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Differential pulse polarographic investigation of micafungin and anidulafungin using a dropping mercury electrode

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The electrochemical behavior of the echinocandin antifungals anidulafungin (AF) and micafungin (MF) has been investigated by differential pulse polarography (DPP). The measurements were carried out in a supporting electrolyte solution consisting of Britton-Robinson buffer and methanol at various substance concentrations and pH values. An amperometric cell with a three electrode system consisting of a dropping mercury electrode (DME) as working electrode, an auxiliary platinum electrode and an Ag/AgCl reference electrode was used in all experiments. AF was electrochemically reduced at potentials between -1.3 and -1.5 V. MF showed a first reduction peak (a) between -1.0 and -1.4 V and a second peak (b) between -1.5 and -1.8 V. A strong pH-dependence was observed, with optimal results at pH 2.0-3.0 for the AF peak, pH 2.0 for the MF peak (a) and pH 5.0 for the MF peak (b). A linear correlation between the concentration and the peak current has been demonstrated for all reduction peaks. MF peak (a) showed a similar behavior to the AF peak regarding shape, peak current and pH-dependence. Therefore, it can be assumed that both reductions are based on the same mechanism, a two-step reduction of the N-acyl group.

1. Introduction

Anidulafungin (AF) and micafungin (MF) are echinocandin antifungal agents approved in Europe and the United States for the treatment of invasive candida infections (Pfizer Limited 2017; Astellas Pharma Europe 2018). Both substances are semisynthetic lipopeptides (Figs. 1, 2). The antifungal action is based on an inhibition of the production of 1,3-β-D-glucan, a component of the fungal cell wall which is necessary for the fungus to continue living and growing (Kofla and Ruhnke 2011). The fungicidal effect depends on an intact ring structure and a covalent N-acyl group.
Various methods have been reported for the quantification of AF and MF (Farowski et al. 2010; Martens-Lobenhoffer et al. 2011; Sutherland et al. 2011; Uranishi et al. 2011; Alebic-Kolbah and Modesitt 2012; Ventura et al. 2017). All methods are based on high performance liquid chromatography or liquid chromatography-tandem mass spectrometry, which requires extensive equipment, particularly in terms of instrumentation. Due to the chemical structure with peptide bonds, phenolic and acylic groups and carboxy hemiaminal diols, it is assumed that both substances have electrochemical activity (Figs. 1, 2). Electrochemical methods for analysis of AF and MF have not yet been reported. Therefore, we investigated the electrochemical behavior of these echinocandin antifungals using DPP on a DME.

2. Investigations and results

2.1. Peak profile of AF and MF in dependence on pH value

At each pH value between 2.0 and 8.0 polarograms of AF were determined in a concentration range of 0.05 mg ml^{-1} - 0.15 mg ml^{-1}. A pH-dependent reduction peak was detected at potentials between -1.3 and -1.5 V. MF was determined under the same conditions at concentrations between 0.025 mg ml^{-1} and 0.075 mg ml^{-1} and showed a first reduction peak between -1.0 and -1.4 V (a) and a second peak between -1.5 and -1.8 V (b). Other peaks were not reproducible and therefore not considered further. Figure 3 shows the relevant polarogram extracts of AF at pH 3.0 and MF at pH 5.0. With increasing pH value there was a shift of all peak potentials to more negative values (Table). AF and MF peaks (a) were detected in the whole range of pH 2.0-8.0, while the MF peak (b) initially occurred at pH 4.0 and was beyond the detection limit of -1.8 V above pH 6.0. Between pH 2.0 and 3.0 the absolute value of the AF peak current increased from 287 to 373 nA, and then with increasing pH value decreased to 2 nA. The absolute value of MF peak (a) current decreased with increasing pH from 498 nA (pH 2.0) to 28 nA (pH 8.0). The MF peak (b) showed a maximal absolute peak current value with 293 nA at pH 5.0 (Table).

