NOTE

A new assay to determine Oritavancin concentrations

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Oritavancin (The Medicines Company, St Laurent, Quebec, CA, USA) is an investigational parenteral lipoglycopeptide antibiotic that demonstrates activity against a large variety of gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase-negative *S. aureus*, *Streptococcus pneumoniae* and vancomycin-susceptible and resistant Enterococci.^{1–4} With a rapid concentration-dependent activity and the ability to accumulate intracellularly, oritavancin represents a promising candidate for the treatment of serious and/or persistent gram-positive infections.^{5,6} A phase 2 clinical trial evaluating single and infrequent doses of oritavancin with once-daily dosing in the treatment of complicated skin and skin structure infections caused by MRSA started in January 2007 in the United States, and positive results have been already reported.⁷ Several studies mimicking expected human dosages have also been performed to evaluate oritavancin *in vitro* and *in vivo* activities with the issue of confirming whether the dosing regimen was appropriately simulated.^{4,8,9} Indeed, owing to its large molecular size, the diffusion into agar is difficult and standard biological assays cannot be easily performed to measure the concentration of oritavancin in experimental or human samples. So far, available options include expensive and complicated procedures, such as scintillation counting of radioactive labeled drug or liquid chromatography/mass spectrometry, respectively.^{4,8,9}

Bioassays are relatively accurate techniques that represent attractive alternatives due to their easy and rapid performance at a very low cost. Usually performed by paper disk diffusion on agar, several factors such as stability of the antibiotic solutions, diffusion of the drug into the agar, the organism's susceptibility, inoculum size, pH, incubation time and temperature can affect the performance and the accuracy of this assay. By taking into consideration most of these parameters, we developed and validated a biological assay that can be easily performed to measure oritavancin concentrations in experimental samples with or without albumin.

Bacillus subtilis ATCC 6633 and *Micrococcus luteus* ATCC 9341 were evaluated as test organisms to develop the assay. Mueller–Hinton broth (Difco, Detroit, MI, USA), adjusted at 25 mg l⁻¹ of calcium and

12.5 mg l⁻¹ of magnesium (25 SMHB, for 25 supplemented MH broth) and containing 0.002% of polysorbate-80, was used for standard dilutions and susceptibility testing. Antibiotic medium 11 (AAM 11, Difco) was used as assay medium. As pilot experiments performed in our laboratory demonstrated improved diffusion kinetics of oritavancin in alkalinized agar medium, the pH was adjusted with 1 M sodium hydroxide to a pH ranging between 9 and 10 immediately before pouring the plates. Oritavancin standard concentrations of 1, 10, 25, 50 and 100 mg l⁻¹ were freshly prepared on the day of experiments from lyophilized powder appropriately diluted in 25 SMHB with or without serum. An appropriate volume of albumin (Red Cross, Detroit, MI, USA) was added to the broth in order to evaluate if the presence of albumin (at a final concentration of 4 g dl⁻¹) affects the performance of the assay. Polysorbate-80 was added to any solvents, diluents and broth used to achieve a final concentration of 0.002% in the assay. A volume of 20 µl of each standard concentrations of oritavancin (LY333328, Lot 103RM6, Eli Lilly Research Laboratories, Indianapolis, IN, USA) was loaded in duplicate into the 4 mm wells previously drawn into the agar using a sterile punching device. Plates (15 cm diameter) were left at room temperature (25 °C) for 24 h to allow for the diffusion of the drug into the agar. The suspensions of *M. luteus* or *B. subtilis* at 10⁵–10⁸ CFU ml⁻¹ were lawned onto the surface of the plates until complete and homogeneous dispersion of the suspension. Plates were incubated at 37 °C for 24 h and resulting zone sizes were measured either by hand using a dial Manostat caliper with 0.05-mm scale or by computerized plate reader (Protocol, Synoptic Limited, Frederick, MD, USA). Mean and s.d. values were plotted using SigmaPlot software (Version 10.0. 2006 Systat. Software, Washington, IL, USA), and linear regression curves were graphed to determine the coefficients of variation.

