Available online at www.sciencedirect.com
SCIENCE DIRECT®

Talanta 64 (2004) 929–934

Talanta

www.elsevier.com/locate/talanta

Preparation and characterization of perhydroxyl-cucurbit[6]uril bonded silica stationary phase for hydrophilic-interaction chromatography

Si-Min Liu, Li Xu, Cheng-Tai Wu*, Yu-Qi Feng*

Department of Chemistry, Wuhan University, Wuhan 430072, PR China

Received 30 January 2004; received in revised form 7 April 2004; accepted 14 April 2004

Abstract

In this paper, perhydroxyl-cucurbit[6]uril ($(HO)_{12}CB[6]$) was, for the first time, grafted to silica gel as a hydrophilic-interaction chromatographic stationary phase. Several alkaloids were used as probes to investigate the retention mechanism on the new stationary phase. The effect of mobile phase variables such as acetonitrile content, ionic strength and pH on their chromatographic behavior was investigated. The results indicate that the stationary phase behaves as a hydrophilic-interaction chromatographic packing. Finally, several alkaloids were separated on the stationary phase.

Keywords: Hydrophilic-interaction chromatography; Perhydroxyl-cucurbit[6]uril; Alkaloids

1. Introduction

Hydrophilic-interaction chromatography (HILIC) was first proposed by Alpert [1] in 1990 as a new chromatographic concept, which is to describe the combination of hydrophilic phases and hydrophobic, mostly organic mobile phases. The most important characteristic of HILIC is that the mobile phase contains high content organic solvent and low content water, while the mobile phase in normal phase chromatography (NPC) contains no water. Though the retention mechanism of HILIC has not been definitely established due to the fact that complex interactions may occur during the partitioning process, separations by HILIC are in a manner similar to NPC. Therefore, generally it can regard that retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase [1]. So in contrast to reversed-phase chromatography (RPC), polar compounds have stronger retention than non-polar compounds in HILIC mode.

The advantages of HILIC lie in following aspects: (1) it is particularly well available for the separation of polar biological solutes like proteins, peptides, amino acids and carbohydrates; (2) it can analyze the water soluble polar compounds to overcome the problem that separations cannot be accomplished in NPC mode if solutes can only be dissolved in aqueous; (3) its coupling to mass spectrometry for quantification and identification purposes is relatively easy because it can realize the separation in the absence of involatile inorganic salts and in the presence of high organic solvent concentration. In a word, it can be complementary to the conventional NPC and RPC.

Many applications of HILIC have been reported. A diol column was employed to separate carbohydrate [2]; Dallet et al. [3] determined urea, allantoin and lysine pyroglutamate in cosmetic samples using a polyhydroxyethyl A column; Tolstikov and Fiehn [4] analyzed the highly polar compounds of plant origin on the Amide 80 stationary coupling to the electrospray ion trap mass spectrometry; glucosinolates from broccoli and other cruciferous vegetables were also analyzed on a polyhydroxyethyl column [5]; some polar pharmaceutical compounds, such as tromethamine [6], pyrimidines and purines, have been determined using an aminopropyl column [7]; a mixed-mode anion–cation exchange/hydrophilic interaction chromatography–electrospray mass spectrometry has been used for small molecule drug discovery [8]; mixed-mode hydrophilic interaction/cation-exchange chromatography is

---

* Corresponding authors. Tel.: +86-27-87867564; fax: +86-27-87647617.
E-mail addresses: yqfeng@public.wh.hb.cn (Y.-Q Feng), ctwu@public.wh.hb.cn (C.-T. Wu).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.04.012

a novel technique for peptide separation [9] on the polysulfoethyl A strong cation-exchange column; the chiral separations of drug substances and underivatized amino acids were demonstrated through the employment of multi-modal chromatography utilizing cyclodextrin- or teicoplanin-derivatized stationary phases by the HILIC mechanism [10].

