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An alternative capillary electrophoresis method for the quantification of caspofungin in lyophilisate powder and its measurement uncertainty

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Recently, the use of capillary electrophoresis (CE) methods in pharmaceutical practice has gradually increased. Moreover, measurement uncertainty has been used to guarantee the traceability and the reliability of analytical results obtained. The aim of this work was to develop and validate a CE method for the quantification of caspofungin in lyophilised powder for injection, as well as to study the main sources of uncertainty associated with the method proposed and establish a procedure to estimate uncertainty in routine analysis. The results obtained during the validation procedure were statistically evaluated and demonstrate the specificity, robustness, linearity (r = 0.9999; y= 7.9014x-0.0107, concentration range: 20 to 300 μg/ml), precision (repeatability, RSD: 0.40%; intermediate precision, RSD: 0.54%) and accuracy (recovery range: 95.80 to 100.45%). Without using internal standard correction, almost all uncertainty is associated to repeatability of sample and standard peak areas (more than 90%). On the other hand, using internal standard reduced variability significantly. The results allow us to affirm that EC method is suitable for analysis of caspofungin and it may be applied in routine quality control laboratories. In addition, this study confirmed that the equations proposed in the paper may be used for the measurement of uncertainty estimation in routine analysis.

Key words: Caspofungin, capillary electrophoresis, measurement uncertainty.

INTRODUCTION

In recent years, there has been an increase in systemic fungal infections, either by population aging, increasing the number of surgical interventions, even by the number of patients with compromised immune system, among others. Caspofungin is an antifungal that belongs to the echinocandin class, with significant use in hospitals worldwide (Bennett, 2006).

Capillary electrophoresis has been employed in the drugs and medicines analysis due to its analytical technique features. The advantage of CE in comparison with other methods of analysis is the speed, easiness of implementation, low consumption of solvents and reagents, small

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amounts of sample, among others (Ekiert et al., 2010).In addition, capillary electrophoresis may use several mechanisms of separation, which allow the separation of a broad kind of chemical substances (Tavares, 1997). The direct implication of this versatility defines one of the most important aspects of this technique, which is the possibility of analyzing structures from small ions to macromolecular dimensions (Tavares, 1997).

Despite the importance of echinocandins, there are a few number of methods described in literature and none in most important official compendia and pharmacopeias. Caspofungin in biological samples and in pharmaceutical products is often analyzed by high performance liquid chromatography with mass spectrometry (Egleet al., 2004; Rochatet al., 2007; Neoh et al., 2010), electrochemical (Traunmuller et al., 2006) and UV detector (Ghisleni et al., 2014b) or by microbiological assay (Ghisleni et al., 2014a). However, a capillary electrophoresis method of analysis of echinocandins, including caspofungin, was not found in literature.

Since the ISO 17025 publication, major importance has been given to the traceability and reliability of analytical results (Mueller, 2002; ISO 17025, 2005). An analytical result is not complete unless it has been reported with its measurement uncertainty (Desimoni and Brunetti, 2011). Measurement uncertainty is a parameter that, when associated with a measurement result, characterizes values dispersion that can be fundamentally attributed to a result (Eurachem, 2012; Traple et al., 2014).

The main sources of uncertainty should be considered in the final uncertainty estimation, including sampling (Wunderli, 2003), matrix effects and interferences, environmental conditions (Leito et al., 2002; Lourenço et al., 2012), uncertainties of mass and volumetric equipment, uncertainties of spectrophoto-metric (Sooväli et al., 2006; Hsu and Chen, 2010) and chromatographic equipment (Anglov et al., 2003; Lourenço, 2012), uncertainties of biological and microbiological responses (Niemi and Miemelã, 2001; Lourenço, 2012), purity of reagents and chemical reference substances (Weitzel, 2012), method validation (Brüggemann and Wennrich, 2002) and random variability (Chui et al., 2002).

Several works regarding the measurement uncertainty from high performance liquid chromatograph methods (Okamoto et al., 2013; Ghisleni et al., 2014b), UV spectrophotometric methods (Saviano and Lourenço, 2013) and microbiological assays (Lourenço, 2012; Ghisleni et al., 2014a) have been reported in literature. However, as found in literature, there is no work describing the identification and quantification of main sources of measurement uncertainty from capillary electrophoresis methods.

