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# Impact of solvent conditions on separation and detection of basic drugs by micro liquid chromatography–mass spectrometry under overloading conditions

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ABSTRACT

In this study the impact of solvent conditions on the performance of μLC/MS for the analysis of basic drugs was investigated. Our aim was to find experimental conditions that enable high-performance chromatographic separation particularly at overloading conditions paired with a minimal loss of mass spectrometric detection sensitivity. A focus was put on the evaluation of the usability of different kinds of acidic modifiers (acetic acid (HOAc), formic acid (FA), methansulfonic acid ($CH_3SO_3H$), trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA)). The test mixture consisted of eleven compounds (bunitrolol, caffeine, cocaine, codeine, diazepam, doxepin, haloperidol, 3,4-methylendioxyamphetamine, morphine, nicotine, and zolpidem). Best chromatographic performance was obtained with the perfluorinated acids. Particularly, 0.010–0.050% HFBA (v/v) was found to represent a good compromise in terms of chromatographic performance and mass spectrometric detection sensitivity. Compared to HOAc, on average a 50% reduction of the peak widths was observed. The use of HFBA was particularly advantageous for polar compounds such as nicotine; only with such a hydrophobic ion-pairing reagent chromatographic retention of nicotine was observed. Best mass spectrometric performance was obtained with HOAc and FA. Loss of detection sensitivity induced by HFBA, however, was moderate and ranged from 0 to 40%, which clearly demonstrates that improved chromatographic performance is able to compensate to a large extent the negative effect of reduced ionization efficiency on detection sensitivity. Applications of μLC/MS for the qualitative and quantitative analysis of clinical and forensic toxicological samples are presented.



## 1. Introduction

Liquid chromatography (LC) hyphenated to mass spectrometry (MS) represents one of the most powerful methods for the characterization of bioorganic molecules. Electrospray ionization (ESI) is the most widely used interface for LC/MS [1]. LC/MS is considered to represent a sensitive and specific analytical method highly suitable for qualitative and quantitative analysis. In an attempt to decrease the detection limits of LC/MS miniaturized separation techniques are often applied [2,3]. Principally, chromatographic columns with inner diameters in the range 20–500 μm offer a gain of detection sensitivity of 64–40,000 in comparison to 4 mm columns. Thus, either by keeping the sample concentration constant the injected volume can be reduced or by keeping the injected volume constant lower limits of detection in terms of sample concentration can be reached. Miniaturized LC/MS has become the method of choice for the analysis of biopolymers including peptides, proteins and nucleic acids [4–10]. The number of reports on the utilization of capillary LC/MS for the analysis of small bioorganic molecules is limited [11–16]. For this class of molecules the development of efficiently working miniaturized chromatographic systems seems to be particularly challenging. Utmost gain of detection sensitivity can only be reached if effects causing peak distortion (i.e., extra-column band broadening, overloading) are completely eliminated. Whereas the negative effects of extra-column band broadening can be avoided by using properly designed chromatographic systems, overloading effects are much more difficult to control. There are two types of overloading that cause deterioration of column performance: volume overloading and mass, or concentration, overloading. In volume overloading, the sample injected is so large that the eluted peaks are broadened, and the conventional Gaussian peak is replaced by a flat-topped profile. However, the solutes concentrations are low enough to fall within the linear portions of their respective adsorption isotherms. Problems related to volume overloading can often be circumvented by using "on-column focusing" [17–21]. "On-column focusing" occurs when solutes are concentrated onto the top of the chromatographic column by injecting the sample in a solvent of lower eluting strength than that of the mobile phase. Applications of large-volume injection have been reported [21–23]. Such approaches represent "a

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logical way of lowering the concentration limits of detection in trace analysis by chromatography and related methods in all cases where sufficient quantity of liquid sample or extract of solid sample is available" [21]. In mass or concentration overloading, the eluted peaks are broadened and usually show right-angled triangle shapes, even though the sample volume may be small, because at least one of the solute concentrations is high enough to involve nonlinear adsorption. Characteristics of mass overloading of small molecules have been extensively studied and reviewed recently [24]. It is widely recognized that ionizable analytes such as basic drugs have very much lower sample loading capacities compared to low polarity neutral compounds [25–27]. Mass overloading of ionized compounds on standard 4.6 mm i.d. columns can occur if the sample mass exceeds ~0.1 μg. Due to the reduction of the amount of stationary phase, mass overloading is even more difficult to avoid on miniaturized chromatographic columns. Problems related to mass overloading can often be circumvented by proper choice of the mobile phase composition. For basic compounds high pH and high ionic strengths [28–32] were shown to diminish the effect of load on chromatographic efficiency [33]. Another attempt to improve peak shapes for ionizable compounds both in the linear region of the isotherm as well as in the overloaded region involves the addition of strong ion-pairing or chaotropic reagents to the mobile phase [30,34,35]. Due to their negative effects on ionization efficiency, however, such mobile phase additives are often disfavored for LC/MS [36]. Mallet et al. have tested the impact of trifluoroacetic acid (TFA) on the detection of basic and acidic drugs and reported ion suppression effects causing a more than 40% reduction of signal intensity [37]. Kamel et al. reported a 2- to 8-fold reduction of the sensitivities for nucleoside antiviral agents with 0.1% TFA in comparison with 1% formic acid (FA) [38]. Much more knowledge on the impact of ion-pairing agents on mass spectrometric detection sensitivity is available for peptides and proteins [39]. Huber and Premstaller observed a 35- to 160-fold reduction of protein detectability when using 0.1% TFA in comparison to 0.1% FA [40]. Liu et al. reported a three-fold decrease in mass spectrometric sensitivity with 0.1% TFA in comparison with 2% FA [41]. In both cases, however, a significant improvement of separation efficiency was reported, which can facilitate the comprehensive analysis of complex samples. Poor chromatographic performance can lead to ion suppression particularly hampering the detection of low-abundant compounds. Thus, for successful application of μLC/MS assays to biological samples the use of selective chromatographic separation offering best achievable peak capacity at the cost of a moderate loss of mass spectrometric detection sensitivity is often obligatory. Such methods have been developed for peptides and proteins. Corradini et al. [42] as well as Walcher et al. [43] reported for instance that 0.05% TFA as mobile phase additive was more useful for the separation of complex membrane protein samples than FA. For small bioorganic molecules no detailed studies exist which evaluated the impact of mobile phase additives on μLC/MS performance or give recommendations or cautions on the use of ion-pairing additives.

In the present study, we have investigated the impact of mobile phase additives on the separation and detection of basic drugs with μLC/MS. The aim of this work was to find appropriate chromatographic conditions that enable high-performance chromatographic separation paired with satisfying detection sensitivity under volume and mass overloading conditions. Large-volume injection was performed on purpose to reach low limits of detection in terms of sample concentration. Chromatographic separations were performed on a commercially available instrument designed for high-performance μLC of biopolymers. A mixture of eleven compounds commonly observed in analytical toxicology was used as sample set (Table 1 and Fig. 1). The drugs covered a broad range of hydrophobicities as well as basicities. Chromatographic separations were accomplished by using linear gradients of organic modifiers in acidified aqueous solutions. Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH) or acetone were used as organic modifier. Acidic modifiers tested included acetic acid (HOAc) and FA as well as the ion-pairing additives methansulfonic acid ($CH_3SO_3H$), TFA, pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA). Retention times ($t_r$), peak widths at half height ($w_{1/2}$) and peak heights were used as parameters to characterize the performance of μLC/MS at varying experimental conditions. Applications of μLC/MS for the qualitative and quantitative analysis of human plasma and urine samples are presented.

