Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1036 (2004) 135–143
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Monolithic silica columns with chemically bonded *tert*-butylcarbamoylquinine chiral anion-exchanger selector as a stationary phase for enantiomer separations

Dieter Lubda [a,*], Wolfgang Lindner [b]

[a] *Merck KGaA, LSP R&D MDA, Frankfurter Stasse 250, Darmstadt D-64271, Germany*
[b] *Institute of Analytical Chemistry, University of Vienna, Währinger Str. 38, A-1090 Vienna, Austria*

Received 11 November 2003; received in revised form 2 March 2004; accepted 5 March 2004

Abstract

An enantioselective silica rod type chiral stationary phase (CSP) is presented as a novel combination of the well-known enantiomer separation properties of immobilized *tert*-butyl-carbamoylquinine chiral anion-exchanger selector with the unique properties of monolithic silica material. The chromatographic behavior of the *tert*-butyl-carbamoylquinine silica rod was studied and compared with a similar prepared particulate material. Good selectivities were achieved for a spectrum of chiral test components like N-derivatized amino acids (DNB- Ac-, DNZ-, Bz-, Z-amino acids) and for Suprofen. The influence of mobile phase parameters, as well as the effect of serially coupling up to six 10 cm monolithic silica columns was studied and put in context to conventional columns of particulate 5 μm type CSP. Using that 60 cm long monolithic column it was possible to improve the enantiomer separation of Suprofen and achieve a baseline separation in less than 10 min of total separation time.

Keywords: Enantiomer separations; Chiral stationary phases, LC; Column coupling; Monolithic column; *tert*-Butyl-carbamoylquinine; Silica

1. Introduction

The separation of enantiomers by high-performance liquid chromatography (HPLC) has proven to be one of the most useful methods for the analytical analysis up to preparative scale separations of numerous differently structured chiral compounds. Among diverse separation concepts, enantioselective HPLC became a very effective method for the resolution of racemic drugs or chiral synthons but also to control the enantiomeric excess (ee) down to levels of 99.9:0.1. Today it is still a growing field due to the importance of enantiomerically pure drugs, intermediates, and fine chemicals.

Several articles deal with the wide variety of chiral stationary phases (CSPs), many of them commercially available, and with the attempts to interpret how these chiral stationary phases may operate with respect to the underlying molecular recognition mechanisms in analytical or preparative chromatography [1].

Among the various chiral stationary phases the high selectivity of chemically bonded quinine (QN) and quinidine (QD) derivatives as chiral anion exchanger selectors for enantiomer separations of chiral acids has proven validity [2,3]. One of the chiral selectors, namely *tert*-butylcarbamoylquinine (*t*-BuCQN), was found to offer in its immobilized form as chiral stationary phase a high enantioselectivity for N-protected amino acids and other acids with a very good success rate.

Applications of *t*-BuCQN type CSP based on porous silica particles for separations of positional and stereoisomers have received significant attention in practice.

A new approach to develop faster and high throughput HPLC methods can be attributed to the use of monolithic columns composed of a continuous bed with through-pores of either organic or inorganic skeleton matrix. Over the last years different research groups [4–7] have tested organic and inorganic monolithic materials for their use in HPLC.

Nakanishi and Soga [8] developed a novel sol–gel process for the preparation of monolithic silica columns with a bimodal pore structure (i.e. with through-pores and mesopores). The method is based on the hydrolysis and poly-

* Corresponding author. Tel.: +49-6151-72-7642; fax: +49-6151-72-5096.
E-mail address: dieter.lubda@merck.de (D. Lubda).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.03.016

condensation of alkoxysilanes in the presence of water-soluble polymers. Tanaka et al. [9] demonstrated that such silica materials could also be surface modified eq. to RP materials, thus allowing the preparation of chromatographic columns with high efficiencies and low column backpressures as a result of an independent control of the sizes of the silica skeleton and the through pores. The hydrodynamic behavior of silica-based monoliths (and monoliths in general) can be expressed in terms of equivalent particle (sphere) dimensions [10] for the permeability (d_{perm}) and band broadening (d_{disp}).

Regarding enantiomer separation a number of different approaches to use enantioselective monolithic columns particularly in the capillary format in CEC have been summarized in a review of Lämmerhofer et al. [11]. Along this line the preparation of chiral polymeric organic monoliths incorporating the cinchonane type chiral selector moiety has been reported by copolymerizing a mixture of methacrylates consisting of a chiral monomer, comonomers, and a crosslinker within the confines of a fused-silica capillary [12,13]. Chen et al. [14,15] published a series of papers, which developed chiral monolithic silica column with chemically modified ligand exchange stationary phase for micro-LC and CEC.

First results to bridge the silica-based monolithic technology of conventional format (4.6 mm i.d.) with the development of silica-based CSPs employing β-CD as chiral selectors show encouraging results [16], leading to similar performances as comparable porous particle based columns but with the advantage to assure faster enantiomer separation.

In order to extend this promising concept of monolithic CSPs, in the conventional or capillary format, we want to describe in this work the preparation of *tert*-butylcarbamoylquinine based monolithic silica columns and to characterize their performance.

Thus, focus was given to the evaluation of these columns, with respect to speed of eluent flow, and eluent compositions in comparison to a conventional silica particle based CSPs column. Finally, the opportunity to couple several monolithic columns in series to improve the overall total plates numbers and thus the resolution of a racemate will be shown.

