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Comparison of the chromatographic behavior of levofloxacin, ciprofloxacin and moxifloxacin on various HPLC phases

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A simple and rapid HPLC method with UV detection was developed for the separation of ciprofloxacin, levofloxacin and moxifloxacin. Chromatography was carried out using a BDS Hypersil® C18 (100 × 4.6 mm, 2.4 μm) HPLC column and an isocratic mobile phase consisting of MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3 and flow rate 1 ml·min^{-1}. The effect of mobile phase variables such as methanol content, pH and buffer concentration on the chromatographic behavior of the three fluoroquinolones was investigated. The retention behavior on a sub 3 μm C18 column was also compared with that on three different calixarene-bonded and on monolithic stationary phases. The results indicate that some differences exist between these three types of stationary phases, particularly in the effect of buffer concentration on the retention mechanism of the three used FQs on calixarene-bonded stationary phases.

1. Introduction

Fluoroquinolones (FQs) are an important group of broad-spectrum synthetic antibacterial agents used for the treatment of different bacterial infections in both human and veterinary medicine. Ciprofloxacin, levofloxacin and moxifloxacin are important FQs because of their broad range of therapeutic indications, particularly active against gram-negative pathogens and against a few gram-positive cocci. These antibiotics are frequently used for the treatment of serious respiratory tract infections, such as severe community-acquired pneumonia (CAP) and acute exacerbations of chronic bronchitis (AECB) (Moran et al. 2008). A need to develop a simple and rapid analytical method for the separation of the three most important FQs for routine analysis of the antibiotics is certainly warranted. The development of one single global method for all three FQs which can be used irrespective of which of the three FQs is administered is favored.

Difficulties may be met in the analysis of FQs. These molecules are weak heterocyclic amino acids with two reactive sites: an amino group which can be protonated and a carboxyl group which can lose a proton. Due to this amphoteric nature of FQs, they may exist in cationic HFQ^+, neutral HFQ^o, zwitterionic $HFQ^{\pm}$ and anionic FQ^- forms (Ross and Riley 1992). Fig. 1 shows the chemical structures and the pK_a values of the studied FQs.

Fluoroquinolones have been determined in many different matrixes as drug formulations, biological, animal and environmental samples. Most of methods apply chromatographic techniques and mainly that of HPLC for the determination of the FQs.

Several different methods describing the separation of FQs have previously been reported. C18 or C8 analytical columns were mainly used (Liang et al. 2002; De Smet et al. 2009; Shervington et al. 2005; Pena et al. 2010). In two studies, monolithic columns were employed (Pena et al. 2007; Seifrtová et al. 2008). UV or fluorescence detection was the technique most used.

In all cases, separation was done with a mobile phase of acidic pH (below the first pK_a value). Seifrtová et al. (2010) described the separation of FQs with a basic mobile phase on phenyl analytical column at pH 10.5 (higher than pK_{a2}).

In addition, other attempts have reported the separation and/or simultaneous quantification of the three FQs described in this paper (Liang et al. 2002; De Smet et al. 2009). However, almost all of these papers describe the separation of FQs with the use of ion-pairing agents such as alkylsulfonates $R\text{-}SO_3^-$ and/or tetraalkylammonium salts R_4N^+. Nevertheless, the use of these agents has certain problems that are either absent from RPC separation or differ in some respect for ion-pair chromatography IPC, such as the slow column equilibration (approximately 1.5 h at a flow rate of 1 ml/min (Shervington et al. 2005)). When an IPC reagent is to be replaced, removal of the previous IPC reagent from the column with a special wash solvent, followed by equilibration of the column with the new mobile phase is needed. Due to the slow equilibration of the IPC reagent with columns, it is possible that not all of the IPC reagent will be washed from the column, even with extensive washing procedures. A trace of IPC reagent remaining on such a column could cause differences in selectivity that would not be reproduced upon replacement with a new column. For this reason it is recommend that columns that have been used with IPC not be used subsequently for RPC separations.