**Table 1**
Properties of pharmaceutical compounds used to evaluate the influence of mobile phase composition on LC/MS performance.

| Compound | $c$ (ng/μl) | Molecular formula | $m/z$ of $[M+H]^+$ | p$k_a$ | log Pow[a] |
|---|---|---|---|---|---|
| Bunitrolol | 0.5 | $C_{14}H_{20}N_2O_2$ | 249.1598 | 9.1 | 2.00 |
| Caffeine | 1.0 | $C_8H_{10}N_4O_2$ | 195.0876 | 0.7 | 0.07 |
| Cocaine | 0.5 | $C_{17}H_{21}NO_4$ | 304.1543 | 9.0 | 2.30 |
| Codeine | 1.0 | $C_{18}H_{21}NO_3$ | 300.1594 | 8.2 | 1.14 |
| Diazepam | 1.0 | $C_{16}H_{13}ClN_2O$ | 285.0789 | 3.4 | 2.82 |
| Doxepin | 1.0 | $C_{19}H_{21}NO$ | 280.1695 | 9.2 | 2.37 |
| Haloperidol | 1.0 | $C_{21}H_{23}ClFNO_2$ | 376.1474 | 8.3 | 3.36 |
| MDA | 1.0 | $C_{10}H_{13}NO_2$ | 180.1019 | 9.9 | 1.60 |
| Morphine | 1.0 | $C_{17}H_{19}NO_3$ | 286.1437 | 8.3 | 0.76 |
| Nicotine | 0.5 | $C_{10}H_{14}N_2$ | 163.1229 | 8.0 | 0.28 |
| Zolpidem | 1.0 | $C_{19}H_{21}N_3O$ | 308.1757 | 6.0 | 2.50 |

[a] log Pow stands for octanol–water partition coefficient. The partition coefficient represents a measure for the hydrophobicity of a compound. Compound-specific values were extracted from the LOGKOW databank.

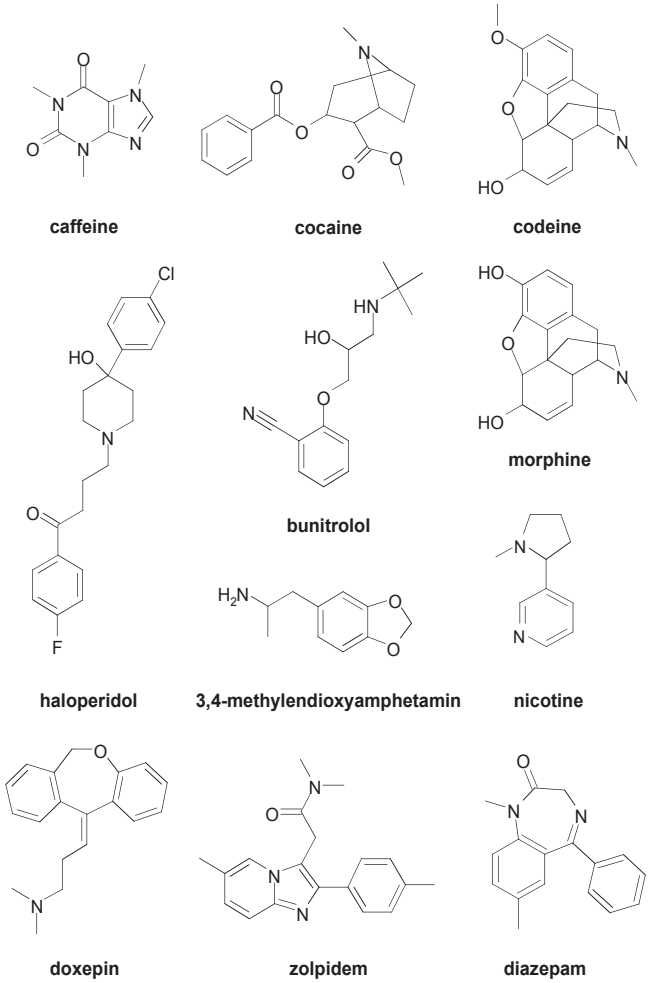


**Fig. 1.** Chemical structures of the test compounds.

## 2. Materials and methods

### 2.1. Chemicals

ACN (gradient grade), water (gradient grade), MeOH (gradient grade), EtOH (gradient grade), acetone (gradient grade), HOAc (puriss p.a.), FA (puriss p.a.), $CH_3SO_3H$ (puriss p.a.), TFA (puriss p.a.), PFPA (puriss p.a.), and HFBA (puriss p.a.) were obtained from Sigma Aldrich (St. Louis, MO, USA).

A mixture of eleven pharmaceutical compounds was used to evaluate the influence of the mobile phase composition on LC/MS performance. Bunitrolol hydrochloride was obtained from Chemicals International (Holte, Denmark). Caffeine, diazepam, doxepin hydrochloride and haloperidol were purchased from Sigma Aldrich. Nicotine was supplied by Merck Schuchardt (Hohenbrunn, Germany). Zolpidem was purchased from Ratiopharm (Vienna, Austria). Cocaine, codeine, 3,4-methylendioxyamphetamine (MDA), and morphine were obtained from Cerilliant (Round Rock, TX, USA). Properties of the test compounds are summarized in Table 1. Chemical structures are shown in Fig. 1. For preparation of the test mixture, methanolic stock solutions of the drugs (1.0 μg/μl) were mixed and subsequently diluted with aqueous solutions of acidic modifiers (eluent A).

A urine sample containing benzoylecgonine (500 pg/μl), chlorprothixene (600 pg/μl), ketamine (1200 pg/μl), nordazepam (550 pg/μl), nordoxepine (1000 pg/μl), methadone (500 pg/μl), and zolpidem (700 pg/μl) was supplied by Arvecon (Walldorf, Germany) as part of a proficiency test on the detection of drugs in urine.

### 2.2. Sample preparation by solid-phase extraction (SPE)

SPE was accomplished according to a previously described procedure [14]. 4.0 ml of urine were diluted with 4 ml of distilled water and 4 ml of phosphate buffer (pH 6.0) and vortexed. After centrifugation (5 min, 4500 turns/min) the supernatant was used for SPE. The SPE column (SPE-ED Scan ABN, Applied Separations, Allentown, PA) was conditioned with 2 ml MeOH and 2 ml 0.1 M phosphate buffer (pH 6.0). The sample was passed through the column with 1.0–1.5 ml/min. Next, the column was washed with 3 ml distilled water, 1 ml 1.0 M acetic acid and an aqueous MeOH solution (5%, v/v), centrifuged for 5 min (4500 turns/min) and dried with a nitrogen stream. Elution was performed with 2.0 ml of a mixture of dichloromethane (78%, v/v, puriss p.a., Sigma–Aldrich), isopropanol (20%, v/v, gradient grade, Sigma–Aldrich) and ammonium hydroxide (2%, v/v, puriss p.a., Sigma–Aldrich). The eluate was evaporated to dryness at 60 °C under a nitrogen stream and reconstituted in 4.0 ml 0.05% HFBA solution containing 5% acetone.

