Characterisation of stationary phases for the liquid chromatographic analysis of basic pharmaceuticals

CHARACTERISATION OF STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS

PROEFSCHRIFT

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1. INTRODUCTION AND SCOPE

1.1 INTRODUCTION

In the pharmaceutical industry of today development of new drugs in a short period of time is required to improve the treatment of diseases and make it economically beneficial. In the last decade the introduction of new products as well as better insight in diseases and treatment thereof has resulted in increased sales. However, despite these results, research and development of pharmaceuticals still is a long term and high-risk challenge.

Development of new drugs is important since today numerous diseases cannot be treated effectively or sufficiently. Depression, cancer, aids and cardiac diseases are well-known examples in which the development of new drugs can improve the treatment. Inadequate treatment of diseases has both an economic and social effect. As an example, the situation for depression can be taken. Today depression is one of the most common mental disorders, affecting approx. 340 million people in the world. Depression accounts for 10% of productive years lost throughout the world. It occurs to people of all social classes, all countries and all cultural settings. It is estimated that depression costs e.g. the United States approximately $ 53 billion each year. The World Health Organisation predicts that by the year 2020 depression will be the greatest burden of ill-health to people in the developing world, and that by then depression will be the second largest cause of death and disability.

Efficiency and speed of research and development in order to meet growing competition in the pharmaceutical industry is another challenge. Any registered medicine represents approx. 10 years of research and development and an investment of approx. 500 million US dollars.

Table 1.1: World sales of pharmaceutical products by market sector, 1992 - 2002 (millions of U.S. dollars) [1].

					Growth (%/year)	
World total	1992	1996	1997	2002*	1992-1997	1997-2002*
Prescription pharmaceuticals	186,000	251,000	265,000	340,000	7.3	5.1
Over-the-counter drugs	35,000	43,000	45,000	56,000	5.4	4.5
In vitro diagnostics	13,000	18,000	19,000	23,000	6.8	4.8
Total	**234,000**	**312,000**	**329,000**	**419,000**	**7.0**	**5.0**

*: predicted

Moreover, a new product is no guarantee of commercial success: typically in the pharmaceutical industry, only one in four new products brought onto the market recoup its research and development investment [1]. Sales of pharmaceutical products in the last decade have increased, i.e. in 1997 world wide sales of

pharmaceutical products totalled $329 billion, an increase of approx. 6% compared to the sales from 1996. From 1992 to 1997, global sales of pharmaceutical products increased by approx. 40%, which is an annual growth rate of 7%. Moreover, it is estimated that world wide sales will rise to $420 billion in the year 2002. In Table 1.1 the world-wide sales are broken down by market sector: prescription pharmaceuticals, over the counter drugs (obtained without prescription) and in vitro diagnostic products. In Fig. 1.1 the world-wide pharmaceutical products sales are shown by region in 1992 and 1997. As can be seen, each regions share of the total market changed little over the five-year period. North America (in which the biggest market is the United States) has the largest share, followed by Europe, Asia and the rest of the world [1].

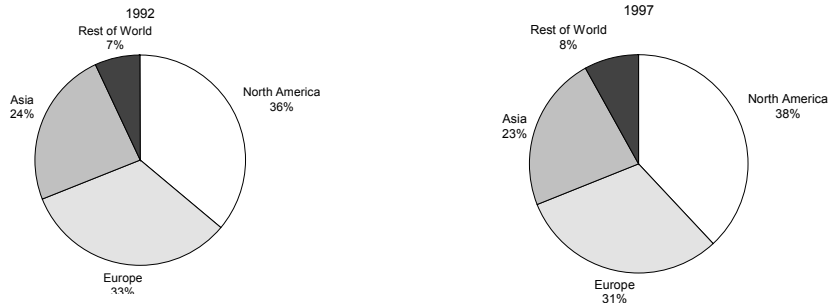

Figure 1.1: Shares of pharmaceutical products by geographical market, 1992 and 1997 [1].

1.1.1 Research and Development of Pharmaceuticals

Research and development of new drugs is a difficult and time-consuming process. Because of the arguments described in the introduction (investment of 500 million $, high-risk) the time pressure on research, development and investment is large. The research and development pathway takes a pharmaceutical compound through three stages: the drug discovery research phase, the pre-clinical development phase and the clinical development phase.

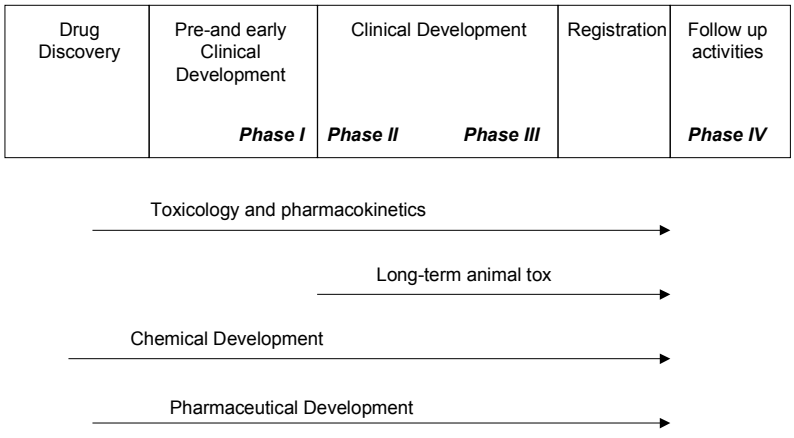

Figure 1.2: Example of a drug research and development pathway [2].

In Figure 1.2 an example of the pathway of drug research and development is shown schematically. The drug discovery research phase primarily involves the design of a biological model. In the pre and early clinical development phase safety is the crucial issue. Also pharmacokinetics, dosage forms and stability are studied in this stage. In the clinical development stage the compound is studied in different phases. In clinical phase I the compound is tested in healthy volunteers given dosages which for safety reasons start much lower on a mg/kg basis than those used to study the safety, pharmacokinetics and pharmacodynamics in animals. After the behaviour of the compound has been assessed in a limited number of volunteers, resulting in a detailed profile, the compound is studied on a larger scale in patients. In Phase II a group of usually 100 – 200 carefully selected patients receive the lead compound in its expected therapeutic dose to establish the efficacy and safety. In Phase III the medication efficacy and safety is compared against placebo and active compounds in typically a group of 3,000 – 5,000 patients who represent the population that will ultimately receive the treatment. This leads on to submitting detailed files to registration authorities with a request for marketing authorisation. In Phase IV the registration of the medication is followed with a view to identify rare, unforeseen side effects in a real clinical situation by means of post-marketing surveillance, cost effectiveness and real-life efficacy studies [2].

1.1.2 Analytical Chemistry in Pharmaceutical Research and Development

Development of a pharmaceutical product involves both the optimisation of the chemistry and manufacturing, as well as determination of the bio-medical profile. The quality of drug substances (active compound) and drug products (e.g. tablet, liquid for injection) is determined by the design, development, and in-process control. Additionally, tests, procedures and acceptance criteria, play a major role in assuring the quality. Establishment of global tests and specifications for drug substances and drug products is described in guidelines [3-6]. The bio-medical profile is determined through e.g. pharmacokinetic and metabolism studies.

These development activities are supported by analytical techniques which can be divided into specific (on a case by case basis) and universal (generally applicable) techniques. Table 1.2 shows a selection of techniques applied to characterise drug substances and drug products. As can be seen separation methods play an important role in determining impurities and active compound(s) and, coupled on-line with spectroscopic techniques structural information can be obtained. Limits for the presence of impurities in drugs are established by the International Conference on Harmonisation (ICH) which is active in the coordination of technical requirements for the registration of pharmaceuticals in the United States, Europe and Japan [7,8]. Table 1.3 shows an extract of these ICH guidelines with respect to impurity identification limits. From a development point of view it is advantageous to know the chemical structure of impurities in an early stage of development.

Table 1.2: Selection of tests for drug substances and drug products [3].

	Test parameters
Drug substance	physical chemical properties; pH in solution, melting point, refractive index, pKa, log P
	particle size distribution
	polymorphic forms; differential scanning calorimetry (DSC), thermogravimetry (TGA), solid state nuclear magnetic resonance spectrometry (SS-NMR), infrared spectrometry (IR), X-ray powder diffraction
	identity; ^{1}H-NMR, ^{13}C-NMR, mass spectrometry (MS), infrared spectrometry (IR), LC-UV (diode array), LC-MS, GC-MS, CE-MS
	content inorganic impurities; atomic absorption spectrometry (AAS), ion chromatography (IC)
	content active compound; LC-UV, GC-FID, CE-UV, titration
	content organic impurities; LC-UV, GC-FID, CE-UV
	residual solvents; GC-FID
	content water; Karl-Fisher titration, loss on drying
Drug product	physical properties; pH, visual inspection of particulates, functionality testing of delivery system, osmolarity, dissolution, disintegration, hardness
	sterility; antimicrobial preservative content, antioxidant preservative content,
	identity; LC-UV (diode array), LC-MS, GC-MS, CE-MS
	content active compound; LC-UV, GC-FID, CE-UV
	content impurities; LC-UV, GC-FID, CE-UV
	residual solvents; GC-FID

Furthermore, especially for the analysis of samples from e.g. metabolism studies, separation methods are coupled on-line with techniques such as mass spectrometry for selective and sensitive detection.
From the separation methods, in the pharmaceutical industry reversed phase liquid chromatography (RPLC) is most widely applied because of the broad range of compounds that can be analysed, i.e. non-ionic, ionisable and ionic compounds [9]. Moreover, when a pharmaceutical product is on the market, LC is the most widely used analytical technique in quality control to determine the identity and content of drugs in production batches.

1.1.3 Liquid Chromatography in Pharmaceutical Development

In the pharmaceutical industry, LC has become an indispensable tool. From the initial development to the routinely use in quality control, LC is applied to analyse pharmaceutical compounds. For a pharmaceutical company like NV Organon, in The

Netherlands (approx. 2500 employees in the Netherlands) approx. 200 LC systems are operating.

Table 1.3: Table extracted from ICH guidelines on identification of impurities [7,8]

Maximum daily dose	***Identification threshold***
Drug substance	
< 2 g/day	0.1% or 1 mg per day intake (whichever is lower)
> 2 g/day	0.05%
Drug product	
< 1mg	1% or 5 μg TDI*, which ever is lower
1 mg – 10 mg	0.5% or 20 μg TDI, which ever is lower
> 10 mg – 2 g	0.2% or 2 mg TDI, which ever is lower
> 2 g	0.1%

*: TDI: Total Daily Intake

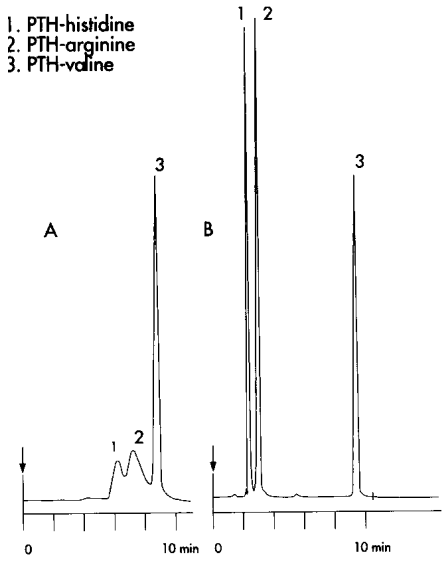

Figure 1.3: Effect of asymmetrical (A) and symmetrical peaks (B) on separation and quantitation of PTH-histidine, PTH-arginine and PTH-valine. Reprinted from ref. [9].

The applicability of LC in pharmaceutical development and quality control can be subdivided into two areas, i.e. quantitative and qualitative analysis.

Both for quantitative and qualitative analysis it is important to develop efficient separations with narrow symmetrical peaks since in daily practice symmetrical peaks are a prerequisite for high sensitivity and resolution. In Fig. 1.3 the influence of asymmetrical (A) and symmetrical peaks (B) on a chromatographic analysis is shown. As can be seen in Fig. 1.3A sensitive analysis and resolution is hampered by the asymmetrical shape of peaks 1 and 2.

In quantitative analysis, the contents of pharmaceutical analytes are determined in batches of drug substance, in drug products, in samples from metabolism studies, in samples from stability studies etc. Besides the influence on the separation, the peak shape also influences the determination of the peak area. Integration of asymmetrical peaks is difficult because determination of the end of a peak is more difficult [10,11]. This results in more dispersion of the analytical data reflected by a higher relative standard deviation, and thus a less accurate determination of the contents of analytes. Moreover, measurement of a narrow symmetrical peak requires 20-30 data points, whereas measurement of a broad asymmetrical peak may require as many as 350 data points to achieve 0.1% accuracy [12]. In qualitative analysis drugs are often screened for impurities using hyphenated techniques. LC-UV and LC-MS are used routinely [13], the on-line coupling of LC with NMR is receiving increasing attention [14-16], whereas also LC-IR can be used [17]. Acquisition of UV, MS, IR and NMR spectra of eluting peaks will improve with increasing efficiency and resolution.

In the development of LC methods for basic pharmaceutical compounds the peak shape is often problematic. Many pharmaceutical compounds contain basic nitrogen atoms in the molecular structure, e.g. central nerve system drugs, cardiovascular drugs, amino acids, peptides, proteins etc. These basic nitrogen atoms can interact with the silica backbone of reversed phase packing materials, which are commonly used in LC. Therefore, besides the reversed phase interaction also ionic and other polar interactions can occur between the analyte and the packing material, which can result in asymmetrical peaks and irreproducible retention [18]. Optimisation of the peak shape of basic analytes can be obtained through optimising the mobile phase, and/or selection of a suitable stationary phase. Optimisation of the mobile phase involves the selection of a suitable eluent pH, addition of silanol blockers to the eluent and selection of a suitable buffer. Selection of a suitable stationary phase generally means selection of a phase with low polar sites-analyte interaction, responsible for asymmetrical peaks. As discussed before LC is used for quantitative (determination of content) and qualitative (detection of impurities) analysis. For the identification or structure elucidation of compounds LC is often coupled on-line to spectroscopic techniques (hyphenated techniques). In hyphenated techniques very often conflicts occur, e.g. buffers favourable for the LC analysis often cannot be applied in combination with MS, whereas buffers favourable for the MS often result in asymmetrical peaks. This contradiction demonstrates the necessity for good

stationary phases, were the demands for the buffer are less, enabling the development of on-line LC-MS separations for basic compounds with acceptable peak shapes.

1.1.4 Liquid Chromatography Theoretical Aspects

LC is a method of separation in which the analytes to be separated are distributed between two phases: a stationary phase and a mobile phase. The chromatographic process occurs as a result of repeated sorption/desorption steps during the movement of the sample components along the stationary phase. The separation is due to the differences in interaction of the individual sample components with the stationary phase [9]. The quality of the chromatographic separation is given by the resolution (Rs) and can be calculated using:

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{k_2 + 1}\right) \quad \{1\}$$

in which N is the plate number, α is the selectivity and k_2 is the retention factor of the later eluting peak of a peak pair.

The efficiency of a separation system, expressed as the plate number (N), can be calculated using the half width method:

$$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \quad \{2\}$$

in which t_R is the retention time and $w_{0.5}$ the width at half height of the peak. It is then assumed that the chromatographic peak elutes from the column as a symmetrical Gaussian peak [9]. In daily practice, however, the peak shape of a chromatographic peak is only in few cases symmetrical. Especially for compounds containing basic nitrogen atoms often asymmetrical peaks are obtained with RPLC stationary phases. To calculate chromatographic parameters from non-Gaussian peaks, several methods have been developed based on the Exponentially Modified Gaussian (EMG) model [19-21]. From these, the method developed by Foley and Dorsey can be applied by using data easily obtained from the chromatographic peak [21]. The method is developed to calculate the efficiency of asymmetrical peaks, taking into account the peak asymmetry, approximating the true plate number, N [9]. The equation is:

$$N = \frac{41.7\left(t_R / w_{0.1}\right)^2}{B/A + 1.25} \quad \{3\}$$

in which t_R is the retention time, $w_{0.1}$ the width of the peak at 10% of the peak height, and A and B are the front and the rear side of the peak at 10% of the peak height, respectively. Since the width of the peak affects the plate number, it also affects the resolution.

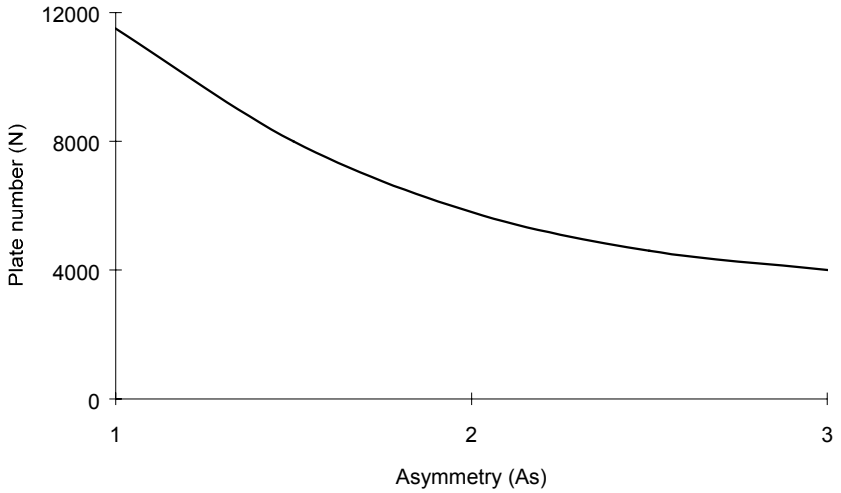

Figure 1.4: Relation between peak asymmetry (As) and efficiency (N) simulated using the Foley & Dorsey equation. t_R= 10 min, A = 0.2 min. and $w_{0.1}$=A+B.

In Fig. 1.4 the relation between peak asymmetry and efficiency is shown using the Foley & Dorsey equation for N: a higher As results in lower N.
Determination of the shape of a chromatographic peak can be performed using various methods. Generally, the asymmetry factor, which is determined at 10% of the peak height, is used. However, especially in the pharmaceutical industry often the tailing factor calculated according to the United States Pharmacopeia (USP) is used, which is determined at 5% of the peak height [4,22]. In Fig. 1.5 both the asymmetry factor (As) and the USP tailing factor (Tf) are shown. It is clear that both the asymmetry factor and the tailing factor are closely related. For a Gaussian symmetrical peak both the asymmetry and tailing factor are 1.0. The relation between As and Tf is given by [23]: Tf=0.6As+0.4

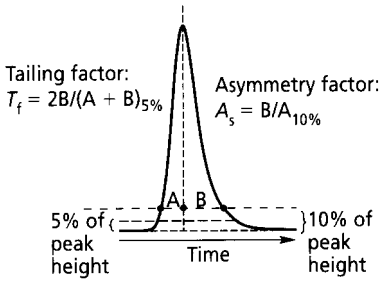

Figure 1.5: Asymmetry factor (As) and USP tailing factor (Tf). Reprinted from ref. [22].

1.2 SCOPE OF THIS THESIS

In the development of a LC separation method, the choice of a suitable column is very important. World-wide approx. 600 different stationary phases are commercially available, from which the analyst can choose [24]. Therefore the choice can be difficult and is often based on experience and expertise of the analyst. Due to the varying column manufacturing procedures, including various types of ligands and end-capping procedures, differences exist between the various brands of stationary phases. Moreover, also between brands of nominal identical phases differences exist, e.g. between various C_{18} phases [25-27]. The advantage of the various columns is that they can be used to solve diverse separation problems. A disadvantage is that analysts do not recognise that nominally identical materials often show very different chromatographic properties.

Today the RPLC analysis of basic solutes in many cases still is problematic. Due to interaction between the analyte, and residual silanols and eventual other polar sites, asymmetrical peaks and irreproducible retention can be obtained. To improve the peak shape both the mobile and stationary phases can be optimised to reduce ionic and other polar interactions. Optimisation of the mobile phase can be achieved by using a suitable eluent pH or addition of silanol blocking compounds. Optimisation of the stationary phase can be achieved by minimising the presence of accesible residual silanols and eventual other polar sites responsible for asymmetrical peaks. This has resulted in the development, improvement and manufacturing of many new stationary phases. The main problem for the analyst now is to select phases suitable to analyse the basic compound of interest from the enormous number of phases commercially available. The properties of the stationary phase substantially influence the chromatographic performance, e.g. retention and plate number, but especially peak shape of basic compounds. Therefore, one column is better suited to analyse basic analytes than another column. Moreover, chemical stability of the stationary phases as well as reproducibility of batches of packing material and columns are also of great importance for the development of robust and reproducible analysis methods. Many test procedures have been suggested to test RPLC columns. In addition, testing columns with respect to selecting a suitable column [28-33], determination of chemical stability [34-37] and determination of batch-to-batch and column-to-column reproducibility [22,38-40] can require different approaches. However none of these procedures gained broad acceptance.

To classify stationary phases for the LC analysis of basic analytes, in the literature various tests are described, which can be divided into *i*: empirical test methods [28-30], *ii*: thermodynamically based test methods [32] and, *iii*: methods based on a retention model [31,33]. In this thesis tests for the selection of suitable columns for the analysis of basic pharmaceutical compounds are evaluated. Also the influence of column-to-column and batch-to-batch reproducibility of the stationary phases on the analysis of basic pharmaceuticals is described.

In **chapter 2** the current status of RPLC analysis of basic pharmaceuticals is reviewed. Options to optimise the analysis are discussed, i.e. eluent optimisation (pH, silanol blockers) and optimisation of the stationary phase (development of new columns with minimised ionic and other polar interactions responsible for asymmetrical peaks). The applicability of empirical based, thermodynamically based and test methods based on a retention model to characterise stationary phases is discussed. The influence of eluent composition on the analysis of basic compounds as well as the applicability of chemometrical techniques to classify stationary phases is shown.

The development and applicability of an empirical stationary phase test method is described in **chapter 3.** This method was developed to enable the selection of suitable stationary phases for the RPLC analysis of basic pharmaceuticals of NV Organon. In section 3.1 the analysis of 32 basic pharmaceuticals using various stationary and mobile phases is discussed. The compounds differed in basicity, polarity, number of nitrogen atoms and type of nitrogen atoms. In section 3.2 the chemometrical analysis of the data described in section 3.1 is discussed. The chemometrical analysis allowed a selection of a test mix of 5 drugs which can be taken as representative of 32 drugs. Chemometrical techniques were used to distinguish between the columns. In section 3.3 the applicability of principal components analysis is shown to graphically present various RPLC columns.

Chapter 4 describes the applicability of previously discussed test procedures to select stationary phases for the RPLC analysis of basic pharmaceuticals. In section 4.1 the applicability of various empirical tests is described. The influence of mobile phase composition, i.e. nature of buffer and modifier, on the chromatographic performance (retention and peak shape) is shown. In section 4.2 a thermodynamically based test method is used to determine interactions between basic analytes and the stationary phase, and if possible to classify RPLC columns. Section 4.3 shows the applicability of quantitative structure-retention relationships (QSRRs) to characterise RPLC stationary phases.

In **chapter 5** the transfer of LC methods developed with conventional RPLC columns to methods with microcolumns to improve on-line LC-MS analysis is discussed. In section 5.1 the influence of several common volatile buffers used with various column diameters for the on-line LC-MS analysis of basic analytes is described. Section 5.2 shows the influence of column hardware, column-to-column and batch-to-batch reproducibility on retention and peak shape of basic analytes.

In **chapter 6** concluding remarks and future perspectives for column characterisation procedures are given.

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2. OPTIMISATION AND CHARACTERISATION OF SILICA BASED REVERSED PHASE LIQUID CHROMATOGRAPHY SYSTEMS FOR THE ANALYSIS OF BASIC PHARMACEUTICALS

2.1 SUMMARY

Reversed phase liquid chromatography using silica-based columns is successfully applied in many separations. However, also some drawbacks exist, i.e. the analysis of basic compounds can be hampered by ionic and other polar interaction of the basic analytes with residual silanols and eventual other polar sites present on the silica surface, which can result in asymmetrical peaks and irreproducible retention. In this chapter, options to optimise the LC analysis of basic pharmaceutical compounds are discussed, i.e. eluent optimisation (pH, silanol blockers) and stationary phase optimisation (development of new columns with minimised ionic and other polar interactions responsible for asymmetrical peaks). The applicability of empirical, thermodynamically based and test methods based on a retention model to characterise silica-based reversed phase stationary phases, as well as the influence of the eluent composition on the LC analysis of basic substances is described. Finally, the applicability of chemometrical techniques in column classification is shown.

R.J.M. Vervoort, A.J.J. Debets, H.A. Claessens, C.A. Cramers and G.J. de Jong, J. Chromatogr. A, 897 (2000) 1-22.

2.2 INTRODUCTION

World-wide approx. 75% from the total amount of liquid chromatographic (LC) analysis is performed using the reversed phase mode, i.e. using C_8 or C_{18} modified silica columns [1]. Especially in the industry the large majority of chromatographers are using LC systems of conventional size, i.e. columns with internal diameters of 4 - 5 mm and 10 - 25 cm length. The broad range of compounds that can be analysed, i.e. non-ionic, ionisable and ionic compounds has caused this widespread applicability. Other main contributions are the short equilibrium times, the possibility to perform gradient analysis and the possibility to use water rich eluents and samples [2]. Although applied in most chromatographic separations, reversed phase (RP) LC has also some drawbacks. Many pharmaceutical compounds contain basic nitrogen atoms, e.g. central nerve system drugs, cardiovascular drugs, amino acids and peptides. The ability of basic nitrogen atoms to interact with residual silanols and eventual other polar sites on the packing material can hamper the RPLC analysis of these compounds and asymmetrical peaks and irreproducible retention can be the result. The use of different packing materials often results in different chromatographic performance. Since world-wide approx. 600 different brands of packing material are available, differences exist between type of ligand, end-capping, type of silica, residual silanols, bonding density, pore size etc. [3-5]. Analysts often do not recognise that a large group of sometimes nominally identical materials often show very different chromatographic properties [6]. Moreover the nomenclature of RPLC stationary phases is not clear and therefore confusing. It is obvious that test procedures enabling a well considered selection of a column are necessary to select a column to solve a specific separation problem and to develop reliable and validated analysis protocols. However, also for test procedures, various approaches are available each with their advantages and disadvantages. As with the choice of the optimal column, the chromatographers get confused which test method to select to characterise columns. In the literature empirical, thermodynamically as wel as test methods based on retention models have been described [7-11], whereas also test methods specially developed for specific classes of compounds have been reported [12,13].

In this chapter the RPLC analysis of basic pharmaceutical compounds is discussed. First, the optimisation of the mobile phase is discussed. Secondly, optimisation of stationary phases with respect to the preparation of silica and the manufacturing of modern packing materials optimised for the analysis of basic pharmaceutical compounds, is described. Thirdly, various column characterisation procedures, divided into empirical methods, thermodynamically based methods, and model based methods, are discussed for the applicability in classifying columns. Fourthly, the influence of the eluent composition on eluent pH, and on pKa of the basic analytes is shown. Finally, the use of chemometrical techniques to analyse the data obtained

when classifying columns, enabling categorisation of columns with equal characteristics, is discussed.

2.3 APPLICABILITY OF LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF BASIC PHARMACEUTICALS

2.3.1 Silica based packing materials

Compounds with a basic nitrogen atom in the chemical structure often cause problems when analysed with RPLC. Asymmetrical peaks, irreproducible retention and non-robust separating methods can be obtained. Depending on the pKa of the analyte and the pH of the eluent, basic nitrogen atoms can be protonated. Ionic and other polar interactions can occur between basic compounds with residual silanol groups and eventual other polar sites of the stationary phase. As an example equation {1} shows the ion-exchange interaction:

$$XH^+ + SiO^-Na^+ \Leftrightarrow Na^+ + SiO^-XH^+ \quad \{1\}$$

Thus, besides the reversed phase retention mechanism also ionic and other polar retention mechanisms occur which can result in distorted peak shapes. Asymmetrical peaks can be explained in terms of kinetic phenomena, i.e. if the mass transfer of one type of column site is slower than from the other. Fornstedt et al showed that the most pronounced peak tailing occurs when the slow sites provide a smaller contribution to retention than the fast ones and if the rate constant of mass transfer for the slow sites is between 20 and 2000 times smaller than that of the fast sites [14]. For basic analytes, the ionic and other polar interactions with silanol and eventual other polar groups may be slower than those with the alkyl ligands, giving rise to peak tailing. Moreover, overloading of ionised silanols by the basic solute can occur which also will contribute to tailing [15].

To improve the peak shape, optimisation of the mobile phase can be considered. Several approaches are suggested to reduce the interaction between analyte and silanols groups on the column packing [16]:

- selection of a suitable mobile phase pH, i.e. pH < $pKa_{silanol\ groups}$, or pH > $pKa_{basic\ analyte}$, to suppress ionisation of either the silanol groups or the basic analytes,
- addition of a silanol blocker to the eluent, i.e. $pKa_{silanol\ blocker}$ >> $pKa_{basic\ analyte}$, the protonated silanol blocker will strongly interact with residual silanols allowing the basic compound to interact solely with the alkyl ligand of the stationary phase, i.e. hydrophobic interaction,
- increasing the ionic strength of the mobile phase in order to drive the reaction in the equation to the left and/or replacement of Na^+ by more strongly retained ions such as K^+, Rb^+ etc., and,
- reduction of the sample concentration to alleviate saturation of the acidic sites.

Improvements of the stationary phase can also lead to improved peak shapes for basic analytes. As a result, a new generation of newly developed stationary phases is available and still increasing. Today analysts can choose a column from a large number of stationary phases [3,4,17]. Stationary phases specially developed for the analyses of basic compounds are phases in which the interaction between basic analytes, and residual silanols and eventual other polar sites, responsible for asymmetrical peaks, are minimised. In the literature various approaches of stationary phase manufacturing have been described:

- High purity silica [18-21]: conventional silica gels contain approx. 10 ppm of trace metals. However, the level of trace metals may differ from batch to batch. Residual metals can influence the acidity of adjacent silanols, and thus indirectly the interaction between silanols and basic analytes. Removal of metals can be achieved via extensive acid treatment of the silica before preparation of the alkyl modified stationary phase. Using the acid treatment, generally two thirds of the metals can be removed still leaving significant amounts of metals in the silica.
- End-capping procedures [22-25]: preparation of alkylsilalyted silica's means that approx. 50% of the silanols reacted, meaning that still approx. 50% of the silanols are left. Removal of some of these silanols can be achieved by end-capping, i.e. reaction of short chain alkylsilanes with these silanols. Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) are known as suitable end-capping agents. HMDS was found to be suitable to end-cap stationary phases for the analyses of bases, whereas TMCS was found to be suitable to end-cap stationary phases for the analysis of acids. It was shown that end-capping of stationary phases could be performed efficiently and sharp peaks for some basic drugs were obtained using these stationary phases [26].
- Polymer encapsulation [27-30]: coverage of the silica with a thin layer of organic silicone polymer and subsequent introduction of long alkyl chains. The polymer coating process solves problems generating from the original silica surface, i.e. an inert surface free from residual silanols and metal impurities. The inertness of such a polymer encapsulated stationary phase was demonstrated showing good peak profiles of pyridine (residual silanols) and hinokitiol (metals). However, from NMR studies it was concluded that after the coating still residual silanols were present [31]. The polymer encapsulated stationary phase was found to be stable when using a mobile phase containing borate buffer pH 10.
- Horizontal polymerisation [32-34]: with this method of stationary phase synthesis a high density is achieved at the silica surface by mixing long chain and short chain trifunctional silanes. Horizontal polymerisation refers to the notion that there is significant Si-O-Si bridging parallel to the silica substrate. The reaction of trifunctional silanes is performed in an anhydrous environment, except for a monolayer of water on the silica. A humidity of approx. 50% was found to be optimal for stationary phase synthesis. Phases prepared by horizontal

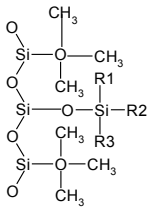
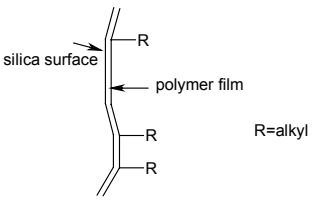
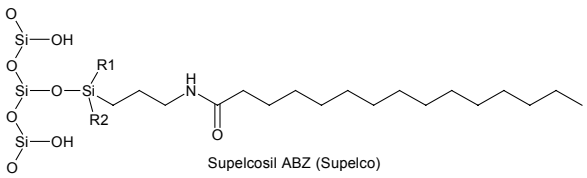
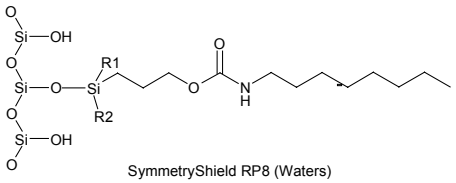
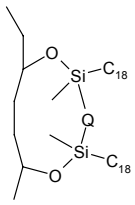
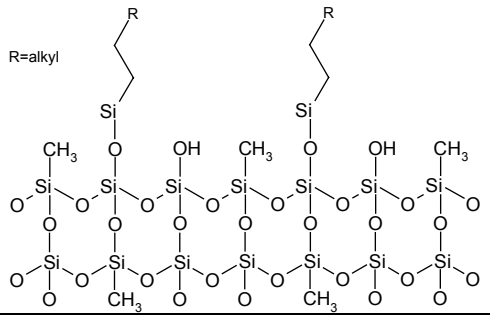
polymerisation were found to be stable at acidic (at least 2400 column volumes at pH 1.8) and basic (at least 900 column volumes at pH 10.0) eluent conditions.

- Embedded polar groups [5,35]: a stationary phase is obtained showing different selectivity compared to ordinary alkyl based stationary phases. The polar function shields the silica surface, preventing the interaction of analytes with residual silanols and other active sites on the silica surface. An additional advantage is that stationary phases with embedded polar groups can be used with eluents containing high amounts of water, without the problem of folding of the stationary phase chains.
- Bidentate stationary phases [21,36]: main advantage of bidentate stationary phases, compared to ordinary alkyl phases, is column stability. It is shown that C_{18}/C_{18} bidentate synthesized columns with a propylene bridge exhibit excellent stability at low and intermediate pH. However, the main advantage is the excellent stability of bidentate phases especially at high pH. This means that basic analytes can be analysed using a mobile phase pH > $pKa_{basic\ analyte}$. Analysing basic analytes as uncharged compounds will reduce the interaction with residual silanols. Other advantages of analysing bases at high pH are situations in which the analyte is unstable at low pH, the desired selectivity cannot be obtained at low pH, and at low pH protonated basic compounds are poorly retained.
- Stationary phases prepared using surface modified silica [37,38]: with this type of stationary phases the silica backbone is modified, resulting in a reduction of residual silanols. In preparing the silica, organic functional groups have become a constituent of the silica backbone as well as of the surface. Compared to ordinary silicas, a part of the OH groups have been replaced by CH_3 groups. After introduction of the alkyl chains, for the ordinary phases the ratio between OH and alkyl chains is 1:1. Using the surface modified silica, the ratio is 1/3 OH, 1/3 alkyl chain and 1/3 CH_3, leading to a reduction of residual silanols. Another advantage is the improved pH stability, i.e. this phase has been used up to mobile phase pH 12 showing good stability.
- Monolithic silica phases [39,40]: these phases are prepared according to a new sol-gel process, based on hydrolysis and polycondensation of alkoxysilanes in the presence of water soluble polymers. The method results in silica rods made of a single piece of porous silica with a defined pore structure. Compared to conventional silica based columns, the porosity of silica rod columns is approx. 15% higher. Moreover, silica rod columns maintain high performance at high flow rates. Recently silica rod columns are commercially available, allowing LC analysis at high flow rates and thus short analysis times. The applicability of silica rod columns in preparative analysis was also shown [41]. Due to the high efficiencies obtained at high flow rates, high throughput and productivity was

demonstrated. The applicability of silica rod columns in the analysis of basic pharmaceuticals to our knowledge sofar has not been reported in the literature.

In Table 2.1 some of the structures of the stationary phases described are shown.

Table 2.1: Structures of silica based stationary phases specially developed for minimised interactions with residual silanols.

endcapping	
polymerisation	
embedded polar groups	 Supelcosil ABZ (Supelco)  SymmetryShield RP8 (Waters)
bidentate	
surface modified silica	

2.3.2 Preparation of silica based reversed phase packing materials

The manufacturing procedure and quality of the silica used to synthesise reversed phase packing materials ultimately determine amongst other parameters the quality of the stationary phase. The silica used for column packings is porous and non-crystalline with the general formula $SiO_2 \cdot xH_2O$, and water is chemically bound forming Si-OH bonds which is most important for use in LC packing materials [42-45]. These silanol groups and siloxane bridges (Si-O-Si) compose the surface of silicas. In Fig. 2.1 the possible types of surface silanols are shown, i.e. isolated, geminal and vicinal silanols, together with siloxane bridges. The silanol groups are hydrophilic, i.e. they can interact with polar groups, whereas the siloxane bridges posses slightly hydrophobic properties [42]. In chromatography the silica particles today in most cases are spherical and the main characteristics of silica as chromatographic material are particle size and specific area (typically in the range of 200 - 400 m^2g^{-1}) [46].

The concentration of surface silanols of a fully hydroxylated surface is approx. 8 $\mu mol/m^2$. Modification of the silica with C_{18} or C_8 results, due to steric hindrance effects, in the reaction of approx. 50% of the silanols, leaving approx. 4 $\mu mol/m^2$ of residual silanols. For the silanols present, isolated silanols can be responsible for strong, undesired interactions with basic compounds in RPLC [47,48]. Furthermore, the existence of clusters of residual silanols on the silica surface results in a small population of silanols, which are able to strongly interact with bases. This population is less than 1% of the total amount of residual silanols [49].

Depending on the manufacturing route of the silica, two types of silica can be distinguished which can be defined by their different physical and chromatographic properties. Particles made by gelling soluble silicates are characterised by higher

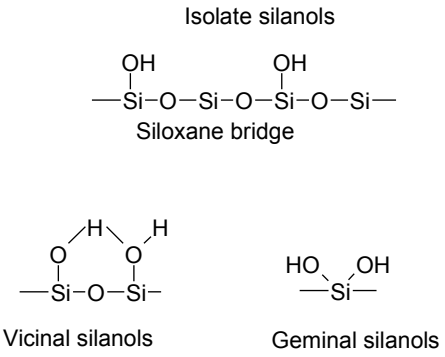


Figure 2.1: Types of surface silanols.

surface areas, higher porosities and irregular pore shapes with variable wall thickness, and are named SilGel silicas. Particles made by aggregating silica-sol particles have lower surface areas, lower porosities, and more regular pores with thicker walls defined by surrounding silica sol micro particles, and are named SolGel

silicas [50]. In LC, the majority of separations is performed in the reversed phase mode, i.e. an apolar stationary phase with a polar mobile phase. Manufacturing of the reversed phase packing material is generally performed by reaction of an organosilane with a silanol group at the silica surface yielding octyl (C_8) or octadecyl (C_{18}) modified packings. Both SolGel and SilGel type silicas are used to manufacture these reversed phase packing materials. Comparison of stationary phases prepared from both silicas showed that phases prepared from SolGel silicas are more durable, compared to phases prepared from SilGel silicas [51].

2.3.3 Alternative solutions

Besides silica based reversed phase packing materials, also alternative solutions have been proposed. The use of bare silica in combination with a reversed phase like mobile phase showed acceptable performance [52,53]. Main disadvantage of this option is the disability to use gradient elution. Other alternative solutions are the use of columns based on alumina, titanium oxide or zirconium oxide. Main advantage of these materials is the, compared to silica, better pH stability, i.e. approx. from pH 0 to 13. This enables the chromatography of strong bases as unprotonated compounds [54,55]. However, with these packings also secondary retention mechanisms occur. Polymer based column packings are also pH stable, e.g. for a polystyrene-divinylbenzene packing from pH 0 to 14. Main disadvantages, however, are the limited pressure resistance, hindered mass transfer and swelling/shrinking properties [5]. Using polymer based columns, symmetrical peaks were obtained for basic analytes, however, due to the hindered mass transfer properties a reduction in plate number must be taken into account [13]. Taking the drawbacks of the alternative solutions into account, for many applications silica based packing materials are still preferred.

2.4 CHARACTERISATION OF SILICA BASED PACKING MATERIALS

A disadvantage of the large number of columns available is how to select the optimal column for the chromatographers application. Classification of columns into groups with equal characteristics would make the choice of a column easier. Most characterisation studies have been performed using either spectroscopic or chromatographic methods.

Spectroscopic characterisation of stationary phases is performed using IR and NMR spectroscopy. Bonded phase, silanols and silanes on the solid support can be identified and information about the type of bonding can be qualitatively obtained using diffuse reflectance infrared fourier transform IR [56]. The various types of silanols (isolated, geminal and vicinal) can be determined using ^{29}Si solid state NMR, whereas the type of bonding (mono, di or trifunctional) and type of endcapping can be determined using ^{13}C solid state NMR [57]. Hetem showed the applicability of various ^{29}Si NMR techniques to study the chemical modifications of non-porous and

porous silica supports, as well as the stability of various modified silicas under conditions normal for daily chromatographic practice [31]. Scholten presented evidence of the decreased hydrogen bonding of residual silanols of di-isobutyl-n-octadecylsilane derivatized silica (Zorbax Stable Bond phases), compared to dimethyl-n-octyl derivatized silica, using NMR. This illustrated the increased steric protection of the ligand siloxane bonds, which is assumed to be the reason for improved hydrolytic stability in aqueous mobile phases with low pH [58].
A disadvantage of spectroscopic techniques, however, is that they can only be used to determine bulk properties of the stationary phase. Since especially in relation to the analyses of basic compounds subtle differences between the phases can have a large impact on the shape of the chromatographic peak, a chromatographic characterisation is preferred. Another advantage is that the stationary phases can be tested in the same condition as they are used in daily practice, i.e. as packed columns. The majority of chromatographic methods used to characterise silica based reversed phase stationary phases determine roughly two stationary phase properties, i.e. hydrophobic and polar properties. The chromatographic characterisation of stationary phases can generally be divided into three classes:

- Empirical evaluation methods: this section can be subdivided into two groups: *i*: evaluation based on chromatographic data of test compounds chosen according to a certain line of thought, and *ii*: evaluation based on chromatographic data of dedicated test compounds. In the case of testing columns for the applicability in analysing basic compounds, basic compounds are used as test probes;
- Thermodynamically based methods: the obtained information is based on studying enthalpies and entropies of transfer of solutes from the mobile to the stationary phase;
- Evaluation methods based on a retention model: the obtained information is based on a specific retention model such as Quantitative Structure Retention Relationship (QSRR) studies.

In this chapter various methods for characterising columns with respect to the analysis of basic pharmaceutical compounds are discussed.

2.4.1 Empirical evaluation methods

2.4.1.1 Hydrophobic Properties of Stationary Phases

With respect to the determination of hydrophobic properties, most tests described in the literature are based on retention of benzene derivatives. To determine hydrophobic stationary phase characteristics Tanaka et al [7] used amylbenzene and butylbenzene, Walters et al [8] used anthracene and benzene, Engelhardt et al [9] used toluene and ethylbenzene, Gonnet et al [59] used benzene and toluene, Eymann [60] used nitrobenzene and toluene, and Neue et al [61] used toluene, acenaphtene and naphtalene. Although the experimental part of these test methods

is comparable, the calculations are not unambiguous. Determination of amount of alkyl chains, carbon content, hydrophobicity, hydrophobic selectivity and surface coverage are mentioned. In several cases different stationary phase characteristics were determined. Hydrophobicity, which can be considered as the retentivity for compounds, based on interactions between the compound and the ligand on the silica, is calculated as the relative retention of two benzene derivatives by some researchers [7-9]. However, relative retention values of many reversed phase columns were found to be comparable, whereas difference in % carbon load, ligand length and applied bonding chemistry must lead to different hydrophobicity [6]. Other researchers use the retention values of aromatic hydrocarbons as a measure of hydrophobicity [62,63]. Recently a good correlation between the absolute retention values of the aromatic hydrocarbons of the Engelhardt and Tanaka test was obtained, resulting in column classification independent of the applied test. Furthermore, it was concluded that the relative retention values should be considered as methylene selectivity i.e. the ability of a given stationary phase to separate structurally closely related compounds [63].

2.4.1.2 Silanol Activity of Stationary Phases

Determination of the silanol activity of stationary phases is important for the analysis of basic compounds. Determination of silanols activity of reversed phase stationary phases is, compared to hydrophobic properties, a more difficult topic. As reviewed by Nawrocki, silanol activity comprises a number of polar stationary phase - solute interactions such as ion-ion (ion exchange), ion-dipole, dipole-dipole (e.g. hydrogen bonding), dipole-induced dipole and induced dipole-induced dipole (London forces) [49]. Since large differences between packing materials exist due to different manufacturing processes used, large differences between peak shapes obtained with various stationary phases can be obtained. Since symmetrical peaks are favourable for a high selectivity and sensitivity, determination of peak symmetry of a given stationary phase is important in order to select a suitable stationary phase for a specific separation.

The empirical tests described in the literature are mainly based on relative retention values between compounds of which the retention is assumed to be caused by hydrophobic and polar interactions, and compounds of which the retention is assumed to be based on hydrophobic interaction only. Furthermore, the nomenclature used in these tests is not identical. Tanaka differentiates between hydrogen bonding and ion exchange using the relative retention of caffeine and phenol, and benzylamine and phenol, respectively [7]. For silanol activity without further specification in the literature Walters [8], Engelhardt [9], Gonnet [59] and Verzele [64] used the relative retention values of N, N-diethyl-m-toluamide and anthracene, aniline and phenol, theophyline and caffeine, and naphtalene and nitronaphtalene, respectively. Additional experiments are included in the Engelhardt

test e.g. the separation of three isomeric toluidines, and recently the peak symmetry of 4-ethylaniline was introduced as a measure of ionic and other polar interactions [65]. Daldrup [66] used the relative retentions of diphenhydramine, 5-(p-methylphenyl)-5-phenylhydantion and diazepam to determine silanol interactions. Another test was developed by Neue for silanol interactions based on the relative retention of a basic compound and a hydrophobic compound, at neutral and acidic eluent pH. The test was used to assess the reproducibility of column material [61] and to classify 50 different commercial available stationary phases [67]. It was shown that modern packing materials could be manufactured reproducible. Kele and Guiochon [68-70] used a mix of the tests based on the tests of Tanaka [7] and Engelhardt [9] to evaluate the column-to-column and batch-to-batch reproducibility of modern packing materials. They found that packing materials were reproducible with respect to analysing neutral compounds. However, contradictory to the results of Neue et al [61], differences between batches of identical packing material were observed with respect to analysing basic substances. These data confirmed our own findings i.e. batch to batch differences can be obtained in the analysis of basic pharmaceutical compounds [71]. In Table 2.2 a selection of the previously discussed tests is summarised. The large variety of the tests described in the literature make it difficult to the user to judge the quality of these tests. Claessens recently showed the low correlation between the tests of Walters, Tanaka and Engelhardt [63], meaning that these tests describe different ionic and other polar properties. Moreover, as discussed in ref. [72], the correlation of these tests with peak shape data obtained when analysing basic pharmaceutical compounds using seven modern stationary phases is low: the tests of Engelhardt and Tanaka were found to be less specific to differentiate between columns of the latest generation for the analysis of basic pharmaceutical compounds. Other empirical tests are based on measuring, besides retention, also other chromatographic parameters such as peak symmetry and efficiency. These tests are dedicated to the analysis of a specific class of compounds, and therefore basic compounds are used as test probes to classify columns for the analysis of basic compounds. The test of Eymann [60] used the retention and peak shapes of basic amines to determine polar interactions. In a number of papers McCalley described the development of a test using generally available basic analytes as pyridine, benzylamine, codeine, diphenhydramine, amphetamine, nicotine, quinine and nortryptyline [12,73-77]. It was found that at mobile phase pH 3, deterioration of the peak shape often is caused by overloading. It is suggested to use low amounts of analyte (0.1 μg) for testing of stationary phases, and that stationary phases should be compared using efficiency and peak shape data. Although these test compounds were claimed to be generally available, both amphetamine and codeine are compounds which belong to the class of controlled drugs and therefore are not generally available to many analysts world-wide. A convenient way of developing a test method is to use compounds structurally closely

related to the compounds for which the stationary phases have to be used. In ref. [13] the selection of test compounds out of a large number of basic compounds is described. The disadvantage of this approach is that the test compounds usually are not generally available and therefore cannot be used by other chromatographers.

Table 2.2: Selection of empirical tests to determine ionic and other polar properties of stationary phases.

Calculation	Nomenclature	Eluent %(V/V)	Reference
$k_{caffeine}/k_{phenol}$	hydrogen bonding capacity	MeOH+H_2O/30+70	Tanaka [7]
$k_{benzylamine}/k_{phenol}$	ion exchange capacity pH > 7	MeOH+0.02M phos. pH 7.6/30+70	
$k_{benzylamine}/k_{phenol}$	ion exchange capacity pH < 3	MeOH+0.02M phos. pH 2.7/30-70	
$k_{DETA}/k_{anthracene}$	free SiOH test	acetonitrile	Walters [8]
$k_{nitrobenzene}$	free SiOH test	dry n-heptane	
$k_{aniline}/k_{phenol}$	silanol activity	MeOH+H_2O/55+45	Engelhardt [9]
α ortho-, meta- and para-toluidine			
$k_{N,N\text{-dimethylaniline}}/k_{phenol}$			
$k_{theophyline}/k_{caffeine}$	test on residual silanols	MeOH+H_2O/40+60	Gonnet [59]
k and α of: 1 and 3-methyl uric acid, 1 and 3-methyl xanthine, 1,3-dimethyl uric acid		MeOH+NaH_2PO_4(10g/l)/9+91	
k and α of: 1 and 3-methyl uric acid, 1 and 3-methyl xanthine, 1,3-dimethyl uric acid, theobromine, para xanthine, theophyline		<u>Solvent 1</u>: MeOH+0.01M acetate/ 50+50 TBA $5\cdot10^{-3}$M <u>Solvent 2</u>: 0.01M acetate, TBA $5\cdot10^{-3}$M; 10 to 30% of solvent 1 in 15 minutes	
k of dopamine, tyramine, theophyline, caffeine, bamifylline and M119		MeOH/0.1M NaH_2PO_4 pH 2 45/55; $5\cdot10^{-2}$M NaCl, $8\cdot10^{-3}$M heptane sulph.	
k and peak shape of: benzylamine, 2-(4-methoxy-phenyl)-ethylamine, N-naphtyl-	elution of amines	A: water; B: acetoni./ water 65/35; C acetoni. /water 40/60 + 10mL 1N H_2SO_4 $1L^{-1}$; D acetoni. /water 40/60 + 10mL 1M pH 7	Eymann [60]

ethylene-1.2-diamine		buffer $1L^{-1}$	
k and peak shape of: Bayer research compound, 4.4'-bipyridine, 2.2'-bipyridine, 2.3-dihydroxynaphtalene	elution of chelating compounds	t_0: %A: 90, %B 0, %C or D 10; t_{20}: %A: 0, %B 90, %C or D 10	
k and peak shape of: 4-hydroxy benzoic acid, 2-nitrobenzoic acid, 2-hydroxy benzoic acid	elution of acids		
k and α diphenydramine, 5-(p-methylphenyl)-5-phenylhydantoin (MPPH), diazepam	"DMD" test	156 g acetonitrile + 340 g buffer; buffer: 6.66 g KH_2PO_4+4.8g of 85% H_3PO_4 in 1L water, pH 2.3	Daldrup [66]
$\alpha_{naphtalene/nitronaphtalene}$	degree of activity or deactivation	MeOH+5% sodium acetate/60+40	Verzele [64]

From a historical point of view, clear differences in the development of column test methods can be observed. The tests of Tanaka [7] and Engelhardt [9] are a decade old, meaning that these tests were developed using stationary phases of the same period. The test methods developed by Gonnet, Daldrup, Verzele, Neue and Walters [8,59,60,64,66] were developed from 1982 – 1987.

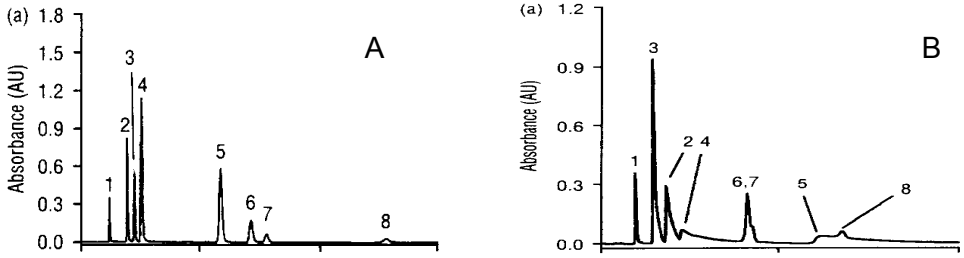


Figure 2.2: Separation of the Engelhardt test mixture using a Symmetry C_8 (A) and a Hypersil MOS (B) column. Eluent methanol-water/49-51%w/w. Analytes: 1: thiourea, 2: aniline, 3: phenol, 4: m- and p-toluidine, 5: dimethylaniline, 6: benzoic ester ethylester, 7: toluene and 8: ethylbenzene. Reprinted from ref. [65].

Since that period many new stationary phases have been developed showing improved performance in the analysis of basic solutes. These phases were developed with respect to minimising ionic and other polar interactions responsible for asymmetrical peaks and as a result the differences between these phases will be small compared to the "older phases". Therefore it is believed that the "older" tests are more suitable to differentiate between generations of columns, as is

demonstrated in Fig. 2.2 were clearly is shown the differences between a Symmetry C_8 and a Hypersil MOS column using the test of Engelhardt. For the modern phases of today only subtle differences are present, which are more difficult to be visualised using tests like the Engelhardt and Tanaka test, and can best be detected using basic compounds as test substances. The comparison of test methods for the suitability to characterise stationary phases for the analysis of basic pharmaceutical compounds was performed recently. Column classification using the test developed by McCalley was in fair agreement with column classification based on the data obtained with a set of seven basic pharmaceuticals from daily practice [72]. Therefore it was concluded that classification of stationary phases for the analysis of basic pharmaceuticals is best performed using basic compounds as test probes.