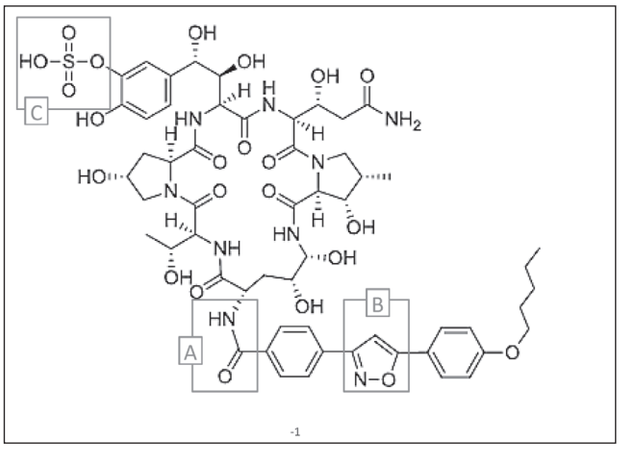

Fig. 1: Micafungin; M_r = 1270,3 g mol^{-1} (A = N acyl group; B = isoxazole ring; C = sulfonic acid)

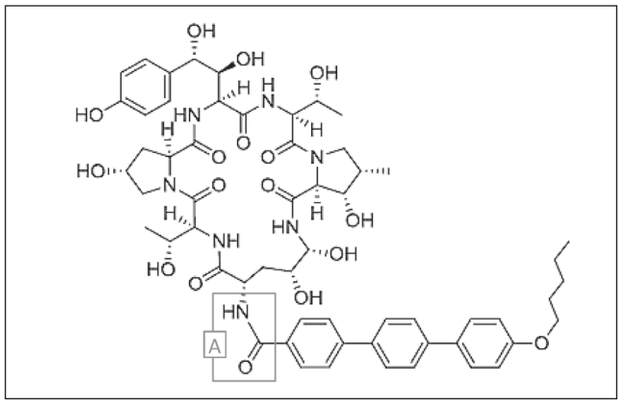

Fig. 2: Anidulafungin; M_r = 1140,2 g mol^{-1} (A = N acyl group

Table: Peak potentials and peak currents of anidulafungin, micafungin (peak a) and micafungin (peak b) as a function of pH-value

	I [nA]			U [V]		
pH	Anidula-fungin	Micafungin (a)	Micafungin (b)	Anidula-fungin	Micafungin (a)	Micafungin (b)
2.0	-287	-498	N/A	-1.29	-0.99	N/A
3.0	-373	-389	N/A	-1.42	-1.12	N/A
4.0	-250	-275	-127	-1.46	-1.19	-1.58
5.0	-86	-259	-293	-1.53	-1.24	-1.60
6.0	-20	-235	-76	-1.52	-1.27	-1.76
7.0	-7	-116	N/A	-1.49	-1.33	N/A
8.0	-2	-28	N/A	-1.47	-1.34	N/A

2.2. Determination of linear ranges

Linear ranges were investigated at pH values with the maximal absolute peak current value for each reduction peak. The AF peak was analyzed at pH 3.0 and showed a linear range of 9 – 100 µg ml^{-1} (R^2=0.9827). At lower concentrations, linearity for AF could not be proven on a DME or HMDE. At pH 2.0, the MF peak (a) showed a linear range from 1 – 40 µg ml^{-1} (R^2=0.9991) using a DME and a linear range at lower concentrations from 0.1 – 0.6 µg ml^{-1} (R^2=0.9888) using a HMDE. The MF peak (b) was analyzed at pH 5.0. Linear ranges from 1 – 40 µg ml^{-1} (R^2=0.9810) on a DME and from 0.05 – 0.4 µg ml-1 (R2=0.9745) on a HMDE were detected. Figure 4 shows exemplarily the peak currents as a function of concentration for AF and MF on a DME.

2.3. Stability of analytes in the test solution

Stability of the analyte in the test solution is essential for obtaining reproducible results. Therefore, polarograms of one concentration of AF and MF were conducted every 5 min over a period of 60 min. Figure 5 shows the results of the stability investigations for AF at pH 3.0 and MF at pH 2.0 and 5.0.

In the case of AF, the proportion of peak current after only 20 min fell below 90 % of the initial value. The current value of MF peak (a) at pH 2.0 was over the whole testing period above 90 %, but showed an initial decrease of 5.8 % after the first measurement. At pH 5.0, MF peak (b) was stable for at least 45 min with a proportion of initial peak current above 95 %, after 60 min the mean value was still 94.7 %.