The cucurbit[n]urils (CB[n]s) are a kind of cyclic methylene-linked glycoluril oligomers. They possess a characteristic annular shape, with two identical carbonyl-fringed portals [11–13]. CB[6], as the most common cucurbituril, has been widely studied in supramolecular chemistry by Mock [14], Kim [15] and their co-workers. Moreover, the molecular recognition properties of other CB[n]s ($n = 5$, 7, 8) arouse much more attention and demonstrate their broad utility in molecular recognition and self-assembly studies [16]. Although cucurbit[n]urils are potentially as useful as well-known host molecules such as crown ethers, cyclodextrins, and calixarenes, their practical applications are still limited mainly due to the difficulty in introducing functional groups on their exterior and poor solubility in common solvents. Recently, Jon et al. [17] reported the facile synthesis of CB[n] derivatives via direct functionalization. This research is highly expected to expand the scope of the chemistry of cucurbit[n]urils, especially to introduce cucurbit[n]urils to the separation science.

The aim of the present work is to explore the potential application of CB[n]s in high performance liquid chromatography. Perhydroxyl-CB[6] ($(HO)_{12}$CB[6]) was chemically grafted to silica gel through a silylating reagent ((3-isocyanatopropyl)triethoxysilane). The HILIC retention behavior of the bonded phase was investigated using some alkaloids as probes. Parameters affecting on the separation, such as buffer pH, acetonitrile content and ionic strength, were investigated. Furthermore, the separation of alkaloids was achieved under optimal condition.

2. Experimental

2.1. Chemicals, reagents and instrumentation

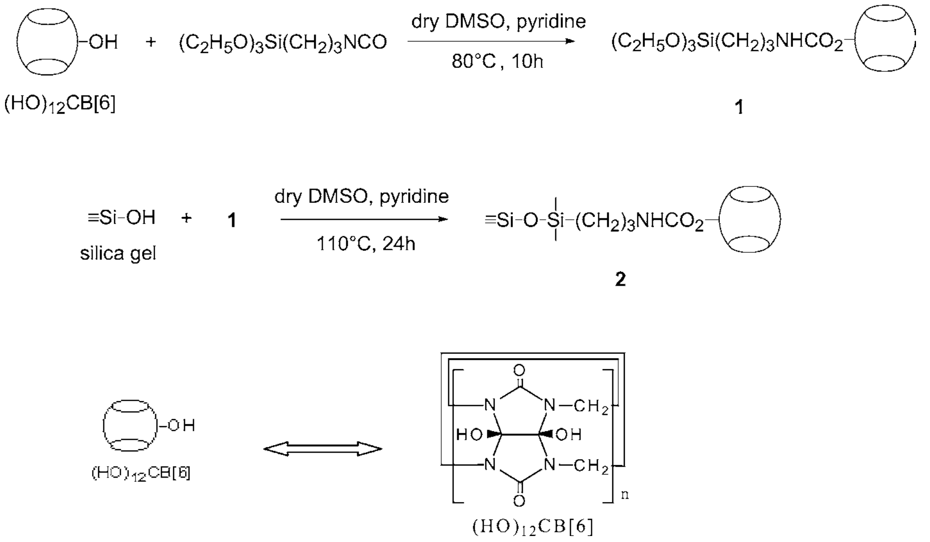
Spherical silica was home-made [18], with 5–7 μm particle size and 290 $m^2 g^{-1}$specific surface area. Silylating reagent, (3-isocyanatopropyl)triethoxysilane, was purchased from Kasei (Tokyo, Japan). Narceine, berberine, ephedrine, atropine, brucine and nicotine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). Other reagents were obtained from various commercial sources and were analytical grade unless otherwise indicated. Water used in this work was re-distilled.

^{1}H NMR spectra were recorded on a Mercury VX-300 (Varian, 300 MHz) or an Inova-600 (Varian, 600 MHz) spectrometer. ESI–MS measurements were performed on Thermo Finnigan LCQ Deca XP at room temperature. The FTIR instrument was AVTAR-360 (Thermo Nicolet, Madison, USA).

2.2. Preparation of stationary phase

Perhydroxyl-CB[6] was synthesized according to the literature [17] with a minor modification. ^{1}H NMR (600 MHz, DMSO-d_6): $\delta = 8.047$ (s, 12H), 5.334 (d, $J = 15.0$ Hz, 12H), 4.433 (d, $J = 14.9$ Hz, 12H); ESI–MS: m/z 1188.0 [M^+], 1226.9 [$M + K^+$]. The result was in agreement with the literature [17].

