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Short communication

A new method for the analysis of amikacin using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatization and high-performance liquid chromatography with UV-detection

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Abstract

A simple and sensitive reversed-phase liquid chromatographic method has been developed for the determination of amikacin (AMK) by derivatization. The method is based on the pre-column derivatization of AMK with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). The derivatization reaction proceeds in aqueous solution at room temperature with a borate buffer of pH 8.0. The formation of the corresponding derivative of AMK is instantaneous and it is stable for more than 36 h. Detection was performed by UV-absorption instead of fluorescence. Several factors influencing the derivatization reaction yields were studied and optimized. The system offered the following analytical parameters: limit of detection (LOD) of 0.068 $\mu g\,ml^{-1}$ (3σ), linear correlation coefficient of 0.9998 and linear range response from 2 to 50 $\mu g\,ml^{-1}$. The precision of the method was <1%. As a preliminary application, the method has been successfully applied to the amikacin determination in parenteral pharmaceutical formulations.

Keywords: Reversed-phase liquid chromatography; Amikacin; 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC); Derivatization

1. Introduction

AMK is a semi-synthetic aminoglycoside antibiotic derived from kanamycin [1]. The AMK molecule lacks any chromophores capable of giving a general and reliable signal in the UV region, lacks volatility and is very hydrophilic. This means that a direct HPLC analysis of AMK using UV-detection is not straightforward. Nevertheless, AMK contains functional groups that can be derivatized to give efficient absorption in the UV–vis wavelength range or efficient fluorescent properties. In fact, various derivatization reagents have been developed and used for the analysis of AMK in several matrices [2–8]. There are more than 30 members of this compound family and several reviews have been published during the last two decades on current methodologies for the analysis of aminoglycosides including AMK [9–12]. Liquid chromatography in conjunction with pre- and post-column derivatization is perhaps the most used technique for AMK determination [13,14]. However, most of the derivatization reactions used to date are not suitable for quality control, in which rapid formation of a stable derivative under mild conditions, preferably at room temperature and in aqueous phase, is required [15].

The 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was introduced initially as a specific reagent for analyzing amino acids [16] and later applied to derivatizing other amine compounds [17,18]. The aim of the present investigation was the determination of AMK by RP-HPLC using the AQC as derivatization agent. The proposed method has distinct advantages over the official USP-assay [19], regarding simplicity and mild conditions for the derivatization. UV-absorbance detection instead of fluorescence was proposed, since low concentrations of AMK are not

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relevant for analysis of pharmaceutical dosage forms. To our knowledge, no paper has been published on the use of AQC as derivatizing agent of AMK.

2. Experimental

2.1. Materials

A pure reference standard of AMK was purchased from Sigma (A 3650 Lot 68F0809, St. Louis, MO, USA) labeled as Amikacin base ($C_{22}H_{43}N_5O_{13}$) 910 μg mg^{-1} with 7.2% water content. HPLC grade methanol was obtained from J.T. Baker (Phillisburg, NJ, USA). HPLC grade water was obtained with a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Boric acid was obtained from J.T. Baker (Phillisburg, NJ, USA). Sodium borate and sodium hydroxide were from Merck (USA). A Waters AccQ-Tag chemistry package containing AccQ-Fluor borate buffer, AccQ-Fluor reagent powder, AccQ-Fluor reagent diluent, AccQ-Tag eluent A concentrate, and 6 mm × 50 mm derivatizing sample tubes, were all purchased from Waters (Milford, MA, USA). The AccQ-Fluor reagent powder was dissolved with AccQ-Fluor reagent diluent and stored at −20 °C in a desiccator for up to 1 month. Standard solution of AMK was prepared by dissolving equivalent amount to 0.0500 g of powder of the pure compound in 50 ml of Milli-Q grade water. This solution was stored in the dark at 4 °C. Working solutions were prepared daily by dilution of these stock solutions with the appropriate volumes of Milli-Q grade water.

2.2. Samples

Commercial formulations of amikacin Biklin (Bristol-Myers Squibb, Venezuela) and generic amikacin (Genven, Leti, Venezuela) were purchased from drug stores in the locality (Mérida, Venezuela). Sample solutions of AMK were prepared by diluting 1 ml of the commercial formulation (ampoule of 250 mg ml^{-1}) to 250 ml with Milli-Q grade water. The sample solutions were stored in the dark at 4 °C. Working solutions were prepared daily by dilution of these stock solutions with the appropriate volumes of Milli-Q grade water.

