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Short communication

Quantification of voriconazole in human bronchoalveolar lavage fluid using high-performance liquid chromatography with fluorescence detection

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ABSTRACT

The quantification of voriconazole concentration in lung epithelial lining fluid to facilitate the management of pulmonary fungal colonisation or aspergillosis is of increasing interest. An accurate and reproducible high-performance liquid chromatography method to quantify voriconazole in human bronchoalveolar lavage (BAL) fluid was developed and validated. BAL samples were concentrated by freeze-drying and reconstituted with water prior to deproteinisation. Separation was achieved with a C18 column employing fluorescence detection (excitation: 260 nm, emission: 370 nm). The calibration curves were linear from 2.5 to 500 ng/mL. The intra- and inter-day precisions were within 7%. Accuracies ranged from 102% to 107%. The clinical applicability was established by successful measurement of voriconazole concentrations in lung transplant recipients. The assay provides an alternative approach for those with negligible access to liquid chromatography–tandem mass spectrometry instrumentation.

1. Introduction

Voriconazole (VRC, Fig. 1) is a triazole antifungal agent with an extended spectrum of activity against various pathogenic yeasts and moulds, including *Scedosporium* and *Fusarium* species [1]. It is widely used for antifungal prophylaxis, and empirical and targeted treatment of invasive fungal diseases. In adults, VRC displays non-linear pharmacokinetics [2], such that an increase in daily dose may result in a disproportionate increase in plasma or tissue concentration. The high variability of serum/plasma VRC concentrations [3] is secondary to CYP2C19 polymorphism, drug–drug interactions [3] and the marked variation in oral bioavailability, which remains unpredictable and poorly defined [4–6].

Plasma VRC trough concentration (≥1 μg/mL) has been associated with a higher rate of successful treatment response [7,8] and improved survival [9] while elevated concentrations (≥5.5 μg/mL) are associated with neurotoxicity [7,10,11] and hepatotoxicity [12–15]. Assessment of plasma VRC concentrations is therefore recommended to optimise clinical management [16]. Noting that the lungs are particularly common target for fungal disease [17,18], development of a robust and simple assay to measure VRC levels in pulmonary tissue is potentially useful clinically. Bronchoalveolar lavage (BAL) [19,20] is a simple non-invasive technique to sample epithelial lining fluid (ELF) of the lungs; however, quantification methods for VRC in BAL samples are sparse. To our knowledge, there are only two published HPLC methods utilising mass spectrometry (MS) detection to measure VRC concentration in BAL samples [21,22]. Of these, the full assay protocol and validation results were not provided in the brief report by Capitano et al. [21]. Crandon et al. provided a more detailed description of their liquid chromatography (LC)–tandem MS assay, which involved dilution of BAL samples with methanol and direct injection into the chromatographic system [22]. Good sensitivity (1 ng/mL) was achieved and the sample volume required was small (200 μL). The widespread use of LC–MS methods, however, is restricted by high acquisition and running costs of these instruments.

The natural fluorescent property of the triazole functional group of VRC represents an opportunity to obtain adequate selectivity and sensitivity with simple sample preparation using a fluorescence detector. Accordingly, the aim of this study was to establish a HPLC-fluorescence method for quantifying VRC in human BAL using freeze-drying prior to chromatography.

2. Experimental

2.1. Chemicals

VRC was kindly provided by Pfizer (Australia). Ketoconazole, acetonitrile (HPLC grade), ammonia 25% solution and ammonium

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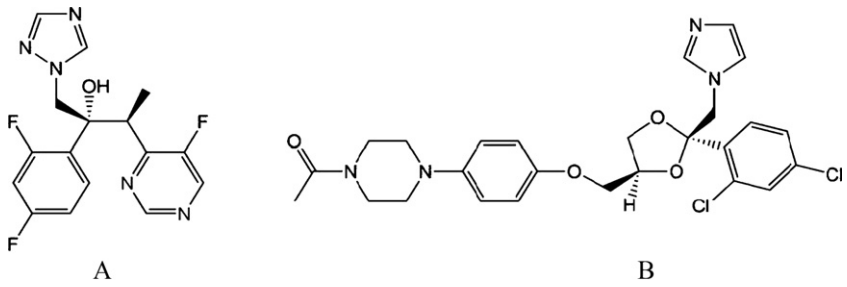

