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Original Research

HPLC Analysis of Amoxicillin Using AccQ-Fluor Reagent for Pre-Column Derivatization

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Abstract

A simple procedure to perform for the determination of amoxicillin is presented, based on reaction with 1-[(quinolin-6-ylcarbamoyl)oxy]pyrrolidine-2,5-dione (AccQ-Fluor) in borate buffer medium at pH of 7.4. The resulting amoxicillin+AccQ-Fluor derivative was determined by reversed-phase chromatography with combined fluorescence (λ_{ex}=245 nm/λ_{em}=368 nm) and UV detection (λ=245 nm) for control. The calibration graph was linear (r=0.9992) over the range 5.0-30 μg/mL. The method was applied to the determination of amoxicillin in pharmaceutical formulations with acceptable precision and accuracy.

Keywords: amoxicillin, HPLC, fluorescence-UV detection, derivatization

Introduction

Amoxicillin (AMO), 6-{[amino(4-hydroxyphenyl) acetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxylic acid is a semi-synthetic, β-lactam antibiotic that acts by inhibiting the synthesis of bacterial cell walls. It is a moderate-spectrum antibiotic active against a wide range of Gram-positive, and a limited range of Gram-negative organisms. Amoxicillin is used to treat certain infections caused by bacteria, such as pneumonia, bronchitis, gonorrhea, and infections of the ear, nose, throat, urinary tract, and skin.

Amoxicillin molecules exhibit hydrophobic and polar properties. A recent study [1] has reported the retention behavior of amoxicillin as a molecule containing hydrophobic skeleton and polar groups in reversed-phase conditions.

Amoxicillin molecules exhibit weak absorption in the UV-Vis wavelength range and a lack of fluorescent properties. Therefore, for analyzing this antibiotic, a derivatization procedure was often used in order to give efficient absorption and fluorescent properties [2-4].

The 1-[(quinolin-6-ylcarbamoyl)oxy]pyrrolidine-2,5-dione (AccQ-Fluor) was originally developed as a specific reagent for amino acid analyses [5-7]. The AccQ-Fluor-derivatives are fluorescent and as a consequence have their greatest utility in sensitivity and selectivity of detection in chromatography. They are very stable, in contrast to most other pre-column derivatives, due to their chemical structures, which are those of N-substituted ureas.

Recent studies utilizing derivatization with AccQ-Fluor for analyzing other amine compounds [8-14], including aminoglycoside antibiotic amikacin [15], have suggested that this reagent could also be useful for analysis of amoxicillin, which contains amine group.

We herein describe the application of pre-column derivatization with the AccQ-Fluor reagent for quantitative determination of amoxicillin using a rapid, sensitive, and selective high-performance liquid chromatographic (HPLC) method with combined UV and fluorescence detection.

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Experimental

Chemical and Reagents

Amoxicillin trihydrate pure substance was obtained from Polfa Tarchomin (Warsaw, Poland).

Amotaks Dis tablets (Polfa Tarchomin, Warsaw, Poland) containing 500 mg amoxicillin as the trihydrate in tablet and Amoxicillin granulate for oral suspension containing 125 mg amoxicillin as the trihydrate in 5 mL suspension (Pliva Kraków, Poland) was purchased commercially.

The AccQ-Fluor reagent kit, chemically 1-[(quinolin-6-ylcarbamoyl)oxy]pyrrolidine-2,5-dione was from Waters (Milord, USA).

Acetonitrile, methanol (gradient grade for liquid chromatography LiChrosolv®) was purchased from Merck (Darmstadt, Germany). Water was purified and deionized by a SolPure-7 water purification systems, from Poll-Lab (Bielsko-Biała, Poland). All other chemicals (boric acid, sodium hydroxide, and glacial acetic acid of analytical-reagent grade) were obtained from POCh (Gliwice, Poland).

Borate buffer was prepared by mixing 100 ml of 0.1 moL/L solution of boric acid with 100 ml of the distilled water and adjusting to pH 7.4 with 0.05 moL/L solution of sodium hydroxide.

AccQ-Fluor reagent at the concentration of 0.25 mmoL/L was prepared by transferring 0.05 mL of AccQ-Fluor reagent kit (5 mmoL/L) into a 1.0 mL volumetric flask and diluting to the mark with acetonitrile.