2.4. Cyclovoltammetric investigations

Cyclic voltamograms were recorded on a HMDE. The results are presented on the example of MF in Fig. 6. In the right cyclic voltamogram extract the relevant measuring ranges are shown in more detail. At pH 2.0, the cathodic course of MF showed a reduction peak at -1.08 V, followed by a minimum at -1.02 V and a further reduction peak at -1.4 V. In the anodic course, no clear signal was detected. AF showed a similar pattern with a reduction peak at -0.98 V, a minimum at -1.12 V and a reduction peak at -1.7 V in the cathodic run (data not shown). At pH 5.0, two reduction peaks of MF at -1.26 V and -1.68 V and an oxidation peak at -1.88 V were detected. Signals at higher potentials were not reproducible and therefore not subject of this investigation.

2.5. Reaction mechanisms

At potentials between -1.0 and -1.5, AF and MF showed a reduction peak with a similar behavior regarding shape, peak current and pH-dependence. Maximum peak currents were detected in an acidic medium and decreased with higher pH-values. The peak potentials shifted to more negative values with increasing pH. The strong pH-dependence of the reduction indicates a reaction mechanism with the involvement of protons.

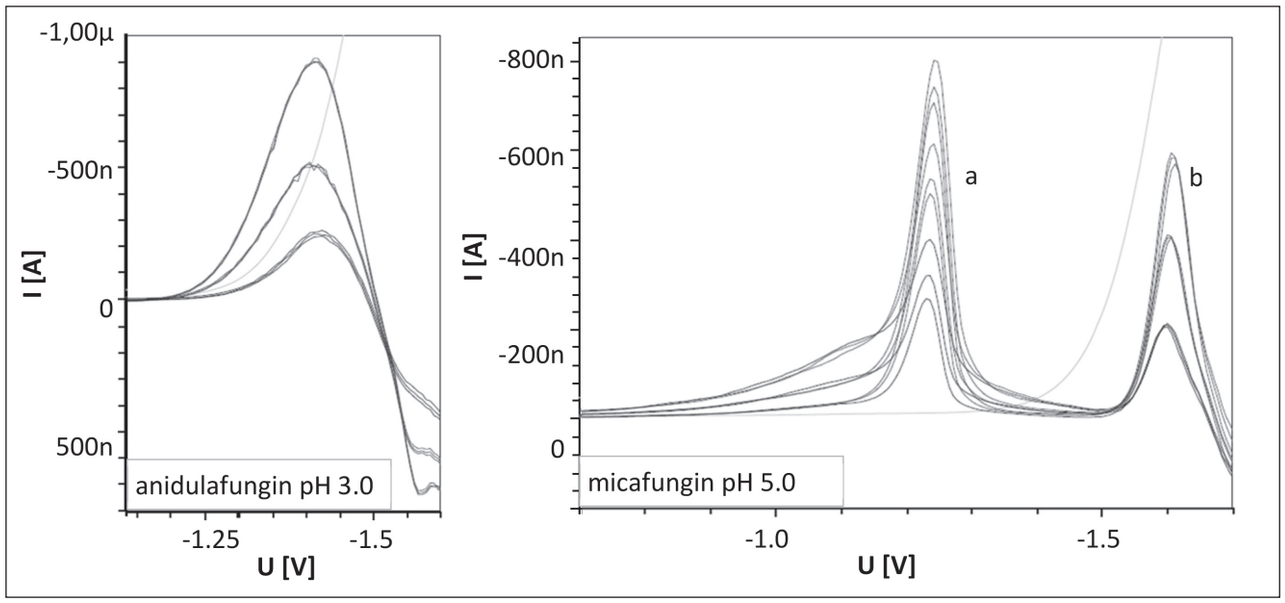

Fig. 3: Polarogram extracts of anidulafungin (c=0.05 mg ml^{-1} – 0.15 mg ml^{-1}) and micafungin (0.025 mg ml^{-1} - 0.075 mg ml^{-1})