2. Experimental

2.1. Reagents and instrumentation

Chromolith® Performance Si 100 mm × 4.6 mm silica monolithic columns cladded with PEEK are commercially available from Merck KGaA (Darmstadt, Germany). The (8*S*,9*R*)O-9-(*tert*-butyl-carbamoyl)-quinine (*t*-BuCQN) as well as the chiral test compounds were contributed from the University of Vienna (Lindner group) and were used without further purification. The preparation of the *tert*-butyl-carbamoylquinine has been described previously [17]. α,α′-Azo-bis-isobutyronitril (AIBN) and dry pyridine, all of analytical grade, were purchased from Merck KGaA. The 3-mercaptopropyl-trimethoxy-silane was from ABCR-GmbH Karlsruhe, Germany. Analytical grade dry toluene was used as solvent for the synthesis of the CSP. Mobile phases for chromatography were prepared from glacial acetic acid and ammonium acetate of analytical grade, and methanol and HPLC water (both of LiChrosolv® grade) were purchased from Merck KGaA. The mobile phases were filtered through a 0.2 μm nylon membrane filter and degassed by sonification prior to use.

HPLC experiments were performed with a Hitachi-Merck LaChrom® HPLC system which consisted of L-7100 intelligent pump, L-7420 UV-Vis detector, L-7200 autosampler, L-7300 Column Oven, and HSM chromatography data station software for the processing of raw-data, all from Merck KGaA. The HPLC columns were kept at a constant temperature of 25 °C. The mobile phase compositions are specified in the tables and figures, respectively. All void times for the calculation of the k' values were measured by injecting thiourea, solved in the eluent mixture, detecting the retention time. The specific surface area of the silica monoliths was determined by measurement of the nitrogen adsorption and desorption isotherms using the ASAP 2400 instrumentation from Micromeritics. The specific surface area (S_{BET}) was calculated according the theory of Brunauer, Emmett, and Teller (BET).

2.2. Preparation of a silica monolith based t-BuCQN CSP column

Conceptually there are two fundamental ways to modify silica gels used as chromatographic stationary phases. In most cases, particulate silica materials are first chemically modified in so-called batch reactions followed by the packing of the modified materials into the chromatographic column. For such a reaction scheme the particles are dispersed in a solvent and after addition of a reaction partner (in most cases a silane), the suspension is refluxed under inert gas atmosphere for several hours following extensive washing steps.

The second approach refers to the in-situ modification of unmodified silica gel already packed into the stainless steel tubing; the in-situ modification is then performed by pumping the reaction-partner solutions at elevated temperature through the column. Although this approach seems to be straightforward, several disadvantages such as the risk of a lower reproducibility, problems in scale-up and danger of damaging the hardware after using aggressive reaction-partners have to be encountered.

However, in comparison of the modification of particulate materials the chemical modification of monolithic silica supports has to be realized more or less exclusively by the through pump and thus in-situ way.

That this concept is possible was proven and confirmed by comparing both methods for the preparation of a monolithic β-CD silica-based CSPs [16]. As a consequence, the in-situ modification process for monolithic silica supports seems

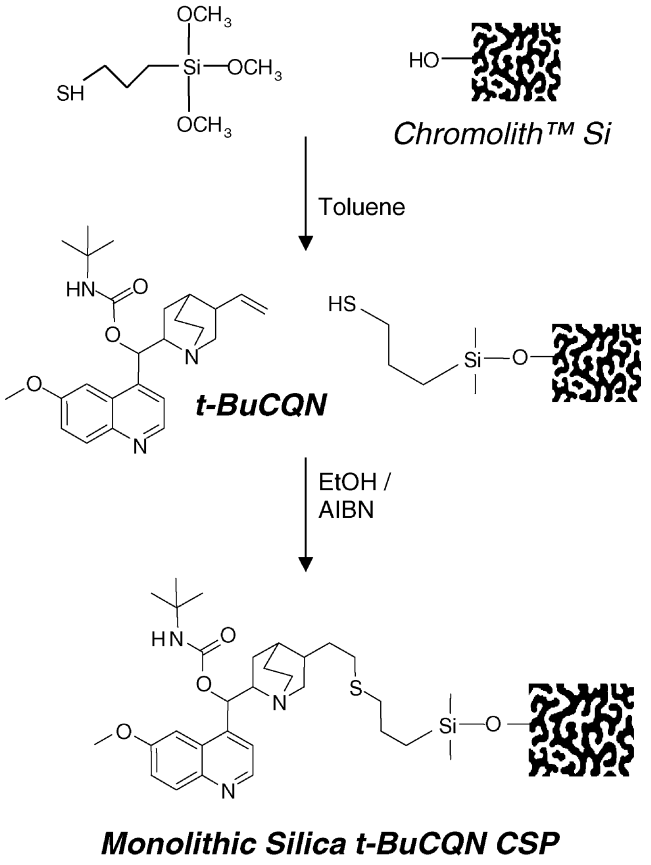

Fig. 1. Preparation strategy for the monolithic silica *t*-BuCQN chiral stationary phases utilized for the present study.

more demanding, particularly for a multi step-wise modification process, as it is the case in the preparation of *t*-BuCQN monolithic stationary phases or silica beads (Fig. 1).

2.3. In-situ modification of silica monoliths

2.3.1. Preparation of a 100*mm* × 4.6 *mm silica monolith column modified with mercaptopropyl groups (SH)*

Prior to the reaction, a Chromolith® Performance Si 100 mm × 4.6 mm column (10 cm in length containing approximately 0.5 g of silica gel), encased in a PEEK plastic cover, was dried for 5 h under vacuum at 100 °C. The surface modification of the dried Chromolith® Si column was carried out with 10 ml of a solution of 0.7 ml (corresponding to a concentration of 24 μmol/m^2 calculated on the measured specific surface area of 300 m^2/g of the unmodified silica rod) of 3-mercaptopropyltrimethoxysilane in dry toluene, containing 0.2 ml of dry pyridine, by pumping the reaction solution through the monolith at a volumetric flow rate of 0.2 ml/min for 1 h. During the reaction, the monolithic silica column was stored inside an HPLC column oven at 80 °C.

