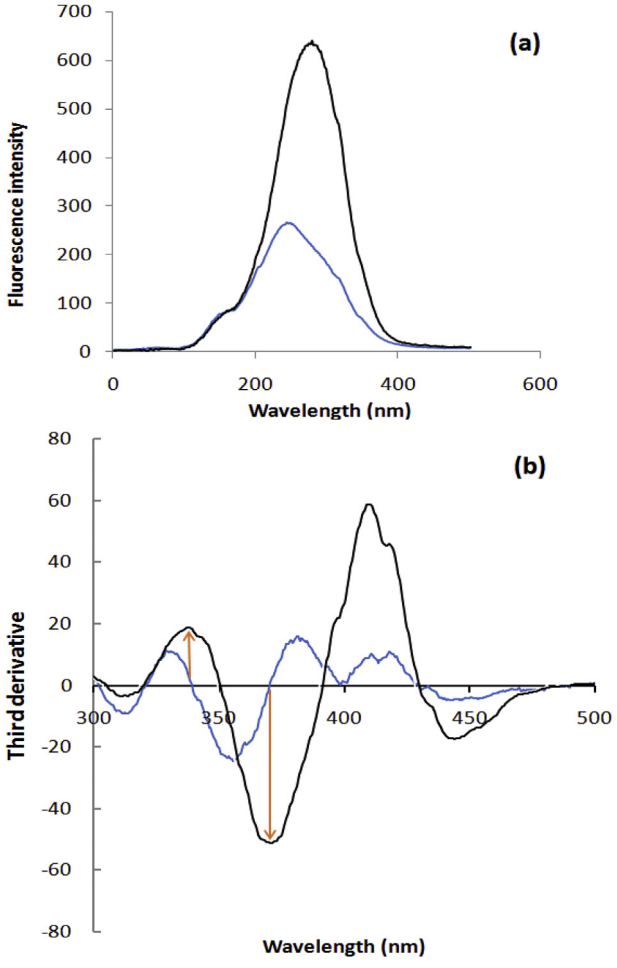


Fig. 3 – Synchronous fluorescence spectra (a) and third derivative synchronous fluorescence spectra (b) of ALN reaction product with fluorescamine (——) and reagent blank (——) recorded by scanning both monochromators at Δλ = 100 nm and derivative spectra were generated using Δλ = 20 nm, for 1 μg mL^{-1} ALN.

In addition, results revealed that the synchronous fluorescence spectral peaks of ALN and the reagent blank were not well separated during the Δλ below or above 20 nm. Therefore, Δλ of 20 nm was selected for recording the synchronous spectrum of the mixture solution (ALN and the reagent blank).

3.2.3. *Optimization of the reaction conditions*

3.2.3.1. *Effect of fluorescamine concentration*. Different volumes of 0.6 mg mL^{-1}fluorescamine reagent from 0.2 to 1.2 mL were studied using the procedure mentioned above. Optimum values of the relative fluorescence intensity (RFI) were obtained with 0.8 mL of fluorescamine reagent (Fig. 4a).

3.2.3.2. *Effect of pH*. The pH effect on the reaction was studied by carrying out the reaction in borate buffer solution in the pH range of 4.0–10.5, in addition to using 0.1M NaOH with pH 13. The results shown in Fig. 4b indicated that the RFI increased initially as the pH increased and maximum readings were attained at pH 9.8 ± 0.3. At higher pH values, sharp decrease in the readings occurred. This was probably attributed to the hydrolysis of the reaction product between ALN and fluorescamine in alkaline medium. Concerning the effect of buffer volume, the highest fluorescence reading was obtained upon adding 2.5 mL buffer pH 9.8 (Fig. 4c).

3.2.3.3. *Effect of time*. To study the optimum time needed for reaction completion, the derivatization reaction was performed at room temperature (25 ± 2 °C) and the RFI was recorded instantly after the addition of fluorescamine and observed for 30 min. The results indicated that the reaction was very fast as the fluorophore is formed immediately. RFI was constant by time and remained stable for a minimum of 2 h at room temperature.

