J IRAN CHEM SOC
DOI 10.1007/s13738-015-0635-7

ORIGINAL PAPER

Reversed-phase high-performance liquid chromatographic separation of some 2-arylpropionic acids using vancomycin as chiral stationary phase

Nabila Bouchair[1,2] · Michel Righezza[2] · Abderrezak Hamdi[1]

Received: 14 August 2013 / Accepted: 18 February 2014

Abstract A rapid, sensitive and reproducible HPLC method has been developed for enantioseparation of six non-steroidal anti-inflammatory drugs, which are acidic compounds: carprofen, fenoprofen, flurbiprofen, ibuprofen, indoprofen and ketoprofen. The effects of the mobile phase composition on retention times and resolutions of the analytes were studied. A column based on vancomycin immobilized by reductive amination to aldehyde functionalised silica was prepared in house and used. The prepared sorbent shows a great stability and selectivity over a range of pH (4–6), and the separation was carried out using the mobile phase composed of a mixture of 40 % of methanol in ammonium nitrate buffer (50 mM) at pH 5.0. Another mobile phase consisted of 50 % of methanol in phosphate buffer (5 mM) at pH 5.0 was also prepared and tested. The two mobile phases are the optimum conditions obtained. All experiments were conducted at flow rate 0.6 ml/min, using a UV detector wavelength at $\lambda = 254$ nm.

Keywords Chiral selector · Enantioseparation · Profens · Reversed-phase high-performance liquid chromatography · Vancomycin

✉ Nabila Bouchair
nabila_bouchair@yahoo.fr

[1] University of Jijel, City Ouled Aissa, B.P. 98, 18000 Jijel, Algeria

[2] Aix Marseille Université, CNRS, iSm2 UMR 7313, 13397 Marseille, France

Introduction

Usually, the enantiomers of racemic drugs show different pharmacological effects. Thus, one enantiomer may be the effective agent while the other isomer is probably inactive or even toxic. The consequences of chirality may extend to drug metabolism and enantiomeric interconversion, where one enantiomer is converted into another in vivo. Thus, 2-arylpropionic acids which are widely used as anti-inflammatory agents provide an example of stereoselective activation. When the drug R-ibuprofen was administered in man, there was a rapid appearance of the S-ibuprofen in the blood [1–4].

These consequences of chirality have increased attention paid by researchers to the studies in the field of stereoselective synthesis and catalysis, as well as many techniques for chiral separations. On the other hand, in regard to the manufacture of chiral drugs, it is very important to examine the chiral purity when a single enantiomer is to be used. Thus, it is important during initial testing of a drug, to be able to isolate and separate the enantiomers to assess which is responsible for the potency, the toxicity and/or unwanted side effects.

Chromatographic methods have become very popular in HPLC where most of these methods involve the use of either a chiral selector chemically bonded to the stationary phase, or added to the mobile phase. In both cases there is formation of reversible diastereoisomeric complexes with the solute enantiomers. The separation results from the differences in stability between the complexes and therefore leads to a difference in retention times.

Currently, a number of chiral stationary phases have been described in the literature for HPLC, which have been shown to be efficient for the separation of enantiomeric drugs and other compounds [5–22]. However, there is at

Published online: 28 March 2015

Table 1 Effect of buffer pH on retention and enantioseparation

pH	Ketoprofen	Indoprofen	Ibuprofen	Flurbiprofen	Fenoprofen	Carprofen
4						
k_1	3.06	3.02	1.71	4.67	3.26	12.54
α	1.66	1.33	1.27	1.80	1.28	1.24
R_s	2.13	1.01	0.57	2.45	1.08	1.13
5						
k_1	3.79	3.75	2.38	6.88	4.43	14.36
α	2.02	1.62	1.74	2.54	1.59	1.57
R_s	3.62	2.19	1.97	4.1	2.04	1.81
6						
k_1	2.63	3.47	2.21	6.45	4.00	16.12
α	1.62	1.46	1.60	2.46	1.47	1.60
R_s	1.73	1.71	1.42	2.78	1.79	1.84