### 2.3. Micro liquid chromatography (μLC)

The μLC system consisted of a K-1001 pump from Knauer (Berlin, Germany) and a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 2.0 μl (200 μm i.d. columns) or a 10.0 μl (500 μm i.d. columns) injection loop, respectively. Chromatographic columns with a length of 200 mm were prepared as described elsewhere [14,44]. A retaining frit was made at one end of either a polyimide coated fused-silica capillary tubing (200 μm i.d., Polymicro Technologies, Phoenix, AZ) or a stainless steel tubing (500 μm i.d., Vici, Schenkon, Switzerland) by sintering a thin slug of 5.0 μm silica particles (Spherisorb S5W, Phase Sep, Queensferry, Clwyd, UK) wetted with a small droplet of sodium silicate solution (Sigma–Aldrich). Eurospher 100-5 C18 particles (Knauer) were used as stationary phase. The particles were suspended in methyl t-butyl ether (Scharlau, Barcelona, Spain). The column was packed with MeOH at a constant pressure of 40 MPa. No frit was made at the inlet end of the column. Aqueous solutions of acidic modifiers were used as eluent A; organic solvents containing the same amount of the acidic modifier served as eluent B. Any adjustment and measurement of the eluent pH was performed on the neat aqueous solution (eluent A). Chromatographic separations were accomplished with linear gradients of eluent B in eluent A. The column temperature was held at 50 °C with a column oven (Thermotechnic Products GmbH, Langenzersdorf, Austria). The flow rate was set to 2.0 μl/min (200 μm i.d. columns) or 10.0 μl/min (500 μm i.d. columns). The column outlet was connected online to the ESI source.

### 2.4. Electrospray ionization mass spectrometry

ESI-MS was performed in the positive ion mode. For almost all experiments a QSTAR XL mass spectrometer (AB Sciex, Foster City, CA) equipped with a modified TurboIonSpray source was used. Modifications included the replacement of the Peek tubing transfer line and of the stainless steel sprayer capillary by fused silica capillaries (transfer line: 375 μm o.d., 20 μm i.d., sprayer capillary: 90 μm o.d., 20 μm i.d., Polymicro Technologies) [14,45]. The spray voltage was set to 4.5 kV. Gas flows of 3 arbitrary units for the nebulizer gas and 20 arbitrary units for the turbo gas were employed. The temperature of the turbo gas was adjusted to 200 °C.

The mass spectrometer was operated either in MS mode or in MS/MS mode under data-dependent acquisition control. A duty cycle in the data-dependent acquisition mode included a single MS scan followed by MS/MS scans on the 5 most abundant precursors (processed in reverse order of abundance) not subject to pre-determined or dynamic exclusion. The intensity threshold for triggering MS/MS experiments was set to 50 counts. Isolation of precursor ions was accomplished with Q1 set to unit resolution. A collision gas flow ($N_2$) of 5 arbitrary units was used. For each precursor, MS/MS spectra were acquired using two different collision energies for fragmentation (25 and 35 eV). Accumulation times were set to 1.0 s for MS scans and to 0.5 s for MS/MS scans. All mass spectra were acquired from 50 to 700 and recorded on a personal computer with the Analyst QS software (1.0, service pack 8, AB Sciex).

For evaluation of the usability of different organic modifiers for μLC/MS of pharmaceutical compounds a QTrap 3200 mass spectrometer (AB Sciex) equipped with a Turbo V ion source was used. The spray voltage was set to 5.5 kV. A gas flow of 20 arbitrary units for the nebulizer gas was employed. Mass spectra were obtained from scanning Q1 in the $m/z$ range 100–700 within 0.5 s. Q1 was set to unit resolution. Ion chromatograms and mass spectra were recorded on a personal computer with the Analyst 1.5 software (AB Sciex).

### 2.5. Library search

Tandem mass spectra were extracted from raw data using the following settings: mass tolerance for combining MS/MS spectra of 0.10, centroid height percentage of 50%, and centroid merge distance of 0.050. The extracted spectra were searched against the positive ion mode collection of the "MSforID" library, which consisted of 8252 spectra corresponding to 836 compounds. A detailed description of the mass spectral library is provided on www.msforid.com. The principles of the library search approach have been described elsewhere [46–48]. The following parameters were applied to the search: mass tolerance of ±0.010, cut-off factor of 0.05. The following criteria were used for validation of search results: relative average match probability (ramp) of >50, precursor ion mass tolerance of ±0.010.

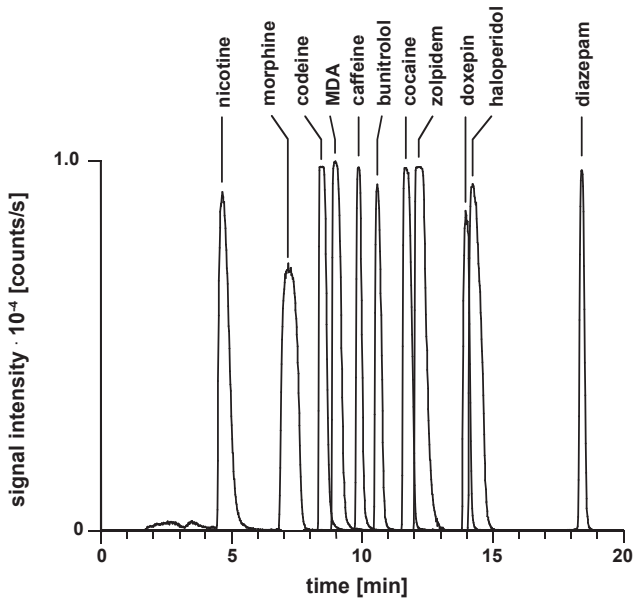


**Fig. 2.** RP-LC/ESI-MS analysis of drugs using HOAc as solvent modifier. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.50% HOAc in water, (B) 0.50% HOAc in ACN; linear gradient, 5–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 2.0 ng each, bunitrolol, cocaine, nicotine, 1.0 ng each.

## 3. Results and discussion

### 3.1. μLC/MS of pharmaceutical compounds using acetic acid as mobile phase additive

One of the most commonly applied mobile phase additives for LC/MS of basic drugs is HOAc. The content is usually in the 0.1–1% range [49]. Due to its importance, HOAc-containing mobile phases were selected as starting point of our study, to which changes introduced by varying chromatographic conditions were referenced. Chromatographic separations were accomplished on a commercially available instrument designed for high-performance μLC of biopolymers. The injection volume was 2.0 μl. The test mixture consisted of eleven compounds commonly observed in analytical toxicology exhibiting different hydrophobicities as well as basicities (Table 1). To minimize the negative effects of volume overloading on chromatographic performance, no organic solvent was added to the sample solution. Overall, 19 ng of sample (1.0–2.0 ng of each compound) were analyzed. The sample amount exceeded the maximum load of <0.25–10 ng for a 200 μm i.d. column. Thus, the occurrence of overloading effects such as peak broadening and tailing was expected. Elution of the drugs was accomplished by using a gradient of ACN in 0.50% (v/v) aqueous HOAc. In Fig. 2 representative selected ion current chromatograms obtained from μLC/MS analysis of the test mixture are shown. A closer look at peak shapes suggested that the HOAc-containing mobile phase might not represent the best choice for μLC particularly under overloading conditions. Peak broadening and tailing caused by mass overloading were observed for almost all compounds. Furthermore, codeine, cocaine MDA, and zolpidem showed flat-topped profiles indicating volume overloading. The $w_{1/2}$-values ranged between 12 and 30 s. The lowest peak widths were obtained for diazepam and caffeine. Both compounds are very weak bases (Table 1). They were almost uncharged under the experimental conditions applied, and therefore their chromatographic performance might have been less affected by overloading than that of ionized species. Asymmetry factors ($A_s$), which were calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peaks to characterize the peak shape, were in the range 1.3–3.1. Nicotine, which was the most polar compound in the test mixture, was hardly retained on the chromatographic column. Some of the analyte was carried through with the injection plug.