The aim of this work was to develop and validate a capillary electrophoresis (CE) method for the quantification of caspofungin in lyophilised powder for injection. In addition, the purpose of this study was to study the main sources of uncertainty associated with the proposed method and to establish a procedure to estimate measurement uncertainty in routine analysis.

MATERIALS AND METHODS

Caspofungin acetate chemical reference substance (CRS) and Cancidas® 50 mg lyophilized powder were kindly supplied by Merck Sharp and Dohme Pharmaceutical Laboratories (São Paulo, Brazil). Other chemical reagents were purchased commercially: sucrose (Synth, Brazil), mannitol (Carlo Erba, Italy), glacial acetic acid (Sigma-Aldrich, Brazil), phosphoric acid (Carlo Erba, Italy), triethylamine (Merck, Germany), peroxide 30% hydrogen (Merck, Germany), sodium hydroxide (Mallindckrodt, Mexico), and loratadine (Zydus Pharmaceuticals Ltd., USA).

The equipment and instruments used were: water purifier system (Milli-Q, Millipore, USA), pHn meter (PG1800, Gehaka, Brazil), fused silica capillary (Polymicro Technologies, total length 50 cm, 40 cm effective length with internal diameter of 75 µm), and capillary electrophoresis equipment (Beckman Coulter, Proteomelab ™ model 800 PA) equipped with high-speed system controller 32 Karat ™ version 9.0 and photodiode detector (PDA) monitoring wavelengths between 190 and 350 nm.

Method development

Several buffer solutions with pH values ranging between 2.0 and 6.0 were tested as electrolytes during the method development. Several concentrations of caspofungin in a variety of solvents were also tested. Different substances were tested as internal standard candidates. Best electropherograms were obtained using 0.2% triethylamine with pH adjusted to 2.50 $\pm$ 0.05 with 10.0% phosphoric acid as electrolyte. Standard and sample solutions were diluted in electrolyte to final concentration of 100 µg/ml caspofungin and 40 µg/ml loratadine (internal standard). Before injection all solutions were filtered with 0.45 µm cellulose filter.

Caspofungin reference standard solution

Caspofungin reference standard stock solution having 0.5 mg/ml was prepared using ultrapure water as diluent. Loratadine stock solution having 0.5 mg/ml diluted in methanol was used as internal standard stock solution. Aliquots of 5.0 and 2.0 ml of caspofungin reference standard stock solution and loratadine internal standard stock solution, respectively, was transferred to a 25 ml volumetric flask and diluted with electrolyte (S100%).

Cancidas® sample solution

The content of one vial (50 mg of caspofungin) of Cancidas® was suspended and transferred to a 100 ml volumetric flask and diluted with ultrapure water (sample stock solution). Aliquots of 5.0 and 2.0 ml of caspofungin reference standard stock solution and loratadine internal standard stock solution, respectively, was transferred to a 25 ml volumetric flask and diluted with electrolyte (U100%).

Method validation

Method validation was carry out using the analytical conditions described as shown in Table 1 and it was performed according to the recommendations of the current official codes (ICH, 2005; Farmacopéia Brasileira, 2010; US Pharmacoepia, 2013).

Robustness

The main parameters affecting CE resolution are capillary dimensions and nature, separation electrolyte composition (pH,

Table 1. Robustness conditions during EC method validation.

Parameter	Analytical conditions	Modification	
Temperature (°C)	25	23	27
Separation voltage (kV)	25	23	27

Table 2. Dilution scheme used to assess EC method accuracy (recovery).

Recovery solution	Aliquot of caspofungin reference standard stock solution (ml)	Aliquot of Cancidas® stock solution (ml)	Aliquot of loratadine internal standard stock solution (ml)	Final volume (ml)
REC80%	4.0	5.0	2.0	25.0
REC100%	5.0	5.0	2.0	25.0
REC120%	6.0	5.0	2.0	25.0
U100%	-	5.0	2.0	25.0
S100%	5.0	-	2.0	25.0

ionic strength, salt nature, additives), applied electric field and capillary temperature. Robustness was assessed by analyzing caspofungin reference standard and sample solutions using the analytical conditions as shown in Table 1.

