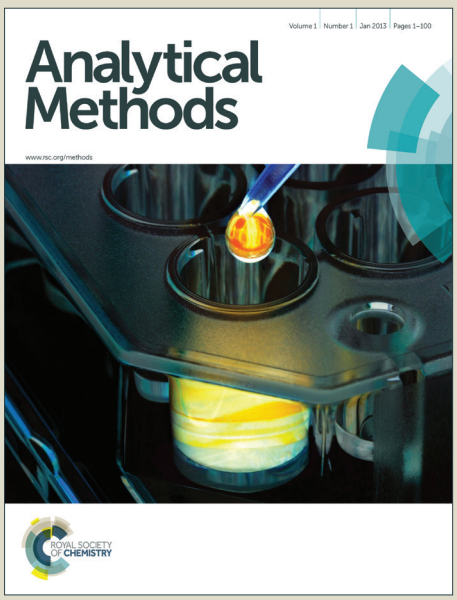
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Journal Name

ARTICLE

"One-pot" click access to triazole bridged cyclodextrin chiral phases for differentiation of clopidogrel enantiomers

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Qing Kang♀, Xiaobin Yao♀, Lifang Zhang, Zhihua Wu and Yong Wang*

The current work demonstrates a "one-pot" click synthetic procedure for the preparation of triazole bridged cyclodextrin (CD) chiral stationary phases (CSPs) and their application for efficient chiral differentiation of clopridogrel enantiomers under reversed-phase high performance liquid chromatography (HPLC). It was found that native-CD-CSP and methylated-CD-CSP afforded good enantioselectivity towards clopridogrel enantiomers with acetonitrile(ACN) /water and methanol(MeOH) /water as the mobile phases, respectively, while the more versatile phenylcarbamoylated-CD-CSP cannot resolve the enantiomers due to the steric hindrance of the bulky phenylcarbamate moieties. Solvent selection plays an important role in CSPs' chiral recognition ability towards clopridogrel enantiomers. The 6-hydroxyl moieties on CD rims were found to participate in the chiral recognition process. The optimal chiral resolution (R_s) was improved to 1.95 by transferring the separation from 5 μm native-CD-CSP to 3 μm native-CD-CSP with ACN/buffer as the mobile phases.

1 Introduction

Clopidogrel (brand name Plavix® and Iscover®), a thienopyridine derivative, acts as an antiplatelet agent which selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and blocks the subsequent ADP-mediated activation of the glycoprotein complex.[1] The clinical benefits of clopidogrel have been demonstrated in trials involving thousands of patients and it has been employed for the long term prevention of ischemic stroke myocardial infarction and stroke.[2]

Figure 1 shows the molecular structure of the enantiomers. It has been confirmed that the active compound clopidogrel is the enantiopure carboxylic ester of *S*-configuration. Its counter-configuration could lead to convulsions in higher doses and has no antithrombotic activity.[3] This indicates that the enantiopurity of drug product clopidogrel must be strictly controlled for health issue. By far, clopidogrel has been one of the hottest drugs treating platelet aggregation in many countries.[2b,4] There is a great demand for the manufacturers to seek economic ways to determine and control the enantiopurity of their products.

Cyclodextrins (CDs) are one of the most versatile and economic chiral selectors in either chromatographic field or electrophoresis. CDs are non-reducing naturally occurring oligosaccharides with a unique hydrophobic cavity. This character endows CDs with a special capacity in accommodating a large variety of compounds, which results in their comprehensive applications in chiral separation.[5] Up to now, numerous CD chiral stationary phases (CSPs) have been developed and employed for enantioseparation in high performance liquid chromatography (HPLC).[5b,6] However, there are only limited publications demonstrating chiral resolution of clopidogrel enantiomers with CD CSPs. Nikolic et al ever used a commercial CD column (ChiralDex) successfully resolving *S*-clopidogrel and its *R*-enantiomer.[7]

Previously, Ng et al have developed a series of triazole-bridged native-CD, methylate-CD and phenylcarbamoylated-CD CSPs by attaching azido-CD derivatives onto alkyne modified silica via click chemistry and employed them for enantioseparation of various enantiomer pairs in reversed-mode HPLC and CEC.[6a,8] Herein, we improved the synthetic pathway by using a "one-pot" anchoring procedure to fabricate five triazole-bridged CD-CSPs based on different CD rims, linkages as well as silica particle size and developed an efficient approach for chiral differentiation of clopidogrel enantiomers with these CD-CSPs.