Mobile phase: 50 mM ammonium nitrate buffer:MeOH (60:40, v/v %), pH 5.0, flow rate: 0.6 ml/min, detection wavelength 254 nm

k_1 retention factor of the first-eluted enantiomer, α selectivity, R_s resolution

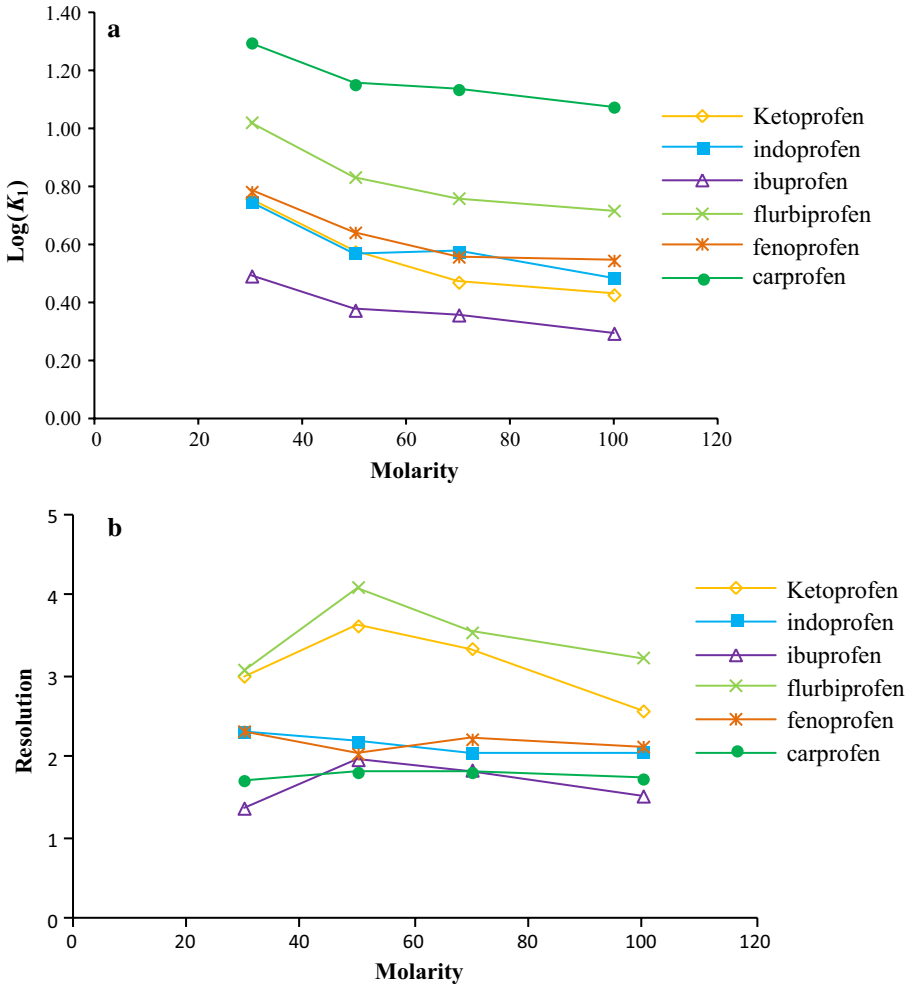

Fig. 1 Effect of buffer molarity on: **a** retention; **b** resolution

present no chiral stationary phase in HPLC that is capable of separating all classes of drugs, so that analysts have to select a convenient phase for a particular separation from a large selection of chiral stationary phases.

Since the introduction of macrocyclic antibiotics as chiral selectors by Armstrong et al. [15], these compounds, especially vancomycin, teicoplanin and ristocetin A, have served as chiral stationary phases for a wide range of drugs.

