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Preliminary study on the effect of miniaturisation and use of volatile mobile phases in LC for the on-line LC–MS analysis of basic pharmaceuticals

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Abstract

To enhance to compatibility of the on-line coupling of liquid chromatography (LC) with mass spectrometry (MS) for the analysis of basic pharmaceuticals, the use of volatile mobile phase systems in combination with miniaturised LC was investigated. Multifactor analysis of variance (MANOVA) was used to evaluate the data obtained for the various variables (modifier, stationary phase, buffer, buffer pH and buffer concentration) on the resolution, peak symmetry and retention of four basic compounds analysed using LC columns with internal diameters (I.D.) of 0.3, 1.0 and 4.6 mm (conventional). Preliminary results obtained with the investigated micro and conventional columns showed similar behaviour with respect to ruggedness. The various investigated variables showed that miniaturisation by simply downscaling dimensions can result in varying selectivity and peak shapes for basic compounds. When comparing volatile mobile phases (containing ammonium acetate or ammonium citrate) and a conventional non-volatile mobile phase (containing sodium phosphate) under pH 3 conditions, similar separation performances were observed. In the present study, ammonium citrate as the buffering salt, a high buffer concentration and methanol as the modifier showed the best peak symmetry.

Keywords: Miniaturisation; On-line LC–MS; Basic drugs; Volatile buffers

1. Introduction

In the vast field of pharmaceutical analysis, liquid chromatography combined with mass spectrometry (LC–MS) is a powerful tool in analysing compounds of low volatility and/or thermal lability. LC–MS is used in drug discovery, impurity

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profiling, metabolite identification and quantitative analysis [1–3]. Although the interest in practical applications is still growing and various LC–MS interfaces are available [4], the use of LC–MS for the analysis of pharmaceuticals has limitations. Most of the LC–MS interfaces do not allow the use of mobile phase additives, such as buffers and ion-pairing agents, whereas these are frequently used in the daily practice of pharmaceutical analysis. Several routes can be followed to remove the non-volatile mobile phase constituents, e.g. valve-switching techniques [5], post-column extraction [6], ion-suppression [7] and post-column phosphate suppression [8]. Nevertheless, the direct coupling of LC with MS is to be preferred to prevent problems concerning sample carry-over, low analyte recoveries and discrimination between the various analytes of interest during the removal step of the non-volatile mobile phase constituents. For this, the use of mobile phase systems in LC containing volatile buffer components has to be considered.

LC using C_8 and C_{18} modified silica as stationary phases in combination with mobile phases containing phosphate buffers are widely used from the initial development of a pharmaceutical compound up to quality control of drugs in various formulations. Since many of the pharmaceutically active compounds, e.g. central nerve system drugs and cardiovascular drugs, contain basic nitrogen groups, the LC analysis using silica based stationary phases can be problematic due to ionic interactions between residual silanols on the silica substrate and the basic drugs, i.e. asymmetric peaks can be obtained which hamper efficient separations and detection at low concentration levels. The different sources and synthesis routes applied in the manufacturing of reversed phase (RP) stationary phases for LC have resulted in a large number of phases, with different chromatographic properties [9–11]. Recent studies revealed that such differences between silica based RP stationary phases occur with respect to the LC analysis of basic compounds [12,13]. Since most studies, when using buffered mobile phase systems, were performed using phosphate buffers little is known about the applicability and mobile phase–stationary phase interactions when using volatile (non phosphate) buffer components.

Many LC applications are performed using columns with inner diameters between 4 and 5 mm. The use of columns with smaller inner diameters, however, is rapidly growing. Reduction of the inner diameter has a number of advantages: less solvent consumption, potential higher mass sensitivity, smaller sample amounts needed and enhanced compatibility (e.g. due to lower flow rates) with less convenient detectors such as mass spectrometry. Moreover, the enhanced permeability of capillary LC columns with the stationary phase packed in fused silica capillaries allows an increased column length and/or the use of smaller stationary phase particles, which improves the separation efficiency [14,15]. Recent studies evaluated the high mass sensitivity of miniaturised LC and demonstrated the applicability for, e.g. the quantitative analysis of drugs in biological fluids [16,17] and pesticides in water [18]. The high mass sensitivity was achieved by large volume injections and subsequent on-column focusing to maintain the separation efficiency.

In order to study the applicability of the use of volatile mobile phase systems in combination with miniaturised LC systems for the on-line coupling with MS, preliminary results of a study on the effect of microcolumn LC systems in combination with volatile mobile phase constituents on the separation performance of four pharmaceutical basic compounds are presented in this paper. Resolution, peak symmetry and retention data are discussed for two different micro LC (0.3 and 1.0 mm I.D.) and conventional (4.6 mm I.D.) LC columns in the LC analysis of the four representative basic pharmaceuticals. Besides the use of micro columns, mobile phases were investigated consisting of the volatile salts (electrolyte) ammonium acetate and ammonium citrate, and sodium phosphate for comparison. The chromatographic performance of the basic compounds like resolution, peak symmetry and retention is discussed with respect

to column diameter, stationary phase and mobile phase composition like organic modifier, type and concentration and pH of the electrolyte solution. The data are statistically evaluated using multifactor analysis of variance (MANOVA).

2. Experimental

2.1. High performance liquid chromatography equipment

The LC experiments for the conventional and micro columns of 1.0 mm I.D. were carried out on a Hewlett Packard 1090 M liquid chromatograph (Palo Alto, CA). Data processing were performed and chromatograms were obtained using a HPLC 3^D Chemstation, also of Hewlett Packard. Using the conventional LC columns, detection was performed using a Hewlett Packard 1040 M Diode Array detector (cell volume 6 μl). For the 1.0 mm I.D. columns, detection was performed using a model 785A Programmable Absorbance detector of Applied Biosystems (Norwalk, CO, USA) equipped with a Z-shaped flow cell (cell volume 35 nl), LC Packings (Amsterdam, The Netherlands).

The experiments with the 0.3 mm I.D. column were performed using a model 140C Microgradient pump (Applied Biosystems), an model AS800 autosampler of Fisons Instruments (Milan, Italy) modified with a Valco 4 Port injector with a fixed 60 nl internal loop (Valco Instruments, Houston, TX) and a 785A Programmable Absorbance detector (Applied Biosystems) using on-capillary detection. Chromatograms were obtained using Turbochrom 4 software (Perkin Elmer, Norwalk, CO). Data processing was performed using a HPLC 3^D Chemstation.

In Table 1, the chromatographic conditions, e.g. flow rate and injection volume used in the MC and conventional LC experiments, are shown. The LC columns were thermostatted at 40°C ± 0.5°C. The applied linear flow rates and injected amounts of the samples onto the different columns were related to the cross sectional area of the various column diameters. When using the conventional and 1.0 mm I.D. columns UV detection was performed at 254 nm. Since UV detection with the 0.3 mm I.D. columns was on-column, the detection wavelength was set to 230 nm to improve the sensitivity.

MS detection was performed by connecting the outlet of the LC to the Perkin Elmer Sciex API-100 LC–MS which was equipped with an Ionspray interface. The Ionspray Voltage was set to 6000, air was used as nebulizer gas. The orifice and ring potential were set at 10 and 225 V, respectively. The MS was scanned in positive ion-mode from 230 to 395 amu in 0.035 amu s^{-1} using a step size of 0.1 amu.

2.2. Chemicals

The basic pharmaceuticals were synthesised by the Department of Medicinal Chemistry, N.V. Organon (Oss, The Netherlands). In Fig. 1, the molecular structures of the test compounds are shown.