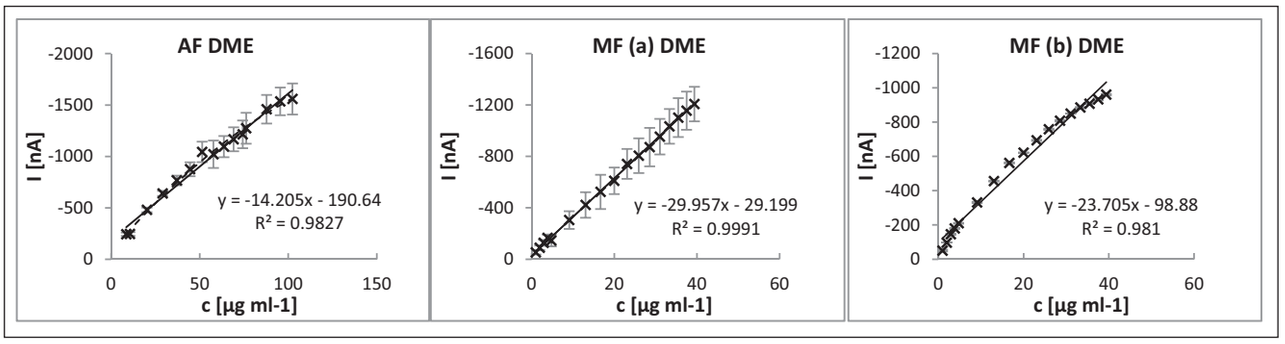

Fig. 4: Linearity of anidualifungin peak (pH = 3.0), micafungin peak (a) (pH = 2.0) and micafungin peak (b) (pH = 5.0)

Reduction reactions of activated ketones usually run at peak potentials between -1.0 and -1.8 V (Henze 2001a). The peaks of AF and MF (a) occur in the potential range of unsaturated, aliphatic ketones (Holleck and Mahapatra 1972). Therefore, it is likely, that the reduction reaction takes place at the N-acyl group in both substances (Fig. 1, partial structure A; Fig. 2, partial structure A). The proposed reduction mechanism is shown in Figure 7.

In addition, MF has further reducible groups. Possible structures for the reduction peak (b) are the isoxazole ring or the sulfonic acid with conjugated double bonds (Fig. 1, partial structures B and C).

3. Discussion

The differential pulse polarographic investigation of the antifungal agents AF and MF revealed, that both substances are electrochemically reducible on a dropping mercury electrode. The similarity of the AF peak and MF peak (a) indicates the same reaction mechanism, a reduction of the N-acyl group. Due to the lack of anodic peaks in the cyclic voltammograms at more positive potentials, an irreversible reduction must be assumed (Henze 2001b). A similar polarographic reaction is described for the beta blocker Acebutolol (Al-Ghamdi et al. 2012). The reduction is a two-step reaction with the uptake of two electrons and two protons. At low pH values both steps occur in one reduction peak, because the first protonation step is supported by the surplus of protons. Therefore, less energy is needed and the reaction runs at less negative potentials. At higher pH values a peak fronting as indication of the two steps was observed.

For MF, a further reduction peak was detected. Two possible reaction regions are the isoxazole ring or the sulfonic acid. Sulfonic acids are reducible in the presence of conjugated double bonds (Henze 2001a).

The reduction of AF showed a linear range on a DME at concentrations, which may be relevant for pharmaceutical analyses. At lower concentrations relating to expected plasma levels, linearity could not be proven for this substance. The investigation of MF revealed for both reduction peaks linear ranges on a DME at higher concentrations and on a HMDE at lower concentrations. A HMDE is characterized by a smaller drop size and an accumulation of the analyte on the surface, enabling measurements with higher sensitivity. But the small surface of the electrode also is the reason for quick analyte saturation and a limitation of the linear range. On the other hand, a DME allows measurements at higher concentrations with a broad concentration range with high linearity.

