Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 5, pp. 469–476, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Design and synthesis of a theophylline bonded-phase column for HPLC — application to separation of aromatic carboxylic acids

M. APFEL, L. A. STERNSON,* A. J. REPTA and D. MILLS

Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, USA

Abstract: A stationary phase has been designed and synthesized in which theophylline residues are covalently bonded to a silica support through an eight carbon hydrocarbon linkage. The phase offers improved resolution in the separation of aromatic carboxylic acids over that available with conventional reversed phase supports. The column is relatively stable. Retention can be modified by adjusting mobile phase composition with respect to pH, electrolyte type and concentration, and organic modifier as well as by manipulating the temperature at which chromatography is carried out. The capacity factors, k', for a series of ring substituted benzoic acids were correlated with the complexation constants previously reported for these compounds with theophylline in bulk phase solution.

Keywords: *Theophylline stationary phase; HPLC; aromatic acids.*

Introduction

Retention behaviour in HPLC is based on the relative interactions of a given solute with the stationary and mobile phases. The addition of species to the chromatographic system that exhibit varying degrees of association with the components in a mixture will aid in their overall separation. Most commonly, this has involved the addition of associative species to the mobile phase, e.g. ion-pairing agents, complexing agents. The presence of such agents in the mobile phase may interfere with the detection of eluting bands or complicate the isolation and purification of separated components. Thus, the availability of stationary phases offering a high degree of selectivity for the separation of selected analytes is desirable.

Recently, the authors [1] reported the development of a new stationary phase in which a xanthine base, theophylline, was chemically bonded to a silica support through an amide linkage. This phase was synthesized through the reaction of theophylline-7-acetic acid with a commercially available aminopropyl silica stationary phase. This stationary phase exploited the complexation known to take place [2] between xanthines and substituted benzoic and naphthoic acids and provided the basis for enhanced retention

*Author and mailing address to whom correspondence should be addressed: Smith Kline & French Laboratories, P.O. Box 7929, Philadelphia, PA 19101, USA.

and selectivity in the separation of these analytes, relative to that achieved with common reversed-phase materials. A number of problems were encountered [1] in the routine use of this phase, all apparently attributable to the amide linkage. In aqueous or aqueous–alcoholic mobile phases column lifetime was short due to erosion of the stationary phase. Accordingly, reproducible retention behaviour was difficult to achieve. Furthermore, the interaction between the bonded theophylline and aromatic carboxylic acids that was known to occur [3, 4] through dispersion forces, pi–pi and hydrophobic interactions was overwhelmed by other solute-stationary phase interactions, producing inadequate selectivity. Accordingly, a new stationary phase has been designed and synthesized in which theophylline is covalently bonded to spherical 5-μm silica particles through a hydrocarbon spacer without the need for polar linkages. Its ability to separate substituted benzoic acids by an apparently unique mechanism is the subject of this report.

Materials and Methods

Apparatus

Chromatography was performed using a Waters Associates (Milford, MA) Model 6000A pump, a Model 440 ultraviolet detector operated at 254 nm and a Model U6K injector. Separations were carried out on 30 × 4.6 mm (i.d.) columns slurry packed (with a Haskel air driven fluid pump, Burbank, CA) with either 5-μm ODS-Hypersil (HETP, Cheshire, England) or 5-μm Hypersil silica which had been derivatized with theophylline through an octyl bridge (Si-O-$[CH_2]_8$-*N*-Theo).

Materials

8-Bromo-octyltrichlorosilane was purchased from Petrarch Chemicals (Bristol, PA) and was used as received but stored under argon. All chemicals were of reagent grade and obtained from commercial sources. HPLC solvents were purchased from Fisher (Fair Lawn, NJ) and electrolytes for inclusion in mobile phases were obtained from Mallinchrodt Chemicals (St. Louis, MO). Toluene was dried over calcium hydride, distilled and stored over molecular sieves (4A).