The mercaptopropyl modified silica rod was washed with toluene for 1 h at 80 °C at a volumetric flow rate of 0.5 ml/min and one additional hour at a volumetric flow rate of 0.5 ml/min at ambient temperature.

The surface coverage of mercaptopropyl ligands (α SH) was obtained by using Eq. (1), which has been introduced by Jeroniec et al. [19].

$$\alpha\,\mathrm{SH}[\mathrm{mol/m^2}] = \frac{[P_C/M_{\mathrm{Element}} \times 100 \times n_C - P_C(M_{\mathrm{Ligand}} - 1)]}{S_{\mathrm{BET}}} \quad (1)$$

Where P_C, P_N, and P_S are the percent carbon, nitrogen, or sulfur number measured in the mercaptopropyl phase; M_{element}, the mass of the calculated element and M_{ligand}, the molecular mass of the attached ligand, n_C, n_N, and n_S are the numbers of carbon, nitrogen, or sulfur atoms per bonded ligand, S_{BET} is the specific surface area in meter square per gram of the unmodified silica.

Elemental analysis of C was 4.3% and calculation of the surface coverage (per meter square of unmodified silica) of mercaptopropyl ligands bonded to the surface of the modified silica rod reached α SH = 4.54 μmol/m^2 revealing that the surface modification was successful. For measuring the surface coverage within different parts of the monolith, we took three parts from the top, middle, and bottom of the in-situ modified mercaptopropyl silica monolith and measured the amount of bounded spacer using elemental analysis. Due to the fact that we found for the elemental analysis with C = 4.2% within the top part, C = 4.3% in the middle, and C = 4.3% on the bottom a satisfying homogeneity of the surface modification over the length and diameter of the mercaptopropyl silica monolith was accomplished.

2.3.2. Preparation of a silica monolith modified with *t-BuCQN in a* 100*mm* × 4.6 *mm column*

The *t*-BuCQN stationary phase was prepared by a reaction of the above described mercaptopropyl modified silica monolith SH 100 mm × 4.6 mm column with a solution of (8*S*,9*R*)O-9-(*tert*-butyl-carbamoyl)-quinine using a radical addition procedure. *t*-BuCQN (0.10 g) [MG = 423 g/mol] (corresponding to approximately 500 μmol/g silica) and 10 mg AIBN were dissolved in 6 ml ethanol and then flushed through the ethanol pre-washed silica monolith SH column kept at 80 °C over a period of 100 min. In order to remove the unreacted amount of modifying reagents, the column was flushed with ethanol at a volumetric flow rate of 0.5 ml/min for an additional 60 min at room temperature.

Elemental analysis of C = 10.5% (increase of 6.2% in the carbon content) and calculation of the surface coverage (per meter square of SH modified silica) by taking just the *t*-BuCQN ligands into account have shown that 0.76 μmol/m^2 of the *t*-BuCQN could be bonded to the surface of the SH pre-modified silica rod. This corresponds to a coverage rate of surface modification with *t*-BuCQN of about 20% based on the calculated amount of mercaptopropyl groups.

3. Results and discussion

Over the last years a set of different cinchona alkaloid based chiral selectors with various modifications have been developed and used for the chromatographic separation of various compounds [2,15]. In the present study, we investigated the combination of a monolithic support modified to a quinine-based CSP and compared its chromatographic efficiency of similarly modified particulate materials.

One goal of this work was also to combine the unique behavior of monolithic silica columns namely to take advantage use of the low column backpressure to allow the coupling of several columns together to realize a higher total number of plates to achieve better resolutions of critical substance pairs. By counting on an additional optimization parameter, namely speeding up the flow rate without too much loss of efficiency by employing a quasi 60 cm long stacked monolithic column, fast separations should be achievable.

3.1. Comparison of monolithic and particulate silica t-BuCQN CSPs

Elemental analysis of a 5 μm particulate material, prepared using a similar method as for the monolithic silica described before, has shown a carbon content for the mercaptopropyl modification of C = 4.7%, and corresponds to a surface coverage of 4.9 μmol/m^2 bonded mercaptopropyl spacers onto the surface. Further a radical addition procedure, using *t*-BuCQN, leads to an increase of 7.5% in carbon content and gave a calculated content of 0.91 μmol/m^2 (or 273 μmol/g) *t*-BuCQN spacer bonded. In contrast to the immobilization protocol of particulate material we were not yet able, to reach this high level for the in-situ modification of the mercaptopropyl pre-modified monolithic silica. However, there is room for further optimization of the immobilization protocol if needed, as the reached coverage was sufficient to prove the principle.

Although the total porosity of the silica monolith is with about 80% higher in comparison with a separation column packed with particles (ca. 65%) the total amount of *t*-BuCQN, which could be bound to the surface of the monolith, was only 120 μmol/g and thus less than half of that value reached with a particular material.