3.2.3.4. *Effect of diluting solvent*. Various solvents were tried to choose the most appropriate diluting solvent which allows both formation and stability of ALN reaction product. These solvents were: water, DMF, acetone, methanol, ethanol, and acetonitrile. The highest fluorescence intensities were obtained with acetonitrile as a diluting solvent (Fig. 4d).

3.3. *Validation*

The ICH guidelines [27] for method validation were followed for the developed pre-column derivatization HPLC-DAD and

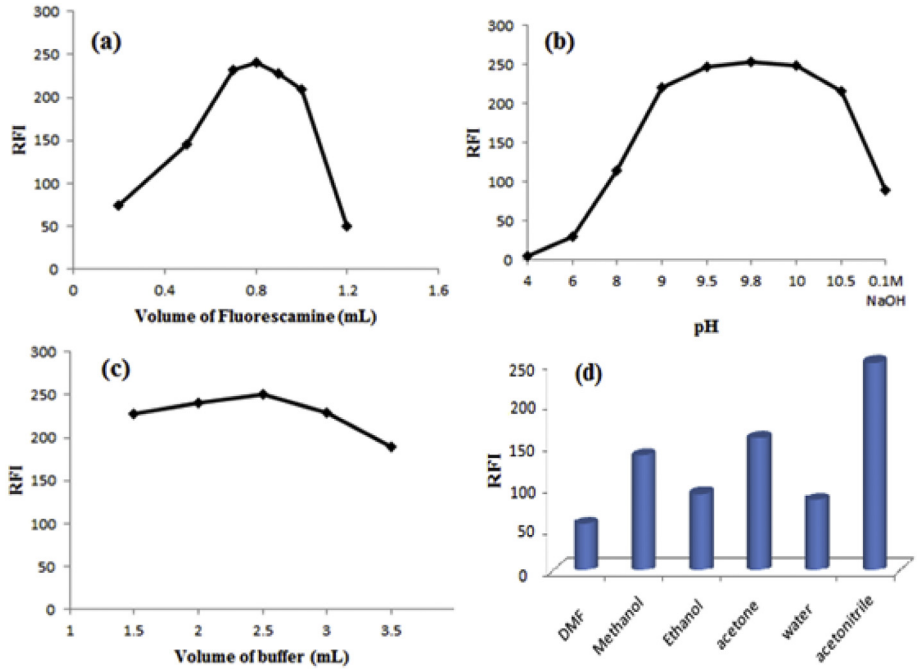


Fig. 4 – Effect of (a) volume of 0.6 mg mL^{-1} fluorescamine, (b) pH of the buffer, (c) volume of buffer and (d) different diluting solvents on the relative fluorescent intensity of reaction product of 0.5 μg mL^{-1} ALN with fluorescamine.

third derivative synchronous fluorometric methods. All validation parameters are shown in Tables 1 and 2.

3.3.1. *Linearity*

The linearity of the proposed methods was evaluated by analyzing series of different concentrations of each of ALN alone or with ABA. According to ICH, a minimum of five points must be involved. Under the experimental conditions described, the graphs obtained by plotting peak area (for method I), third derivative synchronous values (for method II) versus concentrations (in the ranges stated in Table 1) show linear relationships. All linearity parameters obtained by the linear least squares regression treatment of the results are also given. Regression lines with high F-values (low significance F) are considered better compared to those with lower ones. Good regression lines show high values for both (r) and (F) values [28].

Table 1 – Analytical parameters for the regression equations of the proposed methods (n = 5).

