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A micellar electrokinetic capillary chromatography method for monitoring mycophenolic acid in serum of transplant recipients

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Dedicated to Prof. Dr. F. Zymalkowski on the occasion of the 90th anniversary of his birthday

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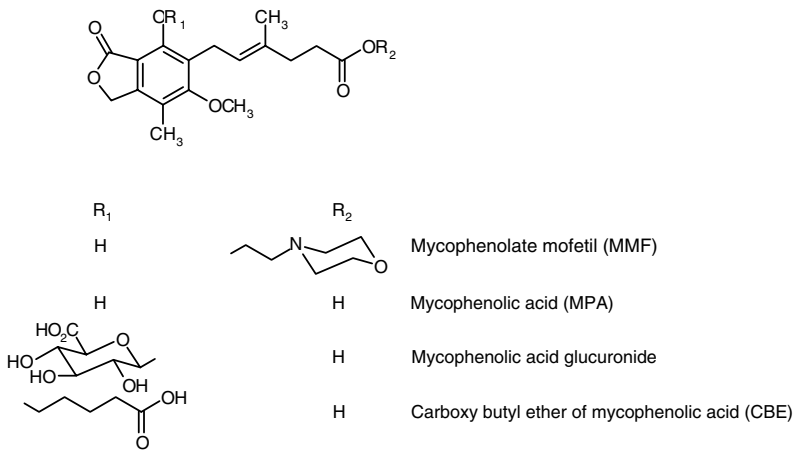
Mycophenolic acid (MPA), the active metabolite of the immunosuppressive agent mycophenolate mofetil (MMF), was for the first time quantified in the serum of transplant recipients using micellar electrokinetic capillary chromatography (MEKC). Sample preparation was carried out with solid phase extraction (SPE) using octadecyl-modified endcapped silica (C_{18} EC) as sorbent. Extremely varying recovery rates in preliminary experiments showed both the importance of pH monitoring during the single SPE steps and the necessity of an internal standard. MPA carboxy butyl ether (CBE), a specifically developed reference standard, was employed. Furthermore, optimisation of the MEKC parameters detection wavelength and injection time was of primary importance in order to enable the quantitation of therapeutic trough serum levels of MPA in the range lower than 5 $\mu g \cdot mL^{-1}$. Under optimised conditions, a limit of quantitation of 1.0 $\mu g \cdot mL^{-1}$ was achieved allowing the determination of MPA in the serum of patients.

1. Introduction

Mycophenolic acid (MPA) is the active metabolite of the immunosuppressive agent mycophenolate mofetile (MMF) (Cellcept®) used in adjunctive therapy with cyclosporin A and corticosteroids for the prevention of graft rejection after solid organ transplantations (Fulton and Markham 1996; Roche Bioscience 1997; Shoker 1997a, 1997b). MPA inhibits inosine monophosphate dehydrogenase (IMPDH), the key enzyme of the *de novo* purine synthesis pathway, reducing the proliferation of especially B and T lymphocytes (Hupe et al. 1986; Langman et al. 1995; Nowak and Shaw 1997).

The recommended dose of MMF is 1.0–1.5 g given twice a day (Hübner et al. 2000). MMF is rapidly absorbed after oral administration and almost completely (94%) hydrolysed to MPA which is extensively bound to albumin (97–98%) and conjugated to MPA glucuronide, respectively (Bullingham et al. 1996a, 1996b; Shipkova et al. 2001). This phase II metabolite undergoes intestinal hydrolysis with consecutive reabsorption of the resulting free MPA which is finally eliminated with the urine (93%) and to a

Scheme



significantly less degree with the faeces (6%) (Fulton and Markham 1996). The highest MPA serum level is observed 1 h after oral administration followed by a second maximum 5–11 h later due to the enterohepatic circulation of the MPA glucuronide (Bullingham et al. 1996a, 1996b; Fulton and Markham 1996).

Monitoring of MPA serum levels is necessary for the optimisation of MMF therapy since the accepted therapeutic range of 1–3 $\mu g \cdot mL^{-1}$ (Holt et al. 2002) is not well established (Budde et al. 2000; Brunet et al. 2000; Hale et al. 2002) and thus the prediction of the efficacy of MMF use in transplantations is still uncertain. Additionally, the relationship between serum level and the corresponding side effects of MPA is extensively investigated, but not fully understood so far (Gummert et al. 1999a; Jacqz-Aigrain et al. 2000; Mourad et al. 2001; Shaw and Nowak 1995).

