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Hydrophilic interaction liquid chromatography in the separation of a moderately lipophilic drug from its highly polar metabolites—The cardioprotectant dexrazoxane as a model case

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ABSTRACT

This paper presents a systematic study of the retention behavior of a model bisdioxopiperazine drug, dexrazoxane (DEX) and its three polar metabolites (two single open-ring intermediates—B and C and an EDTA-like active compound ADR-925) on different stationary phases intended for hydrophilic interaction liquid chromatography (HILIC). The main aim was to estimate advantages and limitations of HILIC in the simultaneous analysis of a moderately lipophilic parent drug and its highly polar metabolites, including positional isomers, under MS compatible conditions. The study involved two bare silica columns (Ascentic Express HILIC, Atlantis HILIC) and two stationary phases with distinct zwitterionic properties (Obelisc N and ZIC HILIC). The chromatographic conditions (mobile phase strength and pH, column temperature) were systematically modified to assess their impact on retention and separation of the studied compounds. It was found that the bare silica phases were unable to separate the positional isomers (intermediates B and C), whereas both columns with zwitterionic properties (Obelisc N and ZIC HILIC) were able to separate these structurally very similar compounds. However, only ZIC HILIC phase allowed appropriate separation of DEX and all its metabolites to a base line within a single run. A mobile phase composed of a mixture of ammonium formate (0.5 mM) and acetonitrile (25:75, v/v) was suggested as optimal for the simultaneous analysis of DEX and its metabolites on ZIC HILIC. Thereafter, HILIC–LC–MS analysis of DEX and all its metabolites was performed for the first time to obtain basic data about the applicability of the suggested chromatographic conditions. Hence, this study demonstrates that HILIC could be a viable solution for the challenging analysis of moderately polar parent drug along with its highly polar metabolites including the ability to separate structurally very similar compounds, such as positional isomers.

1. Introduction

Chromatographic analysis of polar drugs remains a significant challenge due to the poor retention of these analytes on the most commonly used reverse phases (RP) [1]. To resolve this issue hydrophilic interaction liquid chromatography (HILIC) has been developed and currently dominates this field of separation sciences. The term HILIC was introduced by Alpert in 1990 [2] as a variant of normal phase chromatography which utilizes a polar stationary phase along with a binary, mostly organic mobile phase, where water plays the role of a stronger eluent [3]. Based on Alpert's definition, separation in HILIC mode is primarily driven by partitioning, which is in contrast to normal phase chromatography, where compound retention is predominantly governed by surface adsorption. Nevertheless, the outcomes of several experimental studies have pointed to the considerably more complex nature of HILIC retention where partitioning, adsorption and ion exchange interactions might be involved to various degrees, depending on the particular analyte, stationary phase and overall chromatographic conditions used [4–6]. The mixed-mode character of the interactions involved in HILIC can indeed be assumed to be an advantage which may potentially provide improved separation efficacy or modify its selectivity. However, peak tailing and the inability to predict elution order have been reported as the main drawbacks of this approach [6].

The analytical evaluation of polar drugs is relatively complicated, but is nowadays generally a manageable issue. However, analysis of a moderately lipophilic parent drug alongside its highly hydrophilic related compounds still represents a particular challenge [7]. Unfortunately, this is a very typical situation

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in drug bioanalysis as most of the drugs are at least moderately lipophilic, enabling them to pass through biological membranes easily, and they tend to be metabolized into polar metabolites to enhance drug elimination. Nevertheless, separation of such analytes in RP mode requires a highly aqueous mobile phase, addition of an ion-pair reagent or a gradient profile to obtain both the proper retention of polar metabolites and the elution of a parent drug within an acceptable time [8]. However, these conditions are principally not unfavorable for MS detection, which is rightfully a mainstay in current drug analysis and bioanalysis. It should be noted that, despite the evident difficulties, simultaneous analysis of a parent drug and its metabolites is crucial for drug development, dosage schedule optimization and safety monitoring, especially when active metabolites are responsible for either pharmacological or toxicological effects of the drug [9].

Despite the fact that HILIC separation became widely applied in recent decades, there are only a few studies in the current literature dealing with separation of moderately lipophilic drugs from their highly hydrophilic metabolites. Among these, the most significant analytical contribution is an elegant separation of morphine and buprenorphine from their glucoronides on a ZIC HILIC column under gradient conditions [10]. Besides this study, isocratic ultrafast separations of morphine from its glucoronides and midazolam from its hydroxylated metabolites published by Shou et al. demonstrated the high separation ability of Betasil silica using highly organic mobile phases [11]. However, there are certainly more candidates waiting for employment in this approach. Furthermore, different novel columns have been recently introduced to the market providing various unique separation characteristics. Hence, although HILIC may evidently provide significant benefits for analysis of moderately lipophilic drugs in the presence of their polar metabolites the lack of relevant experience in the available literature, hinders estimation of real advantages as well as limitations of this approach.

Dexrazoxane (DEX), an effective cardioprotectant, is a typical example of moderately lipophilic drugs, which is metabolized to strongly hydrophilic metabolites. DEX must be lipophilic enough to penetrate the cell membranes of cardiomyocytes, but the cardioprotective effects are attributed to its hydrophilic metabolite ADR-925. The metabolic bioactivation of DEX is based on the hydrolytic opening of dioxopiperazine rings in two steps. The first step yields single ring-opened intermediates B and C, while in a second step the highly polar EDTA-like active metabolite—ADR-925 is formed, see Fig. 1 [12–14]. Simultaneous chromatographic analysis of DEX and all its metabolites is relatively complicated for several reasons. Besides the highly polar nature of the metabolites, which accounts for their poor retention on common RP columns, there is also high structural similarity of intermediate metabolites B and C. These are the position isomers that differs each other only by the position of a methyl group. Furthermore, the metal chelating ability of ADR-925 might also cause difficulties associated with partial formation of chelates with the trace amount of metals and this may affect the separation efficiency [15]. Moreover, the low absorption coefficients of DEX and particularly of ADR-925, hinder a sensitive and selective UV detection [16]. So far, only RP analytical methods have been developed and applied for the simultaneous analysis of DEX and its metabolites. Indeed, these have utilized highly aqueous mobile phases with addition of ion-pair reagents [17]. Such mobile phase compositions are, however, hardly compatible with MS detection and therefore no HPLC method coupled on line with MS detection for simultaneous analysis of DEX and its metabolites has been published so far. On the contrary, the low aqueous/highly organic volatile mobile phase typical for HILIC analysis is favorable for MS detection, promising a high detector response, especially when ESI–MS is used [6].

The aim of this study was to systematically investigate the retention behavior of the model drug DEX and its three hydrophilic metabolites on different HILIC stationary phases under mass spectrometry compatible conditions. This model is a practical example of a challenging analysis of a parent drug of medium lipophilicity along with its hydrophilic metabolites, including structurally close position isomers. Hence, using this clinically relevant model we sought to reveal benefits and limitations of the HILIC approach in this field of separation science.

2. Experimental

2.1. Chemicals

HPLC gradient grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ammonium formate, ammonium acetate, formic acid, ammonium hydroxide, hydrochloric acid were obtained from Sigma–Aldrich (Schnelldorf, Germany). Milli-Q water was produced by a Millipore purification system (Schwalbach, Germany). Dexrazoxane (DEX) was obtained from Huaren Chemicals (Changzhou, China).