Fig. 1. Molecular structures of voriconazole (A) and the internal standard, ketoconazole (B).

dihydrogen orthophosphate were purchased from Sigma–Aldrich (Australia). HPLC-grade water was from a Milli-Q Gradient system (Millipore Corp., Bedford, Mass).

2.2. Preparation of calibration standards, quality control samples and internal standard

The stock solutions (1 mg/mL) of VRC and ketoconazole (internal standard (IS), Fig. 1) were individually prepared in methanol and stored for a maximum of three months at −20 °C based on reported stability data [23,24]. The series of VRC working solutions (200–1 μg/mL) were freshly prepared from VRC stock solutions by serial dilutions with methanol–water (50:50, v/v). The IS working solutions (3.2 μg/mL) were prepared in methanol–water (50:50, v/v). Normal saline was used as a surrogate matrix [22,25] to construct the calibration standards at 2.5, 5, 10, 20, 40, 80, 200 and 500 ng/mL. These standard samples were used to generate regression equations for BAL calibration curve.

All routine saline-based (7.5, 75, 400 ng/mL) quality control (QC) samples were prepared on the day of analysis. These QC samples were used for back-calculation of VRC concentrations by the regression equation, which acted as the criteria to accept or reject the analytical run.

To validate the saline-based calibration curve, blank BAL samples (i.e. VRC-free) were collected from lung transplant (LTx) recipients under polymedication but not receiving VRC at the time of bronchoscopy, to prepare BAL-based low (7.5 ng/mL) and high (400 ng/mL) QC samples. Saline-based QC samples were also prepared for storage stability tests.

The collection and use of BAL samples from LTx recipients were approved by the institutional Human Research Ethics Committees of Alfred Health and Monash University. Informed consent was obtained.

2.3. Sample preparation

BAL samples collected were immediately centrifuged at 1500 × *g* for 5 min to harvest BAL supernatant, which was stored at −80 °C until analysis. On an assay day, frozen BAL supernatant was thawed and 4 mL of the BAL supernatant (or saline-based sample) was pipetted into a 10 mL polypropylene tube. After addition of 10 μL IS working solution, the resultant mixture was vortex-mixed and freeze-dried (for ~48 h). A benchtop freeze dryer (VirTis AdVantage 2.0, SP Scientific, USA) was used, with the shelf temperature set to −40 °C and the process condenser temperature at −80 °C. The resultant lyophilised powder was reconstituted in 200 μL of water and briefly vortex-mixed, after which 200 μL of acetonitrile was added. The resulting mixture was then centrifuged (4 °C) at 3200 × *g* for 10 min. An aliquot (70 μL) of the upper phase (predominantly acetonitrile) was injected into the HPLC system.

2.4. Instrumentation and chromatographic conditions

The HPLC system used was a Shimadzu system, with two LC-20AD pumps, a SIL-20ACHT autoinjector (Shimadzu) and a RF-10A*xl* variable wavelength fluorescence detector (Shimadzu, Kyoto, Japan). Data collection and processing were carried out by a Multichrom chromatography data acquisition and processing system.

The chromatographic separation was performed using a Phenosphere-Next C18 5-μm (250 mm × 4.6 mm) column, protected by a 4 mm × 3 mm C18 guard cartridge (Phenosphere-Next) filled with the same material. Column temperature was maintained at 35 °C (to optimise chromatographic efficiency), with the autosampler set to 4 °C (as per standard practice in our laboratory). The mobile phase was adapted from Gage and Stopher [26], consisting of 0.04 M ammonium dihydrogen phosphate buffer (adjusted with ammonia 25% to pH 5.8) and acetonitrile (50:50, v/v). The flow rate was 1 mL/min. Fluorescence detection was conducted with excitation wavelength 260 nm and emission wavelength 370 nm. The chromatographic run time was 13.5 min per injection.