Methanol (MeOH, Lichrosolv) and acetonitrile (ACN, Uvasol) were obtained from Merck (Darmstadt, Germany). Sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), acetic acid (~99–100%), ortho-phosphoric acid (85%) and citric acid monohydrate were purchased from Baker (Deventer, The Netherlands) and ammonium ac-

Table 1
Instrumental settings used in micro column and conventional LC

Column diameter (mm I.D.)	Flow-rate (ml min^{-1})	Injection volume (μl)	UV detection wave length (nm)
4.6	1.0	14	254
1.0	0.05	0.7	254
0.3	0.005	0.06	230

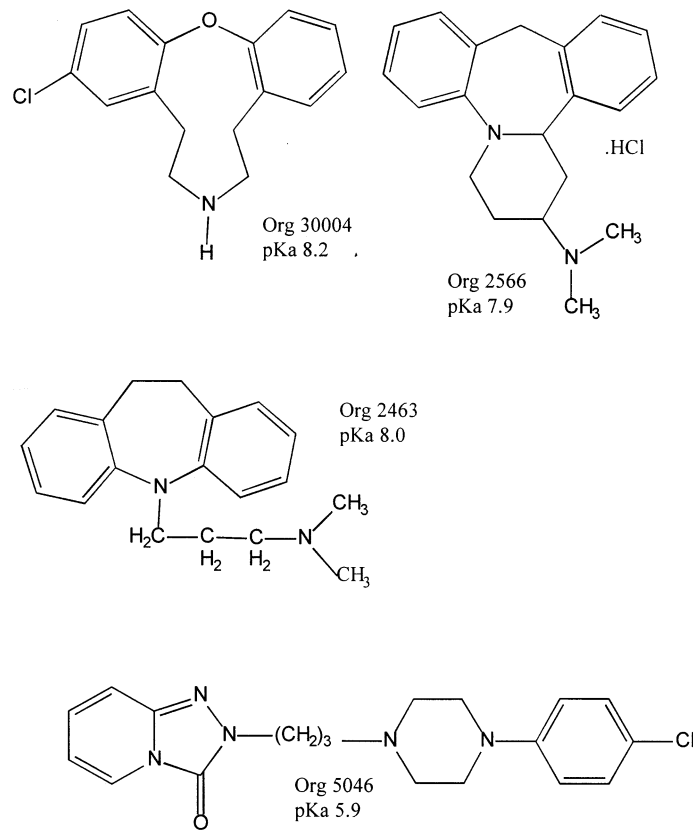

Fig. 1. Chemical structures of the basic pharmaceuticals used in this study.

etate p.a. (NH_4Ac) from Jansen Chimica (Geel, Belgium). Ammonium citrate was purchased from Aldrich (Milwaukee, WS).

Aqueous solutions were prepared using demineralised water. 1 M aqueous electrolyte solutions were prepared with demineralised water to obtain 10 and 25 mM sodium phosphate, ammonium acetate and ammonium citrate solutions of pH 3 and 7. Since the used buffer solutions not always have an optimal buffering capacity in the pH ranges, the buffer salts are referred to as electrolyte solutions.

The LC columns used in this study are listed in Table 2.

As test solutions, 1 mg of the basic solutes was dissolved in 1 ml methanol and diluted 1:10 with the mobile phase used in the experiment.

2.3. Ruggedness testing of liquid chromatography columns

Since LC columns can be subjected to degradation during use [19], the performance of the columns was monitored before and after the various experiments. To test the initial status and to detect stationary phase characteristic differences between the various LC columns, Engelhardt [20] and Sander [21] tests were performed to check changes in silanol activity, hydrophobicity and metal activity.

The solution to test the ruggedness, consisted of 1 μl acetyl acetone, 0.01 μl ortho-toluidine, 0.01 μl meta-toluidine, 0.3 mg para-toluidine, 0.05 μl toluene and 0.2 μl ethyl benzene, added to 1 ml methanol. The mobile phase consisted of a methanol/water mixture (55 + 45, v/v).

2.4. Separation performance: calculations

In order to monitor the separation performance of the studied LC systems, the asymmetry factor, plate number, retention factor and normalised resolution of the analytes were determined.

The asymmetry factor (As) was calculated at 5% of the peak height:

$$\mathrm{As} = \frac{w_{0.05}}{2w_{a,\,0.05}}$$

where $w_{0.05}$ is the width of the peak at 5% of the peak height, and $w_{a,\,0.05}$ is the width of the front side at 5% of the peak height.

To calculate the column efficiency, the half-width method was used to calculate plate numbers (N) [22]:

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

where t_R is the retention time and w_h is the peak width at half height of the peak. The retention factor (k) was calculated using:

$$k = \frac{(t_R - t_0)}{t_0}$$

were t_R is the retention time of the compound and t_0 is the column dead time (dead time of the column was measured as the first disturbance in the baseline after sample injection).

To determine the separation performance, the resolution was determined. Since the resolution of more than one peak pair has to be determined, the sum of resolution could lead to non relevant results. To prevent this, the normalised resolution product (r) was used [23]:

$$r = \prod_{i=1}^{n-1}\left(\frac{\mathrm{Rs}}{\bar{\mathrm{R}}\mathrm{s}}\right) \quad \text{with} \quad \bar{\mathrm{R}}\mathrm{s} = \left(\frac{1}{n-1}\right)\sum_{i=1}^{n-1}\mathrm{Rs}i,\, i+1$$

where Rs is the resolution between subsequent peaks and $\bar{\mathrm{R}}\mathrm{s}$ is the average resolution taken over all peak pairs in a chromatogram. r will vary from 0 (no resolution for at least one of the peak pairs) to 1 when the resolution is equal for all peak pairs in the chromatogram.

2.5. Data evaluation

To statistically evaluate the analytical data, MANOVA was performed by using Statgraphics plus version 2.1 software, Manugistics (Rockville, MA), installed on a personal computer with a Pentium/75 MHz Intell processor.

3. Results and discussion

In order to study the applicability of the use of volatile mobile phase systems in combination with miniaturised LC systems for the on-line coupling with MS, a set of four pharmaceutical basic compounds was selected.

The four compounds were selected from a study using a larger set of 32 pharmaceutical compounds [12]. Using these pharmaceuticals, the following chromatographic variables were studied

Table 2
Overview studied LC columns

Stationary phase	Dimension (length × I.D., mm)	Manufacturer stationary phase	Packed by
Zorbax SB-C_{18}	150 × 0.3	Hewlett Packard	LC Packings
	150 × 1.0		LC Packings
	150 × 4.6		Hewlett Packard
Symmetry C_{18}	150 × 0.3	Waters (Milford, MA)	LC Packings
	150 × 1.0		LC Packings
	150 × 4.6		Waters
Prodigy 5 ODS-2	150 × 0.3	Phenomenex (Torrance, CA)	Phenomenex
	150 × 1.0		Phenomenex
	150 × 4.6		Phenomenex

Table 3
Studied variables and conditions

Variable	Condition		
Modifier	Methanol	Acetonitrile	
Electrolyte	Ammonium citrate	Ammonium acetate	Sodium phosphate
Electrolyte concentration (mM)	10	50	
pH	3	7	
Stationary phase	Zorbax SB-C_{18}	Symmetry C_{18}	Prodigy 5 ODS-2
Column diameter (mm)	0.3	1.0	4.6

(Table 3): LC column inner diameter (I.D.), type stationary phase and the mobile phase parameters type of modifier, type of electrolyte, electrolyte (mobile phase buffer salt) concentration and pH.

To describe and evaluate the effects of the chromatographic variables, LC experiments with the factor combinations were performed. Also, to avoid day to day differences and to average the influence of possible column deterioration, the different mobile phase experiments were randomised and with every mobile phase variation the columns were tested. In total, 24 experiments were performed with each column.

The modifier–electrolyte solution ratio was adapted with respect to the type of modifier (methanol or acetonitrile) in order to elute the compounds with comparable retention ($1 < k < 10$): the amount of acetonitrile used in the mobile phase was 10% less compared to the amount of methanol. When using electrolyte solution pH 3, 30% acetonitrile or 40% methanol, and when using electrolyte solution pH 7, 60% acetonitrile or 70% methanol were used in the mobile phases, respectively.

The electrolyte solutions (pH 3 and 7) were not used at their optimal buffering pH values. However, since only small amounts of basic compounds were injected onto the LC columns, the ionisation of the analytes will be controlled and the pH of the mobile phase unaffected.

3.1. Ruggedness liquid chromatography columns during the study

As can been extracted from Table 3, a total of 24 different mobile phase conditions were used with nine different LC columns. With each mobile phase condition, the sample solution, comprising a mixture of the four basic pharmaceuticals, was injected in duplicate. Since LC columns could degrade during usage, and thus during the presented study, the column characteristics silanol activity, metal activity, hydrophobicity and efficiency were monitored before and after finishing the experiments with the basic drugs, using the test procedures as described by Engelhardt and Sander.