Table 2 Variation of retention factor (k_1), selectivity (α) and resolution (R_s) of the enantiomers with percentages of different organic modifiers in ammonium nitrate buffer (50 mM, pH 5.0)

	Percentage of OM	Ketoprofen	Indoprofen	Ibuprofen	Flurbiprofen	Fenoprofen	Carprofen
THF	20 %						
	k_1	4.76	4.18	3.51	7.87	5.52	13.12
	α	1.96	1.73	1.50	2.61	1.47	1.50
	R_s	2.42	2.35	1.25	3.37	1.71	1.71
	20 %[a]						
	k_1	4.58	4.25	4.16	8.79	6.34	14.78
	α	1.98	2.06	1.94	3.09	1.66	1.77
	R_s	2.92	3.18	1.94	4.46	1.99	2.17
	25 %						
	k_1	3.13	3.05	2.62	4.51	3.77	6.90
	α	2.07	2.31	2.09	3.50	1.82	1.97
	R_s	2.19	2.83	1.87	3.01	1.81	1.90
	30 %						
	k_1	1.78	1.76	1.54	2.41	2.03	2.84
	α	1.96	2.26	2.03	3.37	1.68	1.90
	R_s	2.42	2.71	2.24	4.15	1.68	2.19
ACN	20 %						
	k_1	4.04	4.18	4.15	9.56	5.34	15.71
	α	1.50	1.32	1.35	1.73	1.30	1.31
	R_s	2.16	1.51	1.22	2.39	1.35	1.37
	25 %						
	k_1	2.35	2.28	1.96	3.60	2.93	6.93
	α	1.43	1.31	1.30	1.64	1.27	1.28
	R_s	1.30	1.21	1.00	2.17	1.33	1.36
	30 %						
	k_1	1.49	1.55	1.19	2.09	1.75	3.58
	α	1.40	1.28	1.28	1.57	1.24	1.25
	R_s	1.27	0.98	0.88	1.70	0.89	0.97
MeOH	40 %						
	k_1	2.98	3.79	2.29	5.82	3.65	13.82
	α	2.06	1.64	1.68	2.42	1.57	1.50
	R_s	3.34	2.05	1.84	3.54	2.22	1.81
	50 %						
	k_1	1.68	1.74	1.02	2.40	1.81	4.69
	α	1.95	1.61	1.77	2.35	1.56	1.49
	R_s	2.50	1.50	1.31	1.50	1.43	1.48

OM organic modifier, k_1 retention factor of the first-eluted enantiomer

[a] 0.1 % TEA

The high enantioselectivity of these compounds may be attributed to their abilities to form various interactions, for example. electrostatic, hydrogen bonding, π–π interactions, etc. Drugs which have been extensively studied are the 2-arylpropionic acids. A common pharmacological aspect of this group of non-steroidal anti-inflammatory compounds is the enantiomeric interconversion due to metabolism.

Vancomycin has been used as a mobile phase additive in capillary liquid chromatography for stereoselective separation of seven racemic non-steroidal anti-inflammatory drugs (NSAIDs) and most of the drugs were successfully enantioseparated [23]. However, it proved difficult to separate using the vancomycin-bonded chiral stationary phase, the majority of profen derivatives using a single mobile phase [16, 23].

In this work, we have sought to explore the capacity of the sorbent vancomycin, as prepared by Svensson et al. [24, 25], to separate six profen drugs. Vancomycin is a tricyclic glycopeptide antibiotic used to treat