2.2. Preparation of metabolites

2.2.1. Preparation of ADR-925

ADR was prepared according to Schroeder and Hasinoff [18] with some modifications. In particular, the acid form of ADR-925 was released from its salt by an ion-exchange resin instead of HCl yielding highly pure product. 200 mg (0.75 mM) of DEX was added to 60 mg (1.50 mM) of NaOH in 2 mL of Milli-Q water and stirred for 48 h at room temperature [18]. The reaction process was monitored using RP-TLC (Merck, Darmstadt, Germany) with phosphate buffer pH 7.4/ACN 4:6 (v/v) as a mobile phase, R_f = 0.26. Then, the reaction mixture was adjusted at pH 6–7 with Amberlyst 15. The mixture was filtered and the filtrate evaporated. The residue was dried under reduced pressure to give a white solid. Yield: 92%. Melting point: 128–130 °C (Kofler apparatus, uncorrected). IR (KBr, Nicolet Impact 400 spectrophotometer): ν_{max} 3321; 3167; 1670; 1655; 1383; 1220; 1176 cm^{-1}. ^1H NMR (300 MHz, DMSO, Varian Mercury-Vx BB 300 instrument): δ 7.71 (1H; s; NH_2); 7.49 (1H; s; NH_2); 7.25 (1H; s; NH_2); 7.15 (1H; s; NH_2); 3.31–3.07 (9H; m; 4× CH_2, CH); 2.92–2.85 (1H; m; CH_2); 2.68–.61 (1H; m; CH_2); 0.90 (3H; d; J = 6.5 Hz); ^{13}C NMR (75 MHz, DMSO): δ 173.4; 173.1; 172.9; 172.5; 57.6; 55.6; 55.2; 54.4; 53.6; 53.3; 12.9.

2.2.2. Preparation of a mixture of intermediates B and C

A mixture containing intermediates B and C was prepared by alkaline hydrolysis as described previously [19]. After quenching the hydrolysis, the presence of the intermediates was tested by direct infusion of the diluted sample solution (approx. 2 μg/mL) into the ESI–MS source. $[M+H]^+$ at 287 m/z together with the sodium adduct $[M+Na]^+$ at 309 m/z were detected as main ions in the spectra.

2.3. Preparation of stock and working solutions

The stock solutions of DEX and ADR-925 (1 mg/mL) were prepared by dissolving the appropriate amount of the substance in either MeOH (DEX) or water (ADR-925). These solutions were further diluted with a water/ACN mixture (30:70, v/v) to obtain the working solutions (DEX either 100 μg/mL or 5 μg/mL and ADR-925 either 500 μg/mL or 5 μg/mL) for HPLC–UV and LC–MS analysis, respectively. The sample solvent composition was selected as a compromise between the analyte solubility and the solvent strength.

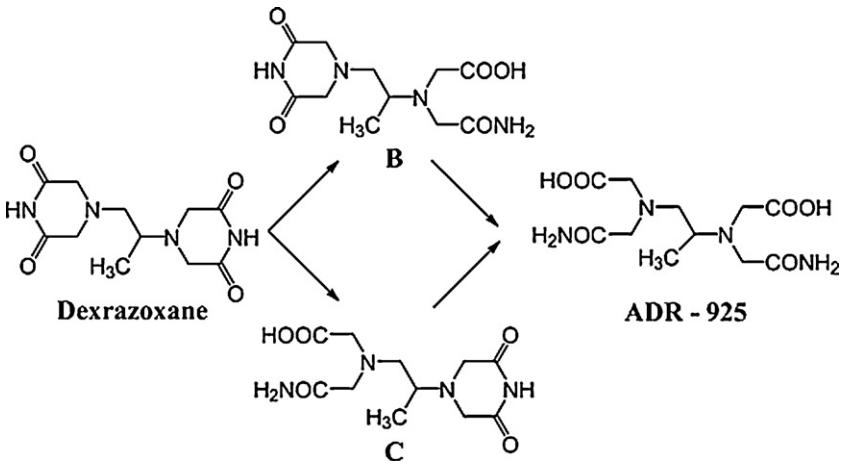

Fig. 1. Scheme of DEX bioactivation.

Stock solution containing both intermediates B and C (approx. 1 mg/mL) was prepared by diluting the sample exposed to alkaline hydrolysis (part 2.2.2) with a mixture of water and ACN (30:70, v/v). The concentrations of B and C were estimated from the aliquot of DEX that was exposed to alkaline hydrolysis. This solution was further diluted with the same solvent to obtain working solutions of B and C (approx. 100 and 5 μg/mL) for HPLC–UV and LC–MS analysis, respectively. B and C were identified after separation on the columns, according to the distinctive MS^n fragmentation.

2.4. Instruments

HPLC–PDA analyses were performed on a chromatographic system LC 20A Prominence (Shimadzu, Duisburg, Germany) which consisted of a DGU-20A3 degasser, two LC-20 AD pumps, a SIL-20 AC auto-sampler, a CTO-20AC column oven, a SPD-M20A photodiode array detector and a CBM-20AC communication module. The data were processed using LC solution software, version 1.21 SP1 (Shimadzu, Duisburg, Germany). LC–MS analyses were performed using a similar Shimadzu chromatographic system coupled with LCQ Max advantage mass spectrometer (Thermo Finnigan, San Jose, USA) equipped with ESI source.

2.5. Study design, chromatographic conditions and settings

2.5.1. Study design

The retention behaviors of DEX and its metabolites were characterized to systematically investigate the separation properties of selected HILIC stationary phases. For this purpose, the HPLC–PDA system was employed in the routine analyses, while selected chromatographic conditions were tested thereafter on the LC–MS instrument. The following chromatographic parameters remained constant throughout the study: A flow rate of 0.3 mL/min, UV detection at 205 nm, and the injection volume of 2 μL.

The range of experimental conditions employed in this study was selected in order to reach the best available separation of all analytes under isocratic HILIC conditions compatible with MS detection. The acceptable time for reproducible retention of all analytes was arbitrarily set to ≤40 min, while the desirable total time for final analysis was considered to be ≤10 min. The range of chromatographic conditions tested in this study reflected the manufacturers' recommendation to ensure long-term stability of the columns.

The standard testing procedure was based on obtaining the basic retention characteristics of the compounds in a first step of experiment to select the composition of the initial mobile phases. Thereafter, the initial conditions were systematically modified to assess the impact of mobile phase strength, pH, buffer concentration and column temperature on the retention characteristics.

2.5.2. HILIC columns employed in this study and their characteristics

Stationary phases evaluated in this study were selected to represent two major groups of phases (bare silica and silica-based zwitterionic stationary phases) intended for HILIC analysis of polar drugs. These columns have different surface chemical properties enabling determination of the involvement of distinct retention mechanisms.

Ascentis Express HILIC (150 mm × 2.1 mm; 2.7 μm particle diameter, 90 Å, Sigma–Aldrich, Schnelldorf, Germany) and Atlantis HILIC Silica (150 mm × 2.1 mm; 3 μm particle diameter, 100 Å, Waters, Milford, USA) represent bare silica columns. Atlantis HILIC is made from totally porous silica, while Ascentis Express HILIC is formed from the fused-core particles. Both stationary phases are made from silica B type. The pH operation range for Ascentis Express HILIC is 3.0–8.0 and the upper temperature limit is 60 °C. On Atlantis HILIC the pH of the mobile phase is limited to 1.0–5.0 and the recommended temperature range is 20–45 °C.

Silica-based zwitterionic columns were represented by ZIC HILIC (150 mm × 2.1 mm; 3.5 μm particle diameter, 100 Å, Merck, Darmstadt, Germany) and Obelisc N (150 mm × 3.2 mm; 5 μm particle diameter, 100 Å, Sielc, Prospect Heights, USA). ZIC HILIC contains a sulfoalkylbetaine moiety (with distal position of sulfonyl group) covalently bonded to the silica surface. The 1:1 ratio between strong cation and anion exchange groups results in the zero surface charge. ZIC HILIC pH range is 3.0–8.0; the upper temperature range is 70 °C. Obelisc N carries anionic groups close to the silica surface that are separated from the cationic groups in the periphery by the hydrophilic chains. The exact chemical nature of the zwitterions is not specified by the manufacturer. The stability of Obelisc N is limited to pH range of 2.5–4.5 and temperature range of 20–45 °C.