Parameter	Method I		Method II
	ALN	ABA	ALN
Linearity range (μg mL^{-1})	10.0–500	0.20–40	0.1–1.5
Correlation coefficient (r)	0.9997	0.9999	0.9997
Intercept (a)	0.408×10^2	0.035×10^2	0.6979
Slope (b)	0.127×10^2	0.098×10^2	0.679×10^2
S_a	0.243×10^2	0.011×10^2	0.770
S_b	0.1198	0.0491	0.908
$S_{y/x}$	0.539×10^2	0.018×10^2	1.030
S_b%	0.94	0.50	1.34
Significance F	4.77×10^{-11}	3.75×10^{-9}	5.27×10^{-6}
LOD (μg/mL)	0.12	0.06	0.024
LOQ (μg/mL)	0.40	0.20	0.073

S_a: Standard deviation of the intercept, S_b: Standard deviation of the slope, $S_{y/x}$: Standard deviation of residuals, %S_b: Percentage relative deviation of the slope, LOD: Limit of detection and LOQ: Limit of quantitation.

3.3.2. *Limit of detection and limit of quantitation*

For method I, limit of detection (LOD) is considered as the concentration which has a signal-to-noise ratio of 3:1. For limit of quantitation (LOQ), the ratio considered was 10:1 with RSD% value less than 10% [27]. For method II, LOD and LOQ were calculated using the formulae given by Miller [29] where the limit of detection, LOD = 3.3 S/b and the limit of quantitation, LOQ = 10 S/b, where S is the standard deviation of replicate blank responses (under the same conditions as for sample analysis) and b is the sensitivity, namely the slope of the calibration graph. Using the proposed methods, LOD and LOQ for each compound were calculated and are presented in Table 1.

3.3.3. *Accuracy and precision*

For method I, the accuracy and precision were evaluated by analyzing three laboratory-prepared mixtures of ALN and ABA at various concentration ratios within the working range of each compound (Table 2). On the other hand, for method II, solutions containing three different concentrations of ALN were prepared and analyzed in five replicates to evaluate the accuracy and precision. Good results were obtained upon checking method repeatability (intra-day precision) using the RSD% values obtained from repeating the assay five times on the same day (Table 2). Intermediate precision was evaluated by the assay of the sample sets on three different days (inter-day precision). Satisfactory recoveries, small percentage

Table 2 – Accuracy, Intra-day and inter-day precision for the determination of ALN and ABA by the proposed methods.

Method I						
ABA: ALN (μg mL^{-1})	Mean % recovery ± SD		RSD%[a]		Er (%)[b]	
	ALN	ABA	ALN	ABA	ALN	ABA
	Intra-day precision					
0.2: 10	101.69 ± 1.34	99.29 ± 1.64	1.32	1.65	1.69	−0.70
0.5: 100	99.72 ± 0.67	100.53 ± 0.85	0.67	0.85	−0.28	0.53
20: 500	100.21 ± 0.10	99.99 ± 1.32	0.10	1.32	0.21	−0.01
	Inter-day precision					
0.2: 10	101.74 ± 1.87	100.56 ± 1.92	1.83	1.91	1.74	0.65
0.5: 100	100.13 ± 0.925	100.42 ± 1.10	0.92	1.10	0.13	0.42
20: 500	100.19 ± 0.068	100.07 ± 1.05	0.07	1.05	0.19	0.07

Method II						
ALN conc. (μg mL^{-1})	*Intra-day*			*Inter-day*		
	Mean % recovery ± SD	RSD%[a]	Er (%)[b]	Mean % recovery ± SD	RSD%[a]	Er (%)[b]
0.1	101.55 ± 2.03	2.00	1.55	100.87 ± 1.91	1.89	0.87
0.5	98.72 ± 0.81	0.82	−1.28	98.39 ± 0.92	0.92	−1.61
1.5	100.61 ± 0.42	0.42	0.61	100.63 ± 0.71	0.70	0.63

[a] Percent relative standard deviation.
[b] Percent relative error.

relative errors (Er%) and small relative standard deviations (RSD%) were obtained, which indicated the high accuracy and precision of the proposed methods.