Chromatographic Conditions

The HPLC system (Waters, USA) equipped with a 2475 fluorescent detector, a 2489 spectrophotometric detector, model 515 isocratic pump, a Rheodyne valve with a 20-μL loop and Millenium software for collecting data was used. Chromatographic analysis was performed on a LiChrospher 100 RP-C18 (125 mm × 4.0 mm, 5 μm particle size) column from Merck (Darmstadt, Germany) at 25ºC. Void volume of the column measured by injection of sodium nitrate, as inorganic void volume marker [16] was 0.945 mL. A mixture of acetonitrile, glacial acetic acid and deionized water (67:1:32, v/v/v) was applied as mobile phase at an isocratic elution with a flow program, from 1.0 mL/min initially to 4 min, then increased to 3 mL/min from 4 to 8 min. Measurements were made using fluorescence detection at an excitation wavelength of 245 nm and at an emission wavelength of 368 nm, and using UV-detection for control at 245 nm.

Preparation of Samples

Calibration Solutions

The stock solution of AMO at a concentration of 1.0 mg/mL was prepared by dissolving 10 mg of the substance to be examined in 10.0 mL of borate buffer pH 7.4, aiding ultrasound. Calibration solutions at concentrations of 5, 10, 15, 20, 25, and 30 μg/mL were prepared by transferring the appropriate volumes of the stock solution into 10 mL volumetric flasks and diluting to the mark with the same buffer. Then these solutions were subjected to derivatization procedure.

Preparation Samples

An accurately weighted quantity of Amotaks Dis tablet mass or of Amoxicilline granulate equivalent to 5.0 mg of amoxicillin trihydrate was placed in a 50 mL volumetric flask, added 40 mL of methanol, extracted 10 min in a ultrasound both, diluted to 50 mL with the same solvent and filtered (0.1 mg/mL). A 2.0 mL of this solution was introduced into a 10 mL volumetric flask and complied to the volume with the borate buffer pH 7.4. The resulting solution (20 μg/mL) was subjected to a derivatization procedure.

Derivatization Procedure

The calibration solutions (0.1 mL; corresponding to 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg of AMO) and preparation samples (0.1 mL; corresponding to 2.0 μg of AMO) were transferred separately into a series of glass test-tubes. To each test-tube, 0.1 mL of AccQ-Fluor reagent was added and allowed to stand for 30 min. The resulting solutions were subjected to HPLC analysis.

Results and Discussion

Amoxicilline after pre-column derivatization was determined by HPLC method with combined fluorescence and UV detection for control. As the derivatizing compound was used, AccQ-Fluor reagent was developed for the amine analysis, especially for the amino acid analysis. Chemically, AccQ-Fluor reagent is 1-[(quinolin-6-ylcarbamoyl)oxy]pyrrolidine-2,5-dione and it is the highly reactive amine-derivatizing compound that converts both primary and secondary amines to fluorescent derivatives. An excess reagent hydrolyzes to produce the non-interfering by products such as quinolin-6-amine, 1-hydroxypyrrolidine-2,5-dione and carbon dioxide.

An amoxicillin molecule in its native form does not exhibit luminescent properties. Therefore, amoxicillin as a primary amine was converted with AccQ-Fluor reagent in a simple reaction to the derivative which exhibited fluorescent and UV-absorption properties. The AMO+AccQ-Fluor product was formed when AMO solution in borate buffer, pH 7.4 was treated with AccQ-Fluor reagent prepared in acetonitrile.

In order to optimize the derivatization conditions, several factors were investigated by controlling the peak area of the resulting AMO+AccQ-Fluor derivative. Satisfactory results were obtained using a 0.25 mmol/L AccQ-Fluor concentration in the borate buffer medium at pH value of 7.4. The derivatization reaction time with a good reaction yield was reached after 30 min. at room temperature (Fig. 1).

Under chromatographic conditions, the derivatized AMO was eluted, forming a satisfactorily shaped peak with a retention factor of 1.65 (1<k<5 generally is preferred).

The flow-rate was increased (0-4 min: 1 mL/min, 4-8 min: 3 mL/min) to faster elute the other peaks (probably the hydrolysis products of AccQ-Fluor reagent) that appeared after the peak corresponding to AMO+AccQ-Fluor derivative, thereby reducing total analysis time.

To select the analytical wavelengths, the excitation and emission spectra for AMO+AccQ-Fluor adduct were generated (Fig. 2).

Peak areas of AMO+AccQ-Fluor product (stored at 22ºC, recorded at the λ_{ex}=245 nm, λ_{em}=368 nm) as a function of time (0-12 h) were determined repeatedly at definite time intervals. No significant difference in the peak area and shape was observed as a result of the stable concentration of amoxicillin.