2.5. Specificity and selectivity

The interference from commonly co-administered drugs in the target patient population was investigated by analysing blank BAL samples from six LTx recipients who were receiving multiple medications, but not VRC.

2.6. Accuracy, precision, recovery and lower limit of quantification

In accordance with the US FDA Guidance for Industry-Bioanalytical Method Validation [27], each analytical run included one blank, eight standards and three replicate QC samples of low, medium and high. Calibration curve was constructed by plotting the chromatographic peak area ratios of VRC to IS against concentrations of VRC. The slope-intercept linear regression model: $y = mx + b$ was selected to express linearity and a weighting factor of 1/concentration^2 was used to achieve homogeneity of variance. The best-fit line was generated without forcing through zero. For each calibration curve, a coefficient of determination (r^2) of greater than 0.99 was desirable. The unknown concentrations of VRC were calculated from the linear regression equation for the calibration curve. One set of calibration curve samples was prepared for each inter-day run; calibration (and QC) samples were randomly distributed in the autosampler.

Accuracy was defined by the difference between the observed (C_{obs}) and nominal (C_{nom}) concentrations of low, medium and high QC samples, expressed as percent bias: $\%bias = [(C_{obs} - C_{nom})/C_{nom}] \times 100$. Precision was estimated as the percent coefficient of variation (%CV) of each QC, which was calculated by standard deviation (SD) of the observed concentration over the mean concentration: $\%CV = (SD/C_{mean,obs}) \times 100$. Intra-day accuracy and precision were evaluated for the three levels of

QCs with six replicate measurements within the same run, and in three replicates per run on five separate days. The accuracies and precisions should be within ±15% of the nominal value. The precision of the BAL-based QC samples was calculated using the saline-based calibration curve.

Absolute recoveries were evaluated by comparing the chromatographic peak areas of extracted samples (A_{ext}) with those of the unextracted samples ($A_{un\text{-}ext}$), which represent 100% extraction efficiency, for the three QC samples (low, medium and high), using $(A_{ext}/A_{un\text{-}ext}) \times 100\%$. The unextracted samples were prepared by spiking corresponding VRC and IS working solutions in 9% sodium chloride solution, vortexing the resultant mixture, centrifuging the mixture and then injecting the upper phase. Mean recoveries of VRC and IS were obtained from six determinations of each QC level.

The lower limit of quantification (LLOQ) was defined by the lowest concentration on the calibration curve that can be quantified within ±20% accuracy and precision, and was determined by analysing six replicates of samples spiked to LLOQ concentration of analyte.

2.7. Stability

A stability study was performed to examine the effect of long-term storage at −80 °C on VRC in saline-based samples. The effect of repeated freezing–thawing was investigated by assaying saline-based QC samples over three cycles. Each freeze–thaw cycle consisted of storage for at least 24 h at −80 °C, followed by a complete thaw at room temperature. Samples were analysed before being re-frozen and after cycle 3. Post-preparative stability was tested by storing the upper phase after sample preparation of saline-based QC samples in the autosampler (4 °C) for 24 h prior to analysis. For all stability experiments, three replicates at each QC level were analysed. Stability was validated if measured concentrations did not deviate by more than 15% from freshly processed samples.

3. Results

3.1. Chromatography

Fig. 2A shows a representative chromatogram for a BAL extract from a LTx recipient, who was not receiving VRC at the time of sampling. Fig. 2B shows the chromatogram of VRC spiked at the LLOQ concentration (2.5 ng/mL) while Fig. 2C displays a chromatogram of a BAL sample from a LTx recipient receiving VRC; the sample contained 0.31 μg/mL VRC. The retention times for VRC and IS were 6.64 ± 0.01 and 11.6 ± 0.04 min, respectively. The retention times for VRC in BAL or saline-based samples were identical. No 'ghost peaks' or carryover were observed.