The determinations of MPA thus far described in plasma are based on HPLC techniques (Gummert et al. 1999b; Jones et al. 1998; Li and Yatscoff 1996; Na-Bangchang et al. 2000; Seebacher et al. 1999; Shipkova et al. 1998; Sugioka et al. 1994; Svensson et al. 1999; Tsina et al. 1996), especially in the reversed phase mode (Beal et al. 1998; Jones et al. 1998; Svensson et al. 1999), and on the enzyme multiplied immunotechnique assay (EMIT) (Beal et al. 1998; Brunet et al. 1999; Shipkova et al. 2000; Vogl et al. 1999; Yeung et al. 1999). Although CE generally yields a higher separation efficiency and requires smaller sample volumes than HPLC, only one capillary zone electrophoretic (CZE) method has been established for the determination of MPA in plasma working with a bubble cell in order to achieve a low limit of quantitation (LOQ) of 0.5 $\mu g \cdot mL^{-1}$ (Ünsalan et al. 2001). Protein precipitation with MeCN and concentration of the supernatant was employed as sample preparation.

Based on the previous work carried out in our group (Noe et al. 2000), in this paper we will focus on the development of a solid phase extraction (SPE) and a rapid MEKC method for the routine determination of MPA in the serum of transplant recipients.

2. Investigations, results and discussion

2.1. Previous work

First, our group investigated whether MEKC was in principle applicable to the determination of MPA from serum (Noe et al. 2000). Deviating from the MEKC conditions mentioned in section 2.2, the injection time was 0.06 min, the separation voltage 30 kV and the detection wavelength was 254 nm. For the SPE, 1.0 mL serum acidified with 50 µL 1 M HCl was employed, the washing step was carried out at pH 7 with H_2O and the residue of the eluate was dissolved in 50 µL MeOH. Although the results indicated that the method seemed to be generally feasible, various parameters had to be optimised in order to employ the method for routine analysis. First experiments using direct serum injection (DSI) instead of SPE showed that this technique gives an LOQ of 20 $\mu g \cdot mL^{-1}$ with a signal to noise ratio (S/N) of 2 : 1. However, sample preparation with SPE lowered the LOQ down to 4 $\mu g \cdot mL^{-1}$ (S/N: 3 : 1) which was still insufficient against the background of MPA trough levels in the range of 4–5 $\mu g \cdot mL^{-1}$. Furthermore, recovery rates (RR) of merely 60% were obtained with high relative standard deviations (RSD) of about 30%.

Due to the high LOQ and the bad reproducibility, both the SPE and the MEKC conditions had to be improved.

Table 1: Optimisation of MEKC parameters for a methanolic MPA solution: variation of the detection wavelength λ and injection time t_i

λ (nm)	t_i (min)	h[a] (mV)	RSD[b] (%)
254	0.06	8.02	28.1
251	0.06	9.29	14.3
217	0.06	19.97	8.0
254	0.10	13.64	9.1
254	0.15	20.68	6.3
251	0.10	15.21	10.9
251	0.15	22.17	5.4
217	**0.10**	**34.30**	**4.1**
217	0.15	52.71	7.8

[a] h: peak height
[b] RSD: relative standard deviation
Investigations were carried out with a methanolic MPA solution of the concentration 200.0 $\mu g \cdot mL^{-1}$ without preceding sample preparation. The parameters peak height and RSD were calculated from five injections.

2.2. Optimisation of the MEKC conditions

From the set of MEKC conditions the detection wavelengths (217, 251, 254 nm) and the injection time t_1 (0.06 to 0.15 min) have been optimised simultaneously by direct injections of a methanolic MPA solution with a concentration of 200.0 $\mu g \cdot mL^{-1}$ (corresponding to a serum concentration of 10.0 $\mu g \cdot mL^{-1}$ before SPE).

The previously chosen wavelength of 254 nm taking into account the low absorbance of the immunosuppressive agent cyclosporin A in this range administered together with MMF was altered to the two UV absorption maxima at $\lambda = 216.6$ nm and $\lambda = 250.6$ nm of MPA in MeOH affording larger peaks with significantly lower RSD values compared to those at 254 nm. Best reproducible results were obtained with $\lambda_{max} = 217$ nm and $t_1 = 0.10$ min (Table 1) with a low relative standard deviation RSD of 4,1%. In consequence, further experiments were carried out with these MEKC parameters.