2 Experimental

2.1 Chemicals and materials

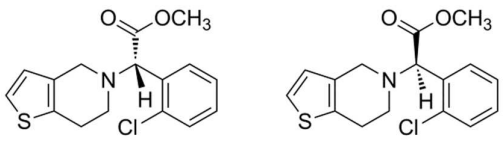


Figure 1 Structures of clopidogrel and its *R*-enantiomer

[a] Department of Chemistry, School of Sciences, Tianjin University, Tianjin 300072, China.
[b] Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, China.
† Corresponding author: Yong Wang; E-mail: wangyongtju@tju.edu.cn.
DOI: 10.1039/x0xx00000x
♀ Qing Kang and Xiaobin Yao Contribute equally.

Sodium azide, Sodium hydride and triphenyl phosphine were purchased from Tianjin Chemical Reagents (Tianjin, China). phenyl isocyanate, cuprous iodide, methyl iodide, propiolic acid and 3-aminopropyltriethoxysilane were purchased from Energy-Chemical (Shanghai, China). β-Cyclodextrin, HPLC-grade methanol (MeOH), acetonitrile (ACN), triethylamine, acetic acid were provided by Guangfu chemical reagents (Tianjin, China). Ultra-pure water was prepared by Milli-Q water purification system (Billerica, MA, USA). Anhydrous N,N-Dimethylformamide (DMF) and toluene were purchased from Heowns (Tianjin, China) Clopidogrel enantiomers and *S*-clopidogrel as well as other analytes (flavanone, bendroflumethiazide, dansyl-*DL*-Leucine and Tröger's base) were purchased from Sigma-Aldrich (Shanghai, China). Kromasil spherical silica gel (5 μm, 100 Å) and Fujisilica SPS100-3 (3 μm, 100 Å) were obtained from Eka Chemicals (Bohus, Sweden) and Fuji Silysia Chemical (Aichi, Japan).

2.2 Instruments

Fourier-transform infrared (FTIR) spectra were collected on an AVATR360 supplied by Thermo Nicolet (USA). Elemental analysis was performed on a VarioMICRO CHNOS elemental analyzer (Elementar Analysensysteme, Hanau, Germany). Chromatographic analyses were performed on a Laballiance HPLC system with a diode array detection (DAD) system (State college, PA, USA).

The mobile phases (MP) were prepared using different amounts of ACN or MeOH mixed with ultra-pure water or 0.5% (v/v) triethylammonium acetate buffer (denoted as 0.5% TEAA). The samples were dissolved in MeOH/H_2O (v/v = 1:1) at a concentration of 1 mg mL^{-1} and the injection volume was 3 μL. All buffers and samples were filtered through a 0.22 μm membrane before usage. The detection wavelength was 240 nm and each solution was injected in triplicate. Calculations (capacity factor, *k*; selectivity, α and resolution, R_s) follow USP standards.

2.3 Preparation of triazole-bridged CD-CSPs

In the current work, five CSPs were prepared following our previously published procedures with slight modification. Their structures are depicted in Figure 2.

General synthetic procedure: The corresponding azido-CDs in excess amount were reacted with N-3-(triethoxysilyl)propyl-2-propynamide (synthesized according to our previous report[8d], detailed synthetic procedure and characterization data are included in supporting information (SI)) in dry DMF in the presence of cuprous iodide-triphenylphosphine [CuI(PPh_3)] for 24 hrs at 85 °C, followed by adding activated silica and the reaction mixture were heated at 90 °C overnight. The crude products were washed with DMF, ethanol and extracted by acetone for 24 hrs before vacuum drying. CSP1,2,3,4 were based on Kromasil 5 μm silica with three single triazole-bridged CSPs (CSP1,2,3) and one multiple trazole-bridge CSP (CSP4), while CSP5 were based on Fuji SPS100-3 silica (3 μm).