2.5.3. Effect of mobile phase strength

The effect of eluent strength was investigated using different mobile phases composed of components A (aqueous part) and B (organic solvent), mixed together to yield a total aqueous part content within the HILIC range (5–40%). Component A was either ammonium formate (2.5 mM) or formic acid (0.053 mM) to achieve full compatibility with MS detection, high solubility in ACN and low UV cutoff. ACN was chosen as an organic solvent. To assure the constant ionic strength, component B was composed of an aque-

ous solution of either ammonium formate (50 mM) or formic acid (1.1 mM) and ACN (5:95, v/v).

The following chromatographic parameters were calculated using HPLC Lab Solution software (Shimadzu) (using Eqs. (1) and (2)): the peak tailing factor (T_f) of the most retained analyte - ADR-925, and the resolution (R_s) between the critical pair—isomers B and C.

$$T_f = \frac{W_{0.05}}{2 \times a_{0.05}} \quad (1)$$

$$R_s = 2 \times \frac{t_R - t_{RP}}{W + W_P} \quad (2)$$

W is a peak width at a baseline level, $W_{0.05}$ is the peak width at 5% height of the peak, $a_{0.05}$ is the width of the front half of the peak at 5% height of the peak and W_P is a width of the previous peak, t_R is a peak retention time and t_{RP} is a retention time of the previous peak.

The retention factor (k) was determined using toluene (a void volume marker) dissolved in a water/ACN (30:70, v/v) mixture in a ratio 1:10,000 (v/v).

2.5.4. Effect of temperature

Effect of the column temperature was examined within the range of 20–50 °C and was expressed using van't Hoff plots ($\ln k$ versus $1/T$) based on Eq. (3).

$$\ln k = \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi \quad (3)$$

ΔH° and ΔS° are retention enthalpy and entropy, R is the gas constant, T is the absolute temperature in K and Φ is a phase ratio.

The linearity of the plots was tested using linear regression analysis by GraphPad Prism, (version 5.02; GraphPad Software, La Jolla, CA, USA). In addition, the interaction between the analytes and the stationary phases were basically characterized by the enthalpy calculated from the slope of the plots (slope = $-\Delta H^\circ/R$) [20].

2.5.5. Influence of mobile phase pH and buffer strength

The influence of pH was examined on Ascentis Express HILIC and ZIC HILIC columns that are able to operate from acidic to neutral range. This particular pH range of the column was found essential for a systematic study. Ammonium formate was replaced by ammonium acetate in these experiments, due to the higher buffering ability near neutral pH. The impact of pH was evaluated using ammonium acetate of different pH (^w_wpH 4.0–6.8) and the effect of buffer strength was investigated at pH ≈ 6 using 0.5–5 mM concentration of ammonium acetate (in total). Only one solvent strength (75% of ACN) was used in these experiments. The mobile phase pH is expressed as ^w_wpH measured in aqueous part, before mixing with ACN.

2.5.6. Investigation of the prevalent retention mechanism

The general equations describing retention based on partitioning (Eq. (4)) and surface adsorption (Eq. (5)) were employed to analyze the retention mechanism [4,21]. The logarithm of retention factor (k) was plotted against the volume aqueous fraction of the mobile phase in linear and logarithmic scales. Linearity of both plots was evaluated using linear regression analysis by GraphPad Prism (version 5.02, GraphPad Software, Inc. USA) and was expressed as r^2.

$$\log k = \log k_W - S\varphi \quad (4)$$

k_W is the retention factor for the pure weaker eluent, φ is the volume fraction of the stronger eluent, and S is a slope of $\log k$ versus φ.

$$\log k = \log k_B - \frac{A_S}{n_B} \log N_B \quad (5)$$

k_B is the retention factor of solute when the pure stronger eluent (B) is used as mobile phase, A_S and n_B are cross-sectional areas occupied by the solute on the surface, N_B is a mole fraction of the stronger eluent B.

2.5.7. MS setting and LC–MS/MS analysis

MS and MS^n experiments were performed using ESI^+ ionization. The following settings were used for MS and LC–MS/MS analysis: capillary voltage of 4.0 kV, capillary temperature of 250 °C, sheet and auxiliary gas flows of 40 and 20 units, respectively. Full scan spectra were recorded from 100–800 m/z, MS^n experiments were carries out using collision energy from 25% to 30%. The final chromatograms were recorded in selected ion monitoring (SIM).

3. Results and discussion

3.1. General description of the retention behavior

The retention times of all compounds involved in the study (DEX, ADR-925, intermediates B and C) decreased with increasing the aqueous fraction of mobile phase (see Fig. 2). The elution order was identical on all columns tested. It was found that DEX was eluted close to the void volume ($k < 0.35$) on all HILIC columns, even if weak mobile phase (5% of the aqueous fraction) was employed. In contrast, ADR-925 was relatively strongly retained on all HILIC columns and hence, its analysis was somewhat troublesome. Using mobile phase containing less than 15% of the aqueous fraction, ADR-925 failed to be eluted in a manageable time (RT > 40 min) or an unacceptable peak shape was observed. The highest retention was observed on Obelisc N, where ADR-925 was not eluted unless mobile phase contained at least 27% of the aqueous fraction. The strong retention of this compound was the main factor that hindered simultaneous analysis of the analytes using weak mobile phases. Furthermore, it was found that addition of buffer (>0.5 mM in total) to the mobile phase is crucial to reach ADR-925 elution in an acceptable time.

Separation of the position isomers B and C was achieved on both zwitterionic stationary phases in the study (Obelisc N, ZIC HILIC), while on both bare silica columns (Ascentis Express HILIC and Atlantis HILIC) these metabolites co-eluted either with DEX or with each other. These co-elutions could not be overcome despite the wide range of chromatographic conditions tested. In addition, the mobile phase of pH near to neutral (^w_wpH 6) was generally favorable for the analysis of all compounds.

3.1.1. Retention behavior on bare silica columns

Due to the co-elution of isomers B and C the retention behavior was systematically followed only for DEX and ADR-925 on bare silica columns (Ascentis Express HILIC and Atlantis HILIC). The retention profile of these compounds was similar on both columns, even though ADR-925 was slightly more retained on Atlantis HILIC than on the Ascentis Express HILIC column (Fig. 2a and b). Peak tailing factors calculated for ADR-925 using different aqueous fraction contents in the mobile phase are given in Table 1. The highest peak symmetry of ADR-925 was reached on both columns with the medium content of the aqueous fraction (25–30%).

3.1.2. Retention behavior on zwitterionic columns

Sufficient separation of all given analytes using a single mobile phase was not feasible on Obelisc N. While a mobile phase composed of a mixture of an aqueous solution of formic acid (0.053 mM) and ACN was able to separate DEX, B and C, acceptable peak shape and reproducible retention of ADR-925 could not be reached without presence of buffer ions. However, this was accompanied by a loss of separation of the intermediate metabolites (B and C).