3.3.4. *Robustness*

Robustness of the proposed methods was evaluated by analyzing ALN alone or with ABA at the same concentration levels used for accuracy and precision studies. For method I, the parameters studied were acetonitrile % in mobile phase, pH of the acetate buffer, wavelength of detection and flow rate. However, for method II, the parameters included: volume of fluorescamine reagent, pH value of borate buffer, volume of buffer used and different lots of acetonitrile. It was found that variation in the above-mentioned parameters had no significant influence on the determination of the drug alone or with ABA using the proposed methods. The low values of RSD% of peak areas along with nearly unchanged k' values (for method I) and the low RSD% of third derivative synchronous peak values (for method II) obtained after introducing small deliberate changes in the method parameters indicated the robustness of the developed methods.

3.3.5. *Selectivity and specificity*

For method I, the selectivity was checked by analyzing synthetic mixtures containing different ratios of both drugs, where good percentage recoveries were obtained indicating that they did not interfere with each other (Table 2). The peak purity of ALN and ABA condensation products was checked by using a G1315D photo diode array detector where Fig. 5 shows the superimposed UV spectra for the peaks of ALN and ABA condensation products for standards and samples' solutions obtained at different times through their elution indicating purity of the obtained peaks. In addition, the obtained purity angle was not exceeding the threshold limit which indicates that no additional peaks were co-eluting with each of ALN and ABA providing evidence of the ability of the method to assess the analyte of interest in the presence of potential interferences.

The selectivity of method II was checked by measuring the ratio of the third derivative values of synchronous spectra for different concentrations of ALN standard solutions and pharmaceutical formulations. The value of D_3 370 nm/D_3 339 nm for ALN bulk powder was found to be 3.01 with a relative standard deviation (RSD) of 1.84% (n = 5), for ALN tablet solution, this ratio was 3.00 with RSD of 2.06% and in ALN oral solution this ratio was 3.02 with RSD of 2%. This ratio value is concentration independent and of reasonable reproducibility. Furthermore, it is specific for the drug and can be used for its identification as well as for testing its purity.

3.4. *Analysis of pharmaceutical formulations*

The proposed methods were successfully used to determine ALN in tablets and oral solution. HPLC chromatograms of Fosamax® tablet and Borgalendro® oral solution are shown in Fig. 2. Five replicate determinations were performed. Satisfactory results were obtained for ALN, and they were in a good agreement with label claims (Table 3). The results obtained from the two methods were compared statistically by the use of Student's *t*-test (for accuracy) and the variance ratio *F*-test (for precision). The results in Table 3 show that the calculated *t* and *F* values were smaller than the critical values, indicating there were no significant differences between the two proposed methods.

3.5. *Comparison with reported methods*

Table 4 presents a comparison between our proposed methods and the other reported ones for the determination of ALN in pharmaceutical dosage forms. This comparison reveals that the proposed spectrofluorimetric method (method II) shows comparable [19,21] or better sensitivity [6–9,11–14,16,20] to

