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Caspofungin infusion solutions (50 mg/100 mL): chemical stability and antifungal activity against Candida spp.

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Background: Ready to use caspofungin infusion bags are centrally prepared in the Hospital Pharmacy, University Hospital of Heidelberg, for economic reasons and possibly occurring problems with drug shortages. The aim of this study was a quality control of the in-house preparation of caspofungin infusion bags and the preparation process. Caspofungin concentration with regard to chemical stability and antifungal activity of caspofungin preparations were defined as quality parameters. *Methods:* Three caspofungin infusion bags (50 mg in 100 mL 0.9% sodium chloride) were examined every seven days for a total of four weeks. Chemical stability of caspofungin solutions was analyzed using a validated high performance liquid chromatography (HPLC) method. Antifungal activity was assessed by microdilution tests according to the EUCAST protocol. Additionally, concentration and sterility were determined in returned caspofungin infusion bags. *Results:* The amount of caspofungin in the infusion solutions still exceeded 90% after four weeks (2-8 °C). Antifungal activity was consistent over 28 days with a MIC ≤2 mg/L for different *Candida* spp. In returned infusion bags, caspofungin concentration was found to be ≥90% in 12 out of 13 bags and sterility was given in all preparations. *Conclusion:* These results show that chemical stability of caspofungin infusion solutions (50 mg/100 mL) can be guaranteed for four weeks at 2-8 °C and are confirmed by corresponding results regarding sterility and antifungal activity.

1. Introduction

Caspofungin diacetate, CANCIDAS®, is a cyclic lipopeptide (echinocandin) acting as antifungal agent inhibiting beta (1,3)-D-glucan synthase in the fungal cell wall. Caspofungin shows antifungal activity against *Candida* spp., including fluconazole resistant *C. glabrata* and *C. krusei*, and is widely used for the treatment of invasive candidiasis, especially in critically ill and neutropenic patients. Caspofungin may also be considered for second line therapy of invasive aspergillosis (Merck Sharp & Dohme 2015).

During antifungal therapy with caspofungin (CANCIDAS®), adult patients usually receive an initial dose of 70 mg on the first day, followed by 50 mg once daily, or continued by 70 mg once daily if bodyweight is above 80 kg. In a drug shortage situation as well as from an economic view point, hospital pharmacies play an important role to ensure the supply of medicinal products. For reasons of cost, caspofungin infusion solution (50 mg/100 mL) is centrally prepared in the Hospital Pharmacy of the University Hospital of Heidelberg. Under aseptic conditions, a stock solution is prepared from CANCIDAS® 70 mg vials and 50 mg are added to 100 mL infusion bags of 0.9% sodium chloride. This procedure is of advantage as the price per mg caspofungin is lower when using 70 mg vials. According to the manufacturer, the infusion solution can be kept for 24 h at 25 °C or for 48 h at 2-8 °C (Merck Sharp & Dohme 2015).

Concentration and chemical stability of caspofungin can be determined by HPLC-UV (Neoh et al. 2012), HPLC with fluorescence detection (Schwartz et al. 1997) or LC-MS/MS (Neoh et al. 2010). Nevertheless, activity is an important issue in the characterization of antifungal agents, especially of echinocandins. For the evaluation of *in vitro* antifungal activity, sensitivity tests for caspofungin (M27-A3) were published (Clinical and Laboratory Standards Institute 2008). Standardized techniques for susceptibility testing of yeasts were established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), however, official breakpoints have not been approved for caspofungin. In general, a minimum inhibitory concentration (MIC) of >2 mg/L is referred to as reduced susceptibility (Nguyen et al. 2004; Pfaller et al. 2008).

From previous analyses, caspofungin infusion solution (70 mg/100 mL) is known to be chemically stable for 30 days. Antifungal activity against several *Candida* spp. was tested for up to 14 days after reconstitution (Nguyen et al. 2004). Stability of caspofungin solutions (0.2 to 0.5 mg/mL) in elastomeric infusion devices was monitored by Tsiouris et al. (2010) reporting ≥90% of the initial concentration after 14 days at 5±3 °C. Based on this data, the in-house caspofungin infusion solutions (50 mg/100 mL) prepared under controlled clean room conditions are issued with an expiry date of four weeks.