Specificity

The specificity was assessed by analyzing caspofungin samples submitted to stress conditions, such as acidic (0.1 N HCl) hydrolyses, alkaline (NaOH 0.1 N) hydrolyses, thermal (45°C) degradation, oxidative (H_2O_2 0.3%) degradation, and UV (254 nm) degradation. Solutions submitted to acidic and alkalinehydrolyses were neutralized before completing the volume with electrolyte. Aliquots of caspofungin stock solution were submitted acidic and alkaline hydrolyses for 1, 2 and 3 h. Caspofungin stock solution was placed in a water bath temperature of 45°C. Aliquots were removed after 20, 40, 60, 80 and 120 min and placed in an ice bath. Aliquots of loratadine internal standard were added in each solution submitted to stress condition. Interference of excipients was also evaluated by analyzing a placebo solution, prepared according to the package information and diluted in electrolyte. Placebo solution was prepared using sucrose, mannitol, acetic acid and sodium hydroxide solution (Merck Sharp & Dohme, 2013).

Linearity

Linearity was assessed by analyzing caspofungin standard solutions having 20, 40, 60, 80, 100, 120, and 300 µg/ml. Three calibration curves were constructed in different days. Statistical analysis was performed using least square linear regression analysis.

Precision

Precision of EC method was determined under repeatability and intermediate precision conditions. Repeatability relative standard deviation (RSD) was calculated from six independent Cancidas® samples having 100 µg/ml analyzed in the same day. Intermediate precision RSD was calculated from six independent Candidas® samples having 100 µg/ml analyzed in different days.

Accuracy

Accuracy was assessed by analyzing Candidas® samples spiked in known amounts of caspofungin reference standard. For this purpose, recovery solutions (REC80%, REC100% and REC120%) were prepared as presented as shown in Table 2. Accuracy was determined as the ratio of recovered and spiked amounts of caspofungin.

Measurement uncertainty

The main sources of uncertainty were identified and their contributions were estimated as standard deviations (standard uncertainties). Combined and expanded uncertainties were calculated as described in Eurachem/Citac guide (Eurachem, 2012). A Monte Carlo simulation was performed in order to evaluate the applicability of Eurachem/Citac procedure in the quantification of caspofungin by CE method.

RESULTS AND DISCUSSION

Method development

Among all the tested electrolytes, 0.2% triethylamine solution with pH 2.5 showed suitable electrophoretic behavior with respect to migration time and asymmetry. The results obtained using electrolyte diluent of reference standard and sample solutions showed a reduction of peak asymmetry values, and a baseline with less noise. The changes in area and retention time observed between runs were significantly reduced with the use of internal standard. Among all tested internal standards, loratadine showed the best electrophoretic profile, with good resolution from the peak of interest (greater than 2.0), besides causing significant improvement in the repeatability of results. Electropherogram of the caspofungin and internal standard is as shown in

Table 3. Optimazed analytical conditions used in caspofungin analysis in lyophilisate powder by EC method.

Parameter		Electrophoretic condition
Capillary	Description	Fused silica capillary (Polymicro Technologies) total length 50 cm, 40 cm effective length with internal diameter of 75 µn
	Activation	30 min with NaOH 1 M + 20 min with electrolyte
	Temperature	25°C
Detection		UV 210 nm – PDA (190 - 350 nm)
Electrolyte	Description	0.2% triethylamine with pH adjusted to 2.5 ± 0.05 with 10% phosphoric acid
Separation voltage		25 kV
Inject pressure		0.5 PSI for 4.0 s
Pressure cleaning		20 PSI for 1 min (between each run using the electrolyte)

Table 4. Results of CE method robustness, obtained with small changes in the nominal analytical conditions.