Only one study referred to a calixarene-bonded stationary phase (p-tert-butyl-calix[6]arene), with γ-(ethylenedi amino) propyltriethoxylsilaneas coupling reagent, for the separation of quinolone antibiotics by HPLC (Xiao et al. 2002). However, quinolone peaks were overlapped and peak tailing was extremely serious, in spite of the use of tetrabutylammonium bromide (TBABr), due to the secondary interaction between

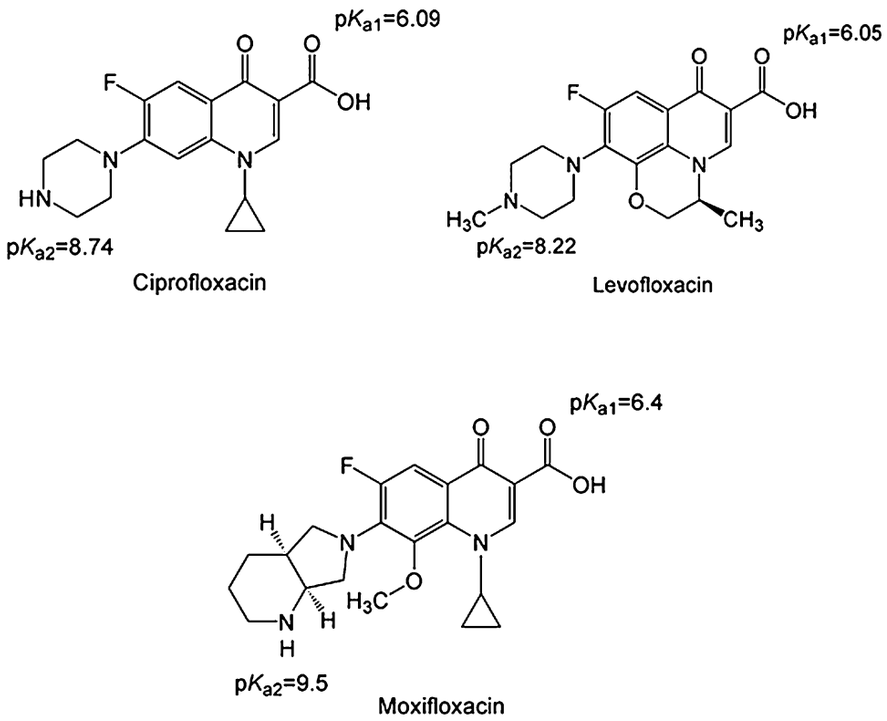

Fig. 1: Chemical structures of fluoroquinolones

silanol groups on column packing material and amino groups on quinolones.

The aim of this study was to develop a single, simple and rapid method for the separation of ciprofloxacin, levofloxacin and moxifloxacin. Initially, the separation of FQs was performed in a short time (analysis was performed within 10 min) without using ion-pairing agents. On the other hand, comparison of different types of RP columns under the same chromatographic conditions should be showing the differences between the investigated stationary phases.

2. Investigations, results and discussion

2.1. Development of separation method on sub 3 μm column

The separation of the three FQs was carried out using a Thermo BDS Hypersil® C18 (100 × 4.6 mm, 2.4 μm) HPLC column and an isocratic mobile phase consisting of MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3 and flow rate 1 ml·min^{-1}. Shorter columns packed with 2.4 μm particles give equivalent efficiency to longer columns packed with 5 μm particles, therefore faster analysis and solvent savings are possible. The smaller particle size also yields 25% greater efficiency than 5 μm particles. The advantage of using 2.4 μm particle size columns is that high speed and high efficiency separations are achievable using conventional HPLC systems (the backpressure was within the limits for a conventional HPLC system and did not exceed 230 bar). To avoid dispersion which can lead to peak broadening, injection volume using columns packed with sub 3 μm particles should be minimized.

The effect of mobile phase variables such as methanol content, pH and buffer concentration on the chromatographic behavior of the three fluoroquinolones on a sub 3 μm C18 column was investigated.

2.1.1. Effect of pH

The pH of the mobile phase is a major factor influencing the chromatographic behaviour of FQs, since these FQs are amphoteric drugs because of the presence of carboxylic acid and piperazinyl (ciprofloxacin and levofloxacin) or diazabicyclo (moxifloxacin) groups in their structures (Fig. 1). The apparent pK_a values of FQs associated with the carboxylic acid function for the compounds studied here ranged from 6.05 to 6.4. It is very likely that the dissociation of the carboxylic acid function is affected by the presence of fluorine atoms. Levofloxacin had the lowest pK_{a1} of this group (6.05) (Ross and Riley 1992).