The silanol activity was expressed as the relative retention of aniline and phenol, and as the selectivity between ortho-, meta- and para-toluidine. The hydrophobicity of the LC columns was expressed as the relative retention of toluene and ethylbenzene whereas the metal activity was expressed as the width at half height of the acetyl acetone peak [20,21]. For the metal activity, however, it was recently shown that the peak width of the acetyl acetone peak was also affected by keto-enol tautomerism effects. Therefore, the observed effects cannot be ascribed solely to changes in metal activity [24]. Concerning the present ruggedness testing, however, only the differences between the LC columns before and after finishing the effects are discussed.

Regarding the stationary phase properties, the results for the silanol activity (relative retention of aniline and phenol, and toluidines selectivity), metal activity and hydrophobicity are presented as bar charts (Fig. 2). As can be seen, with the exception of the width of the acetyl acetone peak, minor changes of the stationary phase properties of the LC columns before and after finishing the study were observed. Compared to the Prodigy 5 ODS-2 and Symmetry C_{18} LC columns, slightly more residual silanol activity was observed for the

Zorbax SB-C_{18} LC columns, as shown by the relative retention of aniline and phenol and by the selectivity of the toluidines. Furthermore, the width of the acetyl acetone peaks of the Symmetry C_{18} and Zorbax SB-C_{18} 1.0 mm I.D. columns increased significantly after performing the experiments with the basic analytes, while for the 0.3 and 4.6 mm I.D. columns the width of the acetyl acetone peaks of both these columns remained the same and on a significant low level compared to the Prodigy column. In Fig. 3 also the plate numbers obtained for ethyl benzene at the initial situation and after finishing the experiments are shown. In general, most LC columns showed decreased plate numbers (down to 50%) after performing the experiments with the basic compounds. Since only minor changes in stationary-phase properties were observed during usage (Fig. 2), these test results indicate that probably the structure of the packed beds of the columns changed, resulting in void volume formation and decreased efficiency. Since the various column

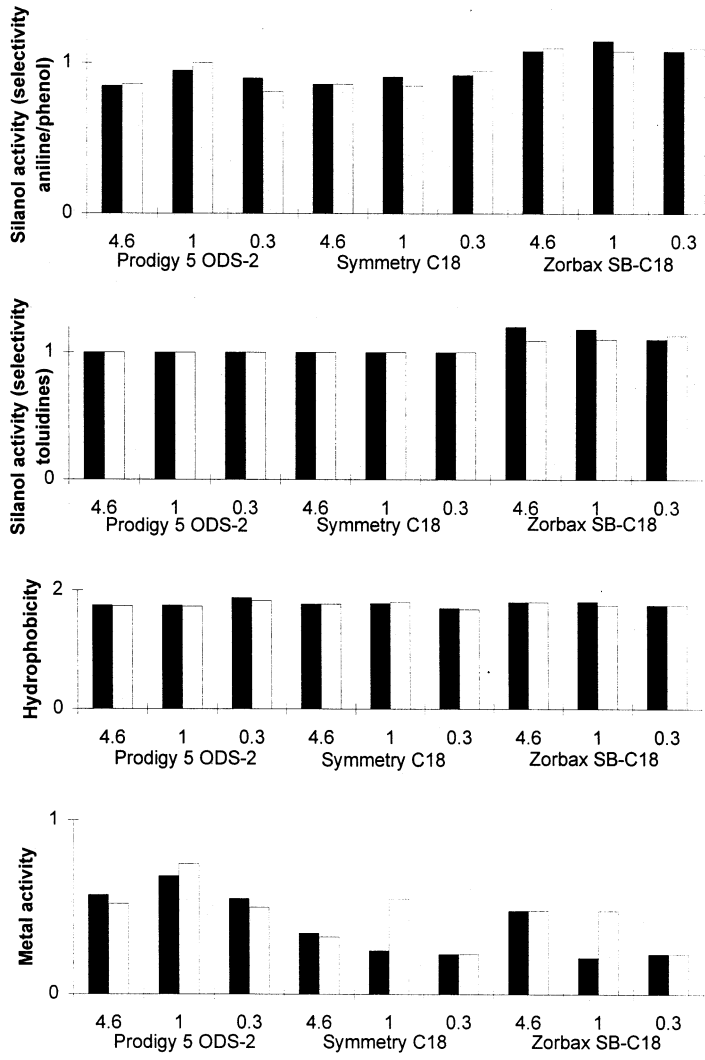

Fig. 2. Silanol activity, by selectivity aniline/phenol and selectivity toluidines, hydrophobicity and metal activity before (■) and after the experiments (□).

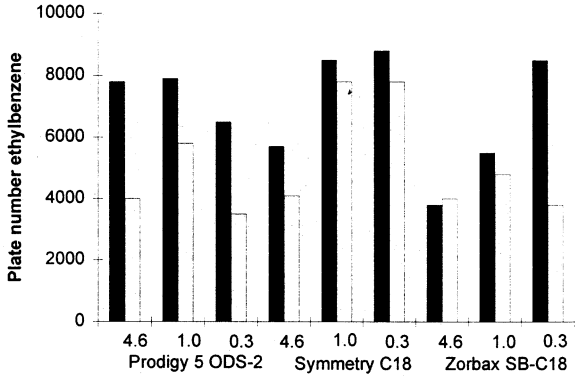

Fig. 3. Plate numbers ethylbenzene before (■) and after (□) experiments.

experiments were randomised, the variations in resolution, peak symmetry and retention of the basic compounds were mainly attributed to the parameters investigated (different column diameters, stationary phases, organic modifiers, type and concentration and pH of the electrolyte solutions used), and less to LC column degradation.

In summary, since only minor changes in stationary phase properties are observed and the experiments are randomised, possible column degradation phenomena will probably have no significant effect on the present study.

3.2. Liquid chromatography analysis of basic pharmaceuticals

Using the different LC columns (9) and the different mobile phases (24), the separation performances of the various LC systems was investigated for the separation of the four selected basic pharmaceuticals.

In Table 4 for the micro and conventional LC systems, the obtained peak shapes and resolutions for the four basic compounds were found to be different (correlation ≤ 0.5) for the 0.3, 1.0 and 4.6 mm I.D. columns. A higher correlation (> 0.8) for the retention factors of the test substances was observed. From the general chromatographic theory, however, a high correlation (as shown by retention) is expected rather than a low correlation (as shown by resolution and peak shape). Low correlation generally means that miniaturising LC separations by simply downscaling the dimensions can result in varying selectivity and peak shapes for basic compounds. These correlations, were calculated using the total data set. To reveal the origin of the low correlation the influence of the individual variables on resolution, peak shape and retention were studied.

To reveal the influence of the various variables on the chromatographic performance of the basic compounds MANOVA was performed. With MANOVA, the variability of normalised resolution, asymmetry and retention is split-up into contributions of the various variables (type, concentration and pH of the electrolyte solution, stationary phase, modifier and column diameter). Using MANOVA, the contribution of each variable is determined excluding the effects of all other variables. The calculated P-values using MANOVA show the significance of each of the variables, i.e. a value of $P < 0.05$ indicates a statistically significant effect of the variable at the 95% confidence level. The residuals of the models of normalised resolution, asymmetry and retention for the 0.3, 1.0 and 4.6 mm I.D. columns were normally distributed with a mean of zero [25,26].