In order to be able to get an idea of the maximum surface coverage of the monolithic and particulate prepared silica *t*-BuCQN CSPs we tried to calculate, on the base of the structure of the mercaptopropyl spacer as well as for the *t*-BuCQN, the needed surface area and volume of these both part of the modification. Using the Spartan software (Version 5.0) from Wavefunction Inc. (Irvine, CA) we obtained for the mercaptopropyl spacer a volume of 102 Å^3 and a surface area of 127.4 Å^2. The *tert*-butyl-carbamoylquinine was calculated to need a surface area of 487.1 Å^2 covering a total volume of approximately 500 Å^3 assuming a non-solvated status. At this point we have to mention, that the calculated values are only valid for one structural conformation and have thus to be handled with care. By only taking into account that the *t*-BuCQN needs about four times more space in comparison to the thiol-spacer, a maximum concentration on the surface of the silica materials in the range of 1 μmol/m^2 seems to be possible as we used a mercaptopropyl modified support with a surface coverage of about 4 μmol/m^2 thiol-groups for the preparation of the CSP. Due to this result, the modification of the particulate material was nearly complete (as calculated before with 0.91 μmol/m^2 *t*-BuCQN-groups) and should cover the remaining thiol-groups in a more sufficient way as this can be the case for the silica monolithic CSP. However, the non-modified remaining mercaptopropyl groups do not interfere with the overall and enantioselective binding events with chiral acids, as shown previously [18].

3.2. Reproducibility of the surface modification of the t-BuCQN monolithic silica CSP

The reproducibility of the surface modification of a stationary phase is an often-discussed topic and an important requirement for the transfer and acceptance of the used methods to industrial laboratories. In order to check if the multi step in-situ modification method and protocol (used for the preparation of the monolithic CSP) was reproducible enough, we compared seven *t*-BuCQN monolithic silica columns. For the evaluation of the lot-to-lot reproducibility,

Table 1
Reproducibility of the in-situ preparation procedure of seven *t*-BuCQN modified monolithic silica columns using acetyl-phenylalanine as a test sample

Column	k'		N (m)		α	Backpressure (bar)
	Ac-D-Phe	Ac-L-Phe	Ac-D-Phe	Ac-L-Phe		
Lu1517	2.09	3.22	56,970	60,230	1.55	16
Lu1519/1	2.10	3.24	48,210	50,660	1.54	15.5
Lu1519/2	2.04	3.15	48,320	48,310	1.54	16
Lu1519/3	2.08	3.21	48,630	51,610	1.54	16
Lu1521/1	2.23	3.48	52,600	61,500	1.56	16
Lu1521/2	2.22	3.47	53,800	62,200	1.56	16.5
Lu1521/3	2.24	3.45	56,670	63,910	1.54	17

Conditions: CSP, *t*-BuCQN based CSP silica monolith; mobile phase, MeOH / glacial acetic acid 99/1 (v/v); temperature, 25 °C; flow rate, 1 ml/min; Injektionvol., 10 μl.

we performed two more preparations by modifying three columns in parallel using a crosspiece.

As test samples racemic *N*-acetyl-phenylalanine and a mixture of methanol/glacial acetic acid 99:1 (v/v) as a mobile phase with a flow rate of 1 ml/min was used. The experimental results of the different fully characterized monolithic CSP are summarized in Table 1.

The column-to-column reproducibility of the surface modification within one preparation of three parallel-coupled monoliths is in good agreement with each other and also between different preparation campaigns. The lot-to-lot reproducibility has shown a very acceptable variation proving that the modification protocol is based on robust conditions.

3.3. Comparison of hydro-organic and polar–organic mobile phases

The chromatographic characterization of the prepared enantioselective *t*-BuCQN silica rods was performed using different mobile phases. For the polar–organic mode, non-aqueous conditions were adopted employing methanol as mobile phase with glacial acetic acid as an acidic modifier, which represents the counter-ion in the ion-exchange process being necessary to facilitate elution within reasonable time. In the hydro-organic mobile phase, a methanol/water acetate buffer eluent (pH adjusted) was used.

For the comparison with a particulate *t*-BuCQN material, a spectrum of chiral test components like racemic N-derivatized amino acids (DNB- Ac-, DNZ-, Bz-, Z-amino acids) and Suprofen were selected. Due to the selector–selectand model of the *tert*-butyl-carbamoylquinine modification, depicted in Fig. 2, using *N*-(3,5-dinitrobenzoyl)leucine as model enantiomer there seem at least four different interactions possible; (1) ionic interaction, (2) hydrogen bond, (3) π–π-interaction, (4) steric interaction via van der Waal increments. The respective chromatographic results and conditions are summarized in Table 2 for the particulate *t*-BuCQN CSP and the *t*-BuCQN monolithic silica CSP.

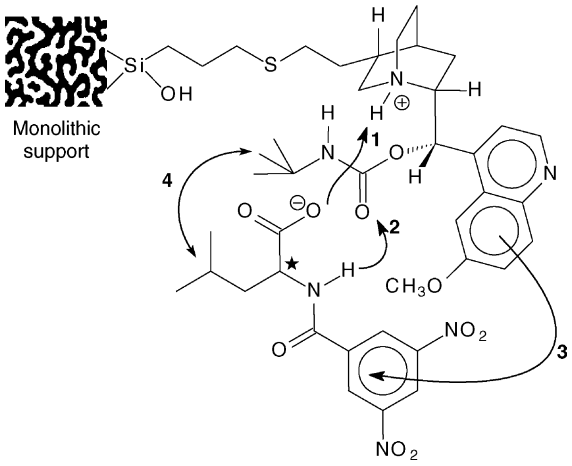

Fig. 2. Selector–selectand interaction model of *tert*-butyl-carbamoyl-quinine (1*S*,3*R*,4*S*,8*S*,9*R*) modification and *N*-(3,5-dinitrobenzoyl)leucine. (1) ionic interaction; (2) hydrogen bond; (3) π–π-interaction; (4) steric interaction.