Synthesis of xanthine-modified stationary phase

(a) *8-Bromo-octylsilica.* A three-neck, round bottom flask fitted with a condenser, Drierite drying tube and nitrogen inlet (equipped with an 18 cm Drierite tower) was flame dried. The flask was charged with silica (4.97 g; 6.8 meq SiOH) that had been previously dried at 150°C under vacuum, toluene (10 ml), pyridine (1 ml; 12.4 mmole) and bromo-octyltrichlorosilane (1.45 ml; 5.9 mmole). The solution was heated to 70°C in a Dubnoff shaker and the mixture agitated (140 oscillations/min) for 2.5 h. The silica was allowed to settle, dry toluene (5 ml) was added, and then removed with a pipette. The silica was washed with three 15-ml portions of toluene and after the third wash, the silica was recovered by filtration through a sintered glass funnel. The residue was washed with methanol (2 × 50 ml), water (2 × 50 ml), and then dried overnight at 110°C in a vacuum oven.

(b) *Silylation of 8-bromo-octylsilica.* To a flame dried, three-necked, round bottom flask equipped with a nitrogen inlet and a drying tube, was added pyridine (4.5 ml; 55.9 mmole), 8-bromo-octyl silica (5.3 g) and trimethylchlorosilane (6 ml; 47.3 mmole). The

mixture was agitated (140 oscillations/min) in a Dubnoff shaker for 3 h at room temperature. Dry pyridine (15 ml) was added and the product allowed to settle. The liquid layer was removed and the residue washed with pyridine (20 ml). The product, recovered by filtration through a sintered glass filter, was washed with pyridine (5 ml), methanol (2 × 60 ml) and then dried in a vacuum oven at 110°C for 17 h.

(c) *Preparation of sodium theophyllinate.* Theophylline (32.8 g; 0.18 mole) was added to a solution containing sodium hydroxide (7.22 g; 0.18 mole) dissolved in 320 ml of water. The mixture was stirred overnight with a magnetic stirrer at room temperature. The product, which crystallized on cooling, was recovered by filtration through a sintered glass filter and was then dried for 17 h at 110°C in a vacuum oven.

(d) *Theophylline-modified stationary phase.* Sodium theophyllinate (3.64 g; 18 mmole) was suspended in DMF (20 ml). The mixture was heated to 135°C with agitation (140 oscillations/min) and silylated bromo-octylsilica (5.3 g) was added. After allowing the reaction to proceed for 1.5 h, the hot mixture was filtered through a sintered glass filter. The product was washed with hot DMF (4 × 60 ml) and with methanol (2 × 60 ml). The solid was then dried *in vacuo* and submitted for elemental analysis.

Results and Discussion

An attempt was made to design a stationary phase that would be chemically stable and would maximize the interaction between theophylline appended to a silica backbone and solutes known to form associative complexes with xanthine bases. By minimizing the likelihood of solutes retained through other types of interactions, this stationary phase should offer a high degree of selectivity for appropriate solutes. Accordingly, a stationary phase was designed in which theophylline was chemically bonded to silica through an eight carbon hydrocarbon spacer, placing the xanthine in an apolar environment and thus also examining its associative potential.

Stationary phase

Spherical microparticulate 5-μm silica was derivatized by reaction with 8-bromo-octyltrichlorosilane to form the corresponding 8-bromo-octylsilyl ether. After treatment with trimethylchlorosilane to silylate residual exposed silanol groups, the bromo group was displaced by sodium theophyllinate to yield the corresponding theophylline adduct.

Aqueous suspensions of the final product failed to show a visual indication of turbidity with addition of $AgNO_3$ (test solution U.S.P.) indicating that essentially all the bromine had been displaced. Based on elemental analysis, *ca* 1.25 mmole of theophylline was incorporated per gram of silica in the final product. Although coverage was incomplete, efforts to achieve greater coverage were not pursued. The coverage achieved was presumed to be sufficient, particularly in the apolar environment provided by the hydrocarbon spacer, to impart adequate selectivity to the stationary phase. Elemental analysis of the stationary phase, as shown by Table 1, demonstrates that the phase could be prepared reproducibly. This was supported by the chromatographic retention behaviour of test solutes which also indicated that the phase was relatively stable. New columns did undergo an ageing process, during which retention times of test solutes gradually decreased proportionately to stable values. This decrease in capacity factor (k') was probably the result of some reorganization of the bonded layer rather than a loss