Table 4
Correlation matrix of normalised resolution (r), peak asymmetry (As) and retention (k)

	0.3 (mm I.D.)	1.0 (mm I.D.)	4.6 (mm I.D.)
r			
0.3 mm I.D.	1	0.39	0.24
1.0 mm I.D.		1	0.50
4.6 mm I.D.			1
As			
0.3 mm I.D.	1	0.32	0.29
1.0 mm I.D.		1	0.21
4.6 mm I.D.			1
k			
0.3 mm I.D.	1	0.84	0.87
1.0 mm I.D.		1	0.91
4.6 mm I.D.			1

Table 5
MANOVA results for normalised resolution (r)

Source	P-value		
Main effects	0.3 mm I.D.	1.0 mm I.D.	4.6 mm I.D.
A: Electrolyte	0.1291	***0.0041***	0.1089
B: Concentration	0.2896	0.1336	0.4375
C: Modifier	0.9555	0.0663	0.2841
D: pH	***0.0004***	***0.0000***	0.0908
E: Stat. phase	0.9349	0.3936	0.0903
Interactions			
AB	0.4750	0.5683	0.9620
AC	0.6523	0.3727	0.5017
AD	0.3681	***0.0020***	0.6424
AE	0.0689	0.6058	0.6178
BC	0.0630	0.2887	0.8958
BD	0.8953	0.8523	0.7612
BE	0.4295	0.4724	0.8238
CD	***0.0182***	0.0543	0.8219
CE	0.2686	0.5172	0.9797
DE	0.9050	0.7309	0.4114

3.2.1. Normalised resolution products (r)

Since baseline separation of analytes is one of the major goals in LC, first the influence of the variables on the separation between the four compounds is discussed. In Table 5, the main effects and interactions of the variables on r for the conventional and micro LC columns are shown, covering the various types of stationary phases. In Fig. 4, the significant effects of the type and the pH of the electrolyte solutions on r are illustrated. For the 1.0 mm I.D. columns, best resolution (high r) was obtained using the sodium phosphate buffer, whereas the resolutions obtained using the ammonium acetate and ammonium citrate solutions were comparable. Although the influence was not significant, for the 0.3 mm I.D. LC columns similar results were obtained as for the 1.0 mm I.D. columns. For the conventional LC columns generally the best resolutions were obtained using both the sodium phosphate and ammonium citrate buffers. Furthermore, from Fig. 4 it can be concluded that for these four basic compounds highest r-values were obtained using buffers at pH 3.

In Fig. 5, the significant interactions are shown graphically. For the 0.3 mm I.D., 1.0 mm I.D. and conventional LC columns, the interaction between the type of electrolyte and pH showed a similar trend: using either ammonium citrate, ammonium acetate or sodium phosphate at pH 3 best resolutions between the four basic compounds. As discussed before, highest r-values were obtained using sodium phosphate buffer. From the interaction plots in Fig. 5, however, it is obvious that this is particularly true for the electrolyte solutions used at pH 7. Apparently, the interaction between electrolyte and basic drug with sodium phosphate at pH 7 is different than between ammonium acetate and ammonium citrate at pH 7. Whether this is due to the different cations (sodium, ammonium) or anions (phosphate, acetate, citrate) cannot be concluded from the present data. For the ammonium acetate, ammonium citrate and sodium phosphate solutions similar separations were obtained at pH 3.

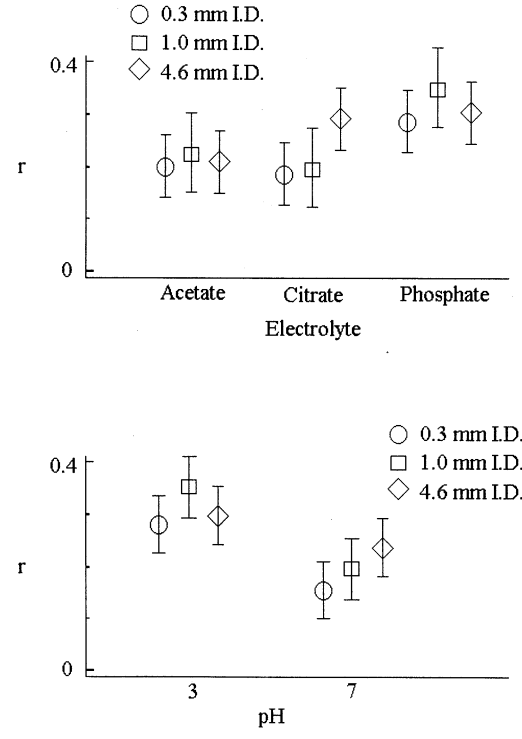

Fig. 4. Main effects normalised resolution product (r); mean and 95% confidence intervals.

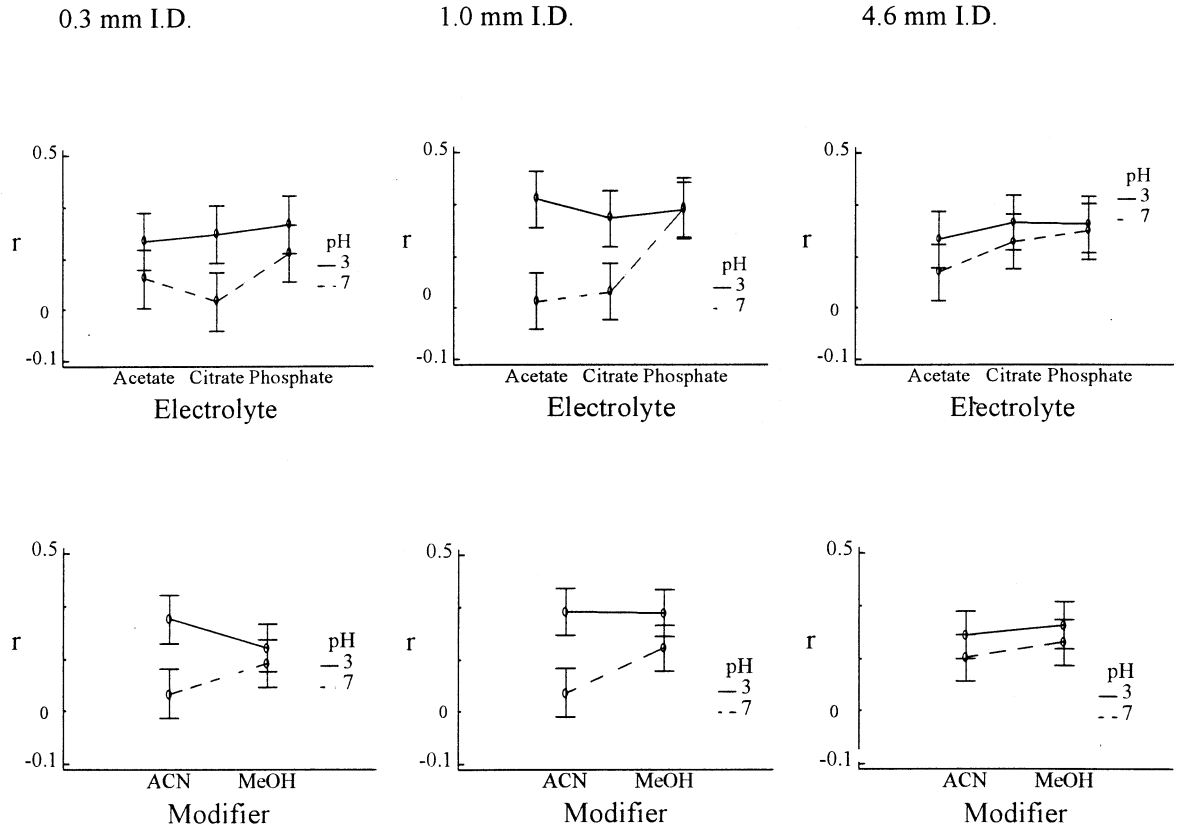

Fig. 5. Interaction effects normalised resolution product (r); mean and 95% confidence interval.

In Fig. 5, the interaction plots between the nature of modifier and buffer pH are also shown. Compared to pH 7, using acetonitrile the resolution obtained on the 0.3 and 1.0 mm I.D., LC columns improved for electrolyte solutions at pH 3. Using methanol in the mobile phases pH 3 and 7, the resolution showed less improvement when going from pH 7 to 3.

In summary, the mobile phase pH and type of electrolyte used to separate the four basic analytes generally influenced the resolution: as experienced from daily practice, at pH 7 using sodium phosphate buffers higher r-values were obtained compared to ammonium citrate and ammonium acetate buffers. However, remarkably at pH 3 the resolutions obtained using ammonium acetate, ammonium citrate and sodium phosphate were comparable. Obviously, using mobile phases at pH 3 the use of volatile buffers (ammonium acetate, ammonium citrate) could be an alternative for the generally used phosphate containing mobile phases.

