Xu, D. et al., 2018. A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography. Journal of Chromatography A, 1557, pp.43–50,

which has been published in final form at DOI:10.1016/j.chroma.2018.05.004

A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography

Dongsheng Xu [a, b, c, 1], Huikai Shao [a, 1], Rongying Luo [a], Qiqin Wang [a], Elena Sánchez-López[c], Salvatore Fanali [d], Maria Luisa Marina[c,e] *, Zhengjin Jiang[a, b, *]

[a] *Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou 510632, China.*

[b] *Department of Pharmacy and Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine & New Drug Research, Jinan University, Guangzhou 510632, China.*

[c] *Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.*

[d] *PhD School in Natural Science and Engineering, University of Verona, Strada Le Grazie, 15 – 37129 Verona, Italy.*

[e] *Instituto de Investigación Química "Andrés M. del Río" (IQAR), Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.*

[1] These authors contributed equally to this work.

[*] Corresponding authors. Tel: +34 91 8854935

[*] Corresponding authors. Tel: +86 2085223604

E-mail: mluisa.marina@uah.es (M. L. Marina), jzjjackson@hotmail.com (Z. J. Jiang)

Abstract: A facile single-step preparation strategy for fabricating vancomycin functionalized organic polymer-based monolith within 100 μm fused-silica capillary was developed. The synthetic chiral functional monomer, i.e 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin, was co-polymerized with the cross-linker ethylene dimethacrylate (EDMA) in the presence of methanol and dimethyl sulfoxide as the selected porogens. The co-polymerization conditions were systematically optimized in order to obtain satisfactory column performance. Adequate permeability, stability and column morphology were observed for the optimized poly(ICNEML-vancomycin-*co*-EDMA) monolith. A series of chiral drugs were evaluated on the poly(ICNEML-vancomycin-*co*-EDMA) monolith in either polar organic-phase or reversed-phase modes. After the optimization of separation conditions, baseline or partial enantioseparation were obtained for series of drugs including thalidomide, colchicine, carteolol, salbutamol, clenbuterol and several other β-blockers. The proposed single-step approach not only resulted in a vancomycin functionalized organic polymer-based monolith with good performance, but also significantly simplified the preparation procedure by reducing time and labor.

Keywords: Vancomycin, Enantioseparation, Organic polymeric monolith, Nano-LC

1. Introduction

Although a large number of chiral stationary phases (CSPs) are available on the market, the development of novel CSPs still attracts considerable interest [1-2]. Recently, increasing efforts have been directed toward the development of organic polymer-based chiral monolithic columns because of their excellent permeability, pH stability, low resistance to mass transfer and high performance [3-4]. So far, various chiral selectors functionalized polymer-based chiral monoliths have been reported, such as cyclodextrin and its derivatives [5-6], quinidine and its derivatives [7-9], cellulose derivatives [10], proteins [11, 12], macrocyclic antibiotics [13-14], crown ethers [15] and chiral ion-exchangers [16].

Over the years, the vancomycin-type glycopeptide antibiotics have been proved to be a versatile class of chiral selectors for enantioseparation in polar organic-phase, normal-phase and reversed-phase modes since their enantioselectivity was demonstrated by Armstrong et al. [17]. However, very few vancomycin functionalized polymer-based monoliths have been reported. So far, only Maruška and coworkers developed a multi-step post-column modification strategy for immobilizing vancomycin onto the surface of organic polymeric monolith at the turn of the century [13-14]. The polymeric support was firstly prepared through *in situ* copolymerization of *N*-(hydroxymethyl) acrylamide, allyl glycidyl ether and piperazine diacrylamide with vinyl sulfonic acid within 100 μm I.D. capillaries. Subsequently, vancomycin was introduced onto the polymeric skeleton *via* reductive amination of the aldehyde groups converted from epoxy groups. Later on, they simplified the preparation procedure by replacing allyl glycidyl ether with *N*, *N*'-diallyltartardiamide, which can be easily cleaved into two aldehyde groups using periodate treatment. The vancomycin functionalized polymer-based monoliths prepared through both ways exhibited good enantioselectivity for racemic compounds in capillary electrochromatography (CEC). However, the authors did not provide any column-to-column and batch-to-batch repeatability data in their studies. To the best of our knowledge, there is no other report about vancomycin functionalized polymer-based monoliths. This may be partially attributed to some disadvantages associated with multi-step preparation strategy, such as time-consuming, laborious and probably dissatisfactory repeatability. Vancomycin functionalized silica-based monoliths were also prepared through multi-step preparation strategy [18-21]. Hsieh et al. recently developed a single-step *in situ* sol-gel approach for preparing vancomycin functionalized silica-based monolith [22]. A

sol-gel precursor containing vancomycin was synthesized and copolymerized with skeleton precursor to form a porous silica-based monolith. The proposed single-step approach not only resulted in a chiral column with good efficiency and enantioselectivity for many basic enantiomers, but also significantly simplified the preparation procedure. Aiming at reducing the time and labor associated with the fabrication of vancomycin functionalized organic polymer-based monoliths, it would be of high interest to develop a single-step copolymerization approach as well.

In this work, a chiral functional monomer, i.e 2-isocyanatoethyl methacrylate (ICNEML) derivative of vancomycin (ICNEML-vancomycin), was first synthesized. It was then *in situ* copolymerized with the cross-linker ethylene dimethacrylate (EDMA) in a binary porogen system of methanol and dimethyl sulfoxide (DMSO). The polymerization conditions were systematically optimized in order to obtain satisfactory permeability, column efficiency and enantioresolution. The enantioresolution capability of the optimized poly(ICNEML-vancomycin-*co*-EDMA) was evaluated by analyzing a series of chiral drugs in either polar organic-phase or reversed-phase modes. The enantioseparation conditions, including the organic solvent type and concentration, the buffer concentration and the pH of the mobile phase, were also carefully optimized.

2. Materials and methods

2.1. Reagents and samples

2,2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)-propylmethacrylate (γ-MAPS), ethylene dimethacrylate (EDMA), 2-isocyanatoethyl methacrylate (ICNEML), DMSO, methanol (MeOH), ethanol, 1,4-butanediol, 1-propanol, tetrahydrofuran (THF), cyclohexane, 1-dodecanol and toluene, acetonitrile (ACN), triethylamine (TEA), acetic acid (HAc), pyridine, acetone and vancomycin hydrochloride were acquired from Aladdin Chemicals (Shanghai, China). Acebutolol, carteolol, sotalol, propranolol, pindolol, tertaolol, clenbuterol, salbutamol and thalidomide were obtained from Energy Chemical (Shanghai, China). Colchicine was purchased from Sigma (Missouri, USA). The fused-silica capillaries (375 μm O.D. × 100 μm I.D.) were obtained from Ruifeng Chromatography Ltd. (Hebei, China). Distilled water was purified using a Milli-Q system (Massachusetts, USA). Polar organic mobile phases were set up by mixing the desired ratio of ACN and MeOH, and then adding various amount of TEA and HAc. All mobile phases were subjected to filtration through a 0.22-μm membrane and sonication degas prior to use.

2.2. Instrumentations

Molecular masses were determined on a Waters Synapt G2 TOF mass spectrometer (Milford, USA). A Jinghong DKS22 water bath (Shanghai, China) was used for thermally initiated copolymerization. Scanning electron microscopy (SEM) experiments were performed with a Zeiss Gemini ultra-55 SEM (Deutschland, Germany) at an acceleration voltage of 5 kV. All nano-LC experiments were conducted on a nano-LC instrument, laboratory assembled. The system consists of a DiNa nano gradient pump (Tokyo, Japan), a Shimadzu SPD-15C UV detector (Kyoto, Japan) with a lab-made on-column detection cell and a Valco four-port injection valve with 20 nL internal loop (Houston, USA). All data acquisition and analysis were carried out with Unimicro Trisep^{TM} Workstation 2003 (Shanghai, China). The pH values of buffer solutions were measured by a Sartorius PB-10 pH meter (Göttingen, Germany).

2.3. Synthesis of the chiral functional monomer ICNEML-vancomycin

The nucleophilic addition of amine or hydroxyl groups were often used for the derivatization of vancomycin [23-24]. In this study, ICNEML was chosen as the derivatization reagent to modify vancomycin through the nucleophilic addition reaction. For the schematic representation of the synthesis of the novel ICNEML-vancomycin monomer, see **Fig. 1**. In brief, vancomycin hydrochloride (60 mg, 0.04 mmol) was dissolved in DMSO (0.3 mL). Then, pyridine (0.4 mL) and ICNEML (10 μL, 0.07 mmol) were added into the mixture and stirred for 24 h under nitrogen at room temperature. After adding acetone (8 mL) and stirring for another 10 min, a white precipitate was collected by centrifugation at 4000 rpm for 5 min and washed with acetone for five times. Finally, the precipitate was dried under vacuum to give the target monomer (light white solid). The molecular formula of ICNEML-vancomycin was established as $C_{73}H_{84}N_{10}O_{27}Cl_2$ from its HR-ESI-MS (m/z: 1603.4965 $[M+H]^+$, calculated for $C_{73}H_{85}N_{10}O_{27}Cl_2$: 1603.4963) in **Fig. S1**.