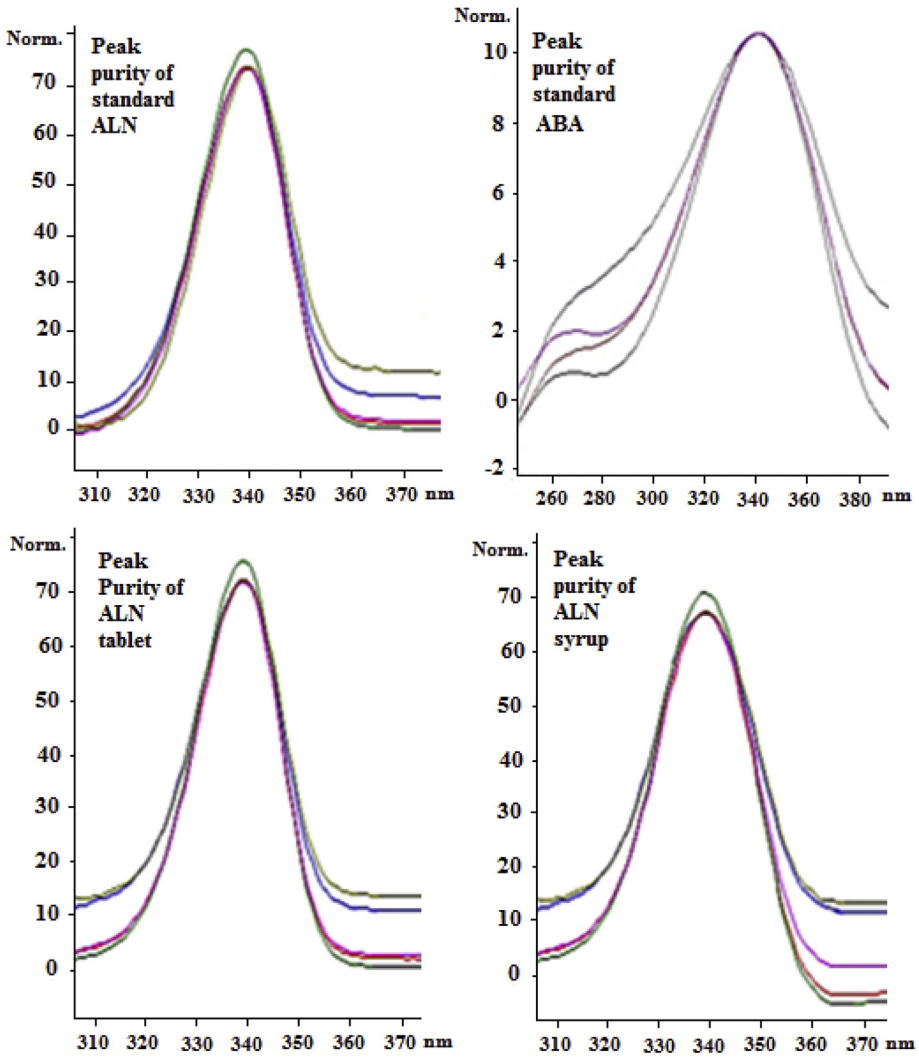


Fig. 5 – Peak purity check of ALN and/or ABA condensation products obtained from standard solutions and pharmaceutical preparations using photo diode array detector.

Table 3 – Assay of the marketed dosage forms using the proposed methods.

Parameter	Fosamax® tablets[a]		Borgalendro® Oral solution[b]	
	Method I	Method II	Method I	Method II
Mean % Recovery ± SD	101.7 ± 1.48	99.72 ± 1.32	99.62 ± 1.95	99.27 ± 1.22
RSD%[c]	1.45	1.33	1.96	1.23
Er (%)	1.70	−0.28	−0.38	−0.73
t[d]	2.25		0.36	
F[d]	1.25		2.57	

[a] **Fosamax® tablets** labeled to contain 10 mg ALN/tablet (MSD, Merck Sharp & Dohme Pharmaceutical Co., new Cairo, Cairo, Egypt).
[b] **Borgalendro® Oral solution** labeled to contain 91.35 mg ALN (equivalent to 70 mg alendronic acid) per 75 mL (BORG pharmaceutical Industries, Borg El Arab New City, Alexandria, Egypt).
[c] Percentage relative standard deviation for five determinations.
[d] Theoretical values of t and F for p = 0.05 are 2.31 and 6.39, respectively.

other derivatization methods reported in literature with several advantages, such as being simple and rapid methods with no need for tedious and elaborate steps (heating, centrifugation or waiting time). On the other hand, the proposed HPLC method (method I) is the first reported method for the separation of ALN and its related impurity ABA at the pharmacopeial limit. Compared to the pharmacopeial method [4], the proposed method I is simpler where separation between ALN and ABA was done at room temperature (RT) after isocratic elution within a shorter run time and using greener reagents and solvents without the need of tedious procedure. In addition, compared to the reported HPLC-FD method [8] for

Table 4 – Comparison of the proposed methods with the reported methods for the determination of ALN in pharmaceuticals.