3.2.2. Comparison peak symmetry

In the LC analysis, the detection at low concentration levels of pharmaceutical compounds is improved by symmetrical sharp peaks: better signal-to-noise ratios can be obtained. As shown in previous studies, however, due to the presence of residual silanols on the silica matrix of RP packing materials, asymmetric peaks are often obtained with basic compounds [12,13].

In Table 6, the main effects and interactions of the investigated variables on peak shape for the conventional, 1.0 and 0.3 mm I.D. LC columns are shown. It was shown that when using the 0.3

mm I.D. LC columns, the type of basic compound did not show any significant effect on peak symmetry. However, the four test compounds were selected because different peak symmetries were obtained when analysed using six different LC columns [12]. The type and basicity of the nitrogen containing group of the basic solute will, amongst other effects, also determine the interaction with residual silanols of the stationary phase [27], and therefore the peak symmetry. It is most likely that the deviation between the obtained peak symmetries of the four compounds prevented the revealing a significant effect of the type compound on peak symmetry when using the 0.3 mm I.D. columns.

In Figs. 6 and 7, the significant effects and interactions are shown graphically. For the 0.3 mm I.D., 1.0 mm I.D. and conventional LC columns, the influence of the type of the electrolyte on peak shape is shown in Fig. 6: chromatographic peaks with the lowest As values were obtained using ammonium citrate as electrolyte solution. Since asymmetrical peaks are often due to interaction with residual silanols, probably the ammonium citrate electrolyte suppresses the interaction between basic compounds and residual silanols. The influence of the electrolyte concentration on the peak shapes of the basic com-

Table 6
MANOVA results for peak asymmetry (As)

Source	*P*-value		
Main effects	0.3 mm I.D.	1.0 mm I.D.	4.6 mm I.D.
A: Electrolyte	***0.0009***	***0.0000***	***0.0000***
B: Compound	0.2841	***0.0000***	***0.0000***
C: Concentration	***0.0064***	***0.0403***	***0.0002***
D: Modifier	0.8448	***0.0000***	0.1308
E: pH	0.8839	***0.0003***	0.5203
F: Stat. phase	***0.0000***	***0.0000***	***0.0001***
Interactions			
AB	***0.0020***	***0.0150***	***0.0020***
AC	0.9677	***0.0237***	***0.0111***
AD	0.8080	0.0845	***0.0003***
AE	***0.0011***	***0.0000***	***0.0064***
AF	***0.0000***	0.1796	***0.0000***
BC	0.5762	0.9336	0.5523
BD	0.9591	0.7879	0.6053
BE	0.1530	***0.0006***	***0.0333***
BF	0.5574	0.7214	***0.0073***
CD	0.7314	0.2299	0.2984
CE	0.9493	0.6150	0.8519
CF	0.1515	0.0564	0.6044
DE	0.8653	0.4791	0.1185
DF	0.2236	0.7449	0.1275
EF	***0.0046***	***0.0196***	***0.0000***

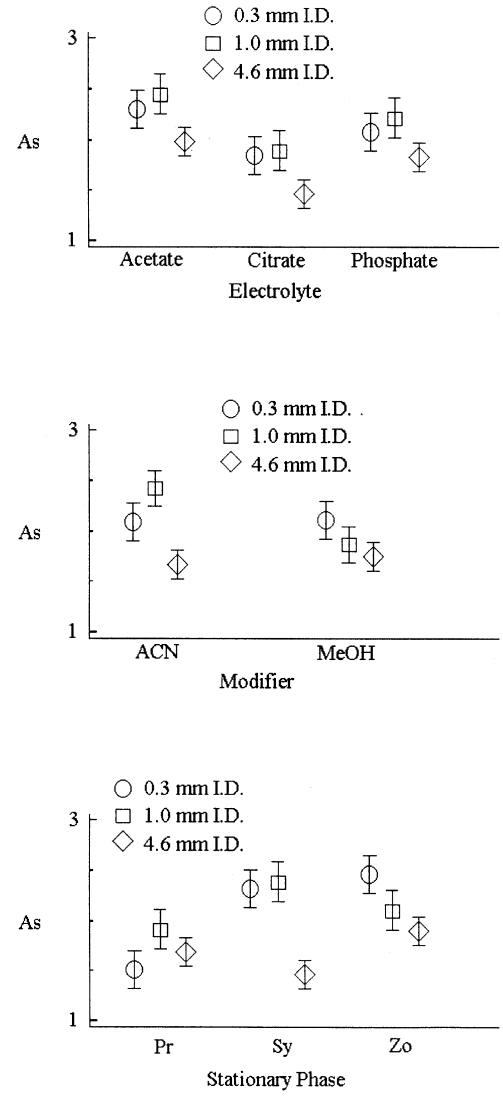

Fig. 6. Main effects peak asymmetry (As); mean and 95% confidence interval.

pounds was evident (data not shown here): compared to the lower buffer concentration more symmetrical peaks were obtained for all LC columns using 50 mM electrolyte solution. This 50 mM mobile phase will suppress more efficiently undesirable interactions of the analytes with the column packing material.

The influence of the type of modifier and stationary phase on peak shape was not comparable between the different LC columns. With respect to the nature of the modifier, a significant effect was observed only for the 1.0 mm I.D. columns. The use of methanol instead of acetonitrile improved the shape of the chromatographic peak significantly. The reason for the improved peak symmetry for the 1.0 mm I.D. columns was not clear, since in the ruggedness testing no differences between the 1.0 mm I.D. and the other columns were observed. Furthermore, to avoid unwanted interference, e.g. day to day variability, the experiments were randomised. With respect to the stationary phases, low As-values were obtained for the Prodigy 0.3 and 1.0 mm I.D. LC columns, respectively. For the conventional LC columns, lowest As-values were obtained for both the Symmetry and Prodigy stationary phases. Furthermore, as can be seen in Fig. 6, peaks with lowest As-values generally were obtained when using conventional LC columns. The data reveal differences between stationary phases packed in LC columns with various diameters. The different packing procedures, batch to batch variability of the packing material and/or column hardware (stainless steel, frits, fused silica capillary) could reveal some insight why the columns show the differences described.

As shown in the interaction plots (Fig. 7) other than the type of electrolyte, peak symmetry is also