The aim of this study was an enhanced quality control of the in-house caspofungin infusion solution and of the preparation process. For this purpose, the concentration of caspofungin with regard to chemical stability and antifungal activity as well as microbial stability were defined as quality parameters and monitored simultaneously over a period of four weeks. In addition, caspofungin infusion bags returned from wards to the pharmacy represent a worst case scenario, as the storage conditions of these preparations are not continuously monitored. Therefore, returned infusion bags were analyzed for caspofungin concentration and sterility of the solution.

2. Investigations and results

2.1. HPLC assay validation

To determine concentrations and chemical stability of caspofungin solutions, we adapted and improved a previously reported HPLC method (Neoh et al. 2012). A simple and sensitive HPLC assay was developed by replacing methanol with acetonitrile and adapting gradient elution to optimize peak shape and analytical run time. With these chromatographic conditions, typical retention times were 2.9 min for caspofungin (Fig. 1). A minor peak at 4.0 min was regarded as a degradation product of caspofungin.

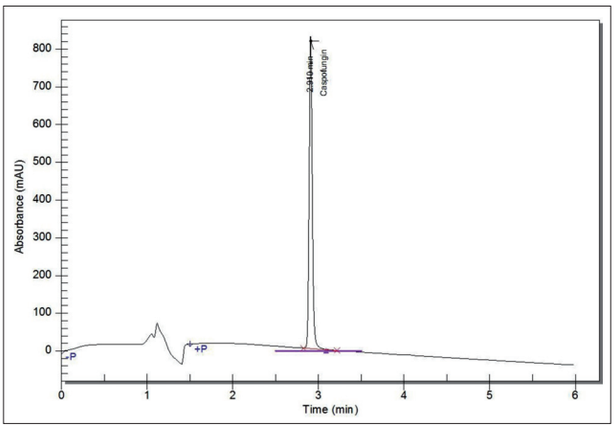

Fig. 1: Typical chromatogram of caspofungin 0.5 mg/mL

Calibration curves of caspofungin were found to be linear for 0.05 to 1 mg/mL (r^2>0.999). Validation data on accuracy and precision are presented in Table 1.

Caspofungin stock solutions were kept at -70 °C and monitored during this study. Our findings indicate that aqueous caspofungin stock solutions could be stored at -70 °C for at least two months without significant changes, confirming previous data (Neoh et al. 2010). A quality control solution of 0.5 mg/mL caspofungin remained chemically stable for 58 days when stored at 2-8 °C. Additionally, caspofungin concentration still exceeded 90% of the initial amount after storage at room temperature (24 °C) for 120 h. However, the peak at 4.0 min was observed to increase over time, accounting for 0.1% of total peak area in freshly prepared solutions up to 1.2% and 2.3% after 48 h and 120 h, respectively.

2.2. Infusion bag stability and sterility tests

Samples of three caspofungin infusion solution bags were aseptically collected shortly after preparation and every seven days for a total of four weeks. The initial amount of the three caspofungin infusion solution bags was 48.2, 50.2 and 51.0 mg. After four weeks of storage at 2-8 °C, caspofungin concentrations were still between 97.4% and 98.3% of the initial amount of the respective bag and >90% of the nominal value (50 mg). Peak area of degradation products ranged from 0.1% of the total peak area (week 0) to 1.6% (week 4).

In addition, we measured the concentrations of caspofungin infusion solutions which passed the expiry date of four weeks. During this study, 13 expired caspofungin infusion bags were returned from the wards to the pharmacy after their expiry date (average 32±6 days) and analyzed by HPLC. In 12 out of 13 cases, contents of caspofungin were >90% of the nominal value. Furthermore, aliquots of each infusion bag were tested according to Ph. Eur. 8.0, 2.6.1 (European Pharmacopoeia 2014) and sterility was given for all samples.

2.3. Antifungal activity tests

In order to monitor potential changes in the activity of caspofungin infusions depending on the time of storage, weekly aliquots of the three caspofungin infusion bags (50 mg/100 mL) were assayed together with pure substance of caspofungin diacetate as reference. The antifungal activity of the three caspofungin infusion solutions showed no evident changes over the study period of four weeks (Table 2) and antifungal activity of the infusion solutions and solutions of the pure substance were similar. For all samples and all *Candida* species, the observed MIC values were ≤2 mg/L.

3. Discussion

In the present study, three caspofungin infusion solutions (50 mg/100 mL) were examined for caspofungin concentration and antifungal activity over a period of four weeks. The HPLC assay described in this paper allows the rapid, simple and precise determination of caspofungin in aqueous solutions.