2.4. Preparation of the poly(ICNEML-vancomycin-*co*-EDMA) monolith columns

Prior to the polymerization, the fused-silica capillaries were pretreated with γ-MAPS to provide the anchoring sites for the bulk polymer [25]. Then, the monomer ICNEML-vancomycin, the binary porogens (DMSO and MeOH), the crosslinker EDMA and the initiator AIBN were accurately weighted and mixed into a homogenous solution in a 2 mL of vial. The mixture was sonicated and degassed for 5 min, and then introduced into 20 cm long pretreated capillaries. Both ends of the capillaries were sealed with rubber plugs and submerged into the water bath at 60 °C for 12 h. The unreacted porogens and

chemicals were removed by flushing the column with methanol. The obtained monolith was cut to 15 cm for nano-LC analysis. A 2-5 mm length of the monolith was used for scanning electron microscopy (SEM) analysis.

3. Results and discussion

3.1. Preparation and characterization of the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column

Porogen selection is a critical step in the preparation of polymer-based monolithic column since the type and amount of porogens influence the porosity, morphology, permeability and even the chromatographic efficiency of the monolith. A suitable porogenic solvent or solvent combination should be able to dissolve all components (including functional monomer, initiator and cross-linker) and does not react each other chemically. In this study, several commonly used polar solvents (DMSO, water, MeOH, ethanol, 1,4-butanediol and 1-propanol) and non-polar solvents (THF, cyclohexane, toluene, 1-dodecanol) were initially investigated. The solubility of monomers, the permeability and visual appearance of the monoliths prepared under each porogen system were inspected using nano-LC and microscopy. Based upon our initial experiments, both ICNEML-vancomycin and EDMA showed good solubility in a binary solvent system consisting of MeOH and DMSO (75/25, w/w). In addition, the resulting monoliths also exhibited a uniform dark structure and good permeability. Therefore, these solvents were selected for the following systematical optimization of the polymerization conditions, including the weight fraction of the porogens, the weight fraction of EDMA and the composition of porogenic mixture. Acebutolol was selected as test analyte using a mobile phase consisting of MeOH/ACN/TEA/HAc (80/20/0.08/0.02, v/v/v/v). The influence of the porogen content was first studied by varying the weight fraction of MeOH/ DMSO (75/25, w/w) at three different percentage, i.e. 71% (Column **C1**), 75% (Column **C2**) and 79% (Column **C3**), while keeping constant the other conditions (see **Table 1)**. The results showed that the porogens content had a significant influence on the column permeability. As the percentage of porogens increased, the backpressure diminished. The column **C1** prepared with 71% porogens exhibited a very high backpressure. When comparing the enantioresolution obtained for acebutolol enantiomers, the column **C2** exhibited a higher enantioresolution, and therefore, it was selected for the following studies.

Second, the content of the crosslinker EDMA in the monomer mixture was optimized since it can also influence both the column permeability and

enantioselectivity. As the weight fraction of EDMA in the monomer mixture increased from 20.8% (column **C5**) to 25.0% (column **C2**), the back-pressure and enantioresolution dramatically increased from 3.6 to 7.5 MPa and 0.51 to 1.45, respectively. However, further increasing the EDMA content to 29.2 % (column **C4**) resulted in a slightly lower R_s value (1.38) and higher backpressure (9.5 MPa) when compared to column **C2**. Thus, 25.0 % EDMA was considered for further optimizations. Finally, the influence of the porogenic mixture composition (MeOH and DMSO) was investigated by varying the weight content of MeOH from 70% (column **C6)** to 80% (column **C7)**. The increase of the MeOH content caused a decrease of the back-pressure from 9.8 to 4.7 MPa. 75 % MeOH (column **C2**) allowed for the highest R_s value under a reasonable backpressure.

Based on these optimization experiments, the polymerization mixture containing 25% monomers (ICNEML-vancomycin/EDMA, 75/25, w/w) and 75% porogens (MeOH/DMSO, 75/25, w/w) were selected for following studies. The morphology of the optimized poly (ICNEML-vancomycin-*co*-EDMA) (column **C2)** monolithic column was evaluated by scanning electron microscopy (SEM). As shown in **Fig. 2**, the SEM images indicated that the column **C2** has a morphology of continuous skeleton and large through-pores, and the monolithic rod is tightly anchored on the inner wall of the capillary column.

3.2. Permeability and reproducibility of the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column

The permeability K of a monolithic column can be calculated according to the following equation [26-27]:

$$K = \frac{u\eta L}{\Delta P}$$

where u is the linear velocity of the mobile phase, L is the length of the column, ΔP is the pressure drop across the column, and η is the dynamic viscosity of the eluent. Toluene (ACN or MeOH as mobile phase) and thiourea (water/ACN (50/50, v/v) as mobile phase) were chosen as the dead time markers. As shown in **Table 2**, the calculated K values for the column **C2** were 2.78×10^{-14}, 4.16×10^{-14} and 1.97×10^{-14} m^2 when using MeOH, ACN and water/ACN (50/50, v/v) as the mobile phases, respectively. It is worth noting that these determined permeability values are quite similar, indicating the swell or shrink of the optimized poly(ICNEML-vancomycin-*co*-EDMA) monolith in solvents with different polarities is little.

Repeatability and reproducibility of some studied parameters on the poly (ICNEML-vancomycin-*co*-EDMA) monolithic column were evaluated through calculating the RSD values for k_1, k_2, α and R_s of the racemic test compound acebutolol using a mixture of MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v) as mobile phase **(Table 3)**. The column-to-column reproducibility (n=6) for retention factors (k_1 and k_2) were 2.36%, while the batch-to-batch (n=6) RSD values were 2.72% and 2.36%. The run-to-run repeatability (n=6) for k_1 and k_2 was also adequate with RSD values of 1.98% and 2.36%, in addition to day-to-day repeatability (n=6) which RSD values were 3.03% and 3.42%, respectively. RSD values of α and R_s were also satisfactory (≤ 5.92%). These data demonstrated that the poly (ICNEML-vancomycin-*co*-EDMA) monolithic column has a satisfactory reproducibility for enantioseparation in nano-LC.

3.3. Application of the poly(ICNEML-vancomycin-*co*-EDMA) monolithic column

3.3.1. Polar organic phase mode

Based on our experience and on the data reported in literature [28] a mobile phase containing ACN-MeOH and TEA-HAc was selected as polar organic mobile phase. It has been reported that in any LC-based enantioseparation, the composition of mobile phase affects the enantioselectivity through changing the charge-charge interaction, hydrogen bonding and π-π interaction, among other factors [29-30]. Therefore, the MeOH/ACN content and additives content (TEA/HAc ratio and their total concentration) were modified to evaluate their effect on the enantioresolution of carteolol, acebutolol and sotalol. Due to the fact that the observed behavior was quite the same for these three compounds, only figure of merits for carteolol will be shown. The influence of the concentration ratio of MeOH/ACN on the retention factor and enantioresolution for carteolol was evaluated by keeping constant TEA/HAc content. As shown in **Fig. 3a** and **b**, with increasing MeOH concentration from 60% to 85 % (v/v), the enantioresolution increased reaching its maximum value, and then decreased when the MeOH concentration further raised from 85% to 100% (v/v). The retention factor (k_1) decreased gradually with the increase of the concentration of MeOH. On the other hand, the enantioselectivity factor (α) increased by raising MeOH content, while the column efficiency increased with the increase of MeOH content from 60% to 90% (v/v) and then diminished at 100% (v/v). As a compromise between enantioresolution and column efficiency, 85% (v/v) MeOH was chosen as the mobile phase. These results also agreed with the previous studies on the vancomycin based chiral stationary phases [22] because higher MeOH content in combination with a small amount of acid/base

additives might contribute to less nonselective hydrogen bonding interactions for carteolol enantiomers and vancomycin stationary phases.