Parameters	Proposed methods		Spectrophotometric methods [6–16]	Spectrofluorometric method [20,21]	HPLC- FD [8]	Anodic stripping voltammetry [19]	Pharmacopeial HPLC method [4]
	Method I	Method II					
Linearity range (μg/mL)	10–500	0.1–1.5	2–10 [6], 1.0–20.0, 4.0–40.0 [7], 14–60 [8], 5–70 [9], 8.1–162.5 [11], 1–60 [12], 6.78–162.72 [13], 3.75–45, 2–24 [14], 2,44–34.1 [16]	0.35–1.1 [20] 0.099–0.597 [21]	10–60 [8]	0.096–0.288	–
LOD (μg/mL)	0.12	0.024	0.19, 0.3 [6], 0.09,1.06 [7], 4.2 [8], 1.7 [9], 2 [11], 0.1 [12], 0.56 [16]	0.019 [20] 0.002 [21]	0.09 [8]	0.0086	–
Heating temperature °C (time)	55 °C (15 min)	RT (immediately)	70 °C (5 min) [6], 70 °C (25 min) or 60 °C (15 min) [7], RT (60 min) [8], 50 °C (10 min) [9], RT [11], 100 °C (60 min) [12], RT [13], 90 °C (20 min) or RT (60 min) [14], 100 °C (15 min) [16]	RT [20,21]	RT (60 min)	RT	RT (30 min)
Diluting solvent	Acetate buffer	Acetonitrile	Acetonitrile [6], methanol [7], water [7,9,13,14,16], NaOH [8], perchloric acid [11], Pyridine [12], H_2SO_4 [14]	Bisphosphonate standard solution [20], NaOH [21]	NaOH	Borate buffer	Acetonitrile and methylene chloride
Comments	- Ecofriendly, no or ganic solvent - Rapid without the use of toxic or expensive reagents.	- No tedious or elaborate steps. - Rapid with no waiting time.	- Heating at very high temperatures [6,7,12,14,16] - Time consuming procedures [7,8,12,14] - Use of toxic reagents [8,12]	- Narrow linearity range [20,21] - Spiked aqueous solution with ALN and no analysis of ALN in pharmaceutical dosage forms [20] - Use of toxic reagents [21] -Tedious procedure for preparation of reagent [20]	- Use of toxic reagents. - Time consuming procedures - Use of specific HPLC column which adds to the cost of the procedure.	- Narrow linearity range. - Use of toxic mercury electrode, not ecofriendly. - Tedious procedure for preparation of reagent.	- HPLC using gradient elution (32 min). - Toxic reagent (9-Fluorenylmethyl chloroformate) and solvent. - Column thermostated at 45 °C. - Tedious procedure.

the determination of ALN alone in tablets, the proposed method I showed wider linearity range, shorter derivatization reaction time without the need of specific column (rigid spherical styrene-divinylbenzene copolymer) or detector (fluorescence detector) which decreases the cost of analysis. Also, the use of water (method I) and acetonitrile (method II) as the diluting solvents promotes these methods to be greener alternatives to other reported methods [8,19,21]. Another advantage is the use of simple instrumentation that are readily available in most pharmaceutical laboratories.

4. Conclusion

This work reports the development of two methods for the analysis of ALN in raw material as well as in pharmaceutical preparations. The first method describes a fast, simple, selective and eco-friendly pre-column derivatization HPLC-DAD method which allows the sensitive determination of ALN in presence of its main related impurity, ABA, at its pharmacopeial limit (0.5%). It is worth nothing that this is the first HPLC report for this purpose. On the other hand, the second proposed method describes a simple, highly sensitive and selective third derivative synchronous fluorometric method that allows the sensitive analysis of ALN in different pharmaceutical dosage forms with no interference from various excipients. Comparing our proposed methods with other published methods reveals that the proposed methods are of comparable or better sensitivity to other derivatization methods with several advantages such as being fast eco-friendly sensitive and are independent on expensive reagents or instrumentation which makes our proposed methods to be superior to other reported methods and more suitable for routine analysis of ALN in quality control laboratories.

Conflicts of interest

The authors have no conflicts to declare.

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