2.4.1.3 Metal Activity of Stationary Phases

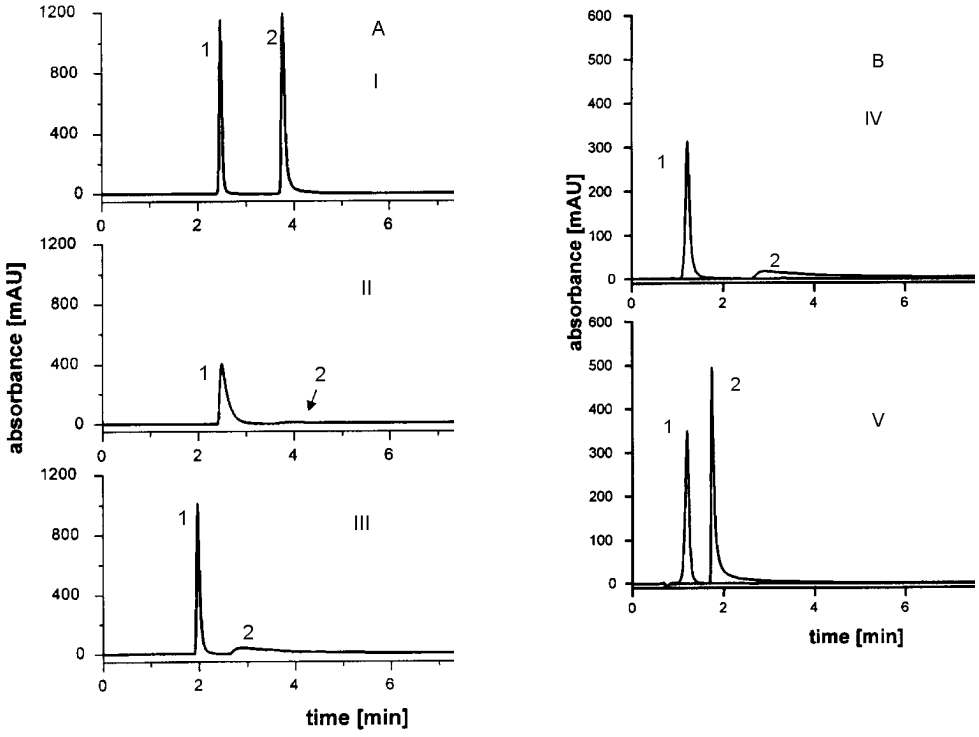


Figure 2.3: Determination of metal activity on RP 18 columns (reprinted from ref. [82]).

A: differentiation between silanol and metal activity; I: low silanol and low metal activity, II: high silanol activity, high metal content, III: low silanol activity and high metal content.

B: Influence of buffer: IV: unbuffered eluent and V: water replaced with 1mM phosphate pH 7.

Compound 1: 4,4'-bipyridil, compound 2: 2,2'-bipyridil.

The eluent used was methanol+water/49+51%(w/w), the flow-rate was 1.0 ml/min, the column temperature was set to 40°C and detection was performed with UV at 254 nm.

As discussed before, metal impurities can influence the chromatographic properties of stationary phases and thus also the performance of a chromatographic analysis.

Metals can increase the acidity of adjacent silanol groups as well as being strong adsorption sites for complexing solutes. To determine the metal content of stationary phases chromatographically, various suggestions have been proposed. The first test solute was proposed by Verzele and Deweale [64]. They suggested that the peak shape and retention of acetyl acetone was a measure of metal contamination. Recently it was demonstrated that the peak shape of acetyl acetone was also affected by keto-enol-tautomerism effects [78] and therefore the effects observed cannot be described to metal activity solely. Other tests use aromatic di- or trihydroxy compounds [79-81], however, as demonstrated by Engelhardt and Lobert [82] these compounds were found to be insensitive in detecting metal impurities. In the same paper, Engelhardt and Lobert described the use of 2,2'-bipyridyl and 4,4'-bipyridyl to determine metal impurities. 2,2'-bipyridyl can form complexes with metals, whereas 4,4'-bipyridyl cannot. Since both compounds are bases, also interaction with residual silanols can occur. However, for both compounds the pKa and basic nitrogen atom are comparable. Therefore, the relative asymmetry of both compounds was found to be a good measure of metal content. In Fig. 2.3A examples of various columns are shown. The test on metal activity was performed using an unbuffered eluent. To demonstrate the influence of buffering the eluent, in Fig. 2.3B the difference between an unbuffered eluent and the use of 1 mM phosphate buffer pH 7 in the eluent is shown. As can be seen, using buffered eluents metal activity is suppressed. Therefore, Engelhardt and Lobert concluded that metal activity should be determined using unbuffered eluents. However, basic analytes are mainly analysed using buffered eluents. Therefore, metal activity data obtained with buffered eluents also show valuable information with respect to the analyses of basic pharmaceutical compounds.

2.4.2 Thermodynamically based evaluation method

Enthalpies and entropies of transfer of solutes from the mobile to the stationary phase can be calculated from retention data by evaluation of the van het Hoff plots [83]. The retention factor can be expressed in terms of standard enthalpies and entropies of transfer from mobile to stationary phase:

$$\ln k = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \beta \quad \{2\}$$

The enthalpy (ΔH^0) represents the measure of energy exchange. Entropy (ΔS^0) represents the chaos of a system. β is the phase ratio of the column.

A plot of ln k vs.1/T shows the van het Hoff plot and is linear if ΔH^0 and ΔS^0 are independent of the temperature. The slope of the van het Hoff plot gives the standard enthalpies of transfer, the standard entropies of transfer are calculated from the intercept and depend on the phase ratio. Comparison of stationary phases

has been performed by studying the changes in mechanism of retention of test analytes with changing column temperature. Examples are the studies of Cole, Sentell and Dorsey [84,85] who examined the influence of bonding density of stationary phases on the retention of nonpolar solutes. They found that partition, rather than adsorption, was found to be the relevant model of retention for non-polar compounds.

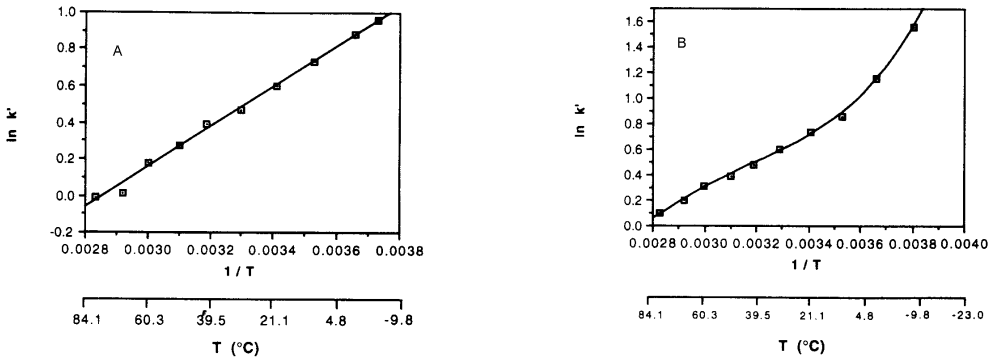


Figure 2.4: van het Hoff plots for benzene on columns with bonding density 2.84 μmol/m^2 (A) and 3.06 μmol/m^2 (B). The eluent used was acetonitrile+water/60+40 %(V/V). Reprinted from ref. [84].

The columns which were compared differed in bonding densities ranging from 1.60 to 4.07 μmol/m^2. The entropic contribution to retention becomes more significant with respect to the enthalpy contribution as the stationary bonding density is increased. Using benzene as test compound for columns with a bonding density ≥ 3.06 μmol/m^2, as shown in Fig. 2.4, a non-linear van het Hoff plots was obtained. Other examples are the studies of Purcell et al [86] using van het Hoff plots to study changes in the secondary structure of peptides and the mechanism of interaction with hydrophobic surfaces, and Philipsen et al [87] using van het Hoff plots to study the retention of polystyrene and polyester oligomers.

For basic solutes, retention is believed to be a combination of hydrophobic and polar interaction [16]. Non-linear van het Hoff plots might be indicative of a change of retention mechanism [87,88]. For basic compounds this means that the ratio of the hydrophobic and polar interaction could change. However, studying the retention of basic compounds linear van het Hoff plots were obtained. As described in ref. [72], no clear deviation from linearity was observed studying the retention from 10 °C to 80 °C for both the McCalley test compounds and a set of basic compounds from daily practice. In Fig. 2.5 the van het Hoff plots for 2 basic compounds are shown using MeOH + 25 mM NH_4Ac / 50 + 50 %(V/V) at buffer pH's 3 and 7 as eluent. It was therefore concluded that detecting changes in mechanisms of retention involved in the analysis of basic solutes was not straightforward by studying the linearity of van het Hoff plots.

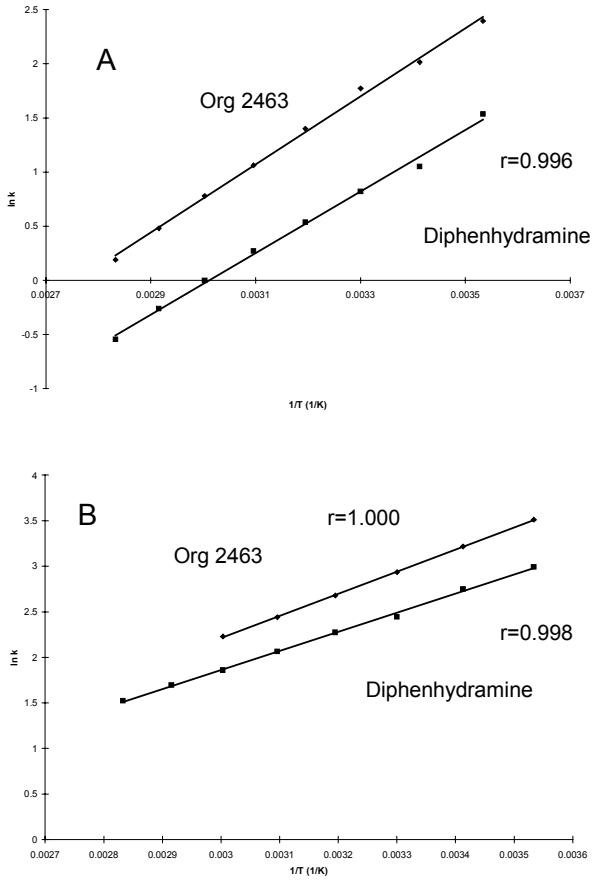


Figure 2.5: van het Hoff plots for 2 basic compounds using MeOH + 25 mM NH_4Ac / 50 + 50 %(V/V) at buffer pH's 3 (A) and 7 (B) as eluent. Alltima C_{18} was used as stationary phase. Reprinted from ref. [72].

2.4.3 Evaluation methods based on retention models

As in most situations for the empirical and thermodynamically based tests, the model based evaluation methods are based on studying the retention of the test compounds, and differences between stationary phases are determined based on differences in retention behaviour of test compounds.

Galushko calculated retention in LC based on the molecular structure of the analyte and characteristics of the stationary and mobile phase. To calculate the retention, a two layer model of a chromatographic system is proposed [89,90]. The surface of a sorbent in reversed phase LC has a surface layer (SL) that involves octadecyl chains and some of the components of a mobile phase, and SL is assumed to be a quasi-liquid that has its own characteristics, i.e. surface tension (γ_s) and dielectric constant (ε_s), and the SL characteristics vary with varying mobile phase and sorbent properties. The molecules of a retained analyte penetrate into the SL and the retention is determined by the difference in molecule solvation energies in the mobile phase and SL. The equation obtained for retention is:

$$\ln k = a\left(\sum_i V_i\right)^{2/3} + b\left(\sum_j \Delta G_{e.s.j,H2O}\right) + c \quad \{3\}$$

in which V_i are the increments of partial molar volumes of the fragments of the test analytes in water, and $G_{e.s.j,H2O}$ are the increments of energy of interaction of bond dipoles in water, respectively. Each column can be characterised quantitatively by using three parameters a, b and c that determines the retention. Using several reference compounds one can calculate the values of the parameters for each chromatographic system (mobile phase - stationary phase). Such a set of parameters can be used to calculate retention of other compounds and for quantitative evaluation of differences between columns [91]. To determine column parameters accurately it is necessary to use test compounds that have substantial differences in V_i and $G_{e.s.j,H2O}$, i.e. compounds differing in surface and polarity. The test mixture of Galushko to initially test stationary phases consists of aniline, phenol, benzene and toluene [91]. Column classification based on Galushko's approach was recently compared with column classification using the empirical Engelhardt and Tanaka tests [63]. For the hydrophobic column properties, good correlations were observed. However, for the polar properties, low correlation between the tests was found. Judgement which test yields the best information is difficult. In ref. [72] various empirical and model based tests are compared and correlated with the peak shapes of a set of basic solutes. It was obvious that the Galushko test revealed only limited information about ionic and other polar properties of stationary phases as shown by the low correlation of the Galushko data with the peak shapes of the set basic compounds. However, the retention data of aniline, phenol, benzene and toluene are only used for an initial characterisation. A final characterisation of column and stationary phase is obtained using the retention data of the compounds of interest.

Quantitative structure - retention relationships (QSRR) are relationships between physical parameters determined for test solutes and molecular descriptors which describe the structures of the test analytes. In fact, equation {3} can also be considered as a QSRR. In the last two decades QSRRs have often been applied to: i: predict retention for a new solute, ii: identify the most informative structural descriptors, iii: gain insight into the molecular mechanisms of separation, iv: evaluate complex physicochemical properties of analytes, and v: predict relative biological activities within a set of drugs [92]. Different approaches have been described in the literature with respect to characterising chromatographic systems, i.e. regression of log k values against 1-octanol - water partition coefficients (log P) [93,94], linear solvent energy relation ships (LSER) using experimentally determined molecular descriptors [95,96], and describing log k values in terms of calculated molecular descriptors [97,98].

Regression of log k against log P was performed by Kaliszan et al [11] and revealed information about the hydrophobic properties of the columns. Log k_w data were obtained for 18 different columns and were linearly regressed against log P, i.e. log $k_w=k_1+k_2$ log P. The value of k_2 reflects the degree to which the analyte is surrounded by the stationary phase. The more the solvated stationary phase is alike to octanol, the closer k_2 should be to 1. In Fig. 2.6 the stationary phases are ordered by their ability to mimic octanol i.e. hydrophobic properties. The Aluspher (Alu) column was found to be the most hydrophobic, whereas the Nucleosil C_8 (NuC8) column was found to be least hydrophobic.

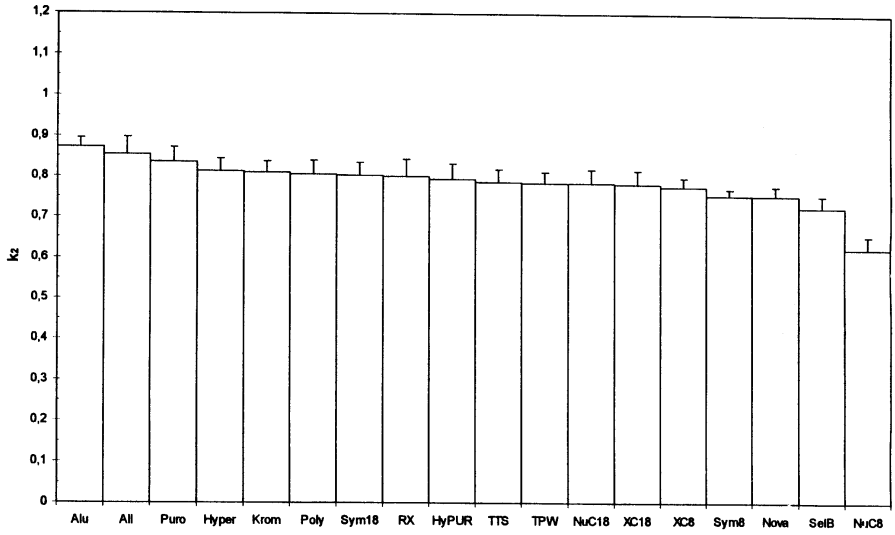


Figure 2.6: k_2 values for 18 columns of log k_w vs. log P relationship. The log k_w data were obtained in methanol + water eluents. Reprinted from ref. [11].

The general equation for LSER is:

$$logSP = c + rR^2 + s\pi_2^H + a\sum\alpha_2^H + b\sum\beta_2^H + vV_x \quad \{4\}$$

where SP is a solute property e.g. solubility, partition coefficient or retention, R^2 denotes an excess molar refraction of the analyte, V_x is a parameter characteristic of the solute size (e.g. molar volume), π_2^H a measure of the solutes capacity for dipole/polarizability interactions and α_2^H and β_2^H characterise the solutes hydrogen donor (acidity) and acceptor (basicity), respectively. The terms c, r, s, b, a and v are related to the chemical nature of the mobile and stationary phase and are unique for the combination of mobile and stationary phase. Therefore these parameters can be suitable for stationary phase characterisation [99]. Tan et al [100] used the retention data of 87 aliphatic and aromatic solutes obtained using five bonded phases and acetonitrile + water / 50 + 50 %(V/V) as eluent in LSER studies. The most important

retention governing solute parameters were found to be solute volume and hydrogen bond acceptor basicity. The relative importance of the solute

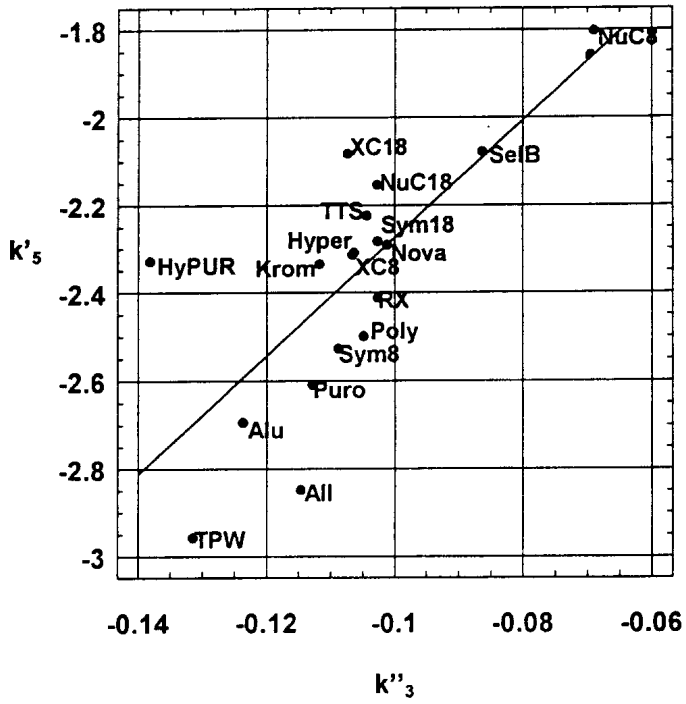


Figure 2.7: Ordering of stationary phases according to their hydrogen-bond donor activity (k'_5) and total dipolarity (k''_3). Reprinted from ref. [11].

parameters in explaining the data did not differ for the five bonded phases studied. Buszewski et al [101] used LSER to show differences between a conventional C_{18} bonded silica and N-acylaminopropyl silica. It was demonstrated that using the C_{18} bonded phase retention was dominated by the reversed phase mechanism. For the N-acylaminopropyl bonded phase, however, a significant effect on retention was shown for the structurally specific dipole - dipole and charge transfer interactions with the solutes. Sandi et al [62,102,103] used the retention factors of 34 solutes obtained under isocratic conditions using 15 different LC columns. Principal components analysis (PCA) was performed to differentiate between the various columns. They found that the first principal component (PC1) described the hydrophobic character, the second PC described the hydrogen bonding acceptor basicity, whereas the third PC described the hydrogen bonding donor acidity of the various columns.

Column classification using calculated molecular descriptors was performed by Kaliszan [11]. The applicability was demonstrated by the ordering of 18 stationary phases according to their calculated total dipolarity properties. The ordering was

found to be comparable to the ordering of the columns by the hydrogen-bond donor properties determined via LSER (see Fig. 2.7). Both total dipolarity and hydrogen-bond donor properties are related with the accessibility of the analyte with free silanols of the stationary phase.

2.5 ELUENT COMPOSITION

In Table 2.2 a selection of the discussed empirical evaluation methods are shown. As can be seen, some chromatographers use buffered eluents, whereas others use non-buffered eluents to test columns for ionic and other polar interactions. Whether the eluent should be buffered or not, is a subject of discussion [9,12,63]. Since buffers can mask silanol and eventual other polar interactions, testing in unbuffered systems can be advantageous for determination of these interactions [65]. In daily practice basic compounds are analysed using buffered eluents to control the ionisation of the analyte to obtain robust separations.

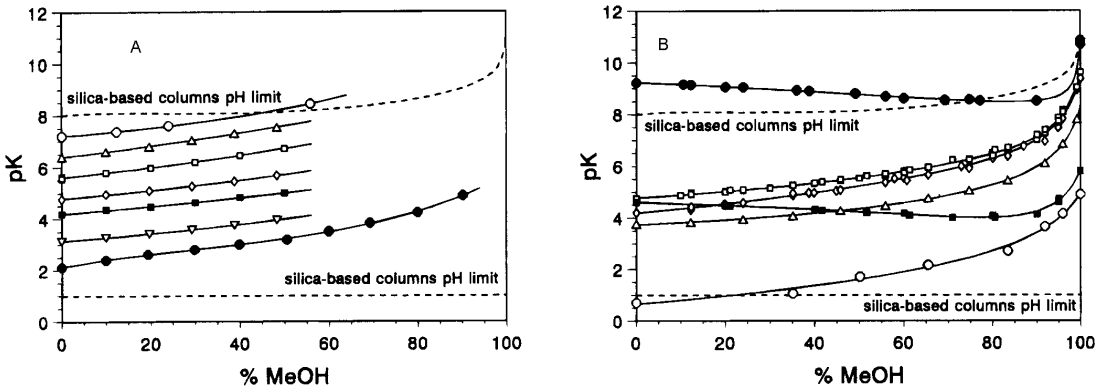


Figure 2.8: Variation of pKa values of acids with methanol-water composition.
A: • pKa1 of phosphoric acid, ∇ pKa1 of citric acid, ■ pKa1 of succinic acid, ◊ pKa2 of citric acid, pKa2 of succinic acid, pKa3 of citric acid and O pKa2 of phosphoric acid.
B: O trichloro acetic acid, formic acid, ◊ benzoic acid, acetic acid, ■ anilinium and • ammonium.
Reprinted from ref. [108].

Moreover, a test that is generally accepted for column characterisation should be rugged and reproducable in many different labs world-wide. This can only be achieved by using a rugged eluent, which can only be obtained using buffered eluents. To obtain rugged eluents, preparation and control of the pH of the eluent must be performed carefully. Therefore the influence of eluent composition on the analysis of ionisable analytes is discussed.
In LC it is common practice to measure the pH of the mobile phase buffer before mixing with the organic modifier. However, it is also recognised that the pH changes after addition of organic solvent since the pKa values of the acids used to prepare the buffers change with the solvent composition [104-106]. In Fig. 2.8 the influence of percentage methanol on the pKa values of acids often used for preparing LC

buffers are shown. The dissociation of electrolytes in binary solvents depends strongly on their preferential solvation and this is different for each solute [107]. Moreover, one should be aware that, dependent on the type of buffer ions, addition of methanol can result in an eluent pH outside the pH range commonly used with silica based LC columns [108]. For organic modifier - buffer mixtures the pH can be calculated from measurements using conventional pH electrodes:

$$pH_x^* = pH_x^{app} - \delta \quad \{5\}$$

in which pH^*_x is the pH value in an aqueous - organic system, pH^{app}_x is the measured value in an aqueous - organic system and δ is a correction factor for the

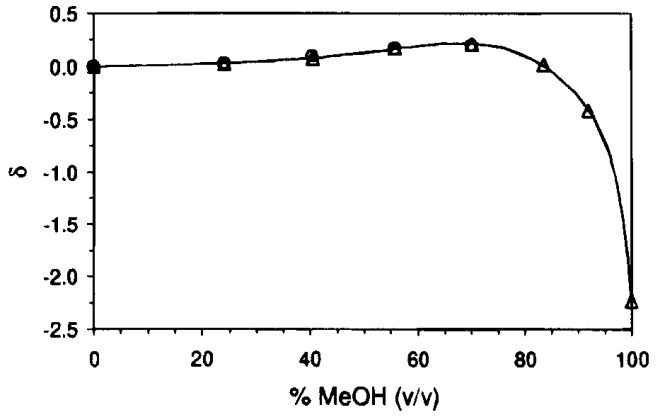


Figure 2.9: Variation of δ with solvent composition. Reprinted from ref. [111].

liquid junction between the electrode and the eluent [105]. Values of δ have been published for several methanol – water ratios, and as shown in Fig. 2.9 it can be concluded that for amounts of organic modifier up to 80% the correction factor is small, i.e. approx. 0.2 [109-111].

Recently Barbosa et al [104] determined pH values for eluents using various organic modifiers as well as various buffers. In Table 2.3 the results obtained with methanol are shown. As can be seen, addition of 50% methanol to the buffers resulted in an increase of approx. 1 pH for the eluent. When using different modifiers and buffer ions, different pH values are obtained. For sodium phosphate the relation between the pH of the aqueous buffer and measured pH of the eluent after preparing a 1:1 mixture with methanol with 25 mM buffers from pH 3 up to pH 7 was found to be approx. linear as shown by the correlation coefficient of > 0.99. Comparable data were found for ammonium acetate, ammonium citrate and ammonium phosphate buffers [72]. However, small differences between the slopes of the regression lines were obtained. Besides the pH and the buffering range of the eluent, the organic modifier will also influence the pKa of the basic analyte. Comparable to the pH of the eluent, the pKa values of the analytes are often measured in pure water. In Fig. 2.10

the influence of the methanol, acetonitrile and tetrahydrofuran concentration on the pKa of the basic pharmaceutical mirtazipine is shown. As can be seen increasing the modifier concentration lowers the pKa value of the basic analyte, which can influence the chromatographic performance of the basic analyte [72]. The effect was comparable for methanol and acetonitrile, but more significant for tetrahydrofuran.

Table 2.3: pH values in methanol+buffer mixtures at 25 °C. Reprinted from ref. [104].

	% methanol				
buffer	0	10	30	40	50
KH_2-citrate	3.77	3.94	4.28	4.47	4.65
KH-phtalate	4.01	4.24	4.63	4.87	5.13
acetate	4.65	4.78	5.13	5.35	5.49
$H_3PO_4/H_2PO_4^-$	2.12	2.38	2.68	2.86	3.12

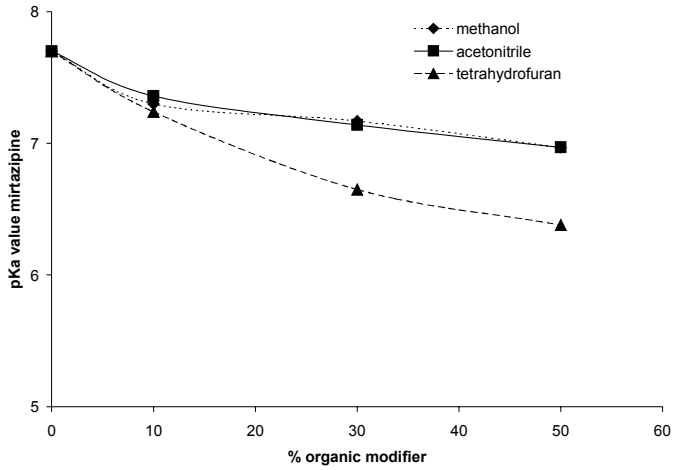


Figure 2.10: Relation pKa vs. % modifier for the basic pharmaceutical compound mirtazipine at 25 °C. Reprinted from ref. [72].

An example of the influence of a changed pKa and eluent pH as a result of addition of modifier to the aquaous solution was recently described by Neue et al [61,67]. A shift in retention observed for propranolol at a given buffer pH did not correspond with the pKa value, which were both measured without modifier. However, the pKa of the analyte will be lower compared to the value measured in pure water, whereas the pH of the eluent will be higher as the pH value of the pure buffer. Therefore, the analyte might be analysed as a non-protonated compound, explaining the shift in retention. Other possible effects that should also be considered are the influence of the eluent pH on the stationary phase inside the column as well as the effect of the organic modifier on the pKa of the residual silanol groups [70].

2.6 CHEMOMETRIC EVALUATION OF CHARACTERISATION DATA

Testing of stationary phases usually results in large amounts of data such as retention factors, peak asymmetries and plate numbers of the test compounds used with the various stationary phases. Extraction of valuable information from these large amounts of data is difficult. Chemometric techniques can be helpful tools to extract information from large datasets, enabling the classification of objects (stationary phases) with comparable characteristics [112]. The most frequently used tool is principal component analysis (PCA). With PCA, a set of new variables (principal components, PC) is defined instead of the original variables. To find the PC of a data matrix, X, the first step is to look for a vector t_1 (score) =Xp_1 that is a linear function of X with maximum variance, where p_1 (loading) is a vector of m constants $p_{11},p_{12},\ldots\ldots,p_{1m}$ and length 1. The second step is to look for a linear function Xp_2, orthogonal to Xp_1, which has maximum variance, etc. A datamatrix can thus be written as:

$$X = t_1 p_1^T + t_2 p_2^T + \cdots\cdots \quad \{6\}$$

Since most of the variability of the data is present in the first PC's, it is possible to present the datamatrix X graphically [113] and the advantage of PCA is the ability to handle a more expansive dataset.

Delaney et al [114] used PCA to classify columns tested using the method developed by Walters [8]. The use of PCA resulted in the same classification scheme as determined by a LC specialist. Schmitz et al [115] and Walczak et al [116] presented differences between stationary phases using correspondence factor analysis (CFA) by which similarities in the structure of the objects (stationary phases) and the variables were presented simultaneously. Hamoir et al [117] used spectral mapping analyses (SMA) to classify sixteen stationary phases. Both CFA and SMA are chemometrical tools related to PCA.

In ref. [118] the classification using PCA of fourteen commercially available stationary phases characterised for the applicability to analyse basic analytes using phosphate buffers pH 3 and pH 7 is described. Brereton and McCalley [119], who classified eight commercially available stationary phases, also with respect to the analysis of basic analytes, used the same approach. In Fig. 2.11 the classification of the columns using buffers pH 3 and 7, and methanol, acetonitrile and tetrahydrofuran as modifiers, is shown. The influence of the nature of modifier on column classification is clear. Sandi et al [62] compared PCA data of the test compounds with data from LSER studies. Solvatochromic parameters were regressed against the principal components (PC) and it was found that the first PC was a measure of hydrophobic strength whereas the second PC was positively correlated with hydrogen bond acceptor and polarizability/dipolarity. A negative correlation of the second PC was observed for the molecular size and hydrogen bond donor. The third PC was only positively correlated with hydrogen bond donor.

Besides the classification of stationary phases, PCA has also successfully been applied to reduce the number of test compounds. In ref. [13] it is shown how PCA is used to reduce a set of 32 test compounds. The test compounds were presented in the score plots in which compounds describing comparable information are clustered. By selecting compounds situated apart from each other in the score plot, the number of compounds in the test set was reduced to five. In Fig. 2.12 the score plot is shown and the underlined compounds were selected and applied to test columns.

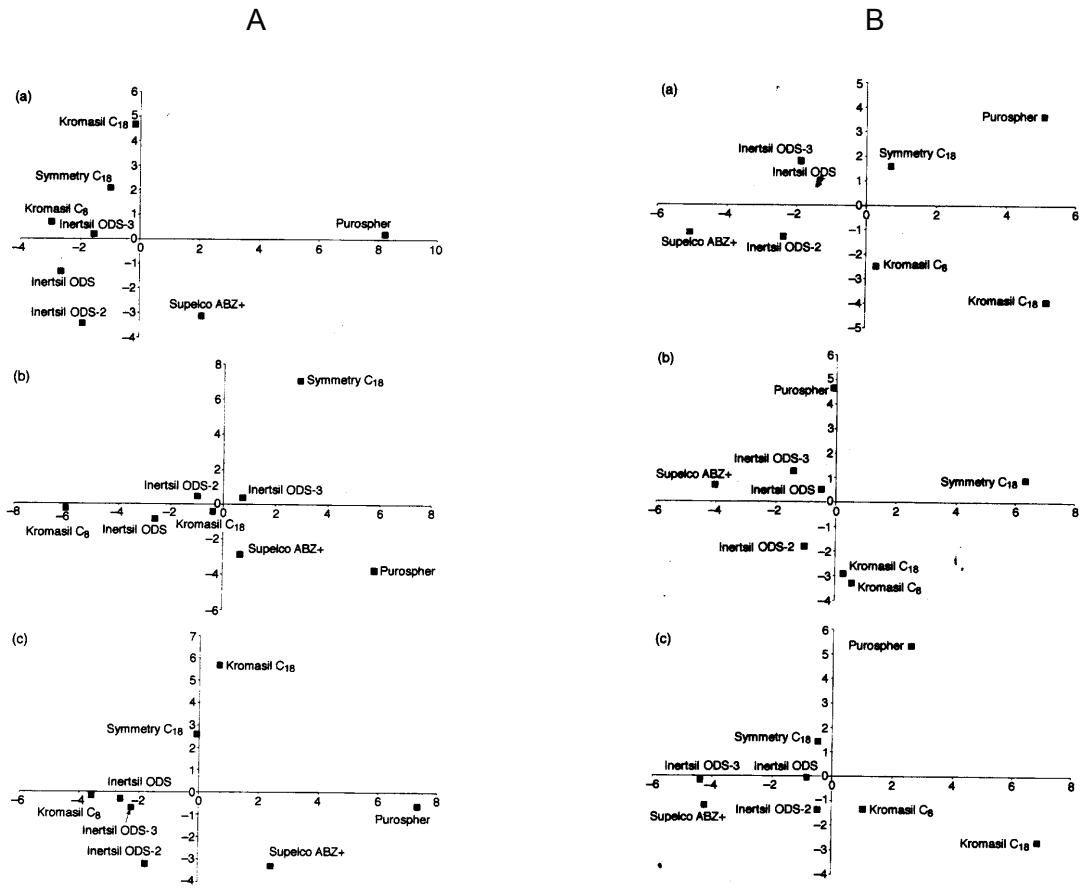


Figure 2.11: Score plots of column classification at pH 3.0 (A) and pH 7.0 (B): (a) methanol, (b) tetrahydrofuran and (c) acetonitrile. Reprinted from ref. [119].

In summary, the discussed examples clearly revealed that a graphical presentation of a large n-dimensional data set by means of chemometrical techniques can be very useful in classification of objects of interest, i.e. stationary phases and test compounds.

2.7 CONCLUSIONS

For the LC analysis of basic pharmaceuticals, optimisation of the mobile phase can be achieved by choosing the optimal eluent pH, addition of silanol blocking compounds etc. Optimisation of the stationary phase is achieved by minimising the interaction between analyte, and residual silanols and eventual other polar sites responsible for asymmetrical peaks. Moreover, stationary phases have been developed which can be used at high eluent pH to enable the analysis of basic analytes as non-protonated compounds.

Due to the large number of columns available to tackle a separation problem, chromatographers could get confused which column should be selected for their application. To allow the selection of suitable columns, the availability of a good and efficient column characterisation procedure would be of great help, e.g. to classify columns into classes with comparable characteristics. For the characterisation of stationary phases various attempts have been reported and it is confusing for the chromatographers which procedure should be used.

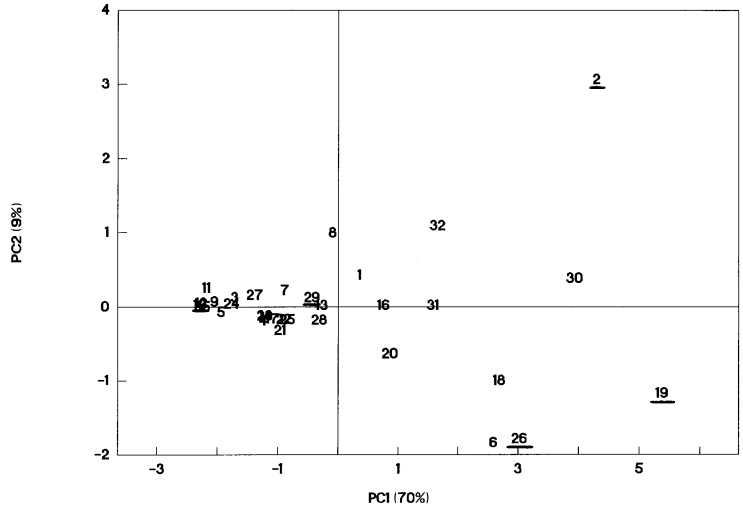


Figure 2.12: PCA score plots for peak symmetry data of 32 basic compounds obtained with 6 different LC columns. Reprinted from ref. [13].

The majority of column characterisation procedures can be divided into empirical methods, thermodynamically based and model based methods. The empirical methods are based on the chromatographic performance of compounds chosen according to a certain line of thought. Most of these methods were developed more than 10 years ago and use the retention of compounds like aniline, benzylamine and phenol to calculate characteristics like silanol activity and ion exchange capacity. The applicability of these methods to characterise the modern phases of today for the

analysis of basic compounds is not straightforward. Other empirical methods use test compounds that are structurally related to the compounds of interest. For instance, the test developed by McCalley makes use of compounds like diphenhydramine and nortriptyline to characterise columns for the applicability to analyse basic pharmaceutical compounds. Tests of this nature were found to be suitable to differentiate between columns with respect to suitability for the analysis of basic pharmaceuticals.

Van het Hoff plots can be used to study a change in retention mechanism caused by a variation of the column temperature. However, studies performed sofar using basic pharmaceuticals showed that the change in retention mechanism cannot be detected investigating the linearity of van het Hoff plots.

Studies using quantitative structure-retention relationships (QSRR) require physical parameters determined for the test analytes. Meaningful classification was obtained using QSRR studies. Significant differences could be quantitated between a C_{18} and a N-acylaminopropyl bonded phase, whereas comparance of C_{18} phases resulted in an ordering of the phases. A comparable ranking of columns was obtained using data obtained from LSER and QSSR studies. A disadvantage of these methods is that laborious experiments are needed to obtain the required information. Therefore, these methods are preferably not used in daily practice. Nevertheless, both LSER and QSRR reveal useful information about mechanisms of retention.

Special attention in the LC analysis of basic compounds should be paid to the eluent composition. Some tests use buffered eluent, whereas others use non-buffered eluents to test columns for ionic and other polar interactions. Since buffers can mask polar interactions, testing in unbuffered systems can be advantageous for determination of these stationary phase properties. However, in daily practice basic compounds are analysed using buffered eluents to control the ionisation of the analyte to obtain robust separations. A test that is generally accepted for column characterisation should be rugged and reproduced in many laboratories and in many countries. This can only be achieved by using a stable eluent, i.e. a buffered eluent. However, one should be aware that the amount and nature of the modifier affects the pH of the eluent, as well as the pKa of the basic analyte. Moreover, possible effects of the stationary phase to the pH of the eluent inside the column should also be considered as well as the effect of the organic modifier on the pKa of the residual silanol groups.

Stationary phase testing usually results in large amounts of data such as retention factors, peak asymmetries and plate numbers of the test compounds used with the various stationary phases. Extraction of valuable information from these large amounts of data is difficult. Chemometric techniques have proven to be useful tools to extract information from such large datasets. Especially the use of principal components analysis or techniques closely related, should be considered when

columns and test compounds are characterised and classified into groups with equal characteristics.

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3. DEVELOPMENT OF A TEST METHOD TO CHARACTERISE STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS

3.1 COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF BASIC DRUGS

3.1.1 SUMMARY

Problems that can be encountered in the high performance liquid chromatographic analysis of basic compounds are severe peak asymmetry and low separation efficiency. In attempting to solve these problems, one can become confused by the variety of suggestions given by the specialists and by the numerous stationary phases available. In this chapter the analysis of basic drugs was studied from two directions. In both approaches a set of 32 basic drugs was used, differing in basicity, polarity and number of and type of N-atom(s).

In the first approach the effect of mobile phase additives and buffers on the performance of a single column was determined. It was found that tertiary and quaternary amines can be applied successfully as silanol blockers. The latter proved to be aggressive towards silica based stationary phases. Addition of triethylamine showed a remarkable improvement in peak shape with different columns. Other aspects, such as pKa, retention and amount injected were systematically studied.

In the second approach, 8 different columns, specially recommended for the chromatography of basic drugs, were evaluated. The chromatographic results showed great variability. As far as peak shape is concerned, an electrostatically shielded stationary phase was most promising for the analysis of basic compounds. This column can even be used without buffers, which can be an advantage in liquid chromatography-mass spectrometry coupling. Because some results were inconsistent with published results, a third approach was to study three columns in more detail.

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3.1.2 INTRODUCTION

Peak asymmetry, which is often observed in the chromatography of basic drugs, is an important performance characteristic for a given stationary phase. In routine analysis system suitability criteria are set for the maximum allowable asymmetry, expressed as asymmetry factor. For quantitative analysis an asymmetry factor of less than 1.5 is preferred. It is generally accepted that the severe peak asymmetry of basic drugs in reversed-phase chromatography can be caused by ionic and other polar interactions of the solutes with silanol and eventual other polar groups of the packing material. Despite these negative effects, reversed-phase liquid chromatography (RPLC) is still the most widely used method because of the fast equilibration time, the selectivity characteristics and the retention reproducibility. As can be seen in Figure 3.1.1 we are in fact in a bad situation. The requirements for a good chromatographic performance of strongly basic compounds conflict with the demands for high stability of the column. Good performance requires a high pH to avoid ionisation of the basic solute or a low pH to suppress the formation of ionised silanol groups.

Over the years, numerous suggestions have been put forward to improve this situation. Special attention has been given (i) to reduce the number of free silanols (end-capping), (ii) to diminishing the effect of free silanols by choosing the right mobile phase pH and reducing the acidity of the silica and (iii) to eliminating silanol effects by adding inorganic and organic salts. The use of blocking agents such as tertiary and quaternary amines proved to be a useful approach [1,2].

Alternative solutions have been proposed by workers who use bare silica, dynamically modified silica or alumina [3,4]. These methods, however, did not gain widespread application. In RPLC the most obvious solution is the choice of a proper column. Stadalius et al [1] pointed to the existence of "acidic" and "basic" columns. The latter clearly are the best for this particular task. Over more than a decade our approach has been to improve the mobile phase composition and to test new stationary phases. As a result of this, μBondapak C_{18} and Nova-Pak C_{18} columns have been used more or less as a standard for the analysis of basic drugs. However, research on LC packing materials is continuing and recently resulted in the introduction of new columns specially designed for the chromatography of basic drugs [5].

In this chapter we present data on the influence of the mobile phase composition (pH, buffers, additives) on peak performance and on the stability of μBondapak C_{18} packing material. We also compared the chromatographic performance of special column materials. For both, a set of 32 basic drugs was used. These drugs differ widely in pKa, polarity, number of N-atoms and structural type of the N-atom.

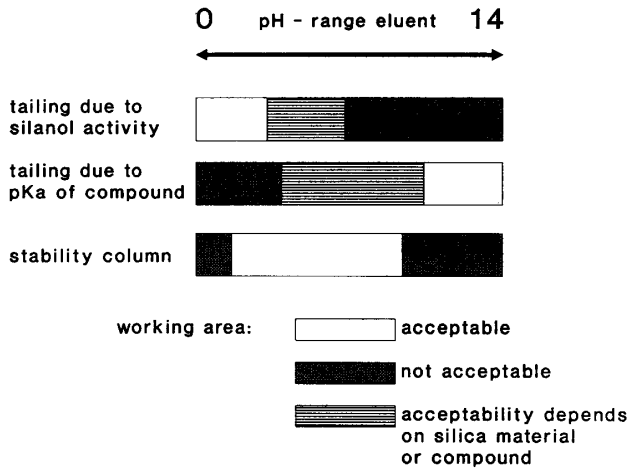

Figure 3.1.1: Qualitative illustration of the working area for the analysis of basic compounds on silica based reversed phase materials.

3.1.3 EXPERIMENTAL

3.1.3.1 Chemicals

All basic drugs were obtained from NV Organon (Oss, The Netherlands). Methanol was freshly distilled and water was obtaind from a Milli-Q purification system (Millipore). Tetramethylammonium hydroxide (TMAH) was obtained from Southwestern Analytical Chemicals Inc. (Austin, TX, USA). Disodiumhydrogen phosphate (Na_2HPO_4), sodiumdihydrogen phosphate (NaH_2PO_4) and ammonium hydroxide 25% (NH_4OH) were supplied by J.T. Baker (Deventer, The Netherlands), acetonitrile (CH_3CN) and concentrated phosphoric acid (H_3PO_4) by Merck (Darmstadt, FRG). Sodium 1-hexane-sulphonate was obtained from Eastman Kodak Company (Rochester, USA) and triethylamine (TEA, sequanal grade) from Pierce (Rockford, USA). The pKa values of the basic drugs were determined in 60% methanol at 37 °C because of the low solubility in water. As a rule these pKa values are approx. 0.5 - 1 unit lower than in water.

3.1.3.2 Apparatus

The experiments were carried out on an HP 1090M liquid chromatograph equipped with a HP 1040M Diode-Array Detector. Data were collected on an HP 79994A HPLC Workstation (Hewlett Packard, Amstelveen, The Netherlands).

3.1.3.3 Experimental set up

The experiments can be divided into three parts. In the first part of our study we used a μBondapak C_{18} column and chromatographed a set of 32 basic drugs with different mobile phases, as explained in Fig. 3.1.2A. The influence of the mobile phase

parameters on asymmetry factor was studied quantitatively, and more qualitatively on plate number. We also checked their influence on the stability of the stationary phase.
In the second part, developments in column technology have been explored. For this we used different stationary phases, specially recommended by the manufacturers for the chromatography of basic drugs. With a constant mobile phase composition we studied the chromatographic performance of these columns with our set of basic drugs. In Fig. 3.1.2B an overview is shown of these experiments. The results will be described in a qualitative way.
In order to show column effects and differences between columns as good as possible we performed our comparative experiments under critical conditions. Therefore, we chose pH values of 3.5 and 7.4 and plain buffer without additives, since the addition of silanol blockers can mask differences.

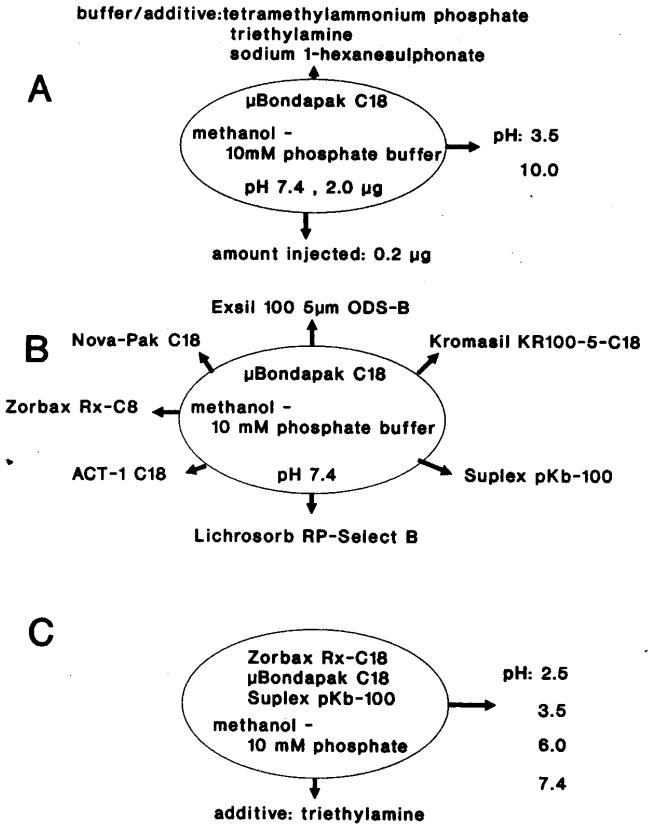

Figure 3.1.2: Overview of the experiments in this study.

For this study a set of 32 basic drugs was selected, and a representative group of them is shown in Table 3.1.1. The selection of 32 compounds was based on the following characteristics: (i) basicity, with pKa values ranging from 3 to 9, (ii) polarity,

with retention indices (RI) [6] between 400 and 1600, (iii) number of N-atoms from 1 to 5 and (iv) different structural type of N-atom.
In the third part the effect of the pH and the addition of triethyl-amine was studied for 7 "difficult" compounds on 3 selected columns (see Fig. 3.1.2C).

Table 3.1.1: Selection of compounds used, with their pKa values and retention index (RI). Asymmetry factors (As) are obtained under conditions as described in Fig. 3.1.3.

structure	pKa	RI	As
1	<3	790	1.0
2	5.0	460	1.7
3	6.5	1120	2.6
4	8.2	1380	5.2
5	8.3	1160	3.6
6	8.7	1550	9.1
7	7.9	1360	4.8
8	8.0	1310	4.0
9	7.7	1310	5.7
10	8.7	690	5.6
11	7.7	(not included in the test set)	

3.1.3.4 Chromatographic conditions

All bonded-phase materials were obtained from the manufacturers as pre-packed columns. An overview of the stationary phases is given in Table 3.1.2. The mobile phases used in this study are shown in Table 3.1.3.
The pH of the buffers was measured before mixing with the organic modifier. They were prepared by dissolving 3.58 g (10 mM) disodiumhydrogen phosphate or 9.05 g (50 mM) tetramethyl-ammonium hydroxide in 1 l of water. Occasionally 2 ml (15 mM) triethylamine was added to the phosphate solution. Concentrated phosphoric acid or 0.5 M sodium hydroxide was finally added until the desired pH was reached.
The amount of basic drug injected was 2.0 μl from a 1 mg/ml solution in methanol, i.e. 2 μg injected, unless indicated otherwise. After 10-20 injections of the basic drugs, a test solution was injected to check the column performance for changes in silanol activity, hydrophobicity and metal activity. The test solution consisted of a mixture of acetylacetone, aniline, phenol, benzene and anthracene.

Table 3.1.2: Stationary phases used.

No.	Column	Manufacturer	Dimensions length x I.D.(mm)	Particle size (μm)
1	μBondapak C_{18}	Waters	300 x 3.9	10
2	NovaPak C_{18}	Waters	300 x 3.9	4
3	Zorbax Rx-C_8	Rockland Techn.	250 x 4.6	5
4	Zorbax Rx-C_{18}	Rockland Techn.	250 x 4.6	5
5	LiChrosorb RP-Select B	Merck	250 x 4.0	5
6	Exsil 100 5μm ODS-B	Exmere	250 x 4.6	5
7	Kromasil KR100-5-C_{18}	Eka Nobel	250 x 4.6	5
8	Suplex pKb-100	Supelco	250 x 4.6	5
9	ACT-1 C_{18}	Interaction	150 x 4.6	10

Methanol+water / 60+40 %(V/V) was used as mobile phase for the test solution. The column temperature was 40 °C and UV detection was carried out at 210 nm. Triethylamine should be of a high-purity grade to prevent high UV offset. For basic drugs the flow rate was set at 1.0 ml/min and for the test solution at 1.5 ml/min.

3.1.3.5 Calculations

The asymmetry factors were calculated at 10% of the peak height using the ratio of the width of the rear and front side of the peak [7]. Because most peaks do not have the ideal Gaussian shape, we used the second moment of the peak for the calculation of the plate numbers [8].

Table 3.1.3: Mobile phases used with the various columns.

Mobile phase	Columns used
CH_3OH + 50 mM TMAH, pH 7.4	1
CH_3OH + 10 mM NaH_2PO_4, pH 2.5	1,4,8
CH_3OH + 10 mM NaH_2PO_4, pH 3.5	1,4,8
CH_3OH + 10 mM NaH_2PO_4, pH 6.0	1,4,8
CH_3OH + 10 mM NaH_2PO_4, pH 7.4	1,2,3,4,5,6,7,8
CH_3OH + 10 mM NaH_2PO_4, pH 10.0	1
CH_3OH + 10 mM NaH_2PO_4+ 15 mM TEA, pH 2.5	1,4,8
CH_3OH + 10 mM NaH_2PO_4+ 15 mM TEA, pH 3.5	1,4,8
CH_3OH + 10 mM NaH_2PO_4+ 15 mM TEA, pH 6.0	1,4,8
CH_3OH + 10 mM NaH_2PO_4+ 4 mM TEA, pH 7.4	1
CH_3OH + 10 mM NaH_2PO_4+ 15 mM TEA, pH 7.4	1,4,8
CH_3OH + 10 mM NaH_2PO_4+ 10 mM IPR, pH 3.5	1
CH_3OH + H_2O	6,8
CH_3CN + 1% NH_4OH, pH 11.2	9

3.1.4 RESULTS AND DISCUSSION

3.1.4.1 Varying the mobile phase composition using a μBondapak C18 column

The aim of this study was to find the best mobile phase conditions to analyse basic compounds. In the first set of experiments we chromatographed all basic drugs on a μBondapak column, using the starting conditions given in Fig. 3.1.2A (methanol+10 mM phosphate pH 7.4, 2 μg inj.). Clearly, as can be seen in Fig. 3.1.3, there is a correlation between the pKa-value of the compound and the asymmetry factor. Generally, peak tailing increases with increasing pKa-values. These results are also confirmed by others [1]. Peak tailing is also a function of the retention factor, as illustrated in Fig. 3.1.4 for a number of basic drugs. Therefore, the data given in Fig. 3.1.3 were normalised to a k-value of 5.

For compounds having a pKa less than 6 we did not observe any problems. Peaks were symmetric with asymmetry factors < 1.5 and plate numbers were about 4000-6000. Asymmetric peaks were obtained for compounds with pKa > 6. These compounds are partly protonated and hence more strongly bound to the acidic silanol and other polar sites.

There are indications that the pKa is not the only parameter influencing peak tailing. It is thought that structural factors also play a role. For example, substances having comparable pKa values show widely differing asymmetry factors. Generally it is observed that the flexibility of the protonated N-atom and thus the possibility of interacting with silanol and eventual other polar sites plays a predominant role.