To assess the selectivity of the developed method, AMO samples were deliberately degraded under stressed conditions such as UV light, acid (1 M HCl), base (1 M NaOH) for 1 h and the appearance of other peaks (degraded products) was observed. Ultraviolet irradiation showed no important changes in the peak area and shape of the tested drug, and no degradation peaks were detected. But exposure to sodium hydroxide and hydrochloric acid solutions caused significant decomposition of amoxicillin (Fig. 3). In these cases, the degraded products were easily separated from the peak of amoxicillin (identified at 2.5 min).

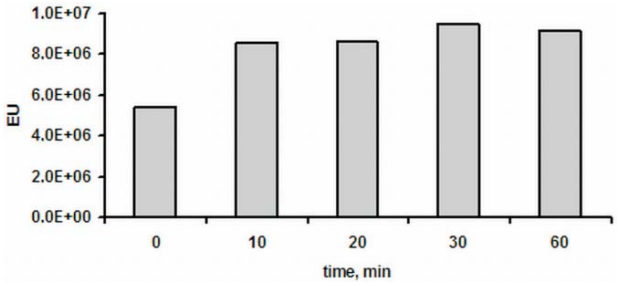

Fig. 1. Influence of time on the derivatization reaction of AMO with AccQ-Fluor reagent at room temperature.

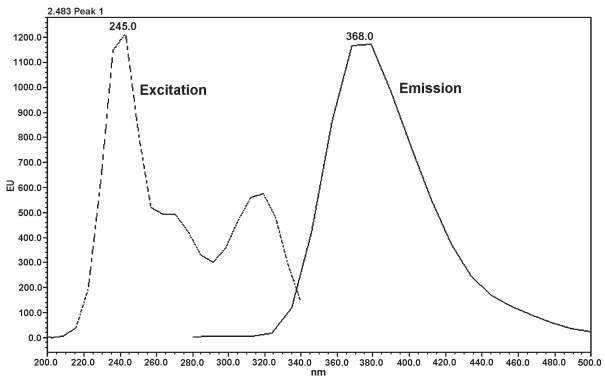

Fig. 2. Fluorescence spectra of AMO+AccQ-Fluor product. Excitation spectrum with emission at 368 nm, emission spectrum with excitation at 245 nm.

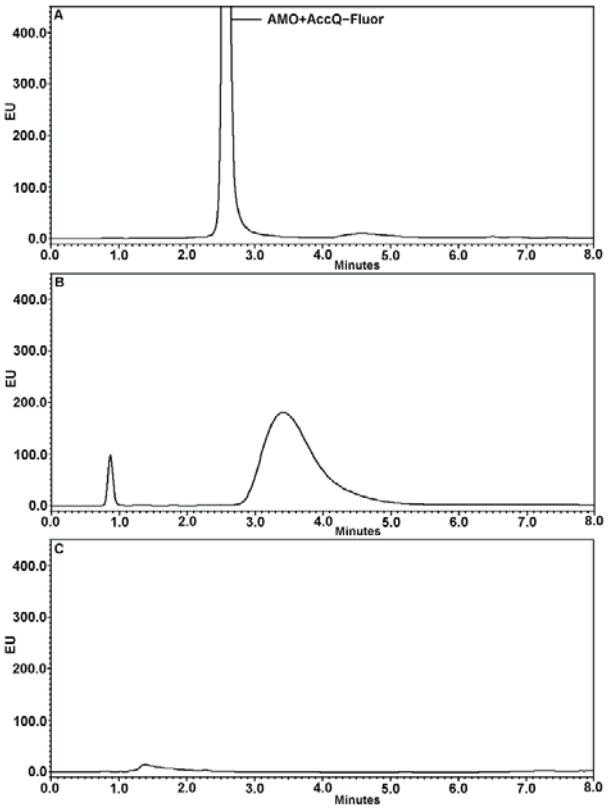

Fig. 3. Chromatograms recorded, after derivation reaction with AccQ-Fluor reagent, for AMO without degradation (A) and for AMO subjected to the chemical degradation using 1 M HCl (B) and 1 M NaOH (C).

In the present study, linearity was studied in the concentration range 5.0-30 μg/mL (25-150% of nominal concentration, n=3) and the following regression equation was found by plotting the peak area (y) versus the AMO concentration (x) expressed in μg/mL: y=3786919x+9208059. The correlation coefficient (r=0.9992) obtained for the regression line demonstrates the most satisfactory relationship between peak area and concentration of AMO. Determination of the drug in calibration solutions resulted in good precision with RSD not exceeding 3%. A verification of linearity according Mandel's test (TV=0.51<$F_{99\%(1,27)}$=7.68 for n=30 and *P*=99%) showed that the first-order function was fitted to the determination results better than the second-order function.