3.2. Accuracy, precision, recovery and lower limit of quantification

The intra- and inter-day results are presented in Table 1. The assay was accurate (mean accuracy ranging from 96.6% to 106.9%) and precise (intra-day CV ranged from 3.82% to 5.79% and inter-day CV from 2.88% to 6.88%). The BAL QC samples gave similar results as the saline-based QC samples, with accuracy and precision within the predefined acceptance limit of 15% (Table 1). The mean recoveries of VRC in saline extract ranged from 72.0% to 81.3%. Mean recovery for IS was 62.4 ± 1.8% (n = 6). The LLOQ of the BAL method was 2.5 ng/mL. The calibration curve (inter-day analysis, n = 5) was linear over the range 2.5–500 ng/mL with mean values for slope = 0.0214 ± 0.0016, intercept = −0.0122 ± 0.0083 and r^2 = 0.9967 ± 0.0016.

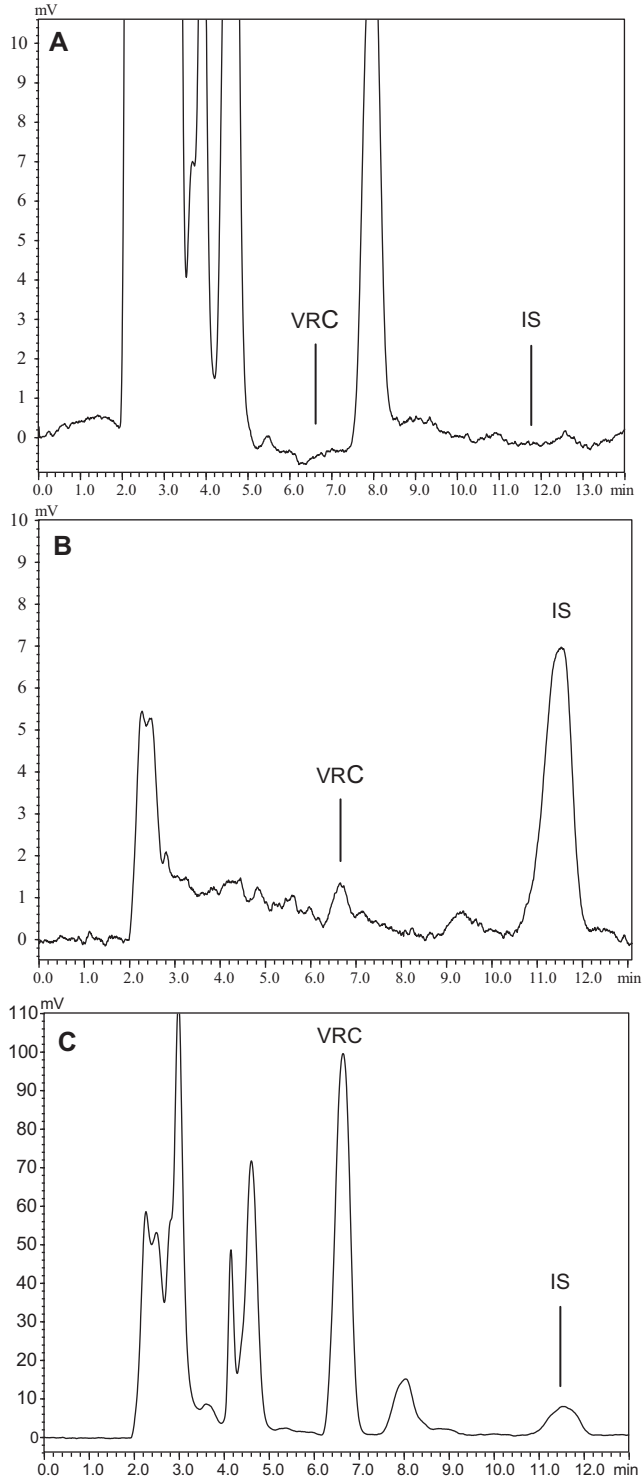

Fig. 2. Chromatograms: (A) blank BAL sample from LTx recipient not receiving VRC; (B) saline spiked with 2.5 ng/mL of VRC (LLOQ) and 8 ng/mL of IS; (C) BAL sample from LTx recipient receiving VRC 200 mg orally twice daily (VRC 0.31 μg/mL).

3.3. Specificity and selectivity

BAL samples collected from six LTx recipients who were not receiving VRC, but were receiving other drugs commonly co-administered in this patient population, showed no interference at the retention times of VRC and IS. These LTx recipients were receiving immunosuppressants (tacrolimus, azathioprine, mycophenolate mofetil, and prednisolone), antibiotics

Table 1
Precision and accuracy data for VRC in saline and human BAL samples.

Parameter	Conc[a] (ng/mL) in saline				Conc[a] (ng/mL) in BAL	
	2.5 (LLOQ)	7.5 (QC-L)	75 (QC-M)	400 (QC-H)	7.5 (QC-L)	400 (QC-H)
Accuracy (% bias)						
Intraday ($n=6$)	8.00	6.89	6.18	2.08	4.44	−10.4
Interday ($n=15$)[b]	2.54	0.00	−0.60	−3.44		
Precision (% CV)						
Intraday ($n=6$)	6.20	5.49	3.82	5.79	5.16	5.21