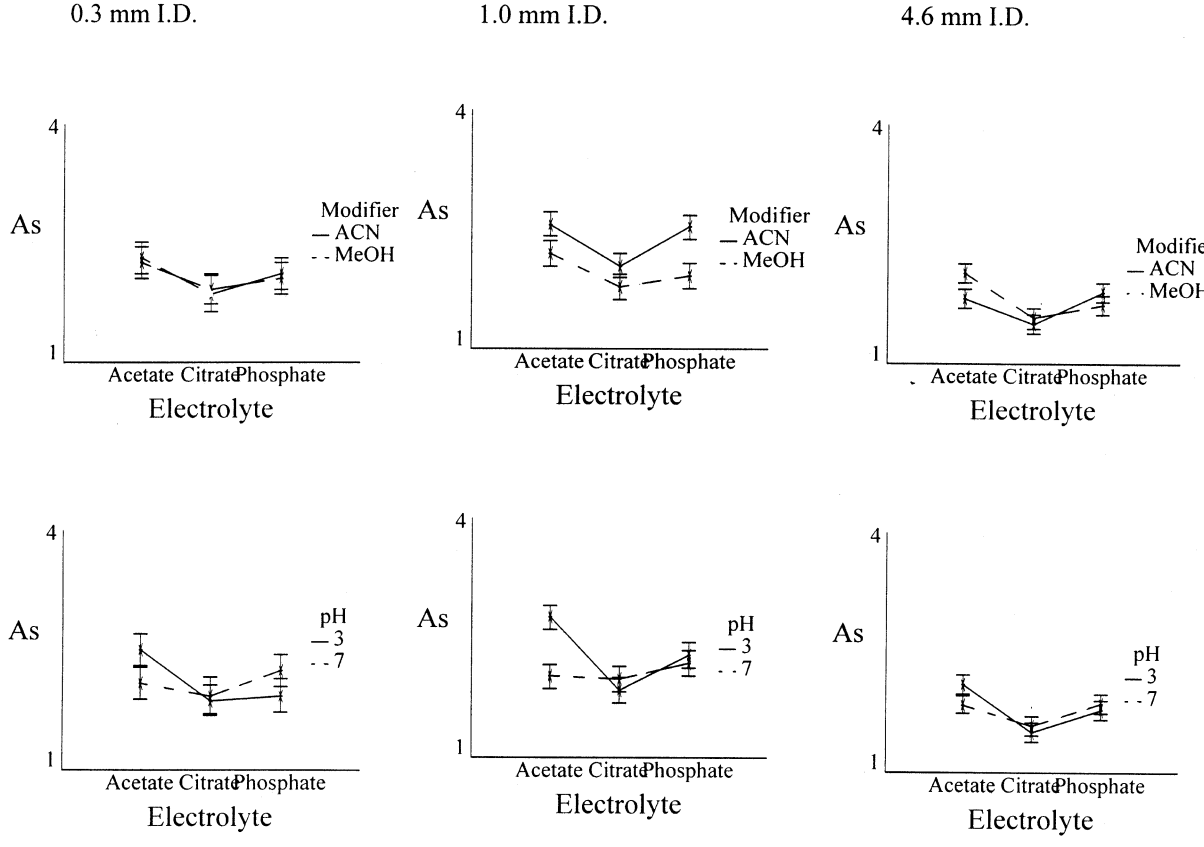

Fig. 7. Interaction effects peak asymmetry (As); mean and 95% confidence interval.

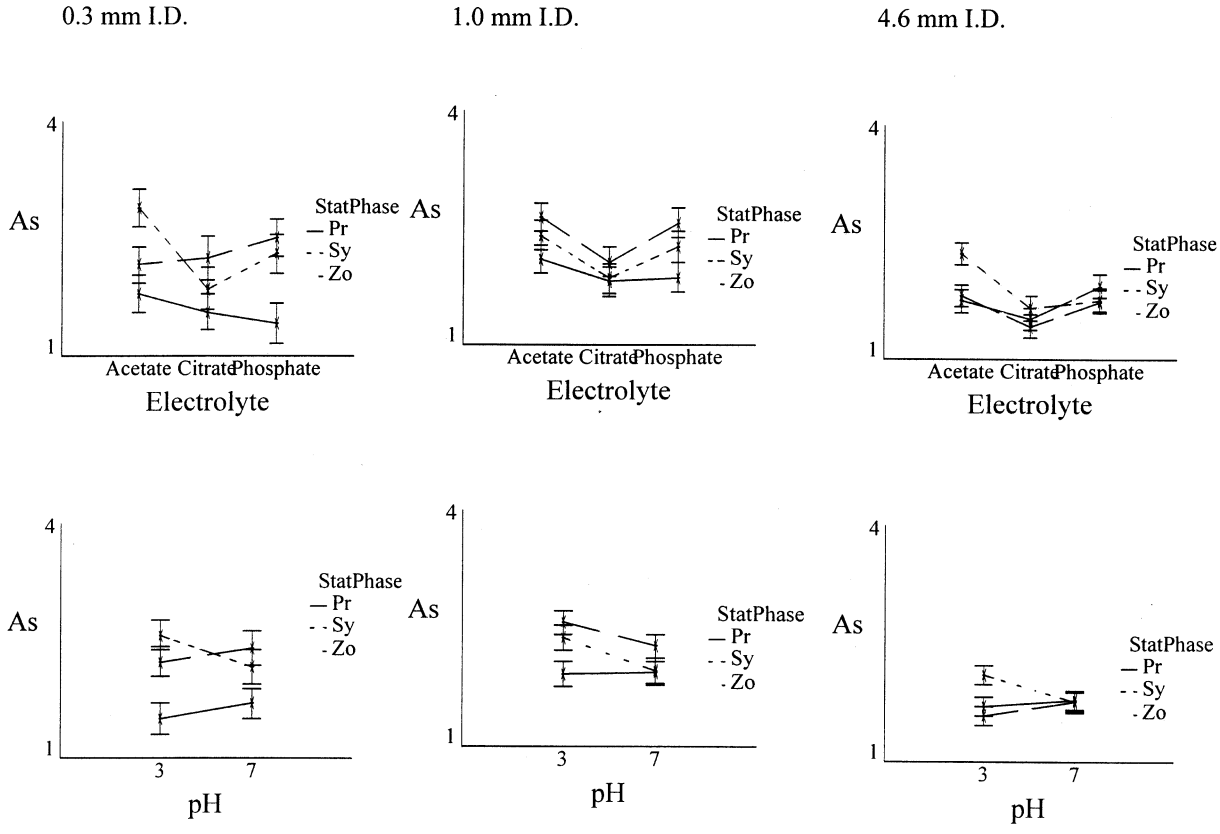

Fig. 7. (*Continued*)

affected by the modifier, pH and type of stationary phase. The effect of the type of modifier for the 1.0 mm I.D. LC columns, as described before, was observed for every type of electrolyte used in this study; the use of methanol instead of acetonitrile improved peak symmetry. Using ammonium acetate at pH 7, the shapes of the peaks became more symmetrical, compared to pH 3. Since these results were obtained for the 0.3 mm I.D., 1.0 mm I.D. and conventional LC columns, this effect can be ascribed to the use of ammonium acetate which at low pH has limited buffer capacity. Insufficient buffering capacity results in poorly controlled ionisation of both the stationary phase and the basic analyte which than can result in adsorption and asymmetrical peaks. Furthermore, using the 0.3 and 1.0 mm I.D. Prodigy columns, low As-values were obtained, compared to the Symmetry and Zorbax columns. For the conventional Zorbax column, slightly higher asymmetrical peaks were obtained compared to the Prodigy and Symmetry columns (Fig. 6). However, as shown in Fig. 7 the observed peak shapes were also affected by buffer pH: the peak shapes when using the 0.3, 1.0 and 4.6 mm I.D. Zorbax columns improved when electrolyte solutions pH 7 were used, whereas with the Symmetry and Prodigy columns improved peak symmetry (low As) was obtained when using mobile phase pH 3. Asymmetrical peaks at low pH point to the presence of ionic sites at the stationary phase which can interact with the, at low pH, protonated basic solutes.

In summary, lower As values were obtained for the four basic substances using ammonium citrate electrolyte solutions in the mobile phase and the Prodigy stationary phase. For every column used in this study, it was evident but also remarkable

that the use of ammonium citrate as buffer in particular, resulted in good peak shapes for the four basic compounds. As expected (and in accordance with theory), the packing of the three investigated stationary phases in LC columns with different inner diameters did not reveal a clear influence of the column diameter on peak symmetry.

3.2.3. Comparison of retention factors

Since the retention factors of the four basic compounds using MC and conventional LC columns were similar (Table 4) only the main effects will be discussed.

In Table 7, the main effects and interaction effects of the variables on retention are shown. It was found that the concentration of the mobile phase electrolyte solution showed no influence on retention for the conventional LC columns. As expected, the other variables, i.e. electrolyte, compound, modifier, column diameter, pH and stationary phase showed a significant effect on retention. The influence of the nature of the basic analyte and modifier on retention is obvious. Protonation and basicity of the compounds and the proton donor/acceptor properties of the modifier are main effects here. Since at low pH the compounds are protonated, the influence of pH on retention also shows a significant effect. As already shown in Table 4, the correlation between the LC columns for retention was high. In Fig. 8, the effects of the type of electrolyte, electrolyte concentration and stationary phase on retention are shown graphically. The effect of the concentration of the mobile phase electrolyte solution was of no significant influence on retention using the conventional LC columns. For the 0.3 and 1.0 mm I.D. LC columns, the retention decreased with increasing electrolyte concentration. Increasing the electrolyte concentration showed a posi-

Table 7
MANOVA results for retention (k)