Proposed change		Migration time (min)		Theoretical plates	Asymmetry	Resolution[b]	Mean (%)[c]
		CASP	IS[a]				
Temperature (°C)	23	6.20	8.09	29020	1.68	11.63	98.47
	27	5.55	7.26	32475	1.68	11.73	97.81
Voltage	23 KV	6.07	8.06	27800	1.63	11.40	98.49
	27 KV	4.98	6.43	25870	1.55	10.62	97.63
Nominal conditions		6.05	8.08	27185	1.69	11.94	99.98

[a]IS: Internal standard; [b]Resolution between CASP and loratadine; [c]Presentation of the average results obtained from the analysis of two independent samples.

Figure 1. The optimized conditions used in validation were presented as shown in Table 3.

Method validation

After optimization of analytical conditions, EC method was validated by assessing robustness, specificity, linearity, precision (repeatability and intermediate precision), and accuracy.

The results of robustness, obtained with small changes in the nominal analytical conditions, were presented as shown in Table 4. These results indicate that the CE method was insignificantly affected by slight changes of temperature and voltage.

Specificity results indicate that there were interference of excipients and impurities/degradation products in the determination of caspofunginin lyophilised powder for injection. Samples submitted to acidic hydrolyses, alkaline hydrolyses, oxidative degradation and UV degradation showed no significant decay in the caspofungin areas, and all electrophoretic parameters were considered satisfactory. On the other hand, samples submitted to thermal degradation showed a significant reduction in caspofungin content, as shown in Figure 2. However, the appearance of peak of impurities/degradation products was not observed. These may be explained due to total degradation of caspofungin and results in degradation products with no chromophore groups in their chemical structure. Other studies conducted in our laboratory (Ghisleni et al., 2014b), in conditions of greater stress, allow us to state that the caspofungin is more sensitive to temperature than other stress conditions, which confirms the results obtained using CE method.

The CE method was linear in the range from 20 to 300 µg/ml, showing a correlation coefficient (r) of 0.9999 and a linear equation of y = 7.90x - 0.0107. In addition, CE method was precise (repeatability RSD of 0.40% and intermediate precision RSD of 0.54%) and accurate (mean recovery of 98.00%). A summary of validation results is as shown in Table 5.

These results indicate that the CE method was robust, specific, linear, precise and accurate regarding the quantitative determination of caspofunginin lyophilized powder

Table 5. Summary of CE method validation results.

Linearity	Range (µg/ml)	20.0 – 300.0	
	r (correlation coefficient)	0.9999	
	Linear equation	y = 7.90x-0.0107	
		Day 1	**Day 2**
Precision	Mean (%)	102.49	102.66
	Repeatability RSD (%)	0.40	
	Intermediate Precision RSD (%)	0.54	
Accuracy	Mean (%)	98.00	RSD% = 1.90
	Recovery range (%)	95.80 – 100.45	

powder for injection.

Measurement uncertainty

The main sources of uncertainties were presented as shown in Figure 3, including those related with sample and standard preparation, repeatability of sample and standard peak areas, and method validation. Standard uncertainties were estimated based on certificate information, calibration results, experimental studies, and method validation (Table 6). Using these results, we estimated combined uncertainty as described by Eurachem/Citac guide (Eurachem, 2012). Although uncertainties associated with internal standard were listed in cause-effect diagram. It is important to notice that these uncertainties should be annulated, because they are equally considered in reference standard and sample preparations.

Using internal standard correction, uncertainties associated with accuracy were the most significant, contributing with 46% of overall uncertainty. Repeatability of sample and standard peak ratios contribute with about 39% of overall uncertainty. On the other hand, without using internal standard correction, almost all uncertainty is associated to repeatability of sample and standard peak areas (more than 90% of overall uncertainty). The individual contribution of each source of uncertainty is as shown in Figure 4.

The measurement uncertainty calculated using Eurachem procedure was evaluated using a Monte Carlo simulation. In Monte Carlo simulation, 50,000 assay's results calculated using random raw data were simulated. Then, the range which includes 95% of the results (95% confidential limits) using the frequency distribution plot was established (Figure 5). The caspfungin content result and its measurement uncertainty obtained using Eurachem procedure was 104.8 ± 2.5% and 101.6 ± 4.9%, with and without internal standard correction, respectively. These results were very close to those obtained using Monte Carlo simulation, 104.8 ± 2.2% and 101.6 ± 4.8%, with and without internal standard correction. As a consequence, the procedure for measurement uncertainty estimation described in this work was acceptable and may be used in routine analysis.