As can be observed in **Fig. 4a**, the use of an appropriate TEA/HAc concentration and ratio could be of paramount importance in influencing both enantioseparation and column efficiency. Therefore, the ratio of the TEA/HAc (%, v/v) in the mobile phase was varied from1:3 to 9:1, while the total concentration of TEA and HAc was kept at 0.1% (v/v) and the ratio of MeOH/ACN kept constant at (85/15, v/v). The increase of the ratio of TEA/HAc from 1:3 to 4:1 caused an increase of enantioresolution. Then this parameter decreased as the ratio further increased to 9:1. This is because the hydrogen bonding is the most important in this mode, so the stronger interaction between the CSP and enantiomer with the Ac^- content decreased in the mobile phase. However, the ionization of the basic compounds was weak as the TEA/HAc ratio increased from 4:1 to 9:1, and this would weaken the interaction. On the contrary, the chromatographic efficiency showed a different trends (decreased almost linearly by increasing the TEA/HAc ratio from 1:3 to 9:1). As a compromise to achieving optimum enantioresolution and column chromatographic efficiency, the ratio of 4:1 for TEA/HAc (0.08%/0.02%, v/v) was chosen as the mobile phase additive.

Fig. 4b shows the effect of total concentration of TEA and HAc on the column efficiency. As can be seen, the increase of the total concentration of TEA and HAc in the mobile phase from 0.01% to 0.2% (v/v) caused a raising of number of theoretical plates, while the highest enantioresolution was obtained at 0.1 % (v/v). Therefore, a total concentration of TEA and HAc of 0.1% (v/v) was selected as the optimum mobile phase modifier mixture.

Under the optimal conditions (mobile phase consisting of MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v)), eight racemic compounds were tested. As shown in **Table 4** and **Fig. 5**, good R_s values were obtained for most of the compounds.

3.3.2. Reversed phase mode

As reported in previous studies [13, 14], the basic compound carteolol was not enantio-resolved on the vancomycin functionalized monolith in the reversed phase elution mode where ACN was mainly used [22]. In our preliminary experiments, no enantioresolution of this analyte was observed employing similar conditions. Therefore, MeOH instead of ACN was chosen for the enantioseparation of carteolol to investigate the effect of MeOH concentration on the enantioseparation of carteolol. As shown in **Fig. 6a**, both retention factor (k_1) and enantioselectivity factor (α) increased by varying

MeOH content in the mobile phase in the range 80-98 % (v/v) with the highest values at 98 % (v/v). This effect can be explained with a consequent stronger interaction of the studied enantiomers with vancomycin CSP because higher MeOH concentration combined with TEAA would lead to less nonselective hydrogen interaction [14]. As shown in **Fig. 6b**, the MeOH content also had a strong influence on the enantioresolution and column efficiency, and a slightly higher enantioresolution and column efficiency was obtained when the mobile phase contained 90% (v/v) MeOH. Hence, 90% (v/v) MeOH was selected as the optimum mobile phase for the enantioseparation of carteolol.

Due to the fact that in reversed phase mode the pH and content of buffer solution also played an important role for enantioseparation, they were investigated. **Fig. 7a** shows the effect of the buffer pH present in the mobile phase on enantioresolution and column chromatographic efficiency. Both parameters increased with increasing the buffer pH value from 4.5 to 6.0 and the optimum pH value was 5.5. In order to improve the enantioseparation, various concentration of TEAA buffer were evaluated (**Fig. 7b)**. A decrease of the enantioresolution factors with increasing TEAA buffer content can be observed, while the column chromatographic efficiency raised when the TEAA buffer content increased from 0.1% to 1% (v/v) and then decreased. As a compromise between enantioresolution and column efficiency, 0.5% TEAA (pH=5.5)/MeOH (10/90, v/v) was selected as the mobile phase. Carteolol enantiomers were baseline separated with R_s value of 1.59 as shown in **Fig. 8a**. Clenbuterol, salbutamol, acebutolol and several other β-blockers were also tested using 0.5% TEAA (pH=5.5)/MeOH (10/90, v/v) as the mobile phase. However, it was found that the enantioresolutions of these compounds were not satisfactory.

Colchicine was also tested under the above optimized conditions, unfortunately, no baseline enantioseparation was achieved. Therefore, a similar optimization process was performed. Under the optimized condition, i.e. 50 mM ammonium acetate (pH=5.5)/water/ACN (5/5/90, v/v/v), a baseline separation with R_s value of 2.92 was obtained for colchicine enantiomers (**Fig. 8b**). Due to the fact that thalidomide enantiomers were separated in previous reports using 0.2 % TEAA, pH 4.5/ACN (80:20, v/v) as mobile phase [22], these conditions were employed for the separation of thalidomide on the poly (ICNEML-vancomycin-*co*-EDMA) monolith column. It was found that the enantioresolution was still good (3.27) but the analysis time was too long (≥80 min). Thus, the mobile phase was re-optimized in which 0.5% TEAA buffer

(pH=5.4)/ACN (70/30, v/v) offered the best output in terms of enantioresolution and analysis time (**Fig. 8c**). As shown in **Table 5**, carteolol, colchicine and thalidomide enantiomers can be completely separated on the poly (ICNEML-vancomycin-*co*-EDMA) monolith column under the reversed-phase mode by nano-LC.

4. Conclusion

This study has demonstrated a novel and facile method to synthesize vancomycin functionalized organic polymeric monolith through a single-step approach, which simplifies the fabrication of previous studies. The prepared monolith has been proven to possess large through-pores and a good mechanical stability. Satisfactory column permeability and good enantioselectivity were obtained on the optimum poly(ICNEML-vancomycin-*co*-EDMA) monolith. The mobile phase composition of different buffer pH, organic modifier content and buffer concentration which could influence the enantioseparation was further investigated both in the polar organic and reversed phase modes for enantioseparation of β-blockers. The vancomycin functionalized organic polymer monolith displayed baseline separation for most of the selected enantiomers in both chromatographic modes.

5. Acknowledgements

M. L. Marina and E. Sánchez-López thank the Ministry of Economy and Competitiveness (Spain) for research project CTQ2013-48740-P. D. Xu and E. Sánchez-López thank the Comunidad of Madrid and European funding from FEDER program for their contracts (AVANSECAL-CM program, ref. S2013/ABI-3028). This work was also supported by the Science and Technology Planning Project of Guangdong Province, China (2016A040403056 and 2015A020211018) and the International Science & Technology Cooperation Program of Guangzhou, China (201807010022).

6. References

[1] S. Fanali, Nano-liquid chromatography applied to enantiomers separation, J. Chromatogr. A 1486 (2017) 20-34.

[2] B. Chankvetadze, Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A 1269 (2012) 26-51.

[3] J.L. Guo, Y.J. Lin, Y. Xiao, J. Crommen, Z.J. Jiang, Recent developments in cyclodextrin functionalized monolithic columns for the enantioseparation of chiral drugs, J. Pharm. Biomed. Anal. 130 (2016) 110-125.

[4] N.W. Smith, Z.J. Jiang, Developments in the use and fabrication of organic monolithic phases for use with high-performance liquid chromatography and capillary electrochromatography, J Chromatogr A, 1184 (2008) 416-440.

[5] Q.X. Zhang, J.L. Guo, F. Wang, J. Crommen, Z.J. Jiang, Preparation of a β-cyclodextrin functionalized monolith via a novel and simple one-pot approach and application to enantioseparations, J. Chromatogr A, 1325 (2014) 147-154.

[6] Y.J. Lin, J.L. Guo, H. Lin, J.C. Wang, G. W. Somsen, J. Crommen, Z. Jiang, Effect of fabrication strategy on the enantioseparation performance of β-cyclodextrin-functionalized polymethacrylate monoliths: A comparative evaluation J. Sep. Sci. 40 (2017) 3754-3762.

[7] Q.Q. Wang, E. Sánchez-López, H. Han, H.H. Wu, P.J. Zhu, J. Crommen, M. L. Marina, Z.J. Jiang, Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column-Analysis of l-carnosine in dietary supplements, J. Chromatogr. A, 1428 (2016) 176-184.

[8] Q.Q. Wang, P.J. Zhu, M. Ruan, H.H. Wu, K. Peng, H. Han, G. W. Somsen, J. Crommen, Z.J. Jiang, Chiral separation of acidic compounds using an O-9-(tert-butylcarbamoyl) quinidine functionalized monolith in micro-liquid chromatography, J. Chromatogr. A 1444 (2016) 64-73.