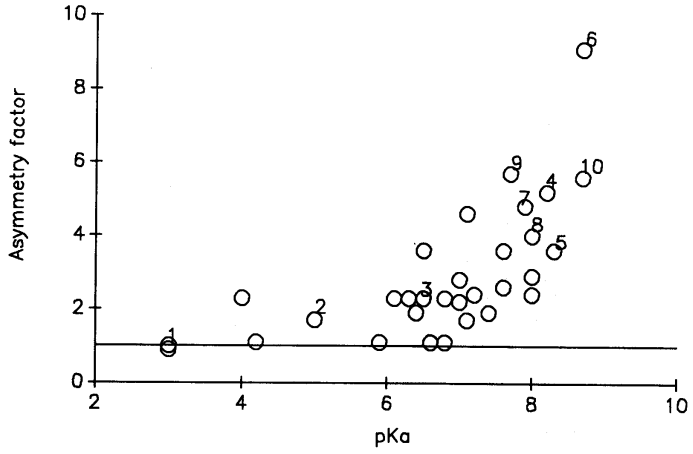

Figure 3.1.3: Scatterplot of pKa vs peak asymmetry, normalised to k = 5. The numbers in the plot correspond to compounds in Table 3.1.1. For HPLC conditions see Fig. 3.1.2A.

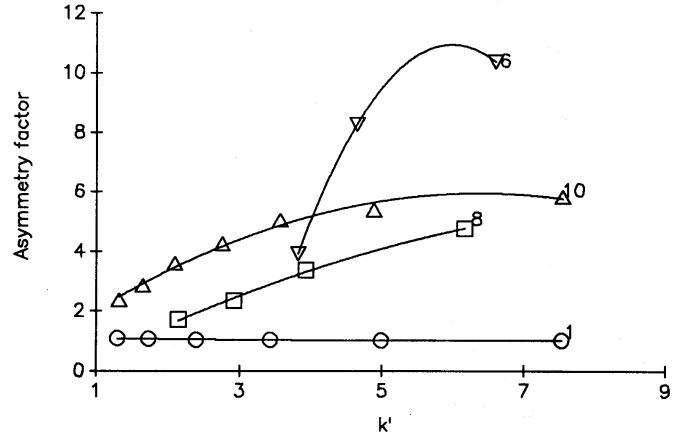

Figure 3.1.4: Relationship between asymmetry factor and k for substances covering the pKa range 3 - 9.

For instance, when we compare substances 6, 4 and 5 (see Table 3.1.1) the decrease of the flexibility in this order of the (protonated) N-atom is significant. The asymmetry factor decreased likewise. Data on the flexibility of protonated and unprotonated N-atoms are taken from NMR measurements.

3.1.4.2 Influence of the pH of the buffer

The influence of the pH of the buffer was tested with 10 mM sodium phosphate at different pH values in the range 3.5-10. Lowering the pH to 3.5 will result in less dissociation of the silanol groups, whereas at pH 10.0 none of the compounds is protonated. In the literature often a pH of less than 3.5 is recommended to suppress silanol activity as effectively as possible. On purpose we have chosen a pH of 3.5, which in our view was sufficiently critical to show differences between columns. The effects of lower pH will be discussed in section 3.1.4.7.

As is shown in Fig. 3.1.5 the overall results on a μBondapak column were in favour of the higher pH values, especially for substances with pKa 6-8. The effects on peak asymmetry are probably not statistically different for compounds with pKa values of 3-6 and 8-9. At pH 7.4 most of the test compounds are not protonated and the effect of dissociated silanols is therefore small.

An effect that is often overlooked is that the pH of a methanol - buffer mixture differs significantly from that of the starting buffer. For instance, 10 mM phosphate buffer pH 7.4 shows a virtual pH of 8.6 in a 1:1 mixture with methanol.

These results conflict with the general opinion that a low pH is the best condition for the chromatography of basic compounds. In order to support our conclusions we have added additional data on this pH effect for 3 different columns in section 3.1.4.7.

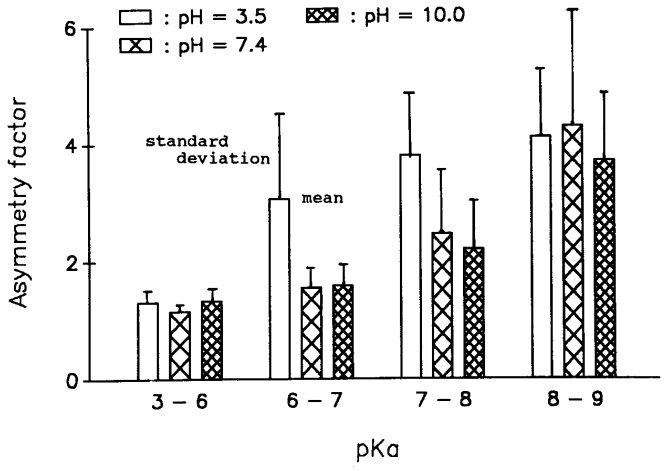

Figure 3.1.5: Influence of the buffer pH on peak asymmetry. Methanol was used as modifier and 2.0 μg were injected. For HPLC conditions see Fig. 3.1.2A. The bars represent the mean and standard deviation (as shown).

The use of a buffer of pH 10.0 led to a small improvement only for very basic substances. This high pH is in fact unrealistic and was only incorporated to see

whether unexpected effects would arise. Summarising, we recommend a pH of around 7.

3.1.4.3 Influence of the type of buffer

To determine the influence of the type of buffer, we compared 50 mM tetramethylammonium phosphate and 10 mM sodium phosphate buffer. A low concentration of sodium phosphate was necessary because of the low solubility in eluents with a high methanol concentration. In our laboratory tetramethylammonium phosphate buffer is being used as a standard for the HPLC analysis of basic drugs. The results obtained with the two buffers are summarised in Fig. 3.1.6. For compounds with pKa values smaller than 8 almost no difference in peak shape was noticed. As far as plate numbers are concerned the use of sodium phosphate resulted in a slight increase. For compounds with pKa values larger than 8 tetramethylammonium phosphate showed better results (although not statistically significant), but asymmetry factors are still larger than 2. Overall it can be concluded that tetramethylammonium phosphate can be used successfully to improve peak shapes by effectively blocking the activity of silanol groups and other polar sites. It should be added, however, that the tetramethylammonium ion is rather aggressive (see section 3.1.4.6).

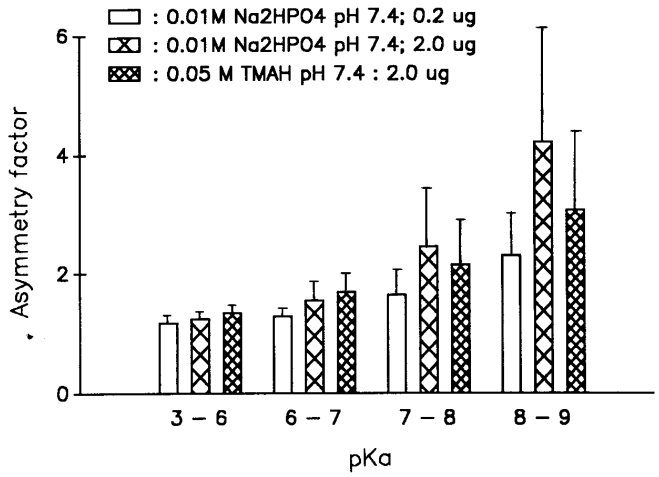

Figure 3.1.6: Influence of the type of buffer and the amount injected on peak asymmetry.

3.1.4.4 Influence of the type of additive

To determine the influence of a silanol blocking agent triethylamine was used with a 10 mM sodium phosphate buffer pH 7.4. The results were compared with those without triethylamine and are shown in Figure 3.1.7 and in Table 3.1.5. Triethylamine (TEA) is a very basic compound (pKa about 11) which will also interact with silanol

groups and so compete with the basic drugs. Dimethyloctylamine (DMOA) has been successfully used by others [1] for the same purpose. A comparison between TEA and DMOA is shown in section 3.3 of this thesis. As can be seen in Figure 3.1.7 the addition of triethylamine resulted in a clear improvement in peak shape. Particularly for compounds with pKa > 6 tailing clearly decreased, resulting in plate numbers of 3000-6000 with acceptable symmetry. However, several basic drugs still have unacceptable peak shapes. Comparing Figs. 3.1.6 and 3.1.7 it can be concluded that the effect of TEA is better than of tetramethylammonium. Whether the addition of TEA is to be preferred at high (ca. 7) or low (ca. 3) pH will be discussed later.
Sodium 1-hexanesulphonate was also tested as an ion-pair reagent (IPR). For this the basic drugs have to be protonated and 10 mM sodium phosphate (pH of 3.5) was used as buffer. Comparing these results with those obtained with 10 mM sodium phosphate buffer (pH 3.5) the addition of IPR did not result in a clear improvement of peak symmetry. Compared with sodium phosphate (pH 7.4) peak symmetry is even worse, except for compounds with pKa 8 - 9, were addition of IPR seems to result in less tailing.

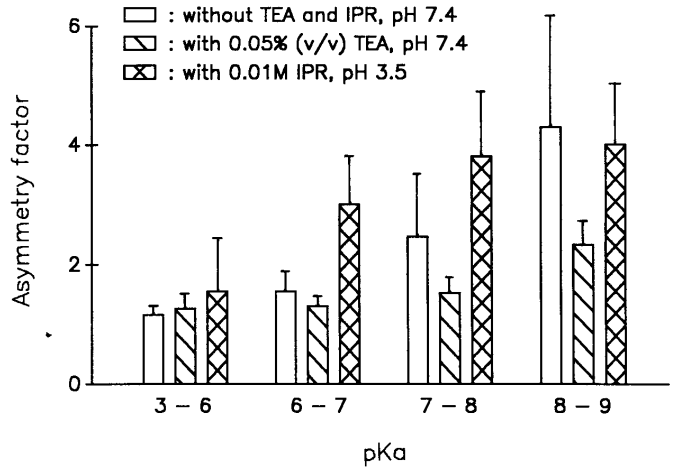

Figure 3.1.7: Influence of triethylamine (TEA) and sodium 1-hexane-sulphonate (IPR) on peak assymetry. Methanol was used as modifier and 2.0 µg were injected. For HPLC conditions, see Fig. 3.1.2A.

3.1.4.5 Influence of the amount injected

For the determination of the influence of the amount of basic drug injected onto the column several experiments were compared. Amounts of 0.2 and 2.0 µg were injected and 50 mM tetramethylammonium phosphate (pH 7.4) and 10 mM sodium phosphate (pH 3.5 and 7.4) were used.
Decreasing the amount injected can dramatically improve peak symmetry (Figure 3.1.6). Although Figure 3.1.6 shows only data at pH 7.4, the effect is also seen at pH 3.5, both for sodium phosphate and tetramethylammonium phosphate. Most

asymmetry factors were less than 2 when 0.2 μg was injected. For very tailing compounds the asymmetry factor was even reduced by a factor 2 for small amounts injected. Especially for substances with low UV absorbance (see compound 11, Table 3.1.1) the large amounts injected are the main reason for peak tailing. When the amount of sample is reduced, the saturation of residual silanol and eventual other polar groups will be avoided.

3.1.4.6 Influence of mobile phase composition on column stability

The analysis of basic compounds with RPLC is often used in quality control and stability studies. This means that many samples have to be analysed and that mobile phase volumes in the order of 1000 ml and more are pumped over the column. Therefore, it is important to use a mobile phase-stationary phase combination that is not destructive.

During the experiments, as shown in Fig. 3.1.2A, the μBondapak column was tested for changes in silanol activity, hydrophobicity and metal activity. To check the change in silanol activity a mixture of aniline and phenol was injected. For well deactivated stationary phases aniline elutes before phenol [9,10]. As a measure of silanol activity the ratio of the retention times of aniline and phenol was used. To check the hydrophobicity a mixture of anthracene and benzene was injected. A change in the ratio of the retention times indicates a change in hydrophobicity [9]. Acetylacetone was used to determine the chane in metal activity [9]. It can form a complex with metal ions resulting in a broad and tailing peak that is retained. In Figure 3.1.8 a chromatogram of the test solution is shown.

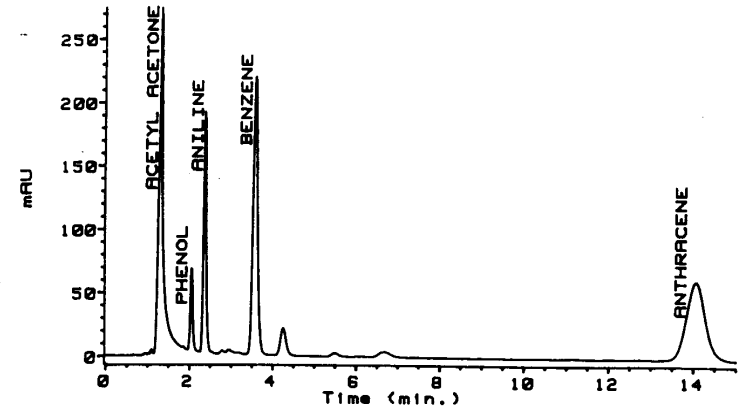

Figure 3.1.8: Chromatogram of the test compounds on a μBondapak C18 column (300 x 3.9 mm I.D.). Methanol + water/60+40 %(V/V) was used as eluent.

When tetramethylammonium phosphate and sodium phosphate (pH 7.4) were compared it was seen that the former is more aggressive and strips of the stationary phase.

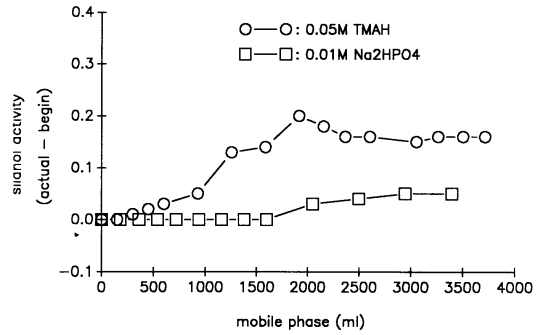

Figure 3.1.9: Influence of the type of buffer and the volume pumped over the column on silanol activity (expressed as the difference between the actual and the starting values). For HPLC conditions see Fig. 3.1.2A and 3.1.5.

As shown in Figures 3.1.9 and 3.1.10 the change in silanol activity and hydrophobicity is less pronounced with sodium phosphate. These conclusions are confirmed by Wherli et al [11] who also found that quaternary ammonium compounds are aggressive towards silica-based stationary phases. Adding triethylamine or sodium 1-hexanesulphonate to the mobile phase did not clearly influence the stability of the column.
As far as the metal activity is concerned, when tetramethylammonium phosphate was used the peak shape of acetylacetone was sharp and eluted after k=1 at the beginning of the experiment. The tailing steadily increased and the retention decreased to k=0.1 after 1000 ml mobile phase were pumped over the column.

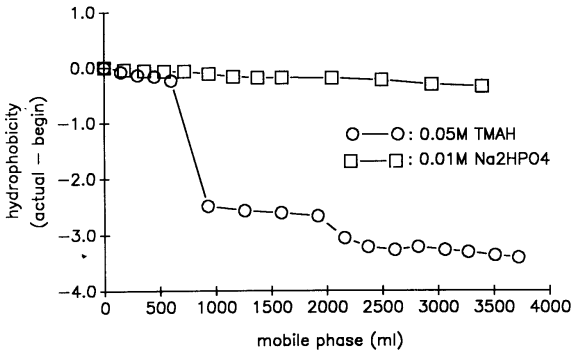

Figure 3.1.10: Influence of the type of buffer and the volume pumped over the column on hydrophobicity (expressed as the difference between the actual and starting values). For HPLC conditions see Fig. 3.1.2A.

Subsequently, the peak symmetry and retention time remained stable. An increase in tailing indicates an increase in metal activity. The decrease in retention indicated a

decrease in metal activity or could be a result of reduced reversed phase activity due to a loss of bonded phase. When phosphate was used the peak shape was asymmetric but stable. The retention time decreased from k=0.4 to k=0.1 after 3400 ml of mobile phase pumped over the column.
Another parameter that can influence the stability of the column is the pH of the mobile phase. In Fig. 3.1.11 the influence of the pH on the change in silanol activity is shown. It was found that the use of high pH resulted in a rapid increase of silanol activity and a decrease of hydrophobicity. This high pH will strip of the stationary phase. The use of pH 3.5 shows no change in silanol activity and hydrophobicity, whereas for pH 7.4 a slight change after about 1000 ml was noticed.

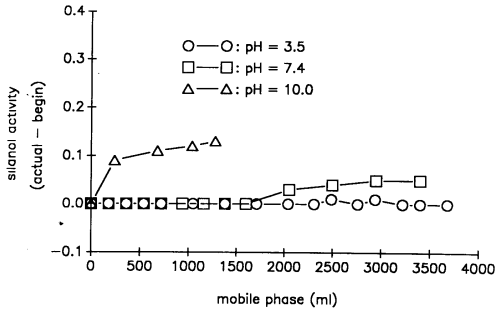

Figure 3.1.11: Influence of the pH of sodium phosphate buffer and the volume pumped over the column on silanol activity (expressed as the difference between the actual and starting values). For HPLC conditions see Fig. 3.1.2A and 3.1.6.

3.1.4.7 Varying the stationary phase

Stationary phases specially developed for the analysis of basic compounds were investigated and the results were compared with the μBondapak column, using methanol+10mM sodium phosphate buffer (pH of 7.4) as the mobile phase. In order to ensure a fair comparison, the columns were compared in the same k range. The results are described in a qualitative way.

Nova-Pak C18

The Nova-Pak C_{18} column showed more tailing for most compounds with a pKa larger than 7. For compounds with a pKa less than 7 there is less difference. Most compounds have asymmetry factors of 2 or less. Owing to the smaller particle size of the Nova-Pak phase, for symmetrical peaks it gives about twice as many plates as μBondapak under comparable conditions.

Zorbax Rx-C8, Kromasil KR-5-C18 and Lichrosorb RP-Select B

These three columns are being offered as stationary phases specially deactivated for basic compounds.

They did not show a clear improvement in peak symmetry compared with a μBondapak C_{18} column. For 9 compounds with a pKa larger than 7 the Kromasil column even showed asymmetry factors of 3 and larger. However, for symmetric peaks the plate number was over 10.000.

Exsil 100 5μm ODS-B

The Exsil phase is also specially deactivated for basic compounds and, according to the manufacturer basic compounds can be eluted even without buffer. For the compounds in our test series this was correct. All compounds, however, had extremely asymmetric peaks with asymmetry factors of 6 and more. The use of 10 mM sodium phosphate (pH 7.4) instead of water did not reduce the peak asymmetry significantly.

Suplex pKb-100

The Suplex column is a silica based C_{18} stationary phase where residual silanols and eventual other polar sites are electrostatically shielded. First this column was tested with methanol+10 mM sodium phosphate (pH 7.4) as eluent. As can be seen in Figure 3.1.12 good peak shapes were obtained. Only six compounds had an asymmetry factor larger than 2 and plate numbers were in the range 3000-11000. Compared with μBondapak this stationary phase is more suitable for the analysis of basic compounds.

The column was also tested without buffer using a methanol+water mobile phase, which can be advantageous when the system is coupled to a mass spectrometer. In that case the column should be "conditioned" by washing with 10 mM buffer. Under these conditions good peak shapes were also obtained. Only 1 compound showed an asymmetry factor larger than 2.

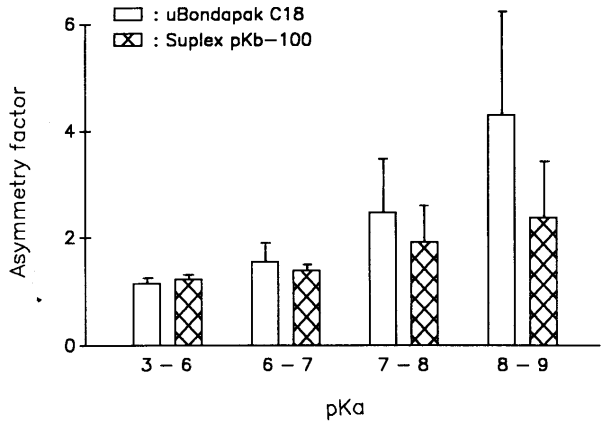

Figure 3.1.12: Comparison between μBondapak C_{18} and Suplex pKb-100. Methanol+10mM sodium phosphate (pH 7.4) was used as eluent and 2.0 μg was injected.

Plate numbers were also in the range of 3000 to 11000. However, 5 compounds with a pKa value larger than 8 showed fronting peaks. This phenomenon was not observed using a phosphate buffer. Of course, for a rugged method involving ionic and ionisable compounds, the mobile phase always should be carefully buffered. In Figure 3.1.13 chromatograms of a compound with a pKa value of 8.7 with and without buffer are shown.
In our experience, it was found that for this column the equilibration time is longer than usual. Reproducibility proved to be good as compared with conventional columns. For better stability acetonitrile should be used instead of methanol.

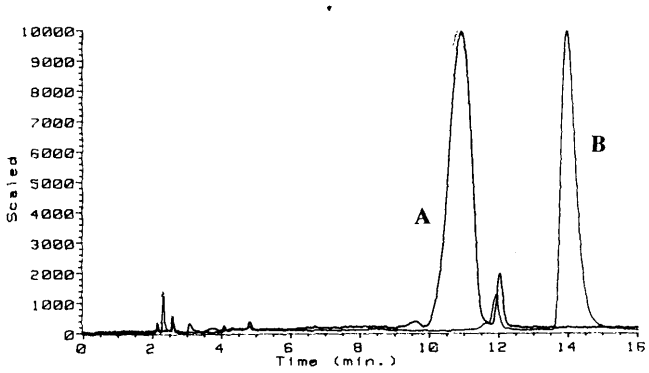

Figure 3.1.13: Chromatogram of a basic compound (pKa = 8.7) (A) without and (B) with 10 mM sodium phosphate (pH 7.4) on the Suplex pKb-100 column.

Table 3.1.4: Comparison of stationary phases.

Column	Peak[a] shape	Plate[a] number	Stability[a]	Remarks
μBondapak C_{18}	0	0	0	
NovaPak C_{18}	-	+	0	
Kromasil KR100-5-C_{18}	0	+	0	
Lichrosorb RP-Select B	0	+	0	
LiChrosorb RP-Select B	0	+	0	
Exsil 100 5μm ODS-B	--	-	0	Can be used without buffer
Suplex pKb-100	+	+	0	Can be used without buffer
ACT-1 C_{18}	0	--	+	Very pH stable

a: --, very bad; -, bad; 0, acceptable; +, good.

ACT-1 C18

This column is based on a C_{18}-polymer stationary phase. The advantage over silica based columns is that there should be no ionic or ionisable species present. Furthermore, these columns are very stable and a pH range of 0 - 14 can be used. Our results with this column showed acceptable tailing, of the order of 1.5 to 3. However, a great disadvantage is the low plate numbers obtained. They are in the order of 50 - 250 which is less than 10% of the plate numbers obtained with a μBondapak C_{18} column. The results obtained with the different stationary phases are summarised in Table 3.1.4.

Comparison of μBondapak C18, Zorbax Rx-C18 and Suplex pKb-100

The experiments in the previous section strongly suggest that at pH 3.5, where the compounds are protonated, the dissociated silanols are still fully active. Even at pH 2.5, a pH recommended by most manufacturers, the peak shape improved only marginally (see Table 3.1.5). Plain buffers are thus unable to mask effectively silanol and eventual other polar effects. A further illustration, also using a Zorbax Rx C_{18} column, is given in Figure 3.1.14. At a buffer pH 7.4 the substances are not protonated and a high portion of methanol is needed to elute the substances from the column. With a buffer of pH 6.0 the compounds are partly protonated (more polar, less methanol needed) which especially for codeine leads to increased tailing.

Table 3.1.5: Comparison of three columns tested under low pH conditions.

Column	Eluent	Compound	pKa	pH 3.5		pH 2.5		pH 2.5 + 15mM TEA	
				k	As	k	As	k	As
Zorbax Rx-C18	A	7	7.9	3.2	5.1	3.1	4.3	3.1	2.1
		6	8.7	2.1	4.1	1.7	4.3	1.8	2.5
		9	7.7	2.6	4.5	2.1	4.0	2.2	2.0
		8	8.0	4.4	5.1	4.1	4.5	4.3	2.2
μBondapak C_{18}	A	7	7.9	3.3	8.6	3.3	7.3	3.2	1.7
		6	8.7	2.2	7.4	2.3	5.6	2.2	2.1
		9	7.7	2.4	7.7	2.5	5.8	2.4	1.6
		8	8.0	4.6	9.7	4.6	7.6	4.5	1.7
Suplex pKb-100	B	7	7.9	4.5	1.9	4.8	1.9	4.1	1.7
		6	8.7	1.9	1.6	2.0	1.5	1.8	1.7
		8	7.7	2.9	1.7	2.9	1.6	2.7	1.5
		9	8.0	6.2	2.0	6.4	1.9	5.4	1.7

Eluent A: methanol+10 mM sodium dihydrogenphosphate / 50+50 %(V/V)

Eluent B: methanol+10 mM sodium dihydrogenphosphate / 25+75 %(V/V)

At a pH of 3.5 the peaks are sharpened and silanol and other polar effects are suppressed. A remarkable effect is the inversion of morphine and oxymorphone as a result of the pH change.

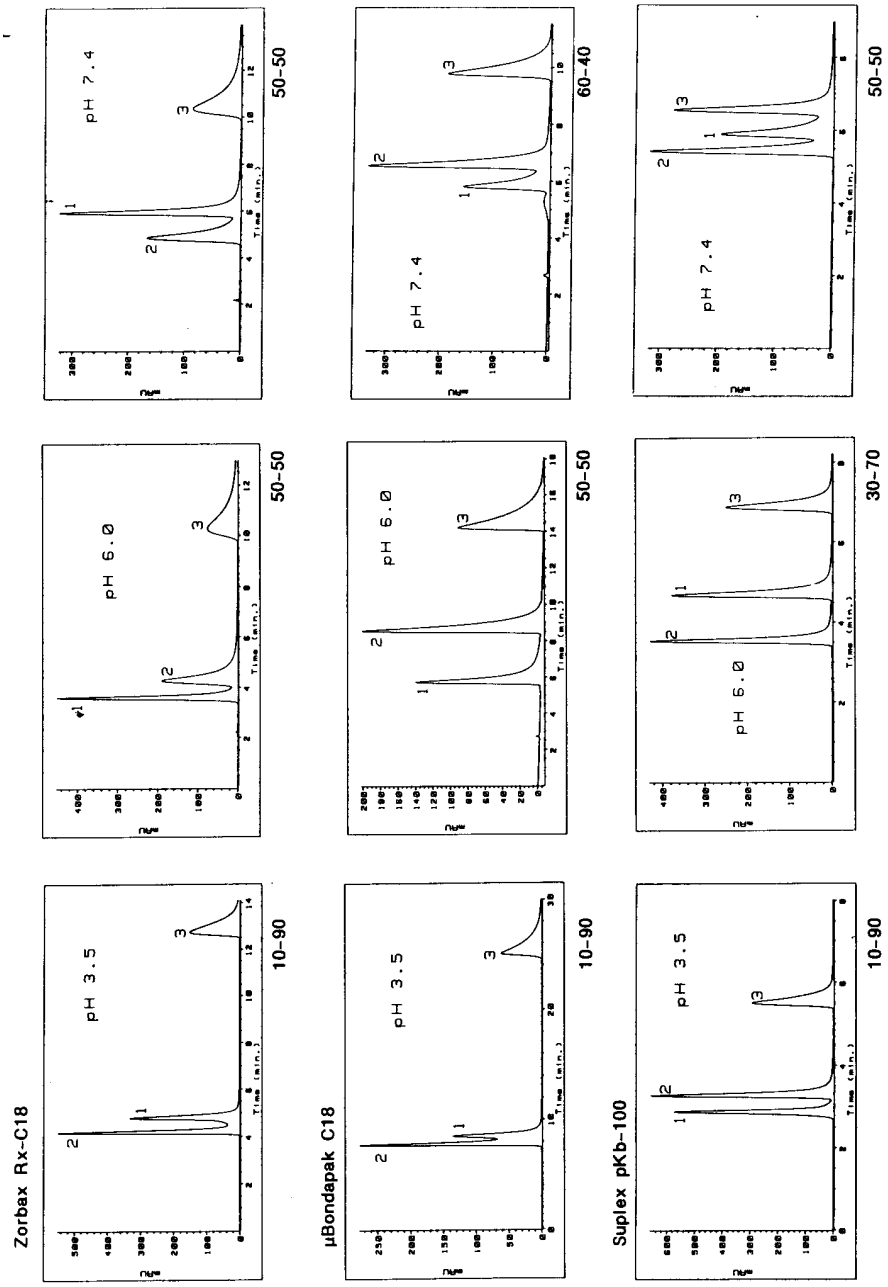

Figure 3.1.14: Comparison of Zorbax Rx-C_{18}, µBondapak C_{18} and Suplex pKb-100 at pH 7.4, 6 and 3.5. Solutes: 1=oxymorphone, pKa=7.65; 2=morphine, pKa=7.08; 3=codeine, pKa =7.00. The methanol+10mM phosphate compensations are given in %(V/V) beneath the plots.

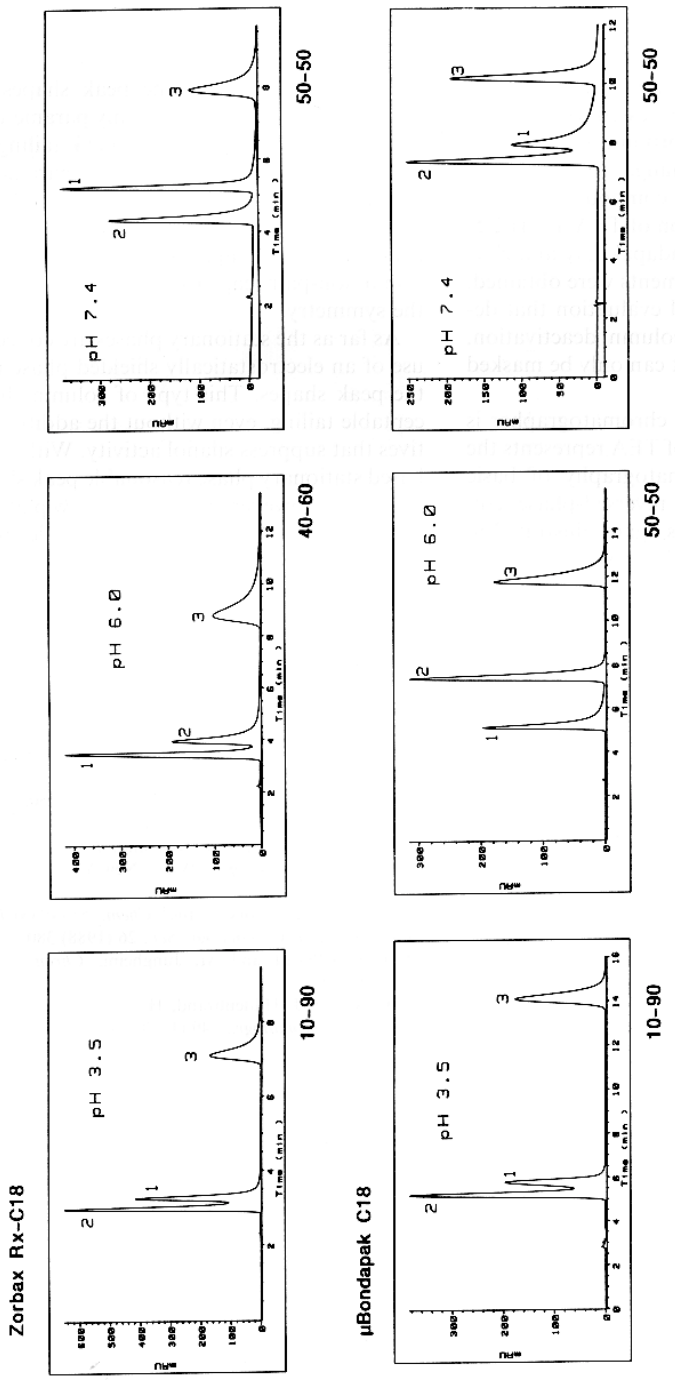

Figure 3.1.15: Chromatograms of the separation of the three morphines at pH 7.4, 6.0 and 3.5 after addition of 15 mM triethylamine to the mobile phase. The methanol+10mM phosphate compensations are given in %(V/V) beneath the plots.

For a set of 4 "difficult" compounds we also studied the effect of the addition of TEA at pH 2.5. As shown in Table 3.1.5 for µBondapak and Zorbax Rx C_{18} dramatic

improvements were obtained. We conclude from this critical evaluation that despite a low eluent pH and column deactivation, many active sites still exist that can only be masked by adding TEA. A major conclusion for the chromatographer is that a low pH plus TEA represents the best condition for the chromatography of basic compounds on conventional reversed phase columns. This recommendation is again illustrated in Figure 3.1.15. The morphines already shown in Figure 3.1.14 were now eluted under the same conditions except for the addition of 15 mM TEA. Also here the results are significant. For the Suplex column a different result was obtained. The special character of this stationary phase can also be deduced from the data in Table 3.1.5. Clearly, the shielding is so effective that addition of TEA is no longer necessary. As a further illustration we refer to Figure 3.1.14 for the separation of morphine, oxymorphone, and codeine. The best condition proved to be pH 3.5, but without TEA.

3.1.5 CONCLUSIONS

The optimisation of the peak shapes of basic compounds is difficult and many parameters can be varied. It was found that the peak tailing depends on the pKa value and on structural parameters of a compound. When tailing occurs the addition of silanol blocking reagents, such as triethylamine, was most effective in suppressing this effect. The addition of ion-pair reagents, however, did not improve symmetry.

As far as the stationary phases are concerned, the use of an electrostatically shielded phase improved peak shapes. This column showed acceptable tailing, even without the addition of additives that suppress silanol activity.

With a polymer based stationary phase reasonable peak shapes were obtained. However, owing to the low plate number, this column has poor separation efficiency.

3.1.6 REFERENCES

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3.2 SELECTION OF STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC COMPOUNDS USING CHEMOMETRIC METHODS

3.2.1 SUMMARY

The analysis of basic compounds by means of reversed phase liquid chromatography (RPLC) is often hampered by poor peak shapes. In this chapter chemometrical methods are used to select and reduce the number of test compounds and to detect differences in applicability of stationary phases designed for the analysis of basic drugs.

In the first part principal component analysis was applied to reduce the number of test compounds necessary to characterise a stationary phase. From a data set of the asymmetry values of 32 test compounds analysed on six different LC columns, five representative compounds were selected. Subsequently, these five compounds were used for evaluation of commercially available columns.

For the column judgement the asymmetry of the test compounds, the efficiency, and the day-to-day repeatability of the retention factor and the plate number, were taken into account. Graphical presentation using bar charts, multi-criteria decision making based on the Pareto optimality and bi-plots were used to distinguish between columns. First of all eight columns were compared at individual pH values of 3.0, 7.0 and 11.0. Finally, all results were combined and revealed that for our test compounds good results were obtained at a pH of 11 using a column containing zirkonium oxide particles coated with polybutadiene (3MZ-18). At low pH values good results were obtained with a Supelcosil LC-ABZ and a Zorbax Rx-C_{18} column.

Overall it can be concluded that a chemometric approach is successfully applied for the development of a method for in-house column testing and evaluation dedicated to the Organon type of compounds. Other columns developed for the analysis of basic compounds can now be efficiently tested with the method described in this chapter. Chemometric methods were useful to efficiently reduce the number of test compounds and for column evaluation. However, the final selection of a column strongly depends on the requirements defined by the expert. The requirements, which are, for example, for routine quality control clearly different than for purity testing of new chemical entities in drug development, can be translated to weighing factors for the variables tested. For this the advice of the expert remains indispensable.

R.J.M. Vervoort, M.W.J. Derksen and F.A. Maris, J. Chromatogr., 678 (1994) 1-15.

3.2.2 INTRODUCTION

In pharmaceutical analysis reversed-phase liquid chromatography (LC) is the most frequently used technique. It is used as a tool to quantify active ingredients in pharmaceutical formulations, to determine impurities, to investigate the stability of a product, etc. For the analysis of basic drugs, however, asymmetric peaks can be obtained due to ionic and other polar interactions of compounds with residual silanol groups and eventual other polar sites of the stationary phase [1,2].

To improve peak shape, optimisation of both the mobile and stationary phase should be considered. In the literature, many suggestions have been given to optimise the mobile phase, e.g. by adding silanol blockers, using ion-pair reagents and by selecting an optimal pH [1,2]. Also the number of stationary phases specially designed for the analysis of basic compounds is exponentially increasing [1,3]. Because many possibilities are available, it is difficult to select the best system.

Recently, we reported about the analysis of 32 basic compounds using several columns with different eluents [1]. Relations between retention, asymmetry and basicity of the compounds were studied for a μBondapak C_{18} column. Together with data of the peak shape of the 32 test compounds on five other columns, a data set was obtained which contains a lot of information. However, to extract useful information from such a set is often problematic and the use of chemometric methods can be helpful.

Multivariate techniques, like principal component, cluster, correspondence factor and discriminant analysis, can be used to analyse and reduce the number of variables of a data set [4-8]. In the literature, Musumarra et al reported results from principal component analysis (PCA) of chromatographic retention data of drugs for identification purposes [6]. Delaney et al used PCA to select a set of test compounds in LC [7]. Schmitz and co-workers compared different multivariate techniques for the characterisation of phases in LC and to select test compounds [8].

From a data set of 32 compounds and different columns, a number of test compounds was selected using PCA. The test compounds were selected on basis of the asymmetry obtained on six stationary phases, which are recommended for the analysis of basic compounds. Subsequently, the test compounds were used for testing of several other stationary phases. For selecting the optimal column, not only the peak shapes are important, but also the efficiency and ruggedness. In these situations methods of multi-criteria decision making (MCDM) can be applied for column judgement [9-12]. Within this approach it is not necessary to make a priori decisions and experiments can be compared easily. Also bar-charts and bi-plots can be helpful tools to characterise stationary phases [13].

For the column judgement presented in this chapter the asymmetry values, the plate height, and the day-to-day repeatability of the retention factor and plate height are used.

3.2.3 EXPERIMENTAL

3.2.3.1 Apparatus

The HPLC experiments were carried out using a HP1090M liquid chromatograph equipped with a HP1040M Diode-Array Detector (Hewlett Packard, Amstelveen, The Netherlands). HPLC chromatograms were collected on a HP 79994A HPLC Workstation.
The software packages Unscrambler 5.03, Camo As (Trondheim, Norway) and Microsoft Excel 4.0, Microsoft Corporation (Redmond, WA, USA) were used to analyse the data.

3.2.3.2 Chemicals

All basic drugs were synthesized by NV Organon (Oss, The Netherlands).
As organic modifiers methanol (MeOH) and acetonitrile (ACN) were used. Methanol was freshly distilled before use. Analytical grade acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands).
For the preparation of the buffers disodiumhydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and boric acid (H_3BO_3), supplied by J.T. Baker were used. To obtain 25 mM buffers, adequate amounts were dissolved in water of Milli Q quality. Sodium hydroxide (NaOH) and concentrated phosphoric acid (H_3PO_4) were obtained from Merck (Darmstadt, Germany) and were added to the buffers until the desired pH value was reached.
The amount of basic drugs injected was 2 μg and was achieved by injecting 2 μl from a 1 mg/ml solution in methanol.
The stationary phases used in this study were obtained from the suppliers as pre-packed columns (Table 3.2.1).

3.2.3.3 Experimental set-up

The experiments carried out to characterise the columns were performed in strict order. Initially, for each column data were collected in duplicate at the highest pH to be tested. Subsequently, this was done for the lower pH. All experiments were repeated on a subsequent day.
Generally, silica-based columns are claimed to be only stable from pH values of 2 to 8. In order to avoid operating the silica-based columns too close to the operating boundaries, they were tested at pH's of 3 and 7 using methanol as modifier. The non-silica based columns were tested, using acetonitrile as modifier, at pH values of 7 and 11, and for the Aluspher RP Select B and the 3MZ-18 column also at pH 3.
The modifier-buffer ratio was adjusted to ensure k larger than 1.

Table 3.2.1: Overview of the stationary phases used for the selection of the test compounds (A) and for the evaluation of the column performance (B).

	Column	Abbreviation	Manufacturer	Dimensions (length x I.D.)	Particle size (μm)
A	μBondapak C_{18}	BON	Waters	300 x 3.9	10
	NovaPak C_{18}	NOV	Waters	300 x 3.9	4
	Kromasil KR100-5-C_{18}	KRO	Eka Nobel	250 x 4.6	5
	Exsil 100 ODS-B	EXS	Exmere	250 x 4.6	5
	Suplex pKb-100	PKB	Supelco	250 x 4.6	5
	Zorbax Rx-C_{18}	ZRX	Rockland Tech.	250 x 4.6	5
B	Zorbax Rx-C_{18}	ZRX	Rockland Tech.	250 x 4.6	5
	Hypersil BDS-C18	BDS	Shandon	150 x 4.6	5
	Chromspher B	CHB	Chrompack	250 x 4.6	5
	Supelcosil LC-ABZ	ABZ	Supelco	150 x 4.6	5
	Polyspher RP-18	POL	Merck	150 x 4.6	10
	Asahipak ODP-50	ASA	Asahi Chem. Co.	125 x 4.6	5
	Aluspher RP-Select B	ALU	Merck	125 x 4.6	5
	3M-Z18	3MZ	Cohesive Techn.	150 x 4.6	6

3.2.3.4 Calculations

The asymmetry factor (As) was calculated at 10% of the peak height and expressed as the ratio of the width of the rear and the front side of the peak. For the calculation of the plate height (HETP) the second moment of the peak was used [14].
For the ruggedness of the column the day-to-day repeatability of the plate height (RH) and the retention factor (Rk) were determined. This was done by repeating the analyses on the next day. The difference between two days divided by the highest value (in most cases the first day) times 100% is reported.

3.2.4 RESULTS AND DISCUSSION

3.2.4.1 Selection of test compounds

Until now, the applicability of stationary phases for the analysis of basic compounds was tested using 32 compounds [1]. However, analysing 32 compounds each time is rather time consuming. The use of a representative set of test compounds, extracted from the 32 compounds, will result in a reduction of experimental work. This extraction, using the asymmetry data obtained for the test compounds on six different columns, was achieved using PCA. The information obtained with the

reduced number of test compounds should be comparable with the information obtained with 32 compounds.

The data matrix used is shown in Table 3.2.2. For the missing values the particular column averages were used. The data were collected using methanol+10 mM phosphate buffer pH 7.4 as eluent [1]. The asymmetry factors (As) for the μBondapak C_{18} column (BON) were calculated at a retention factor of 5.0. On the other columns the asymmetry values were taken at retention factors varying from 2 to 8 with an average value of about 5.

In Fig. 3.2.1 the results of the principal component analysis of the 6 variables (LC columns) and the 32 objects (test compounds) are shown. Before performing principal component analysis the data were autoscaled. With the first two principal components (PC) 79% of the variance was described, while using the first three PC's 86% of the variance could be described. In Figs. 3.2.1A and 3.2.1B the score and the loading plot of the first and second PC, respectively, are given. In the loading plot can be seen that for the six different columns almost the same value is obtained for the first PC. A better distinction between the columns is obtained by plotting the second against the third PC (Fig. 3.2.1D). From this plot can be seen that the BON column and the NovaPak C_{18} (NOV) column behave very similar, which is not unexpected as they are produced by the same manufacturer. Also with the Kromasil C_{18} (KRO) column comparable results are obtained. In Fig. 3.2.1C the score plot is given for the second and the third PC.

Looking at the score plots, the compounds positioned to the left in Fig. 3.2.1A and in the middle of Fig 3.2.1C always gave symmetrical peaks. Compound 10 was selected as a "neutral" test compound, giving symmetrical peaks on all 6 columns tested. This compound is used to calculate the efficiency of the columns. The first PC seems to correspond with a general asymmetry effect; with increasing value for the PC the average asymmetry for the compounds is increasing. Other test compounds were selected from Figs. 3.2.1A and 3.2.1C on basis of their position somewere on the edge of the cluster in order to obtain maximum discrimination. Compounds 2, 19, 26 and 29 were selected. Looking at Fig. 3.2.1C, compounds 30 and 32 would also have been good choices. The selection of the test compounds on basis of PCA fitted very well with the compounds, which would have been selected by the analytical expert [1]. However, other factors involved can easily be overlooked by the expert or can be difficult to interpret. In Fig. 3.2.2 the structures of the selected compounds are shown.

The correlation matrix of the variables (columns) is given in Table 3.2.3. In this Table again the high correlation between the BON and the NOV column can be noticed. For the other columns probably different mechanisms play a role which influence the (a)symmetry. These different elution mechanisms can be a result of the different ways residual silanols and eventual other polar sites are shielded. For instance, with the Zorbax Rx-C_{18} (ZRX) stationary phase highly pure silica was used, whereas

residual silanols and eventual other polar sites of the Suplex pKb-100 (PKB) stationary phase are shielded electrostatically.

Table 3.2.2: Asymmetry factors for 32 compounds (objects) and 6 LC columns (variables).

Objects	BON	NOV	ZRX	KRO	PKB	EXS
1	3.60	5.74	2.76	2.63	1.30	2.02
2	5.60	10.44	4.82	4.19	3.86	.40
3	1.70	1.57	1.57	1.30	1.28	1.47
4	2.30	Missing	Missing	1.44	1.33	Missing
5	1.10	1.05	1.24	1.20	1.43	1.93
6	4.60	6.92	Missing	Missing	1.73	7.49
7	1.90	2.39	1.64	1.84	1.87	2.34
8	2.30	3.22	3.30	2.38	1.40	2.14
9	1.10	1.21	1.49	1.17	1.17	1.46
10	1.00	1.15	1.22	1.12	1.17	1.06
11	1.10	1.19	Missing	1.17	1.19	0.99
12	0.90	1.07	1.21	1.19	1.23	1.08
13	2.80	3.07	2.20	2.32	1.48	2.95
14	2.20	2.35	1.50	2.16	1.29	1.69
15	1.10	1.24	1.23	Missing	1.21	1.23
16	2.60	5.41	1.96	4.08	2.07	3.10
17	2.30	1.89	1.54	1.70	1.50	2.50
18	5.20	5.51	2.77	5.32	1.85	6.57
19	9.10	14.44	2.94	6.11	2.17	6.43
20	3.60	5.81	1.92	Missing	Missing	4.24
21	3.60	1.59	1.55	1.37	1.38	2.52
22	2.40	2.35	1.91	1.08	Missing	3.28
23	2.30	1.56	1.63	1.34	1.46	2.60
24	1.90	1.23	1.51	1.19	1.25	1.54
25	2.30	2.13	1.79	Missing	Missing	2.83
26	4.80	8.02	2.29	4.19	Missing	8.29
27	1.70	1.37	1.53	1.29	1.72	2.24
28	2.90	3.42	1.99	3.31	1.19	2.13
29	2.40	1.95	1.88	1.70	2.55	3.50
30	5.70	6.99	3.11	4.89	3.55	6.51
31	4.00	6.05	2.47	3.02	.35	4.74
32	3.77	4.19	4.16	4.51	1.56	2.99

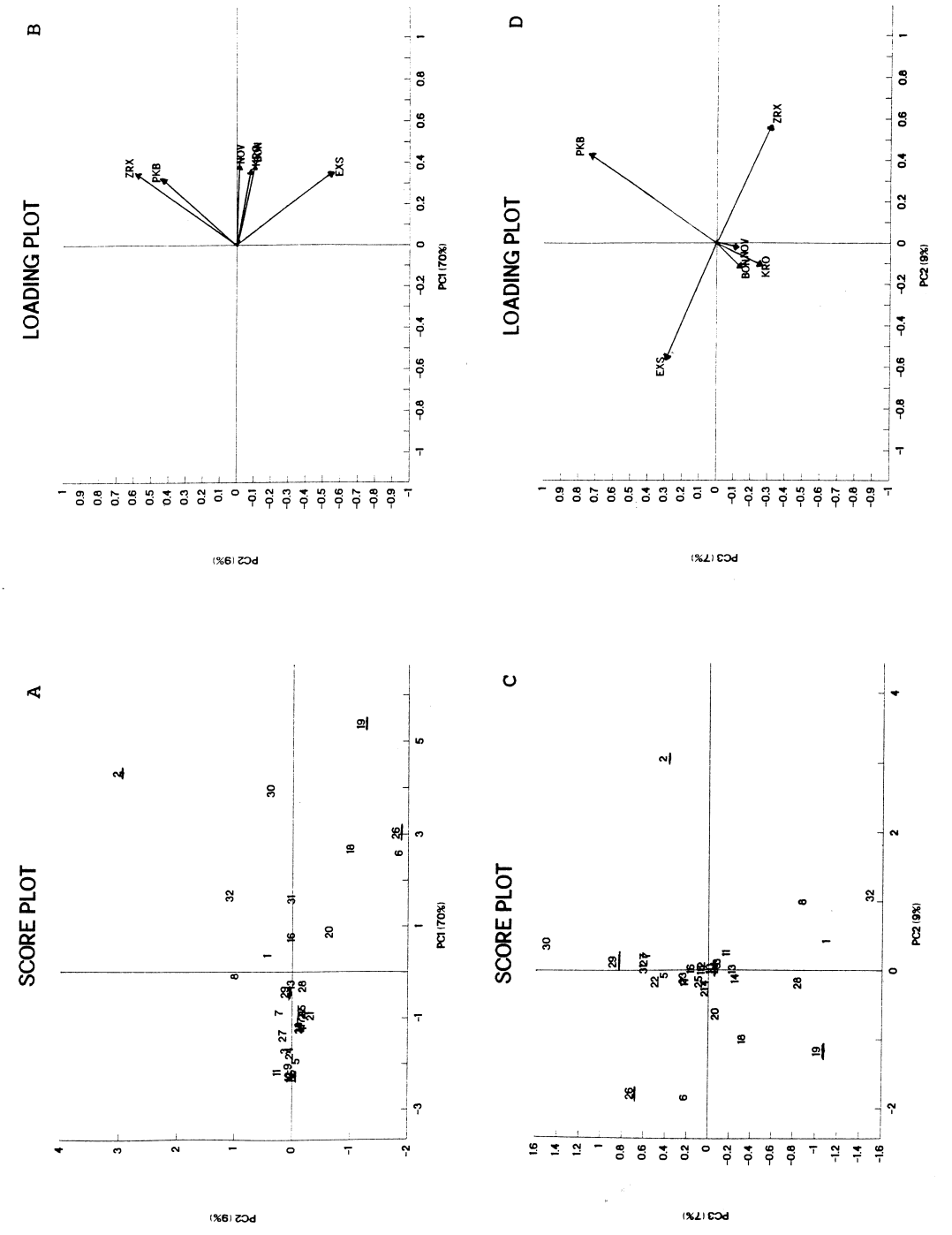

Figure 3.2.1: Results of the principal components analysis. (A) Scoreplot PC1 vs. PC2; (B) loading plot PC1 vs. PC2; (C) scoreplot PC2 vs PC3 and (D) loading plot PC2 vs. PC3. Compounds selected for the test set are underlined in (A) and (C).

Table 3.2.3: Correlation matrix of the variables (columns) calculated from Table 3.2.2.

	BON	NOV	ZRX	KRO	PKB	EXS
BON	1.000	0.935	0.678	0.877	0.636	0.771
NOV		1.000	0.696	0.871	0.674	0.704
ZRX			1.000	0.720	0.658	0.387
KRO				1.000	0.608	0.755
PKB					1.000	0.524
EXS						1.000

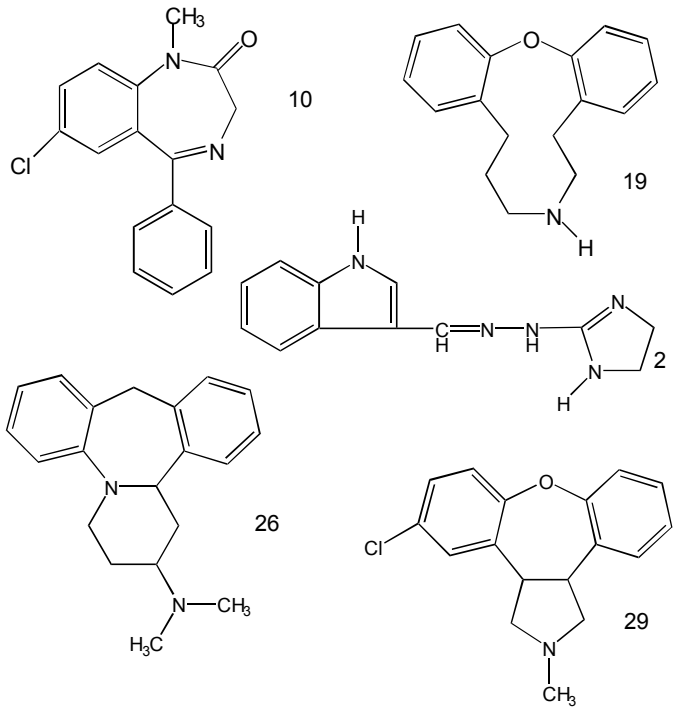

Figure 3.2.2: Structures of compounds selected with PCA.

3.2.4.2 Testing of HPLC columns specially designed for the analysis of basic compounds

In chapter 3.1 eight HPLC columns were compared in a more qualitative way. From these results it was concluded that the PKB column was the most promising column. Later on an improved version of this column appeared on the market, viz. Supelcosil LC-ABZ (ABZ). Also with the ZRX column reasonable results were obtained. Therefore, these two columns and 6 other LC columns were further tested using the five compounds selected in the first part of this study.

The silica-based columns used in the second part of this study (Table 3.2.1B), differed in the manner the residual silanols and eventual other polar sites are

shielded. As mentioned before, very pure silica was used in the preparation of the ZRX column. Residual silanols and other polar sites of the ABZ column are shielded by electrostatic repulsion. The stationary phase used for the Chromspher B (CHB) column are polymer coated silica particles whereas for the Hypersil BDS (BDS) column silica with a homogeneous surface is used which is end-capped after bonding the C_{18} phase.
For the 3MZ-18 (3MZ) and the Aluspher RP-Select B (ALU) stationary phase, polybutadiene was coated on particles of zirkonium oxide and aluminium oxide, respectively. The Asahipak ODP-50 (ASA) column consisted of macroporous particles of polyvinylalcohol based polymer, in which reversed phase chains were introduced by binding stearic ester chains through an ester bond. The Polyspher RP-18 (POL) column consisted of particles of polystyrene - divinylbenzene polymer with C_{18}-chains.
Besides the asymmetry factor an important parameter for the comparison of columns is the efficiency. For example for purity analysis of unknown compounds, i.e. new chemical entities in drug development, high separation efficiency is necessary in an acceptable time. Low efficiency and high asymmetry will lead to poor purity analyses. High asymmetry will also hamper a correct integration. To make it more complicated, columns with low efficiency will mask the factors, which contribute to the tailing.
The primary goal of the column selection is to select the best column, which can be broadly applied. Therefore the average asymmetry of the test compounds was used and not the single values. For the calculation of the efficiency of the column the results of test compound 10 were used. In order to compare the efficiency of the columns and to compensate for difference in the column length the plate height was calculated.
Another factor, which is important for a good column performance, is the repeatability of analysis. In this study the retention factors and the plate heights were measured on two subsequent days. The relative difference between two days is reported for compound 10. The obtained data must be seen as qualitative data in comparison with the more precise data obtained for the asymmetry and the plate height. The day-to-day repeatability of the retention factor is an indication of the stability of the stationary phase, while the day-to-day repeatability of the plate height is an indication of the stability of the packed bed in combination with the stationary phase. The experimental conditions were such that the five test compounds were sufficiently retained (k-values larger than 1).

