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Kinetic microplate bioassays for relative potency of antibiotics improved by partial Least Square (PLS) regression

Fabiane Lacerda Francisco, Alessandro Morais Saviano, Túlia de Souza Botelho Almeida, Felipe Rebello Lourenço *

Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580 — Bloco 13, CEP 05508-000 São Paulo, SP, Brazil

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ABSTRACT

Microbiological assays are widely used to estimate the relative potencies of antibiotics in order to guarantee the efficacy, safety, and quality of drug products. Despite of the advantages of turbidimetric bioassays when compared to other methods, it has limitations concerning the linearity and range of the dose–response curve determination. Here, we proposed to use partial least squares (PLS) regression to solve these limitations and to improve the prediction of relative potencies of antibiotics. Kinetic-reading microplate turbidimetric bioassays for apramacyin and vancomycin were performed using *Escherichia coli* (ATCC 8739) and *Bacillus subtilis* (ATCC 6633), respectively. Microbial growths were measured as absorbance up to 180 and 300 min for apramycin and vancomycin turbidimetric bioassays, respectively. Conventional dose–response curves (absorbances or area under the microbial growth curve *vs.* log of antibiotic concentration) showed significant regression, however there were significant deviation of linearity. Thus, they could not be used for relative potency estimations. PLS regression allowed us to construct a predictive model for estimating the relative potencies of apramycin and vancomycin without over-fitting and it improved the linear range of turbidimetric bioassay. In addition, PLS regression provided predictions of relative potencies equivalent to those obtained from agar diffusion official methods. Therefore, we conclude that PLS regression may be used to estimate the relative potencies of antibiotics with significant advantages when compared to conventional dose–response curve determination.

1. Introduction

The success of antimicrobial therapy depends on the relationship between the plasmatic concentration levels and the antimicrobial activity (or the relative potency) of antibiotic present in the dosage form (Brunton et al., 2006; Guimarães et al., 2010; Butler and Cooper, 2011; Pinto et al., 2015). Several methods for antimicrobial testing have been reported in literature (Wheat, 2001; Liu et al., 2004; Othman et al., 2011; Lee and Chung, 2015; Gottardi et al., 2015). These methods are based on a similar principle, whereby antimicrobial activity is determined by: a) turbidity measurement of microbial growth inhibition in liquid culture medium; b) counting the reduction in the number of colony forming units (CFU) in agar culture medium; and c) measuring the inhibition zone sizes based on agar diffusion method (Wheat, 2001; Liu et al., 2004; Othman et al., 2011; Lee and Chung, 2015; Gottardi et al., 2015). Usually, agar diffusion and counting plate methods are laborious and time expensive, once it requires long periods of incubation (usually from 18 to 24 h) and a high number of plates (Hewitt, 2005; Schmidt et al., 2009; Dafale et al., 2012, 2013). Turbidimetric assay is a reliable alternative because it can easily be performed, use reduced amount of culture medium and antibiotic solutions, and provides results after short periods of incubation (usually from 3 to 6 h) (Tótoli and Salgado, 2013; Pedroso and Salgado, 2014; Francisco et al., 2014; Silva and Salgado, 2015).

Usually, the determination of relative potency of antimicrobial agent present in dosage form by turbidimetric assay is performed using a set of test tubes containing about 10 mL of liquid culture medium inoculated of a sensitive microorganism-test (Hewitt, 2005; Farmacopéia Brasileira, 2010; United States Pharmacopeia, 2014; Pinto et al., 2015). Moreover, the tubes also contain several dilutions of the sample or several concentrations of antibiotic certified reference substance (CRS), and their gradual inhibition growths are compared in order to estimate relative potency (Hewitt, 2005; Farmacopéia Brasileira, 2010; United States Pharmacopeia, 2014; Pinto et al., 2015). A linear relationship between absorbances and log of antibiotic concentration is often obtained using a 3-fold exponential range (*e.g.* from 1 to 4 mg/L), and wide ranges may result in non-linear relationship. This is an issue for estimating the relative potencies of unknown samples (Hewitt, 2005; Farmacopéia Brasileira, 2010; United States Pharmacopeia, 2014; Pinto et al., 2015).