Equations, with (Equation 1) and without (Equation 2) internal standard correction, used in the estimation of measurement uncertainty for caspofungin are described as follows:

$$U = k \times \% \times \sqrt{\left(\frac{u_{ws}}{ws}\right)^2 + \left(\frac{u_{vfs1}}{vfs1}\right)^2 + \left(\frac{u_{vps1}}{vps1}\right)^2 + \left(\frac{u_{vfs2}}{vfs2}\right)^2 + \left(\frac{u_{vfu1}}{vfu1}\right)^2 + \left(\frac{u_{vpu1}}{vpu1}\right)^2 + \left(\frac{u_{vfu2}}{vfu2}\right)^2 + \left(\frac{u_{ARs}}{ARs}\right)^2 + \left(\frac{u_{ARu}}{ARu}\right)^2 + \left(\frac{u_{Lin}}{1}\right)^2 + \left(\frac{u_{Pr}}{1}\right)^2 + \left(\frac{u_{Acc}}{Acc}\right)^2} \quad (1)$$

$$U = k \times \% \times \sqrt{\left(\frac{u_{ws}}{ws}\right)^2 + \left(\frac{u_{vfs1}}{vfs1}\right)^2 + \left(\frac{u_{vps1}}{vps1}\right)^2 + \left(\frac{u_{vfs2}}{vfs2}\right)^2 + \left(\frac{u_{vfu1}}{vfu1}\right)^2 + \left(\frac{u_{vpu1}}{vpu1}\right)^2 + \left(\frac{u_{vfu2}}{vfu2}\right)^2 + \left(\frac{u_{ACASPs}}{ACASPs}\right)^2 + \left(\frac{u_{ACASPu}}{ACASPu}\right)^2 + \left(\frac{u_{Lin}}{1}\right)^2 + \left(\frac{u_{Pr}}{1}\right)^2 + \left(\frac{u_{Acc}}{Acc}\right)^2} \quad (2)$$

According to these results, using internal standard correction, overall uncertainty is significantly reduced (about half of the measurement uncertainty without internal standard correction). These results could be explained due to variability in the injection volume of electrophoresis system. Using an internal standard, the variability of injection volume could be corrected using the ratio of caspofungin and internal standard areas.