Source	P-value		
Main effects	0.3 mm I.D.	1.0 mm I.D.	4.6 mm I.D.
A: Electrolyte	***0.0000***	***0.0000***	***0.0000***
B: Compound	***0.0000***	***0.0000***	***0.0000***
C: Concentration	***0.0024***	***0.0136***	0.6792
D: Modifier	***0.0000***	***0.0000***	***0.0000***
E: pH	***0.0004***	***0.0003***	***0.0061***
F: Stat. Phase	***0.0000***	***0.0000***	***0.0000***
Interactions			
AB	***0.0129***	***0.0000***	***0.0000***
AC	0.4685	0.6357	0.9516
AD	***0.0000***	***0.0000***	***0.0000***
AE	***0.0000***	***0.0000***	***0.0000***
AF	***0.0002***	***0.0005***	***0.0009***
BC	0.4545	0.9555	0.9794
BD	***0.0000***	***0.0000***	***0.0000***
BE	***0.0107***	***0.0000***	***0.0034***
BF	***0.0000***	***0.0000***	***0.0000***
CD	0.3850	0.3392	***0.0033***
CE	***0.0154***	***0.0000***	***0.0000***
CF	***0.0088***	***0.0000***	***0.0003***
DE	***0.0003***	***0.0002***	***0.0000***
DF	***0.0074***	0.2637	0.0746
EF	0.8820	***0.0000***	***0.0017***

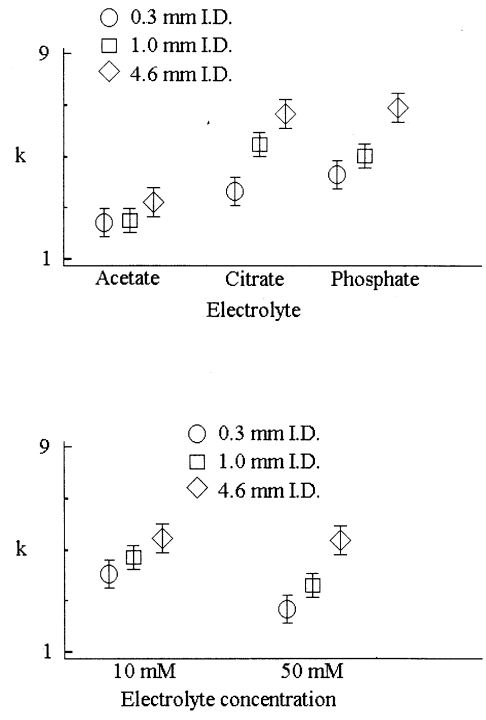

Fig. 8. Main effects retention (k); mean and 95% confidence interval.

tive effect on peak shape: due to less ionic interactions the shape of the peak improved when going from 10 to 50 mM buffers. Since retention is caused through both hydrophobic and ionic interaction, it is obvious that increasing the buffer concentration results in decreased retention. Furthermore, the compounds were most retained using the Zorbax columns, whereas the compounds were least retained using the Prodigy column. Lowest retention was observed using ammonium acetate whereas compounds were most retained using sodium phosphate buffers. Since this latter effect was observed for the 0.3 mm I.D., 1.0 mm I.D. and conventional LC columns, the effect is considered to be a stationary phase feature and not caused by the different column diameters.

In summary, comparable retention factors were found for the various (inner diameters) columns for each of the three investigated stationary phases. As could be expected, similar trends for retention were observed for the studied variables.

As examples, in Fig. 9 the LC–UV chromatograms obtained for the four test substances using the Prodigy 5 ODS-2 MC and conventional LC columns are shown.

3.3. Liquid chromatography coupled on-line with mass spectrometry

From the study regarding the separation of the basic pharmaceuticals, it can be concluded that regarding the use of mobile phases consisting of volatile electrolytes in mobile phases with pH 3, the resolutions obtained when using ammonium acetate, ammonium citrate and sodium phosphate were comparable. Since non-volatile phosphate buffers cannot be used for on-line LC–MS, the present study shows that at pH 3, the volatile ammonium acetate and ammonium citrate buffers can be used as a good alternative without loss in separation performance. However, regarding expected sensitivity, the lower As values obtained when using ammonium citrate electrolyte solutions in the mobile phase will be favourable. The applicability of a miniaturised LC system combined with MS using a volatile mobile phase is demonstrated in Fig. 10.

In the corresponding mass spectra of the basic solutes, high abundance ions at m/z 210 $[M + NH_4]^+$ and m/z 402 2 $M + NH_4]^+$ were found as a result of complexes between citrate and ammonium. These signals could interfere with signals from analyte ions. With ammonium acetate, no ions in this m/z range are observed. Therefore, from an MS point of view, the use of ammonium acetate is preferred. From an LC point of view, however, it is obvious that the use of ammonium citrate is preferred over ammonium acetate.

4. Conclusions

To enhance to compatibility of the on-line coupling of LC with MS for the analysis of basic pharmaceuticals, the use of volatile mobile phase systems in combination with miniaturised LC was investigated. The MANOVA results revealed information about the influence of the various variables (modifier, stationary phase, buffer, buffer pH and buffer concentration) on the resolution, peak symmetry and retention of four basic compounds analysed using 0.3 mm I.D., 1.0 mm I.D. and conventional (4.6 mm I.D.) size LC columns.

With respect to miniaturisation of LC columns, preliminary results obtained with the investigated micro and conventional columns showed similar behaviour with respect to ruggedness. A comparable decrease in efficiency was observed for the 0.3, 1.0 and 4.6 mm I.D. column used in this study.

It was found that miniaturising LC separations by simply downscaling dimensions can yield different selectivity and peak shape for basic compounds, which is not expected from general chromatographic theory. Furthermore, as expected, the nature and pH of the eluent showed a significant impact on the separation of the four basic test drugs.

In general, peak symmetry appeared to be influenced by the buffer, basic compound, electrolyte concentration, modifier, pH and station-

ary phase. However, some differences between the 0.3, 1.0 and 4.6 mm I.D. columns were found. From the three stationary phases lowest peak asymmetry over all investigated column diameters was found for the Prodigy stationary phase.

When comparing volatile mobile phases (containing ammonium acetate, ammonium citrate) and conventional non-volatile mobile phase (containing sodium phosphate), it was found that under pH 3 conditions the applied ammonium acetate, ammonium citrate and sodium phosphate buffers showed similar separations. In particular, the use of ammonium citrate resulted in the best peak symmetry.