Scheme 1

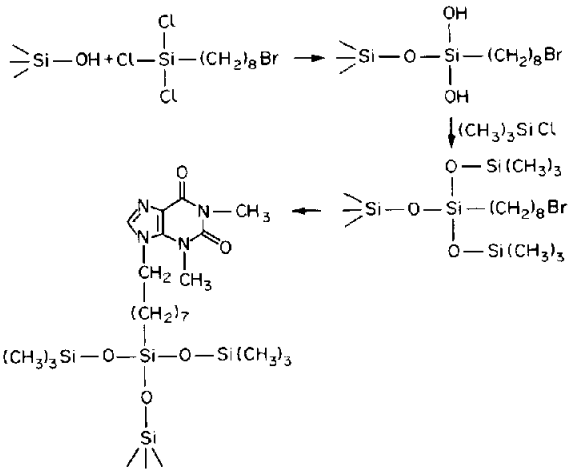

Table 1
Elemental analysis of theophylline-bonded silica phases

%	Batch 1	2	3	2*
C	7.72	7.64	7.80	7.57
H	1.10	1.14	1.30	1.28
N	1.47	1.89	1.90	1.76

* Analysis performed after passing 23 l of mobile phase [K phosphate (5 mM; pH 7) containing methanol (30%)] through a 30 × 4.6 mm column at 1.0 ml/min.

of bonded phase since after passing 22 l of mobile phase (0.5 mM potassium phosphate buffer (pH 7) containing 30% MeOH) through the column at 1.0 ml/min, no change in elemental analysis was observed.

Chromatographic behaviour

The selectivity of the phase was verified by comparing chromatographic retention behaviour on the theophylline phase with that observed on a column of similar dimension packed with ODS. As shown in Table 2, test solutes were retained more strongly on the theophylline phase than on ODS-Hypersil. Relative retention was different on the two phases, supporting the hypothesis that new or additional retention mechanisms were operative with the theophylline phase. Benzoates were retained on the theophylline phase to a greater extent at pH 3 than at pH 7, which is consistent [5, 6] with the pH dependency of theophylline–aromatic acid complexation. Retention decreased with addition of methanol to the mobile phase, consistent with the previously reported [7] effect of organic modifiers on the stability of the xanthine–aromatic acid complexes observed in bulk phase as well as with conventional reversed-phase behaviour. As shown in Fig. 1, addition of methanol to the mobile phase did not effect selectivity, i.e. the order of elution for a series of ring substituted benzoic acid derivatives did not change.

Similar to the effects on column elution observed with methanol, the addition of strong electrolytes to the mobile phase accelerated the elution of test substrates from the

Table 2
Relative retention of solutes on theophylline-bonded phase (TBP) and ODS-Hypersil columns*

Solute	Mobile phase†,‡	k'_{TBP}§	k'_{ODS}§
Benzoic acid	1	2.38	0.36
	2	7.86	1.48
	3	0.94	0.08
p-Hydroxybenzoic acid	1	2.05	0.19
	2	3.83	1.61
	3	0.60	0
p-Chlorobenzoic acid	1	14.39	9.43
	2	4.9	0.54
	3	3.64	0.84
2,4-Dimethylbenzoic acid	1	15.26	9.0
3,4,5-Trihydroxybenzoic acid	1	2.49	0

*Columns were 30 × 4.6 mm and were packed as described under Materials and Methods section.

†Mobile phases: (1) potassium phosphate buffer (5 mM; pH 7); (2) potassium phosphate buffer (5 mM; pH 3) containing 30% methanol; (3) potassium phosphate buffer (5 mM; pH 7) containing 30% methanol.

‡Flow rate: 1 ml/min.

§k' calculated based on $(t - t_o)t_o^{-1}$ where t is the retention time of solute, and t_o is the retention time of the unretained solute D_2O.