3.2.4.3 Testing of columns at pH 7

The results are shown in Table 3.2.4. The first question was whether k-values should be treated as a factor. In ref. [1] a correlation was found for k and As. Because the k-values vary from column to column, for a relevant comparison of the asymmetry factors the k-values should be included. However, in this case there was hardly any

correlation between k and As, as can be seen from the correlation matrix presented in Table 3.2.5. Because in the column evaluation only the asymmetry plays a role, the k-values were excluded.

Table 3.2.4: Chromatographic data obtained at pH 7.

Col.	Asymmetry (As)					Retention factor (k)					Average				
	2	10	19	26	29	2	10	19	26	29	As	k	H (μm)	RH (%)	Rk (%)
ZRX	3.8	1.1	6.8	5.9	3.1	3.4	4.9	7.6	5.2	4.0	4.1	5.0	17.3	1.7	10.3
BDS	7.1	0.9	8.3	7.4	2.5	3.5	6.1	2.3	2.9	2.6	5.2	3.5	19.9	2.5	3.3
CHB	4.7	1.1	7.5	6.5	3.9	5.0	2.6	24.4	16.4	8.6	4.7	11.4	31.4	1.0	1.9
ABZ	5.8	0.9	2.1	2.2	1.3	5.2	7.8	4.1	6.4	5.7	2.5	5.8	20.7	4.1	3.9
ASA	6.1	1.7	4.6	4.5	5.0	2.0	5.1	2.8	2.0	2.9	4.4	2.9	64.3	11.4	2.0
ALU	1.3	0.6	1.6	1.7	1.4	12.9	5.0	5.5	6.2	6.5	1.3	7.2	74.8	14.1	6.1
POL	3.3	1.1	1.8	1.3	1.2	0.5	5.6	1.2	3.2	8.0	1.8	3.7	298.3	69.3	9.0
3MZ	1.6	1.0	1.7	1.4	1.2	4.5	2.6	3.2	3.1	3.1	1.4	3.3	25.2	8.9	3.9

In order to interpret the results, bar charts were made (see Fig. 3.2.3). For four of the columns recommended for basic solutes an average asymmetry for the test compounds of more than 4 was observed. Lowest asymmetry factors were obtained for the 3MZ, the ALU and the POL column. From these three columns only the 3MZ column showed a reasonable low plate height. Poor efficiency was observed for the POL column. However, the day-to-day repeatability of the 3MZ column is moderate in comparison with for example the CHB column. A qualitative interpretation of the bar charts is given in Table 3.2.6. In this table the day-to-day repeatability of the retention factor and of the plate height are combined. It can be concluded that the best results are obtained with the 3MZ column, although there are still doubts about the ruggedness of the column.

Table 3.2.5: Correlation matrix at pH 7 calculated from Table 3.2.4.

	As	k	Rk	H	RH
As	1.00	0.14	-0.34	-0.40	-0.46
k		1.00	-0.21	-0.21	-0.29
Rk			1.00	0.46	0.48
H				1.00	0.99
RH					1.00

Multi criteria decision making using the Pareto optimal (PO) points [15] is another method to interpret the results. A PO-plot reveals the best possible combination of two criteria. Applying four factors, in principle 6 plots of 2 criteria can be made. The most important one is the asymmetry vs plate height. As the asymmetry is the starting point for the optimisation it was decided to add the PO-plots for

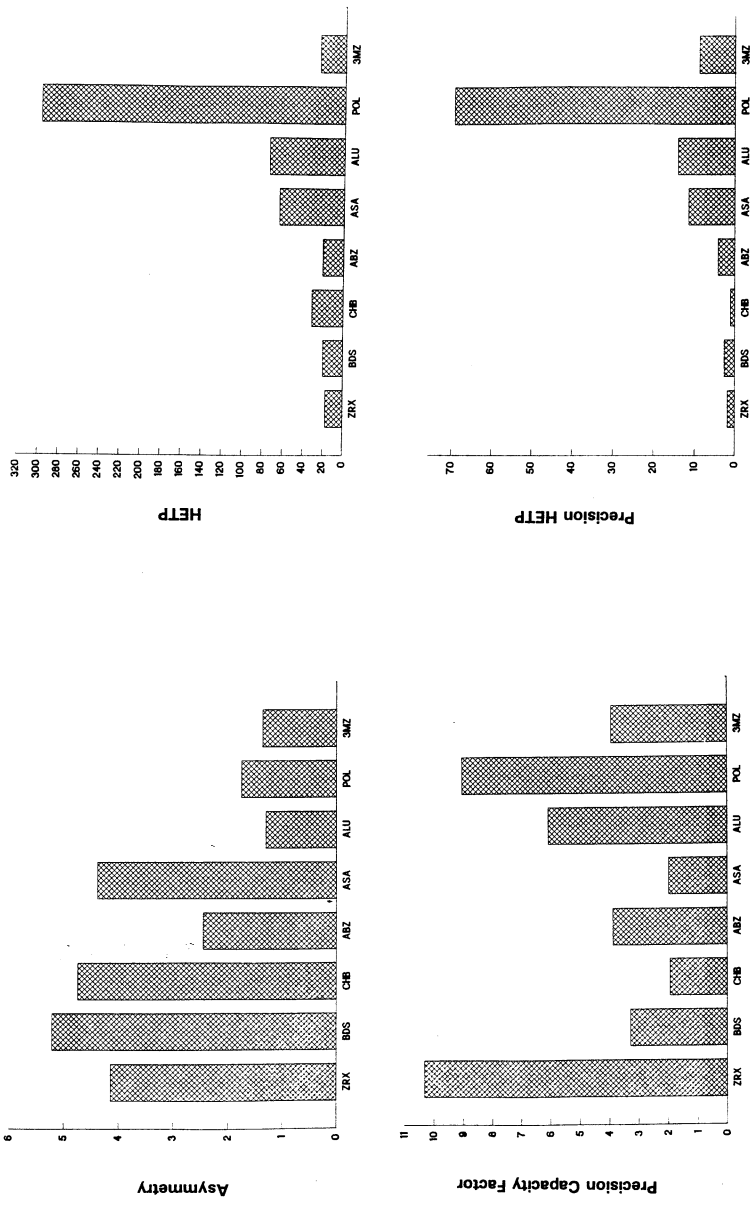

Figure 3.2.3: Bar charts of the asymmetry, the day-to-day repeatability of the retention factor, height equivalent of a theoretical plate (HETP) and the day-to-day repeatability of the HETP obtained at pH 7. The numbers are calculated as described in the Experimental section.

Table 3.2.6: Qualitative interpretation of the results in Fig. 3.2.3.

Column	Asymmetry	Efficiency	Repeatability
ZRX	-	+	0
BDS	-	+	+
CHB	-	+	+
ABZ	0	+	+
ASA	-	0	0
ALU	+	0	0
POL	+	-	-
3MZ	+	+	0

-: bad; 0: acceptable; + good.

asymmetry vs repeatability of the plate height, and asymmetry vs the repeatability of the retention factor. When three or more criteria are involved a stacked PO-plot can be made. A stacked PO-plot is a stack of scatterplots of the individual criteria. The individual plots are placed on top of each other. Points which are exactly vertical to each other (over the different plots) belong to the same stationary phase [15]. The stacked PO-plot is shown in Fig. 3.2.4. The PO-points are indicated (PO between bracket after column). Very good asymmetry values are obtained for the ALU and the 3MZ. Comparing the efficiency, the 3MZ is clearly better. With respect to this aspect the ZRX and ABZ are even better. However, the increased asymmetry is likely to be more substantial than the gain in efficiency. Therefore, the 3MZ is to be preferred.

Still it is interesting to see whether other chemometrical methods can improve the column evaluation. In Figure 3.2.5 a bi-plot is given based on the information presented in Table 3.2.4. The first two PC's explain 83% of the variance. As can be seen the demands on asymmetry and day-to-day repeatability of the retention factor are contradictory to each other, while there is a high correlation between plate height and day-to-day repeatability of the plate height. For the optimal columns these factors must be as low as possible, preferably in the opposite directions of the arrows in Fig. 3.2.5. The best columns are clustered together in the upper right quadrant of the Figure. Also from this graph, taking the asymmetry and plate height as the most important factors, can be concluded that the 3MZ is the most optimal column, although the differences with three other columns is small. An advantage of the bi-plot is that all information can be seen in one graph, while this is not the case for the bar charts and the MCDM plots. However, one has to realise that with the bi-plot information is lost, the interpretation is sometimes difficult and that all factors are treated as being of the same importance.

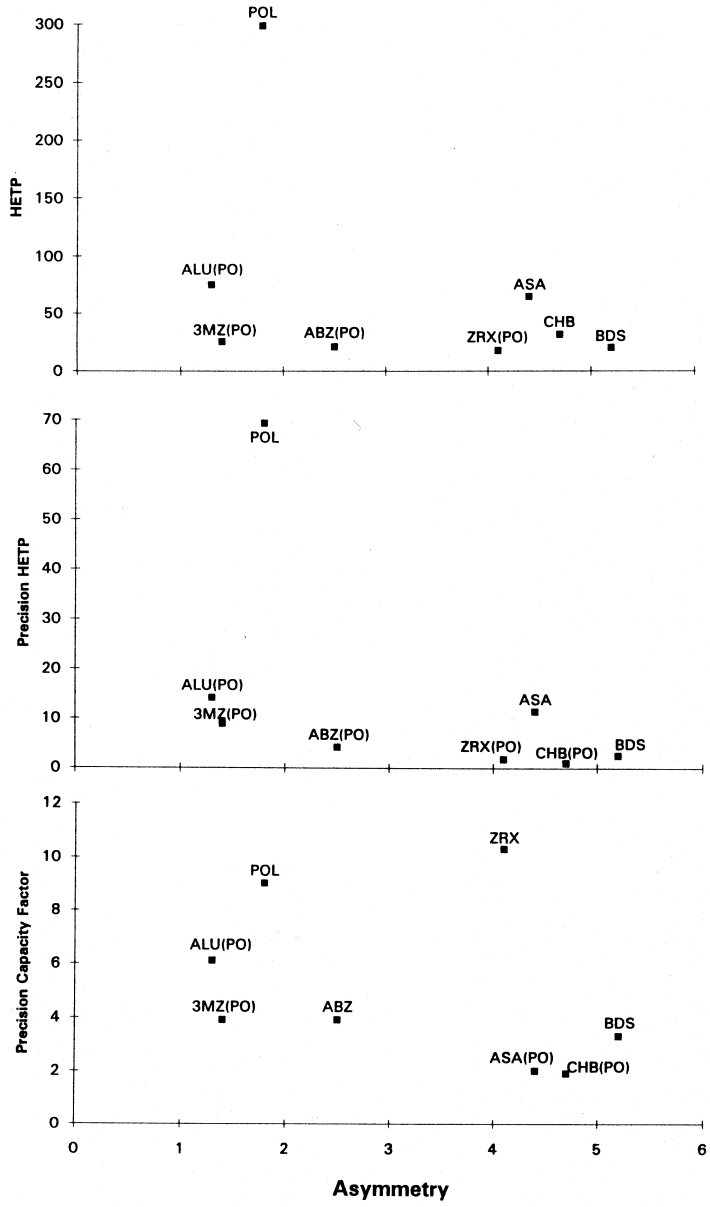

Figure 3.2.4: Stacked PO plot of the asymmetry vs. HETP, day-to-day repeatability of the HETP and day-to-day repeatability of the retention factor, obtained at pH 7. The PO points are indicated between brackets (PO) in the plot.

3.2.4.4 Testing of new columns at pH values of 3, 7 and 11

An overview of the results at pH values of 3, 7 and 11 is given in Table 3.2.7. Again from the original data the correlation between the retention factors and the

asymmetries was so small that the retention factor was excluded as a factor. From the correlation matrices at pH 3 and 11 again surprisingly high correlations of 0.82

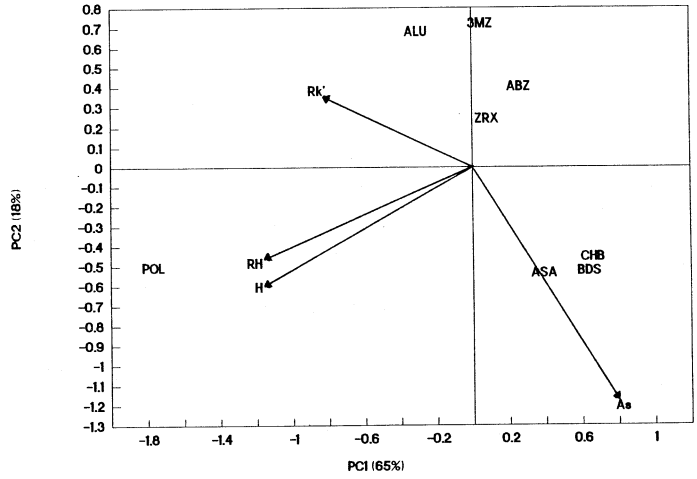

Figure 3.2.5: Bi-plot obtained at pH 7. The data used for the bi-plot are the average values given in Table 3.2.4.

and 1.00, respectively, were found between the plate height and the day-to-day repeatability of the plate height, as was also observed in the previous section at a pH of 7 (see Table 3.2.5).

Table 3.2.7: Chromatographic data obtained at pH 3, 7 and 11.

Col.	pH 3				pH 7				pH 11			
	As[a]	H (µm)	RH (%)	Rk (%)	As[a]	H (µm)	RH (%)	Rk (%)	As[a]	H (µm)	RH (%)	Rk (%)
ZRX	3.3	17.5	5.4	4.7	4.1	17.3	1.7	10.3				
BDS	8.8	20.5	4.4	0.0	5.2	19.9	2.5	3.3				
CHB	5.1	32.6	0.5	4.1	4.7	31.4	1.0	1.9				
ABZ	1.8	21.0	5.7	4.0	2.5	20.7	4.1	3.9				
ASA	3.2	62.0	27.1	6.3	4.4	64.3	11.4	2.0	2.2	64.0	14.9	3.6
ALU					1.3	74.8	14.1	6.1	0.7	73.8	10.6	25.6
POL					1.8	298.3	69.3	9.0	1.4	323.6	89.3	6.2
3MZ	1.6	26.5	13.8	6.8	1.4	25.2	8.9	3.9	1.0	24.7	4.9	7.7

a: Average values of compounds 2, 10, 19, 26 and 29.

Because at pH of 3 and 11 less columns were used than at the pH of 7, the interpretation is easier. The PO-points were determined and are given in Table 3.2.8.

At a pH of 3 the 3MZ column again shows the lowest tailing, although the difference with the ABZ column is very small. Even more, with the ABZ column a smaller plate height and a better day-to-day repeatability is obtained.

Table 3.2.8: Pareto optimal (PO) points at pH 3, 7 and 11.

Col.	PO points at pH 3			PO points at pH 7			PO points at pH 11			PO points all pH values		
	As-H	As-RH	As-Rk	As-H	As-RH	As-Rk	As-H	As-RH	As-Rk	As-H	As-RH	As-Rk
ZRX	**	*		**	*		-	-	-	**	*	
BDS			*				-	-	-			*
CHB		*			*	*	-	-	-		*	
ABZ	**	*	*	**	*		-	-	-	**	*	
ASA						*			*			
ALU	-	-	-	**	*	*	**	*	*	**	*	*
POL	-	-	-						*			
3MZ	**	*	*	**	*	*	**	*	*	**	*	*

- = not tested; ** PO points are considered to be more important than *PO points.

With the ZRX column a further (small) improvement in efficiency is obtained while the other factors are worse. In Fig. 3.2.6 chromatograms of compounds 19, 26 and 29 on the ABZ (Fig. 3.2.6A) and ZRX column (Fig. 3.2.6B) at pH 3 are shown. With the ABZ column the results at pH 3 are better than at pH 7.

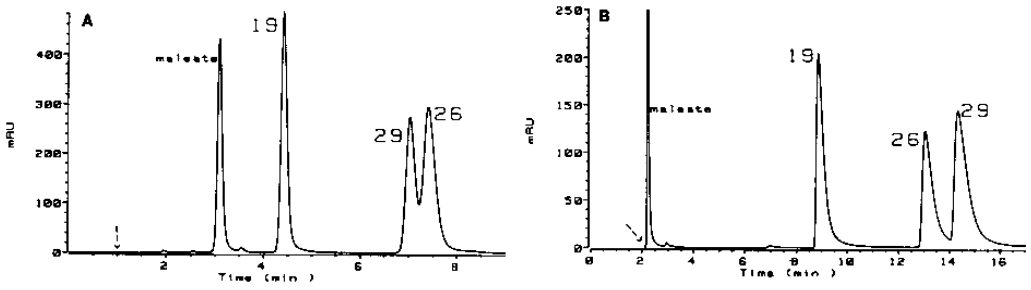

Figure 3.2.6: LC-UV chromatograms of compounds 19, 26 and 29. Detection was done at UV 210 nm. The arrow in the chromatograms indicates the dead time. (A) A 2 µl volume from a 1 mg/ml solution was injected onto a Supelcosil LC-ABZ 150 x 4.6 mm I.D. column. The flow rate was set to 1.0 ml/min and the eluent used was methanol+25mM NaH_2PO_4 pH 3.0 / 40+60 %(V/V). (B) A 2 µl volume from a 1 mg/ml solution was injected onto a Zorbax RX-C_{18} 250 x 4.6 mm I.D. column. The flow rate was set to 1.0 ml/min and the eluent used was methanol+25mM NaH_2PO_4 pH 3.0 / 45+55 %(V/V).

At pH 11, both the ALU and 3MZ column show good characteristics. However, with the ALU column fronting peaks were observed at this pH (Table 3.2.7). Also the plate height is in favour of the 3MZ column. Clearly, at pH 11 the 3MZ column is a good choice for analysing basic solutes. This is confirmed by the day-to-day repeatability data. In Fig. 3.2.7 a chromatogram of compounds 19, 26 and 29 on the 3MZ column at pH 11 is shown.

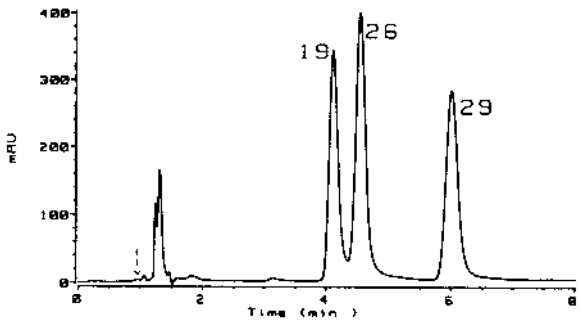

Figure 3.2.7: LC-UV chromatogram of compounds 19, 26 and 29. Detection was done at UV 210 nm. The arrow in the chromatograms indicates the dead time. A 2 µl volume from a 1 mg/ml solution was injected onto a 3MZ-18 150x4.6 mm I.D. column. The flow rate was set to 1.0 ml/min and the eluent used was acetonitrile+25mM borate pH 11.0 / 45+55 %(V/V).

The ultimate goal of course is to select the best column operating at its optimal pH, although in practice there can be reasons to select on forehand a certain pH. In Table 3.2.8 the PO-points at all pH values are given. The results are also graphically presented in Fig. 3.2.8. On basis of this MCDM plot the user can decide which column to use by weighing the importance of the different chromatographic factors. Overall the 3MZ column seems to give the best results of the columns tested. Looking at the individual data for this column (Table 3.2.7), operating at pH 11 is preferred at which symmetrical peaks and acceptable plate heights are obtained.
A bi-plot of the overall results is given in Fig. 3.2.9. For this the data of Table 3.2.7 are used and the data are autoscaled. The first two PCs explain 88% of the variance. A cluster of the optimal columns is encircled. The 3MZ column shows better asymmetry values while the ZRX and ABZ columns show a better day-to-day repeatability of the retention factor. This latter factor, however, is only a qualitative factor while the asymmetry values are accuractely determined. With respect to efficiency and day-to-day repeatability of the efficiency the columns are equally good. It is interesting to see that at different pH values for some columns, e.g the 3MZ, ZRX and ABZ column, only small differences while for other columns, e.g. the ALU and BDS columns, large differences were observed.

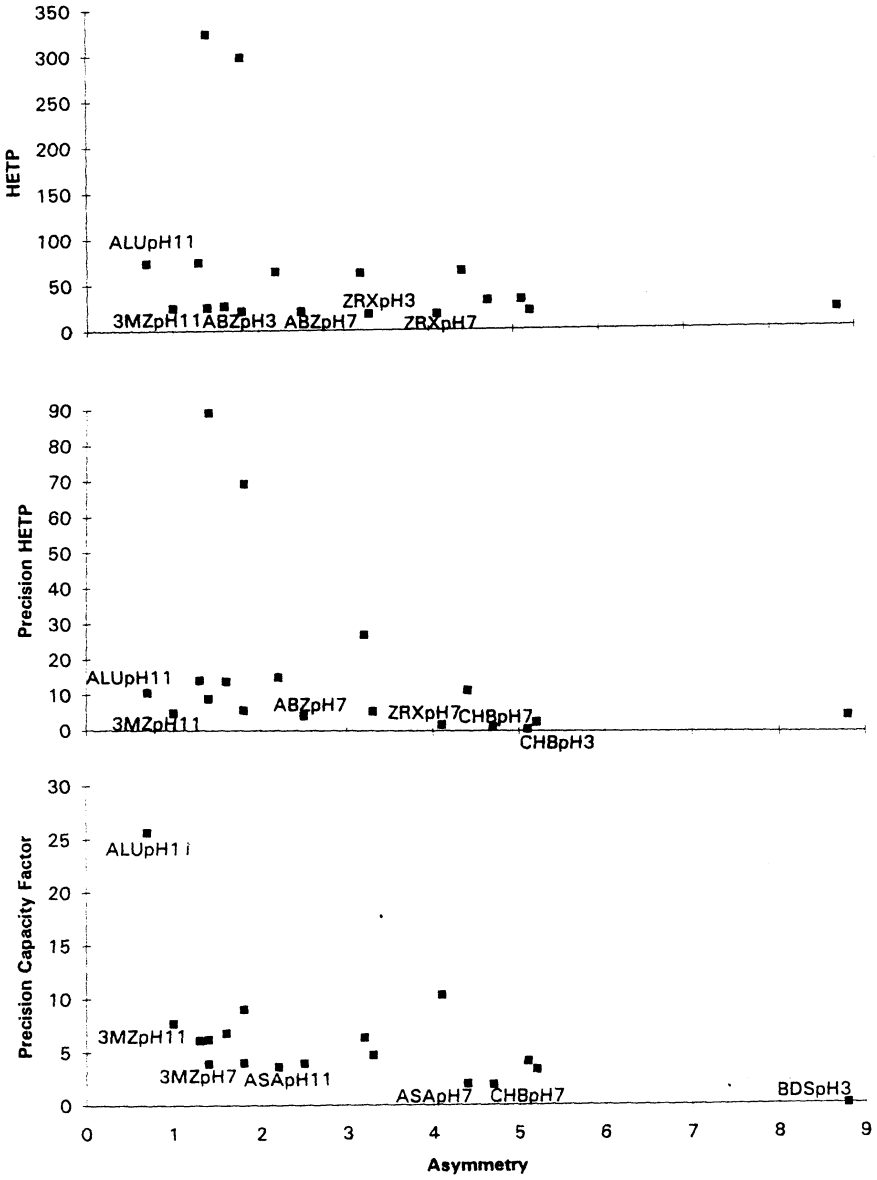

Figure 3.2.8: Stacked PO plot of the asymmetry vs. HETP, day-to-day repeatability of the HETP and day-to-day repeatability of the retention factor, obtained at pH values 3, 7 and 11. Only the PO points are indicated by giving the column abbreviation together with the pH used.

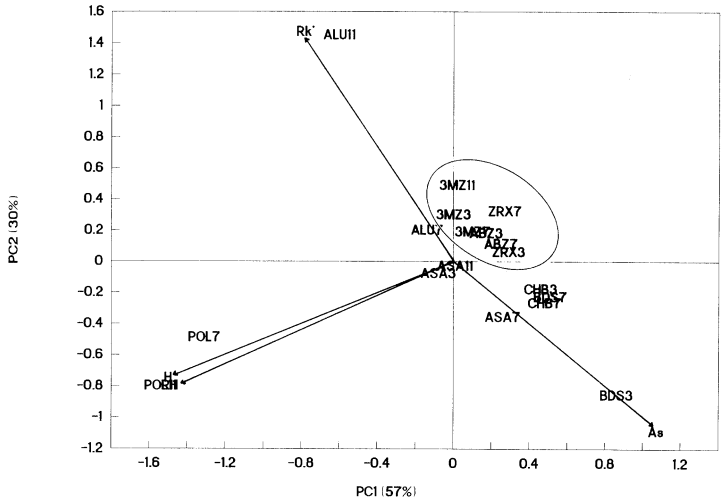

Figure 3.2.9: Bi-plot of the data obtained at all pH values. The data used for the bi-plot are the values given in Table 3.2.7.

3.2.5 CONCLUSIONS

The selection of a set of test compounds out of a larger set with PCA was carried out. Using a test set of only five compounds, information about the applicability of stationary phases developed for the analysis of basic compounds was obtained. A complete column test requires only two LC analyses using two mobile phases for the analysis of 3 and 2 test compounds. Each analysis consists of a duplicate injection and the testing is repeated on the next day.

Using bar charts and several chemometrical techniques such as bi-plots and multi criteria decision making, differences between stationary phases in their applicability for the analysis of basic solutes were successfully made. The advantage of the MCDM plot is that the factors involved can be visually weighed in order to select a column. The bi-plots are more difficult to interpret. However, when comparing a large number of columns we prefer to use bi-plots to visualize columns with comparable and different characteristics. An interesting conclusion is that the columns with a high efficiency generally show a good day-to-day repeatability for the plate height.

A column consisting of zirkonium oxide particles coated with polybutadiene proved to be suitable at all pH values tested. The best results with this column were obtained at pH 11. However, one has to realise that at this moment too limited information is available on the ruggedness of the column. This requires specific testing on this aspect. Furthermore the high price of this column is a limiting factor. At pH 3 a Supelcosil LC-ABZ and a Zorbax RX-C_{18} column showed also promising results.

Polymer based columns showed inferior results because of the high plate heights obtained.

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3.3 CHARACTERISATION OF SILICA BASED REVERSED PHASE STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS USING PRINCIPAL COMPONENTS ANALYSIS

3.3.1 SUMMARY

In this study 14 commercially available reversed phase stationary phases were compared for the high performance liquid chromatographic analysis of basic pharmaceutical compounds. The influence of silanol blocking compounds on retention and peak shape was investigated using phosphate buffers pH 3 and pH 7. Principal components analysis was used to analyse the data set, enabling an evaluation of the various stationary phases.

The commercially available stationary phases showed distinct differences in their suitability to analyse basic compounds whereas addition of N,N-dimethyloctylamine to mobile phase buffer pH 3 showed an improvement of the peak shapes.

R.J.M. Vervoort, M.W.J. Derksen and A.J.J. Debets, J. Chromatogr. A, 765 (1997) 157-168.

3.3.2 INTRODUCTION

In the pharmaceutical industry many compounds of interest contain basic nitrogen atoms e.g. CNS (central nerve system) drugs, cardiovascular drugs, etc. To analyse these pharmaceutically relevant compounds reversed phase high performance liquid chromatography (RPLC) using stationary phases based on silica modified with octadecyl (C_{18}) or octyl (C_8) chains is in many cases the preferred method. The RPLC analysis of pharmaceutical compounds is promoted by efficient separations and symmetrical peaks, which enable the quantification of analytes at a low level of concentration. The RPLC analysis of basic compounds, however, can be problematic. Due to ionic and other polar interactions of residual silanols and eventual other polar sites on the silica material with basic compounds, other retention mechanisms can occur next to the reversed phase retention mechanism. This mixed retention mechanism will depend on the character of the LC column used and can result in varying peak shapes. Also changes in separation and peak shape can occur as the stationary phase ages [1-5].

Today, many stationary phases specially designed for the analysis of basic compounds are available [6]. For many basic compounds, however, still asymmetric peaks are obtained using these stationary phases [7]. Therefore, testing of stationary phases before applying them in development or quality control applications is highly beneficial [8].

Testing of silica-based reversed phase stationary phases can be carried out using numerous test procedures. In the literature examples can be found were test compounds were selected to reveal differences between stationary phases such as silanol activity, hydrophobicity and metal activity [9]. A high number and a high acidity of residual silanols present on the stationary phase silica caused asymmetrical peaks, whereas the presence of metals in the silica influenced the acidity of the residual silanols [1]. Recently a selection of 5 basic compounds was reported, used to discern stationary phases suitable for the LC analysis of basic compounds, which represented a set of 32 pharmaceutically relevant compounds. The set of 5 compounds was selected from a data set containing the asymmetry values of the 32 compounds analysed on six different LC columns, using principal components analysis (PCA). The selection was based on the mutual distance between the compounds in the score plots [7]. In the present study, the selected 5 compounds were used to evaluate the applicability of commercially available stationary phases to analyse pharmaceutically relevant basic compounds.

Since the number of stationary phases is very large and still increasing, classification of stationary phases into groups of equal characteristics can be helpful to select a suitable stationary phase. This can be obtained by chromatographic characterisation followed by chemometric evaluation. Schmitz et al [9] and Walczak et al [10] presented differences between stationary phases using correspondence factor analysis (CFA), by which the similarity structure of the objects (stationary phases)

and the variables was presented simultaneously. Principal component analysis (PCA) was used by Delany et al [11] to discern stationary phases, whereas Hamoir et al used spectral mapping analysis (SMA) to study 16 stationary phases [12]. CFA, PCA and SMA are closely related chemometric analysis tools. In this chapter the comparison of 14 silica based reversed phase stationary phases for the analysis of pharmaceutically relevant basic compounds is reported using PCA as a chemometric evaluation tool. In the study the influence of the addition of the silanol blocking compounds triethylamine and N,N-dimethyloctyl amine to the mobile phase, consisting of phosphate buffers pH 3 and pH 7 with methanol, on retention and peak shape was included. PCA was used to present the objects (stationary phases) in score plots and the variables (asymmetry, retention, plate height) in loading plots.

3.3.3 EXPERIMENTAL

3.3.3.1 HPLC apparatus

The HPLC experiments were carried out using a HP 1090 M liquid chromatograph equipped with a HP 1040 M diode array detector (Hewlett Packard, Amstelveen, The Netherlands). HPLC chromatograms were collected using a HP 79994A HPLC Workstation or a HPLC 3D Chemstation.

3.3.3.2 Chemicals

The basic compounds were synthesised by N.V. Organon (Oss, The Netherlands). In Fig. 3.3.1 the structures are shown. As organic modifier methanol (MeOH), supplied by J.T. Baker (Deventer, The Netherlands) was used. For the preparation of the buffers disodiumhydrogen phosphate (Na_2HPO_4) and sodiumdihydrogen phosphate (NaH_2PO_4) supplied by J.T. Baker were used. The aqueous buffers were prepared by using Milli Q filtered water, (Millipore, Bedford, MA, USA). Triethylamine (TEA, sequanal grade) was obtained from Pierce (Rockford, Illinois, USA) and N,N-dimethyloctylamine (DMOA 95 %) was obtained from Aldrich (Gillingham, England). To obtain 0.1 % (v/v) solutions of the silanol blockers 1 ml (TEA or DMOA) was added to 1 l buffer solution, before mixing with MeOH. Concentrated phosphoric acid (H_3PO_4) was obtained from Merck (Darmstadt, Germany) and was added to the buffers, after addition of TEA or DMOA and before mixing with MeOH, to adjust the pH of the mobile phase. The amount injected was 2 μg and was achieved by injecting 2 μl from a 1 mg/ml solution of the basic analytes in MeOH. The stationary phases studied are shown in Table 3.3.1.

3.3.3.3 Experimental set-up

In this study mobile phases with pH values of 3 and 7 were used. The stationary phases were tested without addition of a silanol blocker, and with addition of 0.1% TEA and 0.1% DMOA to the buffer solution in the mobile phase. The MeOH+buffer

ratio was adjusted to ensure k > 1 without addition of the silanol blockers. After addition of the blocker to the mobile phase buffer, the MeOH+buffer ratio remained unchanged. The sequence of experiments for each stationary phase was performed as shown in Table 3.3.2. All experiments were performed in duplicate.

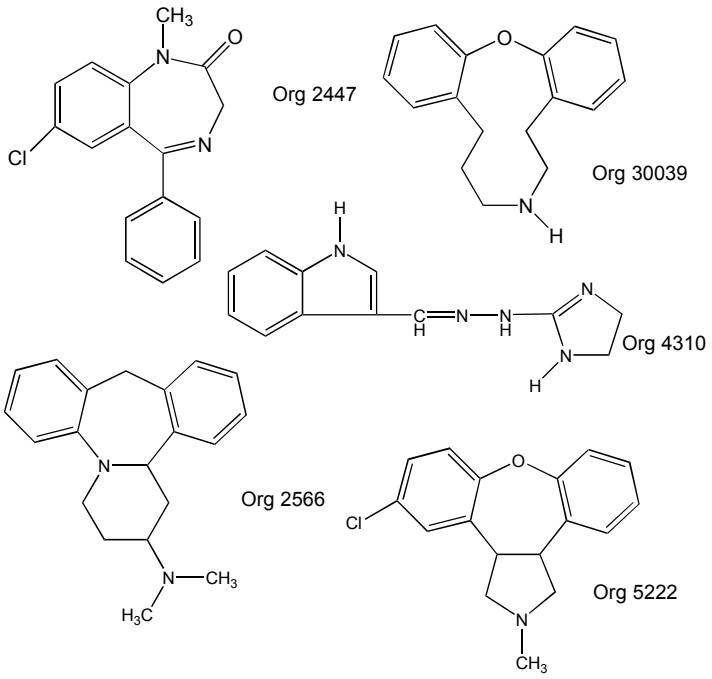

Figure 3.3.1: Molecular structures of the test compounds.

The CHB and INGL stationary phases were not tested at buffer pH 7. Due to the apolar character of these stationary phases the test compounds did not elute using 80% (V/V) of methanol in the mobile phase.

3.3.3.4 Peak performance calculations

The asymmetry factor (As) was calculated for the compounds Org 4310, Org 5222, Org 2566 and Org 30039 at 10 % of the peak height and expressed as the ratio of the width at the rear and at the front side of the peak. To describe the retention of the test compounds, the k values of Org 4310, Org 5222, Org 2566 and Org 30039 were used. The first deviation of the baseline was used to determine the dead volume of the LC column. For the calculation of the plate height (HETP) of the LC column the second moment of the Org 2447 peak was used [13].

3.3.3.5 Principal component analysis

Principal component analysis (PCA) was performed using the software package Unscrambler 5.03, Camo AS (Trondheim, Norway), installed on a personal computer with a 486/66 MHz Intell processor. To enable a graphical presentation the

Unscrambler results were transferred to Lotus 123 3.0 software, Lotus Development Corporation (USA).

Table 3.3.1: Stationary phases used in this study.

Stationary phase	Abbreviation used in this study	Manufacturer	Dimension lenght x I.D.(mm)	Particle size (μm)
Hypersil BDS	BDSSH	Shandon	150 x 4.6	5
Hypersil BDS	BDSHP	Shandon	125 x 3.0	3
Inertsil 5 ODS-2	INCH	GL Sciences Inc.	150 x 4.6	5
Inertsil ODS-80Å	INGL	GL Sciences Inc.	150 x 4.6	5
Supelcosil LC-ABZ	ABZ	Supelco	150 x 4.6	5
Supelcosil LC-ABZ+Plus	ABZ+	Supelco	150 x 4.6	5
Zorbax SB-C_{18}	ZSB	Rockland Technologies	150 x 4.6	5
Zorbax Rx-C_{18}	ZRX	Rockland Technologies	250 x 4.6	5
Chromspher B	CHB	Chrompack	250 x 4.6	5
Purospher RP-18	PUR	Merck	250 x 4.0	5
Symmetry C_{18}	SYM	Waters	150 x 3.9	5
Prodigy 5 ODS-2	PROD	Phenomenex	150 x 4.6	5
YMC Basic	YMC	YMC Inc.	150 x 4.6	5
Nucleosil 100-5C_{18} AB	NUCAB	Machery Nagel	250 x 4.0	5

Table 3.3.2: Sequence of experiments.

Sequence number	Mobile phase
1	MeOH + 25 mM Na_2HPO_4 pH 7
2	MeOH + 25 mM NaH_2PO_4 pH 3
3	MeOH + (25 mM Na_2HPO_4 pH 7 + 0.1 % (V/V) TEA)
4	MeOH + (25 mM NaH_2PO_4 pH 3 + 0.1 % (V/V) TEA)
5	MeOH + (25 mM Na_2HPO_4 pH 7 + 0.1 % (V/V) DMOA)
6	MeOH + (25 mM NaH_2PO_4 pH 3 + 0.1 % (V/V) DMOA)

3.3.4 RESULTS AND DISCUSSION

3.3.4.1 Selection of variables

The first consideration for stationary phase evaluation was the selection of variables known to describe the LC performance, e.g. peak shape and plate height. In Tables 3.3.3 and 3.3.4 data obtained for each stationary phase listed in Table 3.3.1 are shown, when performing the sequence of experiments shown in Table 3.3.2. During the experiments, it was observed that the LC analysis of compound Org 2447 always revealed symmetrical peaks (asymmetry factor ≤ 1.2). Therefore this compound was used to calculate the efficiency (HETP) of the stationary phases. Together with the individual asymmetry values of Org 4310, Org 30039, Org 2566 and Org 5222, the

HETP values were used in PCA evaluation. As can be seen in Tables 3.3.3 and 3.3.4, for an individual stationary phase and compound, a reduction of k showed also a reduction of As. Since the amount of modifier (MeOH) remained unchanged during the experiments, the reduction in peak asymmetry and retention was evidently caused by the addition of TEA and DMOA to the mobile phase buffer. Clearly, as shown in Table 3.3.3, the silanol blocking compounds supressed the interaction between residual silanols and eventual other polar sites, and basic compounds, resulting in improved peak symmetry and a decrease in retention times. The relation between retention and peak shape was also discussed in previous studies [14,15]. The stationary phases presented in this study (Table 3.3.1), however, showed a different chromatographic behaviour: variations in asymmetry and in retention after addition of silanol blocking compounds to the mobile phase buffer. The deviating chromatographic behaviour is specific for a given stationary phase, e.g., the influence of silanol blocking compounds in the mobile phase buffer on peak asymmetry is for the PUR column in Table 3.3.3 neglectable compared to the INCH (and other) columns. To include these stationary phase characteristics into PCA, also the k values of the compounds without and with addition of TEA and DMOA to the mobile phase buffer were used. Since the data differed in magnitude, they were autoscaled prior to PCA.
In this study various stationary phases that can be used for the HPLC analysis of pharmaceutical basic compounds are compared. Stationary phases showing comparable characteristics will cluster in the PCA scoreplots whereas both score and loading plots can show relations between the stationary phases and the variables (retention, peak shape, plate height). First, stationary phases were compared independently from the mobile phase buffer pH using the combined data obtained with phosphate buffers pH 3 and pH 7. Secondly, the stationary phases were compared at phosphate buffer pH 3 and at pH 7 separately.

3.3.4.2 Stationary phase monitoring; phosphate buffers pH 3 and pH 7

To evaluate the stationary phases, the obtained data (Tables 3.3.3 and 3.3.4) were analysed using PCA. With PCA a set of new variables, principal components (PC) are defined instead of using the original variables (retention, asymmetry, plate height). To find the PC's of a data matrix X, the first step is to look for a vector t_1 (score) = Xp_1 that is a linear function of X with maximum variance, where p_1 (loading) is a vector of m constants p_{11}, p_{12}, ...,p_{1m} and length 1. The second step is to look for a linear function Xp_2 orthogonal to Xp_1 which has maximum variance, etc. Summarising for the data in Table 3.3.3, a 14 * 27 data matrix (14 stationary phases * 27 variables) can be written as: $X = t_1p_1^T + t_2p_2^T + ...$. Since the first few PC's contain most of the variability of the data, a graphical presentation of the data is possible i.e. the scores reflect the objects (stationary phases) and the loadings the variables (retention, peak shape, plate height), respectively [16].

Table 3.3.3: Data obtained testing the stationary phases using phosphate buffer pH 3.

column	Asymmetry												Plate height (µm)											
	Org 4310			Org 30039			Org 2566			Org 5222			Org 2447			Org 4310			Org 30039			Org 2566			Org 5222			Org 2447		
	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA
CHB	3.7	2.9	2.5	6.0	2.7	2.4	6.5	3.6	2.8	8.0	3.3	2.7	32.7	39.7	39.5	1.2	1.0	0.9	70	3.1	1.8	1.6	70	4.0	3.0	2.5	70	5.7	2.5	2.2	70
PUR	1.7	1.9	1.5	1.6	1.6	1.4	2.0	1.9	1.6	2.0	2.1	1.5	20.4	20.5	19.8	2.6	3.1	1.7	20	2.4	2.9	2.4	35	6.0	6.5	5.7	35	5.1	6.0	5.1	35
SYM	3.1	2.5	1.5	3.0	2.4	1.2	2.6	2.2	1.4	2.5	2.1	1.4	18.3	19.7	19.2	4.2	3.7	0.9	25	2.4	2.7	1.7	45	4.7	5.2	4.2	45	4.8	4.4	3.1	45
ABZ+	2.2	2.0	1.3	2.3	2.0	1.0	1.9	1.7	1.3	2.0	1.8	1.2	15.3	15.2	15.5	1.3	1.3	0.6	30	1.6	1.7	1.1	40	3.3	3.4	2.4	40	3.0	3.1	2.2	40
ABZ	2.1	2.1	1.9	1.9	1.8	1.6	2.0	2.1	1.8	1.9	1.9	1.8	21.6	17.1	17.8	2.2	2.3	1.3	30	2.3	2.4	1.8	40	4.5	4.5	3.5	40	4.2	4.2	3.3	40
YMC	3.4	2.7	1.3	3.4	2.9	1.2	2.8	2.2	m	2.7	2.2	1.2	13.1	13.0	13.2	4.3	3.7	0.9	20	3.7	3.6	3.5	40	6.7	6.4	m	40	6.2	5.9	3.3	40
BDSHP	6.0	4.5	2.5	6.2	4.7	1.6	8.6	6.4	2.2	7.0	5.1	2.0	16.0	17.3	14.8	5.8	4.0	0.8	25	3.6	3.2	1.7	45	6.4	5.6	3.0	45	5.7	5.1	2.8	45
BDSSH	12.1	2.0	1.4	8.9	2.5	1.2	10.2	3.5	1.5	12.6	2.5	1.3	20.9	19.5	21.4	2.8	2.8	0.7	30	1.0	0.8	0.6	60	4.0	2.0	1.4	55	3.3	1.8	1.3	55
INCH	17.9	5.1	1.4	10.7	3.8	1.2	14.9	4.8	1.6	14.6	4.8	1.5	15.1	15.9	15.1	3.1	4.8	1.0	25	2.5	2.2	1.1	50	4.0	1.8	1.4	60	1.6	1.4	0.9	60
INGL	3.3	2.8	1.6	4.3	2.7	1.7	3.8	2.6	1.6	3.7	2.5	1.6	23.9	29.7	26.9	2.2	1.9	1.1	25	2.2	2.5	1.1	50	4.5	3.7	1.9	50	3.8	3.7	1.9	50
ZSB	4.7	3.2	1.5	3.5	2.4	1.3	4.4	2.9	1.4	3.6	2.4	1.2	15.2	16.1	15.7	2.1	1.9	1.0	25	3.9	3.9	2.0	45	6.3	6.3	3.3	45	6.0	6.2	3.3	45
ZRX	3.9	2.9	1.8	3.9	2.6	1.6	4.1	2.8	1.5	4.1	2.7	1.3	18.0	24.7	24.9	3.4	3.1	0.7	25	3.1	2.5	1.3	45	5.7	4.4	2.5	45	5.1	4.2	2.4	45
PROD	1.5	1.3	1.2	1.5	1.4	1.1	1.2	1.3	1.2	1.4	1.4	1.2	14.7	14.1	16.3	3.2	3.1	0.9	30	2.4	2.5	1.1	50	4.2	4.4	2.9	50	3.7	4.0	2.7	50
NUCAB	2.9	2.5	1.6	2.0	1.8	1.2	1.9	1.7	1.1	2.8	2.2	1.3	19.4	19.3	19.3	3.6	3.4	0.6	25	3.1	3.3	1.5	45	5.6	5.7	2.8	45	4.8	4.8	2.6	45

NS: no silanol blocking compound added to the mobile phase buffer
TEA: triethylamine added to the mobile phase buffer
DMOA: N,N-dimethyloctylamine added to the mobile phase buffer
m: no data

Table 3.3.4: Data obtained testing the stationary phases using phosphate buffer pH 7.

column	Asymmetry												Plate height (µm)					
	Org 4310			Org 30039			Org 2566			Org 5222			Org 2447					
	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA	NS	TEA	DMOA			
PUR	4.1	5.1	3.8	5.8	6.4	6.1	4.8	4.8	2.6	2.0	2.4	1.9	17.0	18.9	19.0			
SYM	2.4	2.0	1.4	2.5	2.4	2.0	1.6	1.5	1.2	1.2	1.2	1.1	21.6	20.0	18.8			
ABZ+	4.4	5.1	4.2	1.6	1.6	1.5	1.5	1.5	1.4	m	1.2	1.1	13.8	15.6	14.9			
ABZ	5.5	5.2	3.9	2.1	2.3	2.0	2.0	3.0	1.7	1.3	1.7	1.4	21.1	17.9	19.2			
YMC	3.3	2.6	1.2	4.3	2.8	1.5	3.3	2.1	1.0	1.3	1.2	1.0	13.0	12.9	13.1			
BDSHP	5.3	4.8	2.4	15.1	5.2	2.8	9.1	4.0	1.2	4.4	2.0	1.2	18.2	15.7	16.1			
BDSSH	7.3	3.6	1.6	9.7	4.1	2.7	11.7	2.6	1.2	3.6	1.3	1.0	20.2	23.8	23.2			
INCH	21.2	2.7	2.7	12.3	7.3	6.2	4.3	3.3	1.9	1.9	1.8	1.4	15.3	15.1	15.9			
ZSB	4.6	3.3	2.1	5.0	3.1	3.2	2.4	1.6	1.2	1.2	1.1	1.1	15.2	15.4	16.1			
ZRX	3.3	m	2.0	6.1	5.2	3.7	5.3	2.4	1.8	2.9	1.3	1.1	17.1	23.6	24.6			
PROD	1.7	1.4	1.2	3.5	2.1	2.1	1.9	1.4	1.2	1.3	1.2	1.2	14.6	13.9	13.3			
NUCAB	4.2	2.7	1.7	10.4	3.7	3.9	6.0	1.6	1.4	4.3	1.5	1.2	19.4	19.5	19.8			

	Retention (k')																	
	Org 4310			MeOH (%)	Org 30039			Org 2566		MeOH (%)	Org 5222			MeOH (%)	Org 2447 k'			MeOH (%)
	NS	TEA	DMOA		NS	TEA	DMOA	NS	TEA		NS	TEA	DMOA		NS	TEA	DMOA	
PUR	3.3	4.2	3.1	40	8.0	7.5	9.0	9.0	8.5	80	8.3	80	7.0	7.0	6.8	80		
SYM	6.3	5.3	4.4	25	2.3	2.8	2.8	5.8	5.6	75	5.6	75	5.8	5.7	5.7	75		
ABZ+	1.4	1.4	1.2	50	2.0	1.8	1.9	3.9	3.9	70	3.9	70	m	4.2	4.2	70		
ABZ	5.3	3.2	2.7	50	4.2	2.9	2.9	6.3	5.3	70	5.0	70	5.6	5.1	5.0	70		
YMC	3.1	2.8	2.2	25	1.8	1.7	1.6	4.6	4.4	65	4.0	65	4.8	4.7	4.4	65		
BDSHP	2.4	2.7	2.2	30	3.0	3.1	2.9	5.4	6.4	70	6.0	70	5.3	6.3	6.0	70		
BDSSH	3.6	3.1	2.3	30	2.2	2.2	2.0	2.7	2.7	80	2.6	80	2.4	2.4	2.4	80		
INCH	3.2	2.8	2.8	35	3.2	3.7	3.5	4.1	5.0	80	4.2	80	3.8	4.6	3.9	80		
ZSB	5.3	4.4	3.0	35	5.9	5.1	5.3	5.8	5.6	80	5.5	80	4.9	4.8	4.8	80		
ZRX	3.1	m	2.6	35	7.6	10.2	6.8	5.4	7.3	80	4.8	80	4.1	5.0	3.9	80		
PROD	3.4	3.2	2.7	30	1.9	1.8	1.9	3.4	3.3	80	3.2	80	3.3	3.3	3.2	80		
NUCAB	3.5	3.3	2.7	25	1.5	0.9	1.0	1.8	1.7	80	1.7	80	1.9	1.8	1.8	80		

NS: no silanol blocking compound added to the mobile phase buffer
TEA: triethylamine added to the mobile phase buffer
DMOA: N,N-dimethyloctylamine added to the mobile phase buffer
m: no data

In Fig. 3.3.2 the score and loading plots are shown using the data in Tables 3.3.3 and 3.3.4. The first three PC's explained 64 % of the variance present in the dataset. To evaluate the stationary phases qualitatively, this was found to be sufficient since conclusions drawn from the score and loading plots could additionally be verified using the data in Tables 3.3.3 and 3.3.4. As can be seen in Tables 3.3.3 and 3.3.4 the PROD stationary phase showed a lower asymmetry and plate height compared to the other stationary phases. These low As and low HETP are favourable for the separation of basic compounds. In the PC1 - PC2 scoreplot the PROD stationary phase was situated in the lower right quadrant. The vectors for As and HETP in the corresponding loading plot pointed to the opposite direction, indicating low As and low HETP for the PROD stationary phase. The stationary phases situated close to the PROD stationary phase (the lower left and lower right quadrants) in the score plots showed also low asymmetry and low plate height, e.g., the ABZ+ and NUCAB stationary phases using phosphate buffer pH 3. Using phosphate buffer pH 7 the YMC and SYM stationary phase showed low asymmetry and low plate height. Stationary phases situated apart from the PROD stationary phase in the score plots were less suitable for the LC analysis of basic pharmaceutical compounds as used in the present study (Fig. 3.3.1). Apparently, not all stationary phases developed for the separation of basic compounds are suitable to analyse all types of basic pharmaceuticals. As can be seen in the literature, other types of basic compounds showed different peak symmetries. For instance, the use of the INCH stationary phase, showing asymmetric peaks when used for the test compounds, showed in another study an acceptable low asymmetry factor [17]. Nevertheless, it should be kept in mind that the results presented in this study were valid for a rather limited set of 32 basic compounds, represented by the 5 compounds shown in Fig. 3.3.1.

For several stationary phases like PROD, YMC and ZSB the obtained peak shapes, when using phosphate buffer pH 3, were similar to the obtained peak shapes when using phosphate buffer pH 7, as was shown by the vectors in the PC1 - PC2 and PC1 - PC3 scoreplots. Other stationary phases showed a different pattern. For the PUR stationary phase a difference could be observed when a buffer pH 3 or buffer pH 7 was used. Using buffer pH 3 relatively symmetrical peaks ($1.4 < As < 2.1$) for the basic compounds were obtained, whereas using buffer pH 7 asymmetrical peak shapes ($1.9 < As < 6.4$) were observed. Generally, for the selected compounds the use of phosphate buffer pH 3 yielded better peak symmetry then the use of phosphate buffer pH 7. Furthermore, differences in peak shape were observed for apparently similar packing materials, e.g., INCH and INGL which were both manufactured from Inertsil silica material, and BDSSH and BDSHP which both were prepared using Hypersil BDS stationary phase.