The bonding reactions are shown in Scheme 1. All reactions were performed under anhydrous conditions. 1.5 g (1.26 mmol) dried $(HO)_{12}$CB[6] was dissolved with stirring in 50 ml of anhydrous DMSO and 5 ml dry pyridine was added to the solution. Under nitrogen atmosphere, 1.2 g (4.85 mmol) (3-isocyanacyanatopropyl) triethoxysilane was added to the stirred solution with a syringe. The



Scheme 1. Preparation of $(HO)_{12}$CB[6]-bonded silica gel phase.

mixture was then kept at 80 °C with stirring for 10 h until absorption at 2250–2300 cm^{-1} (corresponding to the –NCO group) completely disappeared. To this solution was added 2 g of silica gel, which had been dried at 80 °C for 12 h in vacuo, and the suspension was then kept at 110 °C for 24 h under nitrogen. After cooling, the resulting silica was filtered, washed successively with DMSO, methanol, water, methanol, and ether, and then dried at 60 °C for 6 h in vacuo. The amounts of bonded perhydroxy-CB[6] was estimated from the results of elemental analysis. The value was about 70.8–88.6 μmol g^{-1}.

2.3. Chromatographic procedure

The HPLC apparatus was purchased from Dalian Elite Analytical Apparatus Limited Company, which was composed of a P230 pump (Elite, Dalian, China), a UV 230 ultraviolet detector (Elite, Dalian, China) and a Rheodyne 7725i injector with 20 μL sample loop (Cotati, California, USA). The chromatographic data was acquired by chromatography working station Echrom2000 (Elite, Dalian, China). The $(HO)_{12}CB[6]$-bonded silica gel stationary phase was slurry-packed into a 15 cm × 4.6 mm (i.d.) stainless-steel column with isopropanol (25 ml) and methanol was used as eluent. A mixture solution of acetonitrile and phosphate buffer was used as mobile phases. Before use, the mobile phases were generally filtered through a G-4 fritted glass funnel and degassed in an ultrasonic bath for about 5 min. The measured pH refers to the pH of prepared aqueous mobile phase. The flow rate was set at 1 ml min^{-1}. The wavelength used for detection was 214 nm. The retention time of the solvent peak was used as void time for the calculation of capacity factor. All measurements were carried out at ambient temperature (26–28 °C) and repeated at least twice.

3. Results and discussion

3.1. Characteristic of the stationary phase

In order to make sure the immobilization reaction works, the medium product **1** has been separated (The reaction solution was poured into acetone to form a precipitate. The obtained precipitate was filtered, washed with acetone and dried in vacuum). The IR spectra (Fig. 1) show that there is a strong peak in 1082 cm^{-1}, corresponding to the Si–O group. There is not a distinct peak around 1725 cm^{-1} (corresponding to –NHCOO); we think that this peak has been shielded by the stronger peak in 1745 cm^{-1} (corresponding to the –CO group of CB[6]). The formation of the medium product **1** has also been proved by ^{1}H NMR spectrum. ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 7.915$ (HO of $(HO)_{12}CB[6]$), 5.282 (H_a of $(HO)_{12}CB[6]$), 4.374 (H_b of $(HO)_{12}CB[6]$), 3.72 (–CH_2–O–), 2.294 (–CH_2–NH–), 1.408 (–CH_2–CH_2–CH_2–), 1.138 (CH_3–CH_2–), 0.504 (–Si–CH_2–).

