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Fit-for-purpose chromatographic method for the determination of amikacin in human plasma for the dosage control of patients

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ABSTRACT

In this paper, a simple, rapid and sensitive method based on liquid chromatography with fluorimetric detection (HPLC-FLD) for the determination of amikacin (AMK) in human plasma is developed. Determination is performed by pre-column derivatization of AMK with ortho-phtalaldehyde (OPA) in presence of *N*-acetyl-L-cysteine (NAC) at pH 9.5 for 5 min at 80 °C. In our knowledge, this is the first time that NAC has been used in AMK derivatization. Derivatization conditions (pH, AMK/OPA/NAC molar ratios, temperature and reaction time) are optimized to obtain a single and stable, at room temperature, derivative. Separation of the derivative is achieved on a reversed phase LC column (Kromasil C_{18}, 5 µm, 150 × 4.6 i.d. mm) with a mobile phase of 0.05 M phosphate buffer:acetonitrile (80:20, v/v) pumped at flow rate of 1.0 mL/min. Detection is performed using 337 and 439 nm for excitation and emission wavelengths, respectively. The method is fitted for the purpose of being a competitive alternative to the currently used method in many hospitals for AMK dosage control: fluorescence polarization immunoassay (FPIA). The method exhibits linearity in the 0.17–10 µg mL^{-1} concentration range with a squared correlation coefficient higher than 0.995. Trueness and intermediate precision are estimated using spiked drug free plasma samples, which fulfill current UNE-EN ISO15189:2007 accreditation schemes. Finally, for the first time, statistical comparison against the FPIA method is demonstrated using plasma samples from 31 patients under treatment with AMK.

1. Introduction

Amikacin (AMK) is an antibiotic that belongs to the group of aminoglycosides. It is currently an aminoglycoside of first choice for the treatment of serious infections. AMK is commonly parenterally administered for treatment of aerobic gram-negative bacillary infections [1]. Its pharmacological action is based on the irreversible binding to the 30S and 50S ribosomal subunits of bacterial cell, resulting in an ineffective membrane-synthesis of proteins and, consequently, causing the death of pathogen and its antibiotic effect [2].

The major concern in AMK therapy, as other aminoglycosides, is its toxicity. All aminoglycosides are both nephrotoxic and ototoxic. The problems are pronounced in patients with impaired kidney function. Because of the narrow therapeutic range of aminoglycosides, therapeutic drug monitoring is essential [3–7].

Several methods have been applied to the analysis of aminoglycosides in various matrices [8–10]. The main methods described for AMK in plasma are microbiological assays, immunoassays and chromatographic methods [11]. Traditional semi-quantitative microbiological methods are inexpensive but imprecise, non-selective, tedious and time consuming [11]. Therapeutic drug monitoring of AMK and other aminoglycosides antibiotics is mostly done by inmunoassays, mainly fluorescence polarization immunoassay (FPIA), due to their selectivity and accuracy. In addition, the cost and the problems of handling the radioactive material are obvious drawbacks [11]. On the other hand, a major drawback of FPIA in the field of healthcare, is the high cost of patented reagents provided by the manufacturer of the instrument. Recently, a home-made surface plasmon resonance immunoassay biosensor has proved its potencial for the determination of AMK. However, its applicability has not been

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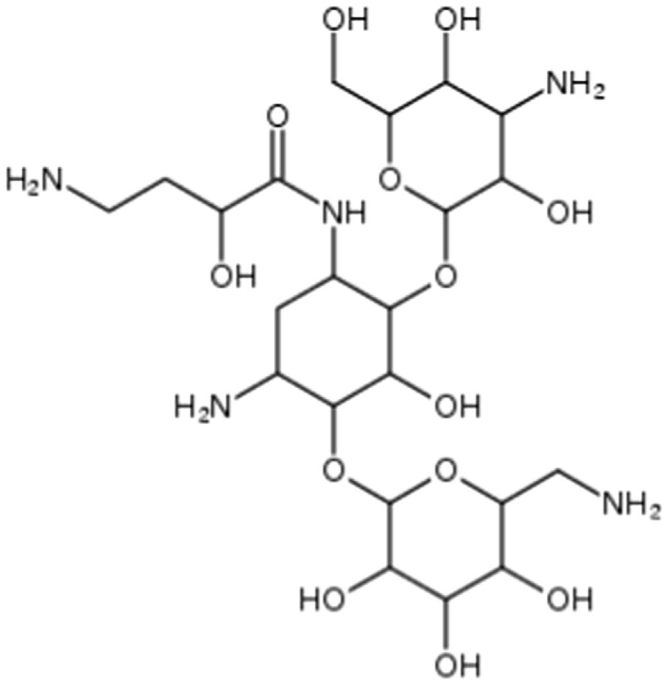

Fig. 1. Chemical structure of AMK.

tested in real plasma samples [12].

Liquid chromatography (HPLC) has been also proposed for the determination of amikacin in plasma samples due to its selectivity and sensitivity [11,13–25]. Compared with other methodologies, HPLC methods are easier to develop, providing reliable analytical results.