[9] M. Lämmerhofer, E. C. Peters, C. Yu, F. Svec, J. M. J. Frechet, Chiral monolithic columns for enantioselective capillary electrochromatography prepared by copolymerization of a monomer with quinidine functionality. 1. Optimization of

391 polymerization conditions, porous properties, and chemistry of the stationary
392 phase, Anal. Chem. 72 (2000) 4614-4622.
393 [10] X.L. Dong, R.A. Wu, J. Dong, M.H. Wu, Y. Zhu, H.F. Zou, Polyacrylamide-based
394 monolithic capillary column with coating of cellulose tris(3,5-dimethylphenyl-
395 carbamate) for enantiomer separation in capillary electrochromatography,
396 Electrophoresis, 29 (2008) 919-927.
397 [11] Y. Zheng, X. Wang, Y.B. Ji, Monoliths with proteins as chiral selectors for
398 enantiomer separation, Talanta 91 (2012) 7-17.
399 [12] E. Machtejevas, A. Maruska, A new approach to human serum albumin chiral
400 stationary phase synthesis and its use in capillary liquid chromatography and
401 capillary electrochromatography, J. Sep. Sci. 25 (2002) 1-6.
402 [13] O. Kornysova, P.K. Owens, A. Maruska, Continuous beds with vancomycin as
403 chiral stationary phase for capillary electrochromatography, Electrophoresis 22
404 (2001) 3335-3338.
405 [14] O. Kornysova, R. Jarmalaviciene, A. Maruska, A simplified synthesis of polymeric
406 nonparticulate stationary phases with macrocyclic antibiotic as chiral selector for
407 capillary electrochromatography, Electrophoresis 25 (2004) 2825-2829.
408 [15] T. Koide, K. Ueno, Enantiomeric separations of primary amino compounds by
409 capillary electrochromatography with monolithic chiral stationary phases of chiral
410 crown ether-bonded negatively charged polyacrylamide gels, J. Chromatogr. A
411 909 (2001) 305-315.
412 [16] B. Preinerstorfer, W. Lindner, M. Lämmerhofer, Polymethacrylate-type monoliths
413 functionalized with chiral amino phosphonic acid-derived strong cation exchange
414 moieties for enantioselective nonaqueous capillary electrochromatography and
415 investigation of the chemical composition of the monolithic polymer,
416 Electrophoresis 26 (2005) 2005-2018.
417 [17] D. W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J. R. Chen, Macrocyclic
418 antibiotics as a new class of chiral selectors for liquid chromatography, Anal.
419 Chem. 66 (1994) 1473-1484.
420 [18] X.L. Dong, J. Dong, J.J. Ou, Y. Zhu, H.F. Zou, Preparation and evaluation of a
421 vancomycin immobilized silica monolith as chiral stationary phase for CEC,
422 Electrophoresis 28 (2007) 2606-2612.
423 [19] G.S. Ding, A.N. Tang, Capillary electrochromatographic separation of racemates

on a norvancomycin-bonded monolithic column under reversed-phase conditions, J Chromatogr. A, 1208 (2008) 232-238.

[20] A.N. Tang, X.N. Wang, G.S. Ding, X.P. Yan, On-line preconcentration and enantioseparation of thalidomide racemates by CEC with the hyphenation of octyl and norvancomycin monoliths, Electrophoresis 30 (2009) 682-688.

[21] E. Pittler, M.G. Schmid, enantioseparation of dansyl amino acids by HPLC on a monolithic column dynamically coated with a vancomycin derivative, Biomed. Chromatogr. 24 (2010) 1213-1219.

[22] M.L. Hsieh, L.K. Chau, Y.S. Hon, Single-step approach for fabrication of vancomycin-bonded silica monolith as chiral stationary phase, J Chromatogr. A, 1358 (2014) 208-216.

[23] A. Berthod, U.B. Nair, C. Bagwill, D.W. Armstrong, Derivatized vancomycin stationary phases for LC chiral separations, Talanta 43 (1996) 1767.

[24] U. B. Nair, S. S. C. Chang, D. W. Armstrong, Y. Y. Rawjee, D. S. Eggleston, Elucidation of vancomycin's enantioselective binding site using its copper complex, J. V. McArdle, Chirality 8 (1996) 590.

[25] B. Xiong, L. Zhang, Y. Zhang, H. Zou, J. Wang, Capillary electrochromatography with monolithic poly (styrene-co-divinylbenzene-*co*-methacrylic acid) as the stationary phase, J. High Resol. Chromatogr. 23 (2000) 67-72.

[26] Z.J. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Hydrophilic interaction chromatography using methacrylate-based monolithic capillary column for the separation of polar analytes, Anal. Chem. 79 (2007) 1243-1250.

[27] J. Lin, S. Liu, J. Lin, X. Lin, Z. Xie, Novel highly hydrophilic methacrylate-based monolithic column with mixed-mode of hydrophilic and strong cation-exchange interactions for pressurized capillary electrochromatography, J. Chromatogr. A 1218 (2011) 4671-4677.

[28] S. M. Zarcero, I. Sierra, Comparative HPLC methods for β-blockers separation using different types of chiral stationary phases in normal phase and polar organic phase elution modes. Analysis of propranolol enantiomers in natural waters, J. Pharm. Biomed. Anal. 62 (2012) 33-41.

[29] T.L. Xiao, R.V. Rozhkov, D.W. Armstrong, Separation of the enantiomers of substituted dihydrofurocoumarins by HPLC using macrocyclic glycopeptide chiral stationary phases, Anal. Bioanal. Chem. 377 (2003) 693-654.

[30] C. Karlsson, L. Karlsson, D.W. Armstrong, P.K. Owens, Evaluation of a vancomycin chiral stationary phase in capillary electrochromatography using polar organic and reversed-phase modes, Anal. Chem. 72 (2000) 4394-4401.

Figures captions:

Fig. 1. Schematic representation of the synthesis of the ICNEML-vancomycin.

Fig. 2. SEM images of the poly (ICNEML-vancomycin-*co*-EDMA) monolithic column at different magnifications.

Fig. 3. Effect of the MeOH content on (a) retention factor and enantioselectivity; (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (at the desired ratio of MeOH and ACN/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 4. Effect of the ratio (a) and content (b) of TEA/HAc on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: mobile phase: (a) MeOH/ACN/TEA/HAc (85/15/at the desired ratio of TEA and HAc, v/v/v/v); (b) MeOH/ACN/TEA/HAc (85/15/at the desired content of TEA and HAc, v/v/v/v); other experimental conditions are the same as in **Fig. 3**.

Fig. 5. Enantioseparation of racemic compounds in polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 6. Effect of the MeOH content on (a) retention factor (k_1) and enantioselectivity factor (α); (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: 0.5% TEAA, pH=5.5/MeOH; UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 7. Effect of TEAA buffer pH (a) and content (b) on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode.

Conditions: mobile phase: (a) 0.5% TEAA/MeOH (10/90, v/v); (b) TEAA, pH=5.5/MeOH (10/90, v/v); other experimental conditions are the same as in **Fig. 6.**

Fig. 8. Enantioseparation of (a) carteolol, (b) colchicine and (c) thalidomide in reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: (a) 0.5% TEAA, pH=5.5/MeOH (10/90, v/v); (b) 50 mM ammonium acetate, pH=5.5/water/ACN (5/5/90, v/v/v); (c) 0.5% TEAA, pH=5.4/ACN (70/30, v/v); UV detection wavelength: 230 nm (a and c) or 243 nm (b); flow rate: 400 nL/min; injection volume: 20 nL.

Figures captions:

Fig. 1. Schematic representation of the synthesis of the ICNEML-vancomycin.

Fig. 2. SEM images of the poly (ICNEML-vancomycin-*co*-EDMA) monolithic column at different magnifications.

Fig. 3. Effect of the MeOH content on (a) retention factor and enantioselectivity; (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (at the desired ratio of MeOH and ACN/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 4. Effect of the ratio (a) and content (b) of TEA/HAc on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the polar organic-phase mode. Conditions: mobile phase: (a) MeOH/ACN/TEA/HAc (85/15/at the desired ratio of TEA and HAc, v/v/v/v); (b) MeOH/ACN/TEA/HAc (85/15/at the desired content of TEA and HAc, v/v/v/v); other experimental conditions are the same as in **Fig. 3**.

Fig. 5. Enantioseparation of racemic compounds in polar organic-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 6. Effect of the MeOH content on (a) retention factor (k_1) and enantioselectivity factor (α); (b) enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: 0.5% TEAA, pH=5.5/MeOH; UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL.

Fig. 7. Effect of TEAA buffer pH (a) and content (b) on enantioresolution and column chromatographic efficiency for carteolol enantiomers in the reversed-phase mode.

Conditions: mobile phase: (a) 0.5% TEAA/MeOH (10/90, v/v); (b) TEAA, pH=5.5/MeOH (10/90, v/v); other experimental conditions are the same as in **Fig. 6.**

Fig. 8. Enantioseparation of (a) carteolol, (b) colchicine and (c) thalidomide in reversed-phase mode. Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: (a) 0.5% TEAA, pH=5.5/MeOH (10/90, v/v); (b) 50 mM ammonium acetate, pH=5.5/water/ACN (5/5/90, v/v/v); (c) 0.5% TEAA, pH=5.4/ACN (70/30, v/v); UV detection wavelength: 230 nm (a and c) or 243 nm (b); flow rate: 400 nL/min; injection volume: 20 nL.