Since the piperazinyl or diazabicyclo nitrogen is not in close proximity to any of the fluorines and is not connected by a conjugated system, fluorines are not likely to have an effect on pK_{a2}. The only structural difference seen with these FQs that would be expected to affect the pK_{a2} is the presence of a methyl substituent on the piperazinyl nitrogen. pK_{a2} value of the tertiary amines (levofloxacin) is 8.22, while pK_{a2} values of secondary amines (ciprofloxacin and moxifloxacin) are 8.74 and 9.5 respectively (Ross and Riley 1992).

The pH and organic modifier content of the mobile phase used for LC analysis of FQs are therefore known to effect the capacity factors of these compounds (Barbosa et al. 1998).

The retention of ciprofloxacin, levofloxacin and moxifloxacin, expressed as k', was monitored as a function of pH using methanol/water 30/70 (v/v) with pH (2.5; 3 and 4) adjusted with phosphoric acid as a mobile phase. The maximum retention was observed at pH 4. However the separation (resolution R_s and selectivity α), especially between the first two peaks (levofloxacin and ciprofloxacin), was similar between pH 3 and 4 (Fig. 2).

This study showed that with the BDS Hypersil® C18 column the best result in terms of resolution, selectivity and time of analysis was achieved at pH 3.

2.1.2. Effect of ionic strength

The remaining free silanol groups of the silanized silica gel material in their ionized state may lead to electrostatic interactions with positively charged analytes, a phenomenon extensively studied (Horvath et al. 1977; Jandera et al. 2004;

Table 1: Influence of KH_2PO_4 concentration in the mobile phase on the chromatographic parameters of FQs on Thermo® BDS C18 column

Analytes	12.5 mM KH_2PO_4			25 mM KH_2PO_4			50 mM KH_2PO_4		
	t_r (min)	k'	R_s	t_r (min)	k'	R_s	t_r (min)	k'	R_s
Levofloxacin	2.91	1.05	–	2,86	1,01	–	2.72	0.91	–
Ciprofloxacin	3.45	1.43	1.74	3,42	1,41	1,87	3.26	1.30	1,87
Moxifloxacin	10.23	6.21	18.53	9,98	6,03	17,09	9.63	5.78	17.39

Mobile phase: MeOH/(12.5; 25; 50) mM phosphate buffer 28/72 (v/v) at pH 3

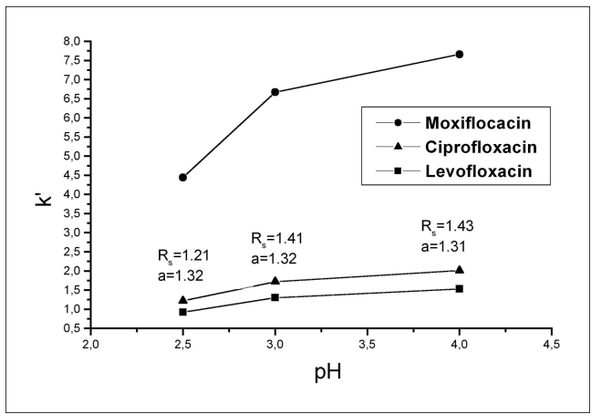

Fig. 2: k'/pH profile of the three FQs (Thermo ®) BDS Hypersil® C18 column) Mobile phase: MeOH/H2o 30/70 (v/v)

Neue et al. 2005). This secondary interaction between the stationary phase and analytes leads to prolonged retention, albeit the resulting peaks are usually broad and not symmetric.

In order to overcome the secondary interaction between free silanol groups on column packing material and amino groups on FQs, 25 mM phosphate buffer at pH 3 was added to the mobile phase, which resulted in a sharper peak shape. The peak width at half height of moxifloxacin $W_{1/2}$ was 0.25 min with 30% methanol (without buffer was 0.42 min). However the previously used percentage of the organic modifier (30% methanol) had to be decreased to 28% to reach baseline separation ($R_s = 1.87$) between ciprofloxacin and moxifloxacin. The retention time of moxifloxacin was 9.98 min (less than that with 30% methanol without the buffer (10.89)) (Fig. 3).