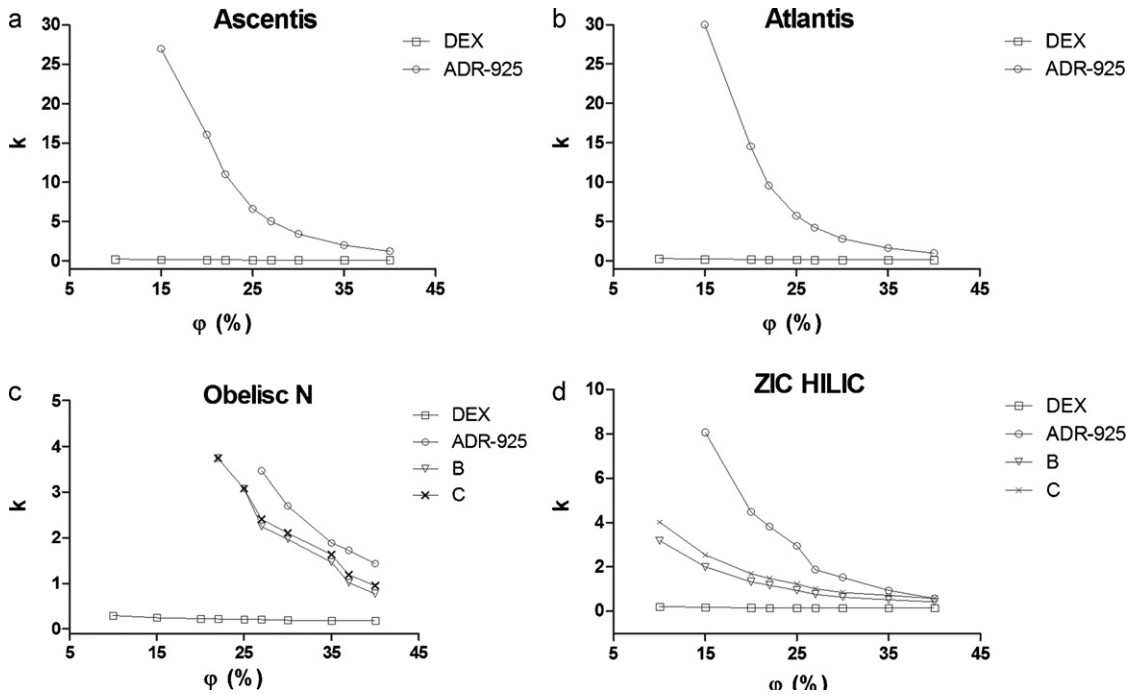

Fig. 2. Retention behavior of the analytes as a function of a volume aqueous fraction (φ) of the mobile phase (a) Ascentis Express HILIC (b) Atlantis HILIC (d) ZIC HILIC columns using mobile phases composed of ammonium formate (2.5 mM in total, pH either not adjusted for Ascentis Express and ZIC HILIC or adjusted to 5.0 for Atlantis HILIC) and ACN. (c) Obelisc N column using either an aqueous solution of formic acid (0.053 mM) or ammonium formate (2.5 mM in total, pH 4.5) and ACN for DEX/B and C and ADR-925, respectively.

The retention behavior of the studied compounds on Obelisc N is shown in Fig. 2c. DEX and the intermediate metabolites (B and C) were analyzed using buffer-free mobile phase, while for analysis of ADR-925 ammonium formate/ACN mixtures were used. Due to co-elution or strong retention of the analytes on this column, retention of B, C and ADR-925 was followed only till 25% and 27% content of aqueous fraction, respectively. The peak tailing factors values for ADR-925 are given in Table 1. The highest resolution of B and C was achieved at 40% of the aqueous fraction (Table 1). Besides the mobile phase strength, its pH (adjusted in this particular case by addition of formic acid) had a marked impact on the B and C resolution. It was found that higher pH enhanced their separation, with the highest possible resolution obtained near the upper pH limit recommended for this column ($^{w}_{w}$pH 4.5).

In contrast to Obelisc N, DEX and all its metabolites were well separated on ZIC HILIC column. The retention profile of the compounds as a function of the aqueous fraction of the mobile phase is shown in Fig. 2d. Peak tailing factors of ADR-925 were within the range 1.37–1.79 with the best available peak symmetry at 25% aqueous fraction of the mobile phase (Table 1). Lower mobile phase strength enhanced B and C resolution (Table 1), however, only up to 15% of the aqueous fraction. Further decrease of the mobile phase strength resulted in the loss of the resolution of the intermediates mainly due to the distortion of their peak shapes. In contrast to Obelisc N, the introduction of buffer ions to the mobile phase was essential to obtain the appropriate shape and, particularly, resolution of metabolites B and C.

3.2. Effect of temperature

It was found that the retention of all compounds decreased with increased column temperature (Fig. 1S). The r^2 values of the van't Hoff plots ranging from 0.972 to 0.999 (Table 2) indicate a linear relationship between $\ln k$ and $1/T$. The lowest r^2 values were obtained for DEX on ZIC HILIC that was caused rather by higher degree of scattering of the k values than curvature of the plot. The positive slopes of the plots and negative enthalpy (Table 2) suggest an exothermic character for the analyte transfer from the mobile

Table 1
The peak tailing factors of ADR-925 and the resolution of B and C as a function of a volume aqueous fraction of the mobile phase (φ). On Ascentis Express HILIC, Atlantis HILIC, ZIC HILIC columns mobile phases composed of ammonium formate (2.5 mM in total, pH either not adjusted for Ascentis Express and ZIC HILIC or adjusted to 5.0 for Atlantis HILIC) and ACN were used; On Obelisc N column either an aqueous solution of formic acid (0.053 mM) or ammonium formate (2.5 mM in total, pH 4.5) and ACN were employed for the analysis of B, C and ADR-925, respectively.

φ (%)	ADR-925 peak tailing				R_s of B and C	
	Ascentis	Atlantis	Obelisc N	ZIC HILIC	Obelisc N	ZIC HILIC
10	–	–	–	–	–	2.03
15	1.85	2.11	–	1.46	–	2.54
20	1.80	2.08	–	1.65	–	1.40
22	1.54	1.34	–	1.46	–	1.14
25	1.60	1.32	–	1.37	0.15	1.06
27	1.40	1.31	1.10	1.71	0.24	1.01
30	1.36	1.30	1.16	1.79	0.53	1.00
35	0.90	1.57	1.13	1.64	0.77	0.88
40	0.71	2.13	1.10	1.65	0.82	0.70

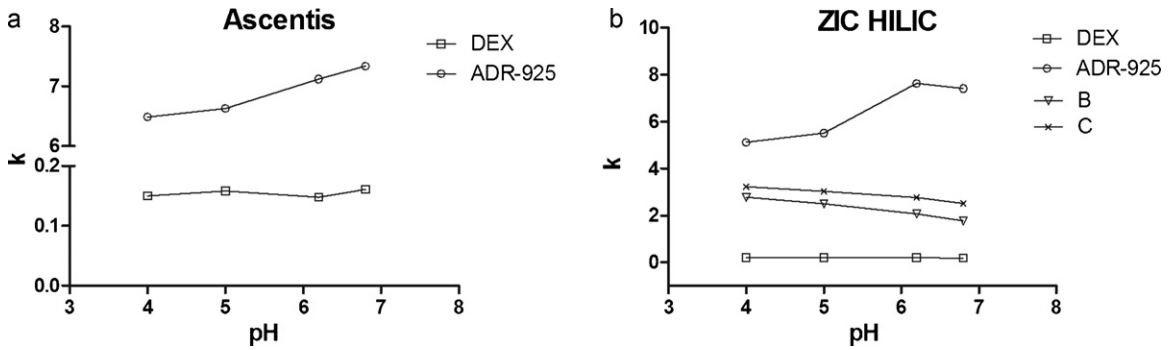

Fig. 3. Retention of DEX, ADR-925 and intermediates B and C as a function of pH: (a) Ascentis Express HILIC (b) ZIC HILIC using ammonium acetate (2.5 mM in total) and ACN in a ratio 25/75 (v/v).