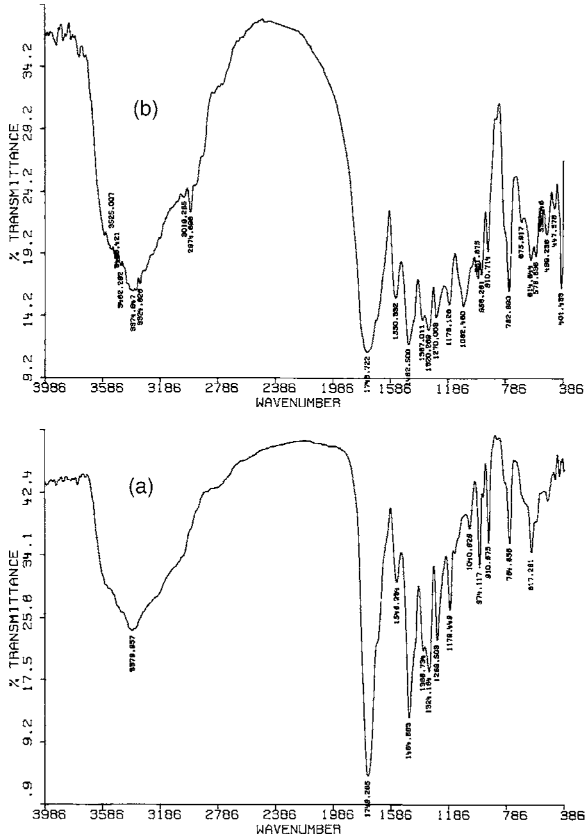


Fig. 1. IR spectra of (a) pure $(HO)_{12}CB[6]$; (b) the medium product **1**.

Diffuse reflectance FTIR measurements were undertaken to confirm the production of the new chromatographic packing. In Fig. 2, two distinct peaks in the 2950–2880 cm^{-1} region arising from C–H stretching; strong absorption in the 1100–1085 cm^{-1} resulting from Si–O–Si stretching bands; and strong absorption of 1745 cm^{-1} due to carbonyl stretching band. Probably, the weak broad absorption observed around 3440 cm^{-1} is caused by the O–H stretching bands

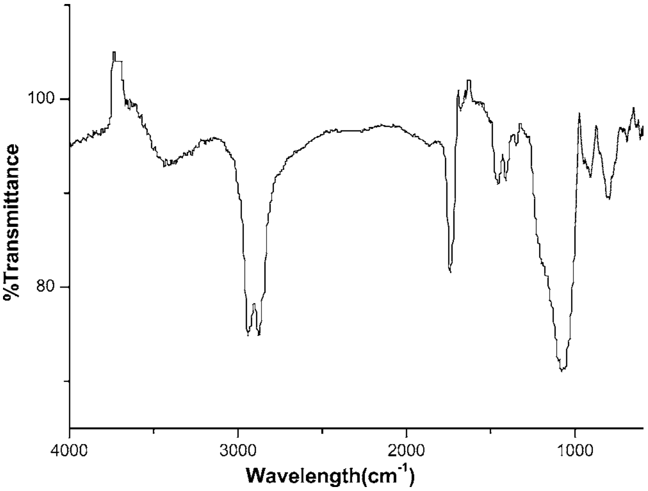


Fig. 2. FTIR spectrum of the $(HO)_{12}CB[6]$-bonded silica stationary phase.

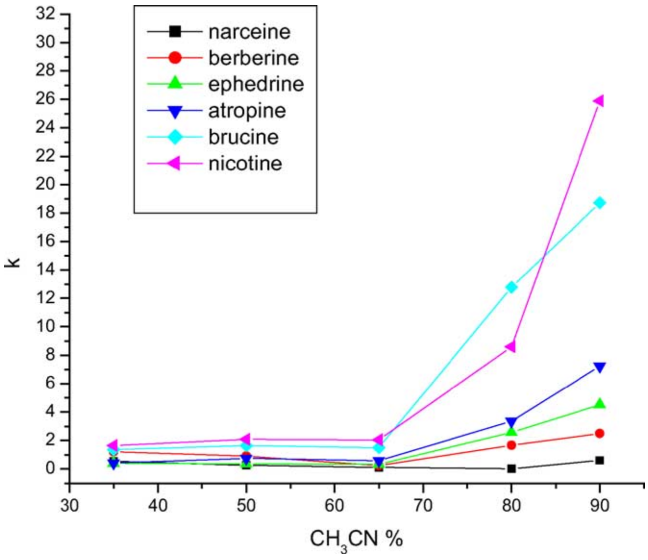


Fig. 3. Influence of the acetonitrile content of mobile phase on the capacity factor of alkaloids on the $(OH)_{12}CB[6]$-bonded stationary phase. Mobile phase: 5 mM $Na_2H\ PO_4$ buffer solution with acetonitrile at pH 3.48. Flow rate: 1 ml min^{-1}.

from adsorbed water and the remained hydroxyls of the perhydroxylation of cucurbit[6]uril.