2.3. Optimisation of the SPE conditions

The optimisation of the SPE parameters towards higher and more reproducible RR values was done with the internal standard MPA carboxy butyl ether (CBE), specifically developed as reference standard by Roche Diagnostic GmbH with UV maxima very similar to those of MPA ($\lambda_{1max} = 214.4$ nm and $\lambda_{2max} = 250.9$ nm). The detection wavelength was changed to the intermediate value of $\lambda = 215$ nm. Investigations with a methanolic solution containing both MPA (200.0 $\mu g \cdot mL^{-1}$) and CBE (100.0 $\mu g \cdot mL^{-1}$) were carried out with a lowered voltage of 25 kV applied during the MEKC achieving migration times for MPA and CBE of 8.2

Table 2: Variation of MeOH volume V_R to reconstitute the residue after SPE

number of SPE procedure	$V_R = 50$ µL		$V_R = 100$ µL	
	RR[a] (%)	RSD[b] (%)	RR[a] (%)	RSD[b] (%)
1	100.7	23.0	81.6	17.4
2	164.1	20.1	90.4	25.0
3	119.7	31.1	100.8	8.5
4	167.2	7.6	80.0	23.9

[a] RR: recovery rate
[b] RSD: relative standard deviation
Investigations were carried out with serum samples spiked only with the internal standard CBE in a concentration of 10.0 $\mu g \cdot mL^{-1}$. RR and RSD values were calculated from five injections for each SPE procedure

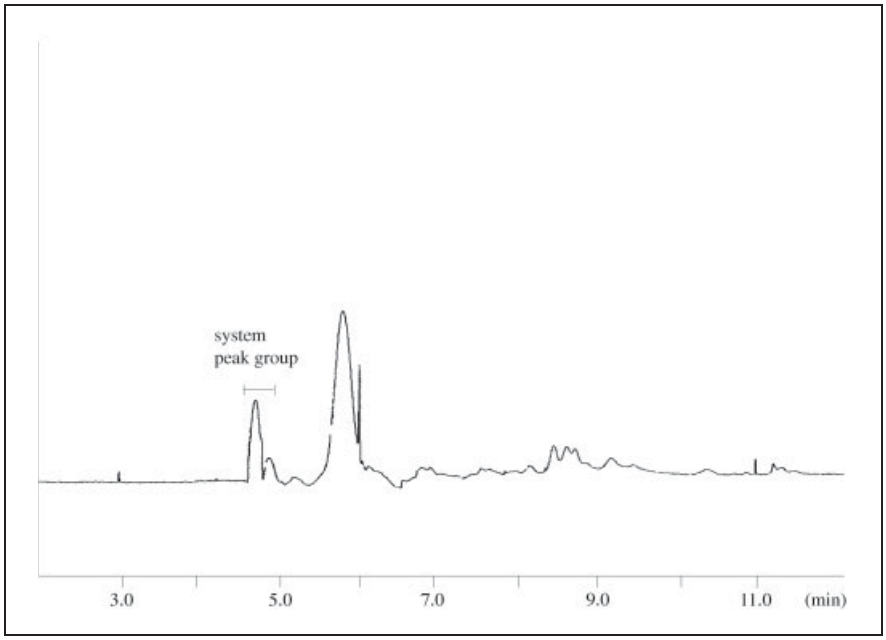


Fig. 1a:
MEKC electropherogramm of blank serum after SPE. The peaks in the range between 5 and 7 minutes are caused by endogenous substances

Table 3: Optimisation of the MEKC parameter injection time t_i after preceding SPE

t_i [min]	peak height	RSD[a] (%)	peak area	RSD[a] (%)	normalized peak area	RSD[a] (%)
0.10	31.77	13.8	217.23	50.1	21.01	36.9
0.15	44.27	11.2	296.66	14.8	31.28	17.9
0.20	64.64	6.3	468.51	12.1	46.49	1.6

[a] RSD: relative standard deviation
Investigations were carried out with serum samples spiked only with the internal standard CBE in a concentration of 10.0 $\mu g \cdot mL^{-1}$. Peak heights, areas, and normalized areas as well as the corresponding RSD values were calculated from three SPE procedures with five injections each

and 9.3 min, respectively, resulting in a sufficient separation with an resolution (R) of 8.11.