Table 2
Coefficients of determination (r^2) for the van't Hoff plots and the calculated retention enthalpy (ΔH°). Mobile phase were composed of ammonium formate (2.5 mM in total, pH either not adjusted for Ascentis Express and ZIC HILIC or adjusted to 5.0 for Atlantis HILIC) and ACN in a ratio 25/75 (v/v) for Ascentis Express HILIC, Atlantis HILIC, ZIC HILIC columns. On Obelisc N mobile phases consisted of either an aqueous solution of formic acid (0.053 mM) or ammonium formate (2.5 mM in total, pH 4.5) and ACN in a ratio 40/60 (v/v) for DEX/B and C and ADR-925, respectively, were employed.

Compound	Ascentis		Atlantis		Obelisc N		ZIC HILIC	
	r^2	ΔH° (kJ/mol)	r^2	ΔH° (kJ/mol)	r^2	ΔH° (kJ/mol)	r^2	ΔH° (kJ/mol)
DEX	0.997	−3.1	0.997	−2.6	0.997	−10.5	0.972	−1.74
ADR-925	0.999	−6.7	0.950	−2.1	0.998	−0.78	0.992	−0.87
B	–	–	–	–	0.987	−1.2	0.974	−1.9
C	–	–	–	–	0.980	−1.3	0.984	−2.2

to the stationary phases. Similar trends are usually observed in RP separations, while in HILIC both negative and positive slopes were found in the plots [22–25]. In our study, the positive slope and the linear character of the plots suggest partitioning as a main retention mechanism [22]. In this study, neither the peak shape of ADR-925 nor the resolution of B and C were improved using higher temperature.

3.3. Effect of mobile phase pH and buffer strength

The effect of mobile phase pH within the range of $^{w}_{w}$pH 4.0–6.8 was investigated on Ascentis Express HILIC and ZIC HILIC as their higher operating range better allowed a systematic study of the impact of this factor. It was found that $^{w}_{w}$pH lower than 4.0 resulted in deterioration of the peak shape, particularly for ADR-925; and therefore it was not evaluated further.

The acidobasic properties of the analytes are as follows. DEX possesses two tertiary amines of weak basicity due to proximity of two carbonyls (p$K_a \sim 2.6$ according to ACD/Labs Software V8.14) and two acidic imide groups with p$K_a \sim 11$. Thus, within the pH range studied, it is unionized and therefore its retention should not be influenced by pH modification. This is well in line with our experimental results (Fig. 3a and b). In contrast, ADR-925 contains two relatively strongly acidic carboxyl groups (p$K_a \sim 2$), which would be fully ionized within the pH range used in this study. In addition, the basicity of the two tertiary amino groups is higher (p$K_a \sim 7.8$) than of those in DEX; therefore, they will be protonated. Thus, the prevalent form of ADR-925 in the pH range 4–6.8 is a zwitterion or, more precisely, it contains two cations and two anions. The acidobasic properties of the partially open intermediates B and C would be a combination of those of DEX and ADR-925 with prevailing zwitterionic character over the studied pH.

Table 3
R^2 values for lin–log and log–log plots of retention factor versus a volume aqueous fraction of the mobile phase. On Ascentis Express HILIC, Atlantis HILIC, ZIC HILIC columns mobile phases composed of ammonium formate (2.5 mM in total, pH either not adjusted for Ascentis Express and ZIC HILIC or adjusted to 5.0 for Atlantis HILIC) and ACN were used; On Obelisc N column either an aqueous solution of formic acid (0.053 mM) or ammonium formate (2.5 mM in total, pH 4.5) and ACN were employed for the analysis of DEX/B and C and ADR-925, respectively.

Column	Lin–log (r^2)	Log–log (r^2)	Range (φ%)	Lin–log (r^2)	Log–log (r^2)	Range (φ%)
	DEX			ADR-925		
Ascentis	0.798	0.936	10–40	0.982	0.999	15–40
Atlantis	0.852	0.966	10–40	0.956	0.994	15–40
Obelisc N	0.967	0.998	10–30	0.993	0.998	27–40
ZIC HILIC	0.939	0.986	10–30	0.993	0.978	15–40
	B			C		
Obelisc N	0.989	0.998	20–40	0.987	0.999	20–40
ZIC HILIC	0.978	0.989	10–40	0.973	0.993	10–40
	DEX			ADR-925		
Ascentis	0.798	0.936	10–40	0.982	0.999	15–40
Atlantis	0.852	0.966	10–40	0.956	0.994	15–40
Obelisc N	0.967	0.998	10–30	0.993	0.998	27–40
ZIC HILIC	0.939	0.986	10–30	0.993	0.978	15–40
	B			C		
Obelisc N	0.989	0.998	20–40	0.987	0.999	20–40
ZIC HILIC	0.978	0.989	10–40	0.973	0.993	10–40