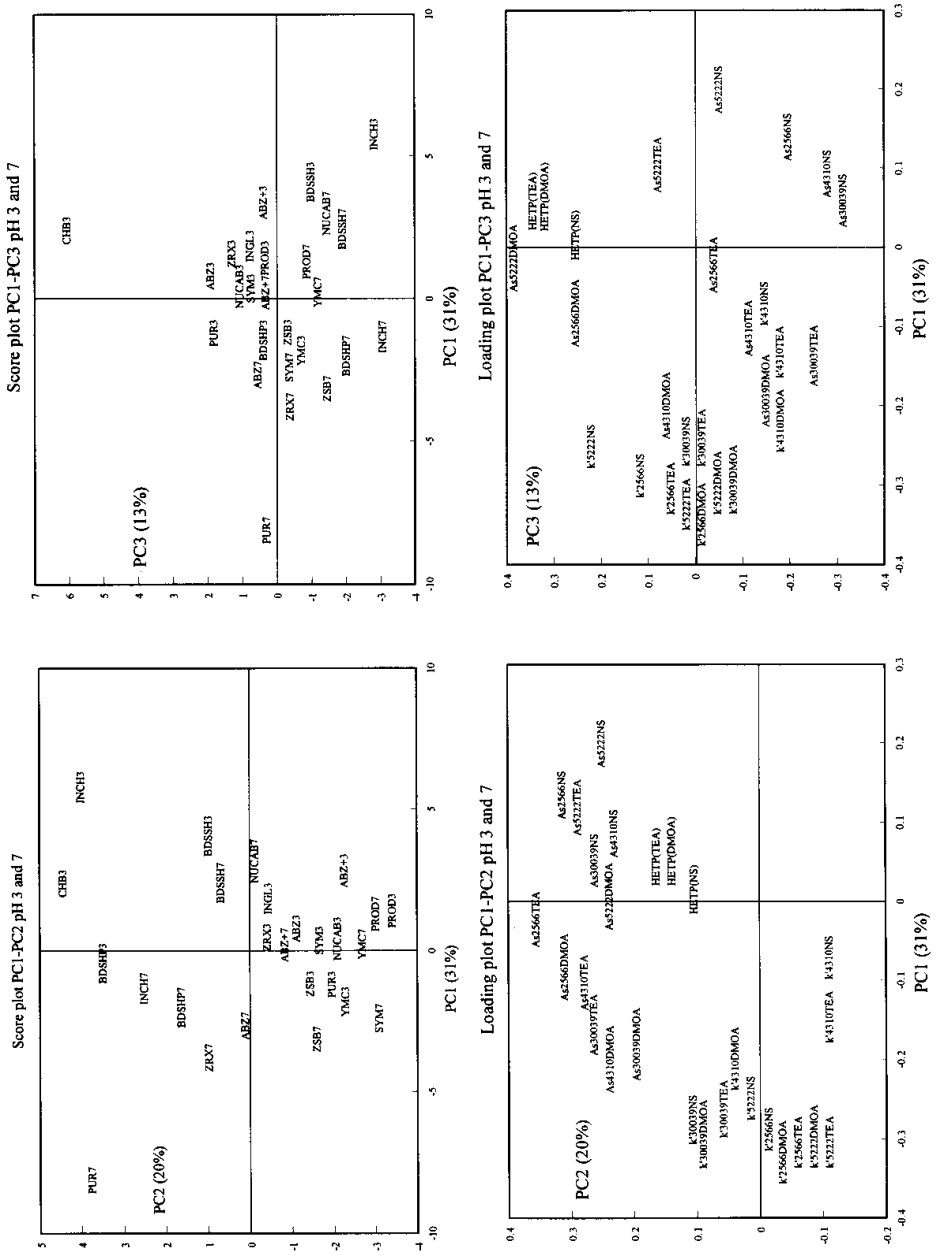

Figure 3.3.2: Score and loading plots using the first three principal components (PC) and the data obtained using phosphate buffers, pH 3 and pH 7 (Tables 3.3.3 and 3.3.4). The numbers behind the column abbreviations (Table 3.3.1) in the score plots are the pH values used. The abbreviations in the loading plots are related to the following; As2566DMOA means the loading of the asymmetry value (As) of Org 2566 (2566) after addition of DMOA.

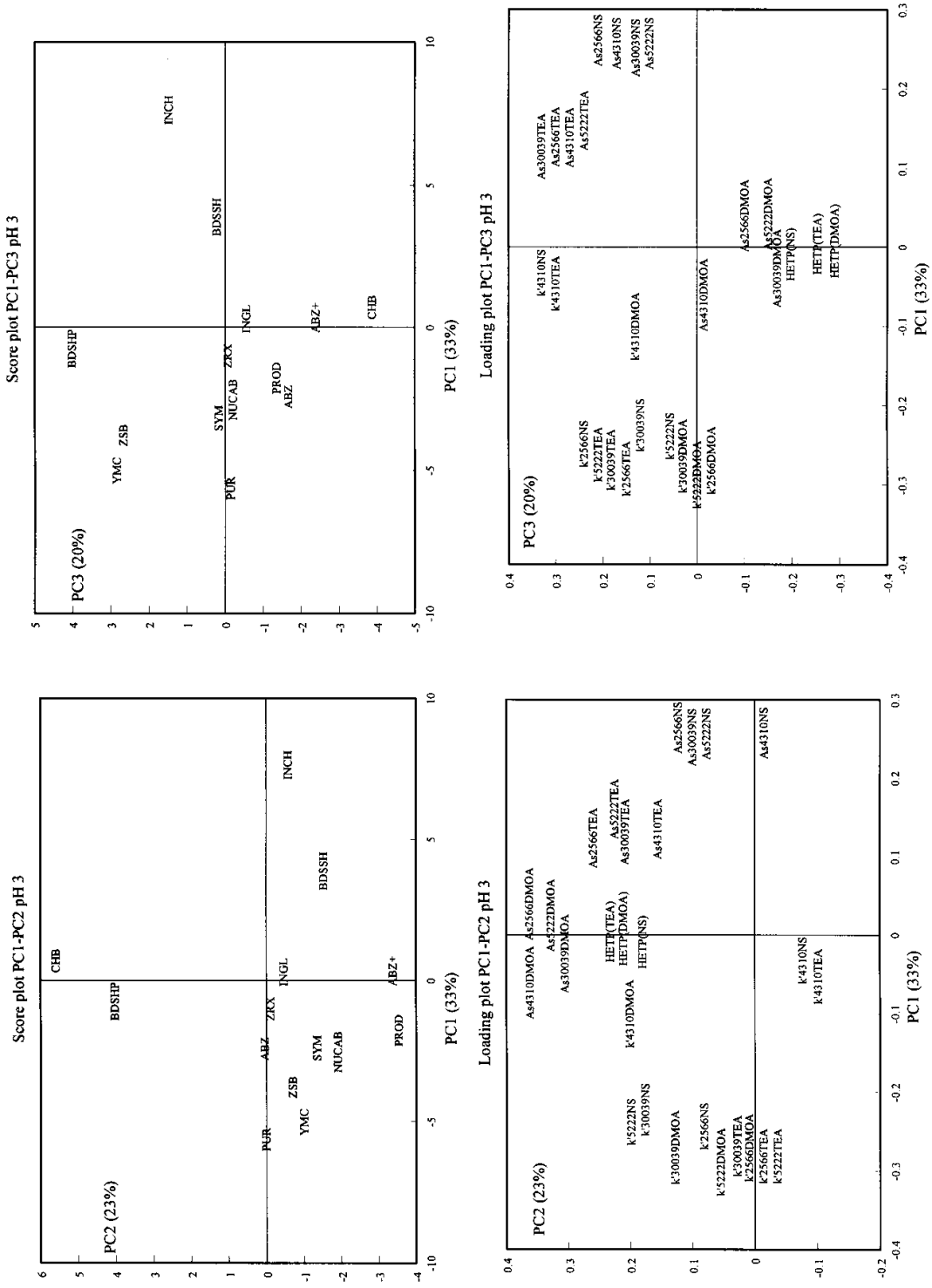

Figure 3.3.3: Score and loading plots using the first three principal components(PC) and the data obtained using phosphate buffer pH 3 (Table 3.3.3). For the column abbreviations, see Table 3.3.1. The abbreviation in the loading plots are as explained in Fig. 3.3.2.

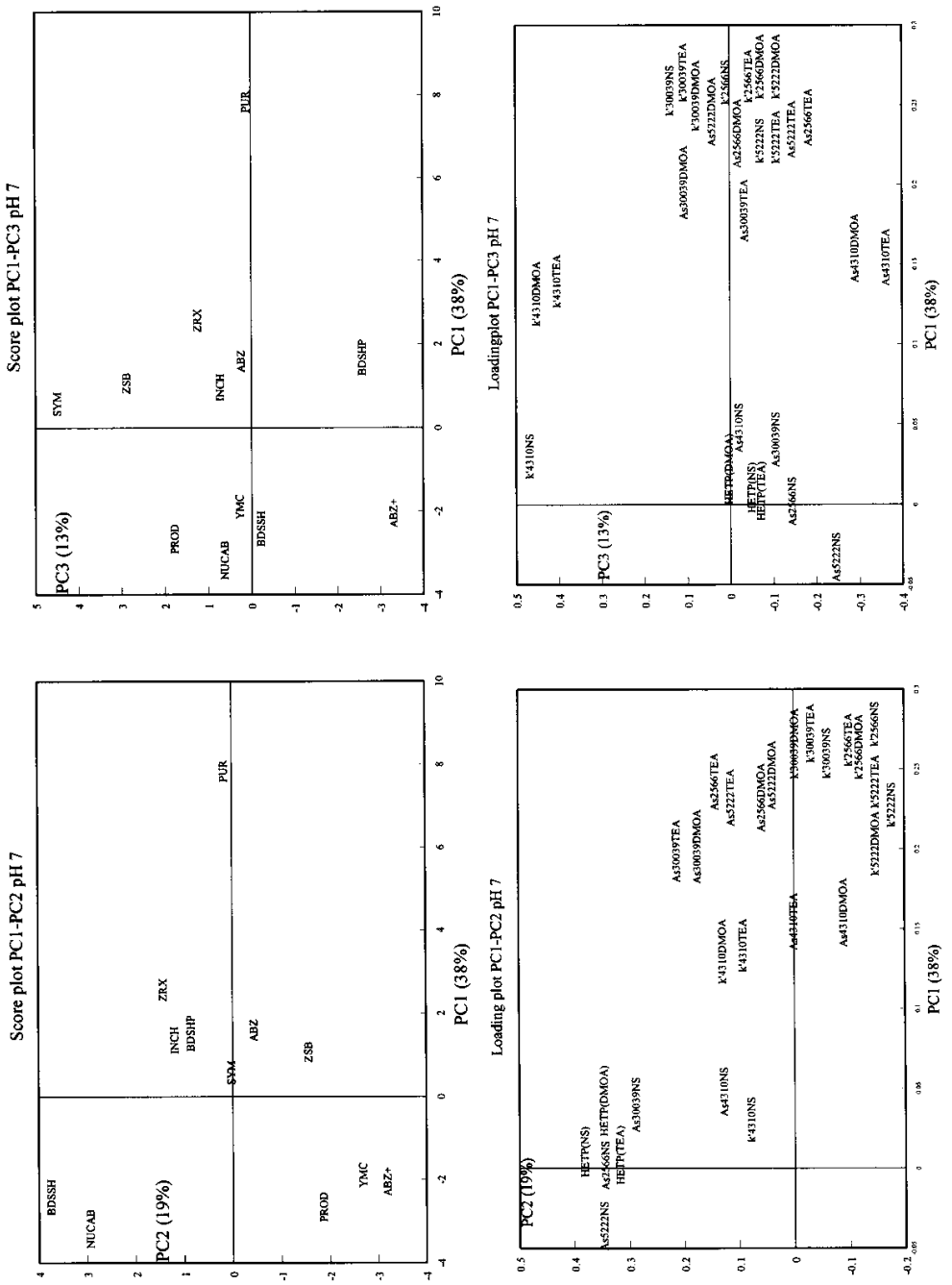

Figure 3.3.4: Score and loading plots using the first three principal components(PC) and the data obtained using phosphate buffer pH 7 (Table 3.3.4). For the column abbreviations, see Table 3.3.1. The abbreviation in the loading plots are as explained in Fig. 3.3.2.

3.3.4.3 Stationary phase monitoring; phosphate buffers pH 3 and pH 7 separately

In Fig. 3.3.3 the score and loading plots of the first three PCs are shown using the data in Table 3.3.3 (phosphate buffer pH 3), explaining 76% of the variance. In Fig. 3.3.4 the score and loading plots of the first three PC's are shown using the data in Table 3.3.4 (phosphate buffer pH 7), explaining 70% of the variance.

To reveal pH effects, Figs. 3.3.3 and 3.3.4 are illustrative. In Fig. 3.3.3 it is observed that the loadings of the asymmetry values of the four compounds obtained without addition of silanol blocking compounds to the mobile phase buffer shows clusters. This is also observed for the loadings of the asymmetry values after addition of TEA, and for the loadings of the asymmetry values after addition of DMOA to the mobile phase buffer. Hence, the four test compounds showed similar chromatographic characteristics, i.e. peak shape. Therefore, it can be concluded that the use of one of the test compounds instead of four, would yield similar information about the performance of the LC stationary phases when phosphate buffer pH 3 was used as mobile phase buffer.

A loading plot reveals information about the relationship between variables since each loading vector describes a different source of variation in the data. The correlation between variables is expressed by the angle between the various variable vectors in the loading plot, i.e., a small angle between the variable vectors means a high correlation between the variables. Low correlations between k-values (retention) and asymmetry values (peak shape) were observed in the loading plots. In particular, this was observed for the data when DMOA was added to the phosphate buffer pH 3, expressed by the orthogonality of the retention and asymmetry vectors in the loading plots, meaning no correlation exists between As and k. Clearly, the addition of DMOA to the mobile phase buffer yielded symmetrical peaks for the basic compounds. Since interactions between the basic solutes and residual silanols yield asymmetrical peaks, symmetrical peaks obtained after addition of DMOA to phosphate buffer pH 3 indicates blocking of residual silanols.

Contradictory to the results in Fig. 3.3.3, in Fig. 3.3.4 the loading vectors for the asymmetry values with addition of TEA and DMOA to phosphate buffer pH 7, shows no clusters in the PC1 - PC2 and the PC1 - PC 3 loading plots. However, without the addition of silanol blocking compounds, in the PC1 - PC2 loading plot the loadings for the asymmetry values of Org 5222, Org 2566 and Org 30039 are clustered and in the PC1 - PC3 loading plot also Org 4310 was included in the cluster. Obviously, the effect of adding silanol blocking compounds to mobile phase buffer pH 7 is different for the test compounds. As can be seen in Table 3.3.4, after addition of TEA to phosphate buffer pH 7 the peak symmetry improved. After addition of DMOA to the phosphate buffer pH 7 symmetric peaks were obtained for Org 5222 and Org 2566, whereas for Org 4310 and Org 30039 the enhancement of the peak symmetry was less significant.

As was shown in the loading plots in Figs. 3.3.3 and 3.3.4, the loadings for the k-values of Org 4310 differed from the loadings for the k-values of Org 2566, Org 5222 and Org 30039. The loadings for retention without addition of silanol blocking compounds, and after addition of TEA and DMOA were highly correlated for Org 2566, Org 5222 and Org 30039 using phosphate buffers pH 3 and pH 7, meaning that for these compounds the influence of silanol blocking compounds on retention was similar. The loadings for retention of Org 4310 were not included in these clusters, meaning the effect on retention compared to Org 2566, Org 30039 and Org 5222 for TEA and DMOA was not similar.
Besides the influence on the symmetry and k-value of the chromatographic peak, also some practical considerations of using silanol blocking compounds should be noticed. Stabilisation times were substantially longer (approx. 1 hr), compared to eluents without silanol blocking compounds. Also the removal of the silanol blocking compounds from the stationary phase by rinsing procedures with methanol+water is difficult and will take some time (approx. 1 hr) compared to eluents without silanol blocking compounds. Additionally, the removal of DMOA from the stationary phase will take approximately twice the time required to remove TEA from the stationary phase.

3.3.5 CONCLUSIONS

The characterisation of stationary phases for the LC analysis of basic compounds was performed by analysing chromatographic data (retention, peak symmetry and plate height) of five basic test compounds by principal component analysis (PCA). PCA was found to be useful to discern stationary phases. Explanation of 64 % of the variation present in the data set was found to be acceptable for graphical presentation and qualitative discernment of stationary phases. Stationary phases with similar chromatographic behaviour (retention, peak shape, plate height) clustered in the score plots. In the loading plots the relations between the variables were depicted.
It was found that the observed peak symmetries of the test compounds obtained with some stationary phases when using phosphate buffer pH 3 were different than when using phosphate buffer pH 7. Other stationary phases showed similar peak symmetries when using phosphate buffer pH 3 and pH 7.
The use of silanol blocking compounds like triethylamine (TEA) and N,N-dimethyloctylamine (DMOA) showed an improvement of the shape of the chromatographic peak. In particular, after addition of DMOA to mobile phase buffer pH 3 symmetrical peaks were obtained using the stationary phases presented in this study.

3.3.6 REFERENCES

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4. EVALUATION OF CHARACTERISATION METHODS FOR THE REVERSED PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS

4.1 CHARACTERISATION OF REVERSED PHASE LIQUID CHROMATOGRAPHY STATIONARY PHASES FOR THE ANALYSIS OF BASIC PHARMACEUTICALS: INFLUENCE OF ELUENT COMPOSITION AND COMPARISON OF EMPIRICAL TEST METHODS

4.1.1 SUMMARY

The reversed phase liquid chromatographic analysis of basic pharmaceuticals can be problematic. Both the properties of the eluent and the stationary phase influence the chromatographic performance. Therefore selection of a suitable stationary phase for the analysis of basic compounds can be difficult. In this chapter data about the influence of mobile phase constituents on the properties of basic analyte and eluent are shown. Furthermore, various test procedures are compared for their applicability to characterise stationary phases for the analysis of basic pharmaceuticals.

It was found that the organic modifier and the nature of the buffer influence the eluent properties. Moreover, the nature and amount of modifier also influence the basicity of the analytes. Test procedures using basic analytes as test probes provided relevant information with respect to selecting columns to analyse basic pharmaceutical compounds. Test procedures using compounds like aniline, phenol and benzene were found to be less suitable.

Investigations showed that the nature of the buffer can have a significant influence on retention and peak shape of basic compounds. In the liquid chromatographic analysis of bases phosphate buffers are often used. Mass spectrometric detection is frequently applied in pharmaceutical analysis. Since phosphate buffers are involatile, testing of columns using e.g. ammonium acetate would add useful information for the selection of LC conditions (i.e. stationary and mobile phase) for modern pharmaceutical analysis.

R.J.M. Vervoort, E. Ruyter, A.J.J. Debets, H.A. Claessens, C.A. Cramers and G.J. de Jong, J. Chromatogr. A, submitted.

4.1.2 INTRODUCTION

Reversed phase high performance liquid chromatography (RPLC) using silica based C_{18} or C_8 stationary phases is used in the pharmaceutical industry to analyse many kinds of samples. From the initial development of a pharmaceutical compound to the routine analysis of pharmaceutical products in quality control RPLC is used to analyse drug substances and drug products, samples of metabolism and pharmacokinetic studies, samples of stability studies etc. The RPLC analysis is promoted by sharp and symmetrical peaks which, compared to asymmetrical peaks, will result in lower limits of detection and quantitation, improved resolution and less dispersion between the calculated quantitative results because of improved determination of the area of the chromatographic peak [1,2].

Many pharmaceutical compounds contain one or more nitrogen atoms and the basicity of these compounds can vary from low (pKa approx. 3) to high (pKa approx. 11). Ionic and other polar interactions between basic nitrogen atoms with residual silanols and eventual other polar sites of the reversed phase stationary phase occur together with the hydrophobic interaction, which can result in asymmetrical peaks and irreproducible retention. These polar interactions comprise a number of stationary phase - solute interactions such as ion-ion (ion exchange), ion-dipole, dipole-dipole (e.g. hydrogen bonding), dipole-induced dipole and induced dipole-induced dipole (London forces) [3,4]. Developments in manufacturing of stationary phases have led to the existence of a large number of different phases. For the analysis of basic compounds these phases can, due to the different manufacturing procedures and silicas used, result in large differences between peak shape and selectivity. The development of stationary phases suitable to analyse basic substances is concentrated on minimising the interaction between residual silanols and eventual other polar sites responsible for asymmetrical peaks, with the basic analyte. Examples of such phases are phases utilising high purity silica [5-7], end-capping procedures [8-11], polymer encapsulation [12-15], embedded polar groups [16,17], bidentate phases [7,18] and phases from which the silica backbone is modified by introducing organic functional groups [19,20].

Besides the stationary phase, the composition of the mobile phase can also affect the chromatographic characteristics, e.g. retention and peak shape. The RPLC-UV analysis of basic analytes is usually performed using buffered eluents to control the ionisation of the analytes and stationary phases to obtain rugged and reproducible separations. For various reasons different buffers can be advantageous in the analysis of basic analytes. For the analysis of bases phosphate buffers often are used. However, phosphate buffers are known to be aggressive towards the stationary phase at neutral pH [21]. Moreover, phosphate buffers are not volatile and therefore less suitable for e.g. liquid chromatography on-line coupled with mass spectrometry (LC-MS) analysis. In the pharmaceutical industry LC-MS is routinely used in biomedical applications, impurity profiling, metabolite identification and

quantitative analysis [22-24]. Therefore, the influence of the nature of the buffer on the eluent properties and the nature of the modifier on basicity of the solutes were determined. Also the applicability of volatile buffers was investigated as an alternative to the involatile phosphate buffers to enhance the compatibility with MS.
The selection of a suitable stationary phase is an important parameter in the development of LC methods. Characterisation of stationary phases using generally accepted test procedures would make the choice of a suitable column for a dedicated application easier. As recently discussed, column evaluation tests can be divided into empirical tests, thermodynamically based tests and tests based on a retention model [25]. Empirical tests have in common that the information is obtained by using test compounds and eluents chosen according to a certain line of thought. Determination of silanol interactions is commonly based on relative retention of compounds of which the retention is mainly expected to be caused by hydrophobic and silanol interaction, and compounds from which the retention is based on hydrophobic interactions [26,27]. Tests based on retention models are e.g. the test of Galushko [28-30] and linear solvent retention relationship studies [31-34], whereas thermodynamically based tests use van het Hoff plots [35-37]. Several of these tests were recently compared with respect to determining polar and hydrophobic properties. The hydrophobic properties determined with the various tests were in good agreement, but the tests determined different ionic properties and other polar activities [25]. Some column characterisation tests are performed using buffered eluents whereas others are performed using non buffered eluents. Both buffering and not buffering of the mobile phase shows advantages and disadvantages. Since buffers can mask silanol interactions, testing in unbuffered systems can be advantageous for silanol activity determination [27]. However, a test generally accepted for column characterisation should be rugged and reproduced in many different labs world-wide. This can only be achieved by using a stable well defined eluent, which can be obtained using buffered solvents. It was recently shown that buffering of the eluent is mandatory for reproducible test results [38,39]. Another aspect is the nature of the buffer. In the majority of tests developed phosphate buffers are used to control the pH of the eluent [38-41]. However, since buffer ions can interact with residual silanols, other polar sites and basic analytes [42,43], it is obvious that the choice of the buffer can influence the results of the column tests.
In the present study several column test procedures are compared. Most test procedures are developed for general column characterisation purposes and the majority of these tests mainly determine two stationary phase characteristics, i.e. hydrophobic and polar properties. In this study especially the applicability of stationary phases in the analysis of basic pharmaceuticals is investigated. The tests were performed to determine polar properties of the stationary phases, which are most important for the analysis of basic compounds. The results were related to the chromatographic data obtained by analysing seven basic pharmaceutical drugs from

NV Organon. The influence of various buffers on the analysis of these basic compounds and on the results of column characterisation tests was included.

4.1.3 EXPERIMENTAL

4.1.3.1 Apparatus

The determinations of eluent pH were carried out using a Methrom 713 pH meter, Metrohm (Herisau, Switzerland) and a combined glass electrode Hamilton (Bonaduz, Switzerland). The pH meter was calibrated using buffers pH 4.00, 7.00 and 9.00. The titrations of the eluent were performed using a Metrohm 670 Titriprocessor.
The HPLC experiments were performed using a HP1100 liquid chromatograph consisting of a quaternary pump, solvent degasser, autosampler, column oven and diode array detector (Agilent Technologies, Amstelveen, The Netherlands). LC-UV chromatograms were collected using a HPLC 3^D Chemstation (Agilent Technologies).

4.1.3.2 Chemicals

The basic compounds of the Organon compound mixture were obtained from N.V. Organon (Oss, The Netherlands). In Fig. 4.1.1 the structures and the related pKa values are shown. The compounds for the Engelhardt, Galushko, Tanaka and McCalley tests were obtained from various manufacturers and are of p.a. quality. The McCalley test compounds and related pKa values are shown in Fig. 4.1.2. As organic modifier methanol (MeOH), supplied by J.T. Baker (Deventer, The Netherlands) was used. The buffers were prepared from disodiumhydrogen phosphate, sodiumdihydrogen phosphate, ammonium phosphate, acetic acid (99-100%), orthophosphoric acid (85%) and citric acid monohydrate supplied by J.T. Baker (Deventer, The Netherlands). Ammonium citrate was purchased from Aldrich (Milwaukee, WS) and ammonium acetate from Janssen Chimica (Geel, Belgium). 1M aqueous solutions of acetic acid, ammonium acetate, citric acid, ammonium citrate, ammonia, ammonium monohydrogen phosphate, ammonium dihydrogenphosphate, sodium dihydrogen phosphate and sodium monohydrogen phosphate were prepared. Adequate amounts were mixed to obtain 25 mM buffers of pH 3 and 7.
To avoid overloading of the stationary phases by basic compounds, as recently discussed [40], for the McCalley and the Organon test compounds amounts of 0.1 µg of each test compound were injected onto the column. For the Galushko test 2 µl of a solution of 0.03 mg uracil, 0.21 mg aniline, 1.78 mg phenol, 8 mg benzene and 8 mg toluene in 5 ml methanol were injected. For the other tests the amounts as described in the literature were injected onto the column [26,27]. The flow-rate was set to 1.0 ml/min. The stationary phases studied are shown in Table 4.1.1. The analytical conditions are given in Table 4.1.2.

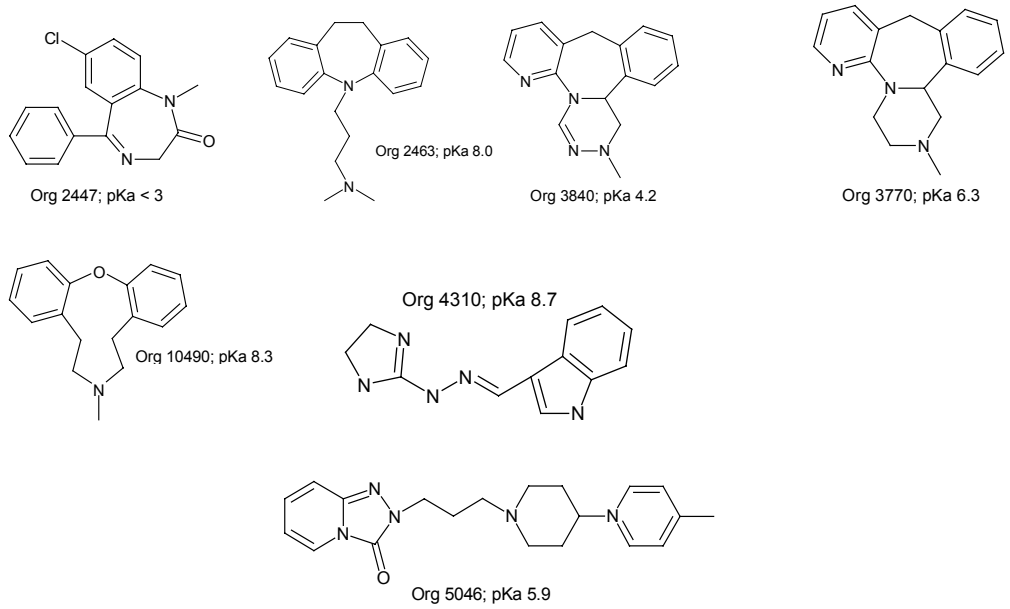

Figure 4.1.1: Structures of the Organon compounds used in this study. The pKa values were measured in MeOH+H_2O/60+40 %(V/V).

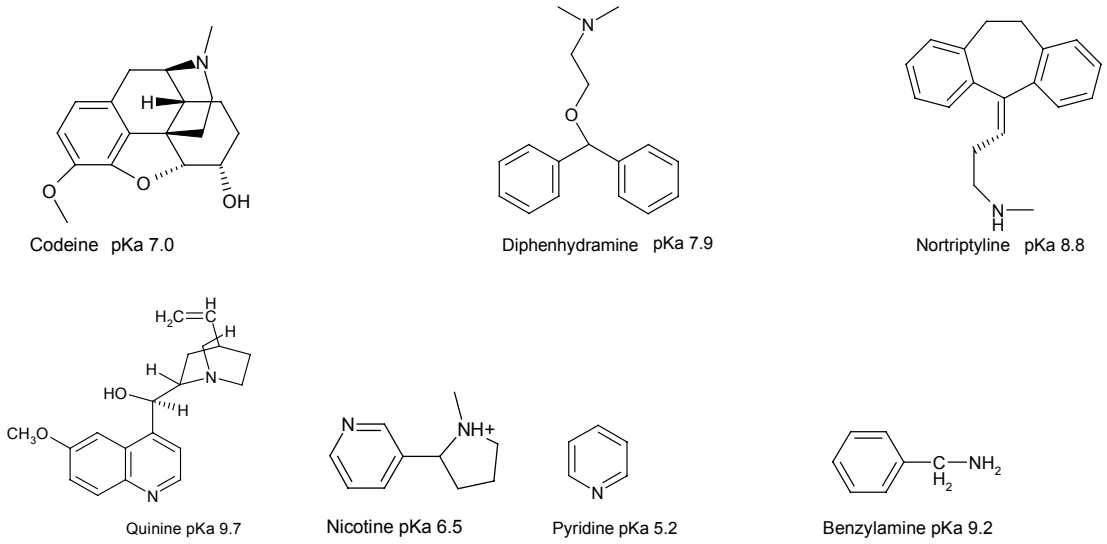

Figure 4.1.2: Structures of the McCalley test compounds used in this study. The pKa values were measured in water.

4.1.3.3 Column tests

First, the columns were tested for polar properties using the test procedures as summarised in Table 4.1.2. Secondly, to study the influence of the nature of buffers on the test results, the water or buffer moiety of the eluents of the original tests was

replaced by 25 mM solutions of ammonium citrate, ammonium acetate, ammonium phosphate and sodium phosphate pH 3 and 7.

Table 4.1.1: Investigated stationary phases; dimensions are 150 x 4.6 mm I.D.

Stationary phase	Manufacturer	Abbreviation	Phase characteristics
Symmetry Shield C_{18}	Waters	SYSH	embedded polar group
Symmetry C_{18}	Waters	SYMM	high purity silica
Zorbax SB-C_{18}	Agilent Technologies	ZOSB	sterically protected
Zorbax Extend-C_{18}	Agilent Technologies	ZOBI	bidentate bonded alkyl chains
Alltima C_{18}	Alltech	ALLT	polymeric bonded phase
Luna C_{18}	Phenomenex	LUNA	high purity silica
Discovery C_{18}	Supelco	DISC	high purity silica

4.1.3.4 Calculations

The column characteristics were calculated according to the literature [26-30,40] and as shown in Table 4.1.2. The results of the Galushko test were calculated using the software program Chromlife, Merck (Darmstadt, Germany). The USP asymmetry values were calculated at 5% of the peak height using the HPLC^{3D} Chemstation software:

$$As = \frac{w_{0.05}}{2w_{a,0.05}} \quad \{1\}$$

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 of the peak at 5% of the peak height. Correlation coefficients between the test variables were calculated using Microsoft Excel 5.0 software, Microsoft.

Table 4.1.2: Tests used in this column test evaluation.

Test	Test compounds	Calculation	Experimental conditions	Reference
Tanaka	caffeine, benzylamine, phenol	hydrogen bonding: $k_{caffeine}/k_{phenol}$	methanol+water / 55+45 %(V/V), column temperature 40 °C	[26]
		ion exchange capacity: $k_{benzylamine}/k_{phenol}$	methanol+25 mM sodium phosphate pH 3 / 55+45 %(V/V) methanol+25 mM sodium phosphate pH 7 / 55+45 %(V/V)	
Engelhardt	aniline, phenol, 4-ethylaniline	silanol activity: $k_{aniline}/k_{phenol}$ $As_{4\text{-ethylaniline}}$	methanol+water / 55+45 %(V/V), column temperature 40 °C	[27]
Galushko	aniline, phenol, benzene, toluene	NH-interaction and polarity, calculated using Chromlife software	methanol+water / 60+40 %(V/V), column temperature 30 °C	[28-30]
McCalley	pyridine, quinine, benzylamine, diphenhydramine, codeine, nortriptyline, nicotine	asymmetry and retention of the test compounds	methanol+25 mM sodium phosphate pH 3: for pyridine, nicotine, codeine, quinine and benzylamine ratio 30+70 %(V/V) ; for diphenhydramine and nortriptyline ratio 55+45 %(V/V) methanol+25 mM sodium phosphate pH 7 for all compounds ratio 65+35 %(V/V)	[40]
Organon	Org 2447, Org 4310, Org 3840, Org 10490, Org 2463, Org 3770, Org 5046	asymmetry and retention of the test compounds	methanol+25 mM sodium phosphate pH 3 / 50+50 %(V/V) methanol+25 mM sodium phosphate pH 7 / 50+50 %(V/V)	[41]

4.1.4 RESULTS AND DISCUSSION

4.1.4.1 Eluent composition

In the analysis of substances that are ionisable, the pH of the eluent will affect the degree of ionisation of the basic analyte. Moreover, the pH of the eluent will also control the degree of ionisation of residual silanols present on the surface of the stationary phase. Therefore, the pH of the eluent will influence the ionic and other polar interactions between the basic analyte and the stationary phase. It is obvious that eluent pH is a very important parameter in the RPLC analysis of basic analytes and can influence the results obtained with the various test procedures applied in this study.

In LC it is common practice to measure the pH of the mobile phase buffer before mixing with the organic modifier. However, it is also recognised that the pH changes after addition of organic solvents [38,44]. For organic modifier/buffer mixtures the pH can be calculated using measurements with conventional pH electrodes:

$$pH_x^* = pH_x^{app} - \delta \quad \{2\}$$

where pH^*_x is the pH value in an aqueous - organic system, pH^{app}_x is the measured (apparent) value in an aqueous - organic system and δ is a correction factor for the liquid junction between the electrode and the eluent [45]. Values of δ have been published for several organic modifier - aqueous systems, and it can be concluded that for amounts of organic modifier up to 80% the correction factor (δ) is small i.e. approx. 0.1 [46,47]. This means that the measured pH is approx. equal to the true pH for eluents with an organic modifier concentration less than 80%.

In Fig. 4.1.3 the relation between the pH of the pure buffer solution and the pH of the methanol+25 mM sodium phosphate buffers / 50+50% (V/V) solutions from pH 3 up to pH 7 is shown. As can be seen, addition of methanol to the sodium phosphate buffers resulted in an increased eluent pH and, in the pH range studied, the relation between the buffer pH and the pH of the methanol+25 mM sodium phosphate buffers / 50+50%v/v solutions is linear (r^2=0.9998). Comparable data were obtained using ammonium acetate, ammonium citrate and ammonium phosphate buffers. Differences were found between the slopes of the curves. For sodium phosphate, the slope of the curve is 1.07, meaning that the pH shift depends slightly on the buffer pH. For ammonium acetate the slope was 0.96, for ammonium citrate 1.06 and for ammonium phosphate 1.01, respectively.

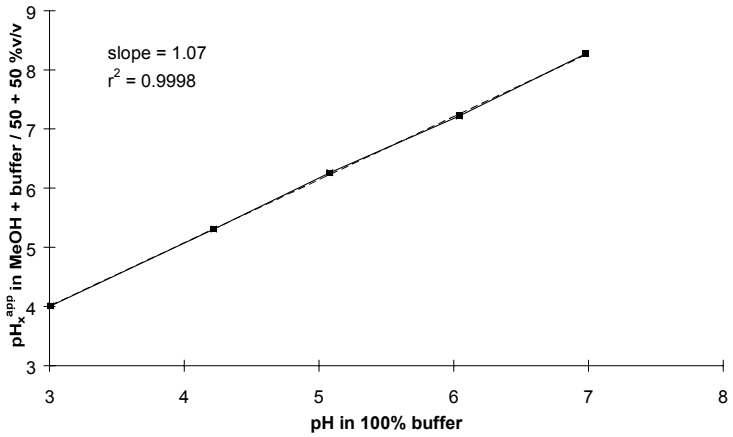

Figure 4.1.3: Relation buffer pH and eluent pH. The buffers used are for 25 mM sodium phosphate and the solvents used are MeOH+buffer/50+50%(V/V).

The discussed data were obtained using methanol. However, Barbosa et al [44] showed that the nature of the modifier also influences the eluent pH. Different eluent pH values were obtained mixing buffers with methanol, acetonitrile and tetrahydrofuran.

The advantage of buffering the mobile phase is maintaining a constant pH during the RPLC analysis, which for ionisable compounds is important to obtain reproducible and robust analyses. A stable pH is obtained when the applied pH is in the buffering range of the buffer. To study the influence of the amount of methanol on the buffering range, mixtures of MeOH+25 mM sodium phosphate/30+70, 50+50 and 70+30 %(V/V) were titrated with 0.5 M hydrochloric acid solution. Fig 4.1.4 shows the results.

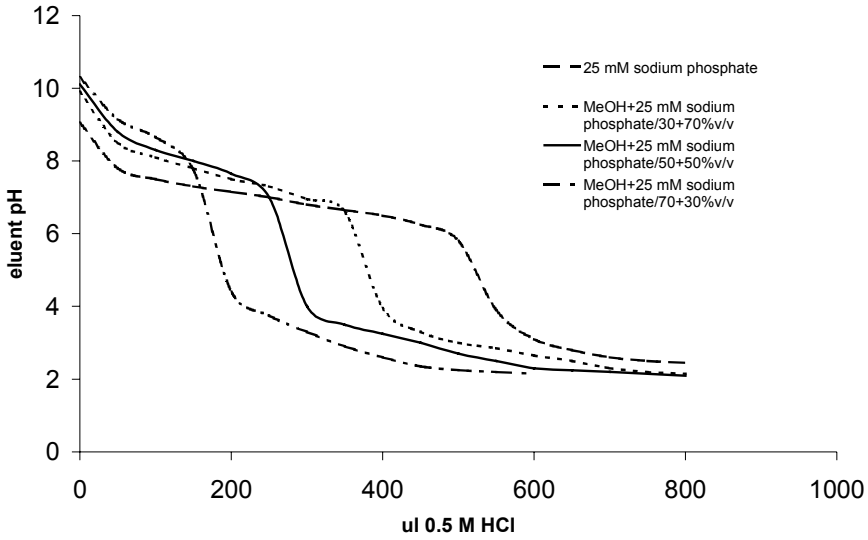

Figure 4.1.4: Influence amount of methanol on buffering range of the eluent.

As can be seen the range in which a stable eluent pH is obtained is influenced by the amount of methanol. An increased amount of methanol results in less hydrochloric acid necessarry to change the eluent pH. This is caused by the lower electrolyte concentration in the eluent at increased methanol concentration. Using ammonium acetate and ammonium phosphate comparable data were obtained. Surprisingly, when using ammonium citrate no clear buffering range was obtained. Addition of hydrochloric acid resulted in a slow but steady decrease of the pH from approx. 8 to approx. 2 for the solutions containing 30, 50 and 70% methanol. Although no clear buffering range was obtained, contradictory to the ammonium phosphate, the ammonium acetate and the sodium phosphate buffers, the slow decrease in pH shows that ammonium citrate is useful as buffer in the entire range studied. In Fig. 4.1.5 the influence of the nature of modifiers commonly used in LC, e.g. methanol, acetonitrile and tetrahydrofuran, on the buffering range of the eluent containing sodium phosphate buffer is shown. As can be seen the nature of the modifier did not significantly influence the buffering range of the eluent.

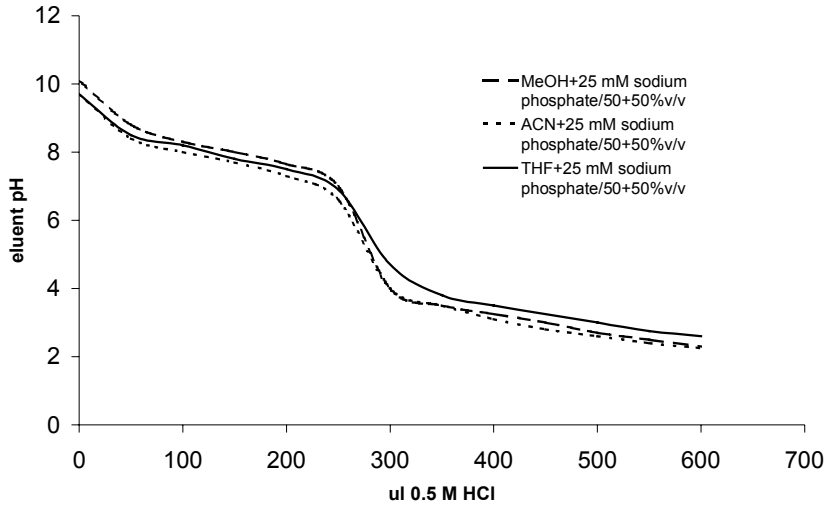

Figure 4.1.5: Influence nature of modifier on buffering range of the eluent.

Besides the pH and the buffering range of the eluent, the organic modifier will also influence the pKa of the basic analyte. Comparable to the pH of the eluent, the pKa values of the analytes are often measured in pure water. In Fig. 4.1.6 the influence of the nature and concentration of organic modifier on the pKa of a basic pharmaceutical, i.e. mirtazipine, is shown. As can be expected, the pKa of the basic substance is influenced: increasing the modifier concentration lowers the pKa value of the basic analyte. The effect was similar for acetonitrile and methanol, however, the effect was significantly different for tetrahydrofuran. The latter can be explained by comparing the normalised hydrogen bond acceptibility (β) of the solvents used. A higher hydrogen bond acceptibility of the solvent will lower the pKa value of the basic analyte. For MeOH and ACN β is 0.29 and 0.25, whereas for THF β is 0.49,

respectively [48]. Therefore, the pKa for mirtazipine with MeOH and ACN is comparable, and with THF significantly lower.
It is clear that the described phenomena should be taken into account when performing and discussing LC analysis of basic analytes. As an example the shift in retention for propranolol as recently described by Neue et al [38,39] can be taken. The shift in retention for propranolol was not expected from the pH of the buffer and pKa of the analyte. Both the pH and pKa values were measured in pure water. In this study it is shown that in the eluent containing organic modifier the pKa of the analyte will be lower, whereas the pH of the eluent will be higher. As a result, the analyte might be analysed as a deprotonated compound, explaining the retention shift. Moreover, the possible influence of the stationary phase on the pH of the eluent inside the column should also be considered as well as the influence of the organic modifier on the pKa of the residual silanol groups of the stationary phase [49].

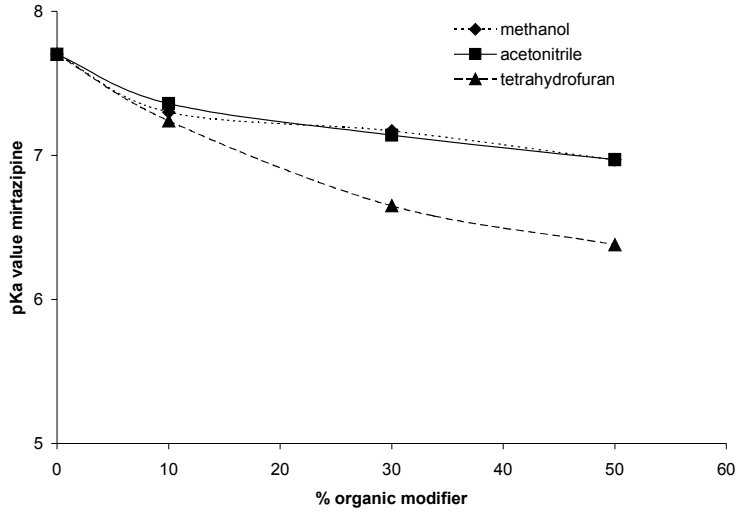

Figure 4.1.6: Influence nature and concentration modifier on pKa value of mirtazipine.

In summary, from the results discussed above it is clear that control and proper usage of the pH of the eluent is important in the analysis of basic analytes. The amount and nature of the organic modifier influence pH of the eluent, pKa of the basic substance and pKa of the silanol groups of the stationary phase. Increasing the amount of modifier will increase the pH of the eluent, whereas the pKa value of the basic pharmaceuticals will decrease. This means that in e.g. gradient analysis, besides a change in modifier concentration, also a change in eluent pH and pKa of the analytes occurs.

4.1.4.2 Comparison of various test procedures

The applicability of the testing procedures described in Table 4.1.2 to characterise stationary phases for the analysis of basic pharmaceuticals is investigated by testing

the stationary phases shown in Table 4.1.1. Comparing the test procedures in Table 4.1.2, differences are observed between the test compounds as well as the eluent conditions used. Analytes like aniline and phenol are frequently used both in empirical (Engelhardt) and model based (Galushko) tests [27-30], whereas Tanaka used caffeine, benzylamine and phenol [26]. McCalley developed a test focused on testing columns for the analysis of basic substances using compounds generally available and structurally comparable to the compounds of interest i.e. basic analytes [40]. One of the McCalley test compounds is amphetamine, which belongs to the class of controlled drugs. For safety reasons these type of compounds are not generally available and can therefore not be included in a test applied worldwide. Therefore, in the McCalley test quinine, nicotine, pyridine, benzylamine, diphenhydramine, codeine and nortriptyline were used as test compounds (Fig. 4.1.2). For comparison and as a reference to daily practice, also seven basic Organon drugs differing in pKa range from <3 to 8.7 were studied (Fig. 4.1.1). For the Organon and McCalley tests the average As values obtained for the test compounds were used. The Engelhardt and Galushko tests are as prescribed not buffered, as well as the hydrogen bonding test procedure of the Tanaka test. With the McCalley test, the ion exchange capacity of the Tanaka test and the Organon test phosphate buffers are used to control the ionisation and retention of the test analytes. Retention (k) and peak asymmetry (As) data are used in the various tests. Since As, compared to k, is more sensitive to extra-column effects and/or column ageing, the neutral compound in the Organon test mix (Org 2447, pKa < 3) was used to control these effects. It was observed that with all eluents used the shape of the Org 2447 peak was symmetrical: 0.95 < As < 1.15 and therefore it was concluded that the columns studied were in acceptable condition when used.

In the first approach a qualitative comparison of the tests was performed by ranking the columns (see Table 4.1.3) based on the parameters calculated as described in Table 4.1.2. A lower ranking number represents a better suitability of the column to analyse basic pharmaceuticals. As can be seen, no uniform ranking of the columns is obtained using the five tests. For the LUNA column, however, the McCalley and Organon data were in agreement i.e. symmetrical peak shapes were obtained using the LUNA column. Using the tests of Engelhardt, Tanaka and Galushko however, the SYSH column generally showed the best characteristics and on the basis of these tests the LUNA column would not be the column of choice. The other columns were ranked differently using the various tests. It should be noted, however, that the differences between the ranking in some cases were relatively small. As shown in Table 4.1.3 testing must be performed at more than one eluent pH. For the McCalley and Organon compounds, the DISC column showed good performance when used with buffer pH 7, but when used with buffer pH 3 asymmetrical peaks were obtained.

Table 4.1.3: Ranking of stationary phases using the tests as described in Table 4.1.2.
The lower the ranking number, the more suitable the column for analysing basic substances.

Test	SYSH	SYMM	ZOSB	ZOBI	ALLT	LUNA	DISC
Engelhardt silanol activity	1	4	6	4	7	2	2
Engelhardt As 4-ethylaniline	4	1	3	5	7	1	5
Tanaka hydrogen bonding	1	2	7	2	6	5	4
Tanaka ion exchange capacity pH 3	1	2	7	4	5	3	6
Tanaka ion exchange capacity pH 7	1	2	7	2	6	5	4
Galushko NH interaction	1	4	6	5	7	2	3
McCalley pH 3*	1	5	1	1	6	1	7
McCalley pH 7*	2	2	5	6	7	1	2
Organon pH 3*	1	1	5	4	7	1	6
Organon pH 7*	3	5	4	6	6	1	2

*: Based on average As values of the test compounds.

As recently discussed by Kele and Guiochon, the interaction and/or the accessibility of the silica surface most probably depends on the molecular structure of the analyte, as illustrated by different trends for aniline and N, N-dimethylaniline [49]. As the structural diversity between the Engelhardt, Tanaka and Galushko test compounds on one side, and the McCalley and Organon test compounds on the other side are relatively large (e.g. aniline vs. diphenhydramine, benzylamine vs. Org 2463), determination of stationary phase characteristics using the various test analytes can result in different test results depending on the test analytes used. In comparison, the data of the McCalley test are more in agreement with the data of the Organon test compounds. As is shown taking both buffer pH 3 and pH 7 into account, both tests classify the LUNA column as most suitable, the SYSH column shows to be a good alternative, whereas the ALLT column is less suitable, for the analysis of basic solutes with regard to peak shape. Moreover, both the McCalley and Organon tests classify the DISC column as second best when used with buffer pH 7, but less useful when used with buffer pH 3. The ALLT column being the highest ranked column, i.e. the worst of the seven columns studied, is similarly ranked by the Engelhardt and Galushko test results. Therefore, it was concluded that differences between generations of columns can be determined using tests like the Engelhardt test. However, often small differences in separation performance exist between the modern phases of today. It was concluded that these small differences can best be detected using basic compounds as test analytes. Using the McCalley test compounds the SYMM, ZOSB and ZOBI were also ranked differently, compared

to the ranking obtained using the Organon compounds. This is also due to the differences between the McCalley and Organon test compounds. When comparing the ranking of the columns using test compounds with comparable molecular structures i.e. diphenhydramine and Org 2463 (both compounds have a tertiary amine situated at the end of an aliphatic chain) a comparable ranking of the columns was obtained (data not shown). To illustrate the importance of a proper column selection for LC method development

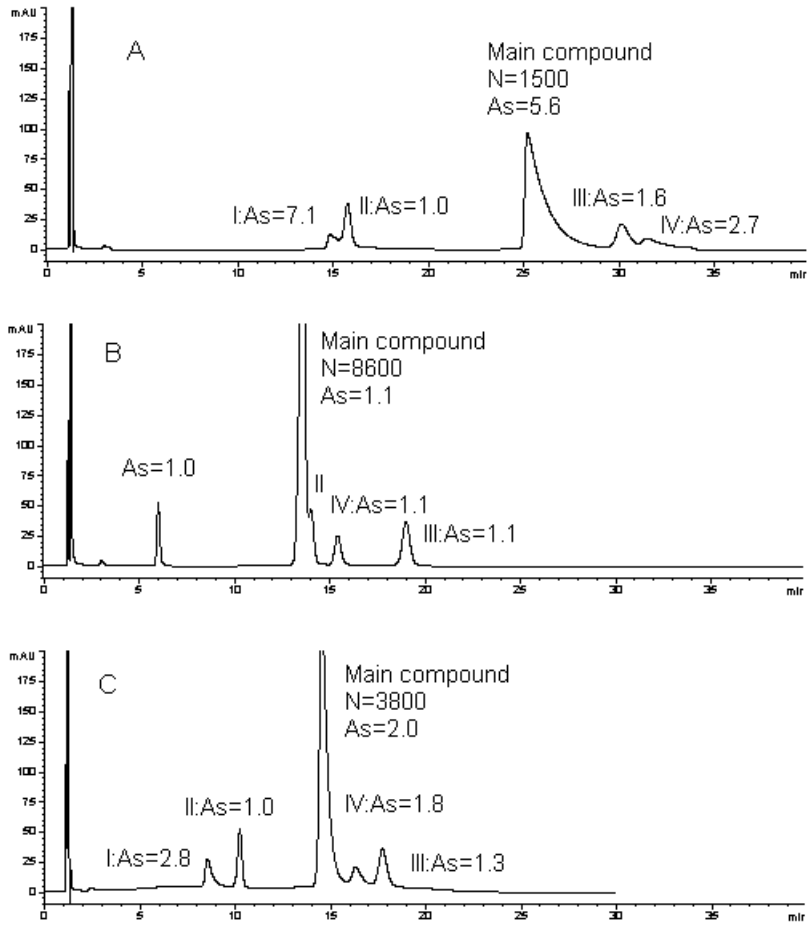

Figure 4.1.7: Comparison of Alltima C_{18} (A), Luna C_{18} (B) and Zorbax SB-C_{18} (C) columns. The eluent used was MeOH+25 mM NH_4Ac pH 7/50+50 %(V/V) at a flow-rate of 1.0 ml/min and the columns were thermostatted at 40 °C. The main compound was spiked with 10% of related substances.

in Fig. 4.1.7 the LC-UV analysis of a candidate drug currently under development and spiked with related substances is shown, using the ALLT, LUNA and ZOSB columns.

In a second approach the influence of the nature of the buffer and mobile phase pH on the test results was studied. Brereton and McCalley already demonstrated that the nature of the modifier influenced the applicability of modern packing materials for the analysis of basic analytes [50]. In this study we investigated the influence of the

nature of the buffer by replacing the water or buffer moiety of the test eluents in Table 4.1.2 by 25 mM ammonium citrate, ammonium acetate, ammonium phosphate and sodium phosphate buffers pH 7 and 3. Using buffers pH 7, the influence of the nature of the buffers on ranking of the columns was small. Using buffers pH 3, the nature of the buffers showed a larger influence on column ranking (data not shown). The McCalley and Organon results were based on average asymmetry data. However, we noticed that the influence of eluent composition on chromatographic performance of single compounds was large. Therefore, the influence of the nature of the buffer on peak shape and retention of single compounds will be discussed in the next part in more detail.

4.1.4.3 Influence of the nature of buffer on peak asymmetry and retention of basic analytes

To study the influence of the nature of the buffer on retention and peak asymmetry both for the McCalley and Organon test compounds, correlations for retention and peak asymmetry values obtained with the seven columns were calculated. Low correlation between the measured variables with the different buffers indicates a significant effect of the nature of the buffer on retention or peak shape.

In several cases for peak asymmetry a significant influence of the nature of the buffer was observed. As an example, correlations between the asymmetry data for the McCalley analytes are shown in Table 4.1.4. Using buffers pH 7, for pyridine, nicotine, benzylamine and nortriptyline besides the stationary phase, the nature of the buffer also affected the shape of the peak. For codeine, quinine and diphenhydramine the peak shape is hardly affected by the nature of the buffers. The peak shape of the latter compounds was affected by the nature of the stationary phase only. Generally when using buffers pH 3, the correlation for peak asymmetry between the buffers was low. Except when comparing the peak shapes obtained when using ammonium phosphate and sodium phosphate buffers e.g. for codeine, quinine, benzylamine, diphenhydramine and nortriptyline the correlation was $\geq$ 0.9 meaning that the effect of the cation on peak shape for these compounds was small. In Fig. 4.1.8 for diphenhydramine examples of a high (ammonium phosphate versus sodium phosphate) and a low (ammonium phosphate versus ammonium acetate) correlation between asymmetry obtained with the seven columns is shown. For the Organon analytes the correlation between the asymmetry data usually was low. Only when analysing Org 10490 using buffers pH 3 the type of buffer hardly influenced the peak shape, i.e. correlation $\geq$ 0.9.

For some McCalley compounds correlation coefficients between the retention factors, obtained with the four buffers at pH 7 were approx. 0.85, but > 0.95 for the other compounds.