The chromatographic tests of both *t*-BuCQN CSP columns (monolithic and particulate) indicated that their enantioselectivities are basically comparable. Using the same mobile phase condition the calculated retention factors, k_1 and k_2, were lower on the *t*-BuCQN monolithic silica CSP then on the particulate column, which is most probably directly correlated with the selector density bound per surface unit (m^2). Using methanol/acetic acid for the separation, the retention factors of the *t*-BuCQN monolithic silica CSP were nearly half in comparison with the particulate CSP corresponding to the ca. 1:2 *t*-BuCQN selector coverage. The decrease in the value of the retention factor was even more pronounced in the methanol/acetate buffer.

From the experiments with the hydro-organic mobile phase in comparison with the polar–organic mobile phases, it became evident that the observed separation and/or the efficiency values may be influenced by diverse factors. Since selector–analyte interactions are widely of an electrostatic

Table 2
Enantiomer separation of a spectrum of chiral test components like N-derivatized amino acids (DNB- Ac-, DNZ-, Bz-, Z-amino acids) and Suprofen

Substances	Hydro-organic mobile phases						Polar–organic mobile phases					
	Particulate column			Monolithic column			Particulate column			Monolithic column		
	k'_2	α	N_2 (1/m)	k'_2	α	N_2 (1/m)	k'_2	α	N_2 (1/m)	k'_2	α	N_2 (1/m)
Z-DL-Leu	7.68	1.29	33,696	2.56	1.26	38,560	3.83	1.32	39,584	1.83	1.31	88,340
BZ-DL-Leu	12.15	2.52	32,352	4.01	2.41	38,100	8.04	2.62	40,904	3.74	2.55	70,470
DNZ-DL-Leu	33.43	2.88	33,072	10.92	2.37	40,123	15.62	3.00	36,864	7.67	2.86	60,550
DNB-DL-Leu	164.64	16.33	29,384	49.64	13.16	34,910	114.24	16.27	38,880	57.10	15.9	62,540
Z-DL-Phe	13.49	1.21	36,016	4.37	1.19	42,520	3.83	1.32	39,584	1.92	1.30	62,460
Ac-DL-Phe	5.73	1.38	32,576	1.52	1.33	39,362	7.88	1.51	39,528	3.38	1.54	60,230
BZ-DL-Phe	15.74	1.89	34,096	4.72	1.82	36,345	8.04	2.62	40,904	4.01	2.59	66,580
DNZ-DL-Phe	36.93	1.90	33,072	11.52	1.73	38,298	30.16	2.10	43,840	15.67	2.02	68,430
Suprofen	9.57	1.13	31,160	3.01	1.10	35,671	3.64	1.15	41,224	1.93	1.14	69,760

Conditions: CSP, *t*-BuCQN based CSP particulate silica material and *t*-BuCQN based CSP monolithic silica material; mobile phase, comparison of MeOH/glacial acetic acid 99/1 (v/v) and methanol—0.1 M ammonium acetate 80:20 (v/v) pH 6.0; temperature, 25 °C; flow rate, 1 ml/min; Injektionvol., 10 μl.

nature, polar–organic conditions (non-aqueous) were found to lead to higher efficiencies. Using hydro-organic conditions a hydrophobic interaction increment becomes more pronounced.

However, the enantioselectivity (α) values are relatively similar although slightly better for the polar–organic mode.

Along this line the efficiencies of the particulate *t*-BuCQN CSP columns were found to be in the same range for hydro-organic and polar–organic mobile phases being also slightly higher for the polar–organic eluents. For the monolithic silica *t*-BuCQN CSP columns, much higher efficiencies and equal or better selectivities were obtained with the polar–organic mode.

The contribution of the underlying remaining mercaptopropyl groups is not fully elucidated, but it seems rather small as the ion-pairing, π–π, hydrogen bounding and hydrophobic interactions are clearly the dominating ones.

3.4. Dependence of efficiency from the eluent flow of particulate and monolith silica CSP

For the application of RP type monolithic silica material columns, it has been shown previously [20] that by increasing the flow rate, the *H*/*u* curve remained relative flat, which results in only a slight decrease in resolution. By combining the monolithic materials with a chiral selector, as β-CD, it was found that the behavior was not so pronounced as for the RP mode separations but the loss of efficiency was still lower as for the comparable particulate β-CD CSP [16]. To be able to optimize the separation time of the enantiomer separation by increasing the flow rate of the eluent, the loss of efficiency as a function of the linear flow (*H*/*u* curve) had to be checked also for the *t*-BuCQN CSPs (Fig. 3).

Comparing a particulate *t*-BuCQN CSP with a particle diameter of 5 μm and a monolithic silica *t*-BuCQN CSP using the enantiomer separation of acetyl-phenylalanine, we were able to get similar separation efficiencies with a minimum plate height of about 20 μm at optimal eluent flow rates. Increasing the eluent flow up to 4 ml/min (5.82 mm/s) leads to a plate height of about 56 μm for the particulate *t*-BuCQN CSP that corresponded to only one third the efficiency of the most efficient flow rate of 0.4 ml/min. The evaluation of the particulate CSP was limited to the maximum flow rate of 4 ml/min due to the limiting backpressure available for the separation of about 200 bars. In contrast to the particulate material, the monolithic CSP column only showed an increase of plate height by using the same flow rate of 4 ml/min from 18 up to 33 μm. Even by using a flow of 7 ml/min, the enantiomer separation of the acetyl-phenylalanine could be attained with a plate height of about 45 μm. The most important result for the further evaluation was that we were able to separate the enantiomers using the monolithic silica *t*-BuCQN CSP, even with a flow rate of 4 ml/min. This offered acceptable efficiencies and at the same time, due to the unique structural properties of the silica monoliths low column backpressures.