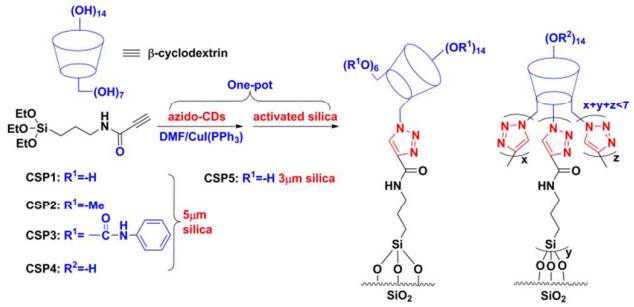


Figure 2 Preparation of triazole-bridged CD-CSPs

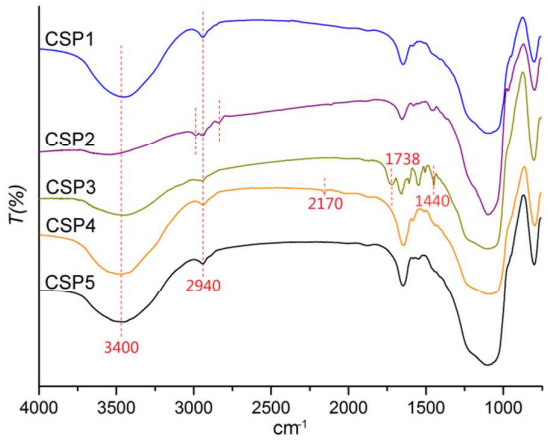


Figure 3 FTIR of CSP1~5

The obtained CSPs were packed into the stainless columns (15 mm × 4.6 mm i.d.) following a conventional high-pressure slurry packing procedure using a packing pump from LabAlliance (State College, PA, USA) with methanol as the packing solvent.

3 Results and discussion

3.1 Synthesis and characterization of CD-CSPs

Our previous synthetic procedure of the triazole-bridged CD-CSPs involves preparation of alkyne silica and the click immobilization of CDs. In the current work, a more convenient "one-pot" synthetic pathway was employed, where N-3-(triethoxysilyl)propyl-2-propynamide was reacted with azido-CDs via click chemistry, after which active silica was added into the reaction mixture directly and the reaction was kept at 90 °C for 12 hrs. The Cu (I) catalyst and other impurities of the crude products can be readily removed by washing the silica beads with DMF, ethanol and acetone to afford the final CD-CSPs. FTIR (Figure 3) shows typical absorptions for the desired CSPs. CSP1,4,5 (cm^{-1}): 2940 (methylene of CDs), 3400 (hydroxyl groups of CDs); CSP2 (cm^{-1}): 2980, 2940, 2850 (methylene and methyl moieties on CD rims); CSP3 (cm^{-1}): 1738 (carbonyls on CD rims), 1448 (phenyl rings). The weak absorption at 2170 cm^{-1} of CSP4 is ascribed to the residual CD azido moieties, which further guarantees the successful immobilization of CDs onto the silica surfaces. Elemental analysis data was shown in Table 1. The surface concentration of CDs were calculated to

be 0.54, 0.45, 0.22, 0.29 and 0.49 μmol·m^{-2} based on the nitrogen content for CSP1~5, respectively. These results proved that the "one-pot" approach can serve as an efficient procedure for the preparation of triazole-bridged CD-CSPs.

3.2 Evaluation of CD R-groups on the differentiation of clopidogrel enantiomers

The enantioseparation was first implemented by using ACN/H_2O (v/v=40:60) as the mobile phases at 25 °C on triazole-bridged CD-CSPs (CSP1~3). It was found that although the enantiomers were fairly retained on CSP1, CSP2 and CSP3, only CSP1 afforded enantioselective abilities towards clopidogrel enantiomers (Figure 4). By closely looking into the structure of clopidogrel, the moieties connected to the chiral carbon are phenyl, tertiaryamine and carbonyl, respectively, where the carbonyl group can act as a hydrogen acceptor forming strong H-bond with the hydroxyl moieties on the CD rims. While CSP2 and CSP3 do not have hydrogen donors on the CD rims, the strong retention was due to the hydrophobic interactions (CSP2) and π–π effects (CSP3). This result reveals that except for the well-known inclusion complexation, H-bonding also plays an essential role in the chiral recognition of clopidogrel.

In order to figure out which hydroxyl groups on the CD rims (-OH on big mouth or on small mouth) take effect in the H-bonding formation, CSP4 (CDs only have 2-OH and 3-OH on the big mouth) was prepared. It was found that no separation was achieved on this CSP. The results indicate that the H-bonding formation might take place between the CD 6-OH and the analytes. Hence, the possible host-guest inclusions should happen as depicted in Figure 4, different from Nikolic's study.[7] A commercial chiral column Cyclobond I 2000 (ASTEC) was also evaluated in this study. It was found the commercial one did not afford any enantioselectivity towards clopidogrel enantiomer pairs, which might be due to the random substitution of the CD –OH groups on 2-position.