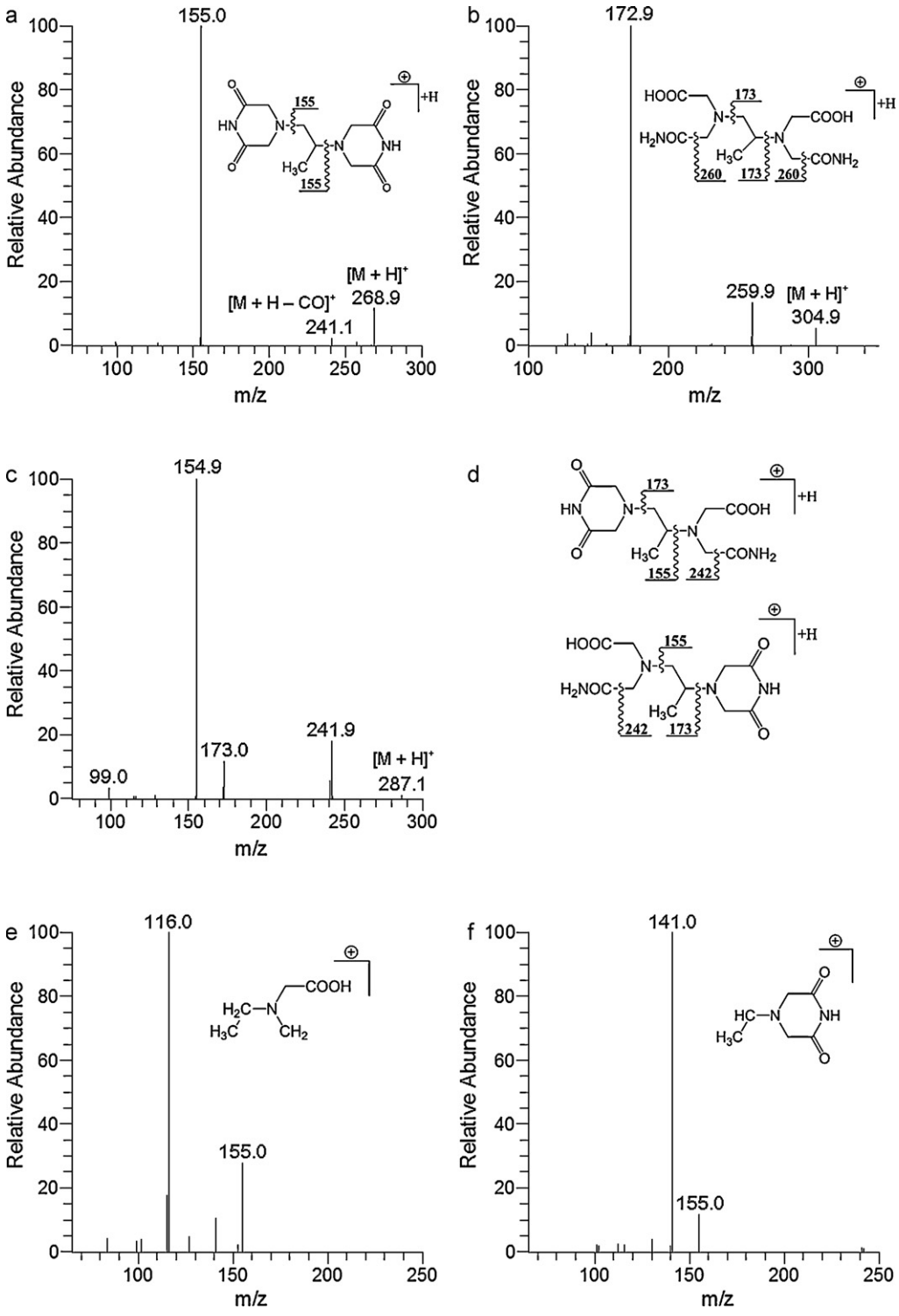

Fig. 4. MS^n experiments: MS^2 spectra recorded after fragmentation of: (a) $[M+H]^+$ at 269 *m/z* for DEX, (b) $[M+H]^+$ at 305 *m/z* for ADR-925, (c) $[M+H]^+$ at 287 *m/z* for intermediates B and C, (d) proposed identity of the fragments of ion at 287 *m/z*. MS^3 spectra recorded after fragmentation of ions at 242 *m/z* for (e) intermediate B and (f) intermediate C.

Retention of ADR-925 increased as a function of mobile phase pH on both columns and reached maxima at $^{w}_{w}$pH 6.8–6.0 on Ascentis Express HILIC and ZIC HILIC columns, respectively (Fig. 3a and b). The pK_a values of ADR-925 (2.1 and 7.8) alone cannot simply explain the retention profile of this compound within the pH range ($^{w}_{w}$pH 4.0–6.8) studied; apparently another mechanism is involved. As the ionization state of bare silica changes as a function of pH (the pK_a of silanols varies from 4 to 7 [26]) a plausible explanation of ADR-925 retention profile might be as follows. With increasing pH, a larger fraction of silanol groups are ionized, leading to a stronger attraction of the positively charged basic groups of ADR-925 and, consequently, its stronger retention. However, the zwitterionic character of ADR-925 with possible electrostatic repulsion between the negative –COO^- groups of the analyte and silanols could also play a role.

Table 4
Summary of the chromatographic conditions suggested for further LC–MS study.

Mobile phase	Ascentis	Atlantis	Obelisc N		ZIC HILIC
	Ammonium formate/ACN (pH ≈ 6)	Ammonium formate/ACN (pH 5.0)	Formic acid/ACN (pH ≈ 4.5)	Ammonium formate/ACN (pH 4.5)	Ammonium formate/ACN (pH ≈ 6)
Ratio	30/70	30/70	40/60	40/60	25/75
$R_{s\ (B/C)}$	–	–	–	0.82	1.06
$T_{f\ ADR-925}$	1.36	1.30	1.10	–	1.37
Time (min)	6	8	6	7	10

Unlike on bare silica, on ZIC HILIC column the silanolic interactions are shielded by the presence of zwitterions embedded on the silica support. Moreover, both positively and negatively charged groups are well balanced. Hence, ZIC HILIC is significantly less charged in comparison with bare silica. However, the distal position of sulfonic groups on the stationary phase still retains the column's ability for weak ionic interactions with positively charged analytes [4]. In contrast to bare silica, the ionization state of this zwitterionic column would not change over the pH studied because the cation is located on a quaternary ammonium nitrogen atom and the negative charge is associated with a strong sulfonic acid. Interestingly, ADR-925 retention as a function of pH showed the profile resembling to that observed on bare silica, with only minor change at pH 6.8.

The retention profile of B and C as a function of pH was studied only on ZIC HILIC where these position isomers could be sufficiently separated. Unlike the case of ADR-925, the retention of both intermediates B and C decreased slightly when the pH of the mobile phase increased. Although this effect may be related to a different ionization of these intermediates compared to ADR-925, this would be rather speculative because the exact basicity of the tertiary nitrogens is difficult to predict. Another possibility is that a slightly different mechanism prevails in the retention of these intermediates. Regarding B and C separation, only a minor improvement of the resolution was observed as a function of mobile phase pH (see Fig. 3b).

In addition, the impact of buffer strength was studied, however using only one pH (ammonium acetate without pH adjustment) and mobile phase strength (75% ACN). Increasing the buffer strength from 0.5 mM to 5 mM (in total volume) led to a slight increase in the retention times of all analytes, with the most pronounced effect on ADR-925 (data not shown).

This is in contrast with our previous findings which suggested involvement of ionic interactions in the retention of this compound. It is difficult to satisfactorily explain this discrepancy, as the influence of buffer strength was studied only at one pH. Nevertheless, it could be hypothesized that at higher salt concentrations, where the ionic interactions might be suppressed, the involvement of hydrophilic partitioning might become more apparent and this could determine the analytes retention. This is in line with results of Marrubini et al. [25], who observed a decrease of retention, when pure water was replaced by a buffer solution of low salt concentration, due to the reduction of the ionic interactions. However, when more salt was added to the mobile phase, the reverse trend was observed, likely due to the reduced solubility of the analyte in the ACN-rich mobile phase leading to the higher susceptibility of the compounds to remain in the aqueous layer on the stationary phase [25]. However, a more complex study would be needed to confirm this hypothesis. As the only one solvent strength (75% ACN) was used in these experiments, the variable interactions between pH, buffer strength and solvent strength were not considered in this study.

3.4. Investigation of the mechanisms of retention and separation

The relative involvement of partitioning and adsorption on the HILIC retention of the analytes was investigated by plotting retention factors versus volume aqueous fraction of the mobile phase (φ) in linear and logarithmic scales. The calculated r^2 values for these plots are given in Table 3 and the corresponding plots are presented in the Supplementary data (Fig. S2).

In the case of DEX, higher r^2 values were obtained for log–log plots than for the corresponding lin–log plots on both bare silica columns, which favors the adsorption mechanism (Table 3). How-

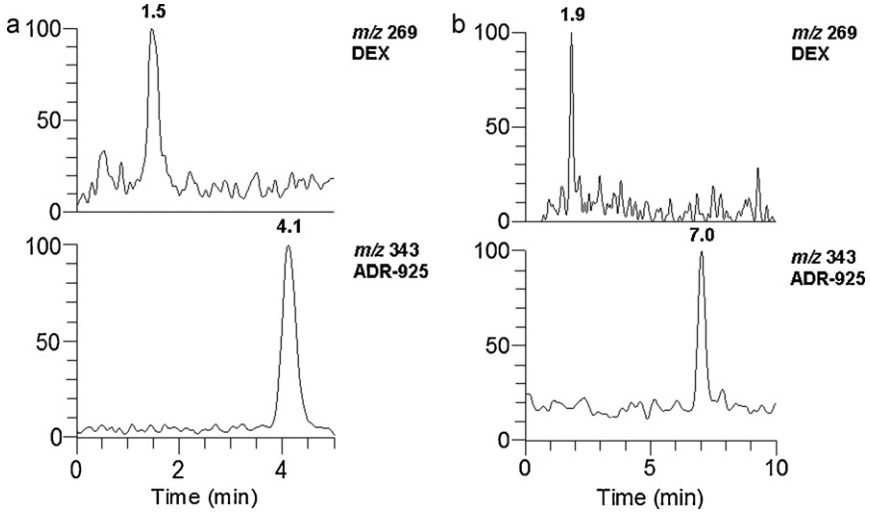

Fig. 5. LC–MS analysis of DEX and ADR-925 on Ascentis Express HILIC and Atlantis HILIC columns Analysis on (a) Ascentis Express HILIC and (b) Atlantis HILIC columns using a mixture of ammonium formate (pH either not adjusted for Ascentis Express or adjusted to 5.0 for Atlantis HILIC) and ACN (0.5 mM total ion strength) in 30:70 (v/v) ratio as a mobile phase. The chromatograms are recorded in SIM, using $[M+H]^+$ at 269 for DEX and $[M+K]^+$ at 343 m/z for ADR-925.