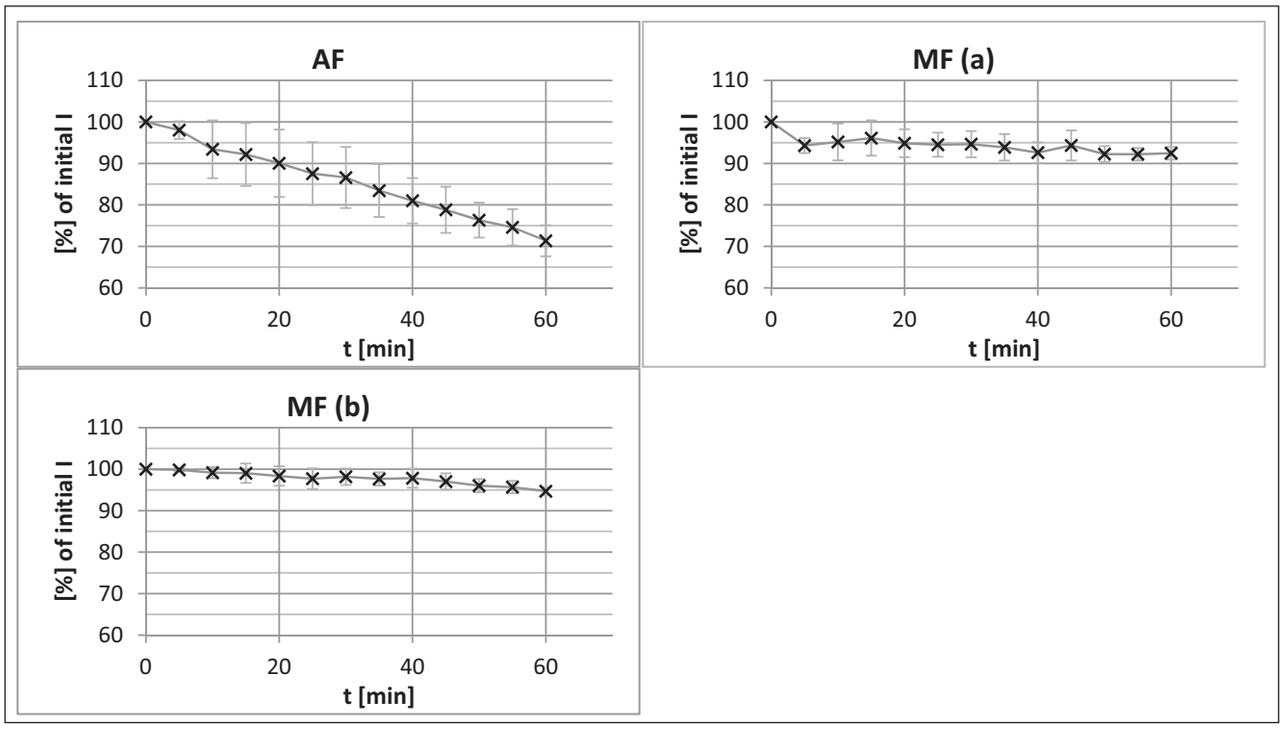

Fig. 5: Stability of anidualifungin (pH = 3.0), micafungin (a) (pH = 2.0) and (b) (pH = 5.0)