Table 1. Composition of the polymerization mixture used for the preparation of the poly (ICNEML-vancomycin-*co*-EDMA) monolith columns and their properties.

Table 2. Permeability of the poly (ICNEML-vancomycin-*co*-EDMA) monolith column

Table 3. Reproducibility of the poly (ICNEML-vancomycin-*co*-EDMA) monolith columns

Table 4. Enantioseparation of eight racemic compounds under the polar organic phase mode.

Table 5. Enantioseparation of three racemic compounds under the reversed phase mode.

Figure 1

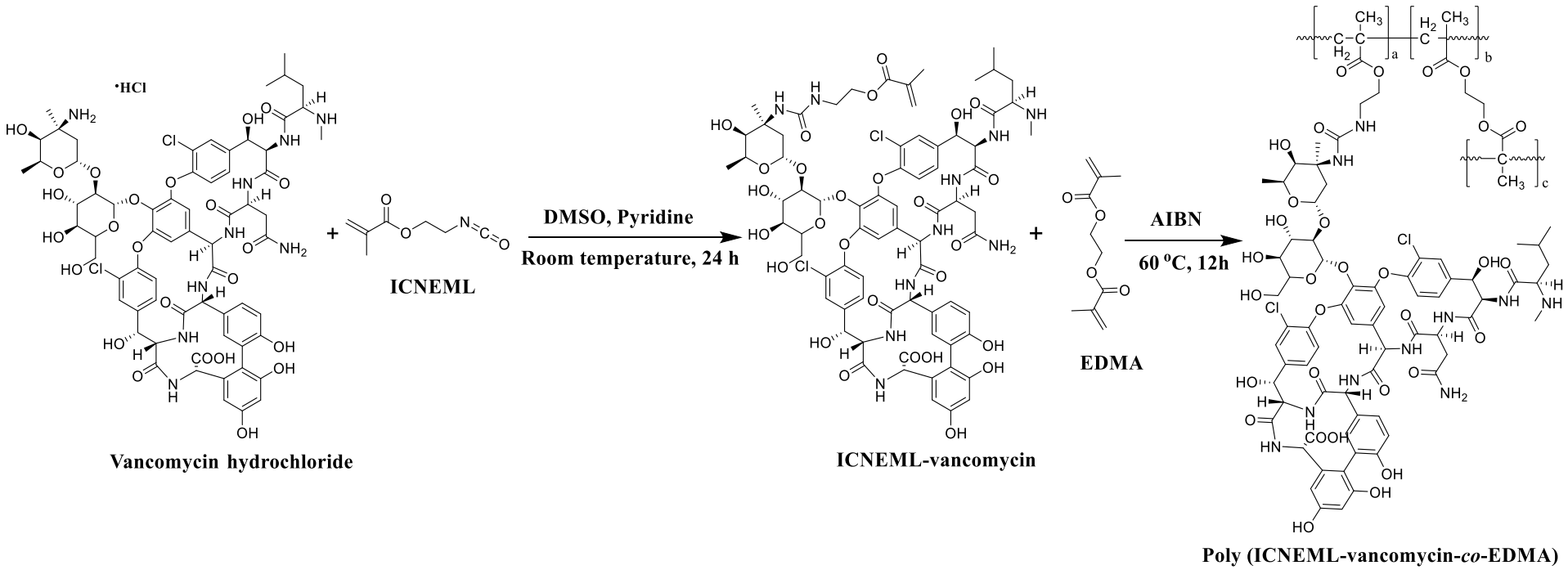
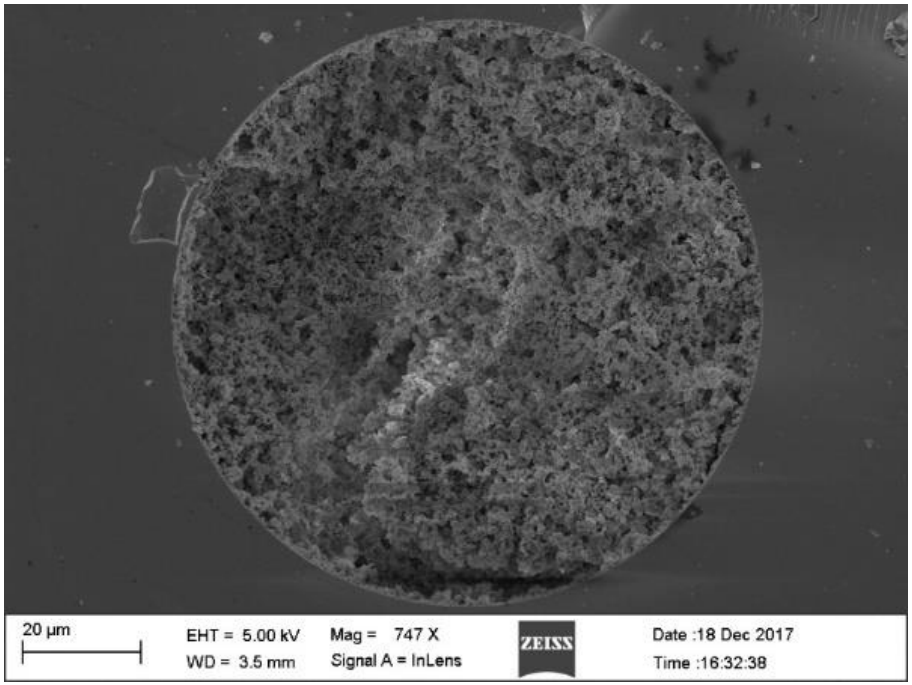

·HCl
Vancomycin hydrochloride
ICNEML
DMSO, Pyridine
Room temperature, 24 h
ICNEML-vancomycin
EDMA
AIBN
60 °C, 12h
Poly (ICNEML-vancomycin-co-EDMA)

Figure 2


20 µm
EHT = 5.00 kV
WD = 3.5 mm
Mag = 747 X
Signal A = InLens
ZEISS
Date :18 Dec 2017
Time :16:32:38

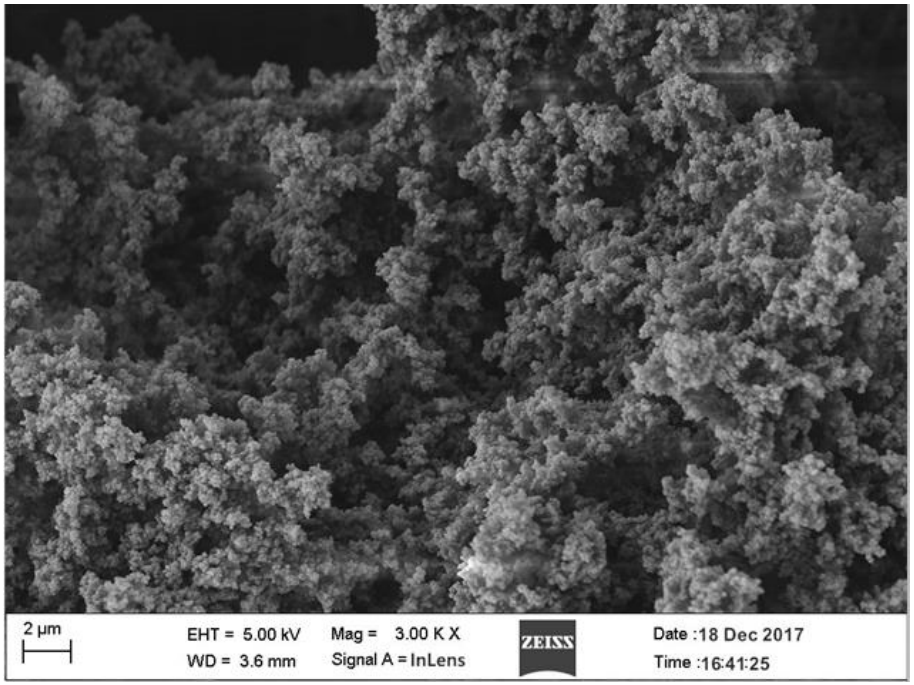

2 µm
EHT = 5.00 kV
WD = 3.6 mm
Mag = 3.00 K X
Signal A = InLens
ZEISS
Date :18 Dec 2017
Time :16:41:25