3.2. *Effect of organic solvent*

The mobile phase used in HILIC is similar to RPC, namely, aqueous–organic mixture. However, water is considered as the stronger solvent and an increase in the organic solvent content would lead to longer retention of polar analytes in HILIC separation [1]. In this study, we investigated the effect of acetonitrile content (35–90%) in the mobile phase on retention.

Fig. 3 illustrates the plot of capacity factor of alkaloids against the volume percentage of acetonitrile in mobile phase at constant pH (3.48) and constant $Na_2H\ PO_4$ buffer concentration (5 mM). As shown in Fig. 3, the retention values of the solutes increased as the acetonitrile content of mobile phase increased above 65%. This result indicates that the new stationary phase can behave as a polar packing and the hydrophilic interaction is one of the factors playing a role in the retention of alkaloids. Hence, retention at high levels of organic solvents is evidently due to HILIC, reflecting the hydrophilic nature of the exterior of the $(HO)_{12}CB[6]$. Another important reason for this high hydrophilic phase is ascribed to the rich acryl groups in the CB[6] molecules, which have been demonstrated to contribute hydrophilic property much as for the conventional HILIC phases [1,19,20].

However, weak retention was obtained for the analytes under 65% acetonitrile content. Though the retention of some alkaloids, such as berberine and ephedrine, slightly increased with the decreasing acetonitrile content while the retention of other studied alkaloids slightly decreased with the decreasing acetonitrile content, retention of all investigated alkaloids was weaker than that above 65% acetonitrile content. The different trends of retention behavior can imply that the separation mechanism might not be completely based on hydrophilic interaction, and secondary interactions might be involved at low acetonitrile content [1], such as hydrophobic interaction and so on. However, the hydrophobic interaction is very weak according to the experiment on the retention of aromatic molecules in the case.

3.3. *Effect of pH*

In order to successfully separate the studied alkaloids, the mobile phase can be modified in two different ways. First, the organic concentration can be adjusted to change the partition coefficient as in Section 3.2 and, second, the pH can be adjusted to alter selectivity. The influence of the pH on the retention of the alkaloids has been studied at pH 2.50, 3.48, 5.48 and 7.05, keeping the acetonitrile content constant at 80% (v/v). The results are shown in Fig. 4.

As can be seen from Fig. 4, retention of alkaloids increased with increasing pH and a reasonably good separation of alkaloids was obtained at pH 3.48. The retention values of alkaloids at low pH (2.50) are found to be smaller than that at higher pH range probably due to the charged stationary phase at low pH. CB[6] can be protonated to some degree at low pH [21,22], thus the molecule can be charged positively. The solutes can also be protonated at low pH because of their basic properties, hence they are all positively charged to different degree. Consequently, static repulsion occurs between the solutes and the stationary phase, resulting in the weak retention under the condition (pH 2.50). At pH 3.48 whether the alkaloids or the bonded $(HO)_{12}CB[6]$ is less protonated than that at pH 2.50, thus

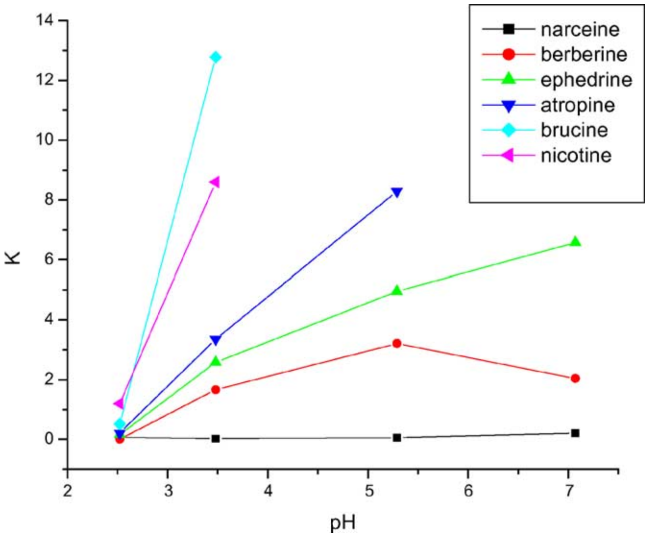


Fig. 4. Influence of the pH of mobile phase on the capacity factor of alkaloids on the $(OH)_{12}CB[6]$-bonded stationary phase. Mobile phase: a mixture of 5 mM $Na_2H\ PO_4$ buffer solution–acetonitrile (20/80, v/v). Flow rate: 1 ml min^{-1}.