Table 4.1.4: Correlation matrix for As for the McCalley compounds using buffers pH 3 and pH 7. AmAc is ammonium acetate, AmCit is ammonium citrate, AmPho is ammonium phosphate and NaPho is sodium phosphate.

Pyridine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	-0.50	0.56	0.43	AmAc	1	-0.50	-0.21	-0.14
AmCit		1	-0.60	0.13	AmCit		1	0.64	-0.16
AmPho			1	-0.17	AmPho			1	0.33
NaPho				1	NaPho				1

Nicotine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	-0.25	-0.33	0.56	AmAc	1	0.77	0.72	0.65
AmCit		1	-0.51	-0.80	AmCit		1	0.91	0.21
AmPho			1	0.40	AmPho			1	0.06
NaPho				1	NaPho				1

Codeine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	-0.30	-0.13	-0.05	AmAc	1	0.90	0.94	0.76
AmCit		1	0.71	0.72	AmCit		1	0.97	0.89
AmPho			1	0.99	AmPho			1	0.97
NaPho				1	NaPho				1

Quinine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	0.46	0.07	0.18	AmAc	1	0.98	0.99	0.96
AmCit		1	0.89	0.95	AmCit		1	0.99	0.97
AmPho			1	0.96	AmPho			1	0.98
NaPho				1	NaPho				1

Benzylamine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	-0.04	-0.05	0.10	AmAc	1	0.81	0.94	0.17
AmCit		1	0.96	0.98	AmCit		1	0.81	0.32
AmPho			1	0.93	AmPho			1	0.25
NaPho				1	NaPho				1

Diphenhydramine

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	0.80	0.11	0.13	AmAc	1	0.99	0.93	0.96
AmCit		1	0.52	0.53	AmCit		1	0.92	0.94
AmPho			1	0.99	AmPho			1	0.80
NaPho				1	NaPho				1

Nortriptyline

pH 3	AmAc	AmCit	AmPho	NaPho	pH 7	AmAc	AmCit	AmPho	NaPho
AmAc	1	0.65	-0.02	0.01	AmAc	1	0.80	0.40	0.92
AmCit		1	0.54	0.55	AmCit		1	0.86	0.89
AmPho			1	0.99	AmPho			1	0.55
NaPho				1	NaPho				1

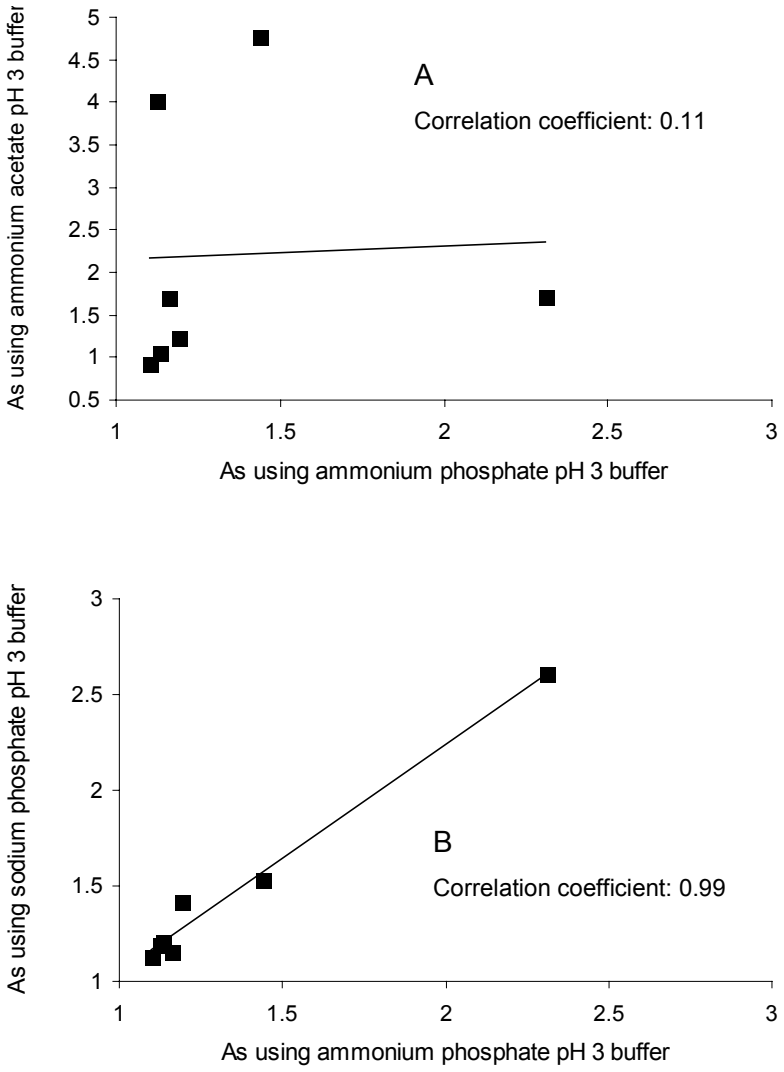

Figure 4.1.8: Example of low (A) and high (B) correlation with calculated regression line. The asymmetry values (As) were obtained for diphenhydramine using the seven columns in Table 4.1.1. The mobile phases used were: methanol+25 mM ammonium phosphate pH 3/55+45 %(V/V), methanol+25 mM sodium phosphate pH 3/55+45 %(V/V) and methanol+25 mM ammonium acetate pH 3/55+45 %(V/V).

This showed that the retention behaviour of the compounds when using the buffers at pH 7 with the seven columns is comparable: the nature of the buffer at buffer pH 7 did not affect the selectivity of the McCalley test compounds using the columns shown in Table 4.1.1. With buffers pH 3, however, an effect of the nature of the buffer was observed. Using ammonium acetate low correlation for retention with the other buffers were obtained with pyridine, nicotine, codeine and benzylamine

(correlation ≤ 0.7), meaning the selectivity for the compounds with the seven columns used with ammonium acetate buffer pH 3 is different. Comparison of ammonium phosphate and sodium phosphate buffers pH 3 showed a good correlation (≥ 0.9) for retention, i.e. the type of cation of the buffer (sodium vs. ammonium) had no significant effect on the retention. For the Organon compounds, comparable phenomena were observed. Using buffers pH 7 comparable selectivity is obtained with the columns in Table 4.1.1. When using buffers pH 3, also for the Organon compounds a low correlation is obtained when comparing ammonium acetate with the other buffers.
From these data it is clear that the mechanism of polar interactions between the stationary phase and the basic analytes is very complex. As discussed by Nawrocki [4], these interactions comprise of a number of stationary phase - solute interactions such as ion-ion (ion exchange) ion-dipole, dipole-dipole (e.g. hydrogen bonding), dipole-induced dipole and induced dipole-induced dipole. Moreover, the influence of counterions of different lipophilicity, size and flexibility on ion-pair partition was recently studied for some drugs [51-53]. The size and flexibility of both analyte and counterion were essential in the interaction between these, whereas the lipophilicity of the analytes could be influenced by the size of the counterions. LoBrutto et al showed that retention of bases depends on the nature of buffer ions and its concentration in the eluent [42,43]. The effects can be attributed to the ionic interactions of protonated analytes with oppositely charged buffer ions, which results in the formation of ion pairs or the disruption of the analyte solvation. It is obvious that various factors affect the interaction between analyte, residual silanols and eventual other polar sites. It depends on the molecular structure of the analyte, the nature of the stationary phase and on the properties of the eluent used.
So far this study showed that characterisation of stationary phases for the analysis of basic pharmaceuticals depends on the type of test analyte and on the properties of the eluent. Stationary phases are usually characterised using phosphate buffer. The data in this study showed that testing under different eluent conditions provides more information. Testing of columns using ammonium acetate buffer would give valuable information for the selection of columns suitable for use in LC-MS analysis.

4.1.5 CONCLUSIONS

For the eluents commonly used in RPLC, it was found that addition of methanol in the eluent affected the pH. For example addition of 50% methanol resulted in an increase of approx. 1 pH unit in the buffer pH range 3 - 7. Besides the pH, the buffering capacity of eluents is also influenced by addition of methanol. In addition, the amount and nature of the modifier also influence the pKa of basic analytes. For example, for methanol and acetonitrile upon addition of these modifiers the pKa shift was approx. 0.7 for mirtazipine. For tetrahydrofuran however, for the same amount

of modifier the change of the pKa value of this compound amounted larger than 1 pKa unit.

In general, comparing the result of the McCalley test with the data of the Organon compounds, the ranking of the investigated RPLC columns was comparable. Comparing the classification of seven RPLC stationary phases of the latest generation, the Engelhardt, Tanaka and Galushko tests resulted in mutual different rankings. In addition, these three latter tests were also in disagreement with the McCalley and basic Organon test compounds.

The mutual replacement of 25 mM ammonium acetate, ammonium citrate, ammonium phosphate and sodium phosphate aqueous buffers pH 3 and 7 in the various eluents did not result in a comparable ranking of the investigated columns.

From the investigated tests only results of the McCalley test could predict suitability of columns for the analysis of the Organon basic compounds. For individual basic compounds a significant influence of the nature of the buffer on peak shape was observed in various cases. In addition, the results of this study revealed the necessity of column testing at different pH-values and various types of buffering salts.

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4.2 CHARACTERISATION OF REVERSED PHASE STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS BY THERMODYNAMIC DATA

4.2.1 SUMMARY

This chapter describes characterisation of reversed phase liquid chromatography (RPLC) columns using thermodynamic measurements. Retention versus 1/T data were used to construct van ‘t Hoff plots. The slope of these plots indicates the standard enthalpy of transfer of the analyte from the mobile to the stationary phase. The standard entropy can be calculated from the intercept and depends also on the phase ratio of the column.

Van ‘t Hoff plots were linear for the investigated RPLC columns, meaning that for basic analytes over the temperature range studied no changes in the retention mechanism occurred. Enthalpies and entropies of transfer of basic analytes from the mobile to the stationary phase revealed information about the types of interaction of protonated and neutral compounds with the stationary phases. A clear view however on how these thermodynamic data may explain the observed substantial differences in peak symmetry cannot be given yet. Addition of N,N-dimethyloctyl amine (DMOA) to the eluent resulted for protonated basic compounds in less negative enthalpy and entropy values. Obviously, for protonated basic compounds it is energetically less favourable to remain in the stationary phase after blocking residual silanols with DMOA.

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4.2.2 INTRODUCTION

Test methods for column characterisation can be divided into three groups: *i*: empirical test methods, *ii*: thermodynamically based test methods and *iii*: test methods based on a retention model [1]. This chapter describes the characterisation of reversed phase liquid chromatography (RPLC) columns for the analysis of basic pharmaceuticals using a thermodynamically based method. For analytes enthalpies and entropies of transfer from the mobile to the stationary phase can be calculated from retention data by evaluation of the van het Hoff plots [2]. The retention factor can be expressed in terms of standard enthalpies and entropies of transfer from mobile to stationary phase. The relation between the logarithm of the retention factor (ln k) and enthalpies and entropies equals:

$$\ln k = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \beta \quad \{1\}$$

where k is the measured retention value, ΔH^0 the enthalpy, ΔS^0 the entropy, T the absolute temperature, R the gas constante and β the phase ratio of the column. Enthalpy (ΔH^0) represents the measure of energy exchange in a system and entropy (ΔS^0) represents the chaos of a system. Van 't Hoff plots are obtained by regressing ln k vs.1/T. Such plots are linear if ΔH^0 and ΔS^0 are independent of the temperature. The slope of the van het Hoff plot indicates the standard enthalpies of transfer. The standard entropies of transfer of the analyte from the mobile to the stationary phase are calculated from the intercept and depend also on the phase ratio. Non-linear van het Hoff plots are indicative for a change of retention mechanism. For example ΔH^0 may not be constant over the whole temperature range studied [2].

Comparison of stationary phases has been performed by studying the changes in mechanism of retention of test analytes with changing column temperature. Examples are the studies of Cole, Sentell and Dorsey [3,4] who examined the influence of bonding density of stationary phases on the retention of nonpolar solutes. They found that partition, rather than adsorption, was found to be the relevant model of retention for non-polar compounds. The columns that were compared differed in bonding densities ranging from 1.60 to 4.07 μmol/m^2. The entropic contribution to retention becomes more significant with respect to the enthalpy contribution as the stationary phase bonding density is increased. Using benzene as a test compound for columns with a bonding density $\geq$ 3.06 μmol/m^2 non-linear van het Hoff plots were obtained. Other examples are the studies of Purcell et al [5] using van het Hoff plots to study changes in the secondary structure of peptides and the mechanism of interaction with hydrophobic surfaces, and Philipsen et al [6] using van het Hoff plots to study the retention of polystyrene and polyester oligomers. For basic solutes, retention is considered to be a combination of hydrophobic, ionic and other polar interactions [7]. Non-linear van het Hoff plots

might be indicative of a change of retention mechanism [6,8]. For basic compounds this means that the ratio of the hydrophobic and polar interactions could change.
This chapter shows the comparison of RPLC stationary phases for the analysis of basic compounds using a thermodynamically based test method. Van 't Hoff plots as well as standard enthalpies and entropies of transfer of neutral and basic analytes from the mobile to the stationary phase were determined for six RPLC columns.

4.2.3 EXPERIMENTAL

4.2.3.1 Apparatus

The HPLC experiments were performed using a HP1100 liquid chromatograph consisting of a quaternary pump, solvent degasser, autosampler, column oven and diode array detector (Agilent Technologies, Amstelveen, The Netherlands). LC-UV chromatograms were collected using a HPLC 3^D Chemstation (Agilent Technologies).

4.2.3.2 Chemicals

The basic compounds of the Organon compound mixture were obtained from N.V. Organon (Oss, The Netherlands). In Fig. 4.2.1 the structures and the related pKa values are shown. The compounds of the McCalley test were obtained from various manufacturers and are of p.a. quality (see Fig. 4.2.2). As organic modifier methanol (MeOH), supplied by J.T. Baker (Deventer, The Netherlands) was used. For the preparation of the buffers acetic acid (99-100%), supplied by J.T. Baker (Deventer, The Netherlands) and ammonium acetate from Janssen Chimica (Geel, Belgium) were used. 1 M aqueous solutions of acetic acid and ammonium acetate were prepared. Adequate amounts were mixed to obtain 25 mM buffers of pH 3 and 7.
To avoid overloading of the stationary phases by basic compounds, as recently discussed [9], for the McCalley and the Organon test compounds amounts of 0.1 µg of each test compound were injected onto the column. The flow-rate was set to 1.0 ml/min. The investigated stationary phases are shown in Table 4.2.1.

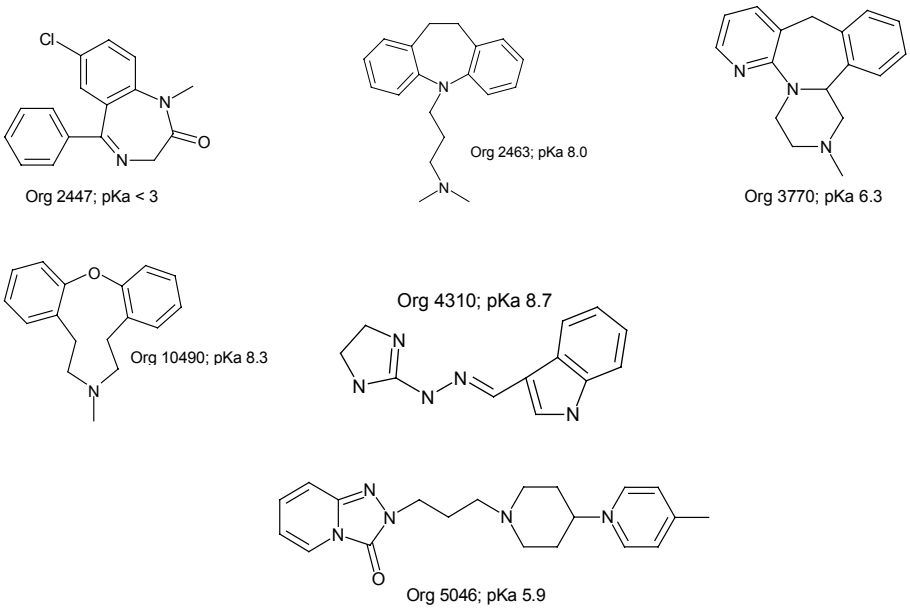

Figure 4.2.1: Structures of the Organon compounds used in this study. The pKa values were measured in MeOH+H_2O/60+40 %(V/V).

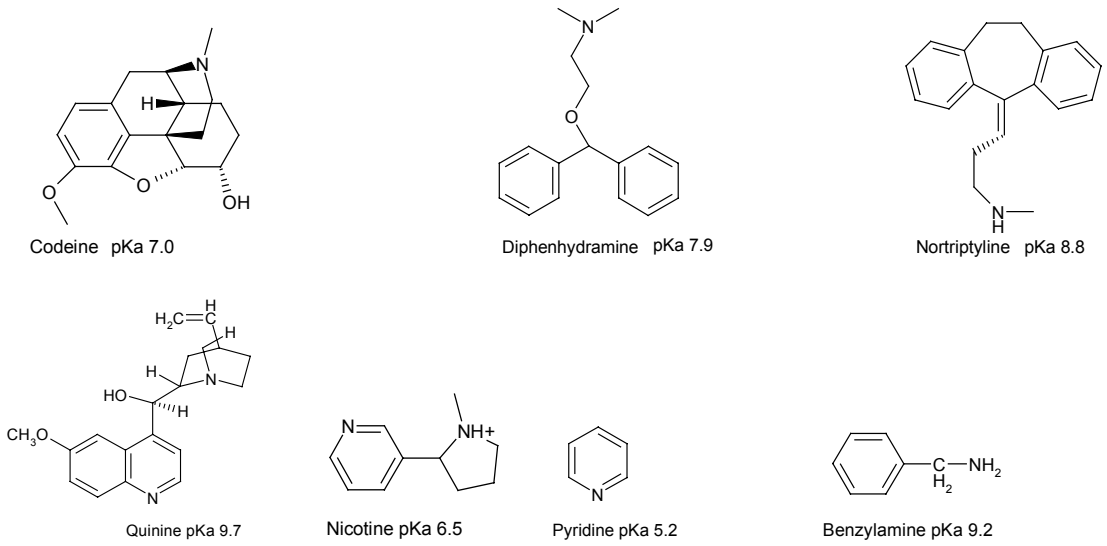

Figure 4.2.2: Structures of the McCalley test compounds used in this study. The pKa values were measured in water.

Table 4.2.1: Investigated stationary phases; dimensions are 150 x 4.6 mm I.D.

Stationary phase	Manufacturer	Abbreviation	Phase characteristics
Symmetry Shield C_{18}	Waters	SYSH	embedded polar group
Symmetry C_{18}	Waters	SYMM	high purity silica
Zorbax SB-C_{18}	Agilent Technologies	ZOSB	sterically protected
Zorbax Extend-C_{18}	Agilent Technologies	ZOBI	bidentate bonded alkyl chains
Alltima C_{18}	Alltech	ALLT	polymeric bonded phase
Luna C_{18}	Phenomenex	LUNA	high purity silica

4.2.4 RESULTS AND DISCUSSION

Enthalpy and entropy of transfer of basic analytes from the mobile to the stationary phase using the columns in Table 4.2.1 were determined to study possible differences in the retention process between the RPLC columns. From van t' Hoff plots enthalpies and entropies were calculated [2,3]. Particularly at higher temperatures limited lifetimes of columns can be expected [10]. Therefore van 't Hoff plots for only one buffer (MeOH + 25 mM NH_4Ac / 50 + 50 %(V/V) at buffer pH's 3 and 7) were determined in the temperature range 10 °C to 80 °C.

4.2.4.1 Linearity of the van't Hoff plots

The van 't Hoff plots showed no significant deviation from linearity ($r>0.99$). Therefore it was concluded that over the studied temperature range no changes in the mixed retention mechanism for basic compounds occurred with the six RPLC columns investigated. As an example the van 't Hoff plots for Org 2463 and diphenhydramine obtained with pH 3 and pH 7 buffers and the ALLT column are shown in Fig. 4.2.3. Comparable data were obtained for the other compounds and columns.

Recently McCalley reported van 't Hoff plots of basic and neutral compounds obtained on an Inertsil ODS-3V column [11]. The eluent consisted of acetonitrile+phosphate buffers pH 3 and 7. The van 't Hoff plots for the neutral and basic compounds were linear, which is in agreement with the results observed in our study. For the neutral analyte benzene ΔH^0 values of –1.93 and –2.05 kcal/mol were found. This is comparable with our data where ΔH^0 values for benzene on the six RPLC columns were in between –2.34 and –2.80 kcal/mol. Depending on the pH of the buffer and the nature of the analyte positive and negative slopes of the van 't Hoff plots were obtained by McCalley. For the six RPLC columns in our study however, only negative ΔH^0 values were obtained. We therefore assume that the nature of the buffered eluent substantially influences magnitude and sign of ΔH^0 values, and thus the mechanism of retention.

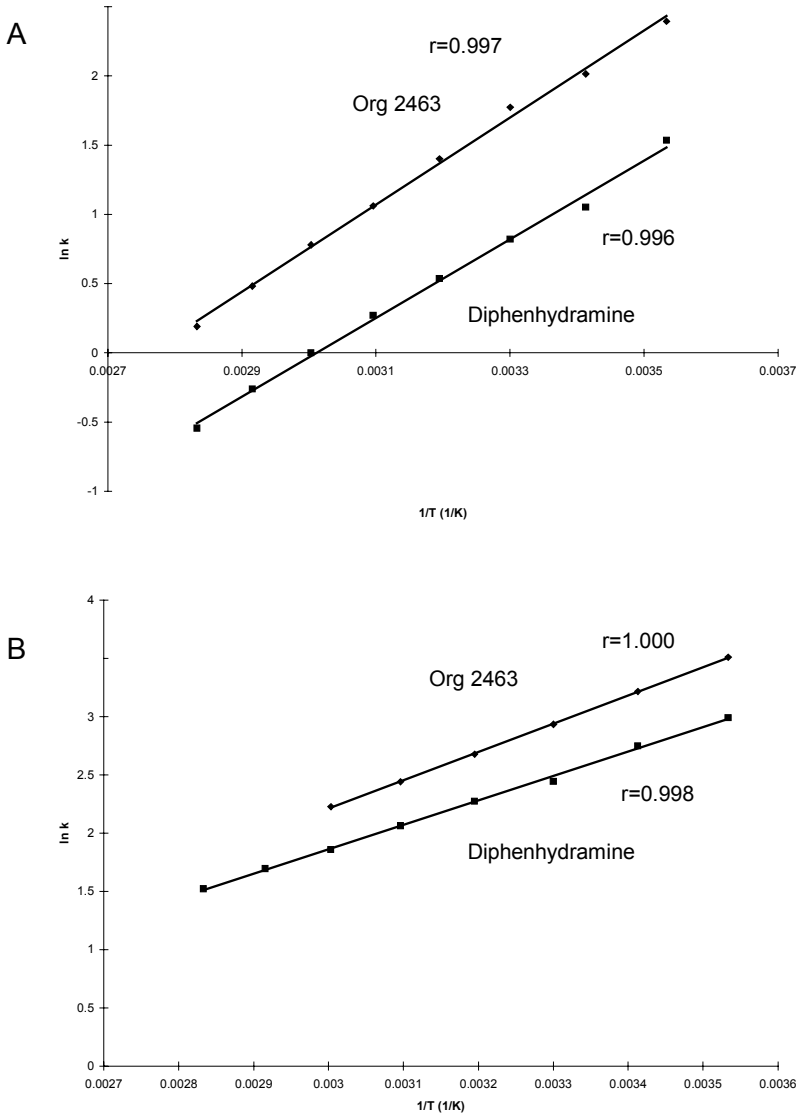

Figure 4.2.3: Typical example of van het Hoff plots for Org 2463 and diphenhydramine using MeOH+25mM NH_4Ac / 50+50 %(V/V) at buffer pH 3 (A) and 7 (B).

4.2.4.2 Enthalpy and entropy of transfer of the analyte from the mobile to the stationary phase

Table 4.2.2 shows the enthalpy data for a selected number neutral and basic test compounds. The eluent conditions were the same for the whole set of columns. Thus the values for the various compounds reflect differences between the stationary phases. In all cases ΔH^0 values were negative under the experimental conditions, demonstrating that retention of basic analytes is an exothermic process. It is energetically more favourable for the analytes to remain in the stationary phase than in the mobile phase. The value of ΔH^0 reflects the degree of interaction between

analyte and stationary phase and a more negative ΔH^0 indicates a higher degree of interaction. For the neutral compounds, i.e. Org 2447, benzene and phenol, the ΔH^0 values were comparable for both buffers pH 3 and pH 7. Typically for the latter compounds on all columns ΔH^0 values in between -10 and -15 kJ/mol were obtained. For the strong basic Org 2463 and nortryptiline, however, significant differences in ΔH^0 -values were obtained for the buffers pH 3 and pH 7 on the LUNA, SYSH and SYMM columns. For the latter columns the ΔH^0 values obtained using buffer pH 3 (protonated compounds) were significantly more negative, compared to the ΔH^0 values obtained using buffer pH 7 (compounds not or partly protonated). Moreover, the enthalpy values at low pH were significantly more negative for protonated compounds than for neutral compounds like Org 2447.

For protonated compounds polar and hydrophobic interactions with the stationary phase will occur. In comparison, for non-protonated compounds the amount of hydrophobic interactions will larger. Combining the results from the Tables 4.2.2 and 4.1.3 (chapter 4.1) obviously the differences in interaction between protonated compounds (pH=3) compared to non or partly protonated compounds (pH=7) with the stationary phase is larger for columns generally showing good peak shapes. This is particularly true for the SYSH, SYMM and LUNA columns. With the ALLT column, which with respect to peak asymmetry was the “worst” column, this effect was not observed. For this latter column comparable ΔH^0 values were obtained with the buffers pH 3 and 7. In addition the ΔH^0 values of the ALLT column are of the same order of magnitude as the data of pH 7 on the other columns. As can be seen in Table 4.1.3 (chapter 4.1) generally the best peak shapes were obtained on the LUNA, SYSH and SYMM columns. Asymmetrical peaks were obtained using the ALLT column, for both buffers pH 3 and 7, which is taken as indicative for more polar interactions between the basic analytes and this column. Therefore, one would expect lower ΔH^0 values (more negative) for the ALLT column compared to the LUNA, SYMM and SYSH columns. However, the present data suggest that peak symmetry improves at larger polar interaction between basic solutes and the stationary phase.

In Table 4.2.3 the intercepts from the van ‘t Hoff plots, representing entropy plus the column phase ratio are presented. For RPLC columns phase ratios (β) are different and difficult to obtain. Therefore, in contrast to ΔH^0 values the absolute entropy data between the various columns cannot be directly compared. ΔS^0 is a measure of ordering of the system, i.e. ordering of the stationary phase chains after bonding the analyte. For a single column R and ln β are constants. Therefore the values in Table 4.2.3 for a single column can be considered as a measure for ΔS^0 and differences between ΔS^0 values obtained at buffer pH 3 and pH 7 can be studied. Again on all columns for the neutral compounds minor differences between the ΔS^0 values obtained with buffers pH 3 and 7 were observed.

Table 4.2.2: ΔH^0 values (kJ/mol) of selected test compounds using MeOH+25 mM NH_4Ac/50+50 %(V/V) as eluent.

	SYSH		SYMM		ZOSB		ZOBI		ALLT		LUNA	
Compound	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7
Diphenhydramine	a	-10.1	a	-13.8	-23.7	-17.4	-26.2	-12.3	-21.2	-17.4	a	-9.1
Nortryptiline	-32.5	-19.2	-33.3	-22.2	-27.7	-24.2	-28.0	-23.3	-24.5	-24.5	-35.3	-21.0
Org 2447	-12.9	-13.8	-13.9	-14.3	-15.9	-15.9	-14.9	-15.6	-15.2	-15.2	-14.6	-15.7
Org 2463	a	-14.6	-35.6	-19.9	-26.6	-42.8	-27.3	-17.7	-25.0	-23.7	-41.9	-14.4
Org 10490	a	-8.3	a	-14.1	-20.9	-17.2	a	-10.1	-20.5	-19.1	a	-7.1
benzene	-10.1	-9.8	-9.8	-11.7	-10.1	-9.8	-11.1	-11.6	-9.8	-10.1	-10.4	-10.4
phenol	-11.1	-10.8	-10.3	-10.4	-10.4	-11.7	-11.3	-11.8	-11.7	-10.4	-10.6	-11.1

a: insufficient data to perform linear regression

Table 4.2.3: $\frac{\Delta S^0}{R} + \ln \beta$ values of selected test compounds using MeOH+25 mM NH_4Ac/50+50%(V/V).

	SYSH		SYMM		ZOSB		ZOBI		ALLT		LUNA	
Compound	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3	pH 7
Diphenhydramine	a	-2.46	a	-3.33	-4.06	-4.42	-10.68	-2.94	-6.91	-4.17	a	-1.66
Nortryptiline	-14.11	-5.17	-13.60	-6.01	-8.83	-6.34	-9.95	-6.51	-7.05	-6.35	-16.76	-5.59
Org 2447	-2.63	-2.99	-2.67	-2.79	-3.48	-3.51	-3.22	-3.40	-2.75	-2.95	-2.78	-3.21
Org 2463	a	-3.33	-15.09	-4.72	-8.67	-5.59	-10.11	-4.13	-7.59	-5.80	-16.98	-2.80
Org 10490	a	-2.21	a	-3.88	-7.98	-4.68	a	-2.62	-7.16	-5.05	a	-1.44
benzene	-2.29	-2.23	-1.95	-1.98	-2.37	-2.28	-2.52	-2.72	-1.89	-1.95	-2.18	-2.23
phenol	-3.76	-3.71	-3.70	-3.81	-4.06	-3.91	-4.30	-4.48	-3.47	-3.57	-3.77	-3.98

a: insufficient data to perform linear regression

However, ΔS^0 values for the more polar compound phenol is slightly more negative in all cases, compared to the other neutral compounds.
For the basic compounds, comparison of the differences in ΔS^0 for each individual column at both pH's shows a certain similarity with the earlier discussed ΔH^0 values. Again columns producing better symmetrical peaks show a larger difference between their ΔS^0 values at pH 7 and 3, compared to columns producing severe tailing peaks. For example $\Delta\Delta S^0$ ($\Delta S^0_{pH3} - \Delta S^0_{pH7}$) values for nortryptiline follow the order: ALLT>ZOBI>ZOSB>SYMM>SYSH>LUNA. This is in fair agreement with column ordering according to the asymmetry values obtained at pH 7 for nortryptiline: ALLT>ZOBI>SYMM>ZOSB>SYSH>LUNA.
To investigate whether the discussed differences were due to polar interactions, the silanol blocking compound N,N-dimethyl octylamine (DMOA) was added to the pH 3 eluent. 0.1%v/v DMOA [12] was added to the buffer solution of the eluent, which resulted in symmetrical peaks with both the LUNA and ALLT columns. Enthalpy and entropy values were determined for Org 2463, nortryptiline and Org 2447. Table 4.2.4 shows ΔH^0 and ΔS^0 values on the columns, with and without the addition of DMOA to the eluent. The addition of DMOA can be considered as a dynamically coating of RPLC columns. It is assumed that addition of this latter reagent result in a dynamically modified more densely bonded RPLC phase. This will result in a further ordering of the system. For the neutral compound (Org 2447) ΔS^0 -values for both columns decrease (more negative) slightly but significantly. This is taken as proof for better ordering of the system upon DMOA addition.

Table 4.2.4: ΔH^0 and ΔS^0 values obtained using the LUNA and ALLT columns, with and without the addition of DMOA to the MeOH+25 mM NH_4Ac pH 3/ 50+50 %(V/V) eluent.

	ΔH^0 (kJ/mol)			
	LUNA		ALLT	
Compound	without DMOA	with DMOA	without DMOA	with DMOA
Org 2447	-14.6	-16.1	-15.2	-15.9
Org 2463	-41.9	-18.2	-25.0	-14.9
Nortryptiline	-35.3	-19.2	-24.5	-16.2
	$\frac{\Delta S^0}{R} + \ln \beta$			
Org 2447	-2.78	-3.40	-2.75	-3.21
Org 2463	-16.98	-6.55	-7.59	-5.03
Nortryptiline	-16.76	-6.57	-7.05	-5.18

As for the neutral compounds, it was expected that addition of DMOA for protonated basic compounds would also result in a better ordering of the system. However, addition of DMOA to the eluent resulted for both the LUNA and the ALLT columns in a reduction (less negative) of both ΔH^0 and ΔS^0 for protonated basic compounds. This is interpreted that for protonated basic compounds energetically it is less favourable to remain in the stationary phase, after blocking residual silanols with DMOA resulting in less negative ΔH^0 -values. Without DMOA protonated basic compounds can interact with ionic sites, polar sites and alkyl chains of the stationary phase. After blocking residual silanols with DMOA, mainly interaction with alkyl chains will take place. As a consequence of reducing ionic and other polar interactions, analytes may increase their degrees of freedom. In turn, this will increase the disorder of the system resulting in less negative ΔS^0 -values of Org 2463 and nortryptiline.
Summarising all the data above, it is recalled that on the ALLT column asymmetrical peaks were obtained without DMOA. From the peak asymmetry data we expected that, compared to the LUNA column, the polar activity with the ALLT column would be higher. From ΔH^0 -values it is obvious that for protonated basic compounds the total amount of ionic and other polar interactions with the LUNA, SYSH and SYMM columns are larger compared to the ZOSB, ZOBI and ALLT columns. However, the interactions responsible for asymmetrical peaks do not necessarily originate from the whole silanol population. We assume that in the case of the ZOSB, ZOBI and ALLT columns a small part of "bad silanols" (< 1% of the total population [13]) may be responsible for the observed worser peak asymmetry for these latter three columns.
From this study it is clear that determination of enthalpies and entropies of transfer from the mobile to the stationary phase for basic analytes reveals information about the types of interaction between protonated and neutral compounds with RPLC stationary phases. A clear view however on how these thermodynamic data may explain the observed substantial differences in peak symmetry cannot be given yet.

4.2.5 CONCLUSIONS

The obtained van het Hoff plots were linear for the investigated set of RPLC columns ($r>0.99$). Therefore it was concluded that over the studied temperature range no changes in the (mixed) retention mechanism for basic compounds occurred. The negative ΔH^0 values obtained for the anaytes showed that retention of basic compounds is an exothermic proces under the actual eluent conditions. For strong basic compounds (nortryptiline and Org 2463) significant differences in ΔH^0 and ΔS^0 values were obtained for the buffers pH 3 and pH 7 on the LUNA, SYSH and SYMM columns. For the same compounds this effect was less observed on the ZOSB, ZOBI and ALLT columns. Using the latter columns generally asymmetrical peaks are obtained for these basic compounds. From the ΔH^0 data it is concluded that the total amount of ionic and other polar interactions with the LUNA, SYSH and SYMM

columns are larger compared to the ZOSB, ZOBI and ALLT columns. It is proposed that in the case of the ZOSB, ZOBI and ALLT columns a small amount of "bad silanols" may be responsible for the observed higher peak asymmetry for the columns.

Addition of N,N-dimethyl octyl amine (DMOA) to the eluent resulted for both the LUNA and the ALLT columns in a reduction (less negative) of both ΔH^0 and ΔS^0 for protonated basic compounds. For the neutral compound the differences in ΔS^0 upon addition of DMOA are relatively small but significant. This latter effect can be interpreted that addition of DMOA results in a dynamical modification of the column. In turn this contributes to a better ordening of the system. Furthermore, for protonated compounds it is energetically less favourable to remain in the stationary phase after blocking with DMOA. As a consequence, by reducing ionic and other polar interactions in the system the degree of freedom for the protonated basic analytes nortryptiline and Org 2463 increases. This is reflected by an increase (less negative) of the corresponding ΔS^0 -values.

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4.3 CHARACTERISATION OF REVERSED PHASE STATIONARY PHASES FOR THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC PHARMACEUTICALS BY QUANTITATIVE STRUCTURE-RETENTION RELATIONSHIPS

4.3.1 SUMMARY

The applicability of quantitative structure-retention relationships (QSRRs) for column characterisation was investigated. The study was performed to characterise stationary phases for the analysis of basic pharmaceutical compounds using basic compounds as test analytes. QSRRs were derived for logarithms of retention (log k) and the intercept of the linear Soczewinski relationship between isocratic log k and the corresponding volume of organic modifier in the eluent (log k_w). The retention data were regressed against calculated molecular descriptors. QSRRs were determined for a set of 6 and a set of 7 silica based reversed phase liquid chromatography (RPLC) columns. The number of test analytes was 29 for the first and 13 for the second set of columns.

The obtained QSRRs revealed effects of calculated molecular descriptors involved in the retention of basic analytes with RPLC columns. The descriptors molar refraction, lowest unoccupied molecular orbital, dynamically calculated polar surface and the number of proton donors were most significant. Furthermore, different trends for regression coefficients of dynamically calculated polar surface and molar refraction were observed using ammonium acetate and sodium phosphate as buffer in the eluent. The latter showed the influence of the nature of the buffer on retention of basic analytes. The QSRRs did not allow the quantitative discrimination between the RPLC stationary phases. The magnitude of the confidence intervals of the regression coefficients was too large. Small confidence intervals will allow the quantitative comparison of stationary phases, which can be of great value for column characterisation.

R.J.M. Vervoort, E. Ruyter, A.J.J. Debets, H.A. Claessens, C.A. Cramers, G.J. de Jong and R. Kaliszan, J. Chromatogr. A, submitted.

4.3.2 INTRODUCTION

In reversed phase liquid chromatography (RPLC) the choice of a suitable column is an important step in method development. Especially in the RPLC analysis of basic pharmaceuticals, large differences with respect to peak shape are observed when using columns from various manufacturers. Therefore, the choice of a suitable column for the analysis of basic compounds is important. Characterisation of RPLC stationary phases enables the selection of columns for a specific application. In the literature various column characterisation procedures are described. They can be divided into *i*: empirical evaluation methods, *ii*: thermodynamically based methods and, *iii*: evaluation methods based on a retention model [1]. The applicability of empirical methods has been investigated recently [2]. In this study data are reported about the applicability of a method based on a retention model to characterise stationary phases i.e. quantitative structure-retention relationships (QSRRs).

In the last two decades QSRRs have been applied to *i*: predict retention for solutes, *ii*: identify the most informative structural descriptors, *iii*: gain insight into the molecular mechanisms of separations, *iv*: evaluate complex physicochemical properties of analytes, and *v*: predict relative biological activities within a set of drugs [3]. Numerous reports have appeared describing the study of retention properties of RPLC materials. Three types of QSRR can be distinguished. One of them is regressing logarithms of retention (log k) or the intercept of the linear Soczewinski relationship between isocratic log k and the corresponding volume of organic modifier in the eluent (log k_w) against logarithms of 1-octanol - water partition coefficients (log P). This approach mainly reveals information about hydrophobic properties of the stationary phases studied [4-6]. The second type of QSRR is based on the so-called linear solvation energy relationships (LSER). LSER is based on regressing log k or log k_w against experimentally determined molecular descriptors [7-11]. For many compounds these descriptors are available in the literature [12]. A third type of QSRR is regressing log k or log k_w against calculated molecular descriptors [5,13,14]. Kaliszan et al recently reported the applicability of these three approaches. It was concluded that meaningful column characterisation was obtained regressing log k_w against log P, against experimentally determined descriptors, and against calculated molecular descriptors [5].

Many QSRR studies are performed using generally available compounds. It was shown recently that three series of in water non-ionised test analytes can be used to compare effects on chromatographic separation of the stationary and mobile phase. Each series was designed specifically for a given QSRR [15,16]. This study is different from other studies for the test compounds used. We wanted to characterise the stationary phases for the analysis of basic compounds. Therefore, basic compounds were used as test compounds. Most of these compounds have been synthesized to determine the pharmacological activity. Other compounds have been reported to be useful to characterise RPLC stationary phases for the analysis of

basic compounds [17]. Data are presented about the characterisation of RPLC stationary phases using QSRR. Calculated molecular descriptors of these compounds were regressed against log k and log k_w values.

4.3.3 EXPERIMENTAL

4.3.3.1 Columns studied

The retention data were determined for the stationary phases shown in Table 4.3.1.

Table 4.3.1: Columns used in this study.

Column number	Column	Abbr. used*	Manufacturer	Dimensions length x ID (mm)
1	μBondapak C_{18}	BON	Waters	300 x 3.9
2	NovaPak C_{18}	NOV	Waters	300 x 3.9
3	Zorbax Rx-C_{18}	ZOR	Agilent	250 x 4.6
4	Exsil 100 5um ODS-B	EXS	Exmere	250 x 4.6
5	Suplex pKb-100	SUP	Supelco	250 x 4.6
6	Kromasil KR100-5-C_{18}	KRO	AKZO Nobel	250 x 4.6
7	Symmetry C_{18}	SYMM	Waters	150 x 4.6
8	Symmetry Shield C_{18}	SYSH	Waters	150 x 4.6
9	Zorbax SB-C_{18}	ZOSB	Agilent	150 x 4.6
10	Zorbax Extend C_{18}	ZOBI	Agilent	150 x 4.6
11	Alltima C_{18}	ALLT	Alltech	150 x 4.6
12	Luna C_{18}	LUNA	Phenomenex	150 x 4.6
13	Discovery C_{18}	DISC	Supelco	150 x 4.6

*: Abbreviation used in this study.

4.3.3.2 Test analytes and experimental conditions

In Fig. 4.3.1 the test analytes are shown. Test analytes 1 - 29 were obtained from NV Organon, (Oss, Netherlands). Test analytes 30 - 36 were obtained from various manufacturers and are of p.a. quality. Methanol (MeOH) was purchased from J.T. Baker (Deventer, Netherlands). For preparation of the phosphate buffers we used disodium hydrogen phosphate (J.T. Baker). The phosphate buffers were adjusted to pH using ortho phosphoric acid (J.T. Baker). Ammonium acetate was obtained from

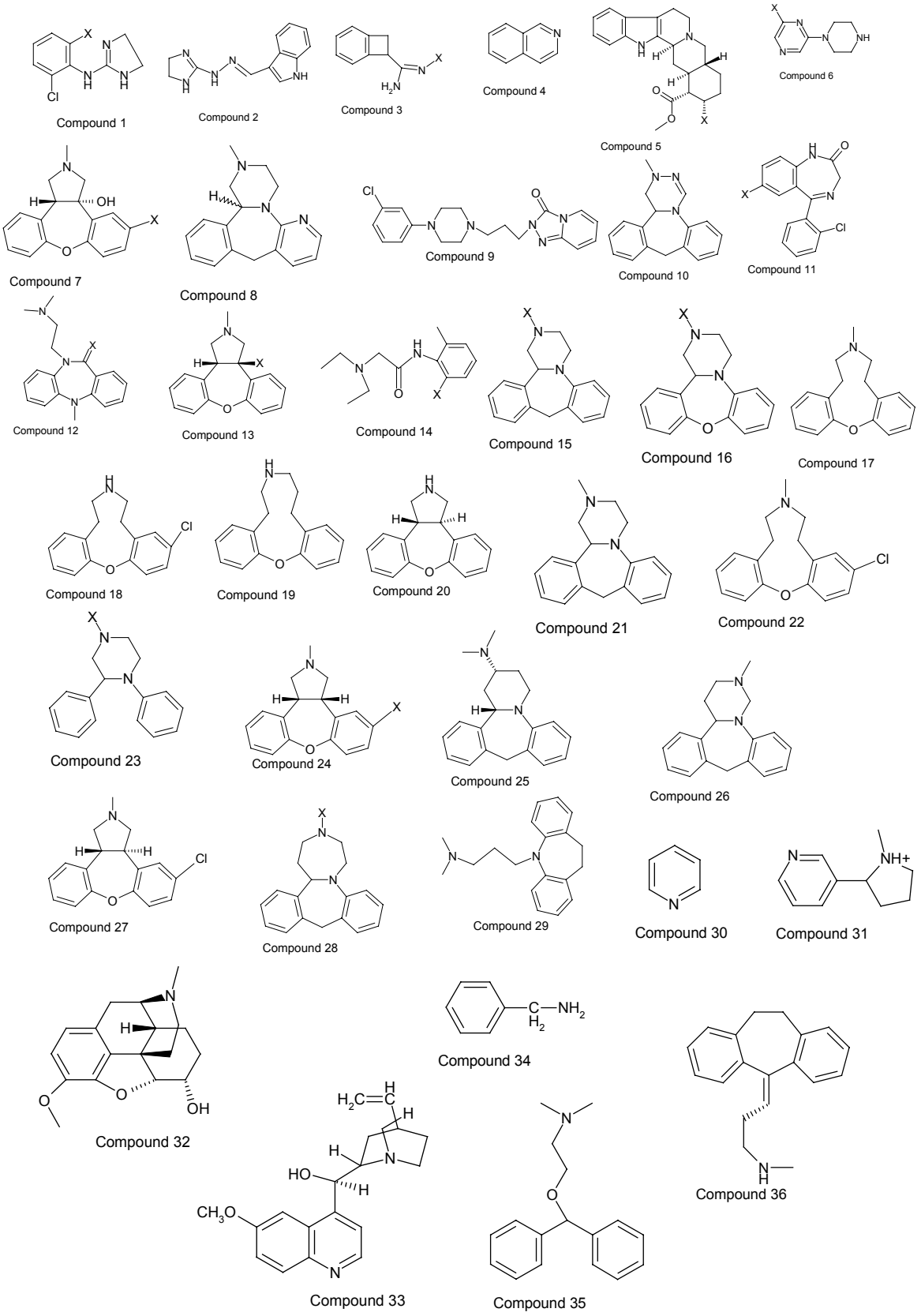

Figure 4.3.1: Test analytes used in this QSRR study. X represents an organic group.

Jansen Chimica (Geel, Belgium). The flow-rate was set to 1.0 ml/min. and 0.1 μg of analyte was injected onto the column. The column temperature was set to 40 °C. To elute the compounds within a reasonable time period different ratios of MeOH+buffer were used. In Table 4.3.2 the MeOH+buffer ratios are shown. The retention data were obtained using a HP1090 or a HP1100 liquid chromatograph equipped with Chemstations from Agilent Technologies (Amstelveen, Netherlands).

Table 4.3.2: MeOH+buffer ratios used to elute the test analytes.

Compound*	Column**	MeOH+buffer/%(V/V)	Descriptor for eluent***
1 - 6	1 - 7	30+70	1
7 - 17	1 - 7	50+50	2
18 - 29	1 - 7	70+30	3
30 - 36	8 - 13	65+35	1
2,8-10,17,29	8 - 13	50+50	2

*: see Table 4.3.3.

**:see Table 4.3.1.

***: used in QSRR.

4.3.3.3 Calculations

The test analytes were subjected to molecular modeling using Cerius^2 software, version 4.5, Molecular Simulations Inc., San Diego (CA, USA).

In effect, a number of structural descriptors were generated (Table 4.3.3). The following descriptors were calculated: ClogP, calculated log P; MW, molecular weigth; Rotlbond, number of rotatable bonds in the molecule; H-donor, number of proton donors; H-acceptor, number of proton acceptors; PolSurf, polar surface; DPS, polar surface dynamically calculated; DAPS, apolar surface dynamically calculated; volume, volume of the molecule; dipole, dipole moment of the molecule; Mref, molar refraction; Vm, molar volume; Apol, atomar polarisability; HOMOM, highest occupied molecular orbital; and LUMOM, lowest unoccupied molecular orbital. Stepwise multiple linear regression was performed using StatGraphics Plus 2.1 software, Manugistics Inc., Rockville (MA, USA).

4.3.3.4 Determination of QSRR using multiple linear regression analysis

Multiple linear regression can be used when the observation y depends on a set of variables [18]. This dependency can be described by:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots \quad \{1\}$$

Table 4.3.3: Calculated molecular descriptors of the test compounds in Fig. 4.3.1.

Comp. number	Compound	ClogP	MW	Rotbond	H-donor	H-acceptor	PolSurf ($Å^2$)	DPS ($Å^2$)	DAPS ($Å^2$)	Volume ($Å^3$)	Dipole (Debije)	Mref (cm^3/mol)	Vm ($Å^3$)	Apol	HOMOM (eV)	LUMOM (eV)
1	2407	1.41	230.10	1	2	3	35.37	41.52	202.38	269.51	6.23	57.3	179.7	9648	-8.40	0.09
2	4310	2.93	227.27	2	3	5	8.29	66.32	200.39	300.47	7.83	66.3	203.4	9284	-6.82	0.18
3	2508	2.30	162.19	1	3	3	60.39	60.11	139.40	219.81	6.25	46.3	149.9	6528	-8.68	0.17
4	7949	1.82	129.16	0	0	1	14.19	11.80	157.35	176.48	4.37	40.3	122.8	5920	-8.86	-0.73
5	5605	2.17	354.45	2	2	5	46.77	51.71	346.55	447.45	6.49	98.4	326.4	13292	-8.08	0.33
6	10192	0.38	198.65	1	1	4	37.77	37.33	187.62	234.99	11.83	53.1	167.5	7582	-9.44	-0.63
7	10499	4.44	301.77	0	1	3	22.77	25.67	293.04	390.70	9.18	82.8	258.6	12468	-9.29	-0.13
8	3770	3.33	265.36	0	0	3	12.63	17.01	295.22	340.73	6.20	82.6	249.7	10926	-8.98	0.17
9	5046	3.17	371.87	5	0	6	35.73	44.77	373.95	472.70	7.50	105.9	326.1	14861	-8.39	-0.35
10	3840	5.42	263.34	0	0	3	15.15	21.95	284.37	322.50	1.40	82.2	246.8	11192	-8.44	0.17
11	10629	3.22	349.61	1	1	3	3.00	42.25	269.95	374.99	3.30	82.5	245.7	11797	-9.46	-0.74
12	4843	3.31	295.38	3	0	4	17.91	21.72	327.81	373.21	4.96	89.5	280.3	12214	-8.56	-0.10
13	10724	3.59	267.33	0	1	3	25.17	26.13	272.95	335.17	4.43	78.0	244.7	10929	-9.06	0.19
14	2443	1.95	234.34	6	1	3	19.89	25.86	287.05	319.44	4.88	72.1	243.5	9306	-9.08	0.34
15	OH46	3.69	250.34	0	1	2	17.07	14.98	276.47	389.64	1.15	79.2	239.8	10627	-8.57	0.38
16	GC46	4.41	266.34	0	0	3	12.93	13.29	294.01	384.48	1.78	81.0	248.7	11017	-8.69	0.17
17	10490	4.69	253.34	0	0	2	9.57	7.04	294.16	356.81	1.41	78.9	246.8	10725	-9.13	0.19
18	30004	5.07	273.76	0	1	2	20.67	15.01	283.15	323.74	8.18	78.4	243.8	11751	-9.15	-0.18
19	30039	4.78	253.34	9	1	2	18.33	13.61	282.79	363.33	1.58	78.2	247.1	10725	-9.12	0.09
20	5649	3.42	237.30	0	1	2	23.19	19.98	249.17	342.87	1.06	71.5	219.6	10071	-9.21	0.17
21	GB94	4.28	264.37	0	0	2	5.19	6.55	309.41	365.64	2.74	84.5	253.9	11186	-8.68	0.37
22	30003	5.54	287.79	0	0	2	9.09	6.76	315.24	372.13	8.29	83.7	260.8	12264	-9.17	-0.12
23	GA43	4.24	252.36	2	0	2	5.19	6.26	313.31	357.92	2.04	80.8	251.6	10717	-9.09	0.50
24	5033	4.74	285.77	0	0	2	12.33	11.06	300.73	339.29	7.97	81.6	250.6	12124	-9.43	-0.15
25	2566	4.00	292.42	1	0	2	6.69	6.40	345.71	384.04	6.12	93.9	288.8	12212	-8.53	0.34
26	2305	4.34	264.37	0	0	2	6.03	6.63	308.12	348.46	2.24	84.2	255.6	11186	-8.02	0.36
27	5222	4.74	285.77	0	0	2	10.41	11.02	304.71	421.50	8.49	81.6	250.4	12123	-9.15	-0.07
28	3367	4.37	278.40	0	0	2	4.05	5.26	324.54	377.82	3.11	89.4	272.3	11698	-8.48	0.36
29	2463	5.04	280.41	4	0	2	5.19	5.090	347.97	411.50	1.31	90.6	283.3	11840	-8.33	0.49
30	Pyridine	0.64	79.10	0	0	1	14.13	11.90	108.07	127.88	5.20	23.9	78.4	3475	-10.08	0.16
31	Nicotine	0.90	162.23	1	0	2	15.75	15.12	207.37	218.67	2.62	49.6	164.3	6382	-9.16	0.22
32	Codeine	0.98	299.37	1	1	4	33.93	35.33	279.98	366.07	4.94	84.6	271.7	11306	-8.75	0.20
33	Quinine	2.79	324.42	4	1	4	8.19	38.50	330.65	428.35	10.13	95.9	307.0	12818	-8.84	-0.84
34	Benzylam.	1.09	107.15	1	2	1	29.31	21.81	131.65	165.66	1.94	34.5	111.3	4729	-9.40	0.51
35	Diphenhydr.	3.54	255.36	6	0	2	4.53	10.24	327.06	378.10	3.73	79.9	258.8	10769	-8.96	0.36
36	Nortryptiline	4.32	263.38	3	1	1	15.45	11.85	320.37	340.36	3.26	87.0	265.6	11721	-9.01	0.10

To determine the estimates ($b_0,\ldots,b_m$) of the unknown parameters ($\beta_0,\ldots,\beta_m$) n observations ar made of the variable y ($y_0,\ldots,y_m$) for n different combinations of the m controlled variables. For QSRR y is log k or log k_w, $x_1,\ldots,x_m$ are the calculated molecular descriptors and $b_0,\ldots,b_m$ are the estimates of the regression coefficients $\beta_0,\ldots,\beta_m$. In vector notation the observations can be written as:

$$y = X\beta + e \quad \{2\}$$

were y is the measurement vector, e is the residual vector, β the vector for the regression coefficients and X the matrix of the descriptors. The estimation of the regression coefficient set β is performed in such a way that the vector e is minimal. The least squares estimates for β (b) can be determined by:

$$b = \left(X^{'}X\right)^{-1}X^{'}y \quad \{3\}$$

and the variance-covariance matrix of the regression coefficients can be written as:

$$V(b) = \left(X^{'}X\right)^{-1}s_e^2 \quad \{4\}$$

In QSRR the 95% confidence interval of the regression coefficient b, the standard error of estimate (s_e), the correlation coefficient (R) and the F-value are used to describe the quality of the equation. The 95% confidence interval of the regression coefficients is given by:

$$b_i \pm t_{0.05,n-m}s_e\sqrt{v(b_i)} \quad \{5\}$$

s_e can be obtained from $s_e^2 = \dfrac{SS_e}{n-m}$ in which $SS_e = \sum_{i=1}^{n}\left(\hat{y}_i - y_i\right)^2$ {6}

R is the correlation between measured log k and log k calculated using the QSRR. The calculated F-value shows whether the predictor variables (descriptors) explain a significant amount of the variance in the y variables (log k or log k_w). F is the ratio of the variation that can be ascribed to the regression line, and the variation that cannot be ascribed to the regression line.