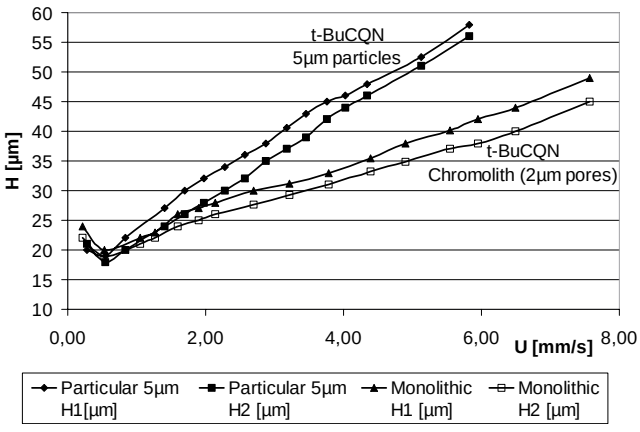

Fig. 3. Comparison of plate height vs. linear flow velocity (*H*/*u* curve) of the mobile phase using a *t*-BuCQN based CSP monolithic silica material and *t*-BuCQN based CSP particulate silica material. Conditions: acetyl-phenylalanine as a test sample; mobile phase, MeOH/glacial acetic acid 99/1 (v/v); temperature, 25 °C; flow rate, from 0.2 to 7 ml/min for the monolithic column and from 0.2 to 4 ml/min for the particulate column.

3.5. Influence of the eluent composition

The effect of the mobile phase composition on retention factor and enantioselectivity was investigated by using methanol with different concentrations of acid as the eluent for the separation of the different compounds as summarized above. This also means that the retention can be readily balanced by the competitive effect of an acidic component equivalent to a counter-ion in the eluent. Here, glacial acetic acid was used as the acidic modifier and was added to methanol in a concentration range of 0.05–2.0% (v/v). In the non-aqueous polar–organic mode, solvophobic interactions are weak, while the electrostatic interactions become dominant although acetic acid can also be classified as polar protic solvent.

It was found, as expected from the results previously published in literature, that the *t*-BuCQN CSP offer very constant separation factor over a wide range of the buffer composition in the eluent [2]. In comparison to the separation factor, the retention factors were however very much affected by the amount of glacial acetic acid used. As summarized in Table 3, we were thus able to easily accelerate the separation of different compounds by using higher concentrations of acetic acid acting as prototic polar solvent with the tendency to also dissociate.

By increasing the concentration of the acetic acid k_2 of 5.69 for Suprofen using methanol/glacial acetic acid 99.95:0.05 (v/v) reduced to 1.23 using methanol/glacial acetic acid 98:2 (v/v). The separation factors stayed nearly constant over the whole range of eluent variations but a slight loss of resolution was found by using higher concentrations of acetic acid.

For the separation time and further optimization of the enantiomer resolution of Suprofen we decided to use an

Table 3
Dependence of retention factors, efficiency and enantioselectivity as well as resolution on the percentage of glacial acetic acid in methanol as mobile phase

Eluent	k'_2	α	N_2 (1/m)	R_s
MeOH/0.05% AcOH	5.69	1.14	65,810	2.25
MeOH/0.1% AcOH	5.04	1.14	65,810	2.17
MeOH/0.2% AcOH	3.86	1.14	69,230	2.11
MeOH/0.5% AcOH	2.95	1.14	67,220	1.95
MeOH/1.0% AcOH	1.93	1.14	69,760	1.74
MeOH/2.0% AcOH	1.23	1.14	66,730	1.38

Conditions: CSP, *t*-BuCQN based CSP monolithic silica material; mobile phase, increase of MeOH/glacial acetic acid 99.05/0.05 (v/v) to and MeOH/glacial acetic acid 98/2 (v/v); temperature, 25 °C; flow rate, 1 ml/min; Injektionvol., 10 μl.

eluent composition of methanol/glacial acetic acid 99.5/0.5 (v/v) as suitable composition.

3.6. Coupling of columns

Since separation time is a major issue today, there is a pronounced trend toward shorter columns filled with small particles (e.g., 3 μm). For complex separations, however, it is still necessary to use long column beds in order to provide the separation efficiency required.

As published before, monolith type columns with reversed phase properties can be run with increased flow rates up to a factor 5–10, leading to much faster separations. Dear et al. [21] investigated the potential of in series coupled alkyl-bonded silica monolithic columns for the isolation and identification of drug-related components in biological fluids. For six columns in series a chromatographic system with more than 40,000 effective plates were achieved. It was reported, that these six coupled columns gave enhanced resolution compared to a high quality silica particulate column packed with 3 μm material, which exhibits the same backpressure.

The reason for the behavior of the monolithic silica columns is an improved bimodal pore size distribution of mesopores and macropores. The macropores of the silica monolith are mainly responsible for the flow resistance. They serve as through pores and enable the substances to be transported with a high flow rate to the activated surface for subsequent chromatographic separation. The skeleton of the monolith contains the surface required for solute–stationary phase interaction.

The goal of this study was to demonstrate the influence of an increase of the effective plate numbers of chirally modified monoliths thus effecting the enantiomer separation of Suprofen.