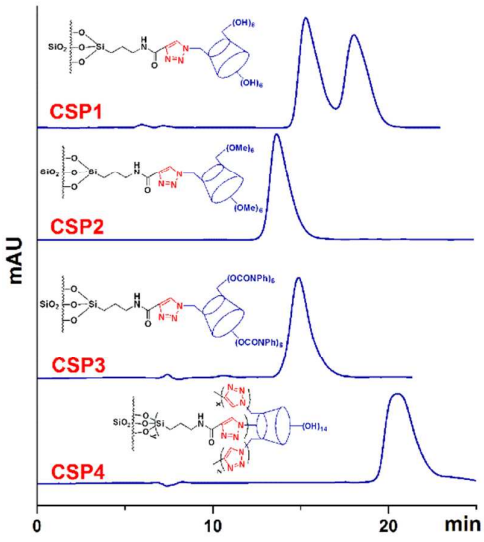


Figure 4 Enantioseparation of clopidogrel enantiomers on CSP1~4 with ACN/H_2O as mobile phase. Conditions: ACN:H_2O (v/v)=25:75, flow rate 0.8 mL min^{-1}, 25 °C

3.3 Evaluation of organic modifiers on the differentiation of clopidogrel enantiomers

It is known that the natures of the commonly used organic modifiers ACN and MeOH are different; MeOH is a protic solvent while ACN is aprotic. It is very interesting to find that the enantiorecognition behaviors of CSP1 and CSP2 towards clopidogrel enantiomers altered significantly when the mobile phases change to MeOH/H_2O from ACN/H_2O (Figure 5).

Table1. Elemental analysis of the CSP1~5

CSP	C %	H %	N %	Surface loading (μmol·m^{-2})
CSP1	8.12	1.95	0.75	0.54
CSP2	7.85	1.80	0.62	0.45
CSP3	11.64	2.53	1.78	0.22
CSP4	9.43	1.79	2.57	0.29
CSP5	7.51	1.91	0.69	0.49

With MeOH/H_2O (60:40) as the mobile phase, CSP2 exhibits good separation capability while CSP1 afforded no recognition ability. As MeOH is a strong protic modifier, the resulted strong salvation effects may significantly influence the differential pairing between CD cavity and solvated solutes hence leading to the different separation results compared to the aprotic ACN. No chiral recognition ability was found with CSP3 either using ACN/H_2O or MeOH/H_2O although the R group of CSP3 could provide more interactions like π–π and dipole-dipole attractions. This might be attributed to the reason that the bulky phenylcarbamate moieties on CD rims may hinder the entry of the analytes into the CD cavity.

3.4 Absolute resolution of clopidogrel enantiomers

The previous CSP2 and CSP3 could only partially separate the enantiomer pairs. Reducing the organic modifiers would significantly delay the elution and broaden the peaks. In order to achieve absolute resolution within acceptable analysis time, we transferred the synthetic pathway to 3 μm silica obtaining

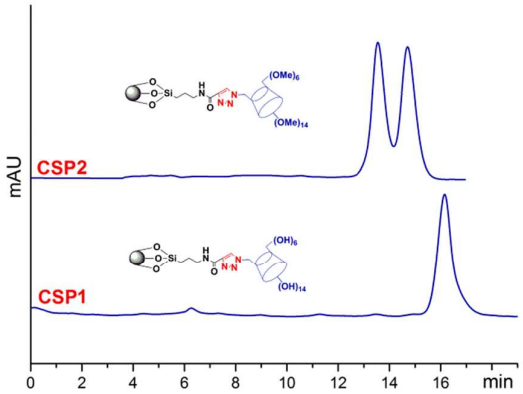


Figure 5 Enantioseparation of clopidogrel enantiomers on CSP1 and CSP2 with MeOH/H_2O as mobile phase. Separation conditions: MeOH:H_2O =50:50 (v/v), flow rate 0.8 mL min^{-1}, 25 °C

CSP5. To optimize the separation, several separation parameters were further evaluated such as temperature, ACN content and buffer effect. The results are summarized in Table 2. As expected, the efficiency and resolution were significantly improved by reducing the particle size to 3 μm with similar selectivity. Higher temperature and ACN content resulted in smaller retention and weakened selectivity and resolution. It is interesting to find that application of 0.5% TEAA buffer is favorable by speeding the analysis while keeping similar resolution. This is due to the competition of TEA with both the enantiomers in occupying the CD hydrophobic cavity. Considering the analysis speed and chiral resolution value, the optimized separation condition was confirmed: ACN/0.5%TEAA (v/v=40/60), temperature 20 °C, 3 μm CSP. Some typical chromatograms were depicted in Figure 6.