Out of the set of SPE parameters, the reconstitution volume for the residue after SPE is of prime importance. Rather high and strongly varying recovery rates from 100.7 to 167.2% with RSD values extending from 7.6 to 31.1% were obtained with 50 µL MeOH. Significantly smaller and less fluctuating RR values from 80.0 to 100.8% with RSD values from 8.5 to 23.9% were achieved with an enlarged volume of 100 µL (see Table 2).

Second, the pH-value of the spiked serum was lowered down to 1.0 to ensure the protonated non-dissociated form of both the MPA and CBE to be completely retained by the hydrophobic C_{18} EC sorbent material. This was finally achieved with an amount of 200 µL 1 M HCl. Additionally, acidified H_2O (3 mL H_2O and 50 µL 1 M HCl) was employed to rinse the column after application of the sample in order to prevent washing out of the dissociated analyte from the column. The results indicate that the reproducibility of the peak heights could clearly be improved whereas the peak areas still strongly fluctuated (Table 3; t_i = 0.10 min) with a non acceptable recovery rate of 73.3%. Therefore the injection time optimised only for a methanolic MPA solution had been readjusted for the the MPA spiked serum after preceding SPE (see section 3.2.).

With a t_i of 0.20 min finally a significantly better RR value of 93.0% was achieved with a further improved reproducibility (RSD = 6.3%) (Table 3). A typical electropherogramm of a separation after preceding SPE from serum is shown in Fig. 1b. Further increase of t_1 caused strong peak broadening resulting in decreasing separation efficiency. The method was finally calibrated with an injection time t_i of 0.20 min via the peak height ratios hr (height of the MPA peak divided by the height of the CBE peak) within the therapeutical range for serum levels up to 5 $\mu g \cdot mL^{-1}$ which is observed after administration of the recommended dose MMF (1.0 g twice a day). The calibration function is given with eq. (1):

$$hr = 0.1456 \cdot c(MPA)[\mu g \cdot mL^{-1}] + 0.0108 \qquad (1)$$

with a correlation coefficient of $r^2 = 0{,}9964$, a standard deviation of the slope sd (b) = 0,0062, and a standard deviation of the intercept sd (a) = 0,0196. The LOQ was found at 1.0 $\mu g \cdot mL^{-1}$ (S/N: 3 : 1). The relative standard deviations of the measured points range from 3.4% to 17.8% corresponding to MPA concentrations of 5.0 $\mu g \cdot mL^{-1}$ and 1.0 $\mu g \cdot mL^{-1}$, respectively (standard deviations were calculated from 3 SPE procedures and 5 injections each). Thus, a sufficient precision for the quantitative determination from a biological matrix is ensured since the acceptable deviation of 20% was not exceeded in any case.

2.4. Determination of MPA from patient samples

The applicability of the developed MEKC procedure with preceding SPE was investigated using serum samples of patients undergoing a combined immunosuppressive therapy including MPA, ciclosporin A, and hydrocortisone after kidney-transplantation. Whole blood of five patients was withdrawn 12 h after the administration of 1.0 g MMF in order to measure trough serum levels. Since the signals of further substances beneath MPA and CBE were observed (Fig. 2), the MPA peak was assigned by spiking each sample with 100 µL of a methanolic MPA solution (50 $\mu g \cdot mL^{-1}$). The results obtained from the analysis of the five patient samples correspond to the range of MPA levels determined with the well established HPLC procedures and thus clearly indicate the applicability of our method.

In our ongoing work, the newly developed MEKC procedure is employed in order to measure complete elimination kinetics from several patients.