Figure 3

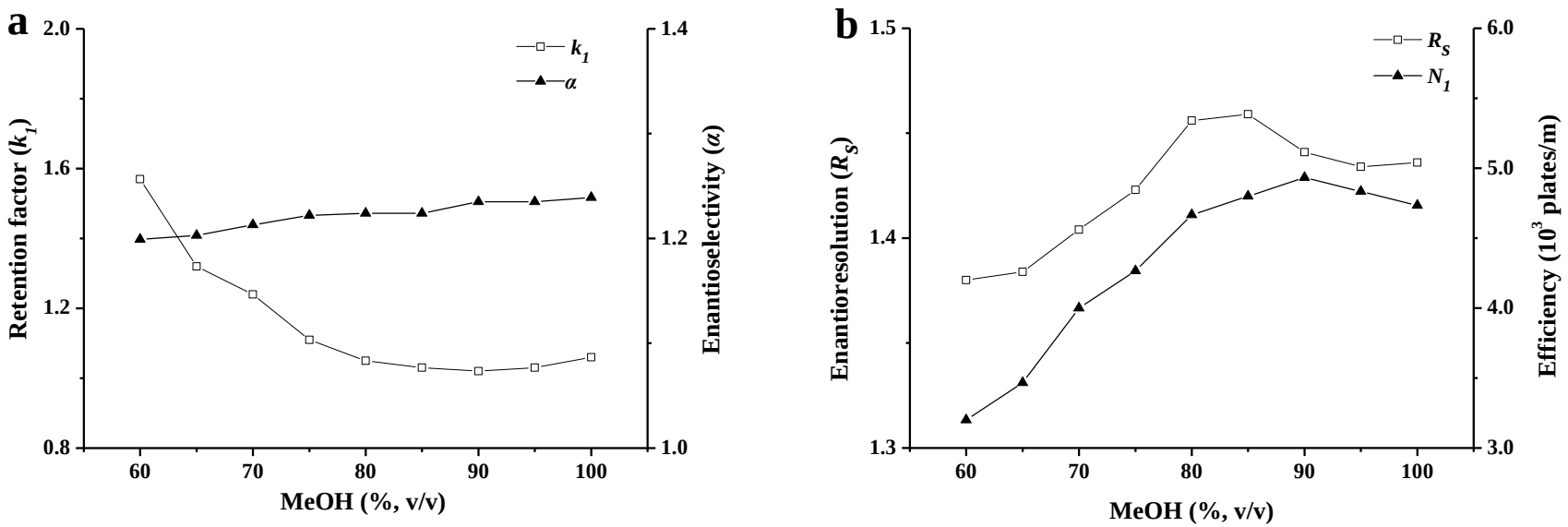

a
2.0
1.6
1.2
0.8
1.4
1.2
1.0
k_1
α
Retention factor (k_1)
Enantioselectivity (α)
60
70
80
90
100
MeOH (%, v/v)
b
1.5
1.4
1.3
6.0
5.0
4.0
3.0
R_S
N_1
Enantioresolution (R_S)
Efficiency (10^3 plates/m)
60
70
80
90
100
MeOH (%, v/v)

Figure 4

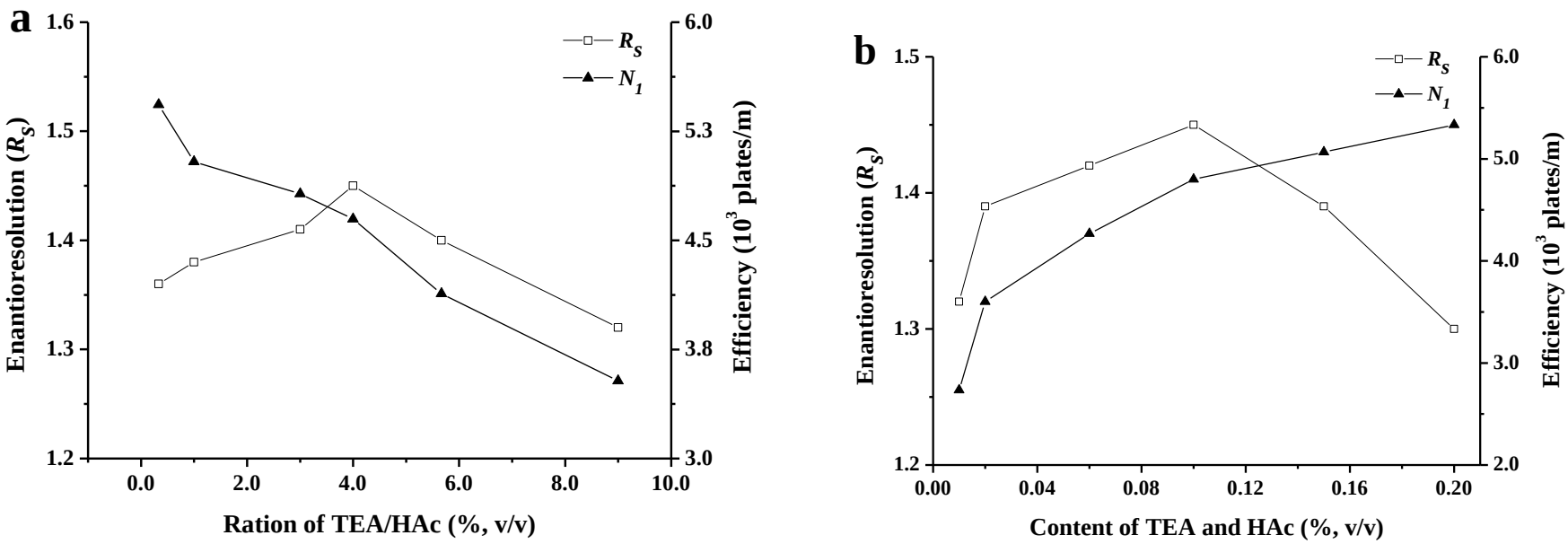

a
b
R_S
N_1
Enantioresolution (R_S)
Efficiency (10^3 plates/m)
Ration of TEA/HAc (%, v/v)
Content of TEA and HAc (%, v/v)
1.2
1.3
1.4
1.5
1.6
3.0
3.8
4.5
5.3
6.0
0.0
2.0
4.0
6.0
8.0
10.0
2.0
3.0
4.0
5.0
0.00
0.04
0.08
0.12
0.16
0.20