By linearly connecting six monolithic columns we were able to get from about 7000 plates, for one 10 cm long column, to a total of 30,000 plates, for the 60 cm long column, with a constant separation factor of 1.15. This was only possible, using the same flow rate for both column lengths, at the expenses of elution time, which increased from 10 to 45 min.

The coupling of six columns was possible due to the low backpressure generated by the monolithic silica HPLC columns. The 60 cm long connected monolithic *t*-BuCQN CSP builds up a column backpressure of only 45 bars (Table 4).

The main reason for the reduction of the possible theoretical plate-number by 30% was the loss of efficiency due to the dead volume of the coupling pieces used for the connection of the 10 cm long Chromolith® columns. But even if there was a loss of efficiency due to the set-up of the column coupling we were able to obtain a baseline separation during the chromatographic separation of the Suprofen enantiomers using this 60 cm long stocked separation column.

3.7. Effect of Increasing flow rates

Enantiomer separation on *t*-BUCQN anion exchangers involves multiple and simultaneously active strong specific non-covalent interactions which are associated with high affinity. A proper balance of these interactions is an ultimate requirement for a sufficient chromatographic performance, but which very often shows slow kinetics of interaction for such type of separations.

For the optimization of the separation with regards to analyses time, it was attempted to take advantage of the combination of the usually stable separation factor at higher flow rates accepting a slight decrease in resolution and the unique

Table 4
Dependence of retention factors, efficiency and enantioselectivity as well as column backpressure on the number of coupled silica monoliths

Numbers of coupled column (= length, m)	Suprofen 1		Suprofen 2		α	R_S	Column backpressure (bar)
	k'_1	*N*/column	k'_2	*N*/column			
1 = 0.1	2.43	7,164	2.79	7,025	1.15	2.10	6
2 = 0.2	2.80	12,370	3.18	11,890	1.14	2.56	14
3 = 0.3	2.82	17,570	3.26	17,060	1.16	3.04	22
4 = 0.4	2.83	21,510	3.26	20,560	1.15	3.08	30
5 = 0.5	2.84	25,040	3.27	24,080	1.15	3.32	37
6 = 0.6	2.87	29,680	3.30	27,970	1.15	3.45	45

Conditions: CSP, *t*-BuCQN based CSP monolithic silica material from 10 to 60 cm column length; mobile phase, MeOH/glacial acetic acid 99.5/0.5 (v/v); temperature, 25 °C; flow rate, 1 ml/min; Injektionvol., 10 μl.

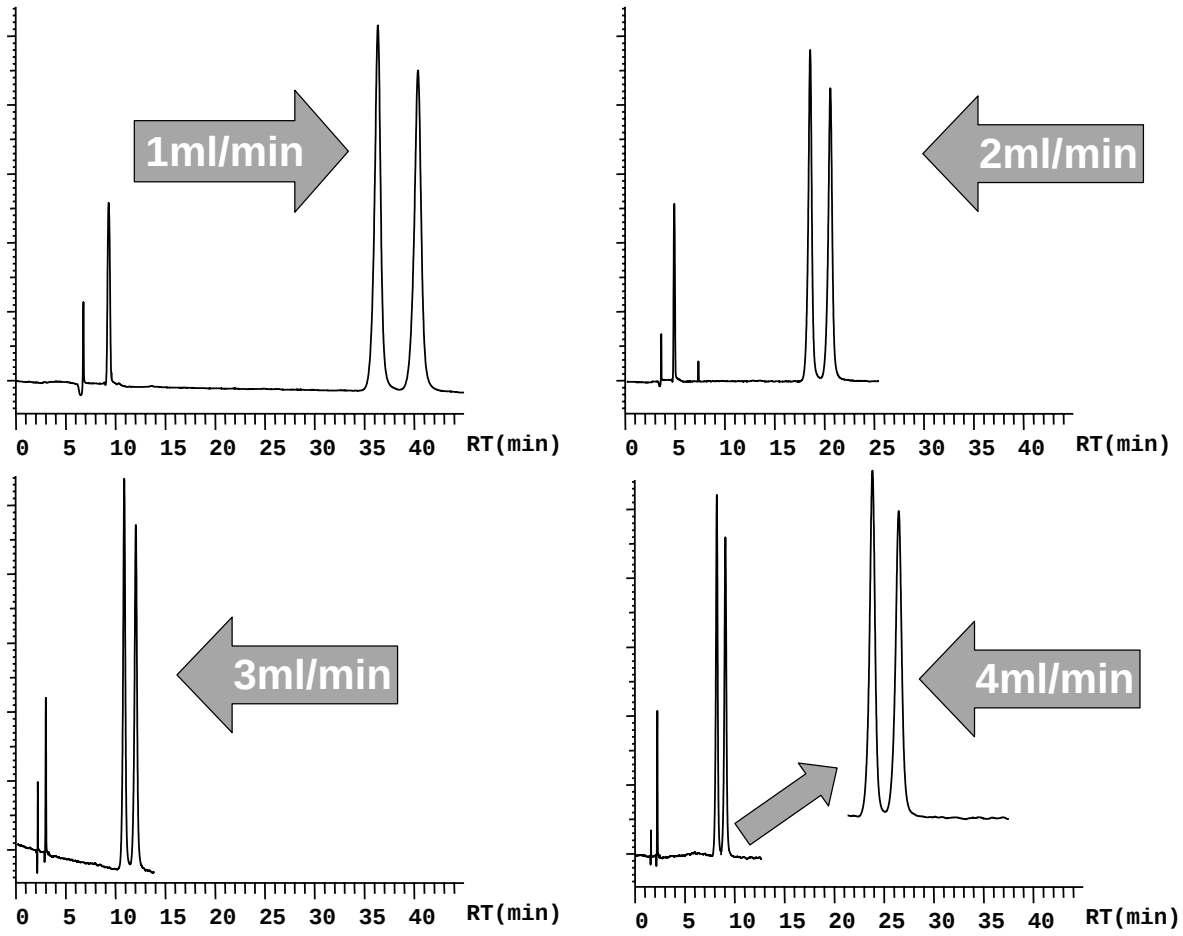

Fig. 4. Effect of increasing flow rate on the separation of Suprofen in MeOH/glacial acetic acid 99.5/0.5 (v/v) using a 60 cm long *t*-BuCQN modified monolithic silica column. Temperature: 25 °C; flow rate: 1–4 ml/min; Injektionvol., 10 μl.