Table2. Optimization data for enantioseparation of clopidogrel

CSP	k_1	R_s	V_{ACN}:$V_{H2O/buffer}$	Temp. (°C)	Flow rate (mL·min^{-1})	
CSP1	3.55	1.22	25:75*		25	0.8
CSP5	3.61	1.82	35:75*		25	0.6
	3.37	1.65	35:75*		30	0.6
	3.76	2.01	35:75*		20	0.6
	3.31	1.93	40:60*		20	0.6
	2.63	1.47	50:50*		20	0.6
	3.09	1.95	40:60**		20	0.6

*ACN and H_2O; **ACN and TEAA (0.5%, pH=6.9)

3.5 Elution order of clopidogrel enantiomers

It is a very important issue to know the elution order of the two enantiomers for quality control in pharmaceutical industry. In this work, the elution order was determined by separating the enantiomers with different amount of *R* and *S* conformation. As shown in Figure 7, the *S*-conformation (clopidogrel) was eluted first on CSP5 indicating stronger affinity of *R*-conformation with CD selectors. This is consistent with the results obtained in Nikolic's study with commercial ChiraDex® column as well as Fayed's results by using sulphated β-cyclodextrin as chiral selectors in capillary electrophoresis.[7,10] Interestingly, the same elution order was also found with CSP2 with MeOH/H_2O as the mobile phase. As

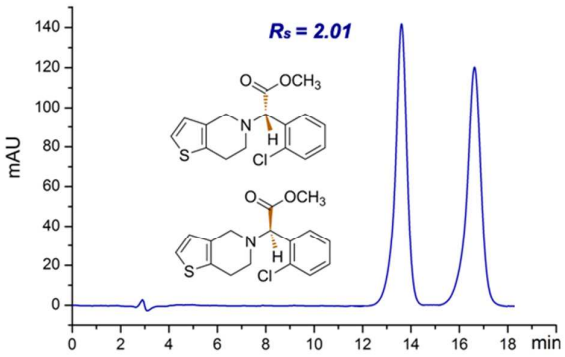


Figure 6 Optimal separation of clopidogrel enantiomers on 3 μm native-CD-CSP (CSP5)

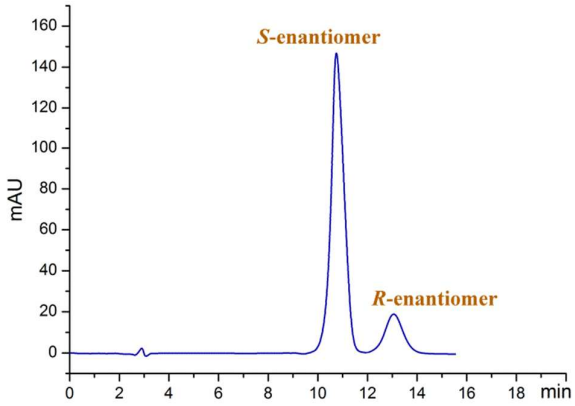


Figure 7 Elution order of *R*/*S*-enantiomers on CSP5. Separation conditions (see figure 6)

stated previously, the solvation of enantiomers by MeOH affords hydrophobic interactions with CSP2 comparable to the H-bonding with CSP1 using ACN/H_2O as mobile phases, which may result in the same steric effect for the inclusion process leading to the same elution order for the two CSPs.

3.8 Enantioseparation of other racemates

The enantioselectivities of the newly prepared CSPs were further verified by enantioseparation of some other analytes such as flavanone, bendroflumethiazide, dansyl-*DL*-Leucine and Tröger's base. By optimizing the separation conditions, all the analytes can be baseline separated and the separation results were depicted in Table S1 (SI) and Figure S1. CSP1 and CSP5 exhibited strong resolving ability toward flavanone, dansyl amino acids and Tröger's base; CSP2 could baseline separate flavanone and partially separate dansyl-*DL*-Leucine; CSP3 afforded superior separation ability toward flavanone and bendroflumethiazide; CSP4 only partially separated flavanone and dansyl-*DL*-Leucine. These results indicate that the immobilization approach and CD functionality produce significant influences on CSP's enantioresolution ability.