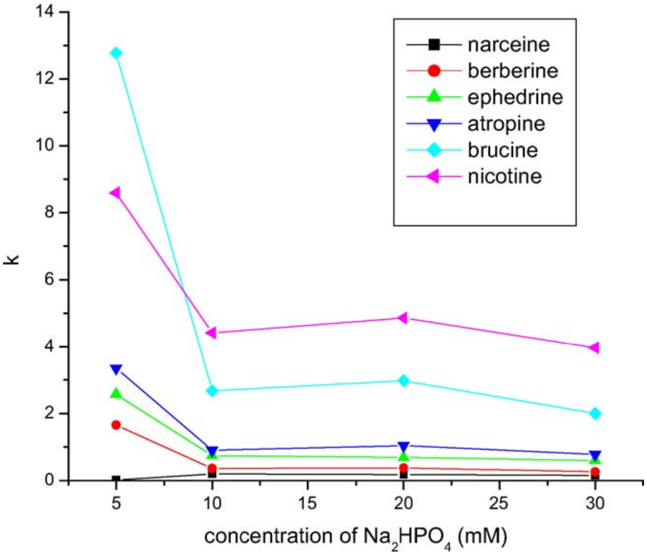


Fig. 5. Influence of Na_2HPO_4 concentration in mobile phase on the capacity factor of alkaloids on the $(OH)_{12}CB[6]$-bonded stationary phase. Mobile phase: a mixture of buffer Na_2HPO_4 solution–acetonitrile (20/80, v/v) at pH 3.48. Flow rate: 1 ml min^{-1}.

hydrophilic interactions become more prominent than electrostatic repulsion, leading to longer retention time of the solutes. Furthermore, the hydrophilic interactions increase with increasing pH. For examples, brucine and nicotine cannot elute out in an hour at pH 5.48 and 7.05, and atropine cannot elute out in an hour at pH 7.05.

3.4. Effect of ionic strength

The ionic strength of mobile phases at constant pH (3.48) and constant acetonitrile content (80%, v/v) increased by increasing Na_2HPO_4 buffer concentration (from 5 to 30 mM). The effect of ionic strength on capacity factor is shown in Fig. 5. When the ionic strength of mobile phase increased, as can be observed, the retention of alkaloids decreased and finally tend to be nearly a constant value. The result is in accordance with that of Alpert's group [1]. They found that the hydrophilic property of the mobile phase increased with the increasing ionic strength, which resulted in the decreasing retention of the solutes. However, the order of the elution keeps the same as the solutes' hydrophilic property whether at high or low ionic strength.

3.5. Separation of alkaloids

The separation of some alkaloids was achieved under the optimal condition with a mixture of acetonitrile–5 mM Na_2HPO_4 buffer solution (80/20, v/v) as a mobile phase at pH 3.48 and flow rate of 1 ml min^{-1}. Their separation chromatogram is shown in Fig. 6. It can be seen that the stationary phase shows good separation selectivity for alkaloids.

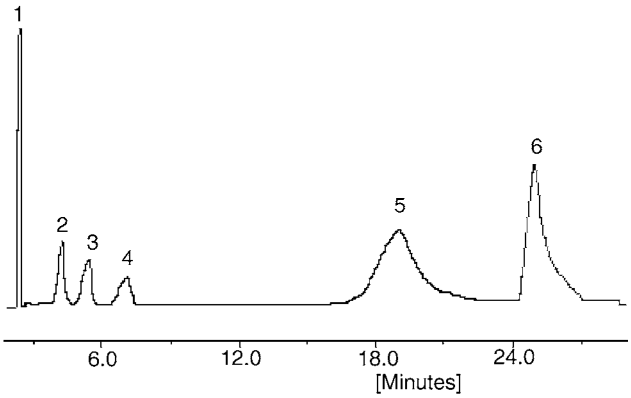


Fig. 6. Separation of alkaloids on the $(OH)_{12}CB[6]$-bonded stationary phase. Mobile phase: 5 mM $Na_2H\ PO_4$ buffer solution–acetonitrile (20:80,v/v), pH 3.48. Flow rate: 1 ml min^{-1}. Samples: (1) narceine; (2) berberine; (3) ephedrine; (4) atropine; (5) nicotine; (6) brucine.