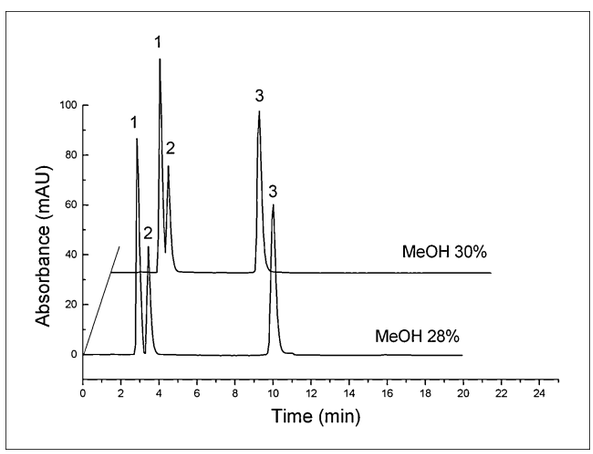

Fig. 3: Separation of FQs (Thermo® BDS Hypersil® C18 column). levofloxacin (1); ciprofloxacin (2); moxifloxacin (3) Mobile phase: MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3, flow rate 1 ml·min^{-1} (A) and MeOH/25 mM phosphate buffer 30/70 (v/v) (B) at pH 3, flow rate 1 ml·min-1

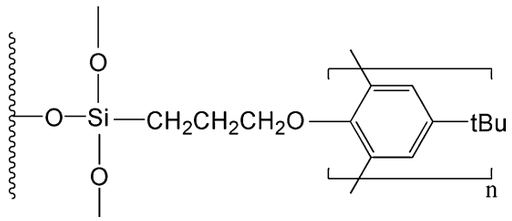

Fig. 4: Schematic structure of the calixarene-bonded stationary phase

The effect of ionic strength of the mobile phase at constant pH value (3) on the separation of the three FQs was studied by increasing the buffer (KH_2PO_4) concentration (from 12.5 to 50 mM). Increasing the ionic strength of the mobile phase, resulted in reduction of retention of the three FQs with enhancement of resolution between levofloxacin and ciprofloxacin (Table 1). Although BDS C18 columns are endcapped, there is still 1% free silanol remaining, which have these electrostatic secondary interactions with the positively charged analytes.

The best separation in terms of resolution with low buffer concentration as possible was achieved with 25 mM (Table 1).

2.2. Retention behavior on calixarene-bonded stationary phases

A comparative study between the retention behavior on a sub 3 μm C18 column and that on three different calixarene-bonded stationary phases was carried out under the same chromatographic conditions. The effect of the ring-size of the calixarenes and the role of the buffer concentration on the chromatographic behavior of the three fluoroquinolones on the calixarene-bonded phases was investigated.

2.2.1. Effect of the ring-size of the calixarenes

Differences as well as similarities between calixarene phases as a function of ring-size of the calixarenes were investigated. The main difference between the three used calixarene-bonded phases is the ring size of the calixarenes (Fig. 4).

As shown in Fig. 5, the separation of the three FQs was achieved on Caltrex® BI and BII, whereas no separation between levofloxacin and ciprofloxacin was possible on Caltrex® BIII. The reason for this is more likely to be due to better formation of π-π interactions in smaller calixarenes. The host-guest supramolecular complexes between calixarene- and analyte-molecules are stabilized by hydrogen bonds, π-π, van der Waals, and solvatophobic interactions between host and guest (Kalchenko et al. 2005). Sokoließ et al. (2000) demonstrated the relationship between the ring size of the calixarenes and the retention process of several analytes. Further investigations about the steric effect, especially the flexible steric, of the calixarene-bonded stationary phases emphasized that this effect on calix[6]arene phases is even larger than the hydrophobic (Schneider et al. 2008). Demonstrated with the longer retention and better resolution

Table 2: Influence of methanol concentration on the chromatographic parameters of FQs on Chromolith® Performance RP-18e column

Analytes	MeOH 28%			MeOH 25%			MeOH 22%		
	t_r (min)	k'	R_s	t_r (min)	k'	R_s	t_r (min)	k'	R_s
Levofloxacin	0,88	0,59		1,03	0,87		1,28	1,33	
Ciprofloxacin	1,00	0,82	1,04	1,23	1,22	1,37	1,61	1,88	1,81
Moxifloxacin	2,39	3,33	7,30	3,65	5,60	9,77	5,98	9,82	12,36

Mobile phase: MeOH/25 mM phosphate buffer (28; 25; 22/72; 75; 78 (v/v)) at pH 3, flow rate 3 ml·min^{-1}