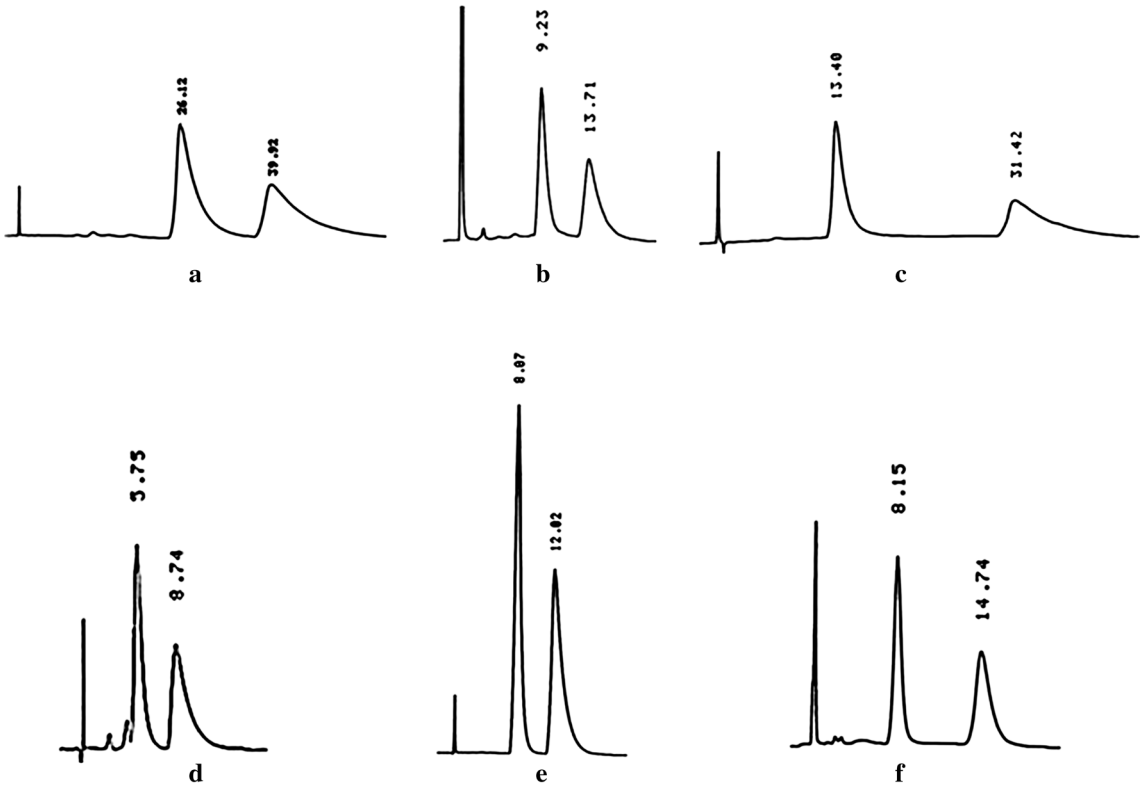

Fig. 2 Chromatograms of the profens in 50 mM, pH 5.0 ammonium nitrate buffer/MeOH (60/40, v/v %); **a** carprofen, **b** fenoprofen, **c** flurbiprofen, **d** ibuprofen, **e** indoprofen, **f** ketoprofen

Table 3 Variation of retention factor (k_1), selectivity (α) and resolution (R_s) of the enantiomers with percentages of methanol in sodium dihydrogen phosphate buffer (50 mM, pH 5.0)

MeOH %	Ketoprofen	Indoprofen	Ibuprofen	Flurbiprofen	Fenoprofen	Carprofen
40						
k_1	5.52	6.13	3.52	9.26	7.20	23.47
α	2.00	1.64	1.72	2.41	1.63	1.65
R_s	2.75	2.1	1.43	3.08	2.07	2.02
50						
k_1	2.70	3.05	1.84	3.86	3.56	8.79
α	1.93	1.66	1.74	2.46	1.57	1.64
R_s	2.36	1.72	1.28	2.40	1.91	2.07

k_1 retention factor of the first-eluted enantiomer

gram-positive infections by inhibiting bacteria mucopeptides biosynthesis. It is produced by the growth of some strains of *Streptomyces orientalis* bacteria and is used for treatment of serious staphylococcal infections in humans.

Vancomycin is reported to have 6 pK_a values: 2.18, 7.75, 8.89, 9.59, 10.4 and 12.0 [26, 27] with 18 chiral centres, three cavities and five aromatic rings in the aglycon part that bears two sugar units. The molecule has a molecular weight of 1449 and contains one carboxylic group, nine hydroxyl groups, seven amido groups and two amino groups [28].