properties of the silica-based monoliths offering a relative flat *H*/*u* curve in comparison with particulate material reported for the RP type system.

In order to reduce the separation time for the analysis of the Suprofen enantiomers, the flow rates were varied from 1 to 4 ml/min using the 60 cm long stacked monolithic column. The respective chromatographic results and conditions can be found in Fig. 4.

By increasing the flow rate from 1 up to 4 ml/min the backpressure rose from 45 to 204 bars but reducing the overall separation time to 10 min achieving a better resolution (resolution of 2.6 and baseline separation) as using only one 10 cm monolithic *t*-BuCQN CSP (resolution of 2.1 but no baseline separation).

4. Conclusions

In this work a concept for the synthesis of *t*-BuCQN type CSP columns based on monolithic silica materials with a reasonable surface coverage is presented. The effect of mobile phase composition, speed of the eluent flow, and linearly coupling of several columns on the retention time and selectivity of different chiral analytes (drugs) was studied.

As expected, the enantioselective silica-based monolith columns (covering a high total porosity) provide, in comparison with the particulate *t*-BuCQN CSPs, a relatively low column backpressure and behave, to a large extent, similar with regard to performance as previously published for non stereoselective RP modified Chromolith® columns [22]. A better separation, within a similar separation time and the same enantioselectivity but with higher resolution factors, was achieved using the 60 cm coupled monolithic *t*-BuCQN CSP by comparing it with the separation of the Suprofen enantiomers using a 10 cm monolithic *t*-BuCQN CSP only.

Acknowledgements

We would like to thank Dr.Cabrera, Mrs. Jung, Mrs. Päsler, and Mr. Kraus from the analytical group of the analytics and reagents department of Merck KGaA for some help during the evaluation of the CSP columns. For the help during the preparation and the analytical tests, we would further thank Dr. Maier and Dr. Lämmerhofer from the

University of Vienna. For their help during the development of the Chromolith® Si silica rods, we would like to thank Dr. Nakanishi from the Dept. of Material Chemistry, Kyoto University and Dr. Minakuchi and Dr. Ishizuka from Kyoto Monotech in Japan.

References

[1] E. Francotte, J. Chromatogr. A 666 (1994) 565.
[2] M. Lämmerhofer, W. Lindner, J. Chromatogr. A 741 (1996) 33.
[3] N.M. Maier, L. Nicoletti, M. Lämmerhofer, W. Lindner, Chirality 11 (1999) 522.
[4] Ch.M. Zeug, J.-L. Liao, K. Nakazato, S. Hjerten, J. Chromatogr. A 753 (1997) 227.
[5] S. Xie, F. Svec, J.M.J. Frechet, J. Chromatogr. A 775 (1997) 65.
[6] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[7] D. Lubda, K. Cabrera, H. Minakuchi, K. Nakanishi, J. Sol–Gel Sci. Technol. 23 (2002) 185.
[8] K. Nakanishi, N. Soga, J. Non Cryst. Solids 139 (1–13) (1992) 14.
[9] N. Tanaka, H. Kobayashi, K. Nakanishi, H. Minakuchi, N. Ishizuka, Anal. Chem. 420 (2001) A-429.
[10] F.C. Leinweber, D. Lubda, K. Cabrera, U. Tallarek, Anal. Chem. 74 (2002) 2470.
[11] M. Lämmerhofer, F. Svec, J.M.J. Frechet, W. Lindner, TRAC 19 (11) (2000) 676.
[12] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Frechet, W. Lindner, Anal. Chem. 72 (2000) 4614.
[13] M. Lämmerhofer, F. Svec, J.M.J. Frechet, W. Lindner, Anal. Chem. 72 (2000) 4623.
[14] Z. Chen, T. Hobo, Anal. Chem. 73 (2001) 3348.
[15] Z. Chen, K. Uchiyama, T. Hobo, J. Chromatogr. A 942 (2002) 83.
[16] D. Lubda, K. Cabrera, K. Nakanishi, W. Lindner, Anal. Bioanal. Chem. 377 (2003) 892.
[17] M. Alexandra, N. Lorenzo, M. Lämmerhofer, W. Lindner, J. Chromatogr. A 858 (1999) 1.
[18] W.R. Oberleitner, N.M. Maier, W. Lindner, J. Chromatogr. A 960 (2002) 97.
[19] C.P. Jaroniec, R.K. Gilpin, M.J. Jaroniec, J. Chromatogr. A 797 (1998) 103.
[20] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[21] G.J. Dear, D.N. Mallett, D.M. Higton, A.D. Roberts, S.A. Bird, H. Young, R.S. Plumb, I.M. Ismail, Chromatographia 55 (3/4) (2002) 177.
[22] D. Lubda, HPLC2001-Lecture Maastricht, 2001.