In the last years, bioassays have been performed using microplates, which allow a high-throughput screening and combination testing with reduced amount of antibiotic solutions and medium culture

* Corresponding author.
E-mail address: feliperl@usp.br (F.R. Lourenço).

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(Lourenço and Pinto, 2011; Botelho et al., 2013; Francisco et al., 2014; Mishra et al., 2015). Besides, several absorbance measurements can be done during the incubation time (kinetic-reading) (Holowachuk, 2003; Lehtinen et al., 2006; Lourenço and Pinto, 2011; Botelho et al., 2013). Relative potencies may be estimated using conventional calculation or using the area under the microbial growth curve *vs.* log of antibiotic concentration (the Integral Method) (Holowachuk, 2003; Lourenço and Pinto, 2011; Botelho et al., 2013; Gottardi et al., 2015). The integral method is a reliable alternative used for estimating relative potencies, once it considers the whole microbial growth curves (not only the absorbances measurements of a single time-point) (Holowachuk, 2003; Lourenço and Pinto, 2011; Botelho et al., 2013; Gottardi et al., 2015). However, the calculation used is laborious, because it depends on the calculation of the area under the curve using trapezoidal method for each segment.

In principle, a predictive model for estimating relative potencies of antibiotics can be constructed using multiple linear regression (MLR). However, if the number of predictors is greater than the number of observations, the model may be over-fitting. In other words, the model will fit the sampled data perfectly, but it will fail to predict new data well (Geladi and Kowalski, 1986; Cramer, 1993; Tobias, accessed in 2015). Partial least squares (PLS) regression is particularly useful to construct predictive models when the predictors are highly collinear and when there is a great number of predictors (Geladi and Kowalski, 1986; Cramer, 1993; Tobias, accessed in 2015). All these are issues in estimating the relative potencies of antibiotics based on microbial growth curves. Thus, PLS (PLS) regression is a suitable alternative to construct a predictive model for estimating relative potencies of antibiotics.

A reduced antimicrobial activity due to subtle changes or adulteration is revealed by microbiological assay, but may not be detected by chemical methods (Adams et al., 1998; Pinto et al., 2013, 2015; United States Pharmacopeia, 2014). Thus, despite the advantages of chemical methods, microbiological assays are still used in the quality control analysis of several antibiotics such as glycopeptides and aminoglycoside antibiotics (Adams et al., 1998; Pinto et al., 2013, 2015; United States Pharmacopeia, 2014). Usually, these antibiotics are obtained from fermentation process and consist in a mixture of very similar substances that may show synergetic or antagonistic effects (Benveniste and Davies, 1973; Pinto et al., 2013).

Apramycin is an aminoglycoside antibiotic that inhibits protein synthesis and is used in the treatment of several infectious diseases such as colibacillosis, salmonellosis, bacterial enteritis and *Escherichia coli* septicemia in animals. Chemically, aminoglycosides are aminosugars linked by glycosidic bonds to an aminocyclitol ring, and can be divided into 1,2-disubstituted and 1,3-disubstituted compounds (Benveniste and Davies, 1973; Pinto et al., 2013). Vancomycin is a glycopeptide antibiotic used in the treatment of Gram-positive infections. It inhibits the peptidoglycan synthesis and alters the selective permeability of cytoplasmic membrane of bacteria. Vancomycin is a large complex cyclic structure that contains amino acids and sugars (Brunton et al., 2006; Guimarães et al., 2010; Butler and Cooper, 2011).

Several methods have been reported in literature for analysis of aminoglycosides and glycopeptides, such as spectrophotometry, high performance liquid chromatography, agar diffusion microbiological assay, turbidimetric assay, among others (Stead, 2000; Hanko and Rohrer, 2007, 2010; Vila et al., 2007; Zhang et al., 2007; Valle et al., 2008; Antunes et al., 2011; Passoni and Salgado, 2012; Xu et al., 2014; Lotfipour et al., 2015; Long et al., 2016). Despite this, microbiological assays are still the official methods for most of aminoglycoside and glycopeptide antibiotics in pharmaceutical dosage forms (Farmacopéia Brasileira, 2010; United States Pharmacopeia, 2014).