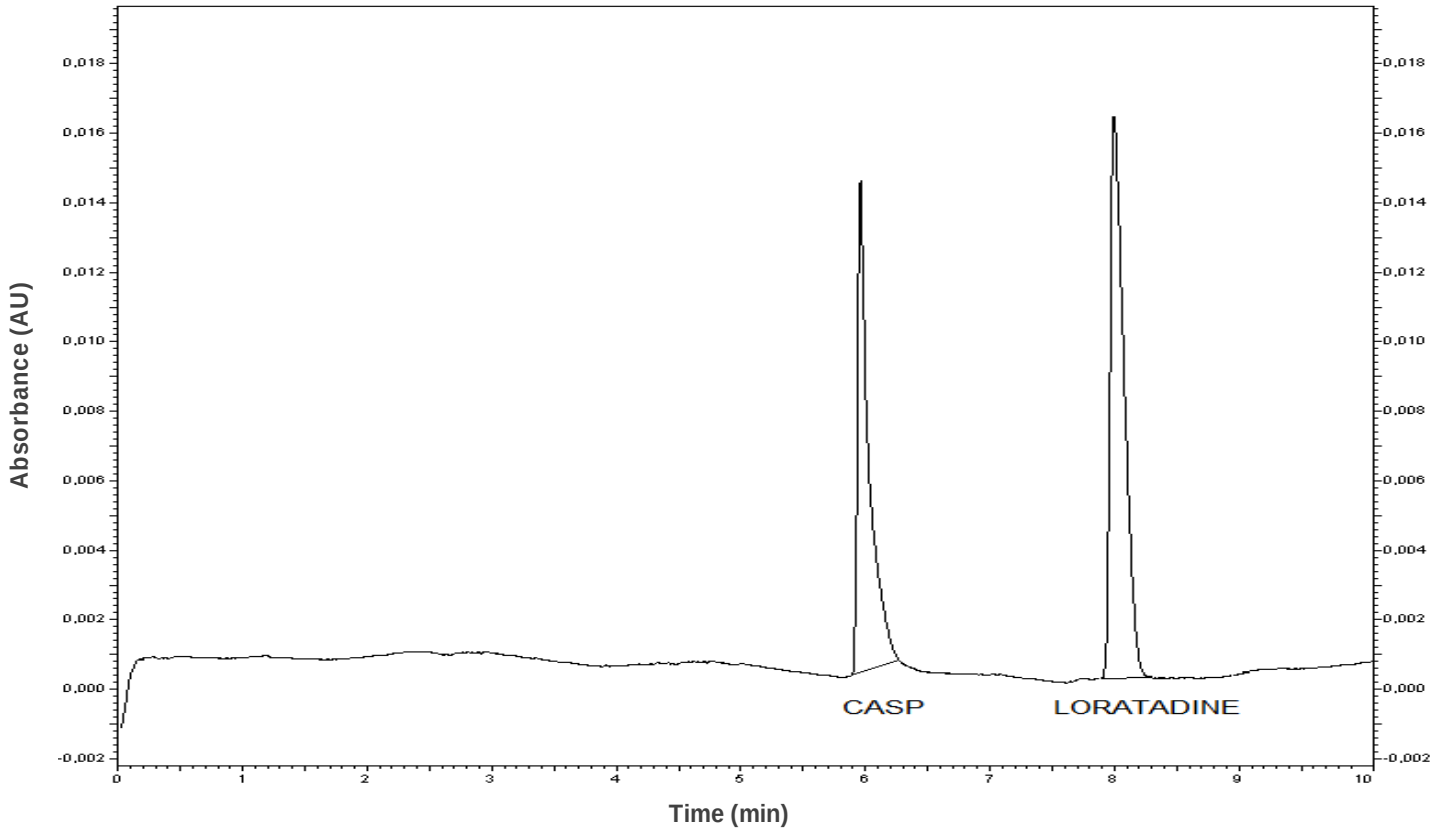

Figure 1. Electropherogramof Candidas® solution using fused silica capillary (Polymicro Technologies), 0.2% triethylamine with pH adjusted to 2.5 ± 0.05 with 10% phosphoric acid as electrolyte, and 25 kV.