2.3. Instrumentation

The HPLC system consisted of a Waters Alliance HT (Milford, MA, USA) coupled to a Waters 996 photodiode array detector and then to a Model Venturis Fx 51665 work station with a MILENIUM software for instrument control and data process. The AMK–AQC derivative was monitored at a wavelength of 247 nm. Chromatographic separations were performed on AccQ-Tag Amino Acid Analysis column, which is a C_{18}, 4 μm Nova pak^{TM} (3.9 mm × 150 mm). The determination of the fluorescent properties of the AMK–AQC derivative was carried out using an Aminco–Bowman spectrophotofluorometer J10-22A PM, microphotometer with a xenon lamp power supply. A Perkin-Elmer Model Lambda 20 UV–vis spectrophotometer was used for the absorbance measurements.

2.4. Derivatization procedure

AMK was derivatized with AQC using the procedure for labeling amino acids introduced by Cohen and Michaud [16]. Nevertheless, each parameter was explored and adjusted to the intended derivatization procedure. AMK standard or sample (20 μl) was first mixed with AccQ-Fluor borate buffer pH 8 (60 μl) in the bottom of a clean 6 mm × 50 mm sample tube on a vortex mixer for 15 s. AQC reagent 5 mM in acetonitrile (20 μl) was added and mixed immediately for 30 s. The reaction mixture was left for 10 min at room temperature (20 °C). The content of the sample tube was transferred to the bottom of an autosampler vial limited volume insert and then it was capped with a silicone-lined septum. Finally, 20 μl of this solution was injected into the HPLC system. The derivative property of the reagent (AQC) was confirmed using proline (Sigma, St. Louis, MO, USA) and spectrophotofluorometry as detection system.

2.5. Chromatographic conditions

Before the analysis, the AccQ-Tag column was pre-equilibrated with the mobile phase for 20 min at 37 °C. Solvent A was prepared from the Waters kit concentrate by dissolving one volume of concentrate in 10 volumes of 5% (v/v) methanol in Milli-Q water. Solvent B was pure HPLC methanol with 0.08% (v/v) acetone. Mobile phase solvents were filtered with Millipore filter (HVLP, 0.45 μm) under vacuum, and degassed before use. The mobile phase was pumped at a flow rate of 1 ml min^{-1}.

2.6. Spectrophotofluorometric conditions

For this study, a 10 μM AQC solution and a 10 μM AMK solution were mixed under the derivatization conditions in order to determine the fluorescent properties of the AMK–AQC derivative. All the measurements took place in a standard 10 mm pathlength quartz cell provided with tap and cooling fan. Excitation and emission bandpass of 5.5 and 11 nm, respectively, and PM microphotometer range switch set to 1.0 position (0–1 relative intensity scale) were used for that assay.

3. Results and discussion

3.1. Fluorescent and UV-detection

The fluorescence properties of AQC and its AMK–AQC derivative were examined in the derivatization medium.

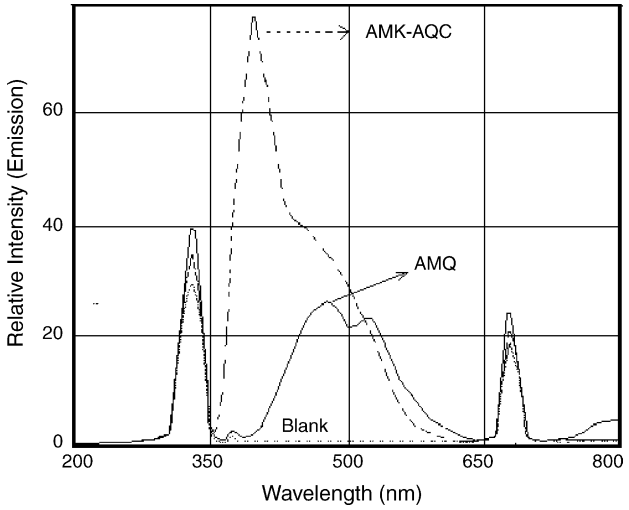

Fig. 1. Emission spectra of a (···) blank containing aqueous borate buffer solution at pH 8.5 and AMK standard; (—) AQC after hydrolysis without AMK standard, where AMQ is the by-product 6-aminoquinoline and (– – –) AMK after derivatization with AQC. Other conditions in the text.