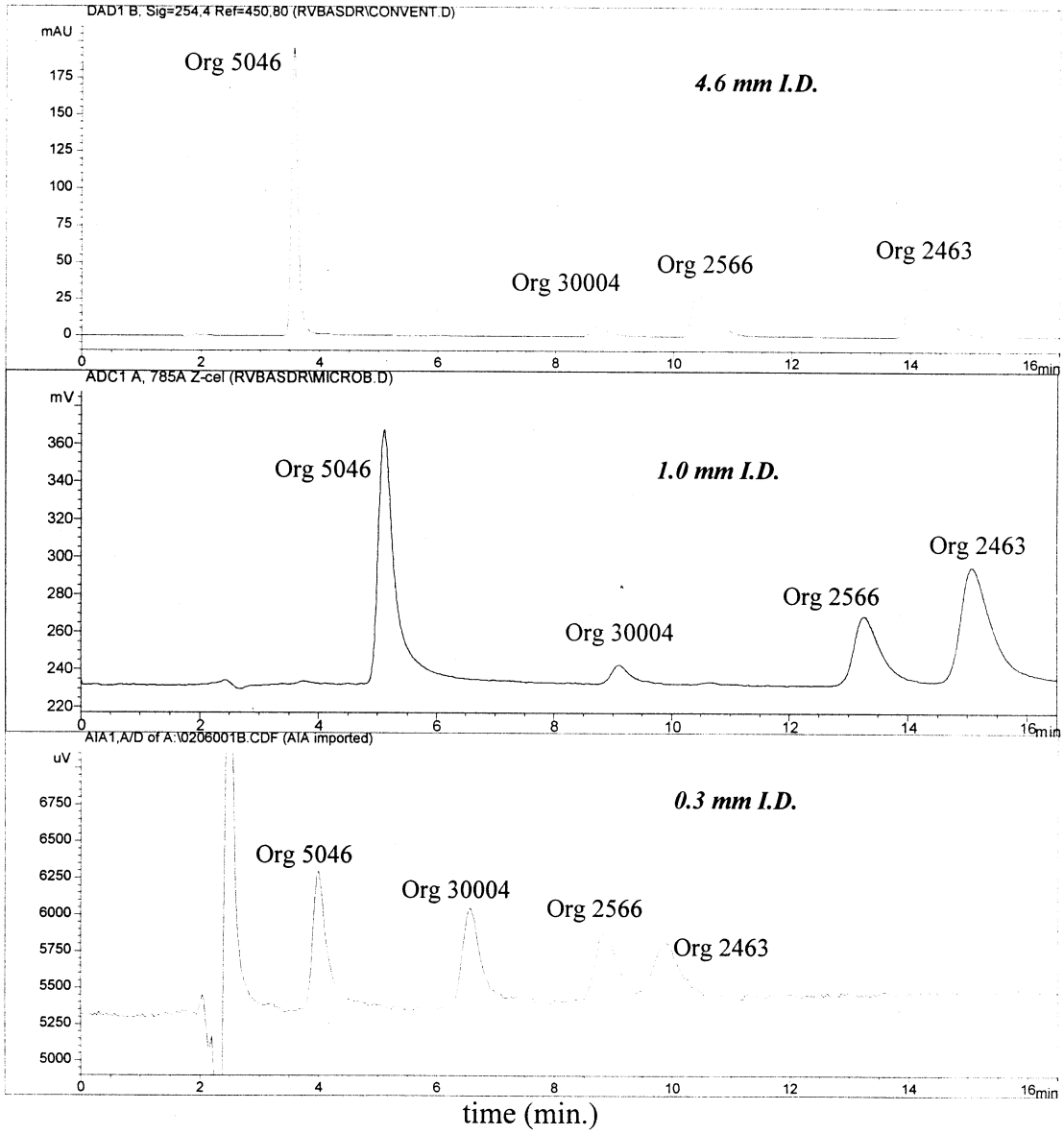

Fig. 9. LC–UV chromatograms of the four basic pharmaceuticals using Prodigy 5 ODS-2 stationary phase and methanol + 50 mM ammonium citrate pH 3/40 + 60 as mobile phase: 0.3, 1.0 and 4.6 mm I.D. columns.

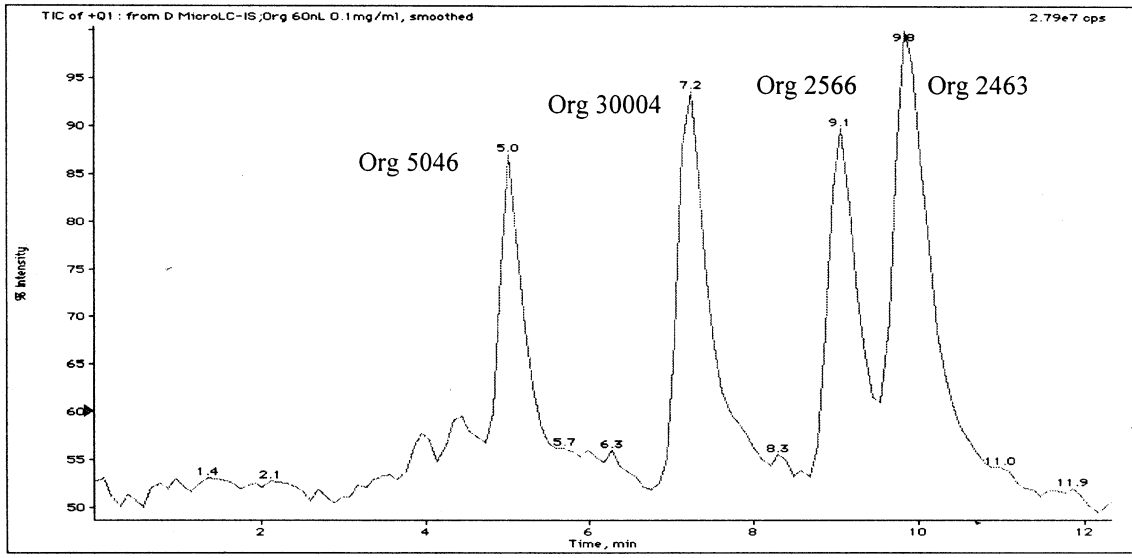

Fig. 10. LC–MS chromatogram of the four basic pharmaceuticals using a Symmetry C_{18} (150 * 0.3 mm I.D.) column and methanol + 50 mM ammonium citrate pH 3/40 + 60 as mobile phase.

References

[1] W.M.A. Niessen, Liquid Chromatography–Mass Spectrometry, second ed., Revised and Expanded, Chromatographic Science Series, Marcel Dekker, New York, 1998.
[2] M.S. Lee, E.H. Kerns, M.E. Hail, J. Liu, K.J. Volk, LC–GC, September 1997, p. 586
[3] S. Gorog, M. Babjak, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, et al., Talanta 44 (1997) 1517.
[4] W.M.A. Niessen, J. Chromatogr. A 794 (1998) 407.
[5] E.R. Verheij, Ph.D. Thesis, Strategies for compatibility enhancement in liquid chromatography mass spectrometry, Leiden University, The Netherlands, 1993.
[6] P. Vouros, E.P. Lankmayr, M.J. Hayes, B.L. Karger, J. Chromatogr. 251 (1982) 175.
[7] J.J. Conboy, J.D. Henion, M.W. Martin, J.A. Zweigenbaum, Anal. Chem. 62 (1990) 800.
[8] A.J.J. Debets, T.J.L. Mekes, A. Ritburg, P.L. Jacobs, J. High Resol. Chromatogr 18 (1995) 45.
[9] C. Gonnet, C. Bouj, G. Rachatze, Chromatrographia 16 (1982) 242.
[10] M.J.J. Hetem, Chemically modified silica surfaces in chromatography. A fundamental study. (Ph.D. thesis, Eindhoven, 1990), ISBN 3-7785-2030-X, Huthig, Heidelberg, Germany, 1993, pp. 175.
[11] M.R.P. Breuer, H.A. Claessens, C.A. Cramers, Chromatographia 38 (1994) 137.
[12] R.J.M. Vervoort, M.W.J. Derksen, F.A. Maris, J. Chromatogr. A 678 (1994) 1.
[13] R.J.M. Vervoort, M.W.J. Derksen, A.J.J. Debets, J. Chromatogr. A 765 (1997) 157.
[14] J.P.C. Vissers, H.A. Claessens, C.A. Cramers, J. Chromatogr. A 779 (1997) 1.
[15] P. Kucera, Microcolumn high-performance liquid chromatography, Journal of chromatography library, vol. 28, Elsevier, Amsterdam, 1984.
[16] J.P.C. Vissers, A.H. de Ru, M. Ursem, J-P. Chervet, J. Chromatogr. A 746 (1996) 1.
[17] B. Malavasi, V. Ascalone, J. High Resol. Chromatogr. 19 (1996) 503.
[18] M.A. Rezai, G. Famiglini, A. Cappiello, J. Chromatogr. A 742 (1996) 69.
[19] H.A. Claessens, M.A. van Straten, J.J. Kirkland, J. Chromatogr. A 728 (1996) 259.
[20] H. Engelhardt, M. Jungheim, Chromatographia 29 (1990) 59.
[21] L. Sander, J. Chromatogr. Sci. 26 (1988) 380.
[22] C.F Poole, S.A. Schuette, Contemporary practice of chromatography, Elsevier, Amsterdam, 1984.
[23] P.J. Schoenmakers, Optimisation of chromatographic selectivity, Journal of chromatography library, vol. 35, Elsevier, Amsterdam, 1986.
[24] H. Engelhardt, M. Arangio, Lecture 54 presented at HPLC'97, Birmingham, 1997.
[25] S.N. Deming, S.L. Morgan, Experimental design: a chemometrical approach, Data handling in science and technology, vol. 11, Elsevier, Amsterdam, 1993.
[26] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics: a textbook, Data handling in science and technology, vol. 2, Elsevier, Amsterdam, 1988.
[27] M. Stadalius, J. Berus, L. Snyder, LC–GC 6 (1988) 494.