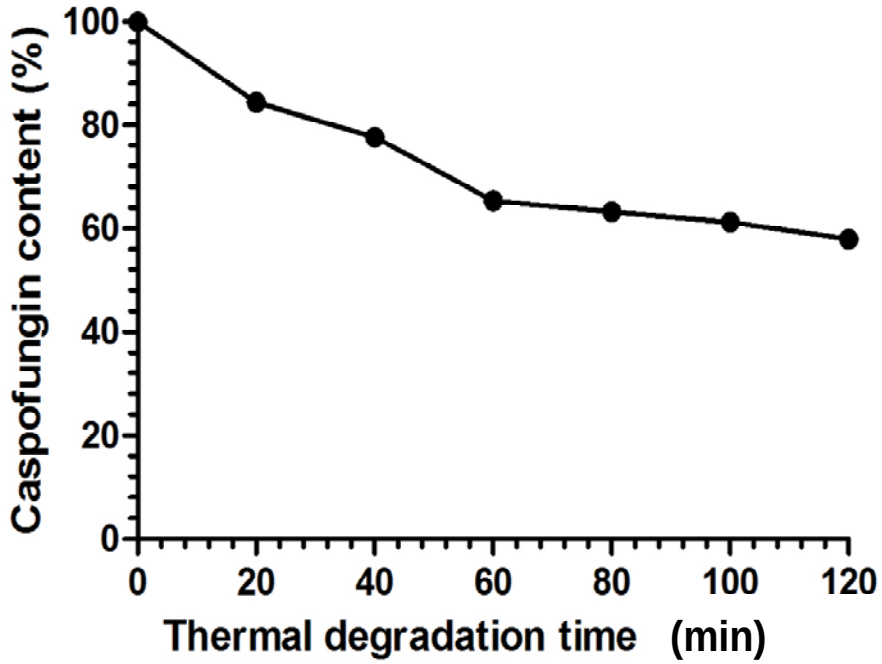

Figure 2. Caspofungin content (%) using CE method in samples submitted to thermal degradation at 45°C.

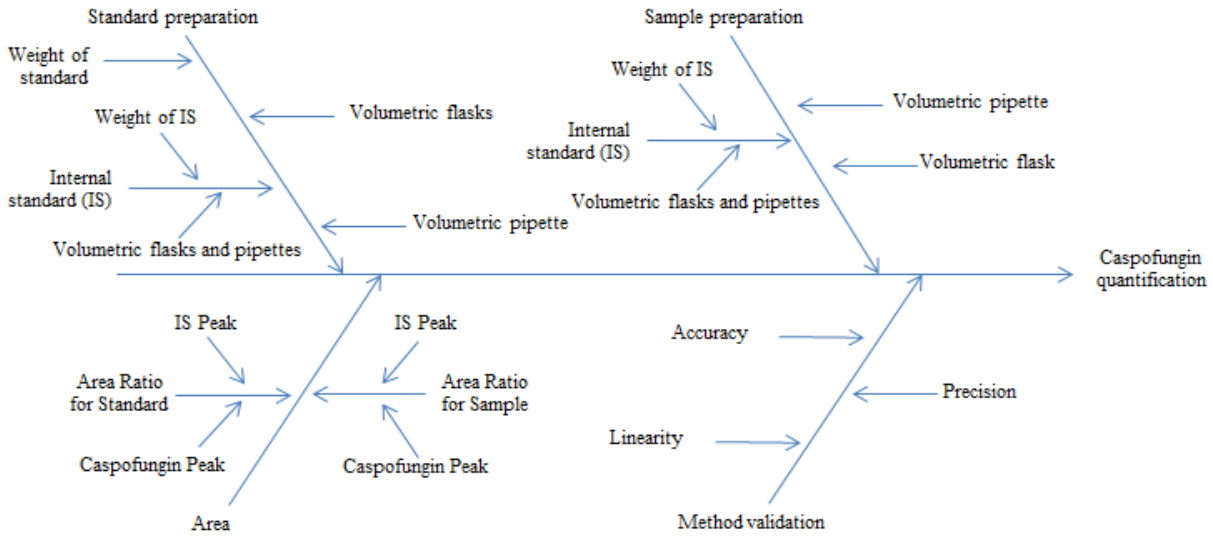

Figure 3. Main sources of uncertainty in the quantification of caspofungin by CE method.