4 Conclusions

The "one-pot" synthetic approach provides a more efficient and easier pathway for the preparation of triazole-bridged CD-CSPs. Native CD-CSP and methylated CD-CSP afford chiral recognition ability towards clopidogrel enantiomers while phenylcarbamoylated-CD-CSP has no chiral selectivity. Selection of appropriate organic modifiers plays an essential role for the resolution of clopidogrel enantiomers. Either on native CD-CSP or methylated-CD-CSP, the stronger retention was found with *R*-enantiomer. Absolute resolution of clopidogrel enantiomers was achieved on 3 μm CSP.

Acknowledgements

The financial support from the National Natural Science Foundation of China (No. 21205086), Tianjin Research Program of Application Foundation and Advanced Technology (13JCQNJC05400) is gratefully acknowledged.

References

1 a) D.D. Shinde, H.-S. Kim, J.-S. Choi, W. Pan, S.K. Bae, C.-W. Yeo, J.-H. Shon, D.-H. Kim and J.G. Shin, *J. Clin. Pharm*, 2013, **53**, 550; b) W.J.M. Dewilde, T. Oirbans, F.W.A. Verheugt, J.C. Kelder, B.J.G.L. De Smet, J.-P. Herrman, T. Adriaenssens, M. Vrolix, A.A.C.M. Heestermans, M.M. Vis, J.G.P. Tijsen, A.W. van 't Hof, J.M. ten Berg and W.S. Investigators, *Lancet*, 2013, **381**, 1107; c) M.T. Furlong, I. Savant, M. Yuan, L. Scott, W. Mylott, T. Mariannino, P. Kadiyala, V. Roongta and M.E. Arnold, *J. Chromatogr. B*, 2013, **926**, 36.

2 K.T. Reddy, K.S. Kumar, G. Omprakash and P.K. Dubey, *Syn. Commun*., 2013, **43**,1387; M. Minarik, M. Kopeckova, M. Gassman, P. Osmancik and L. Benesova, *Electrophoresis*, 2012, **33**,1306.

3 W. Wang, S.Y. Xiang, X.J. Zhou, Y.B. Ji and B.R. Xiang, *Molecules*, 2012, **17**, 303.

4 D. Antic, S. Filipic, B. Ivkovic, K. Nikolic and D. Agbaba, *Acta Chromatogr*, 2011, **23**, 235; X. Delavenne, T. Basset, P. Zufferey, N. Malouk, S. Laporte and P. Mismetti, *J. Sep. Sci*., 2010, **33**, 1968.

5 a) A.R. Fakhari, H. Tabani, S. Nojavan and H. Abedi, *Electrophoresis*, 2012, **33**, 506; b) X.H. Lai, W.H. Tang and S.C. Ng, *J. Chromatogr. A*, 2011, **1218** 3496; c) Z.M. Zhou, X. Li, X.P. Chen and X.Y. Hao, *Anal. Chim. Acta*, 2010, **678**, 208; d) Y. Xiao, Y. Wang, T.T. Ong, L.Y. Ge, S.N. Tan, D.J. Young, T.T.Y. Tan and S.C. Ng, *J. Sep. Sci*., 2010, **33**, 1797.

6 a) Y. Wang, D.J. Young, T.T.Y. Tan and S.C. Ng, *J. Chromatogr. A*, 2010, **1217**, 5103; b) K.K. Ngim, Z. Gu and T. Catalano, *J. Pharm. Biomed. Anal.*, 2009, **49**, 660; c) Z. Guo, Y. Jin, T. Liang, Y. Liu, Q. Xu, X. Liang and A. Lei, *J. Chromatogr. A*, 2009, **1216**, 257.

7 K. Nikolic, B. Ivkovic, Z. Besovic, S. Markovic and D. Agbaba, *Chirality*, 2009, **218**, 78.

8 a) Y. Wang, H. Chen, Y. Xiao, C.H. Ng, T.S. Oh, T.T.Y. Tan and S.C. Ng, *Nat. Protoc*, 2011, **6**, 935; b) Y. Wang, D.J. Young, T.T.Y. Tan and S.C. Ng, *J. Chromatogr. A*, 2010, **1217**, 7878; c) Y. Wang, T.T. Ong, L.S. Li, T.T.Y. Tan and S.C. Ng, *J. Chromatogr. A*, 2009, **1216**, 2388; d) Y. Wang, Y. Xiao, T.T.Y. Tan and S.C. Ng, *Tetrahedron Lett*., 2008, 495, 5190.

9 A.S. Fayed, S.A. Weshahy, M.A. Shehata, N.Y. Hassan, J. Pauwels, J. Hoogmartens and A. Van Schepdael, *J. Pharm. Biom. Anal.,* 2009, **49**, 193.