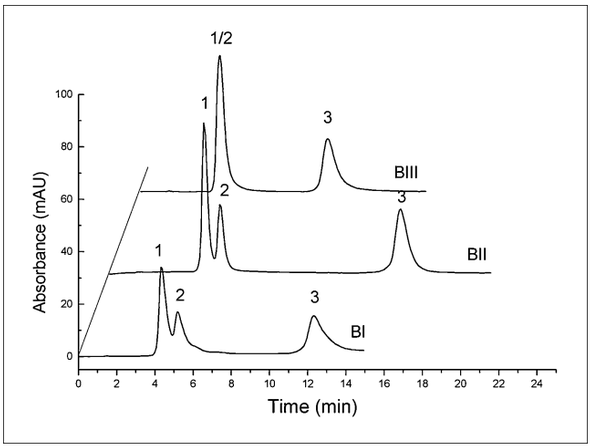

Fig. 5: Separation of FQs on Caltrex® (BI, BII and BIII) columns. levofloxacin (1); ciprofloxacin (2); moxifloxacin (3) Mobile phase: MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3, flow rate 1 ml·min^{-1}

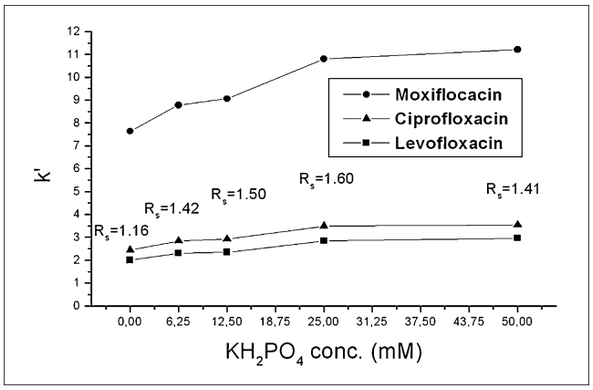

Fig. 6: Influence of KH2PO4 concentration in the mobile phase on the separation of FQs (caltrex ® BII column). Mobile phase: MeOH /(0; 6.25; 12.5; 25; 50) mM phosphate buffer 28/72 (v/v) at pH 3

of the analytes, the inclusion complexes with calix[6]arenes are probably more stable than those with calix[4]arenes. Favorable inclusion of FQs into calix[6]arenes relative to calix[4]arenes might contribute to a better selectivity of silica gels with calixarenes of larger ring size, since the inclusion of the FQs into smaller calixarenes is also hindered by bulky alkyl groups. These could explain the changes in selectivity by changes in ring size of the calixarenes.

2.2.2. *Effect of ionic strength*

The influence of ionic strength in mobile phase on retention factors was investigated by increasing KH_2PO_4 buffer concentration (from 0.0 to 50 mM) at constant pH (3). The retention of FQs on Caltrex® BII increased with increasing the ionic strength of mobile phase as shown in Fig. 6. The reason was probably as follows: the concentration of K^+ increased, the retention of FQs increased because K^+ complexation resulted in a better pre-organization of the basket-like of p-tert.-butyl-calix[6]arene which makes solutes move easily into hydrophobic cavity (Böhmer and McKervey 1991).
Such a similar phenomenon that calix[8]arene stationary phase shows high selectivity to some steroids using KH_2PO_4 as a buffer has been also observed (Liu et al. 2005).
Fig. 6 shows the influence of increasing KH_2PO_4 buffer concentration on the separation of FQs with best result in terms of resolution between levofloxacin and ciprofloxacin achieved with 25 mM KH_2PO_4 ($R_s = 1.60$). Although the retention values of FQs increased with increasing ionic strength of mobile phase, resolution between the first two peaks at high concentration (50 mM) was less than that at 25 and 12.5 mM. That effect is probably due to that at high K^+ concentration, hydrophobic cavity of p-tert.-butyl-calix[6]arene is taken up by K^+, so that it is not easy for positively charged FQs to get close to stationary phase which eventually leads to lower resolution.