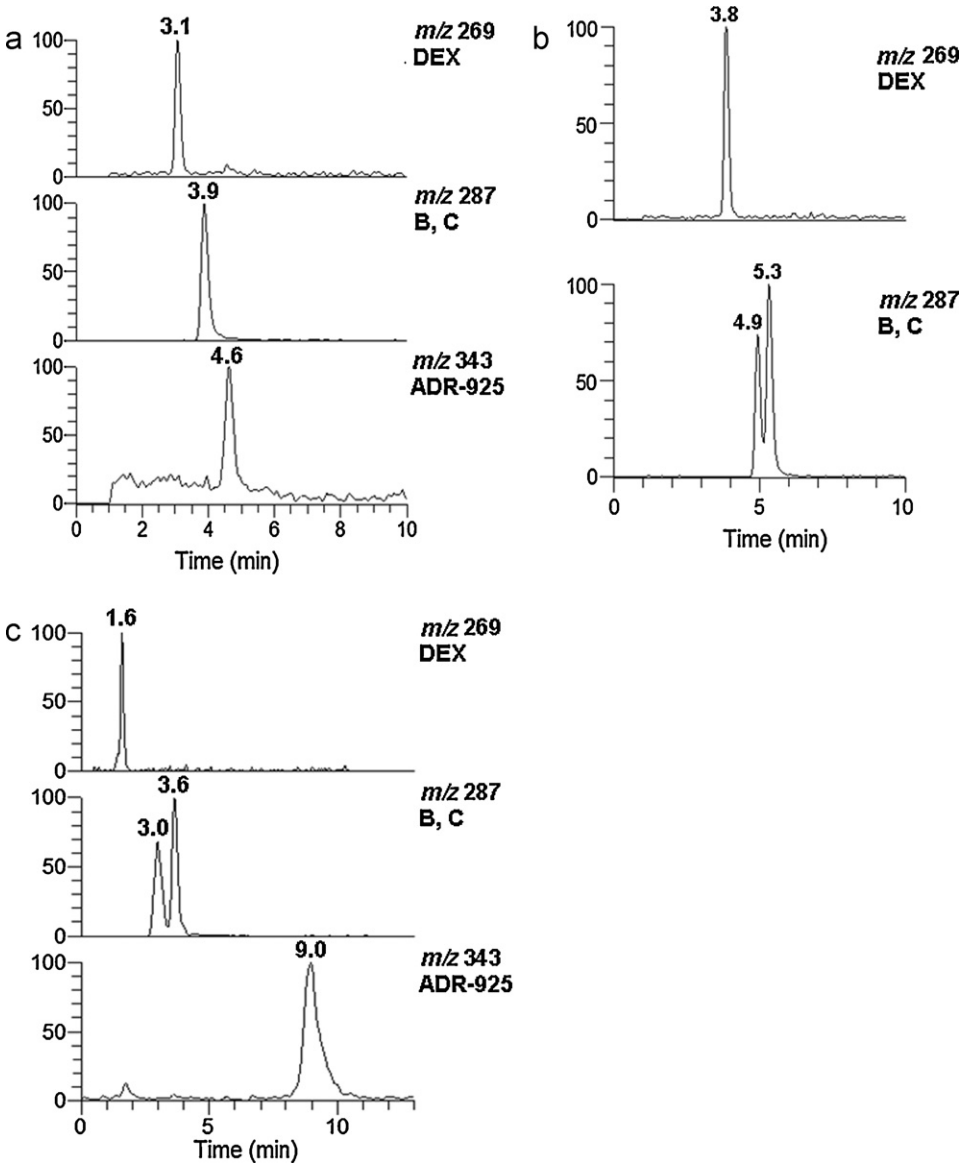

Fig. 6. LC–MS analysis of DEX, intermediates B, C and ADR-925 on Obelisc N and ZIC HILIC columns Analysis on Obelisc N using (a) a mixture of ammonium formate (pH 4.5) and ACN (0.5 mM total ion strength) in 40:60 ratio (v/v), (b) an aqueous solution of formic acid and ACN (0.053 mM of formic acid in total) in 40:60 ratio (v/v) as mobile phases. Analysis on ZIC HILIC using (c) a mixture of ammonium formate (pH not adjusted) and ACN (0.5 mM total ion strength) in 25:75 ratio (v/v) as a mobile phase. The chromatograms are recorded in SIM, using $[M+H]^+$ at 269 for DEX, $[M+H]^+$ at 287 for intermediates B and C and $[M+K]^+$ at 343 *m/z* for ADR-925.

ever, low retention of DEX on the columns might bring higher experimental errors in the measurements, which complicates an exact evaluation. On both zwitterionic stationary phases DEX retention tends to shift from HILIC to RP-like retention at around 30% of the aqueous fraction. Such a retention profile could be classified as aqueous-normal phase behavior [27]. Therefore, the linearity of the plots for DEX on Obelisc N and ZIC HILIC was evaluated only within 10–30% of aqueous fraction. Despite the fact that higher r^2 values favored adsorption the difference in r^2 values are only minor, which means that both mechanisms might be involved to a significant degree [28].

The same results were obtained for ADR-925 and intermediates (B and C), as nearly similar r^2 values for both plots were obtained (Table 3, Fig. S2).

The outcomes of this study also indicated that the zwitterions are likely important to separate these structurally close position isomers B and C, as both bare silica stationary phases in the study failed to resolve this analytical issue. It is important to note that the zwitterionic columns used in this study differ significantly in their surface chemistry – their zwitterions possess different characters and exhibit the opposite orientation of the positive and negative charge on the surface – which likely determines their distinct separation properties with respect to these analytes. It was also found that completely different chromatographic conditions were needed to separate B and C isomers on these columns. While on Obelisc N the presence of buffer ions in the mobile phase hindered B and C resolution, on ZIC HILIC the same mobile phase additive was essential for their separation. This finding might imply that on Obelisc N direct interaction with the sorbent is important for the separation of these isomers. On the other hand, this is not the case of ZIC HILIC, where the shielding of the column surface by solvated buffer ions was essential to achieve B and C separation. Further studies are needed to shed more light on the particular mechanisms involved in the completely different separation properties of these columns.

From the results of this study, we cannot draw a definite conclusion on the particular mechanism predominating in HILIC retention of DEX and its metabolites. Our experiments focused on the temper-

ature effect provided evidence favoring the partition mechanism. In contrast, results of other experiments suggested that adsorption prevailed for DEX on bare silica, and a complex mechanism was involved for other compounds and HILIC columns studied. Similar mixed outcomes have been also presented in the literature [1,24,29]. Hence, HILIC separation is likely determined by multiparametric interactions, where partitioning adsorption, and also ionic interactions for ionic solutes are involved to a significant degree, which is in line with McCalley and others [29,30].