Spectra of the AQC hydrolysis product (AMQ) and the AMK–AQC derivative are shown in Fig. 1. The emission intensity of the AQC hydrolysis product (AMQ) was lower than that of the AMK–AQC derivative. The maximum emission of AMQ–AQC was shifted to shorter wavelengths, with the highest fluorescence intensity appearing at $\lambda_{ex}/\lambda_{em}$ = 334/400 nm. Post-derivatization with AQC agent, clearly, fluorescence is the recommended choice as detection method. However, the absorption spectra obtained for the AMK–AQC derivative showed a strong absorbance at 247 nm. Since not all laboratories have a fluorescence detector, UV-detection represents a good alternative choice. Therefore, UV-detection was selected in this work. After determining the fluorescence and UV-absorption properties of the AMK–AQC derivative, HPLC analysis was carried out in order to optimize the derivatization procedure.

3.2. Optimization of the chromatographic conditions

The chromatographic behavior of the AMK–AQC derivative on the Waters Nova-Pak 3.9 mm × 150 mm column was tested by isocratic elution. With the purpose of maintaining similar conditions to those used in the chromatographic separation of most amino acids and aliphatic amines, diluted (10:110) pure AccQ-Fluor, eluent A, (pH 5.0) was used as aqueous mobile phase, and methanol as organic modifier, eluent B. The optimum HPLC performance was obtained by blending eluents A and B at 52:48 (v/v) at a flow rate of 1.0 ml min^{-1}. Under these conditions, the derivative AMK–AQC eluted at 3.14 min and was well separated from AMQ and other unknown peaks of the AQC reagent. The total elution time was as little as 7 min (Fig. 2). The column temperature was kept constant at 37 °C.

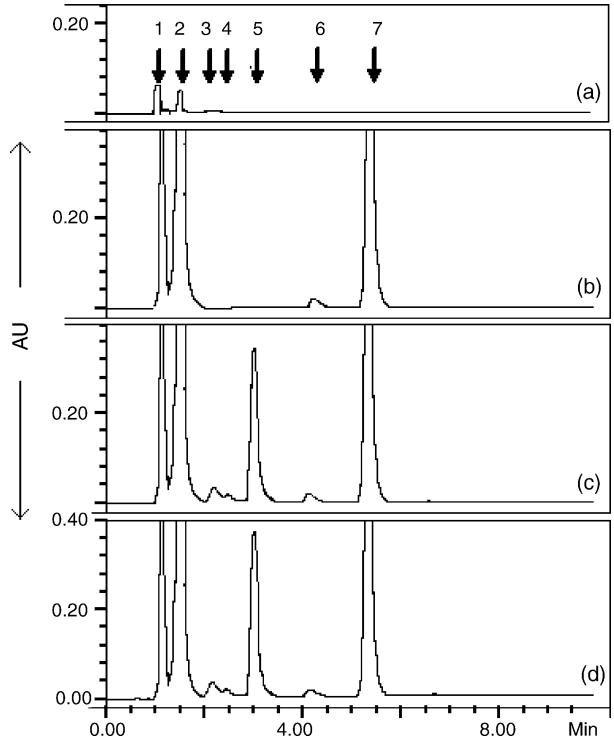

Fig. 2. Typical chromatogram of (a) AMK in borate buffer, (b) AQC reagent, (c) AMK–AQC derivative in standard solution, (d) AMK–AQC derivative in commercial sample solution. Peaks 1, 2, 6 and 7 belong to the derivatizing agent. Peak 5 on chromatograms (c) and (d), respectively, belong to AMK-AQC. Peaks 3 and 4 (unknown) belong to AMK standard and sample, respectively. AMK standard concentration: 30 μg ml^{-1}, 20 μl injected. Other conditions in the text.

3.3. Optimization of the derivatization conditions

To establish the optimum derivatization conditions, several factors were investigated and optimized. The parameters were evaluated by computing the peak area of the resulting AMK–AQC derivative. A good reaction yield was obtained with a 5 mM AQC concentration. The optimum pH value was found at 8.0 using a 0.2 M borate buffer. The derivatization reaction time was very rapid, with a maximum yield reached after 10 min. Consequently, 10 min was chosen as reaction time. Reaction temperature is a critical factor for the derivatization reaction with most *N*-hydroxysuccinimidyl ester reagents [15]. Nevertheless, in this work, when the reaction time was chosen as 10 min, the highest yield of the AMK–AQC derivative was observed at room temperature (20 °C). Furthermore, when a sample solution of AMK–AQC derivative was evaluated over a period of 36 h at room temperature, the results demonstrated that it was stable in dark at room temperature. Therefore, 20 °C was selected as derivatization reaction temperature.

3.4. System suitability

A system suitability test was defined on the results obtained in several representative chromatograms according to

the USP recommendations [19]. The column efficiency for the only peak of interest (AMK–AQC) was 2216, the tailing factor calculated from replicate injections of a standard was ≤1.07 and the R.S.D. for five injections was <1%.