The structure of AMK (Fig. 1) shows four primary amino groups, one secondary amino group and eight primary hydroxyl groups. It presents high polarity and water solubility, and therefore, low retention in reversed phase mode, unless using the technique of ion pair to retain the compound [11,14,15] or hydrophilic interaction liquid chromatography (HILIC) [16].

On the other hand, the absence of chromospheres in the AMK structure requires the use of mass spectrometry (MS) detectors [16–19] or either pre- or post-column derivatization to use UV–vis, fluorescence (FLD) or chemoluminiscence detection. Opposite to MS, UV–vis and FLD detectors are common and accessible in HPLC methods for routine analysis in hospital laboratories. Generally, post-column derivatization requires special instrumental equipment including reaction chambers and thus increases instrumentation complexity [20]. Pre-column derivatization becomes simpler and more efficient, normally improving method sensitivity [11,21–25]. Among the derivatization reagents proposed, ortho-phtalaldehyde (OPA) is the most widely used [11]. OPA reacts with primary amines, in presence of a mercaptan or other strong reducing agents in basic conditions, to form fluorescent derivatives. The most used mercaptan has been mercaptoethanol, but it gives highly unstable derivatives and also involves a potential risk of toxicity due to its manipulation.

In the present study, a new simple HPLC-FLD method for the determination of AMK in human plasma is described, based on reversed phase mode using a pre-column derivatization step. The enhancement of selectivity and sensitivity is based on the use of the OPA, but employing *N*-acetyl-L-cysteine (NAC) for the first time in this determination as reducing agent. NAC was selected because it is not expensive, practically odorless and safe and produces relatively stable and highly fluorescent derivatives [26]. The optimum conditions for the derivatization and chromatographic separation are investigated. The method is fitted for being a competitive alternative to the currently used in many hospitals for AMK dosage control, FPIA. Considering the FPIA features, the HPLC-FLD method is tested for linearity, trueness and precision, using human plasma matrices. In addition, method comparison between FPIA and the proposed method is carried out using plasma samples from patients under treatment with AMK.

2. Materials and methods

2.1. Instrumentation

Spectrophotometric studies were performed with a diode array Hewlett-Packard model 8453 spectrophotometer (Hewlett-Packard, Palo Alto, California, USA). The excitation and emission wavelengths were fixed using a LS 45 Fluorescence Spectrometer (Perkin Elmer, Waltham, Massachusetts, USA).

The chromatograms were obtained using a Jasco LC-2000 plus system (Jasco, Easton, Maryland, USA) equipped with a fluorescence detector (FP-2020 Intelligent Fluorescence Detector) and a Kromasil C_{18} column (5 µm, 150×4.6 mm^2) (Scharlau, Barcelona, Spain). The injection port was equipped with a 20 µL loop for manual injection. Flow rate was adjusted to 1 mL/min. The detection wavelengths were set at 337 and 439 nm for excitation and emission, respectively. A detector gain of 10 was used in all experiments.

Samples were incubated in a thermostatically controlled water bath JP Selecta model Frigiterm-10 (JP Selecta S.A., Abrera, Barcelona, Spain), subjected to agitation in a vortex mixer (Velp Scientific Vortex, Usmate, Italy) and centrifuged in a Heraeus Medifuge Sepatech centrifuge (Heraeus S.A., Boadilla del Monte, Madrid, Spain).

The mobile phases and all the solutions injected into the chromatographic system were filtered through 0.45 µm pore size nylon membranes (Micron Separation, Westboro, MA, USA) prior to injection. Mobile phases were degassed in an Elmasonic S60 ultrasonic bath (Elma, Singen, Germany) prior to use. A Crison Micro pH 2000 pH meter (Crison Instruments, Barcelona, Spain) was employed to adjust the pH of the buffer solutions.

The FPIA assays were performed with an Abbott TDx autoanalyzer (Abbott Scientific, Lake Forest, Illinois, USA).

2.2. Chemicals and reagents

All reagents were of analytical grade. Boric acid, sodium hydroxide, and acetonitrile and ethanol Multisolvent® HPLC grade were from Scharlab S.L. (Barcelona, Spain). *N*-acetil-L-cysteine, NAC, and sodium dihydrogen phosphate were from Fluka (Butch, Switzerland). Ortho-phtalaldehyde, OPA $\geq$ 99% for fluorescence and HPLC was from Sigma-Aldrich Chemie GMBH (Steinheim am Albuch, Germany). Reagents, calibration and control solutions for FPIA were supplied by Abbott Scientific S.A. as commercially patented products.

Ultra Clear TWF UV deionized water (SG Water, Barsbüttel, Germany) was used to prepare solutions. Borate buffer (0.1 M, pH 9.5) was prepared weekly by dissolving the appropriate amount of boric acid in water and adjusting the pH with 2.5 M sodium hydroxide. 0.05 M phosphate buffer at pH 7.5 was prepared in the same way from sodium dihydrogenphosphate. Derivatizing reagent solution, containing OPA 2.55×10^{-3} M and NAC and 2.55×10^{-2} M, was prepared weekly by dissolution of an appropriate amount of OPA and NAC in the minimum volume of ethanol and completing to volume with borate buffer. These solutions were stored at 4 °C. The mobile phase was prepared by mixing phosphate buffer (0.05 M, pH 7.5) and acetonitrile in a ratio of 80:20 v/v.