4. Conclusions

Perhydroxyl-CB[6] has been successfully bonded to the silica gel through a two-step reaction, employing (3-isocyanatopropyl)triethoxysilane as silylating reagent. The influence of mobile phase variables such as content of acetonitrile, ionic strength and pH on the retention of alkaloids on the $(HO)_{12}CB[6]$-bonded silica gel stationary phase has been investigated. The chromatographic behavior of the solutes indicates that the hydrophilic interaction can contribute to the retention of alkaloids at high acetonitrile content and the stationary phase behaves as a polar material. But at low acetonitrile content, other interactions may operate besides the hydrophilic interaction. Finally, the separation of some alkaloids was successfully obtained under optimal condition.

Acknowledgements

This research was supported in part by the National Natural Science Foundation of China (Grant: 20275027 and 20172040) and Excellent Teacher Program of MOE, PRC.

References

[1] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[2] H. Tanaka, X. Zhou, O. Masayoshi, J. Chromatogr. A 987 (2003) 119.
[3] P. Dallet, L. Labat, E. Kummer, J.P. Dubost, J. Chromatogr. B 742 (2000) 447.
[4] V.V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.
[5] J.K. Troyer, K.K. Stephenson, J.W. Fahey, J. Chromatogr. A 919 (2001) 299.
[6] Y. Guo, A. Huang, J. Pharm. Bio. Anal. 31 (2003) 1191.
[7] B.A. Olsen, J. Chromatogr.A 913 (2001) 113.
[8] M.A. Strege, S. Stevenson, S.M. Lawrence, Anal. Chem. 72 (2000) 4629.

[9] E. Hartmann, Y. Chen, C.T. Mant, A. Jungbauer, R.S. Hodges, J. Chromatogr. A 1009 (2003) 61.
[10] D.S. Risley, M.A. Strege, Anal. Chem. 72 (2000) 1736.
[11] W.A. Freeman, W.L. Mock, N.Y. Shih, J. Am. Chem. Soc. 103 (1981) 7367.
[12] J. Kim, I.-S. Jung, S.-Y. Kim, E. Lee, J.-K. Kang, S. Sakamoto, K. Yamaguchi, K. Kim, J. Am. Chem. Soc. 122 (2000) 540.
[13] A. Day, A.P. Arnold, R.J. Blanch, B. Snushall, J. Org. Chem. 66 (2001) 8094.
[14] W.L. Mock, in: F., Võgtle (Ed.), Comprehensive Supramolecular Chemistry, vol. 2, Pergamon, Oxford, 1996, p. 477.
[15] K. Kim, Chem. Soc. Rev. 31 (2002) 96.
[16] J.W. Lee, S. Samal, N. Selvapalam, H.-J. Kim, K. Kim, Acc. Chem. Res. 36 (2003) 621.
[17] S.Y. Jon, N. Selvapalam, D.H. Oh, J.-K. Kang, S.-Y. Kim, Y.J. Jeon, J.W. Lee, K. Kim, J. Am. Chem. Soc. 125 (2003) 10186.
[18] Y.Q. Feng, Chinese Patent, Appl. No. 98121690.O (1998).
[19] B. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 548 (1994) 13.
[20] C.T. Mant, J.R. Litowski, R.S. Hodges, J.Chromatogr. A 816 (1998) 65.
[21] X.X. Zhang, E.K. Krakowiak, G. Xue, S.J. Bradshaw, M.R. Izatt, Ind. Eng. Chem. Res. 39 (2000) 3516.
[22] A. Flinn, G.C. Hough, J.F. Stoddart, D.J. Williams, Angew. Chem. Int. Ed. Engl. 31 (1992) 1475.