Caspofungin concentrations remained at ≥90% of the initial amount throughout this period. These findings for caspofungin infusions bags (50 mg/100 mL) are in compliance with previous investigations on - 0.35 mg/mL (Nguyen et al. 2004). In the chromatographic assay, a degradation product of caspofungin could be separated and monitored as well. With its retention time of 4.0 min, it is likely to be L-747969, the open-ring product as described before (Tsiouris et al. 2010).

The data on chemical stability are confirmed by corresponding results on antifungal activity in this study. Antifungal activity ranged between 0.125 and 2 mg/L for different *Candida* spp. and was found to be consistent in the caspofungin infusion solutions over the four weeks, thus exceeding the period of two weeks investigated before (Nguyen et al. 2004).

The low reproducibility of MIC values for caspofungin (see methods) has become obvious by other studies before (Arendrup et al. 2011; Espinel-Ingroff et al. 2013). Lot-to-lot variability (Arendrup et al. 2011) and the solvent (Arendrup et al. 2011; Espinel-Ingroff et al. 2013) were shown to have an effect but they do not

Table 1: Reinjection reproducibility [relative standard deviation (RSD)], accuracy [100 x ($c_{observed}$ / c_{spiked})] and precision [RSD] for caspofungin (n=6)

Caspofungin	0.1 mg/mL	0.5 mg/mL	1 mg/mL
Reinjection reproducibility, RSD	0.64%	0.15%	0.48%
Intra-day accuracy	98.5%	100.4%	99.5%
Inter-day accuracy	100.9%	101.0%	99.7%
Intra-day precision, RSD	0.58%	0.37%	0.45%
Inter-day precision, RSD	1.32%	0.80%	0.32%

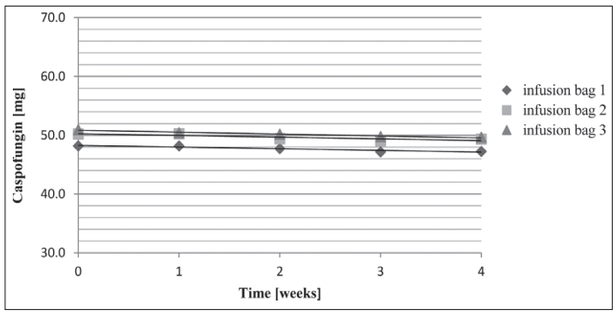

Fig. 2: Contents of caspofungin infusion solutions from week 0 to 4 determined by HPLC.

Table 2: Ranges for the minimum inhibitory concentration (MIC) of caspofungin infusion solutions (week 0 up to week 4) compared to caspofungin pure substance in the microdilution assay

Species and strain	*C. albicans* ATCC 24433	*C. glabrata* JMRC:NRZ: 0069	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750
MIC [mg/L] of					
infusions, t=0	0.25	0.25-0.5	1-2	2	0.125-0.25
infusions, t=1	0.25	0.5	1-2	2	0.25-0.5
infusions, t=2	0.25	0.5	1-2	2	0.25-0.5
infusions, t=3	0.125-0.25	0.5	1-2	2	0.25-0.5
infusions, t=4	0.25	0.5	1-2	2	0.25-0.5
MIC [mg/L] of					
pure substance	0.25	0.5	1	2	0.25-0.5

fully explain the results. As a consequence of this variability of caspofungin MICs, microdilution test for the determination of antifungal activity should be performed with the samples of unknown antifungal activity and the reference substance with known activity on the same plate using the same spore suspension, the same solvent and the same range of concentration for caspofungin.
In conclusion, the results of this study show that the chemical stability and antifungal activity of caspofungin can be guaranteed for four weeks after reconstitution in 0.9% sodium chloride and storage at 2-8 °C when centrally prepared in the pharmacy under controlled aseptic conditions according to GMP guidelines and supported by sterility tests.

4. Experimental

4.1. Preparation of caspofungin infusion solutions and sampling

Caspofungin infusion solution (50 mg/100 mL) is centrally prepared in our hospital pharmacy under aseptic conditions. CANCIDAS® 70 mg vials (MSD Sharp & Dohme, Haar, Germany) are reconstituted with water for injection as recommended by the SmPC (Merck Sharp & Dohme 2015). This concentrate is used to prepare 50 mg caspofungin infusion bags by adding 7.15 mL to a commercial 100 mL infusion bag of 0.9% sodium chloride (Isotonische Kochsalzlösung Fresenius 0.9% free flex®, Fresenius Kabi, Bad Homburg, Germany).
Three caspofungin infusion bags from a single batch were stored at 2-8 °C under monitored conditions. The bags were weighed directly after preparation to account for variability in volume of the sodium chloride bags. Under a laminar air flow, samples of 1 mL were collected from each infusion bag in duplicate every seven days (week 0, 1, 2, 3 and 4) and stored at -70 °C under monitored conditions until analysis.