Interday ($n=15$)[b]	1.94	6.17	6.88	2.88		
Recovery ($n=6$, mean% ± SD)	108.0 ± 6.7	72.0 ± 3.6	81.3 ± 3.1	77.0 ± 4.3		

Conc, concentration; CV, coefficient of variation; SD, standard deviation; QC, quality control samples at low (QC-L), medium (QC-M) and high (QC-H) concentration points.
[a] Nominal concentration of quality control samples in normal saline or BAL samples.
[b] The 15 replicates for inter-day analysis were pooled from five separate assay days (i.e., three replicates for each concentration per day).

Table 2
Stability of VRC in saline.

Nominal conc (ng/mL)	Mean observed conc (ng/mL)	Precision (% CV)
14 days stability at −80 °C ($n=3$)		
7.5	6.83	1.69
75	75.3	0.88
400	404.6	0.11
Three freeze–thaw cycles ($n=3$)		
7.5	6.90	3.83
75	73.8	7.00
400	392.6	2.65
Autosampler stability (4 °C) for 24 h ($n=3$)		
7.5	6.57	4.90
75	74.0	3.02
400	413.5	11.1

Conc, concentration; CV, coefficient of variation.

(sulfamethoxazole and trimethoprim, ciprofloxacin, vancomycin, and nebulised tobramycin), antiulcer agents (ranitidine, pantoprazole, nizatidine, antacid, and esomeprazole), antiemetic agents (metoclopramide and domperidone), supplements (calcium, vitamin D, magnesium and iron), lipid-lowering drugs (atorvastatin, pravastatin, and rosuvastatin) and miscellaneous agents (valganciclovir, terbinafine, aspirin, alendronate, frusemide, perindopril, irbesartan, Seretide® (i.e. fluticasone and salmeterol combination) inhaler, Creon® (i.e. pancrelipase), insulin, oestradiol, oxycodone and Intragam® (immunoglobulin)). Fig. 2A presents the typical chromatogram of BAL extracted from a LTx recipient who was not receiving VRC.

3.4. Stability

VRC in saline (as surrogate matrix for BAL sample) was stable for at least 14 days when stored at −80 °C, throughout the three freeze–thaw cycles and at 4 °C in the autosampler for 24 h (Table 2).