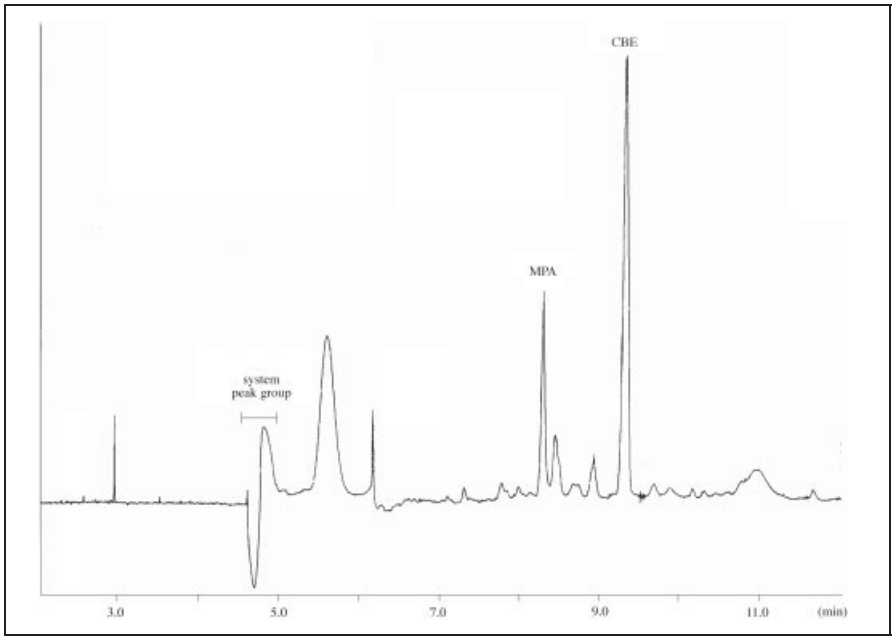


Fig. 1b:
MEKC electropherogramm of MPA and the internal standard CBE after SPE from serum spiked with MPA and CBE of the concentrations 2.0 $\mu g \cdot mL^{-1}$ and 10.0 $\mu g \cdot mL^{-1}$, respectively. Peaks besides those of MPA and CBE and the system peak group are caused by endogenous substances (see Fig. 1a). Separation was carried out under the following conditions: sample injection time 0.20 min with 20 mbar. Buffer: SDS (50 mM) and MeCN (16% (V/V)) containing phosphate-tetraborate buffer (pH* 8.0). Capillary: untreated fused silica (50 µm internal diameter, 49.5 cm effective length, 67.0 cm total length). Voltage 25 kV; resulting current 35 µA. Separation temperature 20 °C. UV detection ($\lambda = 215$ nm)

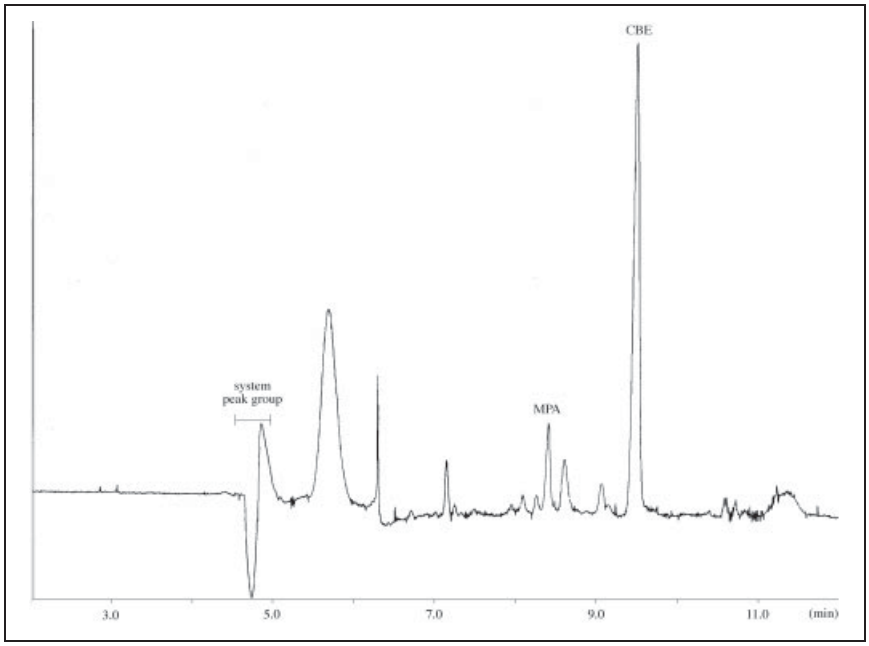


Fig. 2:
MEKC electropherogramm of MPA and the internal standard CBE after SPE from CBE spiked (10.0 $\mu g \cdot mL^{-1}$) serum of a kidney-transplant recipient (see patient No. 1, Table 4, serum concentration of MPA: 1.26 $\mu g \cdot mL^{-1}$) immunosuppressed with MPA, cyclosporin A, and hydrocortisone. Whole blood was withdrawn 12 h after administration of 1.0 g MMF. Separation was carried out under conditions analogous to those of Fig. 1a, b (in addition, see section 2.)