4.2. HPLC assay

Concentrations and chemical stability of caspofungin solutions were determined by HPLC. The qualified HPLC system included a pump, a vacuum degasser, a temperature-controlled autosampler, a column oven and a photodiode array detector (Flexar UHPLC, Perkin Elmer, Überlingen, Germany). Chromera 4.1.0 (Perkin Elmer) was used for instrument control, data acquisition and processing. Separation was achieved using a Gemini C18 column, 150 x 4.6 mm, 5 µm (Phenomenex, Aschaffenburg, Germany) with a C18 pre-column (SecurityGuard ULTRA, Phenomenex). Oven temperature was 35 °C. The mobile phase consisted of (A) 0.1% trifluoroacetic acid (TFA), adjusted to pH 3.0 with diethylamine, and (B) acetonitrile, at a flow rate of 1.5 mL/min. A linear gradient from 30% to 70% (B) in 5 min was applied, followed by equilibrating at 30% (B) for 1 min. The UV detector was set to 225 nm. This method was validated in accordance with current guidelines (ICH 2005, FDA 2001) including the parameters accuracy, precision, linearity and stability.
Stock solutions of caspofungin diacetate (≥97%, Sigma-Aldrich, Steinheim, Germany) were prepared at 1 mg/mL free base in sodium chloride 0.9% and stored at -70 °C. Aliquots were freshly diluted with 0.9% sodium chloride and used as calibration standards and quality control samples, resulting in final concentrations of 0.05 / 0.1 / 0.2 / 0.5 / 0.8 / 1 mg/mL caspofungin. 10 µL were injected into the HPLC system. Calibration curves were obtained by plotting peak area versus concentration.
Samples of three caspofungin infusion bags were collected as described above and assayed in duplicate. In addition, we measured the concentrations of returned and expired caspofungin infusion bags.

4.3. In vitro antifungal susceptibility testing

In order to monitor potential changes in the activity of the caspofungin infusions depending on the time of storage, aliquots of the three caspofungin infusion solutions (50 mg/100 mL) were taken at five different time points (0, 1, 2, 3 and 4 weeks after preparation). As references, solutions of the pure substance of caspofungin diacetate (98.6 wt%, potency 89.6%) provided by MSD Sharp & Dohme (New Jersey) were included in the tests. *In vitro* susceptibilities of the five clinically most important *Candida* species to caspofungin infusion solutions and caspofungin solutions of the pure substance were determined by a broth microdilution technique following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard methodology (Arendrup et al. 2012). Preliminary tests showed that the reproducibility of the minimum inhibitory concentration (MIC) values for caspofungin were low. For a reliable comparison, susceptibility testing of the caspofungin infusion and of the pure substance of caspofungin had to be performed on the same microdilution plate using the same spore suspension, the same solvent and the same range of antifungal concentrations. EUCAST protocol (Arendrup et al. 2012) was completely followed with the exception that water was used as solvent for the pure substance as well. The concentrations of caspofungin tested ranged from 2 to 0.004 mg/L for all preparations. For all tests infusion solutions and solutions of the pure substance were tested on the same microdilution plates and with the same spore suspensions in duplicates.
The following *Candida* species were used for the testing: *Candida albicans* (ATCC 24433), *C. glabrata* (JMRC:NRZ:0069), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), and *C. tropicalis* (ATCC 750). The strains of *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) are EUCAST recommended control strains (Arendrup et al. 2012). Isolates were grown on a 2% yeast peptone dextrose (YPD) medium at 35 °C for 24 h before preparing the inoculum. Yeast cell suspensions were counted with a hemacytometer. MIC endpoints were determined by an automated nephelometer (Nepheloskan Ascent, Type 750, Labsystems Oy, Helsinki, Finland) after 24 h of incubation at 35 °C and defined as 50% or more reduction in growth in comparison to the drug-free wells.

4.4. Test for sterility

For parenteral preparations of our hospital pharmacy, the test for sterility is routinely performed in the Department of Infectious Diseases, Medical Microbiology and Hygiene, Heidelberg University Hospital, according to the current European Pharmacopoeia 8.0, Ph. Eur. 2.6.1 (European Pharmacopoeia 2014).

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