3.5. Interferences and selectivity

The usual excipients (sodium metabisulfite and sodium citrate) of the commercial parenteral formulations do not have amine groups that could produce extra compound-related peaks in the chromatogram. Therefore, one might not expect interferences of that nature. Nevertheless, in order to confirm this statement, a real sample of AMK and a pure standard of AMK were compared using the optimized chromatographic conditions above described. A typical chromatogram for this assay is illustrated in Fig. 2. Peak 5 represents the AMK–AQC derivative. The three large peaks 1, 2 and 7 and the smaller peak 6 belong to the AQC reagent. The peaks 3 and 4 observed in the AMK standard and in AMK commercial samples probably belong to minor additional reaction products. The retention time of these peaks and their respective resolution in regard to the main peak (AMK–AQC derivative) were carefully studied by changing the eluent composition. In fact, these two peaks were well separated from the main peak AMK–AQC which eluted at 3.14 min. Further studies demonstrated that these peaks did not interfere in the quantitative yield of the derivatization reaction of AMK. Consequently, although these compounds could consume part of the AQC reagent, the existence of a large excess of AQC overcomes this influence. In addition, to study the effect of possible matrix interferences, standard addition graphs were prepared by adding various amounts of AMK (from 10 to 50 μg ml^{-1}) to an AMK commercial sample. The regression equation of the standard addition and conventional calibration are shown in Table 1. The fact that the slopes of both calibration curves did not give a significant difference ($P < 0.05$), shows that there was no interference from the common excipients present in the commercial formulations. The impurity peak for the commercial sample and standard of AMK–AQC was examined with a diode array detector. Purity angle (0.358) was less than the threshold (0.701), which was an indication of the homogeneity and lack of impurity of the main peak. All the above studies indicated that the proposed method is feasible for the analysis of AMK in parenteral pharmaceutical formulations.

Table 1
Quantitative aspects of the method

Parameter[a]	Conventional calibration	Standard addition calibration
Concentration range (μg ml^{-1})	2–50	10–50
Number of standards	5	5
Intercept	44698	884464
Slope	56467	56637
Correlation coefficient (r)	0.9998	0.9989
LOD (μg ml^{-1}) ($3S_{y/x}$)	0.068	–
LOQ (μg ml^{-1}) ($10S_{y/x}$)	0.573	–

[a] Confidence limits of the slope and the intercept ($P = 0.05$).

3.6. Validation of the method

To validate the proposed method, different concentrations of AMK over the range of 2.0–200.0 μg ml^{-1} were evaluated. The peak area versus concentration curve was linear at concentrations up to 50 μg ml^{-1} for a 20 μl of injection volume. The linear regression equations are shown in Table 1. The results indicated good linearity of the proposed method. The precision of the method was checked by analyzing five replicate injections of AMK reference solution at low, medium and high concentration levels (2, 10, 50 μg ml^{-1}). The relative standard deviation (R.S.D.) was <1% for all cases. The detection and quantification limit of AMK for a 20 μl of injection volume are shown in Table 1. The accuracy of the proposed procedure was tested on the bases of percentage recovery calculated for AMK commercial samples spiked with the AMK standard at four concentration levels 0, 10, 30 and 40 μg ml^{-1}, respectively. Recovery data ranged from 99 to 101% ($n = 3$) with a R.S.D. of <3%, thus enabling this method to be used for the analysis of real samples.

3.7. Analytical applications

To check the applicability of the proposed method, two parenteral pharmaceutical formulations, amikacin Biklin® (Bristol-Myers Squibb, Venezuela) and generic Amikacin® (Genven, Leti, Venezuela), each with a nominal content of 250 mg ml^{-1} were analyzed. The found concentrations ($n = 3$) were as follows: 242.4 ± 2.4 mg ml^{-1} for Biklin® and 259.5 ± 4.1 mg ml^{-1} for generic Amikacin®. A t-test was applied to compare if nominal values claimed by the manufacturer were similar to the obtained average. The results of the content uniformity test showed no significant differences between the compared values. Consequently, the proposed method could be used for analyzing these parenteral pharmaceutical formulations.

4. Conclusion

In conclusion, a simple, rapid and sensitive HPLC method based on the derivatization of AMK with the AQC reagent, with satisfactory precision and acceptable accuracy has been established and optimized. There is a significant advantage in using the AQC reagent, as the whole derivatization procedure is completed in a few minutes and easy to handle. Furthermore, this method could be used in pharmaceutical quality control using UV-detection instead of fluorescent-detection.

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