3.5. Clinical application

The methods described in this report are currently in use to analyse BAL samples collected from an on-going clinical study involving LTx recipients who are receiving oral VRC therapy and have been successful in the measurement of VRC concentrations. Typically patients receive VRC therapy for an extended period (weeks/months). Thus, the BAL assay turnaround time of 48–72 h (which includes the freeze-drying step) is feasible in a clinical setting and similar to that of many other tests that are used in diagnosis or monitoring.

4. Discussion and conclusion

We present a simple and sensitive analytical method to determine VRC concentration over the ranges clinically encountered in BAL (2.5–500 ng/mL), with negligible interference from a number of medications commonly prescribed to LTx recipients. When coupled with simultaneous measurement of urea levels in BAL supernatant and plasma, these assays can be used to estimate VRC concentration in pulmonary ELF. The use of ketoconazole as the IS controls the variation in sample processing and improves robustness of the methods. The relatively low recovery rates of VRC and IS were most probably related to the large surface areas of the vessels used for freeze-drying. Despite not being 100%, the recoveries were precise and reproducible.

Our method of determining VRC concentrations in BAL samples, involving freeze-drying, allows the BAL supernatant to be concentrated up to 10-fold and therefore enables a LLOQ of 2.5 ng/mL to be reached. Sequential reconstitution of the lyophilised powder with water and acetonitrile was required to firstly dissolve the sodium chloride and then precipitate the proteins, which may be present in a BAL sample. Although acetonitrile is miscible with water, the presence of solvated salt increases the ionic strength and polarity of water, thereby reducing the mutual miscibility, resulting in phase separation (salting out) of acetonitrile from the aqueous phase. Due to the specific physiochemical properties of VRC (weak base, $pK_a = 1.76$ and moderate hydrophobicity, $\log P = 1.8$) [28], this compound tends to partition into the salted-out upper acetonitrile phase. At the nominal pH of 5.5 (4.5–7.0) in sodium chloride solution, ionisation of VRC is very low (0.03%), which enhances its partitioning into the upper acetonitrile phase. Thus as expected, the extraction recovery of VRC in BAL samples was good (~75%).

In conclusion, a sensitive, accurate and reproducible HPLC assay using fluorescence detection for quantification of voriconazole concentrations in BAL samples was developed and validated. The assay can be readily adopted by laboratories, particularly those with limited access to newer MS-based analytical instruments, to support monitoring of VRC concentrations in pulmonary ELF, optimise VRC therapy and/or support ongoing clinical studies.

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References

[1] R. Herbrecht, Expert Rev. Anti Infect. Ther. 2 (2004) 485.