In an attempt to improve the performance of the μLC/MS system mobile phases containing different amounts of HOAc were tested. The HOAc content was varied in the range 0.1–1.0%. Retention times and peak widths were used to characterize the chromatographic performance. Observed changes were moderate; $t_r$- and $w_{1/2}$-values remained almost constant. Peak heights were used to characterize the detection sensitivity. Hardly any effect induced by variation of the HOAc concentration was observed. Only for bunitrolol and morphine a slight decrease of peak heights was observed at HOAc contents beyond 0.50% (v/v).

### 3.2. Selection of an alternative acidic modifier for μLC/MS of pharmaceutical compounds

In another attempt to improve the performance of the μLC/MS system different kinds of acidic modifiers, including FA, $CH_3SO_3H$, TFA, PFPA, and HFBA, were tested. The selected acids are volatile, show chaotropic effects and are able to form ion pairs. The chaotropic action and the tendency of ion-pair formation increase in the order FA < $CH_3SO_3H$ < TFA < PFPA < HFBA [34]. Since chaotropic effects and ion-pair formation can diminish the negative effects of sample load on chromatographic performance, the selected acids find application in RP-LC of ionizable compounds. Due to adverse effects of ion-pair formation on ionization efficiency, ion-pair forming agents are usually avoided for LC/MS of pharmaceutical compounds. The situation might be different for μLC/MS under overloading conditions. Chaotropic or ion-pair forming agents could become applicable if the loss of ionization efficiency would be compensated by increasing the concentration under the peak as a consequence of improved chromatographic performance.

The amounts of the different acids added to the mobile phase were adjusted to reach a pH equal to that of a solvent containing 0.50% HOAc (v/v). In all cases elution was accomplished by changing the ACN content in the mobile phase linearly from 5 to 95% within 15 min. To evaluate the performance of the μLC/MS system with varying acidic modifiers, the following parameters were assessed: $t_r$, $w_{1/2}$, and peak heights. The values measured with 0.50% HOAc (v/v) served as reference for normalization. Performance parameters were extracted from three LC/MS runs. Box plots were used to assess the impact of the kind of acidic modifier on the chromatographic performance parameters (Fig. 3). Box plots are commonly used in statistics to compare groups of numerical data. A boxplot summarizes the following statistical measures: the smallest observation, lower quartile, median, upper quartile, and the largest observation. Furthermore, putative outliers are indicated.

In Fig. 3a the impact of the kind of acidic modifier on retention is shown. Retention times increased in the order HOAc < FA < $CH_3SO_3H \approx$ TFA < PFPA ≈ HFBA. More hydrophobic anions were found to cause a more pronounced increase of retention. Similar effects of the identity of mobile-phase anions on retention were observed for the separation of amines, β-blockers as well as peptides [34,50–52]. With the exception of caffeine the elution order of the pharmaceutical compounds remained constant irrespectively of the kind of acidic modifier used. Caffeine is a very weak base and is uncharged at pH 2.8. Accordingly, chaotropic effects as well as ion-pair formation might have had a lower impact on caffeine retention than on the retention of charged compounds. As exemplified for caffeine relative to the other compounds, depending on the differences in hydrophobicity and basicity of chromatographed compounds, a proper selection of

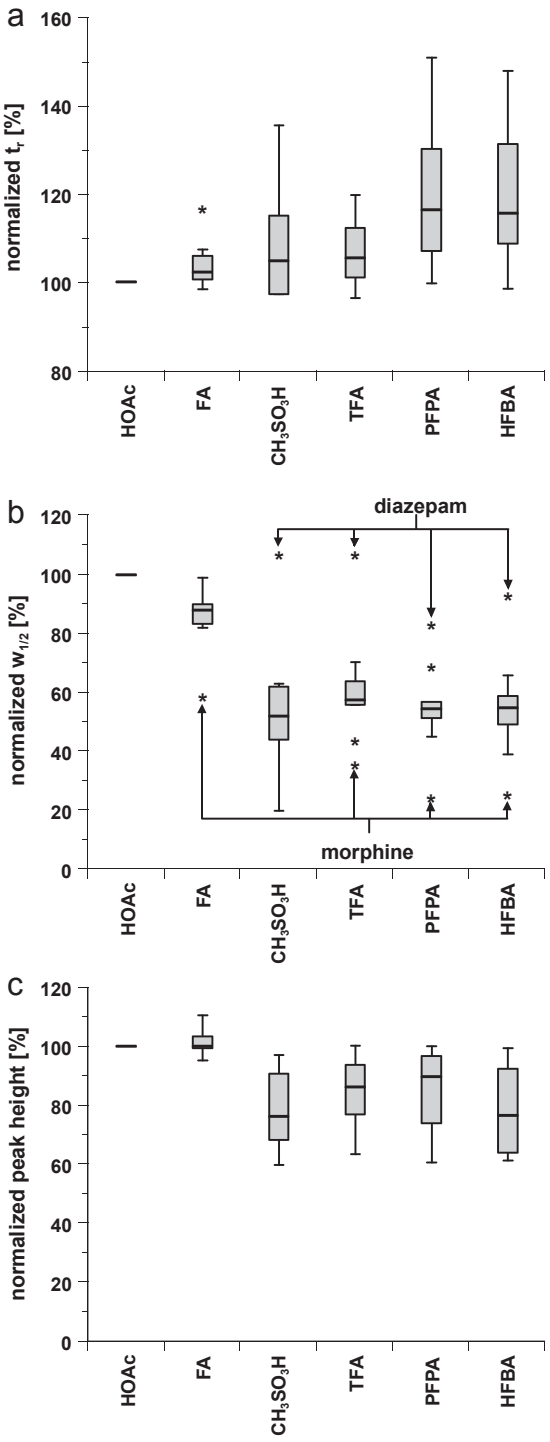


**Fig. 3.** RP-LC/ESI-MS analysis of drugs using different acids as solvent modifiers. The impact of the kind of solvent modifier on (a) $t_r$, (b) $w_{1/2}$ and (c) the peak height are shown. In all cases the pH of solvent A was adjusted to 2.8 with an appropriate amount of the corresponding acid. Results of nicotine were not included. Putative outliers are plotted as stars. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.50% HOAc, 0.035% FA, 0.0080% $CH_3SO_3H$, 0.010% TFA, 0.011% PFPA, and 0.016% HFBA, respectively, in water, (B) 0.50% HOAc, 0.035% FA, 0.0080% $CH_3SO_3H$, 0.010% TFA, 0.011% PFPA, and 0.016% HFBA, respectively, in ACN; linear gradient, 5–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 2.0 ng each, bunitrolol, cocaine, nicotine, 1.0 ng each.