Figure 5

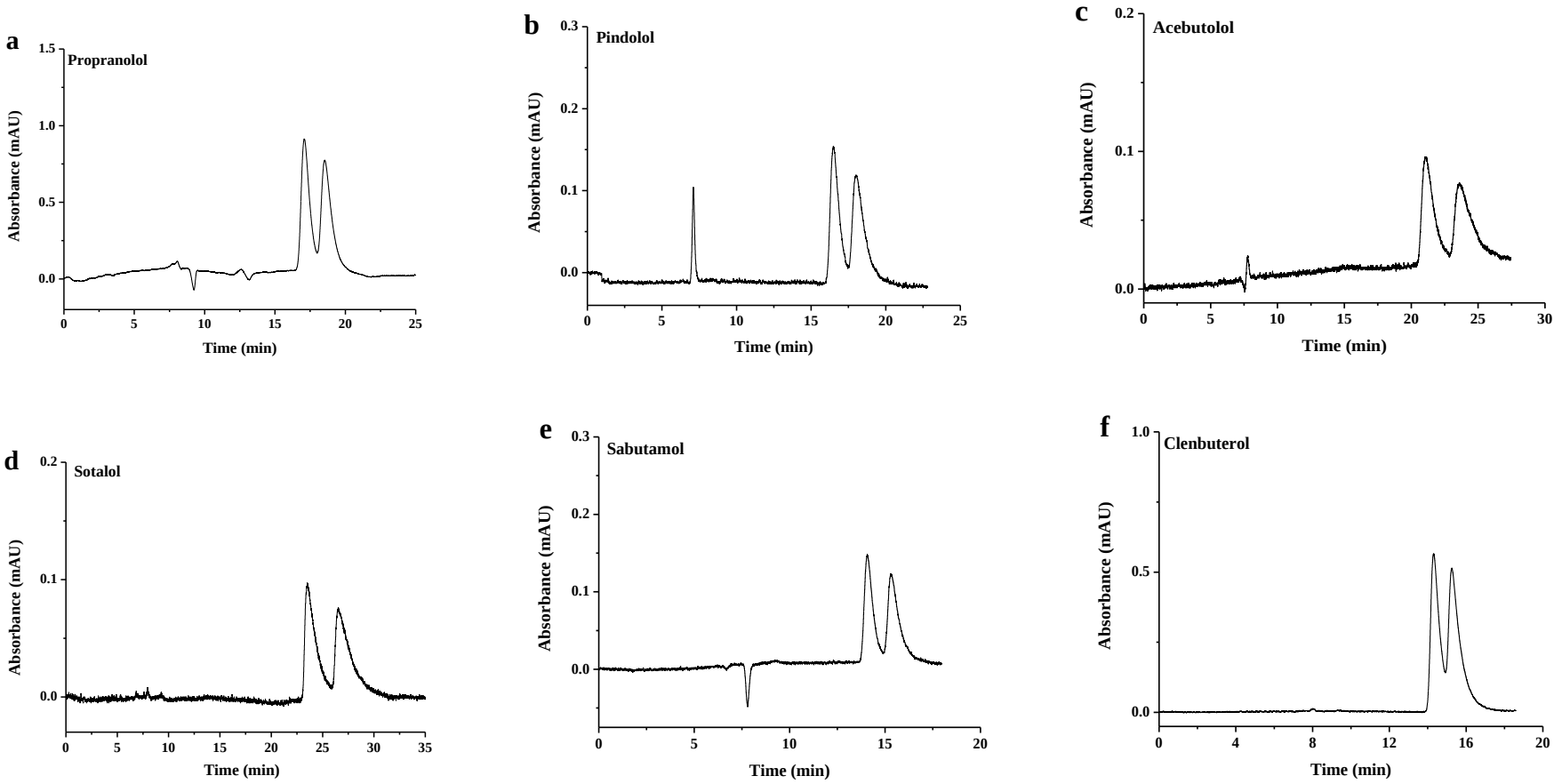

a
Propranolol
Absorbance (mAU)
Time (min)
b
Pindolol
Absorbance (mAU)
Time (min)
c
Acebutolol
Absorbance (mAU)
Time (min)
d
Sotalol
Absorbance (mAU)
Time (min)
e
Sabutamol
Absorbance (mAU)
Time (min)
f
Clenbuterol
Absorbance (mAU)
Time (min)

Figure 6

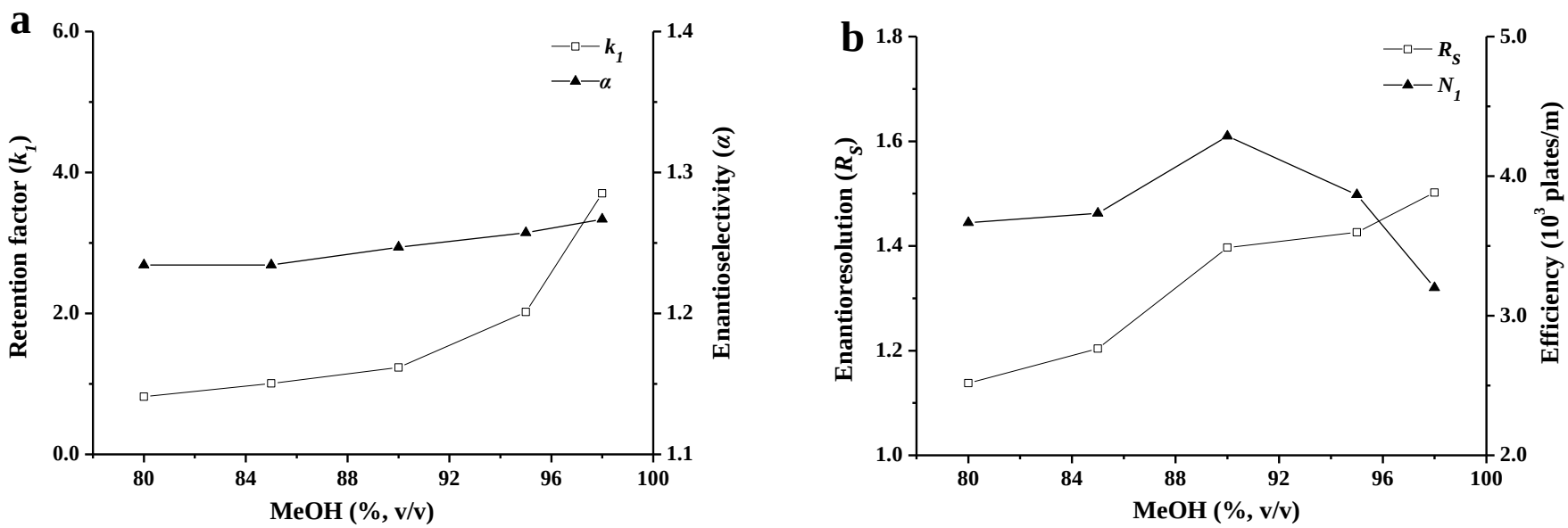

a
6.0
4.0
2.0
0.0
1.4
1.3
1.2
1.1
80
84
88
92
96
100
MeOH (%, v/v)
Retention factor (k_1)
Enantioselectivity (α)
k_1
α
b
1.8
1.6
1.4
1.2
1.0
5.0
4.0
3.0
2.0
80
84
88
92
96
100
MeOH (%, v/v)
Enantioresolution (R_S)
Efficiency (10^3 plates/m)
R_S
N_1

Figure 7

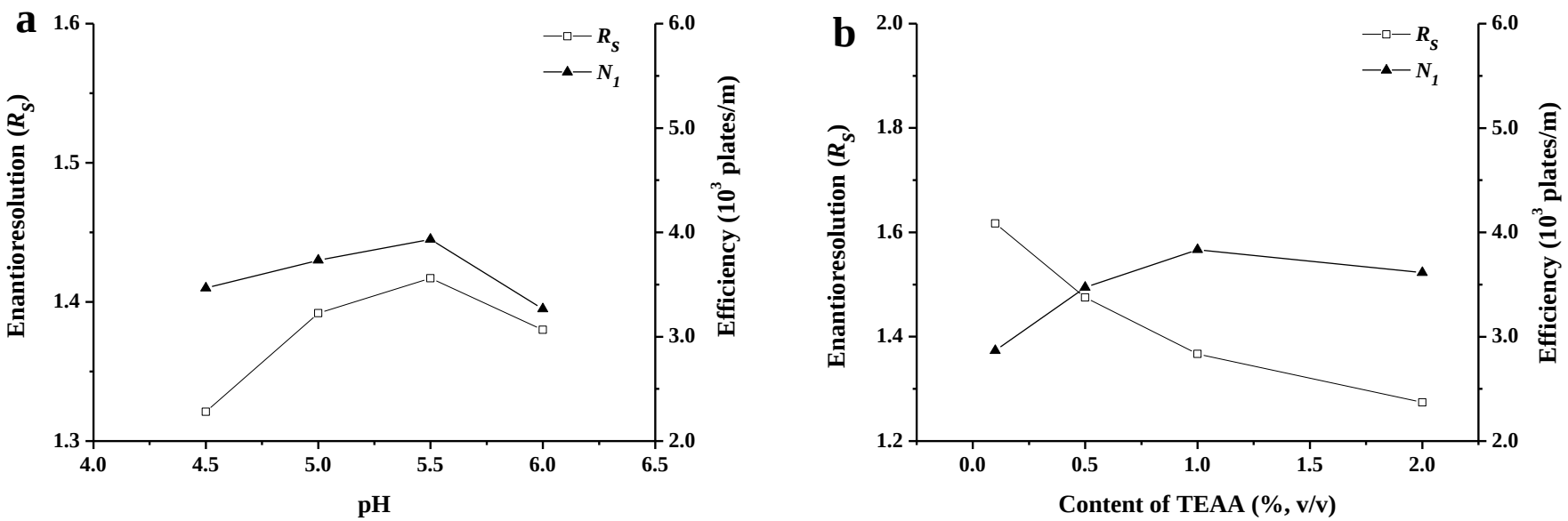

a
b
R_S
N_1
Enantioresolution (R_S)
Efficiency (10^3 plates/m)
pH
Content of TEAA (%, v/v)
1.3
1.4
1.5
1.6
1.2
1.8
2.0
2.0
3.0
4.0
5.0
6.0
4.0
4.5
5.0
5.5
6.0
6.5
0.0
0.5
1.0
1.5
2.0