3.5. LC–MS analysis

The MS behavior of either DEX or its metabolites has not yet been studied. Thus, the main MS characteristics of all compounds were first obtained by direct infusion of the standard solutions into the source. Both positive and negative ionization modes were tested with the former leading to a higher detector response. Protonated molecules $[M+H]^+$ at 269 and 287 m/z were found as base ions for DEX and intermediates B and C, respectively. For the ADR-925 $[M+Na]^+$ adduct at 327 m/z was the most abundant ion in the spectrum, accompanied by $[M+H]^+$ at 305 m/z, $[M+K]^+$ at 343 m/z and $[2M+K]^+$ at m/z 647. The MS^2 fragmentation spectra from $[M+H]^+$ and the proposed identities of the fragments are given in Fig. 4. It was found that B and C could be distinguished utilizing MS^3 analysis. While the ions at 155 and 173 m/z gave the same fragments for both B and C (Fig. 4c and d), the ion at 242 m/z showed different fragmentations for each isomer (see Fig. 4e and f).

Based on the results of systematic investigation of the retention behavior of DEX and its metabolites, the selected chromatographic conditions were applied to pilot LC–MS analysis. The peak tailing factor of ADR-925, resolution of B and C and the total analysis time were set as main criteria. The mobile phase compositions selected for pilot LC–MS analysis together with selected chromatographic parameters are summarized in Table 4. It was found that the presence of buffer ions resulted in the decrease of the detector response for DEX. As either ammonium formate or acetate are pivotal to reach reproducible ADR-925 retention on all columns tested, the concentration of the buffer ions was decreased to 0.5 mM (in total), which had been determined as a useful compromise between the reproducible retention, peak symmetry of ADR-925, and signal suppression for DEX. The retention and selectivity were not significantly altered by this modification. Only a very minor change was detected in the resolution between B and C on ZIC HILIC; however, the resolution was still sufficient. It was found that, when using ammonium formate in the mobile phase, ADR-925 was monitored in all cases as m/z at 343, which corresponds to an $[M+K]^+$ adduct, while $[M+H]^+$ was not detected.

LC–MS simultaneous analyses of DEX and ADR-925 on both bare silica columns using mobile phase composition given in Table 4 are shown in Fig. 5a and b. As discussed above, on Obelisc N, two different mobile phase compositions were necessary for the analysis of DEX and all its metabolites (Table 4). Analysis of DEX, intermediates B and C and ADR-925 using ammonium formate is shown in Fig. 6a. Separation of DEX, B and C using buffer free mobile phase is presented in Fig. 6b. Simultaneous analysis of DEX and all its metabolites on ZIC HILIC column is shown in Fig. 6c. The outcomes of this study clearly demonstrated that ZIC HILIC is the only stationary phase in this study that enables simultaneous analysis of DEX and all its metabolites with sufficient separation.

4. Conclusion

The retention behaviors of DEX and all its metabolites (intermediates B and C and ADR-925) on two bare silica phases (Ascentic Express HILIC, Atlantis HILIC) and two stationary phases with zwitterionic properties (Obelisc N and ZIC HILIC) were studied to evaluate their ability for the simultaneous analysis of the moderately lipophilic parent drug and its highly polar metabolites. The effect of mobile phase composition, pH, and column temperature on the compound's retention and separation was systematically studied. It was found that uncharged DEX was poorly retained on all columns tested and it was relatively resistant to a change of the experimental conditions. The permanently charged ADR-925 was sensitive to modifications in the mobile phase, particularly to the mobile phase strength. This study revealed that both bare silica columns were not able to separate isomers B and C from DEX and from one another. On the contrary, both zwitterionic stationary phases provided at least partial separation of this critical pair. This observation emphasizes the importance of the zwitterions embedded in the stationary phases. These might allow for a specific interaction of analytes and the stationary phase which is likely involved in separation of the isomers. Nevertheless, it was found that these zwitterionic phases significantly differed in their separation ability. While Obelisc N was not able to separate B and C and analyze ADR-925 in one run, on ZIC HILIC sufficient separation of all these compounds (DEX, intermediate metabolites B and C and ADR-925) was achieved. A systematic evaluation of all experimental data obtained in this study underlined the complex nature of the retention of these compounds, where partitioning, adsorption and, in the case of the metabolites also ionic interactions are involved. Our results clearly demonstrated that ZIC HILIC was the only stationary phase in the study, which allowed the simultaneous analysis of DEX and all its metabolites (including the isomers B and C) with the separation of all compounds to a baseline. This study showed that HILIC could be a useful solution for simultaneous analysis of parent drugs and their highly hydrophilic metabolites, which can succeed also in the separation of positional isomers. However, the low retention of the parent compound associated with the risk of signal suppression might limit this approach when MS detection is used.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.11.050.

References

[1] M. Liu, E.X. Chen, R. Ji, D. Semin, J. Chromatogr. A 1188 (2008) 255.
[2] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[3] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka, J. Chromatogr. A 1184 (2008) 474.
[4] P. Hemstrom, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[5] D.V. McCalley, J. Chromatogr. A 1171 (2007) 46.
[6] H.P. Nguyen, K.A. Schug, J. Sep. Sci. 31 (2008) 1465.
[7] E. Goucher, A. Kicman, K. Wolff, N. Smith, S. Jickells, J. Sep. Sci. 33 (2009) 955.
[8] A. Thomas, J. Deglon, T. Steimer, P. Mangin, Y. Daali, C. Staub, J. Sep. Sci. 33 (2009) 873.
[9] B. Dejaegher, Y. Vander Heyden, J. Sep. Sci. 33 (2009) 698.
[10] S. Vikingsson, R. Kronstrand, M. Josefsson, J. Chromatogr. A 1187 (2008) 46.
[11] W.Z. Shou, Y.L. Chen, A. Eerkes, Y.Q. Tang, L. Magis, X. Jiang, W. Naidong, Rapid Commun. Mass. Spectrom. 16 (2002) 1613.
[12] R.S. Cvetkovic, L.J. Scott, Drugs 1005 (2005) 65.
[13] T. Simunek, M. Sterba, O. Popelova, M. Adamcova, R. Hrdina, V. Gersl, Pharmacol. Rep. 61 (2009) 154.
[14] B.B. Hasinoff, Drug Metab. Dispos. 21 (1993) 883.
[15] B.B. Hasinoff, J. Pharm. Sci. 83 (1994) 64.
[16] P.E. Schroeder, B.B. Hasinoff, Cancer Chemother. Pharmacol. 50 (2002) 509.
[17] E. Martin, A.V. Thougaard, M. Grauslund, P.B. Jensen, F. Bjorkling, B.B. Hasinoff, J. Tjornelund, M. Sehested, L.H. Jensen, Toxicology 255 (2009) 72.

[18] P.E. Schroeder, B.B. Hasinoff, Drug Metab. Dispos. 33 (2005) 1367.
[19] B.B. Hasinoff, Int. J. Pharm. 107 (1994) 67.
[20] L.A. Cole, J.G. Dorsey, Anal. Chem. 64 (1992) 1317.
[21] J.J.K.L.R. Snyder, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley-Interscience, New York, 1997.
[22] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[23] Z.G. Hao, B.M. Xiao, N.D. Weng, J. Sep. Sci. 31 (2008) 1449.
[24] L.L. Dong, J.X. Huang, Chromatographia 65 (2007) 519.
[25] G. Marrubini, B.E. Mendoza, G. Massolini, J. Sep. Sci. 33 (2010) 803.
[26] J. Nawrocki, J. Chromatogr. A 779 (1997) 29.
[27] J. Pesek, M.T. Matyska, LC GC N. AM. 25 (2007) 480.
[28] T. Zhou, C.A. Lucy, J. Chromatogr. A 1187 (2008) 87.
[29] D.V. McCalley, J. Chromatogr. A 1217 (2010) 3408.
[30] W. Bicker, J. Wu, M. Lammerhofer, W. Lindner, J. Sep. Sci. 31 (2008) 2971.