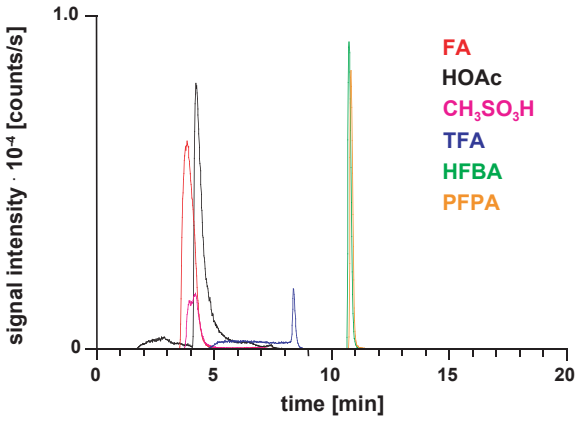


**Fig. 4.** Comparison of elution profiles obtained for nicotine using different acids as solvent modifiers. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.50% HOAc, 0.035% FA, 0.0080% $CH_3SO_3H$, 0.010% TFA, 0.011% PFPA, and 0.017% HFBA, respectively, in water, (B) 0.50% HOAc, 0.035% FA, 0.0080% $CH_3SO_3H$, 0.010% TFA, 0.011% PFPA, and 0.017% HFBA, respectively, in ACN; linear gradient, 5–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 2.0 ng each, bunitrolol, cocaine, nicotine, 1.0 ng each.

the acidic modifier might help to improve chromatographic selectivity.

The dependence of normalized peak widths on the kind of acidic modifier added is shown in Fig. 3b. Chromatographic performance increased in the order HOAc < FA ≪ $CH_3SO_3H$ ≈ TFA ≈ PFPA ≈ HFBA. The average $w_{1/2}$-value changed from 20 s for HOAc to 17 s for FA and to 10–12 s for the remaining acids. In our study TFA, PFPA and HFBA gave almost similar separation efficiencies. This observation is in full accordance with results reported by Shibue et al. on the impact of the kind of acidic modifier on $w_{1/2}$ of peptides [53]. This group reported that the three perfluorinated acids TFA, PFPA and HFBA showed similar magnitudes of peak width values for model peptides over the concentration range 10–30 mM. We observed the overall lowest improvement of separation efficiency by changing from HOAc to an ion-pair forming acid for diazepam. Diazepam is a very weak base and, therefore, almost uncharged under the experimental conditions applied. Furthermore, diazepam is the most hydrophobic compound in the test mixture. For both reasons ion-pair formation showed hardly any effect on diazepam elution [32]. The most pronounced effects were observed for the very early eluting compounds nicotine and morphine. Selected ion chromatograms obtained for nicotine using different kinds of acidic modifiers are depicted in Fig. 4. For FA, HOAc, $CH_3SO_3H$ and TFA, nicotine was hardly retained on the chromatographic column. Furthermore, peak distortion and/or peak splitting were observed. These effects are known to occur for early eluting compounds under volume overloading conditions [20]. With PFPA and HFBA nicotine was retained on the chromatographic column and eluted with almost normal peak shape, which clearly suggests that these hydrophobic ion-pairing reagents can increase the performance of on-column focusing particularly for polar compounds.

In Fig. 3c the impact of the kind of acidic modifier on normalized peak heights is shown. Best mass spectrometric performance was obtained with HOAc and FA. With both acids almost identical peak heights were observed. All other acids induced some loss in peak height. The relative decrease ranged between 0 and 40%. The average reduction was below 20%. The loss of detection sensitivity induced by ion-pair forming agents was moderate in comparison to values reported in the literature [37,38]. Exchange of TFA for FA for instance led to a 2- to 8-fold reduction of sensitivities for nucleoside antiviral agents. Obviously, due to the use of large-volume injection the negative effects of ion-pair forma-

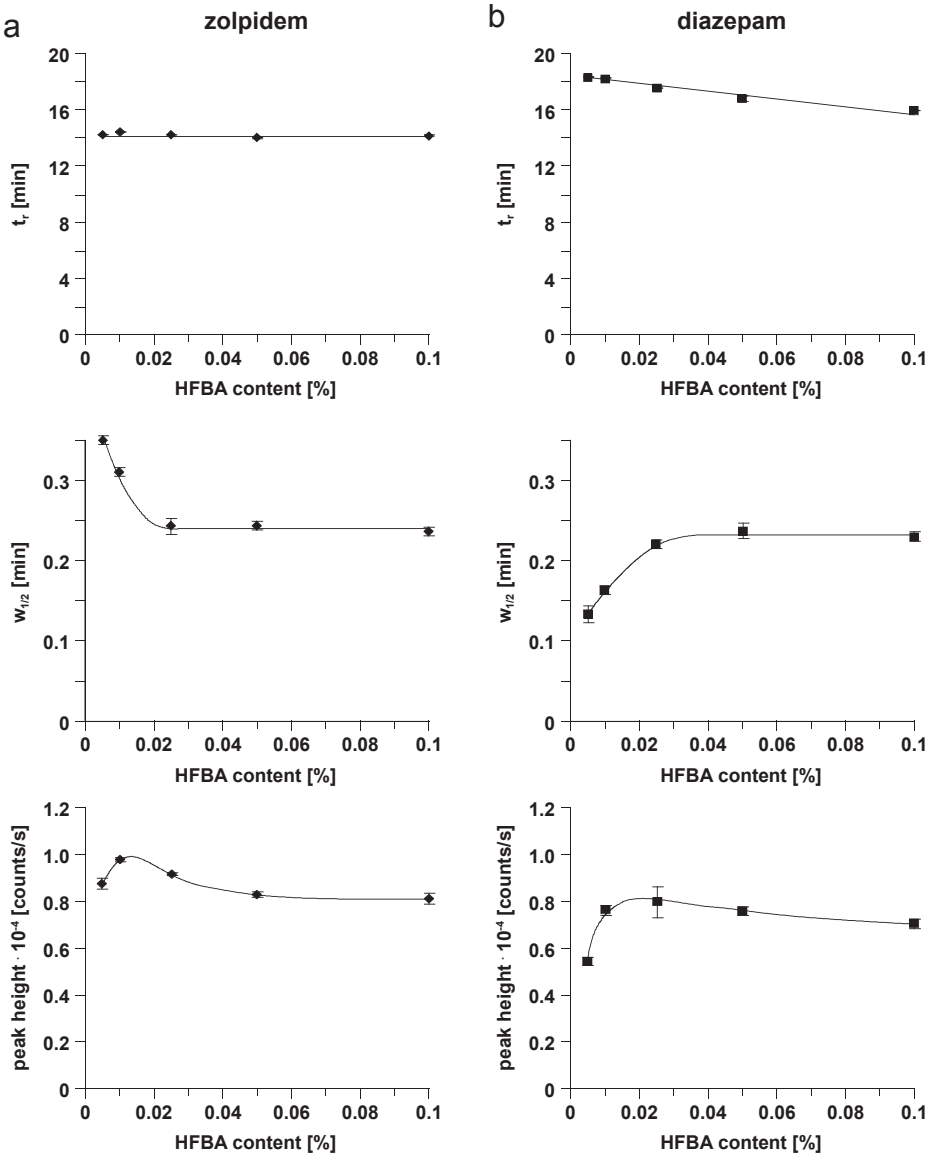


**Fig. 5.** Impact of HFBA content in the mobile phase on the RP-LC/ESI-MS analysis of (a) zolpidem, (b) diazepam. Observed changes of $t_r$, $w_{1/2}$ and the peak height with increasing amount of added HOAc are depicted. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.0010–0.10% HFBA in water, (B) 0.0010–0.10% HFBA in ACN; linear gradient, 10–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 2.0 ng each, bunitrolol, cocaine, nicotine, 1.0 ng each.