[2] L. Purkins, N. Wood, P. Ghahramani, K. Greenhalgh, M.J. Allen, D. Kleinermans, Antimicrob. Agents Chemother. 46 (2002) 2546.
[3] D. Leveque, Y. Nivoix, F. Jehl, R. Herbrecht, Int. J. Antimicrob. Agents 27 (2006) 274.
[4] M.L. Goodwin, R.H. Drew, J. Antimicrob. Chemother. 61 (2008) 17.
[5] W.W. Hope, E.M. Billaud, J. Lestner, D.W. Denning, Curr. Opin. Infect. Dis. 21 (2008) 580.
[6] R.J. Bruggemann, J.P. Donnelly, R.E. Aarnoutse, A. Warris, N.M. Blijlevens, J.W. Mouton, P.E. Verweij, D.M. Burger, Ther. Drug Monit. 30 (2008) 403.
[7] A. Pascual, T. Calandra, S. Bolay, T. Buclin, J. Bille, O. Marchetti, Clin. Infect. Dis. 46 (2008) 201.
[8] J. Smith, N. Safdar, V. Knasinski, W. Simmons, S.M. Bhavnani, P.G. Ambrose, D. Andes, Antimicrob. Agents Chemother. 50 (2006) 1570.
[9] M. Neely, T. Rushing, A. Kovacs, R. Jelliffe, J. Hoffman, Clin. Infect. Dis. 50 (2010) 27.
[10] A. Imhof, D.J. Schaer, U. Schanz, U. Schwarz, Swiss Med. Wkly. 136 (2006) 739.
[11] A.E. Boyd, S. Modi, S.J. Howard, C.B. Moore, B.G. Keevil, D.W. Denning, Clin. Infect. Dis. 39 (2004) 1241.
[12] S. Trifilio, R. Ortiz, G. Pennick, A. Verma, J. Pi, V. Stosor, T. Zembower, J. Mehta, Bone Marrow Transplant. 35 (2005) 509.
[13] D.W. Denning, P. Ribaud, N. Milpied, D. Caillot, R. Herbrecht, E. Thiel, A. Haas, M. Ruhnke, H. Lode, Clin. Infect. Dis. 34 (2002) 563.
[14] K. Tan, N. Brayshaw, K. Tomaszewski, P. Troke, N. Wood, J. Clin. Pharmacol. 46 (2006) 235.
[15] I. Lutsar, M.R. Hodges, K. Tomaszewski, P.F. Troke, N.D. Wood, Clin. Infect. Dis. 36 (2003) 1087.
[16] T.J. Walsh, E.J. Anaissie, D.W. Denning, R. Herbrecht, D.P. Kontoyiannis, K.A. Marr, V.A. Morrison, B.H. Segal, W.J. Steinbach, D.A. Stevens, J.A. van Burik, J.R. Wingard, T.F. Patterson, Clin. Infect. Dis. 46 (2008) 327.
[17] P.G. Pappas, B.D. Alexander, D.R. Andes, S. Hadley, C.A. Kauffman, A. Freifeld, E.J. Anaissie, L.M. Brumble, L. Herwaldt, J. Ito, D.P. Kontoyiannis, G.M. Lyon, K.A. Marr, V.A. Morrison, B.J. Park, T.F. Patterson, T.M. Perl, R.A. Oster, M.G. Schuster, R. Walker, T.J. Walsh, K.A. Wannemuehler, T.M. Chiller, Clin. Infect. Dis. 50 (2010) 1101.
[18] N. Singh, D.L. Paterson, Clin. Microbiol. Rev. 18 (2005) 44.
[19] N.R. Patel, P.S. Lee, J.H. Kim, G.L. Weinhouse, H. Koziel, Chest 127 (2005) 1388.
[20] L. Velez, L.T. Correa, M.A. Maya, P. Mejia, J. Ortega, V. Bedoya, H. Ortega, Respir. Med. 101 (2007) 2160.
[21] B. Capitano, B.A. Potoski, S. Husain, S. Zhang, D.L. Paterson, S.M. Studer, K.R. McCurry, R. Venkataramanan, Antimicrob. Agents Chemother. 50 (2006) 1878.
[22] J.L. Crandon, M.A. Banevicius, A.F. Fang, P.H. Crownover, R.F. Knauft, J.S. Pope, J.H. Russomanno, E. Shore, D.P. Nicolau, J.L. Kuti, Antimicrob. Agents Chemother. 53 (2009) 5102.
[23] C. Michael, J. Teichert, R. Preiss, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 865 (2008) 74.
[24] K.H. Yuen, K.K. Peh, J. Chromatogr. B: Biomed. Sci. Appl. 715 (1998) 436.
[25] J.E. Conte Jr., J.A. Golden, S. Duncan, E. McKenna, E. Zurlinden, Antimicrob. Agents Chemother. 39 (1995) 334.
[26] R. Gage, D.A. Stopher, J. Pharm. Biomed. Anal. 17 (1998) 1449.
[27] Food and Drug Administration, 2001, Available from http://www.fda.gov/cder/guidance/index.htm
[28] A.I. Adams, L.N. Morimoto, L.Z. Meneghini, A.M. Bergold, Braz. J. Infect. Dis. 12 (2008) 400.