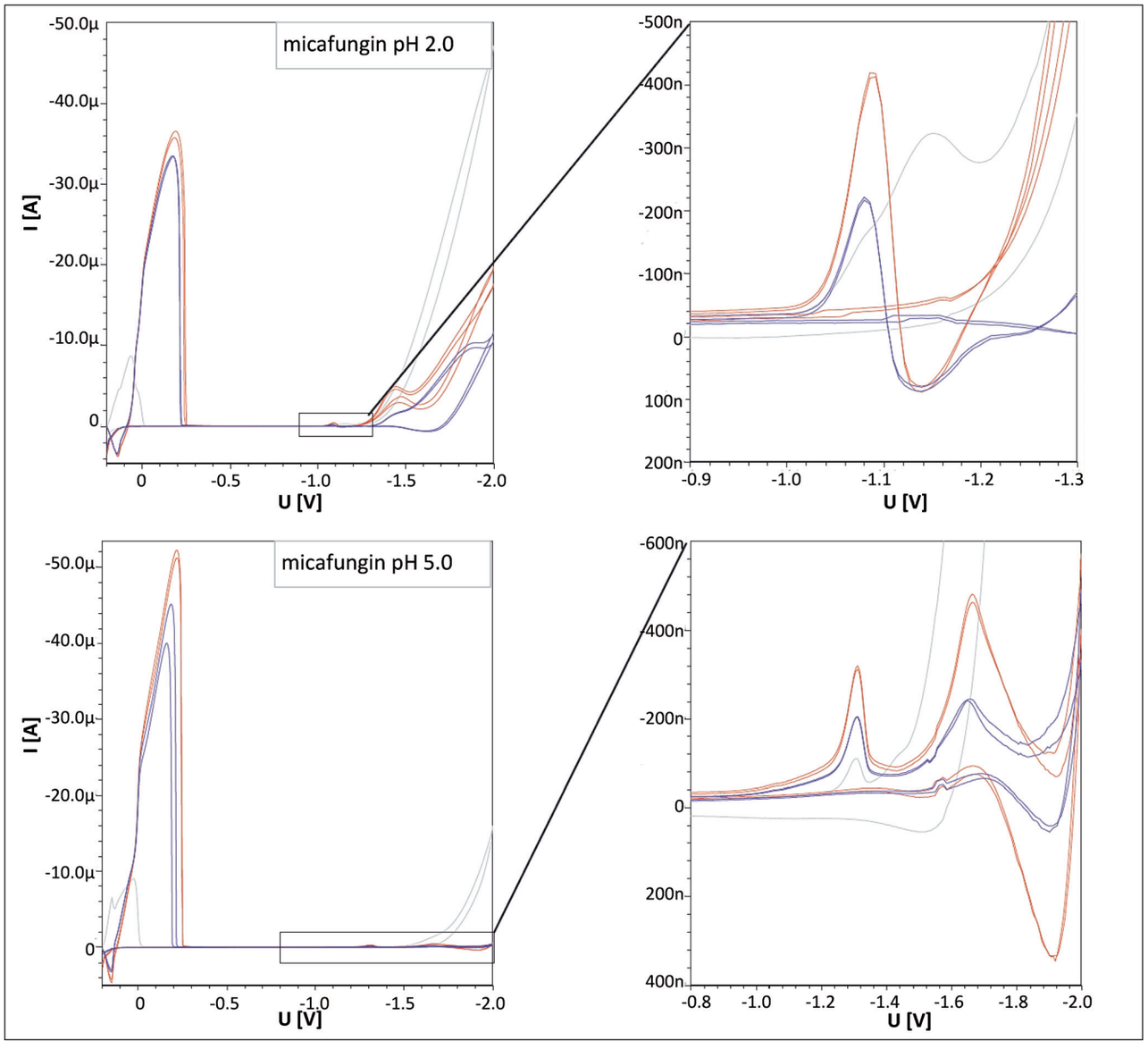

Fig. 6: Cyclovoltamograms of micafungin at ph=2.0 and pH=5.0 (red signal course: 2 cycles of c=0.17 µg ml^{-1}; blue signal course: 2 cycles of c=0.29 µg ml^{-1})

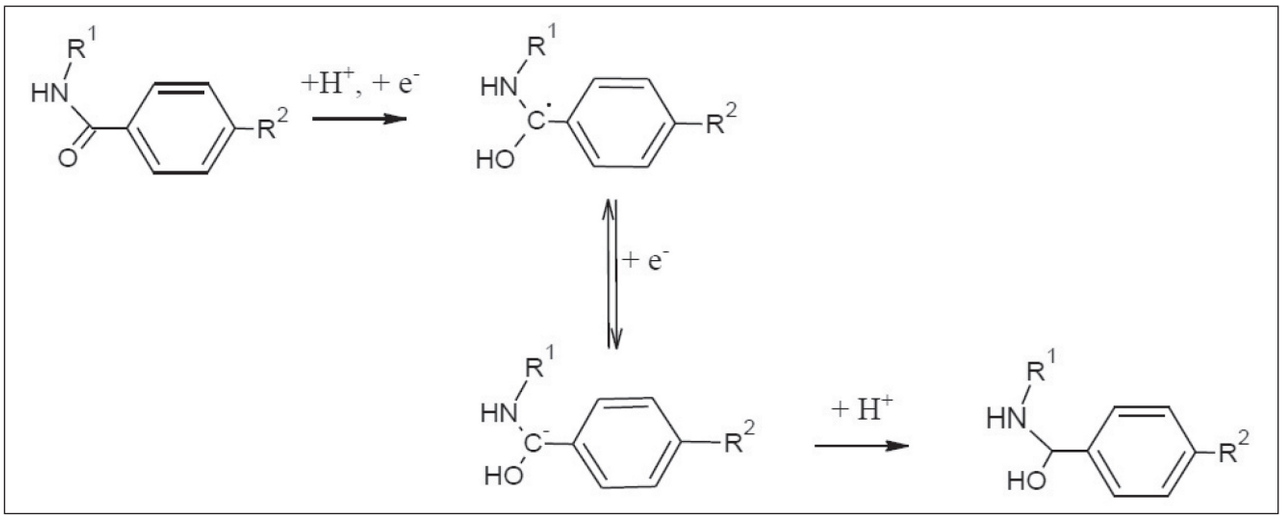

Fig. 7: Proposed reduction mechanism of AF and MF peak (a) on a dropping mercury electrode