Table 4: Determination of MPA in serum of transplant recipients undergoing combined immunosuppressive therapy

Patient No.	MPA serum level ($\mu g \cdot mL^{-1}$)	RSD[a] (%)
1	1.26	2.7
2	2.56	10.3
3	4.78	1.8
4	1.01	2.6
5	1.11	5.4

[a] RSD: relative standard deviation
Samples were taken 12 h after administration of 1.0 g MMF
MPA serum levels and the corresponding RSD values were calculated from five injections each

3. Experimental

3.1. Chemicals

MPA was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The MPA carboxy butyl ether (CBE) was a gift from Roche Diagnostic GmbH (Mannheim, Germany). Both were of pharmaceutical grade. Sodium dodecyl sulfate (SDS) was obtained from BioRad (Richmond, USA). Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \times 1\ H_2O$) and sodium tetraborate decahydrate ($Na_2B_4O_7 \times 10\ H_2O$) were of analytical grade (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Methanol (MeOH) and acetonitrile (MeCN) (Fluka AG, Buchs, Switzerland) were of HPLC grade. All reagents and solvents were used without further purification.

3.2. Solutions and serum samples

Solutions of MPA (1.0, 1.5, 3.0 and 5.0 $\mu g \cdot mL^{-1}$) and CBE (10.0 $\mu g \cdot mL^{-1}$) were prepared in methanol and stored at 8 °C. Whole blood was collected with 10 mL Serum-Monovetten® (Sarstedt, Berlin, Germany) 12 h after the administration of MMF. Serum was obtained from blood of one of the authors by centrifuging for 30 min at 5000 rpm (corresponding to 2 200 × g) and was stored at ca. −20 °C until use. 1.0 mL of the methanolic MPA solution and the methanolic CBE solution each were mixed and evaporated at 70 °C with a home-made evaporator under a stream of nitrogen. The residue was dissolved in 1.0 mL serum, mixed in an ultrasonic bath for 5 min and acidified with 200 µL 1 M HCl. SPE was carried out with 1.0 mL of the resulting solution. In case of a patient sample, the serum was spiked only with the residue of the methanolic CBE solution.

3.3. Solid phase extraction

SPE was carried out with the vacuum workstation IST VacMaster® and IST Isolute® columns from Separtis GmbH (Grenzach-Whylen, Germany) with 200 mg octadecyl-modified endcapped silica (C_{18} EC) as sorbent material (reservoir volume: 3 mL). The dropping velocity used in all extraction steps was 1 mL · min^{-1}. The columns were conditioned with 2 mL MeOH followed by 2 mL H_2O. After application of 1.0 mL of the acidified serum (see section 2.2.), the column was rinsed with 1 mL acidified H_2O (3 mL H_2O and 50 µL 1 M HCl) and allowed to drip dry. The retained MPA and CBE were eluted with 3 mL MeOH, the methanolic eluate was evaporated (see section 2.2.) and the residue dissolved in 100 µL MeOH.

3.4. Separation buffer

All separations were performed with an SDS (50 mM) and MeCN (16% (V/V)) containing phosphate-tetraborate buffer (pH 8.0). It was prepared by dissolving 275 mg $NaH_2PO_4 \times 1\ H_2O$ in 100.0 mL H_2O (solution 1). Solution 2 (762 mg $Na_2B_4O_7 \times 10\ H_2O$ in 100.0 mL H_2O) was added to solution 1 until a pH value of 8.0 was reached (solution 3). 720 mg SDS were dissolved in 20 mL of solution 3, mixed with 8.0 mL MeCN and brought to volume with solution 3 to 50.0 mL.

3.5. CE Equipment and separation technique

MEKC was performed on a Crystal 310 CE instrument (ATI Unicam GmbH, Kassel, Germany). On-column detection was carried out with a Unicam 4225 UV detector at a wavelength λ of 215 nm. Untreated fused silica capillaries (50 µm internal diameter, 49.5 cm effective length, 67.0 cm total length) obtained from Polymicro Technologies (Phoenix, Arizona, USA) were employed. New capillaries were first pretreated by flushing with 1 M NaOH for 10 min, 2 M HCl for 10 min, H_2O for 2 min, and finally with the separation buffer for 20 min. During all conditioning steps a pressure of 1000 mbar was applied. Between runs, the capillary was flushed with 0.1 M NaOH (10 min, 2000 mbar) followed by separation buffer (5 min, 2000 mbar). The temperature of the capillary was maintained at 20 °C. Sample injection was carried out with a pressure of 20 mbar for 0.10, 0.15 and 0.20 min. The voltage during MEKC separation (run time: 16 min) was adjusted to 25 kV resulting in a current of ca. 35 µA. Chromatographic data were collected by means of an Unicam 4880 version 2.04 data system.

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