Figure 8

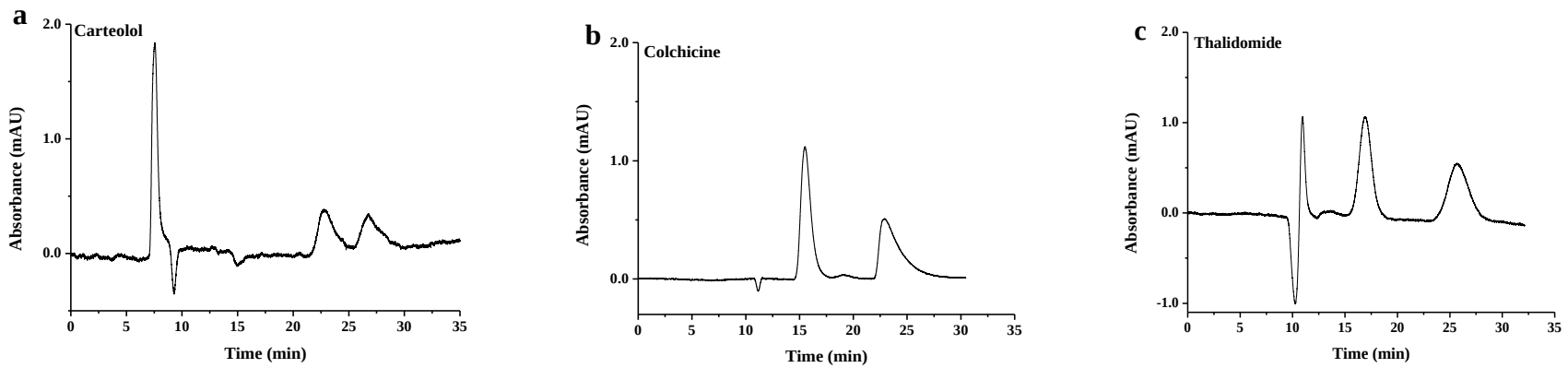

a
Carteolol
Absorbance (mAU)
Time (min)
b
Colchicine
Absorbance (mAU)
Time (min)
c
Thalidomide
Absorbance (mAU)
Time (min)

Table 1. Composition of the polymerization mixture used for the preparation of the poly (ICNEML-vancomycin-co-EDMA) monolith columns and their properties.

Column	Monomers (%, w/w)		Porogens (%, w/w)		Monomers: Porogens (%, w/w)		Backpressure (MPa)	Enantioresolution
	ICNEML-vancomycin	EDMA	MeOH	DMSO	Monomers	Porogens		
C1	75.0	25.0	75.0	25.0	29.0	71.0	Too high	/
C2	75.0	25.0	75.0	25.0	25.0	75.0	7.5	1.45
C3	75.0	25.0	75.0	25.0	21.0	79.0	3.4	0.37
C4	70.8	29.2	75.0	25.0	25.0	75.0	9.5	1.38
C5	79.2	20.8	75.0	25.0	25.0	75.0	3.6	0.51
C6	75.0	25.0	70.0	30.0	25.0	75.0	9.8	0.58
C7	75.0	25.0	80.0	20.0	25.0	75.0	4.7	0.94

Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (80/20/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL; sample: acebutolol.

Table 2. Permeability of the poly (ICNEML-vancomycin-co-EDMA) monolith column

Mobile phase	Relative polarity	Viscosity η ($\times 10^{-3}$ Pa·s)	Permeability K ($\times 10^{-14}$ m^2)
ACN/H_2O (50/50)	/	0.820	1.97
MeOH	0.762	0.544	2.78
ACN	0.460	0.369	4.16

Relative polarity and viscosity data of pure liquids were obtained from Ref. [26-27]

Table 3. Reproducibility of the poly (ICNEML-vancomycin-*co*-EDMA) monolith columns

	Average retention factor (RSD)		Average selectivity	Average resolution
	k_1	k_2	α (RSD)	R_s (RSD)
Column to column (n=6)	1.31 (2.36%)	1.53 (2.36%)	1.17 (2.02%)	1.40 (5.92%)
Run to run (n=6)	1.27 (1.98%)	1.46 (2.36%)	1.15 (1.57%)	1.42 (4.14%)
Day to day (n=6)	1.25 (3.03%)	1.51 (3.42%)	1.21 (1.13%)	1.44 (4.72%)
Batch to batch (n=6)	1.29 (2.72%)	1.49 (2.36%)	1.16 (1.79%)	1.39 (5.33%)

Conditions: column dimensions: 15 cm × 100 μm I.D.; mobile phase: MeOH/ACN/TEA/HAc (85/15/0.08/0.02, v/v/v/v); UV detection wavelength: 230 nm; total flow rate: 400 nL/min; injection volume: 20 nL; sample: acebutolol.

Table 4. Enantioseparation of eight racemic compounds under the polar organic phase mode.

Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
Carteolol	1.04	1.28	1.23	1.45	4500	4100
Propranolol	1.04	1.21	1.17	1.38	5100	4300
Acebutolol	1.26	1.48	1.17	1.43	4400	3900
Pindolol	1.07	1.23	1.15	1.32	3900	3200
Tertaolol	0.66	0.81	1.22	1.39	5500	4600
Sotalol	1.65	2.01	1.22	1.42	4100	3300
Clenbuterol	0.67	0.78	1.16	1.26	4600	4100
Salbutamol	0.74	0.88	1.18	1.47	4900	4100

Experimental conditions are the same as in **Fig. 4**.

Table 5. Enantioseparation of three racemic compounds under the reversed phase mode.

Sample	k_1	k_2	α	R_s	N_1 (m)	N_2 (m)
Carteolol	2.18	2.72	1.24	1.59	3800	2500
Colchicine	0.41	1.08	2.62	2.92	6200	3300
Thalidomide	0.52	1.33	2.55	2.85	5100	4200

Experimental conditions are the same as in **Fig. 7**.

SUPPORTING INFORMATION

A facile and efficient single-step approach for the fabrication of vancomycin functionalized polymer-based monolith as chiral stationary phase for nano-liquid chromatography

Dongsheng Xu [a, b, c, 1], Huikai Shao [a, 1], Rongying Luo [a], Qiqin Wang [a], Elena Sánchez-López[c], Salvatore Fanali [d], Maria Luisa Marina[c,e] *, Zhengjin Jiang[a, b, *]

[a] *Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou 510632, China.*

[b] *Department of Pharmacy and Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine & New Drug Research, Jinan University, Guangzhou 510632, China.*

[c] *Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.*

[d] *PhD School in Natural Science and Engineering, University of Verona, Strada Le Grazie, 15 – 37129 Verona, Italy.*

[e] *Instituto de Investigación Química "Andrés M. del Río" (IQAR), Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.*

[1] These authors contributed equally to this work.

[*] Corresponding authors. Tel: +34 91 8854935

[*] Corresponding authors. Tel: +86 2085223604

E-mail: mluisa.marina@uah.es (M. L. Marina), jzjjackson@hotmail.com (Z. J. Jiang)

Figure S1. HR-ESI-MS of ICNEML-vancomycin.

Elemental Composition Report

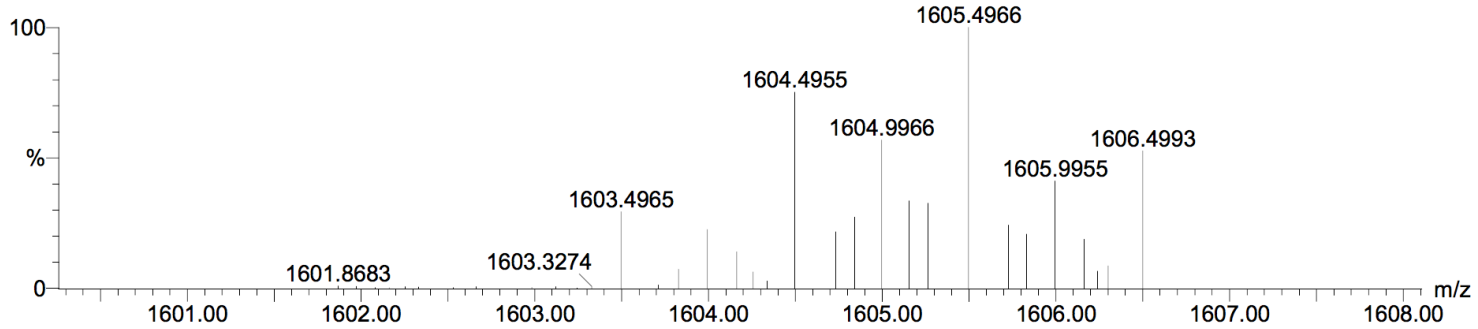
Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
349 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 0-100 H: 0-100 N: 10-10 O: 0-50 Cl: 2-2

170301-1
2016082971 175 (1.421)

1: TOF MS ES+
4.44e+004



Minimum:				**-1.5**				
Maximum:		**5.0**	**10.0**	**50.0**				
Mass	**Calc. Mass**	**mDa**	**PPM**	**DBE**	**i-FIT**	**Norm**	**Conf(%)**	**Formula**
1603.4965	**1603.4963**	**0.2**	**0.1**	**35.5**	**278.3**	**0.388**	**67.87**	**C73 H85 N10 O27 Cl2**
	1603.4986	**-2.1**	**-1.3**	**4.5**	**280.2**	**2.230**	**10.75**	**C48 H97 N10 O45 Cl2**
	1603.4928	**3.7**	**2.3**	**13.5**	**279.5**	**1.543**	**21.38**	**C55 H93 N10 O40 Cl2**