tion on ionization efficiency were to a large extent compensated by an improvement of chromatographic performance giving rise to increased concentrations under the chromatographic peaks. Furthermore, the observed increase in $t_r$ that resulted in elution in mobile phases containing a higher content of organic solvent could represent another factor that might be responsible for some compensation of the negative effects of ion-pair formation on ionization efficiency when using TFA, PFPA or HFBA for LC/MS.

### 3.3. μLC/MS of pharmaceutical compounds using HFBA as mobile phase additive

HFBA represents an appropriate acidic modifier for μLC/MS to preserve chromatographic efficiency and detection sensitivity under overloading conditions. In a series of experiments the impact of the HFBA content on μLC/MS performance was studied. The HFBA content in the mobile phase was varied between 0.0050% and 0.10% (v/v). To evaluate the performance of the μLC/MS system at varying HFBA contents, $t_r$-, $w_{1/2}$-, and peak height values were measured. At each HFBA concentration three LC/MS runs were accomplished. Performance parameters were extracted from selected ion chromatograms. Average values were plotted vs. the HFBA content in the mobile phase (Fig. 5).

For all test compounds but diazepam, nicotine and morphine, $t_r$ remained almost constant over the investigated concentration range. For diazepam a slight decrease (Fig. 5b), for nicotine and morphine a slight increase of retention with increasing HFBA content was observed. A similar effect of the change of the HFBA concentration (1–60 mM) on retention was recently observed by Shibue et al. [51,53]. This group reported an increase of peptide retention time at low concentrations of ion-pairing reagent (ca. 2–10 mM), followed by a general levelling off of the profiles at higher concentrations, presumably due to saturation of the charged groups at high reagent concentrations. It is important to note, however, that a noticeable effect was only observed for multiply charged peptides. The retention of singly charged peptides, which are chemically more similar to our sample set than multiply charged peptides, was

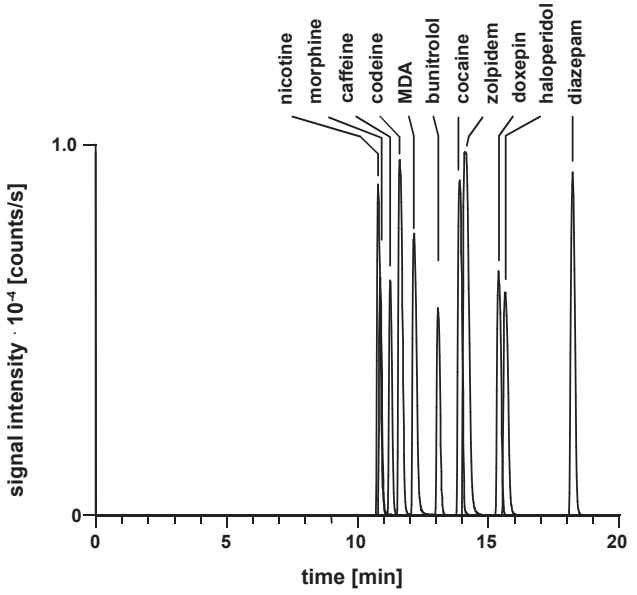


**Fig. 6.** RP-LC/ESI-MS analysis of drugs using HFBA as solvent modifier. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.017% HFBA in water, (B) 0.017% HFBA in ACN; linear gradient, 5–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 2.0 ng each, bunitrolol, cocaine, nicotine, 1.0 ng each.

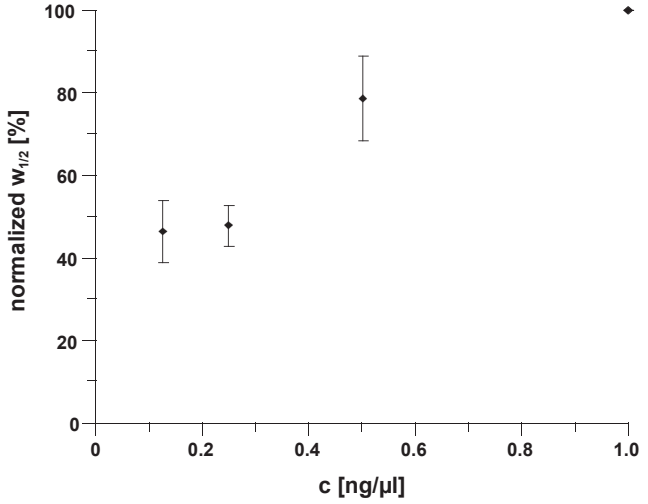


**Fig. 7.** Impact of sample concentration on normalized $w_{1/2}$. For each sample concentration, the average $w_{1/2} \pm$ standard deviation is depicted. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.050% HFBA in water, (B) 0.050% HFBA in ACN; linear gradient, 10–95% B in 15 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, bunitrolol, caffeine, cocaine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, nicotine, zolpidem, 0.125–1.0 ng each.

hardly affected; the observed increase of retention was less than 5%.

Within the range 0.025–0.10% (v/v) peak widths remained constant for all test compounds. Similar results were obtained for singly charged peptides by Shibue et al. [53]. This group reported that $w_{1/2}$-values remained almost constant for HFBA concentrations ranging from 10 to 30 mM (ca. 0.1–0.4%, v/v). At lower HFBA concentrations for several compounds (cocaine, codeine, MDA, nicotine, and zolpidem) we observed an increase of $w_{1/2}$ (Fig. 5a). Only for diazepam, the reduction of the HFBA content led to an improvement of the separation efficiency (Fig. 5b). So with respect to chromatographic performance, the use of >0.025% HFBA (v/v) seems to be convenient.

To evaluate the mass spectrometric performance, the impact of HFBA concentration on peak heights was studied (Fig. 5). For all test compounds, best performance was achieved at 0.010–0.050% HFBA (v/v). In this concentration range the HFBA content is high enough to provide convenient chromatographic performance but low enough to evade severe decrease of mass spectrometric detection sensitivity. In Fig. 6 representative selected ion chromatograms obtained from the analysis of the test mixture at 0.017% HFBA (v/v) are shown. The test compounds eluted from the chromatographic column between 10.7 and 15.4 min. The $w_{1/2}$-values ranged from 7 to 14 s. $A_s$-values of 1.4–2.7 indicated peak tailing. The most plausible explanation for this observation was mass overloading. This hypothesis was proven by studying the impact of sample load on chromatographic performance, and indeed a ten times reduction of the sample load resulted in an average reduction of 50% for the peak widths (Fig. 7).