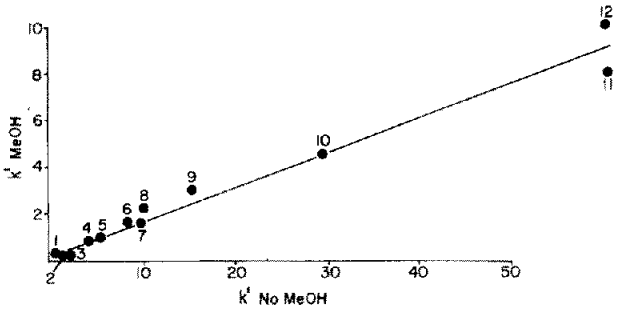

Figure 1
Retention of benzoic acid derivatives on a theophylline-bonded phase column (4.6 × 30 mm) using mobile phases of potassium phosphate buffer (5 mM; pH 7) in the absence and presence of methanol (30% v/v). Flow rate = 1 ml/min. Benzoic acid derivatives: **1** = 2-chloro; **2** = 4-hydroxy; **3** = 3-hydroxy; **4** = 4-methyl; **5** = 3-chloro-4-methyl; **6** = 2,5-dihydroxy; **7** = 3,4-dimethyl; **8** = *p*-chloro; **9** = *p*-bromo; **10** = *p*-iodo; **11** = 3,4-dihydroxy; **12** = 3,4-dichloro.

theophylline phase. Using sodium perchlorate (5 mM) as a representative electrolytic additive, Fig. 2 illustrates that the elution order for a series of analytes was not effected by the presence of electrolyte, but that retention decreases proportionately with addition of salt. Table 3 shows that the strength of the mobile phases increases with salt concentration and that at comparable molarities different salts impart different strengths to the mobile phase. Since no statistical differences were observed in comparing the

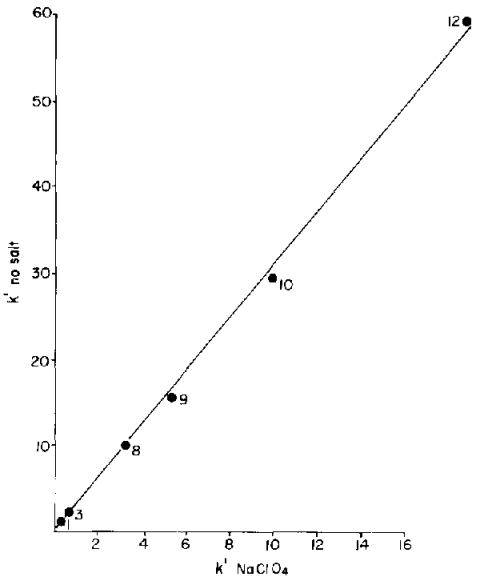

Figure 2
Retention of benzoic acid derivatives on a theophylline-bonded phase column (4.6 × 30 mm) using mobile phases of potassium phosphate buffer (5 mM; pH 7) in the absence and presence of sodium perchlorate (5 mM). Flow rate = 1 ml/min. Solutes identified as in Fig. 1.

Table 3
Effect of electrolytes on the retention of selected solutes on a chemically-bonded theophylline stationary phase

Mobile phase composition				
Electrolyte solution	MeOH (%)	γ*	k'† *p*-Hydroxybenzoate	k'† 3,4-Dimethylbenzoate
None	0	70.6	2.01	15.26
$NaClO_4$, 5 mM	0	69.7	0.99	7.59
$NaClO_4$, 50 mM	0	70.3	0.50	4.33
None	30		0.61	3.01
$NaClO_4$, 50 mM	30		0.45	1.46
$KClO_4$, 50 mM	30		0.45	1.46
NaCl, 50 mM	0	72.3	1.65	11.44
NaCl, 50 mM	30		0.58	2.99
KCl, 50 mM	30		0.64	2.96
$NaNO_3$, 50 mM	0	70.3	1.06	7.91
$NaNO_3$, 50 mM	30		0.55	2.50
KNO_3, 50 mM	30		0.58	2.50

* Surface tension.
† Capacity factors measured as described in Table 2.

elution characteristics of mobile phases prepared with salts composed of the same anion with either sodium or potassium as the cation, the selectivity provided by different salts appears to reside with the anion. Furthermore, this effect does not appear to be related to the surface tension of the resulting mobile phases.