4.3.4 RESULTS AND DISCUSSION

In QSRR studies it is important that the requirements for a meaningful multiple regression analysis are kept in mind. This means *i*: low correlation between the used

structural descriptors, *ii*: 5-6 data points of regressand (log k) should fall per regressor (structural descriptor) and *iii*: the standard error of estimate (s_e) of log k should be less than 0.25 [15].

Table 4.3.4: log k values measured for columns 1 – 6 (Table 4.3.1) using MeOH+10mM disodium hydrogen phosphate pH 7.4 as eluent.

Compound	eluent	BON	NOV	SUP	EXS	KRO	ZOR
2407	1	0.30	0.26	0.00	0.30	0.32	0.40
4310	1	0.56	0.15	0.38	0.88	0.08	0.94
2508	1	0.08	0.00	-0.22	-0.10	0.15	0.08
7949	1	na*	na	0.18	na	0.54	na
5605	1	0.73	0.63	0.72	0.46	1.00	0.59
10192	1	0.54	0.28	0.23	0.86	na	na
10499	2	0.48	0.45	0.74	0.28	0.76	0.49
3770	2	0.45	0.53	0.60	0.34	0.81	0.60
5046	2	0.38	0.48	0.66	0.15	0.81	0.38
2447	2	0.28	0.36	0.54	-0.05	0.70	0.30
3840	2	0.30	0.40	0.49	0.04	0.70	na
10629	2	0.18	0.23	0.54	-0.15	0.59	0.20
4843	2	0.34	0.28	0.40	0.26	0.56	0.46
10724	2	0.32	0.36	0.56	0.15	0.69	0.34
2443	2	0.32	0.46	0.54	-0.04	na	0.34
OH46	2	0.61	0.64	0.89	0.63	0.93	0.69
GC46	2	0.49	0.56	0.74	0.39	0.87	0.47
10490	3	0.58	0.56	0.70	0.47	0.69	0.65
30004	3	0.70	0.60	0.74	0.55	0.67	0.67
30039	3	0.89	0.64	0.65	0.56	0.71	0.85
5649	3	0.57	0.27	na	0.70	na	0.67
GB94	3	0.40	0.47	0.69	0.20	0.63	0.34
30003	3	0.60	0.76	na	0.30	0.80	0.54
2331	3	0.39	0.47	0.69	0.20	0.63	0.34
GA43	3	0.23	0.25	0.38	-0.04	0.43	0.20
5033	3	0.46	0.50	na	0.23	na	0.41
2566	3	0.69	0.66	na	0.57	0.75	0.76
2305	3	0.27	0.36	0.53	0.08	0.50	0.25
4870	3	0.49	0.46	0.70	0.25	0.60	0.49
5222	3	0.51	0.59	0.88	0.32	0.72	0.46
3367	3	0.65	0.59	0.90	0.60	0.70	0.63
2463	3	0.66	0.69	0.91	0.36	0.80	0.62

*: not available.

First, correlation coefficients between the calculated molecular descriptors in Table 4.3.3 were determined for compounds 1 - 29. The descriptors Volume, MW, MRef, Vm and Apol were found to be highly correlated (correlation coefficient > 0.9). These descriptors represent apolar properties of the test analytes. Due to the high correlation only one of these descriptors could be used. Mref was chosen to be used in QSRR analysis. Between the other descriptors representing polar and other properties, low correlations were found.
To elute compounds 1 – 29 within a reasonable time period three different methanol+buffer ratios were necessary (see Table 4.3.2). Therefore, in the QSRR the eluent composition was introduced. Keeping the requirements for a meaningful multiple regression analysis in mind, four other regression coefficients could be determined. Using the log k values in Table 4.3.4 the following QSRR was obtained:

$$\log k = k_0 + k_1 Mref + k_2 DPS + k_3 LUMOM + k_4 eluent + k_5 Hdonor \quad \{7\}$$

In Table 4.3.5 the statistical results are summarised.

Table 4.3.5: Regression coefficients of the fitted model of regression equation {7}. In the Table the regression coefficients, 95% confidence interval, F- value, correlation coefficient (R) and standard error of estimate (s_e) are shown.

column	k_1	k_2	k_3	k_4	k_5	F	R	s_e
BON	0.012	-0.018	-0.230	-0.073	0.322	3.61	0.67	0.15
	(0.004,0.020)	(-0.030,-0.006)	(-0.490,0.031)	(-0.255,0.110)	(0.134,0.511)			
NOV	0.013	-0.018	-0.169	-0.131	0.200	9.97	0.83	0.11
	(0.007,0.018)	(-0.027,-0.009)	(-0.362,0.024)	(-0.266,0.004)	(0.061,0.339)			
SUP	0.019	-0.018	-0.232	-0.071	0.236	13.47	0.88	0.15
	(0.012,0.027)	(-0.030,-0.007)	((-0.481,0.017)	(-0.246,0.104)	(0.050,0.421)			
KRO	0.018	-0.020	-0.243	-0.274	0.178	13.17	0.88	0.12
	(0.003,0.012)	(-0.029,-0.011)	(-0.442,-0.044)	(-0.411,-0.136)	(0.032,0.324)			
EXS	0.007	-0.019	-0.246	-0.142	0.320	0.83	0.40	0.28
	(-0.007,0.020)	(-0.041,0.003)	(-0.718,0.226)	(-0.473,0.189)	(-0.021,0.662)			
ZOR	0.013	-0.012	-0.037	0.024	0.330	1.99	0.58	0.19
	(0.002,0.023)	(-0.028,0.004)	(-0.396,0.321)	(-0.223,0.271)	(0.077,0.584)			

It was found that with 95% confidence the regression coefficients for descriptors Mref, DPS, LUMOM and Hdonor were most significant for columns 1 - 6. From the R and F values in Table 4.3.5 it can be concluded that the predictive power is limited.

However, the obtained QSRR equations are informative about factors that affect retention of basic pharmaceuticals using RPLC packing materials. In this respect, the molecular descriptors Mref, DPS, LUMOM and Hdonor are of interest. Furthermore, the equations in Table 4.3.5 make good physical sense. As can be seen k_1 with the Mref term is positive. This agrees with the fact that the more apolar compounds are more retained. Moreover, the net effects of interactions of a compound with a large DPS value with a non-polar stationary phase, and with the polar components of the eluent, are obviously negative. Hence, a negative value for k_2 is obtained. In addition, the larger the number of proton donor sites of a compound the more the compound is retained.

Table 4.3.6: log k values measured for columns 7 –13 (Table 4.3.1).

MeOH+25 mM sodium phosphate pH 7 used as eluent

Compound	eluent	SYMM	ZOSB	ZOBI	ALLT	LUNA	SYSH	DISC
Pyridine	1	-0.26	-0.17	-0.39	-0.02	-0.26	-0.30	-0.65
Nicotine	1	0.14	0.04	0.00	0.32	0.05	-0.02	-0.25
Codeine	1	0.06	0.19	-0.05	0.35	0.13	-0.03	-0.21
Quinine	1	0.65	0.78	0.59	1.06	0.76	0.52	0.40
Benzyl.	1	-0.01	0.13	-0.06	0.16	-0.29	-0.13	-0.61
Diphen.	1	1.00	1.14	0.94	1.30	0.96	0.76	0.64
Nortript.	1	1.04	1.28	0.97	1.42	0.85	0.80	0.57
2463	2	1.79	1.84	1.77	1.90	1.77	1.49	1.54
3770	2	1.33	1.60	1.14	1.64	1.40	1.16	1.11
3840	2	1.18	1.29	1.14	1.38	1.26	0.93	0.95
4310	2	-0.22	0.25	-0.22	0.34	-0.15	-0.30	-0.39
5046	2	1.35	1.40	1.36	1.55	1.46	1.18	1.14
10490	2	1.26	1.47	1.27	1.60	1.19	1.08	1.00

MeOH+25 mM ammonium acetate pH 7 used as eluent

Compound	eluent	SYMM	ZOSB	ZOBI	ALLT	LUNA	SYSH	DISC
Pyridine	1	-0.56	-0.12	-0.37	-0.13	-0.27	-0.48	-0.67
Nicotine	1	0.06	0.03	-0.14	0.22	-0.06	-0.10	-0.38
Codeine	1	-0.38	0.05	-0.28	0.04	-0.09	-0.33	-0.49
Quinine	1	0.08	0.44	0.16	0.54	0.32	0.11	-0.09
Benzyl.	1	-0.39	-0.18	-0.63	-0.27	-0.56	-0.25	-0.85
Diphen.	1	0.45	0.64	0.33	0.70	0.39	0.34	0.05
Nortrip.	1	0.60	0.82	0.46	0.81	0.54	0.57	0.21
2463	2	1.21	1.52	1.19	1.56	1.25	0.93	0.86
3770	2	1.18	1.44	1.12	1.48	1.22	0.88	0.89
3840	2	1.18	1.26	1.13	1.37	1.25	0.86	0.89
4310	2	-0.22	0.07	-0.30	0.11	-0.09	-0.52	-0.52
5046	2	1.31	1.34	1.00	1.50	1.42	1.02	1.03
10490	2	0.59	0.94	0.56	0.96	0.57	0.47	0.25

The regression coefficient of the number of proton donor sites of the analyte is informative about the proton acceptor sites of the stationary phase. Therefore, this parameter is valuable for the characterisation of RPLC stationary phases for the analysis of basic pharmaceuticals.
Due to the broad confidence intervals, comparison of the regression coefficients did not allow a reliable classification of stationary phases. These data are in agreement with various other QSRR studies. Comparing RPLC stationary phases, calculated regression coefficients in QSRRs explaining isocratic retention often at the 95% confidence level statistically are not significant [5,19]. Statistical significant differences between regression coefficients have only been reported comparing stationary phases with different nature, e.g a C_{18} and a N-acylaminopropyl silica column [20]. To improve the QSRR, calculations were performed using normalised descriptors and 90% confidence intervals. However, both approaches did not result in differences between the calculated regression coefficients for the RPLC columns.

Table 4.3.7: Regression coefficients of the fitted model using equations {8}. Methanol+25 mM sodium phosphate pH 7 was used as eluent. In the Table the regression coefficients, 95% confidence interval, F-value, correlation coefficient (R) and standard error of estimate (s_e) are shown.

column	k_1	k_2	k_3	F	R	s_e
SYMM	-0.023	0.508	0.020	52.77	0.97	0.19
	(-0.030,-0.016)	(0.250,0.767)	(0.014,0.025)			
SYSH	-0.020	0.480	0.018	52.51	0.97	0.17
	(-0.027,-0.014)	(0.245,0.716)	(0.013,0.023)			
ALLT	-0.019	0.479	0.020	50.84	0.97	0.18
	(-0.026,-0.012)	(0.225,0.733)	(0.015,0.026)			
LUNA	-0.020	0.559	0.021	60.29	0.98	0.18
	(-0.027, -0.014)	(0.306,0.812)	(0.016,0.027)			
DISC	-0.021	0.629	0.021	90.49	0.98	0.15
	(-0.027,-0.015)	(0.414,0.843)	(0.017,0.026)			
ZOSB	-0.020	0.582	0.019	40.78	0.96	0.14
	(-0.028,-0.012)	(0.292,0.873)	(0.013,0.025)			
ZOBI	-0.022	0.545	0.020	43.75	0.97	0.21
	(-0.030,-0.015)	(0.255,0.834)	(0.014,0.026)			

Statistically significant differences between the regression coefficients calculated for RPLC columns allow a quantitative comparison. This can be achieved by diminishing the magnitude of the confidence intervals. The 95% confidence interval of a regression parameter is given by equation {5}. For 29 compounds and 5 descriptors

from tabulated t-values it can be concluded that, for instance, a 10-fold decrease of the size of the confidence interval requires an enormous number of test compounds, provided that the diversity within the set of test compounds is not changed. Increasing the diversity within the set of test compounds will most probably also diminish the magnitude of the confidence interval of the regression coefficients. However, the exact number and diversity of test analytes necessary to detect significant differences between the regression coefficients of the RPLC column cannot be determined from this study.

The retention data of compounds 2, 8, 9, 10, 17, 29 and 30 – 36 were determined with columns 7 – 13 (Table 4.3.2). The retention data are shown in Table 4.3.6. Methanol with both 25 mM sodium phosphate and 25 mM ammonium acetate buffers pH 7 were used as eluents. Different ratios of methanol and buffer were necessary to obtain the retention data of the 13 compounds within a reasonable period of time.

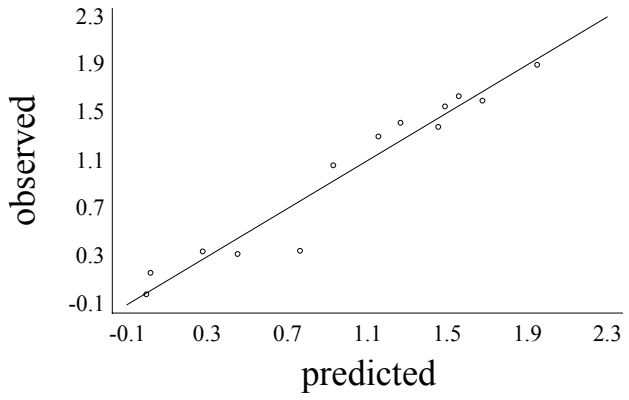

Figure 4.3.2: Relation between predicted and observed log k data obtained for the ALLT column using methanol+25 mM sodium phosphate pH 7 eluent (R=0.97).

Keeping the requirements for a meaningful multiple regression analysis in mind, now, next to the eluent descriptor only two structural descriptors could be used. It was found that DPS and Mref were most significant. The obtained QSRR was:

$$\log k = k_0 + k_1 DPS + k_2 eluent + k_3 Mref \quad \{8\}$$

As shown in Tables 4.3.7 and 4.3.8 equations with good predictive power are obtained. As an example, in Fig. 4.3.2 the relation between observed log k and predicted log k for the ALLT column is shown.

In comparison, using descriptors DPS, Mref and eluent with log k data obtained with compounds 1 – 29 for the BON column, a QSRR equation with small predictive

power was obtained i.e. R = 0.37, F-value = 1.24. Therefore we concluded that the diversity within the latter set of test compounds is larger, compared to compound 1 – 29. As shown this condition is mandatory for the proper use of QSRR.
The obtained confidence intervals for the regression coefficients did not allow the distinguishment of significant differences between the RPLC columns. Trends were found for the ammonium acetate and sodium phosphate buffers used. Using ammonium acetate as eluent buffer, the values for k_1 and k_3 were lower, compared to using sodium phosphate. As shown by LoBrutto et al retention of bases depends on the nature of the buffer ions and its concentration in the eluent [21,22]. Changes in retention using different buffers were attributed to the formation of ion pairs and/or the change of analyte solvation.

Table 4.3.8: Regression coefficients of the fitted model using equation {8}. Methanol+25 mM ammonium acetate pH 7 was used as eluent. In the Table the regression coefficients, 95% confidence interval, F-value, correlation coefficient (R) and standard error of estimate (s_e) are shown.

column	k_1	k_2	k_3	F	R	s_e
SYMM	-0.016	0.697	0.016	16.58	0.92	0.31
	(-0.028,-0.005)	(0.269,1.125)	(0.006,0.025)			
SYSH	-0.016	0.450	0.015	15.42	0.91	0.26
	(-0.026,-0.007)	(0.082,0.817)	(0.007,0.022)			
ALLT	-0.016	0.676	0.016	29.74	0.95	0.23
	(-0.024,-0.007)	(0.356,0.996	(0.009,0.023)			
LUNA	-0.013	0.664	0.016	18.50	0.93	0.28
	(-0.024,-0.002)	(0.267,1.062)	(0.008,0.025)			
DISC	-0.014	0.664	0.016	18.69	0.93	0.28
	(-0.025,-0.004)	(0.269,1.059)	(0.008,0.025)			
ZOSB	-0.016	0.665	0.015	35.86	0.96	0.20
	(-0.024,-0.009)	(0.382,0.945)	(0.009,0.021)			
ZOBI	-0.017	0.663	0.015	25.12	0.94	0.24
	(-0.026,-0.008)	(0.327,1.002)	(0.008,0.022)			

In the literature the use of both log k [10,11] and log k_w [5] data in QSRR studies is reported. Log k_w cannot be considered as a retention parameter that would ever emerge if elution with pure water or buffer was experimentally possible. The log k_w basically is an abstract quantity. It is the intercept of the extrapolated linear Soczewinski relationship [23] between isocratic log k values and the corresponding volume percentage of modifier in the eluent. Apart from the column log k_w depends also on the nature of organic modifier of the binary aqueous eluents used in RPLC.

Table 4.3.9: Log k_w data for the μBondapak C_{18} column using methanol+10 mM sodium phosphate pH 7.4 as eluent.

compound	log k_w	regression coefficient of the linear Soczewinski relationship
2447	2.54	0.998
4310	2.06	0.994
5605	2.27	0.993
10192	1.73	0.992
2508	1.12	0.991
2407	1.52	0.997
GB94	2.22	0.995
30004	3.00	0.991
5649	2.17	0.983
3367	2.76	0.991
2463	3.18	0.994
2566	3.04	0.993
4870	2.76	0.988
5033	2.39	0.988
30039	2.83	0.987
30003	2.94	0.989
5046	2.52	1.000
3840	2.36	0.996
10724	2.43	0.999
10629	2.34	0.995
4843	2.57	1.000
7949	1.56	0.982
GC46	2.20	0.989
OH46	2.38	0.964
2331	2.70	0.985
2443	2.33	0.997
3770	2.47	0.995
10499	2.22	0.993
2305	2.67	0.994
GA43	2.21	0.994
5222	2.33	0.998

The influence of log k and log k_w data on the quality of QSRR equations was determined. In Table 4.3.9 the log k_w data obtained for test compounds 1 – 29 with the μBondapak C_{18} column are shown. Equation {9} shows the obtained QSRR:

$$\log k_w = k_0 + k_1 Mref + k_2 DPS + k_3 LUMOM + k_4 Hdonor \quad \{9\}$$

In Table 4.3.10 the regression coefficients with statistical data are summarised.

Table 4.3.10: Regression coefficients of the fitted model using equation {9}. In the Table the regression coefficients, 95% confidence interval, F-value, correlation coefficient (R) and standard error of estimate (s_e) are shown. The QSRR was obtained for μBondapak C_{18} using log k_w data.

k_1	k_2	k_3	k_4	F	R	s_e
0.029	-0.025	-0.354	0.340	21.51	0.89	0.23
(0.020,0.038)	(-0.037,-0.013)	(-0.722,0.014)	(0.080,0.601)			

From the R and F-values it can be concluded that the predicting power of the QSRR improved using log k_w instead of log k. This is probably due to the fact that when using log k_w the descriptor for eluent can be omitted from the QSRR. The values obtained for R and F were 0.89 and 21.51. In comparison, using log k the values for R and F were 0.67 and 3.61 (see Table 4.3.5). Comparing the confidence intervals for the obtained regression coefficients showed that these were of comparable magnitude when using log k_w and log k.

4.3.5 CONCLUSIONS

The applicability of QSRR to characterise RPLC stationary phase was investigated. This study differed from many QSRR studies because basic compounds were used as test analytes. The log k and log k_w data were regressed against calculated molecular descriptors.

The QSRRs made good physical sense. The molecular descriptors molar refraction, dynamically calculated polar surface, lowest unoccupied molecular orbital and number of proton donors were of interest in retention of basic pharmaceuticals. The regression coefficients with their confidence intervals, however, did not allow a statistical significant classification of the RPLC columns studied. Small confidence intervals of the regression coefficients will allow the quantitative comparison of stationary phases. It can be concluded that the number of test analytes as well as the diversity between the test analytes must be large. However, the necessary number and diversity necessary cannot be determined from this study. Moreover, using log k_w instead of log k data improved the predicting power of the QSRR.

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5. EFFECT OF MINIATURISATION ON THE LC ANALYSIS OF BASIC PHARMACEUTICALS

5.1 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

5.1.1 SUMMARY

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 infuence 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 LC columns with internal diameters (I.D.) of 0.3 mm, 1.0 mm and 4.6 mm (conventional).

Preliminary results obtained with the investigated micro and conventional columns showed similar behaviour with respect to ruggedness. The 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 this chapter, ammonium citrate as the buffering salt, a high buffer concentration and methanol as the modifier showed best peak symmetry.

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5.1.2 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 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 non-volatile mobile phase additives, such as phosphate 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. Many of the pharmaceutically active compounds, e.g. central nerve system drugs and cardiovascular drugs, contain basic nitrogen groups. Therefore the LC analysis using silica based stationary phases can be problematic due to ionic and other polar interactions between residual silanols and eventual other polar sites on the silica substrate, and the basic drugs. 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].

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 chapter. 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, concentration and pH of the electrolyte solution. The data are statistically evaluated using multifactor analysis of variance (MANOVA).

5.1.3 EXPERIMENTAL

5.1.3.1 HPLC 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, USA). 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 Co. Inc., Houston, TX, USA) and a 785A Programmable Absorbance detector (Applied Biosystems) using on-capillary detection. Chromatograms were obtained using Turbochrom 4 software (Perkin Elmer, Norwalk, CO, USA). Data processing was performed using a HPLC 3^D Chemstation.
In Table 5.1.1 the chromatographic conditions, e.g. flow rate and injection volume used in the micro column (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.

Table 5.1.1: Instrumental settings used in micro column and conventional LC.

Column diameter (mm I.D.)	Flow-rate (ml/min)	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

MS detection was performed by connecting the outlet of the LC to the Perkin Elmer Sciex API-100 LC/MS Mass spectrometer 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 V and 225 V, respectively. The mass spectrometer was scanned in positive ion-mode from 230 amu to 395 amu in 0.035 amu/s using a step size of 0.1 amu.

5.1.3.2 Chemicals

The basic pharmaceuticals were obtained from N.V. Organon (Oss, The Netherlands). In Fig. 5.1.1 the molecular structures of the test compounds are shown.

Methanol (MeOH, Lichrosolv) and acetonitrile (ACN, Uvasol) were obtained from Merck (Darmstadt, Germany). Sodium phosphate ($Na_2HPO_4.12H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), acetic acid (p.a. 99-100%), ortho-phosphoric acid (85%) and citric acid monohydrate were purchased from Baker (Deventer, The Netherlands) and ammonium acetate p.a. (NH_4Ac) from Jansen Chimica (Geel, Belgium). Ammonium citrate was purchased from Aldrich (Milwaukee, USA).

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 5.1.2.

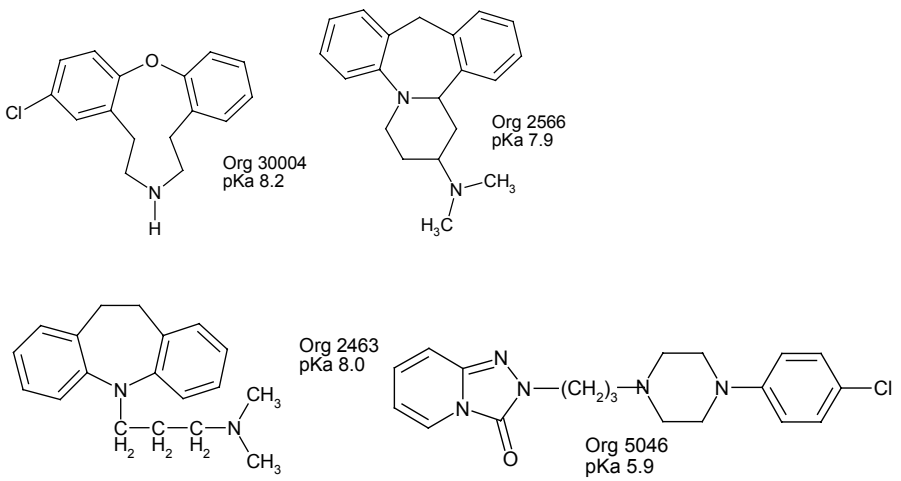

Figure 5.1.1: Chemical structures of the basic pharmaceuticals used in this study.

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.

Table 5.1.2: Overview studied LC columns.

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

5.1.3.3 Ruggedness testing of LC 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 changes of stationary phase properties during the experiments 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 methanol+ water / 55+45 %(V/V).

5.1.3.4 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:

$$As = \frac{w_{0.05}}{2w_{a,0.05}} \quad \{1\}$$

where $w_{0.05}$ is the width of the peak at 5% of the peak height, and $w_{a,0.05}$ 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]:

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

where t_R is the retention time and w_h the peak width at half height of the peak.

The retention factor (k) was calculated using :

$$k = \frac{(t_R - t_0)}{t_0} \quad \{3\}$$

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]:

$$r = \prod_{i=1}^{n-1}\left(\frac{Rs}{\overline{Rs}}\right) \qquad \text{with } \overline{Rs} = \left(\frac{1}{n-1}\right)\sum_{i=1}^{n-1} Rs_{i,i+1} \quad \{4\}$$

were Rs is the resolution between subsequent peaks and $\overline{Rs}$ 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.

5.1.3.5 Data Evaluation

To statistically evaluate the analytical data multifactor analysis of variance (MANOVA) was performed using Statgraphics plus version 2.1 software, Manugistics

Inc. (Rockville, MA, USA), installed on a personal computer with a Pentium/75 MHz Intell processor.

5.1.4 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 4 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 (see Table 5.1.3): 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 detoriation 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.

5.1.4.1 Ruggedness of LC columns during the study

As can been extracted from Table 5.1.3, in total 24 different mobile phase conditions were used with 9 different LC columns. With each mobile phase condition the sample solution, comprising a mixture of the 4 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].

Table 5.1.3: Studied variables and conditions.

Variable	Condition		
modifier	methanol	acetonitrile	
electrolyte	ammonium citrate	ammonium acetate	sodium phosphate
electrolyte concentration	10 mM	50 mM	
pH	3	7	
stationary phase	Zorbax SB-C_{18}	Symmetry C_{18}	Prodigy 5 ODS-2
column inner diameter	0.3 mm	1.0 mm	4.6 mm

Regarding the stationary phase properties, in Fig. 5.1.2 the results for the silanol activity (relative retention of aniline and phenol, and toluidines selectivity), metal activity and hydrophobicity are presented as bar charts. As can be seen, with 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. 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. 5.1.3 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. 5.1.2), these test results indicate that probably the structure of the packed beds of the columns changed, resulting in void volume formation and decreased efficiency.

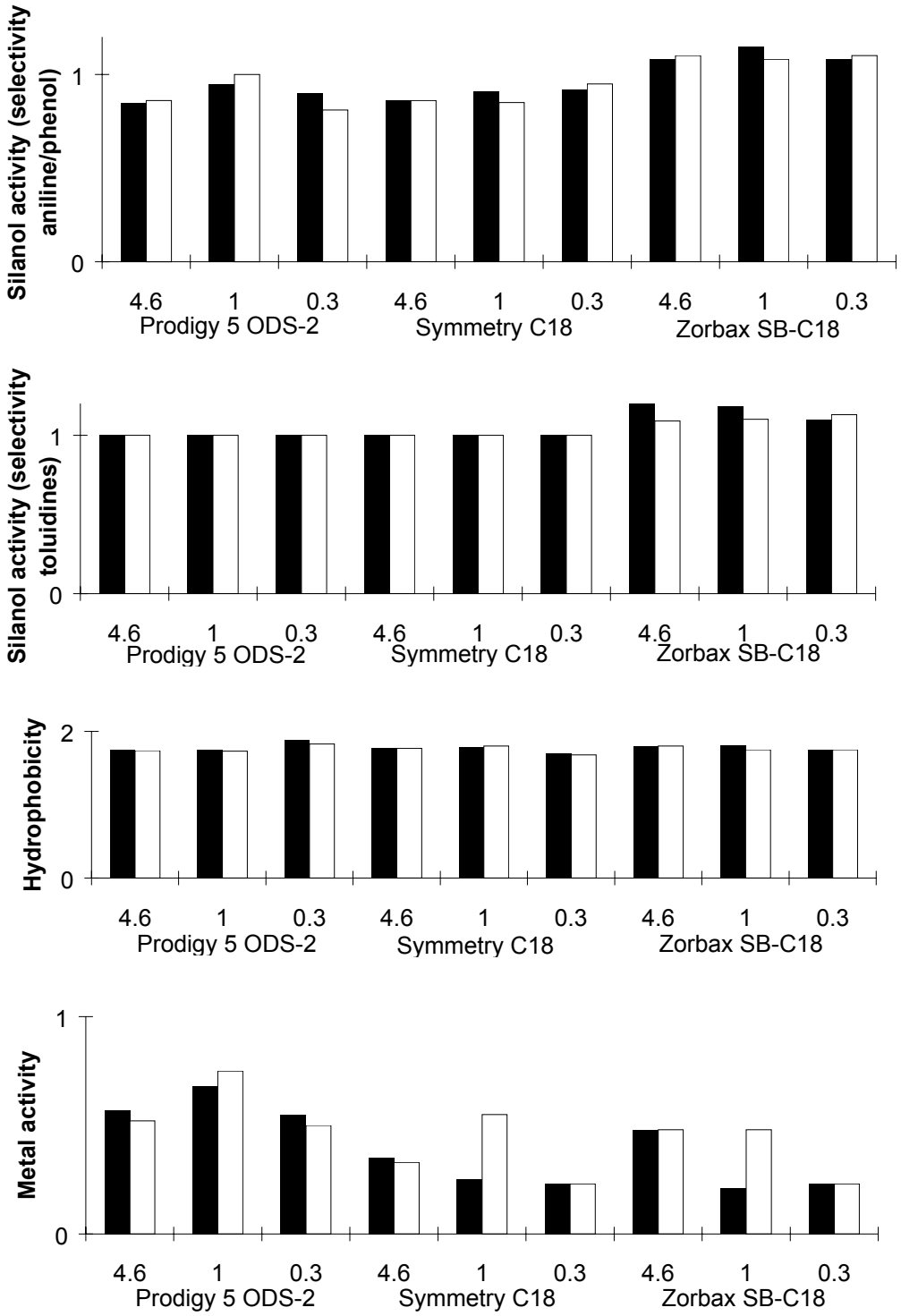

Figure 5.1.2: Silanol activity, by selectivity aniline/phenol and selectivity toluidines, Hydrophobicity and Metal activity before (■) and after the experiments (□).

Since the various column 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.
Summarising, 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.

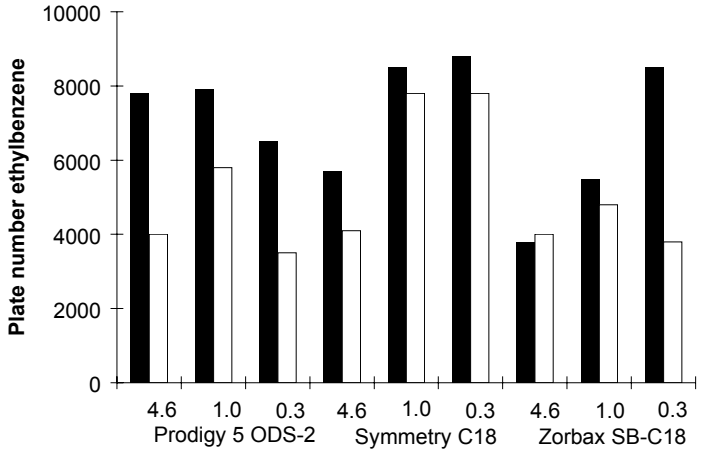

Figure 5.1.3: Plate numbers ethylbenzene before (■) and after (□) experiments.

5.1.4.2 LC 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 4 selected basic pharmaceuticals.
In Table 5.1.4 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 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.

Table 5.1.4: Correlation matrix of normalised resolution (r), peak asymmetry (As) and retention (k)

r	0.3 mm I.D.	1.0 mm I.D.	4.6 mm I.D.
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 mm I.D.	4.6 mm I.D.
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 mm I.D.	4.6 mm I.D.
0.3 mm I.D.	1	0.84	0.87
1.0 mm I.D.		1	0.91
4.6 mm I.D.			1

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). With 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].

5.1.4.2.1 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 5.1.5 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. 5.1.4 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. 5.1.4 it can

Table 5.1.5: 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

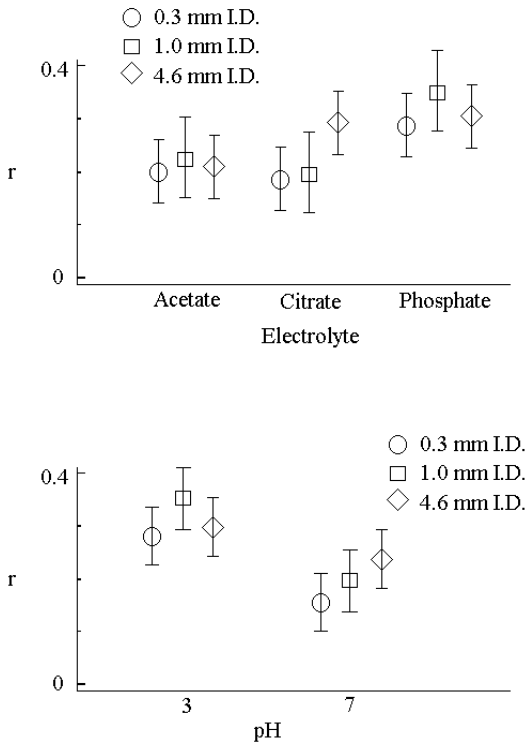

Figure 5.1.4: Main effects normalised resolution product (r); mean and 95% confidence intervals.

be concluded that for these four basic compounds highest r-values were obtained using buffers at pH 3.

In Fig. 5.1.5 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 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. 5.1.5, 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.

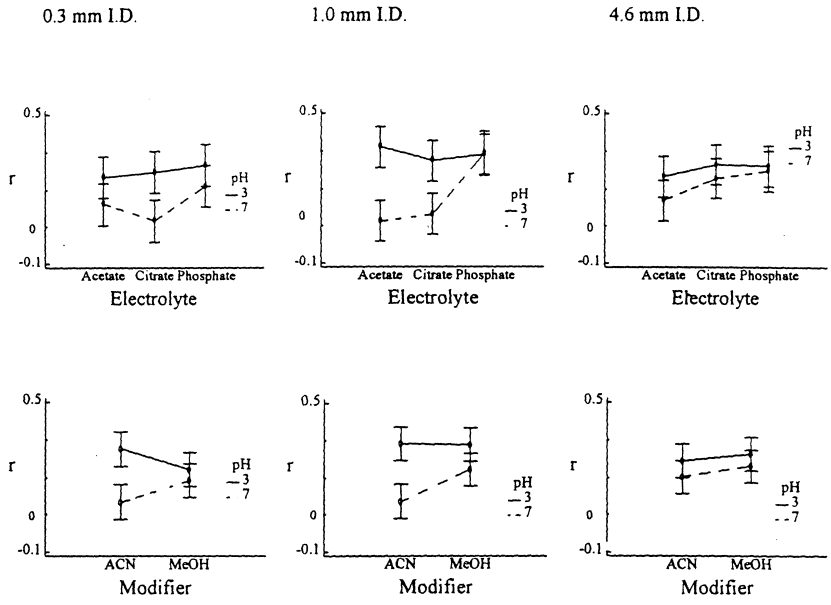

Figure 5.1.5: Interaction effects normalised resolution product (r); mean and 95% confidence interval.

In Fig. 5.1.5 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 mm I.D. and 1.0 mm I.D. LC columns improved for electrolyte solutions at pH 3. Using methanol in the mobile phases pH 3 and pH 7, the resolution showed less

improvement when going from pH 7 to pH 3. At low pH the basic compounds are ionised and apparently the type of electrolyte does not influence the selectivity.
In summary, the mobile phase pH and type of electrolyte used to separate the four basic analytes 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.

5.1.4.2.2 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 5.1.6 the main effects and interactions of the investigated variables on peak shape for the conventional, 1.0 mm I.D. 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 and eventual other polar sites 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 revealing a significant effect of the type compound on peak symmetry when using the 0.3 mm I.D. columns.
In Figs. 5.1.6 and 5.1.7 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 5.1.6: chromatographic peaks with the lowest As values were obtained using ammonium citrate as electrolyte solution. Since asymmetrical peaks are due to interactions with polar sites, probably the ammonium citrate electrolyte suppresses the interaction between basic compounds and these sites.
The influence of the electrolyte concentration on the peak shapes of the basic compounds 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 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, only for the 1.0 mm I.D. columns a significant effect was observed.

Table 5.1.6: 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***

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 interferences, 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 mm I.D. and 1.0 mm I.D. LC columns. 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. 5.1.6, 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.

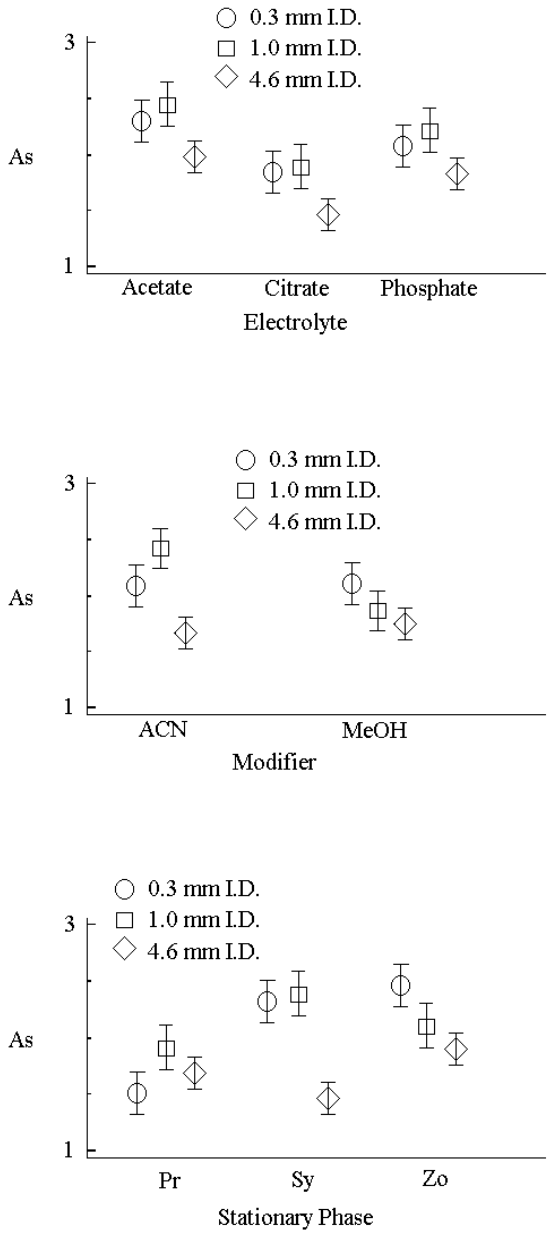

Figure 5.1.6: Main effects peak asymmetry (As); mean and 95% confidence interval.

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.

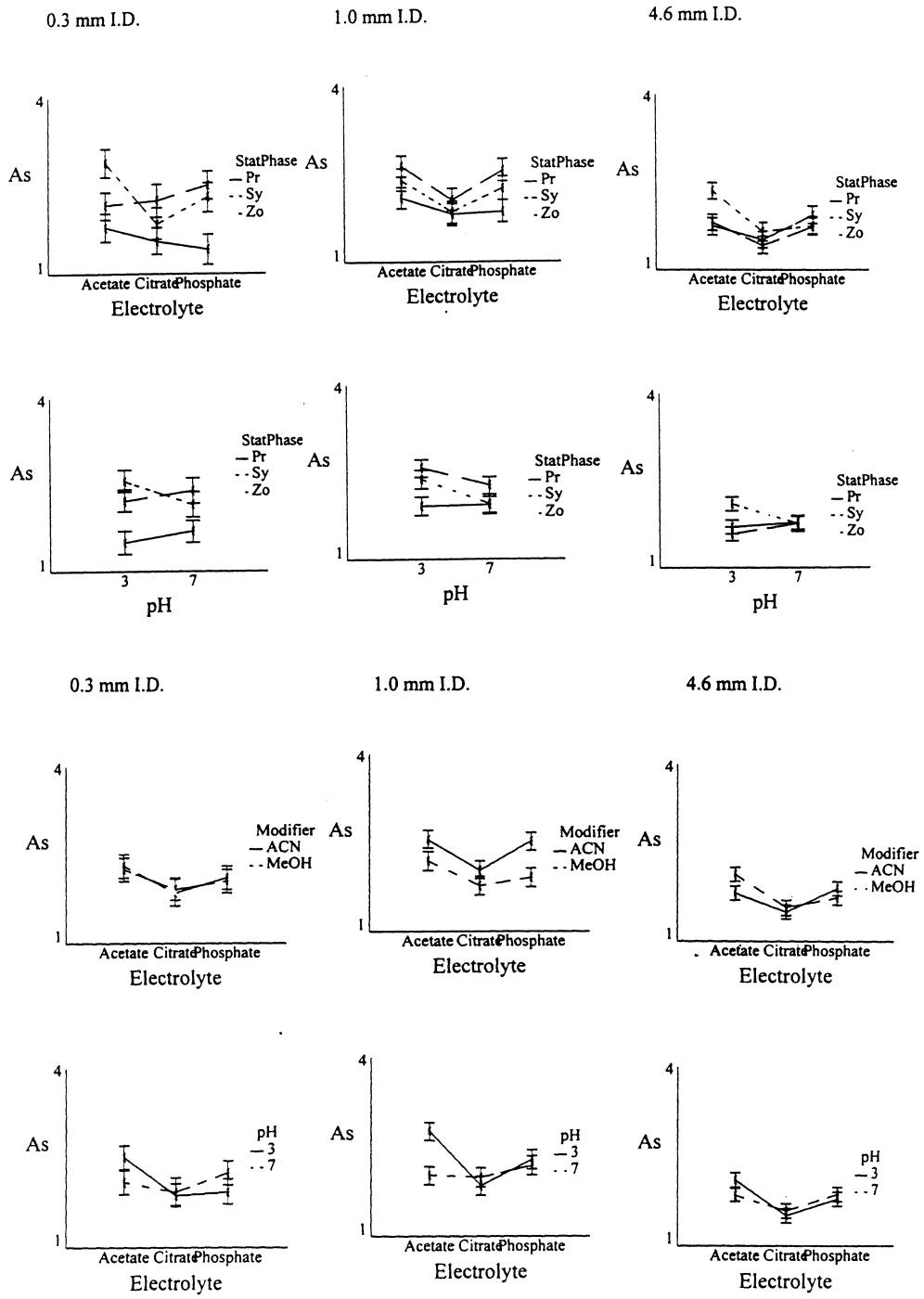

Figure 5.1.7: Interaction effects peak asymmetry (As); mean and 95% confidence interval.

As shown in the interaction plots (Fig. 5.1.7) other than the type of electrolyte, peak symmetry is also 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. Furthermore, using the 0.3 mm I.D. 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. 5.1.6). However, as shown in Fig. 5.1.7 the observed peak shapes were also affected by buffer pH: the peak shapes when using the 0.3 mm I.D., 1.0 mm I.D. 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.
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 especially the use of ammonium citrate as buffer, 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.

5.1.4.2.3 Comparison of retention factors

Since the retention factors of the four basic compounds using MC and conventional LC columns were similar (Table 5.1.4) only main effects will be discussed. In Table 5.1.7 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 shows also a significant effect. As already shown in Table 5.1.4 the correlation between the LC columns for retention was high. In Fig. 5.1.8 the effects of type of electrolyte and electrolyte concentration 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 mm I.D. and 1.0 mm I.D. LC columns the retention decreased with increasing electrolyte concentration. Increasing the electrolyte concentration showed a positive effect on peak shape: due to less polar interactions the shape of the peak improved when going from 10 to 50 mM buffers. Since retention is caused through both

hydrophobic and polar interaction, it is obvious that increasing the buffer concentration results in decreased retention.

Table 5.1.7: 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***

Lowest retention was observed using ammonium acetate whereas compounds were most retained using sodium phosphate buffers. Furthermore, the compounds were most retained using the Zorbax columns, whereas the compounds were least retained using the Prodigy column (data not shown). 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.

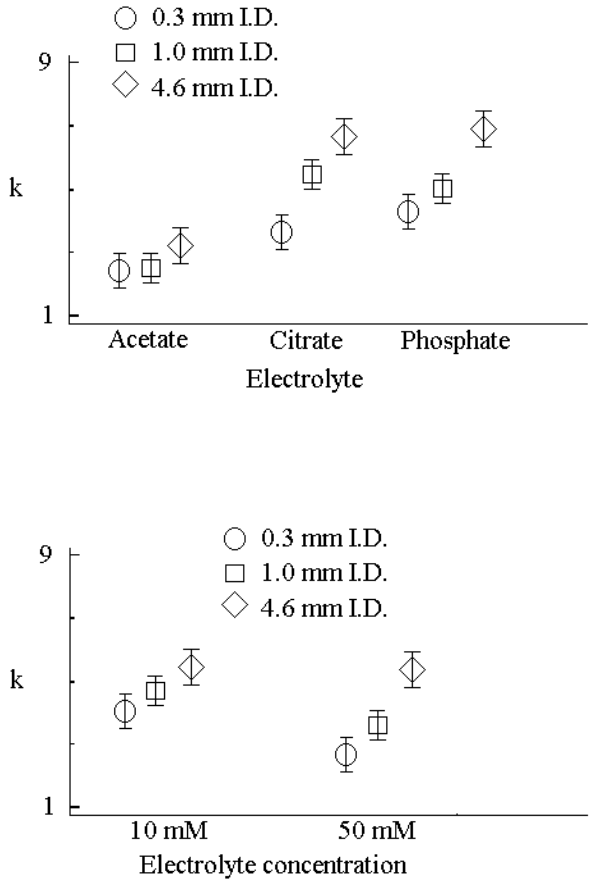

Figure 5.1.8: Main effects retention (k); mean and 95% confidence interval.

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 was observed for the studies variables.
As examples, in Fig. 5.1.9 the LC-UV chromatograms obtained for the four test substances using the Prodigy 5 ODS-2 MC and conventional LC columns is shown.

5.1.4.3 LC coupled on-line with MS

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. 5.1.10.

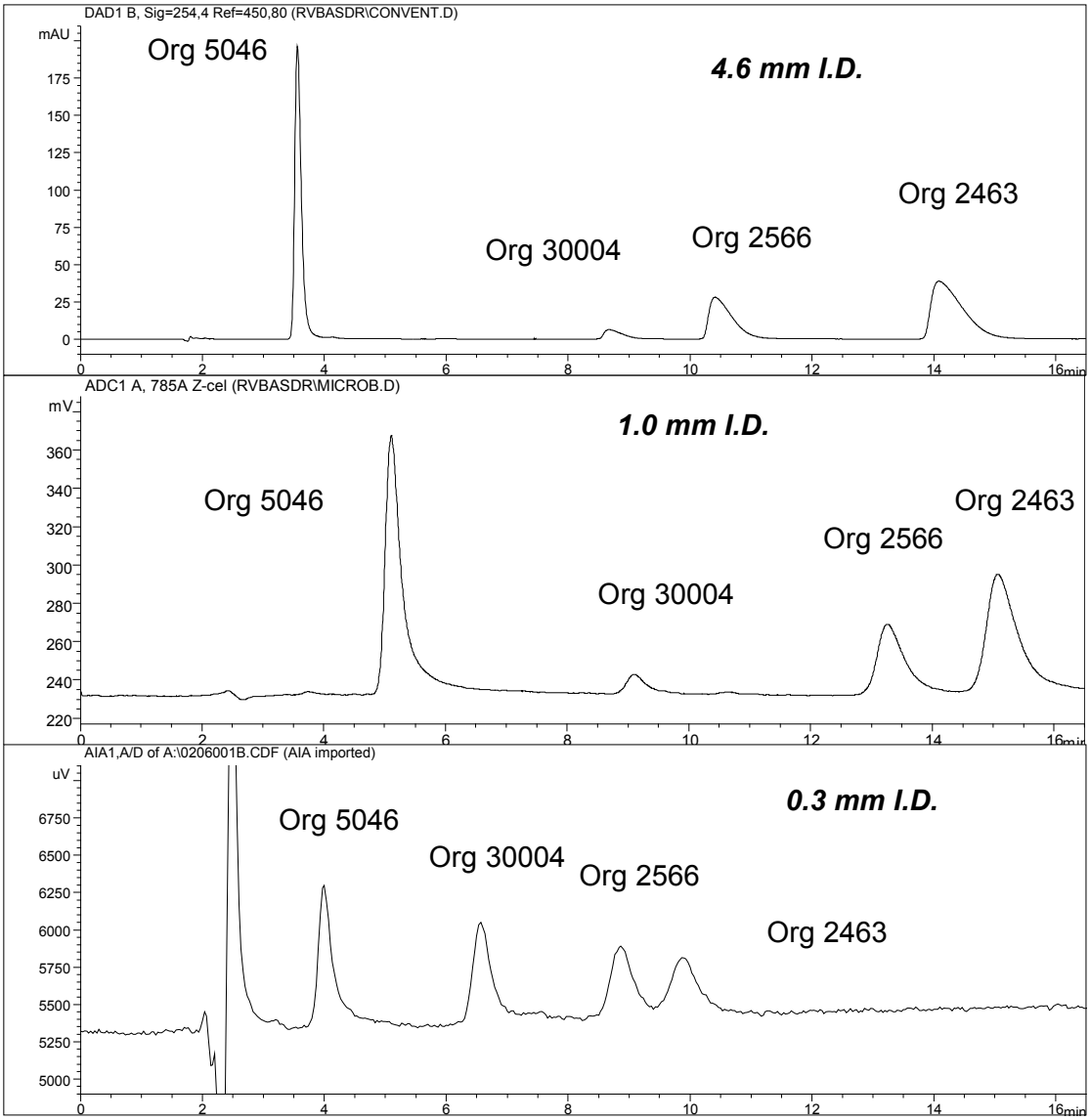

Figure 5.1.9: LC-UV chromatograms of the four basic pharmaceuticals using Prodigy 5 ODS-2 stationary phase and methanol+50mM ammonium citrate pH 3 / 40+60 %(V/V) as mobile phase: 0.3, 1.0 and 4.6 mm I.D. columns.

In the corresponding mass spectra of the basic solutes high abundance ions at m/z 210 $[M+NH_4]^+$ and m/z 402 $[2M+NH_4]^+$ 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.

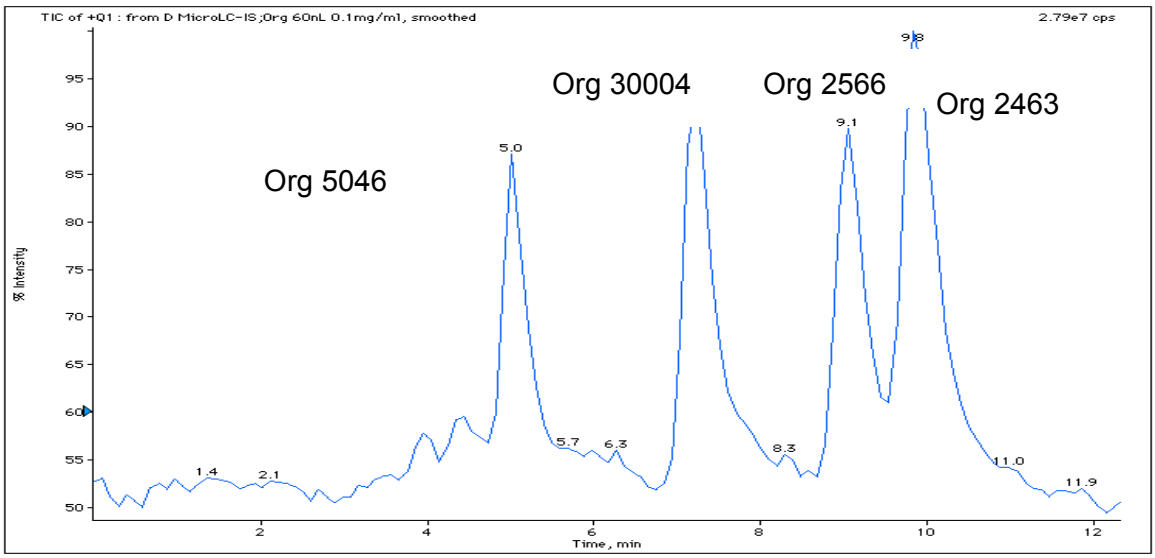

Figure 5.1.10: LC-MS chromatogram of the four basic pharmaceuticals using a Symmetry C_{18} (150 x 0.3 mm I.D.) column and methanol+50mM ammonium citrate pH 3 /40+60 %(V/V) as mobile phase.

5.1.5 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 was 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 stationary 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.

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5.2 INFLUENCE OF BATCH TO BATCH REPRODUCIBILITY OF LUNA C_{18} (2) PACKING MATERIAL, NATURE OF COLUMN WALL MATERIAL AND COLUMN DIAMETER ON THE LIQUID CHROMATOGRAPHIC ANALYSIS OF BASIC ANALYTES

5.2.1 SUMMARY

This chapter discusses aspects arising from transferring liquid chromatography (LC) methods developed on conventional size columns to micro LC, i.e. influence of batch to batch reproducibility of packing material, nature of the column wall material, and the column inner diameter.

It was shown that the nature of the column hardware as well as batches of Luna C_{18} packing material did not influence the retention of basic compounds. The batches of packing material, however, significantly influenced the peak shape for some compounds. The nature of the mobile phase buffer showed also an effect on peak shape.

The column wall material as well as the column inner diameter did not reveal effects on the peak shape. Different packing densities were found for the micro and conventional LC columns, i.e. the micro LC columns were less densely packed than the conventional LC columns

The study demonstrated that for the analysis of basic analytes similar separations are obtained using micro and conventional LC columns, provided that the columns are packed from the same batch of packing material.