Here, we proposed kinetic-reading turbidimetric microplate bioassays for apramycin and vancomycin analysis using partial least squares (PLS) regression. PLS regression allowed us to construct a predictive model for estimating the relative potency of antibiotics present in pharmaceutical dosage forms without over-fitting and improved the linear range of turbidimetric bioassay.

2. Materials and methods

2.1. Reagents, culture media, and microorganism

Apramycin sulfate reference standard (potency 873 μg mg^{-1}), vancomycin hydrochloride reference standard (potency 99,300 μg per vial), and commercial samples of Apralan™ and Vancocin™ were purchased from Eli Lilly do Brasil Ltda. (São Paulo, Brazil). Tryptic soy agar (TSA, Difco) and tryptic soy broth (TSB, Difco) were used in turbidimetric microplate bioassay. Antibiotic medium numbers 2 (Anti 2) and 8 (Anti 8) (as described in U.S. Pharmacopeia) were used in agar diffusion microbiological assay. Phosphate buffer solutions (pH 8.0 and pH 4.5) that were prepared were also used. Culture media and buffer solution were steam sterilized at 121 °C for 15 min. Fresh cultures of *E. coli* (ATCC 8739) and *Bacillus subtilis* (ATCC 6633) were maintained in slants of TSA.

2.2. Instruments and equipment

A microplate reader (Polaris, Celer, Brazil) and a microplate incubator/agitator (Bioshake iQ, Analytik Jena, Germany) were used for turbidimetric microplate bioassay. Agar diffusion microbiological assays were performed using a bacteriological incubator (SP Labor, Brazil) and an antibiotic zone reader (Haloes caliper, IUL). An analytical balance (AUY220, Shimadzu, Japan), a pH meter (PG1800, Gehaka, Brazil), and an autoclave sterilizer (Lutz Ferrando, Brazil) were used to prepare and sterilize culture media and solutions.

2.3. Turbidimetric microplate and agar diffusion bioassays for apramycin

Turbidimetric microplate bioassays for apramycin were performed as described by Lourenço and Pinto (Lourenço and Pinto, 2011). Aliquots of 100 μL of apramycin reference standard solutions (concentrations of 5, 10, 15, 20, 25, 30, and 35 mg L^{-1}) and Apralan™ solutions (theoretical concentration of 20 mg L^{-1}) were transferred to microplate. Also, aliquots of 100 μL of TSB inoculated with *E. coli* (ATCC 8739) (5.0% *v*/v) were transferred to microplate. In addition, negative and positive controls were also included. Microplates were incubated at 37.0 ± 1.0 °C for 180 min, and absorbances were measured at 630 nm at intervals of 15 min. Relative potencies of Apralan™ were determined based on dose–response curve (microbial growth measured as absorbance *vs.* log of apramycin concentration) after incubation, as described in most of official compendia (Farmacopéia Brasileira, 2010; United States Pharmacopeia, 2014). Alternatively, relative potencies were determined according to the integral method (area under the

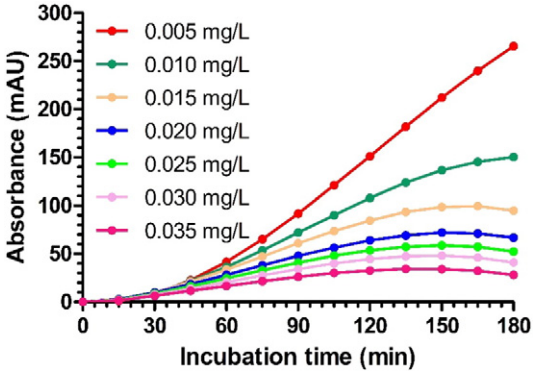

Fig. 1. *Escherichia coli* (5% suspension) growth curves in the presence of apramycin (from 0.005 to 0.035 mg/L), incubated at 37 ± 1.0 °C for 180 min.