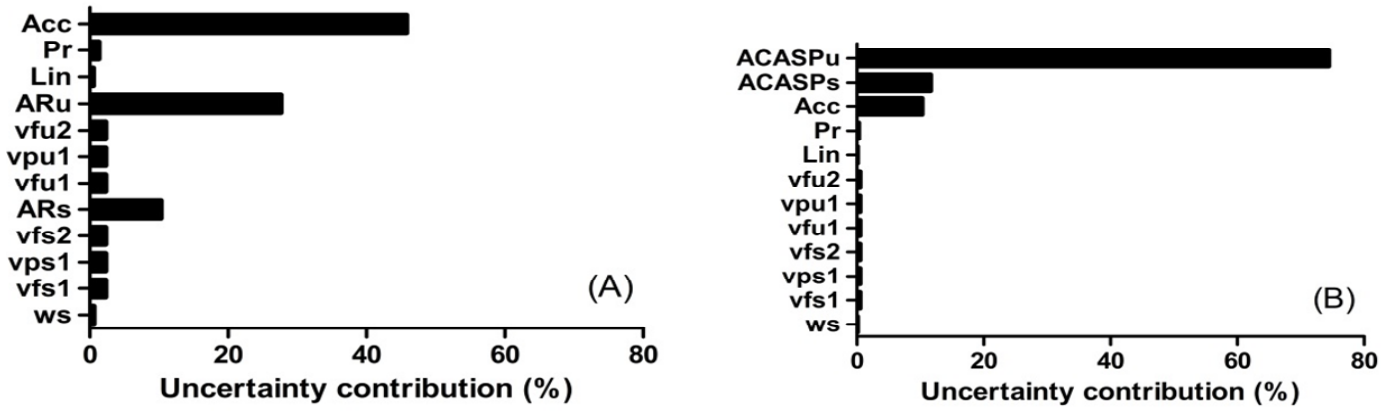

Figure 4. Contribution of each source of uncertainty to the overall uncertainty associated with CASP quantification using HPLC method. Caspofungin determination with (A) and without (B) internal standard correction.ws: Weight of caspofungin reference standard; wis: internal reference standard weight; vf and vp: volumetric flasks and pipettes used in preparation of standard and sample solutions; ACASPs: reference standard areas; ACASPu: sample solutions areas; Ais: internal reference standard in standard solutions; Aisu: internal standard area in sample solutions; ARs: area ratio for standar solutions; Aru: area ratio for sample solutions Lin: uncertainty associated with linearity, Pr: uncertainty associated with precision, and Acc: uncertainty associated with accuracy.

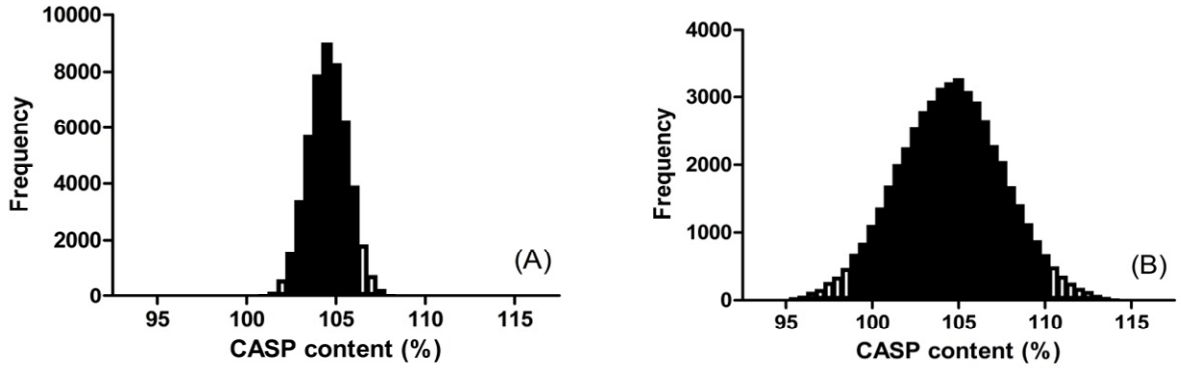

Figure 5. Frequency distribution of 50,000 simulations of caspofungin content. Region in black indicates the range, which included 95% of the results (95% confidential limits). Determination of caspofungin with (A) and without (B) internal standard correction.

Conclusion

During method development, several electrolytes, concentration of caspofungin, and internal standards were tested. After optimization, analytical conditions were defined: (a) fused silica capillary (Polymicro Technologies), (b) 0.2% triethylamine with pH adjusted to 2.5 ± 0.05 with 10% phosphoric acid as electrolyte, (c) 25 kV, (d) caspofungin concentration of 100 µg/ml, and (e) loratadine internal standard concentration of 40 µg/ml. The method validation was conducted according to the official guidelines and validation results indicate that the CE method was robust, specific, linear, precise and accurate. This allows the conclusion that the EC method is suitable for determination of the CASP lyophilisate powder. Furthermore, a procedure was established to estimate measurement uncertainty of caspofungin by CE method in routine analysis. Eurachem procedure was adequate for measurement uncertainty estimation, as proved by Monte Carlo simulation.

Conflict of interest

The authors declare no conflict of interest.

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