Retention of test solutes can also be controlled by temperature changes. In this case, a degree of selectivity can be provided by thermostatting the system at different temperatures (Fig. 3). Slopes of van't Hoff plots are different for some of the solutes reflecting, at least in part, differences in their enthalpies of interaction with theophylline.

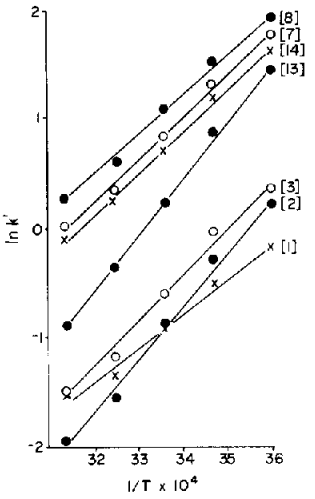

Figure 3
Retention of benzoic acid derivatives determined as a function of temperature on a theophylline-bonded phase column (30 × 4.6 mm) using a mobile phase of potassium phosphate buffer (5 mM; pH 7). Flow rate = 1 ml/min. Solutes identified as in Fig. 1, with **13** = 3-chloro-4-hydroxy and **14** = *p*-nitrobenzoic acid.

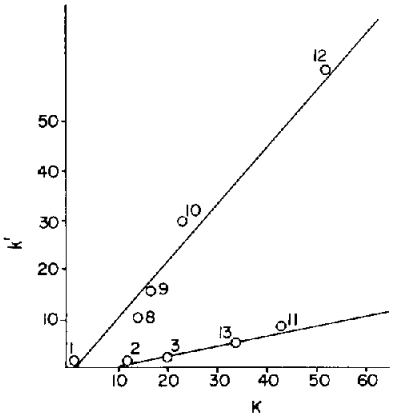

Figure 4
Comparison of chromatographic retention of a series of benzoic acid derivatives (measured in terms of k') on a theophylline-bonded phase column (30 × 4.6 mm) using a mobile phase of potassium phosphate buffer (5 mM; pH 7; flow rate = 1 ml/min) versus complexation constants determined in aqueous bulk phase taken from ref. 2. Solutes identified as in Fig. 1. The lower line refers to hydroxy acids; the upper to halogen substituted acids.

For all solutes retention decreases with increasing temperature — behaviour that is consistent with that described [5] for the complexation phenomena observed in bulk phase.

Further support for the hypothesis that retention on-column is primarily due to association of solute with bonded theophylline residues is provided in comparing relative chromatographic retention and complexation as measured in solution [2, 5]. Figure 4 shows the linear relationship that exists between k' and the corresponding complexation constant (K). Since forces in addition to complexation are presumed to be involved in retention, it is not surprising that different families of benzoates (based on ring substituent) produce curves differing in slope, although the relationship between k' and K within a family is linear.

In summary, a new chromatographic stationary phase is described which is stable and provides an element of additional selectivity through a complexation mechanism involving pendant theophylline residues (covalently bonded to a silica support through a hydrocarbon chain) and appropriate solutes selected for separation.

References

[1] A. J. Repta, L. A. Sternson, K. A. Mareish and N. M. Meltzer, *Int. J. Pharm.* **18**, 277–286 (1984).
[2] T. Higuchi and K. A. Conners, in *Advances in Analytical Chemistry and Instrumentation* (C. M. Reilly, Ed.), Vol. 4, pp. 117–212. Interscience, New York (1965).
[3] T. Higuchi and H. Kristiansen, *J. Pharm. Sci.* **59**, 1601–1608 (1970).
[4] K. A. Conners, M. H. Infeld and B. J. Kline, *J. Am. Chem. Soc.* **91**, 3597–3603 (1969).
[5] T. Higuchi and D. A. Zuck, *J. Am. Pharm. Assoc., Sci. Ed.* **41**, 132–138 (1953).
[6] T. Higuchi and A. Drubulis, *J. Pharm. Sci.* **50**, 905 (1961).
[7] H. Kristiansen, M. Nakano, N. Nakanamo and T. Higuchi, *J. Pharm. Sci.* **59**, 1103–1106 (1970).

[*First received for review 23 July 1986; revised manuscript received 14 January 1987*]