2.3. *Retention behavior on a monolithic stationary phase*

To compare the retention behavior on a sub 3 μm C18 column and that on a monolithic stationary phase the separation of FQs was carried out under same chromatographic conditions. Table 2 shows that some modifications to the mobile phase composition as decreasing the organic modifier (MeOH) from 28% to 22% are necessary to reach baseline separation between the three FQs (The time of analysis with flow rate 3 ml.min^{-1} was about 6.5 min).
Since it is possible to operate monolithic columns at high flow rates without loss of resolution, a baseline separation of the three FQs ($R_s = 1.82$) was achieved at flow rate 5 ml·min^{-1} within 4 min and the backpressure didn't exceed 170 bar (Fig. 7).

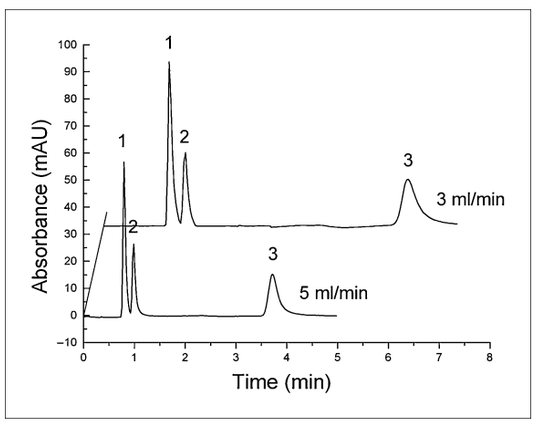

Fig. 7: Separation of FQs (Chromolith® Performacne RP-18e column). levofloxacin (1); ciprofloxacin (2) moxifloxacin (3) Mobile phase: MeOH/25 mM phosphate buffer 22/78 (v/v) at pH 3, flow rate (3; 5) ml·min^{-1}

3. Experimental

3.1. Reagents and chemicals

Ciprofloxacin was obtained from RIEMSER Arzneimittel AG (Insel Riems, Germany), Levofloxacin was kindly provided by Sanofi-Aventis GmbH (Frankfurt am Main, Germany) and moxifloxacin was obtained from Bayer Healthcare (Leverkusen, Germany). Phosphoric acid and potassium dihydrogen phosphate were obtained from Merck KGaA, (Darmstadt, Germany). HPLC gradient grade methanol was purchased from Acros Organics (NJ, USA). Water was obtained by bi-distillation.

3.2. Standard solution

Stock solutions containing 25.0 mg of each FQs (ciprofloxacin, levofloxacin and moxifloxacin) were separately dissolved in 25.0 mL of methanol and stored in the dark at 4 °C. The standard mixture containing 1 $mg.ml^{-1}$ of each FQ dissolved in methanol was used for separation performance studies.

3.3. Equipment

Chromatography was performed on HP 1090 II series (Hewlett Packard, Waldbronn, Germany) equipped with diode array detectors (Agilent Technologies, Waldbronn, Germany). The pH of the solutions was adjusted with a Knick pH-meter (Berlin, Germany).

3.4. Columns

For separation of FQs, Thermo Scientific BDS Hypersil® C18 (100 × 4.6 mm, 2.4 μm) 120 A° pore diameter was used. The study included also three different calixarene-bonded phases (Caltrex® BI: p-tert.-butyl-calix[4]aren; Caltrex® BII: p-tert.-butyl-calix[6]aren and Caltrex® BIII: p-tert.-butyl-calix[8]aren). The ligands were immobilized via hydrophobic spacers on endcapped silica (Kromasil Si 100 A° pore diameter, 5 μm, specific surface area/BET: 300 m^2/g, manufacturer: EKA Chemicals (Bohus, Sweden)). All Caltrex® columns have particle diameters of 5 μm and dimensions of 125 × 4 mm.
The Caltrex® columns were all supplied by Syntrex GbR (Greifswald, Germany). Chromolith® Performance RP-18e (endcapped), 100 × 4.6 mm I.D., was supplied by Merck.

3.5. Chromatographic conditions

The experiments were performed with isocratic elution. The binary mobile phase consisted of different proportions of methanol in aqueous solution (see figures and tables). The two components of mobile phase (aqueous and organic) were mixed 1st time inside the apparatus. pH values were measured in the aqueous component of the mobile phase; phosphoric acid was used for pH adjustment. The eluents were degassed with helium gas before running. In all cases, the column temperature was set at 40 °C. The injection volume was 2 μl. The holdup times (t_0) were determined via uracil. Detection was achieved at 293 nm.

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