A criterion for quantification methods is the analyte stability in the test solution. Stability problems of AF are related to a hydrolytic splitting of the carboxy hemiaminal diol and a ring opening reaction. For an improvement of the AF stability, the use of further test solutions should be tested. It was shown that the analyte is stable over 6 h in a mixture of methanol and sodium chloride 0,9 % (Sutherland et al. 2011). MF showed sufficient stability at room temperature at both pH-values for more than the maximum analysis time of 20 min, which is known from the literature (Sutherland et al. 2011).

In summary, we showed that AF and MF are polarographically active caused by an irreversible reduction reaction. Based on pH-dependence and potential range, a reduction of the N-acyl group can be assumed for both echinocandin antifungals. Compared to chromatographic methods, DPP has the advantage of compact equipment and low investment and operating costs. Therefore, DPP was determined as simple analytical method for the quantification of AF and MF.

3. Experimental

3.1. Materials

AF was obtained from Pfizer (New York, USA) and MF from Astellas (Munich, Germany). Stock solutions of AF 20 mg ml^{-1} and MF 10 mg ml^{-1} were prepared in methanol (Merck, Darmstadt, Germany) and stored at -25 °C until use. Britton-Robinson buffer solutions were used as supporting electrolyte consisting 0.04 M each of phosphoric acid, acetic acid and boric acid (Merck, Darmstadt, Germany). Different pH values were adjusted with appropriate volumes of 1.0 M sodium hydroxide solution (Carl Roth, Karlsruhe, Germany). Dilutions were prepared with water purified by a UO 400 - 1950 System (BWT Pharma & Biotech, 63 Mondsee, Germany) and distillated by a Dewadest System (DEWA, Goslar, Germany). To run the electrode and deaerate the sample solutions, nitrogen gas with a reagent grade of 5.0 was obtained from Messer (Sulzbach, Germany). Mercury 99.9999 % (Merck, Darmstadt, Germany) was used to fill the electrode.

3.2. Instrumental

Polarographic determinations were made using a computer controlled 797 VA Computrace analyser (Metrohm, Herisau, Switzerland) with a multi-mode electrode (MME). A DME or hanging mercury drop electrode (HMDE) as working electrode, an auxiliary platinum electrode and an Ag/AgCl reference electrode, saturated with a 3.0 M KCl solution (Metrohm, Herisau, Switzerland) completed the three electrode cell. The pH measurements were carried out using a Seven Easy S20 pH meter (Mettler-Toledo, Gießen, Germany).

3.3. Procedure

Equal volumes (5.0 ml) of Britton-Robinson buffer and methanol were placed into the polarographic container and deaerated by nitrogen gas with stirring (2000 rpm) for 5 min. A background signal was recorded before adding an adequate sample volume. After further deaeration by nitrogen gas for 10 s and equilibration for 10 s with stirring, a differential pulse polarogram of the analyte was measured. Two replicate measurements of each polarogram have been carried out. The peak current was calculated as the difference between each polarogram and the background signal. A scan rate of 14.9 mV s^{-1}, pulse time of 0.04 s and pulse amplitude of 50 mV were chosen as operating parameters. All measurements were conducted at room temperature.

For determination of pH dependence, polarograms of the stock solutions at different pH values from 2.0 to 8.0 were recorded. Linearity tests were performed on a DME and a HMDE. In order to determine the stability of the analytes in the test solution, replicate measurements of a solution of known concentration were conducted every 5 min for a duration of 60 min. Cyclovoltamograms on a HMDE were recorded with a potential range between 0.2 and -2.0 V to elucidate potential reaction mechanisms.

Conflicts of interest: None reported.

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