The limit of detection defined as the lowest amount of analyte that can be detected above baseline noise was found to be 0.01 μg/mL.

Precision was evaluated at three different concentration levels (10 μg/mL, 17.5 μg/mL, 27.5 μg/mL) that cover the AMO assay method range (5.0-30 μg/mL). Three standard solutions of AMO pure substance at each concentration level were prepared individually and injected two times. The intra-day variation resulting from determinations of AMO solutions during the same day, expressed by the RSD values of the retention time and area of AMO peak, was found to be 3.5%. Inter-day variation as the result of variations con-

Table 1. Results obtained from the determination of amoxicillin in pharmaceutical preparations by HPLC method.

Parameter	Amotaks Dis tablets (500 mg/tablet)	Amoxicillin suspension (125 mg/5 ml)
Mean amount found (mg)	498.4150	120.9500
Standard deviation	32.2495	8.4217
Relative standard deviation, %	6.47	6.96
Relative standard error, %	-0.32	-3.24
95% Confidence interval	±17.1809	±4.4867
Difference between the declared and found amounts (t-Student test, n=16)	TV=-0.048 $<t_{95\%,15}$=2.13	TV=-0.467$<t_{95\%,15}$=2.13

TV – the tested value

cerning determinations of AMO solutions during three different days was found to be from 3.0% to 6.0%.

To check the applicability of the developed method, two pharmaceutical formulations, Amotaks Dis tablets and Amoxicillin granulate for oral suspension, were analyzed. The content of antibiotic in the preparation was found to be 498.41 mg/Amotaks Dis tablet ± 17.18 mg with RSD of 6.47% and 120.95 mg/5 ml of Amoxicillin suspension ± 4.49 mg with RSD of 6.96%. The exemplary chromatograms recorded for AMO pure substance + AccQ-Fluor (A) and AMO suspension extract + AccQ-Fluor (B) derivatives at fluorescence detection and UV detection are shown in Figs. 4 and 5.

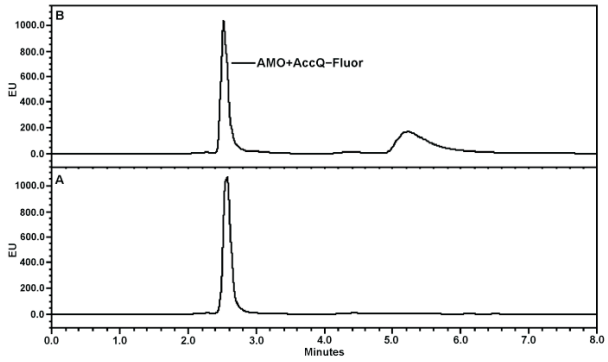

Fig. 4. Chromatograms obtained for AMO pure substance+AccQ-Fluor (A) and AMO suspension extract+AccQ-Fluor (B) derivatives recorded at fluorescence detection.

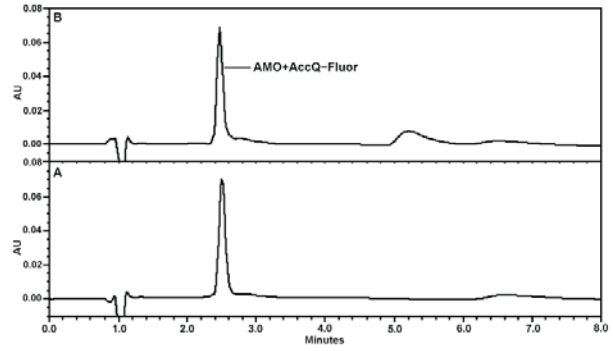

Fig. 5. Chromatograms obtained for AMO pure substance+AccQ-Fluor (A) and AMO suspension extract+AccQ-Fluor (B) derivatives recorded at UV detection.

Accuracy of this method was confirmed after the application of Student's t-test. There was not a significant difference between the recovery of the mean AMO amount found (tablets: 99.68%, suspension: 96.76%) and 100%. The obtained results are summarized in Table 1.

Conclusion

A simple-to-perform, rapid, and selective HPLC method based on pre-column derivatization with the AccQ-Fluor reagent, originally developed for amino acid analyses, with acceptable precision and accuracy has been suggested for analysis of amoxicillin in pharmaceutical quality control. The significant advantage of the developed method is the fact that through the lack of a fluorescence detector, UV-detection represents a good alternative choice. Furthermore, this method could also be extended for the determination of this antibiotic in other, not only pharmaceutical samples.

Acknowledgements

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