Oritavancin minimum inhibitory concentrations were determined according to the CLSI M7-A8 guidelines (CLSI, 2009) and were 0.12 and 0.5 for *M. luteus* at 10⁷ and 10⁸ CFU ml⁻¹, respectively, and 0.015 and 0.12 mg l⁻¹ for *B. subtilis* at 10⁵ and 10⁷ CFU ml⁻¹, respectively.

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Table 1 Performance (r^2) of the bioassay under various conditions

Methods of measurement	Standard concentration (mg l^{-1})	r^2					
		Micrococcus luteus ATCC 9341 at			Bacillus subtilis ATCC 6633		
		10^7 CFU ml^{-1}	10^8 CFU ml^{-1}	10^8 CFU ml^{-1} + albumin (4 g dl^{-1})	10^5 CFU ml^{-1}	10^5 CFU ml^{-1} + albumin (4 g dl^{-1})	10^7 CFU ml^{-1}
Manual	1–100	0.858	0.827	0.642	0.863	0.590	0.926
	10–100	0.989	0.992	0.997	0.980	0.972	0.994
	25–100	0.997	0.987	0.999	0.988	0.951	0.997
Computerized	1–100	0.870	0.861	0.628	0.865	0.739	0.8541
	10–100	0.995	0.988	0.995	0.999	0.987	0.996
	25–100	0.998	0.970	0.999	0.999	0.988	0.989

Abbreviations: ATCC, American type culture collection; CFU, colony-forming unit.

Figure 1 Effect of the test organism, inoculum size and presence of albumin on the performance of the bioassay: (a) with *Bacillus subtilis*, (b) with *Micrococcus luteus*. Lines represent regression curves determined on the basis of three duplicate of oritavancin standard concentrations, namely 1, 10, 25, 50 and 100 mg l^{-1} .

Table 1 summarizes the performance of the bioassay as a function of the different conditions evaluated in this study (test organism, inoculum size, presence of protein and method of zone size determination). Overall, low concentrations of oritavancin (from 1 to 10 mg l^{-1}) led to a greater variability in the assay with lower r^2 values. Best performances ($r^2 > 0.98$) were observed for concentrations ranging from 10 to 100 mg l^{-1} . Both test organisms led to suitable results with r^2 values > 0.9 in the high ranges of oritavancin concentrations in presence or absence of albumin. Although the differences between the two inocula evaluated were only minimal, the use of low inoculum for *M. luteus* (10^7 CFU ml^{-1}) and *B. subtilis* (10^5 CFU ml^{-1}) led to the most reliable and reproducible results (Table 1 and Figure 1). Addition of albumin at 4 g dl^{-1} partially and mainly affected the performance of the assay in the low and unreliable range of concentrations (1 – 10 mg l^{-1}) (Table 1, Figure 1). As reported in the Table 1, the difference between the assay performances using computerized or manual reading were mostly negligible (especially for the highest range of concentrations). Between day and intra-day coefficients of variation were $< 5\%$ for both test organisms, both methods of zone size determination and irrespective of the presence of protein.

In conclusion, we developed an easy and reliable assay to determine oritavancin concentrations in biological samples and demonstrated that the presence of albumin does not represent a major issue for the determination of oritavancin concentrations. Taken together, our results suggest that low concentrations of oritavancin ($< 10 \text{ mg l}^{-1}$)

may not be reliably detected by agar diffusion method. Samples exceeding 100 mg l^{-1} could be easily diluted within 10 and 100 mg l^{-1} to give reliable results using that microbiological assay.

CONFLICT OF INTEREST

Potential conflicts of interest. MJR received research grants from Astellas, Cubist, Forest and Pfizer; consulted for Astellas, Cubist, Forest, Ortho-McNeil and Targanta; and has served on speaker's bureaus for Cubist, Novartis, Wyeth, Pfizer and Targanta.

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