### 3.4. Examination of the usability of different organic modifiers for μLC/MS of pharmaceutical compounds

In RP-LC, ACN is the most commonly applied organic modifier. Because of recent shortage of ACN, however, the search for solvents that are able to replace ACN has gained a lot of interest [54–57]. For LC(/MS) of biopolymers, the use of acetone or EtOH was proposed. For the analysis of small molecules, ACN has often been substituted by MeOH.

In a set of experiments the usefulness of different organic modifiers for μLC/MS of pharmaceutical compounds was evaluated. Tested mobile phase additives included ACN, MeOH, EtOH, and acetone. The HFBA content in the mobile phase was 0.050% (v/v). The elution strength of the organic modifiers increased in the order MeOH < acetone < ACN < EtOH. Gradients were adjusted to keep retention times of analytes almost constant. For all compounds except caffeine, retention time variations were smaller than 10%. The retention time shifts observed for caffeine changed its elution order relative to morphine and nicotine. Obviously, for these three compounds proper choice of the organic modifier can help to improve chromatographic selectivity. The relative retention of caffeine increased in the order acetone < ACN < EtOH < MeOH. With acetone and ACN caffeine was eluted first; with EtOH and MeOH caffeine was eluted last.

To evaluate the performance of μLC/MS, $w_{1/2}$-values and peak heights were measured. Performance parameters were extracted from selected ion chromatograms. For each mobile phase, the results obtained from three consecutive LC/MS runs were averaged and normalized using ACN as reference (Fig. 8). Evaluation of the impact of the kind of organic modifier on separation efficiency revealed that for the early eluting compounds (bunitrolol, caffeine, codeine, MDA, morphine, and nicotine) ACN offered best performance, but for the late eluting compounds (cocaine, diazepam, doxepin, haloperidol, and zolpidem) EtOH and acetone yielded equal or better efficiencies (Fig. 8a). MeOH could not compete at all with ACN regarding the achievable peak widths. This observation is in full accordance with a previous report, where for the chromatographic separation of relatively high-p$K_a$ bases on diverse chromatographic columns ACN was found to yield better performance than MeOH [58]. Concerning peak heights, we obtained overall best performance with MeOH (Fig. 8b). ACN could surpass MeOH only for codeine and MDA; ACN and MeOH showed almost equal performance for doxepine, haloperidol, and nicotine. This observation is in full agreement with results reported for LC/MS of pesticides and drugs, where MeOH was found to give much better sensitivity than ACN [36,59]. Most probably, the lower eluotropic strength of methanol, which causes compounds to elute at

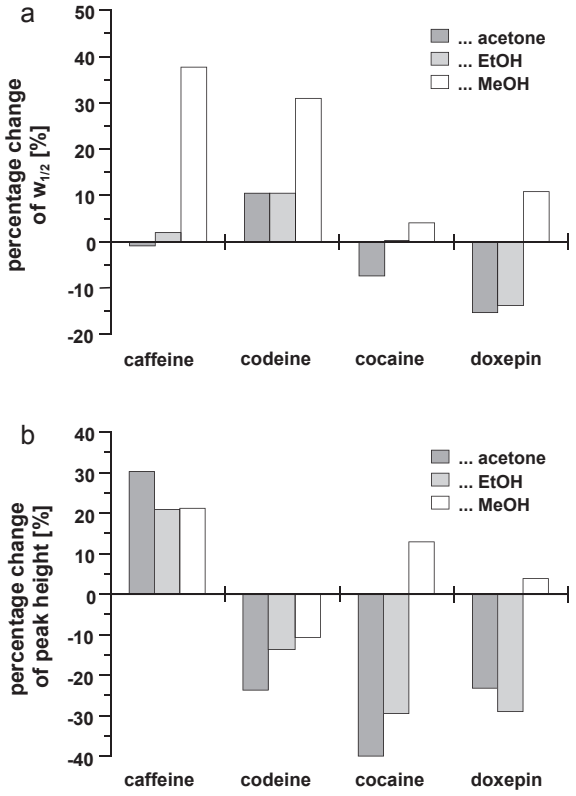


**Fig. 8.** RP-LC/ESI-MS analysis of drugs using different organic modifiers for gradient formation. The impact of the kind of solvent modifier on (a) $w_{1/2}$ and (b) the peak heights of representative compounds are shown. In all cases changes relative to ACN are depicted. Column, Si-C18, 5 μm, 200 mm × 0.50 mm i.d. with silica frit; mobile phase, (A) 0.050% HFBA in water, (B) 0.050% HFBA in ACN, EtOH, MeOH, and acetone, respectively; linear gradient, 10–85% B in 15 min for ACN, 5–75% B in 15 min for EtOH, 18–100% B in 15 min for MeOH, and 10–90% B in 15 min for acetone, respectively; flow rate, 10.0 μl/min; temperature, 50 °C; injection volume, 10.0 μl; sample, caffeine, codeine, diazepam, doxepin, haloperidol, MDA, morphine, zolpidem, 10.0 ng each, bunitrolol, cocaine, nicotine, 5.0 ng each.

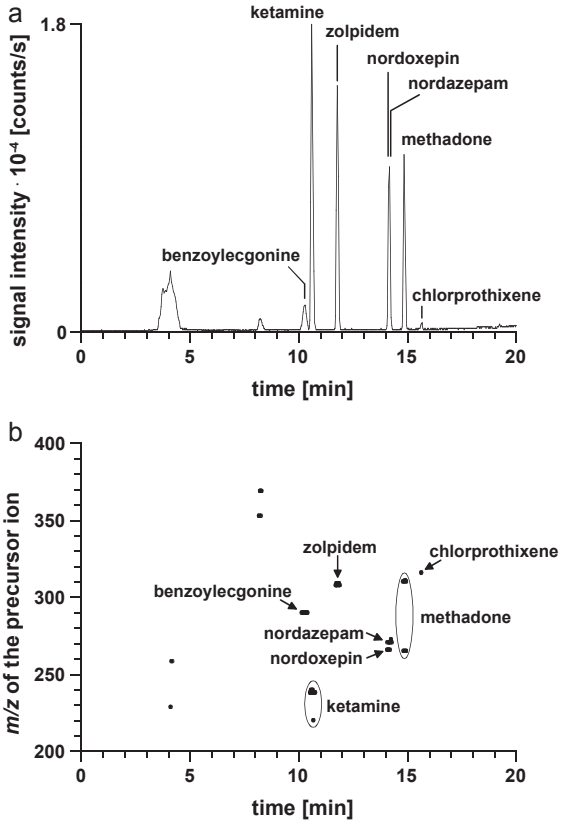


**Fig. 9.** Screening of a urine sample with μLC/MS/MS under data-dependent acquisition control and consecutive library search. (a) The chromatogram as well as (b) a plot of *m/z* of the precursor ion vs. time are shown. Column, Si-C18, 5 μm, 200 mm × 0.20 mm i.d. with silica frit; mobile phase, (A) 0.050% HFBA in water, (B) 0.050% HFBA in acetone; linear gradient, 5–95% B in 20 min; flow rate, 2.0 μl/min; temperature, 50 °C; injection volume, 2.0 μl; sample, extracted urine sample.

a higher percentage of organic solvent, was responsible for the gain in sensitivity observed with MeOH. With the exception of caffeine, diazepam, haloperidol and morphine, acetone and EtOH could not reach the detection sensitivity of ACN or MeOH. Substituting ACN by EtOH or acetone induced an average reduction of 25% for the peak heights.