R.J.M. Vervoort, E. Ruyter, A.J.J. Debets, H.A. Claessens, C.A. Cramers and G.J. de Jong, J. Sep. Sci., 24 (2001) 167-172.

5.2.2 INTRODUCTION

Liquid chromatography (LC) using conventional size columns with inner diameters of 4-5 mm are routinely used, e.g. for the analysis of pharmaceuticals. Complementary to conventional sized LC is microcolumn LC, which is used especially in situations where conventional LC fails or cannot compete. The main advantages of microcolumn LC are the ability to work with small sample volumes, small volumetric flow-rates, and due to reduced chromatographic dilution improved detection performance with concentration sensitive detectors [1-4]. Another advantage was recently demonstrated. The use of columns with an inner diameter ≤ 0.3 mm allowed temperature programming which was found to be a useful tool for optimising separations [5-7]. The development and applicability of micro column LC has been discussed in numerous papers and it is obvious that sample availability and compatibility with mass spectrometric (MS) detection are main driving forces to develop and perform micro column LC analysis methods [8-13].

Recently, the influence of miniaturising of column dimensions in LC on the chromatographic performance of four basic pharmaceutical compounds was studied [14]. The study was performed to investigate whether LC methods developed with conventional columns could easily be transferred to microcolumn LC to improve on-line LC-MS analysis. It was found that transferring conventional LC methods to microcolumn LC can result in different peak shapes and average resolutions of the four basic pharmaceuticals. The columns used were obtained from the manufacturers as commercial pre-packed columns, meaning that the columns were packed from different batches. Furthermore, the 0.32 mm I.D. columns were packed in fused silica tubes and the 1.0 and 4.6 mm I.D. columns in stainless steel tubes. Therefore, batch to batch differences of the packing materials and the different nature of the column wall materials used could have caused the observed differences in chromatographic performance.

The study described in this chapter has been performed to investigate the batch to batch differences of the column packing material and the nature of the column wall material in greater detail. To determine the batch to batch reproducibility three 4.6 mm I.D. columns packed with different batches of Luna C_{18} (2) (particle size 5 μm) were evaluated. To determine the influence of the nature of column wall material we tested various 4.6, 1.0 and 0.32 mm I.D. columns packed from one batch Luna C_{18} (2) material. The column wall materials studied were stainless steel 8 and 16 μuinch, PEEK, glass and fused silica. As test compounds we used seven basic pharmaceuticals and five of the McCalley test compounds. In another study these compounds revealed useful information about the applicability of LC columns for the analysis of basic pharmaceuticals [15]. Retention, efficiency and peak asymmetry values of the test compounds were determined to compare the columns.

5.2.3 EXPERIMENTAL

5.2.3.1 HPLC equipment

The HPLC experiments were performed using a HP 1090 liquid chromatograph, Agilent Technologies (Palo Alto, CA, USA). Data were processed and chromatograms were obtained using a HPLC 3^D Chemstation, Agilent Technologies. With the conventional LC columns, for detection the HP 1090 Diode Array detector (cell volume 6 μl) was used. For the 1.0 mm I.D. and 0.32 mm I.D. columns, a 785A Programmable Absorbance detector, Applied Biosystems (Norwalk, CO, USA) equipped with a Z-shaped flow cell (cell volume 35 nl), LC Packings (Amsterdam, The Netherlands) was used. Injection was performed using a Valco 4 Port injector with a fixed 60 nl internal loop, Valco Instruments Co. Inc. (Houston, TX, USA). With the 0.32 mm I.D. columns, the flow from the HP 1090LC pump was split using a LC Packings flow splitter. The tubing from injector to column and column to detector were fused silica tubing of 75 μm inner diameter.

In Table 5.2.1 the instrumental settings used in the experiments are shown. The LC columns were thermostatted at 40 °C ± 0.5 °C. In all experiments the detection wavelength was UV 210 nm. Table 5.2.2 shows the characteristics of the batches packing material. The length of the columns studied was 150 mm. The hardware used was stainless steel 16 and 8 μuinch, glass lined and PEEK for the 4.6 mm I.D. columns, stainless steel 16 μuinch and glass lined for the 1.0 mm I.D. columns, and glass lined and fused silica for the 0.32 mm I.D. columns, respectively. The values of 16 and 8 μuinch refer to the smoothness of the stainless steel surface inside the column.

Table 5.2.1: Instrumental settings used in micro column and conventional LC.

Column diameter (mm I.D.)	Flow-rate (ml/min)	Injection volume (μl)	UV detection wavelength (nm)
4.6	1.0	2	210
1.0	0.05	0.06	210
0.32	0.005	0.06	210

5.2.3.2 Chemicals

The Organon basic pharmaceuticals were obtained from N.V. Organon (Oss, The Netherlands). In Fig. 5.2.1 the molecular structures are shown. Fig. 5.2.2 shows the McCalley test compounds. In Table 5.2.2 the batches packing material studied are shown. The columns were obtained from Phenomenex (Torrance, CA, USA). For the various columns different packing procedures are used, which often are empirical and based on experience [16].

Methanol (MeOH, Lichrosolv) was obtained from Merck (Darmstadt, Germany). Sodium phosphate ($Na_2HPO_4.12H_2O$) and sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) were purchased from J.T. Baker (Deventer, The Netherlands). Aqueous solutions of 25 mM sodium phosphate pH 3 and pH 7 and ammonium acetate pH 7 buffers were prepared using demineralised water. The sodium phosphate buffers were adjusted to pH 3 and pH 7 using concentrated phosphoric acid from Merck.

Table 5.2.2: Specification and data of the Luna $C_{18}(2)$ batches used in this study.

	Unit	Specification	Batch 5291-9	Batch 5291-10	Batch 5191-13
Particle analysis					
Particle size	μm	5.00 ± 0.30	5.01	4.8	4.90
Particle distribution	90%/10%	1.85 ± 0.30	2.05	2.04	1.96
Pore diameter	Å	100 ± 10	100	101	100
Surface area	m^2/g	400 ± 30	418	390	405
Metal content	ppm	< 55	32.1	33.2	38.4
Bonded phase					
Total carbon	%	17.80 ± 0.7	18.2	18.05	17.57
Surface coverage	$\mu moles/m^2$	3.25 ± 0.50	3.33	3.3	3.36

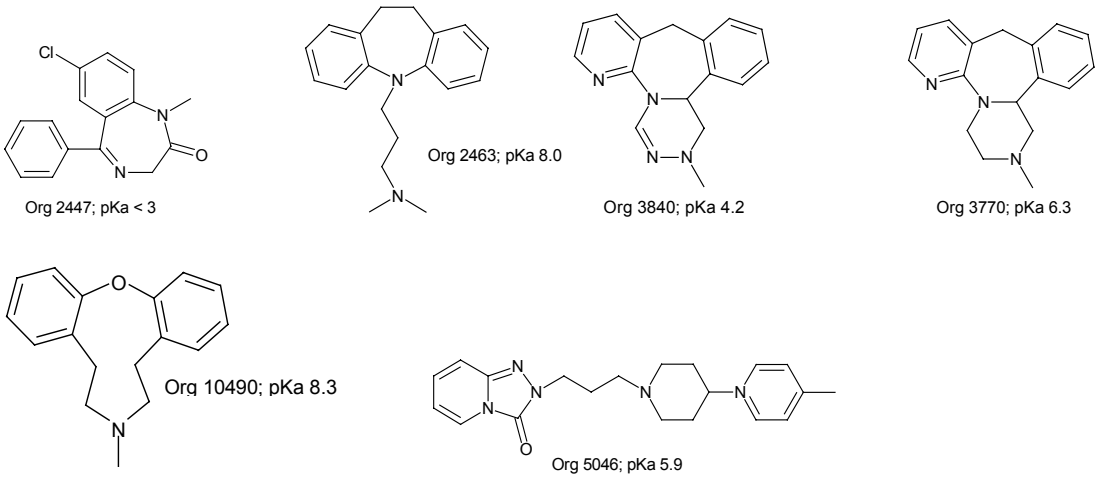


Figure 5.2.1: Structures of the Organon compounds used in this study. pKa values were measured in MeOH+H_2O/60+40 %(V/V).

To prevent influences of the eluent composition in this study, the columns were tested using exactly the same mobile phase composition, achieved by preparing large amounts of eluent.

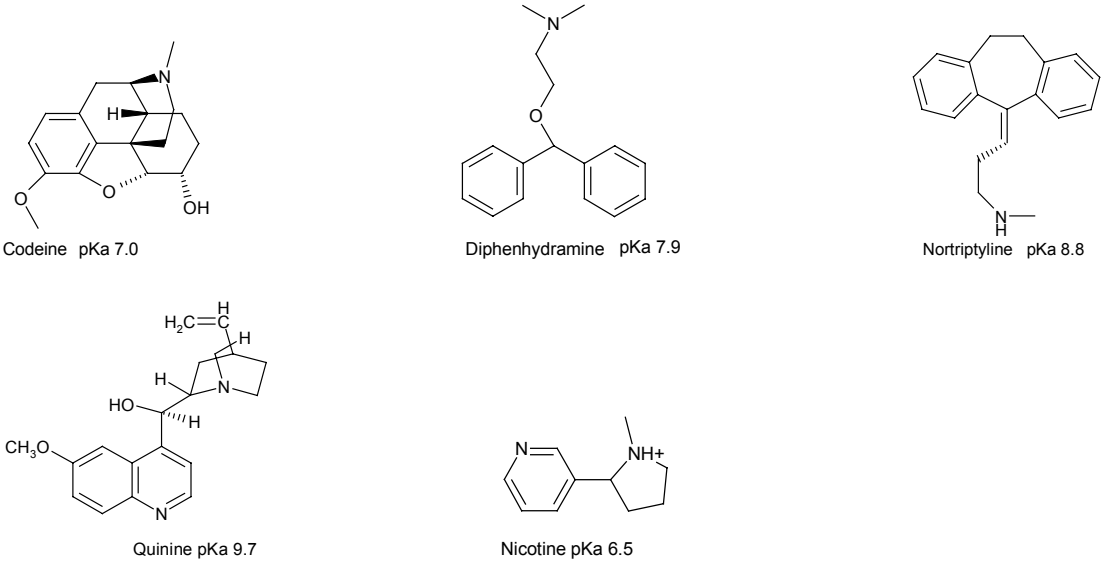


Figure 5.2.2: Structures of the McCalley test compounds used in this study. pKa values were measured in water.

5.2.4 RESULTS AND DISCUSSION

5.2.4.1 Test procedure

Column performance can be divided into thermodynamic and kinetic behaviour [16]. The thermodynamic performance is related to the thermodynamics of the distribution of the solutes between stationary and mobile phase and is related to, inter alia, retention and peak shape. The kinetic performance is related to, inter alia, particle size, permeability and quality of the packed bed. Retention, efficiency and peak shape data of test compounds contain information about either the thermodynamic or the kinetic nature of the column. To enable the comparison of thermodynamic performances of the columns we compensated for possible differences in kinetic performance. This compensation was achieved by calculating relative retention, relative asymmetry and relative efficiency values. Therefore the experimental values of the test compounds were divided by the experimental value of a defined neutral compound. Org 2447 (pKa < 3) was used as neutral compound for the Organon compounds, and toluene was used as neutral compound for the McCalley compounds.

To enable a statistical evaluation of the test results the day to day variability was determined as a measure of reproducibility. For every column dimension studied, one of the columns was measured on three days i.e. at the beginning, in between

and at the end of the experiments. The time period between the beginning and end of the study was approx. 4 months. It was found that the 95% confidence intervals for relative retention, relative efficiency and relative peak asymmetry obtained for the various column diameters were comparable. To enable the determination of significant differences between the measurements 95% confidence intervals were calculated, combining the measurements for the three columns by pooling the standard deviations. The 95% confidence intervals for the mean relative values were: relative k $\pm$ 0.052, relative As $\pm$ 0.102 and relative N $\pm$ 0.054, respectively.

5.2.4.2 Batch to batch reproducibility

In the past, variability in retention and plate number from column to column was found to be a major problem in LC [18]. In the present study the batch to batch reproducibility of a packing material of the latest generation using the compounds shown in Figs. 5.2.1 and 5.2.2. was determined. Three 16 μuinch stainless steel columns were packed with different batches Luna C_{18} (2) packing material (for details see Table 5.2.2).

Fig. 5.2.3 shows the relative k values for retention of the McCalley test compounds. No differences between the batches for retention were observed. Comparable data were found for the Organon test compounds. These data are in agreement with other studies [19-21]. In Fig. 5.2.4 the relative asymmetry values obtained for the basic pharmaceutical compounds in Fig. 5.2.1 are shown. Using basic analytes as test compounds, for peak asymmetry the present study showed significant differences between the batches for Org 2463. With respect to peak symmetry of basic compounds, Kele and Guiochon also observed differences between batches of packing material. RSD values for asymmetry ranging from between 1.5% to 12.5% were reported, demonstrating the extreme sensitivity of the tailing of basic compounds to minute changes in the chemistry of the stationary phase surface. This was observed for different batches stationary phase prepared from one single batch of silica. Larger differences were found between batches stationary phase prepared from different batches of silica [20,21]. The same authors presented comparable data about the batch to batch and column to column reproducibility of Luna C_{18}(2) packing material [22]. However, for this packing material they found that the differences between batches prepared from a single batch of silica was comparable to the differences observed for batches prepared from different batches of silica.

The differences between batches for peak shape are also affected by the nature of the buffer used. For Org 2463 the range for relative peak asymmetry between the three columns was 1.19 - 1.76 using ammonium acetate buffer pH 7 whereas using sodium phosphate buffer 7 the range was much smaller (1.04 - 1.12; difference not significant). Using ammonium acetate buffer pH 7 for Org 10490 the range was 1.15 - 1.25 (difference not significant) and using sodium phosphate 7 buffer the range was 1.10 - 1.46 (difference significant).

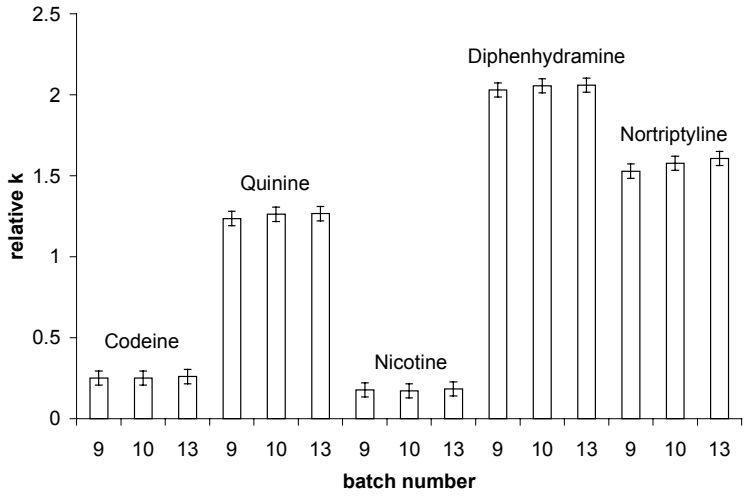


Fig. 5.2.3: Relative k values of McCalley test compound measured on three batches Luna C_{18} packing material. The relative k values are relative to the retention of toluene. As eluent MeOH+25 mM NH_4Ac pH 7 / 50+50 %(V/V) was used.

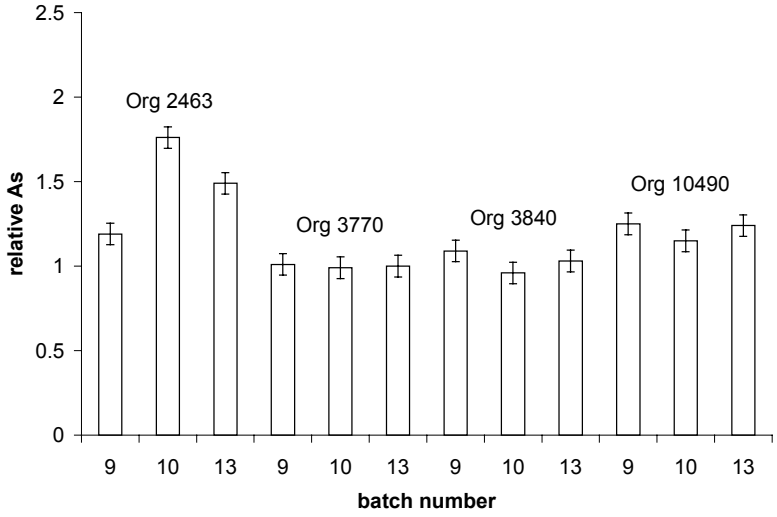


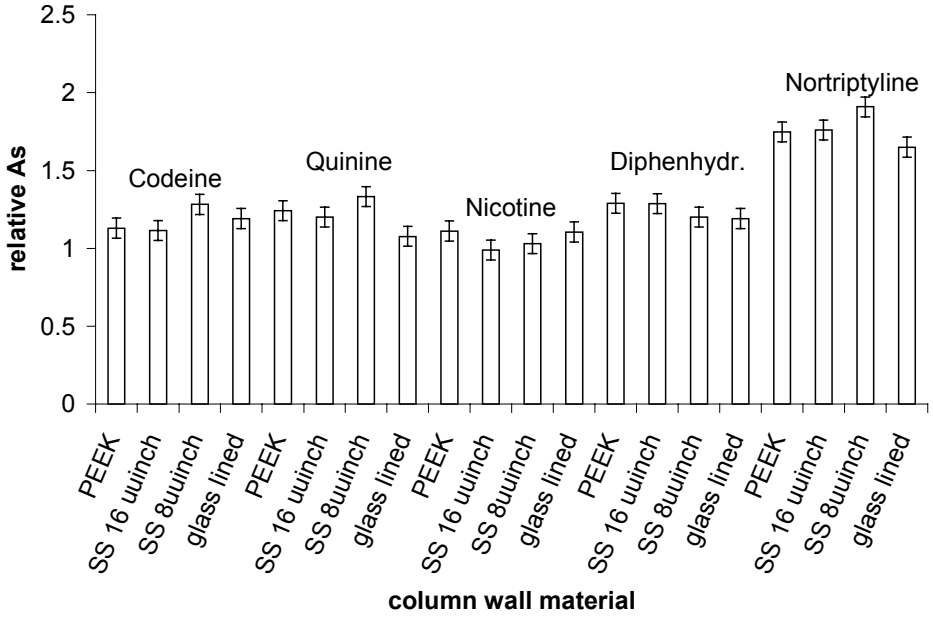
Fig. 5.2.4: Relative As values for the Organon test compound on three batches Luna C_{18} packing material. The values are relative to the peak asymmetry of Org 2447. As eluent MeOH+25 mM NH_4Ac pH 7 / 50+50 %(V/V) was used.

Comparable data were obtained with the McCalley test compounds. Different relative peak asymmetry values with the three batches were found for nortryptiline using sodium phosphate buffers pH 3 and 7, and ammonium acetate pH 7 buffer. In

addition, significant different asymmetry values were observed for diphenhydramine and quinine using ammonium acetate pH 7 buffer. With respect to plate number, no significant batch to batch differences were observed with the Organon and McCalley test compounds using the various buffers.
Section 5.1 described that transferring LC methods developed on conventional LC columns to micro LC by simply downscaling dimensions can yield varying selectivity and peak shapes for basic compounds. From the data in this section and from the literature [20-22] it is clear that these effects are most likely due to batch to batch differences of the used stationary phases.

5.2.4.3 Influence of the nature of column wall material and inner column diameter

Comparison of the 4.6 mm I.D. columns with different nature of wall material showed no significant differences for retention and efficiency. However, for peak asymmetry in few situations significant differences were observed. The differences were small and no clear trend was observed. In Fig. 5.2.5 data for some test compounds are shown.



5.2.5: Relative asymmetry (As) values of McCalley test compounds for the 4.6 mm I.D. columns with different column wall material. The columns were packed with batch 5291-9 Luna $C_{18}(2)$ packing material and the As values are relative to the asymmetry of toluene. MeOH+25 mM Na_2HPO_4 pH 7 / 50+50 %(V/V) was used as eluent.

Explaining the observed differences is difficult. For the various columns different packing procedures are used, which often are empirical and based on experience. The outcome of a packing operation is the result of numerous phenomena like

particle kinetics, friction against walls, and coagulation. Many factors come together to give a more or less homogenous bed that may even change properties [16,23]. From the present study it was concluded that the nature of the column wall has, if present at all, only a small effect on the peak shapes of the compounds used. This conclusion was supported by the data obtained for the variation both in nature of column wall material and inner diameter. Relative asymmetry, relative retention and relative efficiency values obtained with the 4.6, 1.0 and 0.32 mm I.D. columns showed no significant differences. Moreover, the efficiency of the columns was comparable, i.e. for toluene the plate number was in the order of 6000 for the various columns

An important aspect of column performance is its backpressure. The relation between parameters that influence column backpressure is described by the equation for permeability (B_0) [25]:

$$B_0 = \frac{F\eta L}{\pi r^2 \Delta p} \quad \{1\}$$

where F is the flow-rate, η is the viscosity of the eluent, L is the column length, r the radius of the column and Δp the pressure drop across the column.

Table 5.2.3: Permeability (B_0) and interstitial porosity (ε_i) of the 4.6, 1.0 and 0.32 mm I.D. columns calculated using equations {1} and {2}. The length of the columns is 15 cm and the columns are packed with Luna C_{18}(2) packing material batch 5291-9, particle size = 5 μm.

column wall material	inner diameter (mm)	Δp (bar)	B_0 (cm^2)	ε_i
stainless steel 16 μuinch	4.6	120	2.25×10^{-10}	0.39
stainless steel 8 μuinch	4.6	124	2.19×10^{-10}	0.39
glass lined	4.6	123	2.20×10^{-10}	0.39
PEEK	4.6	138	1.96×10^{-10}	0.38
glass lined	1.0	105	2.73×10^{-10}	0.41
stainless steel 16 μuinch	1.0	90	3.18×10^{-10}	0.43
glass lined	0.32	78	3.59×10^{-10}	0.44
fused silica	0.32	87	3.41×10^{-10}	0.43

The specific permeability depends on the particle size d_p and on the interstitial porosity ε_i of the packed bed. The relation is known as the Kozeny-Carman equation [25]:

$$B_0 = \frac{1}{185} \frac{\varepsilon_i^3}{(1-\varepsilon_i)^2} d_p^2 \quad \{2\}$$

Table 5.2.3 shows the calculated permeability and interstitial porosity of the columns used. These data are in fair agreement with data reported in the literature. For the latter columns ε_i of a packed bed is around 0.4 [24,25] whereas for 0.3 mm I.D. column ε_i values of 0.43 – 0.51 have been reported [26]. Less densely packed columns allow the use of longer columns, which results in more efficient separation.

5.2.5 CONCLUSIONS

This study discusses aspects involved in transferring LC methods developed on conventional size columns to micro LC. The study was performed to determine the influence of batch to batch reproducibility of packing material, nature of the column wall material and column inner diameter on the chromatographic performance of basic analytes.

For three columns packed from different batches Luna C_{18} (2) packing material we did not observe different retention and efficiency values. However, different peak shapes were found for basic compounds showing batch to batch differences. Furthermore, the nature of the buffer also influenced the observed batch to batch differences. For Org 2463 the range for relative peak asymmetry between the three columns was 1.19 - 1.76 using ammonium acetate buffer pH 7 whereas using sodium phosphate buffer pH 7 the range was much smaller (1.04 - 1.12). Using ammonium acetate buffer pH 7 for Org 10490 the range was 1.15 - 1.25 and using sodium phosphate buffer pH 7 the range was 1.10 - 1.46. For retention and efficiency no significant differences were found for the three columns. The nature of the column wall did not show a clear effect on chromatographic performance of basic compounds.

The inner column diameter showed no effect on relative peak shape, relative efficiency and relative retention. Similar separations are obtained using micro and conventional LC columns packed from the same batch of stationary phase. Different packing densities were found for the micro and conventional LC columns, e.g. for the glass lined columns the interstitial porosity (ε_i) of the 4.6 mm I.D. column was 0.39, that of the 1.0 mm I.D. column was 0.41 and that of the 0.32 mm I.D. column was 0.44, respectively.

The data in chapter 5.1 showed that miniaturisation of the LC dimensions can result in different selectivity and peak symmetry. This chapter showed that this is most likely due to batch to batch differences of the used packing materials, and not due to miniaturisation.

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6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Due to the different manufacturing procedures of nominally identical stationary phases used in liquid chromatography large differences exist between the properties of these packing materials. As a result different retention and peak shapes can be obtained. The difference in retention behaviour of the many available reversed phase liquid chromatography (RPLC) columns can be used to optimise a separation, whereas for optimal separation and sensitivity sharp and symmetrical peaks are a prerequisite. Therefore, in method development the choice of the column is an important step. To enable the selection of a suitable column for the RPLC analysis of basic pharmaceuticals testing and characterisation is mandatory.

The characterisation of stationary phases using an empirical method with basic pharmaceuticals as test analytes enables the selection of a suitable column within a reasonable time period. It is obvious that for analysts involved in method development this will be the method of choice. Main disadvantage of empirical methods is that a qualitative characterisation of the properties of the stationary phases is obtained. In comparison, quantitative structure-retention relationships (QSRRs) allowing the determination of statistical significant differences between columns enables a quantitative comparison. However, a disadvantage of QSRRs is the circumstantial and laborious experiments are needed to obtain the required retention data. In addition, so far QSRRs in most cases were not able to quantitatively differentiate between RPLC packing materials. To establish QSRR as a useful tool in stationary and mobile phase characterisation several conditions have to be fulfilled. First the required number and diversity of the test compounds must be known. Furthermore, the relevant molecular descriptors of the test compounds are difficult to obtain. To determine descriptors experimentally laborious experiments are required. Until now many workers are using experimental determined so called solvatochromic descriptors. Molecular descriptors can also be calculated using specially designed software. A disadvantage is that for a proper use of this software today the help of an expert is still indispensable. Development of new software that is easy to use will be of great help to establish QSRR as a tool to characterise stationary and mobile phases. When these conditions can be fulfilled there is great potential for QSRRs in characterisation of mobile and stationary phases.
Determining enthalpies and entropies of transfer of basic analytes from the mobile to the stationary phase is helpful to reveal information about differences between types of interactions of protonated and neutral compounds with RPLC stationary phases. Such data can be useful to reveal differences between mechanisms of retention of basic compounds with RPLC columns under a variety of conditions, e.g. buffer salts and modifiers. Whether enthalpy and entropy data can be used to explain the differences in obtained peak shapes for basic compounds with RPLC stationary phases is a subject of investigation. Probably this will require data obtained from many RPLC columns under various eluent conditions.

Chapter 4 showed the influence of the organic modifier on the pKa of the basic analyte and on the eluent pH. In daily practice these effects often are not recognised. It is obvious that analysts must take these effects into account. Furthermore, in various cases for individual basic analytes a significant influence of the nature of the buffer on peak shape and retention was observed. In most tests described in the literature involatile phosphate buffers are used. Since today in the pharmaceutical industry mass spectrometric (MS) detection is frequently applied, testing of stationary phases using volatile buffer salts will allow the well considered selection of stationary phases for LC-MS analysis.

Following the results of chapter 3 a procedure is proposed which can be used for the development of empirical column characterisation methods. As discussed in this thesis the procedure is suitable to develop a method to characterise columns for the analysis of basic pharmaceutical compounds. However, the same approach can be used to develop column characterisation procedures for other compounds, e.g. steroids, proteins or pesticides.

Table 6.1: Steps for the development of an empirical characterisation procedure of stationary phases.

Step	Action
1	Select a number of relevant test compounds related to the application area, based on experience and knowledge.
2	Define eluent conditions, e.g. nature of modifier and buffer, eluent pH. For instance, if mass spectrometric detection is used select volatile eluent constituents.
3	Define the variables to be determined, e.g. peak shape, efficiency, resolution.
4	Select a small number of relevant stationary phases based on experience, knowledge and information from the literature.
5	Characterise the selected stationary phases using the test compounds defined in step 1. The phases are characterised with the conditions selected in step 2 and the variables to be determined are defined in step 3.
6	Present the measured variables obtained in step 5 graphically using principal component analysis (PCA). Compounds with similar chromatographic performance will cluster.
7	Use the PCA results from step 6 to define the set of test compounds. Select a number of compounds representing the total number of compounds, i.e. from each cluster one compound should be selected.
8	Use the restricted set of selected test compounds to characterise a large number of stationary phases within a relatively short period of time.
9	Use principal components analysis to analyse the large number of data obtained by testing stationary phases. PCA enables a graphical presentation and shows clustering of stationary phases with comparable characteristics.

Table 6.1 shows the various action steps. The first step is the selection of a relevant number of test compounds, closely related to the compounds of interest. If a method is developed to characterise columns for the analysis of, e.g. steroids, select a number of relevant steroids, e.g. between 20 and 40, based on available experience and knowledge, or based on information from the literature. Next the eluent conditions used and the variables to be determined are defined. If MS detection is used select volatile eluent constituents. Step 3 defines the variables to be determined. For instance, special attention has to be paid to selectivity if the separation between a peak pair is critical. The following step is to characterise a number of stationary phases. The selection of these phases is based on available experience, knowledge and information from the literature. The obtained results (e.g. peak shape, retention, resolution) are used to reduce the number of analytes resulting in a relatively small set of test compounds. Graphical presentation of the compounds using principal component analysis (PCA) shows clusters of compounds with similar chromatographic performance. This allows a reduction of the number of test compounds, i.e. from each cluster one compound can be selected. The restricted set of test compounds can be used to characterise a large number of stationary phases within a reasonable period of time. PCA is used to graphically represent the characterised stationary phases.

This thesis and also data from the literature showed that for basic compounds differences in peak shape can be obtained with different batches of identical packing material. In industry the developed methods are often used over al long period of time. During this period columns packed from different batches of identical packing material will be used and preferably the separation should not change. Research and development of stationary phases performed by column manufacturers is an on-going process. For many compounds the batch to batch reproducibility often is sufficient. It is to be expected that in the future also for basic compounds more reproducible separations will be obtained using columns packed from different batches of identical material.
New trends in column development can be noticed today. One is the development of RPLC columns that can be used at high eluent pH, e.g. XTerra and Zorbax Extend. High eluent pH allows the RPLC analysis of basic drugs as neutral compounds. However, column stability becomes critical since this is the reason why most RPLC columns are less suitable for a high eluent pH. The stability of these columns at high eluent pH has to be investigated in more detail. If the stability is sufficient there is great potential for such columns. Eluent pH modification is a strong tool to optimise the separation of basic compounds and these columns allow the use of a pH range of 2 - 12. Another new trend in column development is monolithic columns. These columns consist of a single piece of modified porous silica. Main advantage of these columns is the high efficiency maintained at high flow rate, which allows fast

separations. Whether these columns will be useful in the near future has still to be investigated. The column to column reproducibility, batch to batch reproducibility, robustness and chemical stability of silica rod columns must be comparable to conventional RPLC columns. A trend in RPLC analysis is the use of columns with small internal diameters. Comparison of conventional and micro LC columns showed that with both columns similar separations are obtained. Moreover, the robustness of the columns was also comparable. Since MS detection improves with low flow-rates, it is expected that in the future the number of methods developed on micro LC columns will increase.

From this thesis it is obvious that column characterisation for the analysis of basic drugs is mandatory but difficult. In method development the choice of a column is an important step. Today the choice is often based on experience rather than on knowledge of the stationary phase properties. As a result many methods are developed by use of columns which are not optimally suited for the analytical problem. In contrast, most analysts better understand optimisation of the mobile phase, i.e. the influence of changing the amount of modifier or changing the pH of the eluent on a separation. In addition, software programs are available as a helpful tool to optimise the mobile phase, e.g. DryLab (LC Resources, USA) and Chromsword (Merck, Germany). Inclusion of the selection of the stationary phase in such software packages would be very helpful for analysts involved in method development. In fact, at least Chromsword already includes a limited column selection database. However, for a meaningful implementation of a column database into a software program it is mandatory that columns are characterised using a method that is accepted world wide. Today, developments in column characterisation methods have not reached this goal.

SUMMARY

In the pharmaceutical industry research and development (R&D) of new drugs has to be performed in a short period of time to make it economically beneficial. During the R&D stages information about the drug substance and drug product is obtained applying a broad scale of analytical techniques, i.e. spectroscopic analysis, separation methods and physical measurements are performed. From the applied analytical techniques, separation methods play an important role in the entire development and beyond. From the initial development up to and including quality control, separation methods are used to determine the purity and contents of both drug substance and drug product. Many different separation techniques can be used such as gas chromatography and capillary electrophoresis. However, today the majority of analysis in the pharmaceutical industry is performed using reversed phase high performance liquid chromatography (RPLC). RPLC is used to determine impurities and active compounds of drug substance and drug product. The separation of the compounds is based on difference in interaction between the various analytes and the mobile and stationary phase. It is obvious that for efficient separations and sensitive analysis symmetrical and narrow peaks are a prerequisite.

In chapter 2 the characterisation of RPLC systems for the analysis of basic pharmaceuticals is reviewed. Many pharmaceutical compounds contain basic nitrogen atoms in the molecular structure, e.g. central nerve system drugs, cardiovascular drugs, amino acids, peptides and proteins. Inherent to RPLC is the presence of residual silanols and eventual other polar sites on packing materials. The ability of basic nitrogen atoms to interact with these polar groups may hamper the RPLC analysis and asymmetrical peaks and irreproducible retention can be the result. Asymmetrical peaks can be explained in terms of kinetic phenomena. For basic compounds the kinetics of the interactions with polar sites may be slower than the hydrophobic interactions between the neutral part of compounds and the alkyl chains of the stationary phase. Improvement of the peak shape can be achieved by optimising the mobile and the stationary phase as well. Optimisation of the mobile phase can be achieved by choosing the optimal eluent pH, addition of silanol blocking compounds and selection of a suitable eluent buffer. Stationary phases optimised for the analyses of basic compounds are phases in which the number of bad silanols or other polar sites is minimised. Knowledge of the properties of a stationary phase can be of great help to select a stationary phase suitable for a given separation problem.

To obtain information about the properties of packing materials adequate testing is required. Both spectroscopic and chromatographic test methods can be used. The chromatographic characterisation of stationary phases can be divided into three classes: *i*: empirical test methods, *ii*: thermodynamically based test methods, and *iii*: test methods based on a retention model such as quantitative structure-retention

relationships (QSRRs). The applicability of these methods for column characterisation is discussed. In addition, the influence of eluent composition on RPLC analysis and the use of chemometrical techniques to classify columns are described too.

In chapter 3 the development of an empirical column characterisation method is discussed. Based on differences in basicity (pKa), polarity, number of nitrogen atoms and type of nitrogen atom(s) initially 32 basic compounds were selected. Section 3.1 presents the separation of these 32 test compounds. Using a μBondapak C_{18} column various parameters were investigated. It was concluded that peak asymmetry depends on the pKa value and structural parameters of a compound. To improve the shape of chromatographic peaks of basic compounds silanol blocking agents proved to be useful. Section 3.3 shows that especially by the addition of N,N-dimethyloctylamine to mobile phase buffer pH 3 improved peak shapes are obtained. However, addition of ion-pairing compounds to the eluent did not improve the peak shape. Within the set of investigated columns, an electrostatically shielded stationary phase showed most favourable peak shapes. Also for a polymer stationary phase symmetrical peaks were obtained. However, due to the inherent low plate number of polymeric phases the resolution is rather limited.

Section 3.2 shows the applicability of chemometrical techniques to reduce the number of test compounds and to enable a graphical classification of column properties. The data described in section 3.1 were used to reduce the number of test compounds necessary to characterise stationary phases. A limited number of test compounds allows the characterisation of columns in a short period of time. First, from the data set of 32 compounds, a set of five representative test compounds was selected using principal component analysis (PCA). This test set can be used to evaluate commercially available columns. Secondly, several chemometric techniques were compared in their applicability to represent the often large amount of data obtained when characterising stationary phases. Bar-charts, multicriteria decision making and bi-plots were used to compare the columns. It was obvious that for comparison of a large number of columns the use of bi-plots (and thus also PCA) is preferred to visualise columns with comparable and different characteristics.

Section 3.3 shows the applicability of the developed test procedure by demonstrating the characterisation of fourteen different commercially available stationary phases. PCA was used to represent the set of column test data.

In the literature many column characterisation procedures are described. Most tests are developed for general column characterisation purposes. Chapter 4 discusses the applicability of several of these test procedures to characterise columns for the analysis of basic pharmaceutical compounds. In section 4.1 these various empirical test methods are compared.

A set of seven RPLC columns was characterised with the test methods of Engelhardt, Tanaka, Galushko and McCalley. The columns were also characterised

using basic compounds from NV Organon. The latter test is based on the test discussed in chapter 3. However, to cover a larger pKa range, seven compound from pKa < 3 to pKa 8.7 were used. Test procedures using compounds like aniline, phenol and benzene were found to be less informative for column selection for basic drugs. From the investigated tests only results of the McCalley test could predict suitability of columns for analysis of the basic compounds. Obviously, test procedures using basic analytes as test probes provide relevant information with respect to the selection of columns for the analysis of basic pharmaceutical compounds. Furthermore, for individual basic compounds a different and significant influence of the nature of the buffer on peak shape was observed. Moreover, the results revealed the necessity of column testing at different pH-values and various types of buffer salts.

In addition, the influence of the eluent composition on the RPLC analysis of basic compounds is discussed too. For eluents commonly used in RPLC, it was found that addition of e.g. methanol in the eluent affected the pH. For example addition of 50% methanol resulted in an increase of ca. 1 pH unit in the buffer pH range 3 - 7. This effect is also observed for other organic modifiers. Besides the pH, the buffering capacity of eluents is also influenced by addition of methanol or other organic modifiers. The amount and nature of the modifier also influence the pKa of basic analytes. For example, for methanol and acetonitrile upon addition of 50%(V/V) of these modifiers the pKa shift was ca. 0.7 for mirtazipine. However, for tetrahydrofuran the change of the pKa value of this compound amounted larger than 1 pKa unit for the same amount of modifier.

Section 4.2 describes characterisation of RPLC columns by thermodynamic measurements. To obtain deeper insight into retention mechanisms of basic drugs van ‘t Hoff plots were used. The experimentally obtained plots were linear for the investigated set of RPLC columns. Therefore it was concluded that over the studied temperature range no changes in the retention mechanism for basic compounds occurred. The negative enthalpy values obtained for the analytes showed that retention of basic compounds is an exothermic process. Both enthalpy and entropy data revealed information about types of interaction of protonated and neutral compounds with RPLC stationary phases. However, the observed substantial differences in peak symmetry could not be explained by these thermodynamic data.

Section 4.3 shows the applicability of quantitative structure-retention relationships (QSRRs) to characterise stationary phases. The obtained QSRRs revealed effects of calculated molecular descriptors involved in the retention of basic analytes with RPLC columns. The descriptors molar refraction, lowest unoccupied molecular orbital, dynamically calculated polar surface and the number of proton donors proved to be most significant. Furthermore, different trends in the regression coefficients of polar surface and molar refraction were observed using ammonium acetate and sodium phosphate as buffering salts in the eluent. This trend indicates the

substantial influence of the buffer salt on the separation of basic analytes. Finally, because the magnitude of the confidence intervals of the regression coefficients was too large, the QSRRs did not allow a quantitative discrimination between the individual RPLC stationary phases.

The data discussed above were obtained using 4.6 mm I.D. columns. However, todays trend is to use columns with small internal diameters, i.e. ≤ 1 mm. At present it is obvious that the available sample amount and compatibility with mass spectrometric (MS) detection are main driving forces to perform micro column LC. The study described in chapter 5 was performed to investigate whether LC methods developed with conventional columns could be transferred to microcolumn LC. In section 5.1 results obtained with micro and conventional columns (internal diameters (I.D.) of 0.3 mm, 1.0 mm and 4.6 mm) showed similar behaviour with respect to ruggedness. Comparison of mobile phases containing volatile buffers (ammonium citrate and ammonium acetate) with mobile phases containing non-volatile buffers (sodium phosphate) showed that at pH 3 best peak symmetries were obtained using ammonium citrate buffer. To further investigate the effects described in section 5.1 several variables were studied in more detail and are described in the next section.

Section 5.2 shows that different batches of one specific packing material did not influence the retention of basic compounds significantly. However, the different batches of a packing material significantly influenced the peak shape of some of the basic test compounds. In addition, also the nature of the buffer salt showed an effect on peak shape. The column wall material as well as the column inner diameter did not affect peak shape, retention and efficiency. Different packing densities were found for the micro and conventional LC columns. For instance for the glass lined columns the interstitial porosity (ε_i) of the 4.6 mm I.D. column was 0.39. The ε_i-values of the 1.0 and 0.32 mm I.D. glass lined columns were 0.41 and 0.44, respectively. Less densely packed columns allow the use of longer columns with the same backpressure, which results in more efficient separations. It was concluded that similar separations are obtained using micro and conventional LC columns packed with the same batch of a stationary phase.

SAMENVATTING

In de farmaceutische industrie moeten research en ontwikkeling (R&D) van nieuwe potentiele farmaceutische verbindingen uitgevoerd worden in een zo kort mogelijke tijd om het economisch te verantwoorden. R&D bestaat ruwweg uit drie stadia: de ontdekkingsfase, de pre-klinische fase en de klinische ontwikkelingsfase. Tijdens deze drie fasen wordt een breed scala aan analytische technieken gebruikt om informatie over nieuwe verbindingen te verkrijgen. Hiervoor worden spectroscopische analyses, scheidingsmethoden en fysische metingen uitgevoerd. Tijdens de ontwikkelingsfase maar ook daarna worden vooral scheidingsmethoden veelvuldig gebruikt. Vanaf de eerste ontwikkeling tot en met de kwaliteitskontrole worden scheidingsmethoden gebruikt om zuiverheden en gehaltes van verbindingen in zowel de grondstof als farmaceutische produkten te bepalen. Hoewel er verschillende scheidingsmethoden beschikbaar zijn zoals gaschromatografie en capillaire elektroforese, wordt de meerderheid van de analyses in de farmaceutische industrie uitgevoerd met reversed phase-vloeistofchromatografie (RPLC). RPLC wordt vooral gebruikt om zowel de verontreiningen als aktieve stof te bepalen. De scheiding is gebaseerd op het verschil in interaktie tussen de verschillende verbindingen en de mobiele en stationaire fase. Om met RPLC lage concentraties van verbindingen op een efficiente manier te kunnen meting is het belangrijk dat de pieken smal en symmetrisch zijn.

In hoofdstuk 2 wordt een overzicht gegeven van de karakterisering van RPLC systemen voor de analyse van basische farmaceutica. Aangezien veel farmaceutische verbindingen basische stikstofatomen bevatten, zoals stoffen die werken op het centrale zenuwstelsel, cardiovasculaire farmaca, amino zuren, peptiden en eiwitten, is de toepassing van RPLC soms aan beperkingen onderhevig. Basische stikstofatomen kunnen interaktie aangaan met residuele silanol groepen en eventuele andere aanwezige reaktieve groepen van het pakkingsmateriaal. Deze interakties kunnen een nadelig effekt hebben op de kwaliteit van de analyse. Vaak worden dan asymmetrische pieken verkregen en kan het retentiegedrag slecht reproduceerbaar zijn. Asymmetrische pieken zijn het gevolg van het verschil in interaktie tussen basische verbindingen met polaire groepen, en tussen het neutrale gedeelte van de komponent en de alkyl ketens van de stationaire fase. Om de piekvorm de verbeteren is het mogelijk om zowel de mobiele als de stationaire fase te optimaliseren. Optimalisatie van de mobiele fase betekent onder andere het kiezen van een geschikte pH van de buffer, toevoegen van verbindingen die de silanolaktiviteit onderdrukken, en selektie van een geschikte buffer. De optimale stationaire fase is een fase waarbij het aantal aanwezige zeer reaktieve silanolen of andere aktieve groepen die een interaktie kunnen aangaan minimaal is.

Om een geschikte stationaire fase voor de analyse van basische verbindingen te kunnen kiezen is het belangrijk om de eigenschappen van de fase te kennen.

Informatie over deze eigenschappen kan worden verkregen door fasen te testen. Hiervoor zijn zowel spectroscopische als chromatografische methoden beschikbaar. De chromatografische methoden kunnen als volgt worden ingedeeld: *i*: empirische testmethoden, *ii*: testmethoden die gebruik maken van thermodynamische gegevens, en *iii*: testmethoden gebaseerd op een retentiemodel zoals kwantitatieve struktuur-retentie relaties (QSRRs). De toepassing van deze methoden voor kolomkarakterisering wordt verder in het proefschrift beschreven. Tevens wordt de invloed van de mobiele fase samenstelling op de RPLC analyse van basische verbindingen en het gebruik van chemometrische technieken voor kolomklassifikatie besproken.

Hoofdstuk 3 beschrijft de ontwikkeling van een empirische kolomkarakteriseringsmethode. Sektie 3.1 beschrijft de scheiding van een set basische verbindigen. Deze verbindingen zijn geselekteerd op basis van verschillen in basiciteit (pKa), polariteit, aantal stikstof atomen en verschillende typen stikstof atomen. Gebruik makende van een μBondapak C_{18} kolom zijn verschillende variabelen onderzocht. Zowel de pKa als struktuur eigenschappen van de basische verbindingen hebben invloed op de piekasymmetrie. Toevoeging aan de mobiele fase van verbindingen die de silanolaktiviteit onderdrukken heeft een gunstig effekt op de piekvorm, terwijl het effekt van ion-paar vormers te verwaarlozen is. Sektie 3.3 laat zien dat vooral de toevoeging van N,N dimethyloctylamine aan de mobiele fase buffer pH 3 resulteert in symmetrische pieken. Van de set onderzochte kolommen blijkt met name een elektrostatisch afgeschermde kolom goede resultaten te geven. Symmetrische pieken worden ook verkregen als polymere kolommen worden gebruikt. Echter door de lage schotelgetallen, wat typerend is voor dit soort kolommen, is de scheidingsefficientie klein.

Sektie 3.2 beschrijft het gebruik van chemometrische technieken om *i*: het aantal testverbindingen te reduceren, en *ii*: kolomeigenschappen grafisch weer te geven. Met behulp van principale componenten analyse (PCA) en de gegevens van sektie 3.1 is het aantal testverbindingen teruggebracht tot 5. Deze set verbindingen wordt in ons laboratorium gebruikt om commercieel verkrijgbare kolommen te karakteriseren. Voor de grafische weergave van de kolomeigenschappen zijn staafdiagrammen, multi criteria decision making en bi-plots vergeleken. Voor de weergave van een groot aantal kolommen blijkt met name bi-plots (en dus ook PCA) een geschikte techniek.

Sektie 3.3 laat de toepassing van de ontwikkelde testprocedure zien voor de karakterisering van 14 commercieel verkrijgbare RPLC kolommen.

In de literatuur zijn vele kolomtesten beschreven. De meeste van deze testen zijn ontwikkeld om algemene kolomeigenschappen te bepalen. Hoofdstuk 4 beschrijft de toepassing van een aantal van deze testen om kolommen te karakteriseren voor de analyse van basische verbindingen. In sektie 4.1 worden een aantal van deze empirische testmethoden vergeleken. Een set van 7 RPLC kolommen is

gekarakteriseerd volgens de methoden van Engelhardt, Tanaka, Galushko en McCalley. Diezelfde set kolommen is eveneens gekarakteriseerd met basische verbindingen van NV Organon, gebaseerd op de test die is beschreven in hoofdstuk 3. Om een breder pKa gebied te omvatten zijn nu zeven verbindingen van pKa < 3 tot pK 8.7 gebruikt. Het blijkt dat testprocedures die gebruik maken van verbindingen zoals aniline, fenol of benzeen weinig bruikbaar zijn voor de selektie van RPLC kolommen voor de analyse van basische verbindingen. De resultaten van de McCalley testprocedure zijn wel bruikbaar om kolommen voor de analyse van deze verbindingen te selekteren. Hieruit blijkt dat testmethoden waarbij gebruik gemaakt wordt van basische verbindingen het meest geschikt zijn om kolommen voor de analyse van basische farmaceutica te karakteriseren. Bovendien blijkt ook dat voor verschillende verbindingen het type buffer invloed heeft op de piekvorm. Hieruit volgt dat kolommen niet alleen getest moeten worden bij verschillende pH's maar ook met verschillende typen buffer zouten.

In sektie 4.1 wordt ook de invloed van de samenstelling van de mobiele fase op de RPLC analyse van basische verbindingen beschreven. Door toevoeging van b.v. 50% methanol blijkt de pH van het eluens in het pH gebied 3 - 7 met ongeveer 1 pH eenheid toe te nemen. Dit effekt is ook waargenomen voor andere modifiers. Tevens wordt de buffercapaciteit van het eluens beinvloed door toevoeging van methanol en andere modifiers. Behalve invloed op het eluens blijkt hoeveelheid en aard van de modifier ook invloed te hebben op de pKa van de basische verbinding. Voor b.v. mirtazipine verandert de pKa 0,7 eenheden na toevoeging van 50% methanol of acetonitril. Na toevoeging van 50% tetrahydrofuraan verandert de pKa zelfs meer dan 1 eenheid.

In sektie 4.2 wordt de karakterisering van kolommen met behulp van thermodynamische data beschreven. Voor het verkrijgen van een beter inzicht in het retentiemechanisme zijn van 't Hoff plots gebruikt. Voor de onderzochte set van RPLC kolommen werden lineaire plots verkregen. Hieruit blijkt dat in het onderzochte temperatuurgebied het retentiemechanisme niet verandert. De negatieve enthalpie waarden geven aan dat de interaktie tussen basische verbindingen en een RPLC stationaire fase een exotherm proces is. Zowel de enthalpie als entropie waarden bevatten informatie omtrent de verschillen in interaktie van geprotoneerde en neutrale verbindingen met RPLC kolommen. De duidelijke verschillen in piekvorm die verkregen zijn met de kolommen konden hiermee echter niet verklaard worden.

Sektie 4.3 beschrijft de toepassing van QSRRs voor kolomkarakterisering. De QSRRs laten zien welke descriptoren betrokken zijn bij de retentie van basische verbindingen op RPLC kolommen. De descriptoren molaire refraktie, laagste onbezette moleculaire orbitaal, polair oppervlakte en aantal proton donoren blijken het meest signifikant. Bovendien laten de regressiecoefficienten van polair oppervlakte en molaire refraktie verschillende trends zien bij gebruik van ammoniumacetaat en natriumfosfaat buffers. Hieruit blijkt de invloed van het type

bufferzout op de scheiding van basische verbindingen. De grootte van de betrouwbaarheidsintervallen van de regressiecoefficienten laten echter niet toe dat de verschillen tussen de verschillende RPLC kolommen kwantitatief en eenduidig beschreven kunnen worden.

De data die beschreven zijn in hoofdstukken 3 en 4 zijn verkregen met 4,6 mm I.D. kolommen. Tegenwoordig is er een groeiende belangstelling om kolommen met kleinere diameters te gebruiken, b.v. diameters ≤ 1 mm (micro LC). De belangrijkste redenen om micro LC uit te voeren zijn de beschikbare hoeveelheid monster en koppeling met massaspectrometrie (MS). In hoofdstuk 5 wordt beschreven hoe LC methoden die zijn ontwikkeld met conventionele kolommen overgezet kunnen worden naar methoden met micro LC kolommen. Sektie 5.1 laat zien dat de robuustheid van micro en conventionele kolommen (0,3, 1,0 en 4,6 mm I.D.) vergelijkbaar is. Vergelijking van mobiele fasen die vluchtige buffers bevatten (ammoniumacetaat en ammoniumcitraat) met een mobiele fase die een niet vluchtige buffer bevat (natriumfosfaat) laat zien dat de meest symmetrische pieken worden verkregen met een ammonium citraat buffer bij pH 3. De in sektie 5.1 beschreven resultaten geven geen duidelijk inzicht omtrent faktoren die belangrijk zijn voor de vergelijking van conventionele en micro LC systemen. Daarom zijn een aantal variabelen verder onderzocht. De resultaten hiervan zijn in sektie 5.2 beschreven.

Sektie 5.2 laat zien dat de verschillen tussen batches van eenzelfde type pakkingsmateriaal geen invloed hebben op de retentie van basische verbindingen. Voor sommige basische verbindingen blijkt echter dat de verschillen tussen batches van eenzelfde type pakkingsmateriaal een signifikant effekt heeft op de piekvorm. Tevens heeft het type buffer zout een effekt op de piekvorm. De kolom hardware en diameter hebben geen invloed op retentie, efficientie en piekvorm. Voor de pakkingsdichtheid zijn er wel aantoonbare verschillen tussen micro en conventionele kolommen. Voor de glass lined kolommen is de interstitiele porositeit (ε_i) 0,39 voor de 4,6 mm I.D. kolom. Voor de 1,0 en 0,32 mm I.D. kolommen is ε_i respectievelijk 0,41 en 0,44. Een grotere interstitiele porositeit betekent dat langere kolommen kunnen worden gebruikt bij gelijke tegendruk. Het resultaat is scheidingen met betere efficientie. Afsluitend kan er geconcludeerd worden dat vergelijkbare scheidingen worden verkregen met micro en conventionele LC kolommen, mits de kolommen gepakt zijn met dezelfde batch pakkingsmateriaal.

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CURRICULUM VITAE

Ruud Johannes Maria Vervoort is geboren op 21 november 1961 in Afferden, Limburg. In 1979 behaalde hij het HAVO diploma aan het Elzendaalcollege te Boxmeer. In 1983 werd het diploma analytisch chemisch analist HBO-B behaald aan de Analistenschool te Venlo. In 1992 is de verkorte HLO opleiding aan de Hogeschool Arnhem afgerond.

Sinds januari 1984 is hij werkzaam bij NV Organon te Oss. Na drie jaar werkzaam te zijn geweest bij de afdeling Quality Assurance is hij overgestapt naar het huidige Department of Analytical Chemistry for Development. Sinds 1997 is hij groepsleider in de sectie Physical and Chromatographic Analysis.

Het in dit proefschrift beschreven onderzoek is gestart in 1990. Vanaf 1997 neemt de sektie vloeistof chromatogafie, onder leiding van dr. Claessens, van de vakgroep Instrumentele Analyse van de Technische Universiteit Eindhoven, hieraan deel. Hoofd van deze vakgroep is prof. Cramers, bij wie dit promotie onderzoek is afgerond.

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