### 3.5. *Identification of pharmaceutical compounds in urine via μLC/MS/MS under data-dependent acquisition control and library search*

Untargeted analysis of pharmaceutical compounds plays an important role in clinical, forensic as well as doping control laboratories. Accordingly, there has been a great deal of interest in the development of screening techniques, including LC/MS approaches [60–65]. All existing LC/MS assays share a common working principle. Firstly, LC is used to resolve highly complex biological samples prior to mass spectrometric characterization. Secondly, software-assisted interpretation of mass spectrometric data, including high-resolution molecular mass, isotope signature as well as characteristic fragment ions, is used to unequivocal identify drugs and toxic compounds. Due to the recent invention of transferable tandem mass spectrometric libraries [46–48], the combination of LC/MS/MS under data-dependent acquisition control with automated library search seems to be particularly useful. Data-dependent acquisition control is a product ion scan mode providing unattended, automated switching between MS and MS/MS and then returning to MS using data dependent criteria. MS/MS scanning is usually triggered when at least one ion exceeds a preset threshold in MS mode. This method obviates the need to identify any target precursor ion *a priori*, which is particularly valuable in the analysis of samples that may contain a large variety of analytes of interest.

The developed μLC/MS approach, which had been shown to represent a convenient method for separation and detection of pharmaceutical compounds exhibiting a broad range of hydrophobicities and basicities, was converted into a screening tool by implementing MS/MS acquisition under data-dependent acquisition control. After each survey scan, the 5 most abundant precursor ions were processed in reverse order of abundance. The intensity threshold for switching was set to 50 counts. Tandem mass spectra were acquired using two different collision energies (25 and 35 eV) for fragmentation. In a proof of principle experiment, a sample was analyzed that was part of a proficiency test on the detection of drugs in urine (Fig. 9). The sample contained seven drugs commonly observed in forensic toxicological analysis (benzoylecgonine, chlorprothixene, ketamine, nordazepam, nordoxepine, methadone, and zolpidem) at concentrations 0.5–1.2 ng/μl. Chromatographic separation was accomplished using a gradient of acetone in aqueous 0.050% HFBA solution (v/v). As for the analysis of complex samples separation efficiency is of more importance than detection sensitivity, acetone was selected as mobile phase additive. The reconstructed ion chromatogram is shown in Fig. 9a. Precursor ions that triggered MS/MS scans are summarized in Fig. 9b. For methadone and ketamine, the quasi-molecular ion as well as a fragment ion produced by unintended decomposition within the

transfer region of the mass spectrometer served as precursor ions. The measured tandem mass spectra enabled the unequivocal identification of all compounds added to the urine sample as part of proficiency testing via search within the "MSforID"-library. Benzoylecgonine, chlorprothixene, ketamine, nordazepam, methadone, and zolpidem were directly identified. Identities were proven by ramp-values >80 as well as agreement of the precursor ion masses. Nordazepam coeluted with another compound that was identified as nordoxepin. Nordoxepin is a metabolite of doxepine. The "MSforID"-library did not contain any reference spectrum for this compound. In cases, however, where the query compound is not included in the library, database systems can be used to find entries which possess structures or structural features that are similar to that of the query compound. For instance, library search with a tandem mass spectrum of a metabolite often yields a high score for the corresponding precursor drug [44]. Accordingly, the presence of nordoxepin within the sample was approved by a match to doxepin (ramp = 19.7) as well as agreement of the precursor ion mass to the mass of nordoxepin.

### 3.6. Quantitative analysis of pharmaceutical compounds in human plasma via μLC/MS/MS

Quantification is an integral part of clinical and forensic toxicology. Recently, we have shown that μLC/MS/MS can successfully be applied for the quantitative analysis of drugs used for adjuvant breast cancer therapy [66]. Chromatographic separation of tamoxifen, anastrozole and letrozole was accomplished on a 500 μm i.d. column using a gradient of acetone in aqueous 0.050% HFBA solution (v/v). The assay was fully validated for plasma samples and successfully applied to a clinical study, where samples of 310 breast cancer patients were analyzed.

## 4. Conclusion

LC/MS assays are commonly applied tools for the quantitative and qualitative analysis of basic pharmaceuticals. RP-LC represents an integral part of the vast majority of these LC/MS methods. To reach utmost separation efficiency paired with adequate detection sensitivity, mobile phase conditions need to be carefully tuned. The present research work was aimed to determine the impact of mobile phase composition on the performance of μLC/MS under volume as well as mass overloading conditions. The following conclusions can be drawn from the experimental results:

(1) One of the most commonly applied mobile phase additives for LC/MS of basic drugs is HOAc. Nevertheless, our experiments revealed that HOAc-containing mobile phases might not represent the best choice for μLC/MS particularly under overloading conditions. Effects such as peak broadening and tailing caused by mass overloading as well as flat-topped profiles caused by volume overloading severely impair chromatographic performance. Furthermore, polar compounds such as nicotine are hardly retained on the chromatographic column with mobile phases containing HOAc.
(2) Acidic modifiers that show chaotropic effects and/or that are able to form ion pairs offer better chromatographic performance than HOAc. Chromatographic performance increases in the order HOAc < FA $\ll$ $CH_3SO_3H \approx$ TFA $\approx$ PFPA $\approx$ HFBA. Proper choice of the acidic modifier is of particular importance for polar compounds such as nicotine. Only with the very hydrophobic ion-pairing reagents PFPA and HFBA nicotine was retained on the chromatographic column under volume overloading conditions.
(3) Improved chromatographic performance can compensate to a large extent the negative effects of reduced ionization efficiency on detection sensitivity. This effect is particularly pronounced under large-volume injection conditions. Therefore, even mobile phases containing strong ion-pair forming agents such as HFBA can be successfully applied for μLC/MS of pharmaceutical compounds. Particularly, 0.010–0.050% HFBA (v/v) was found to represent a good compromise in terms of chromatographic performance and mass spectrometric detection sensitivity. In comparison to HOAC the average loss of detection sensitivity was only 20%, but peak widths were halved.
(4) Using HFBA as acidic modifier, elution strength of organic modifiers increased in the order MeOH < Acetone < ACN < EtOH. Evaluation of the impact of the kind of organic modifier on separation efficiency revealed that for early eluting compounds (e.g., bunitrolol, caffeine, codeine, MDA, morphine, and nicotine) ACN offered best performance, but for late eluting compounds (e.g., cocaine, diazepam, doxepin, haloperidol, and zolpidem) EtOH and acetone can yield equal or better efficiencies. MeOH could not compete at all with ACN regarding the achievable peak widths. MeOH, however, surpassed ACN in terms of detection sensitivity.

In this paper the impact of solvent conditions on μLC/MS under overloading conditions was studied. Overloading, however, is a problem generally observed in LC/MS. Therefore we believe that conclusions and clues of the present study can be transferred to any kind of chromatographic approach that faces problems with overloading (e.g., LC/MS with analytical columns if the sample volume would exceed 100 μl).

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