The six profens examined are: ketoprofen, indoprofen, ibuprofen, fenoprofen, flurbiprofen and carprofen. The mobile phase constituents: organic modifiers, pH and buffer concentration were all investigated.

Experimental

Instrumentation and chromatographic conditions

HPLC experiments were performed using an isocratic HPLC pump (L-6200, VWR International Darmstadt,

Table 4 Influence of molar concentration of phosphate buffer on retention factor (k_1), selectivity (α) and resolution (R_s) of the enantiomers of profens

Phosphate buffer (mM)	Ketoprofen	Indoprofen	Ibuprofen	Flurbiprofen	Fenoprofen	Carprofen
50[a]						
k_1	1.53	1.85	1.11	2.31	1.91	5.08
α	1.63	1.44	1.55	1.96	1.37	1.36
R_s	1.63	1.38	1.30	2.35	1.20	1.41
25[a]						
k_1	1.90	2.21	1.32	2.84	2.34	6.13
α	1.64	1.45	1.58	2.02	1.38	1.39
R_s	2.07	1.20	1.30	2.73	1.34	1.56
25[b]						
k_1	1.44	1.71	1.02	2.14	1.78	4.73
α	1.59	1.40	1.52	1.91	1.34	1.33
R_s	1.44	1.16	1.14	2.75	1.29	1.35
10[b]						
k_1	2.00	2.35	1.41	2.96	2.49	6.49
α	1.65	1.45	1.58	2.02	1.39	1.41
R_s	2.20	1.29	1.40	1.43	1.64	1.74
5[b]						
k_1	2.54	2.92	1.66	3.65	3.02	7.72
α	1.58	1.40	1.51	1.89	1.35	1.36
R_s	1.83	1.37	1.46	2.63	1.47	1.63

k_1 retention factor of the first-eluted enantiomer

[a] Disodium hydrogen phosphate buffer (Na_2HPO_4):MeOH (50:50, v/v %), pH 5.0

[b] Trisodium phosphate buffer (Na_3PO_4):MeOH (50:50, v/v %), pH 5.0

Germany), a UV–VIS detector (L-4250, VWR International Darmstadt, Germany), EZchrom software (VWR International Darmstadt, Germany) and an autosampler (AS-2000A, VWR International Darmstadt, Germany).

Solvents and reagents

Methanol (MeOH) was purchased from Sigma-Aldrich (France), acetonitrile (ACN) from Merck KGaA (Germany), tetrahydrofuran (THF) from Prolabo (France), triethylamine (TEA) from Fluka Analytical (Belgium), water for HPLC (H_2O) from SDS (France) and acetic acid glacial (HOAc) from Carlo Erba Reagents (France).

Ammonium nitrate (NH_4NO_3, ≥99.5 %) was obtained from Sigma-Aldrich (France), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), disodium hydrogen phosphate anhydrous (Na_2HPO_4) and trisodium phosphate 12-hydrate ($Na_3PO_4{\cdot}12H_2O$) from VWR international (Germany). All profens were purchased from Sigma-Aldrich (France).

Lichrospher 100 DIOL (150 × 3 mm I.D.), 5 µm, was purchased from VWR international (France).

Vancomycin hydrochloride from *Streptomyces orientalis* was purchased from Sigma-Aldrich (France).

All buffer mobile phases were prepared with specified concentrations of salt and TEA. Solution pH was controlled with glacial acetic acid before the addition of organic modifier.

All experiments were performed at room temperature, detected by UV absorbance at 254 nm. The flow rate was 0.6 ml/min.

Preparation of the column

The column was synthesized using Lichrospher 100 DIOL according to the procedure described previously by Svensson et al. [24, 25]. Silica diol stationary phase has a surface coverage of 3.87 µmol/m^2 and a specific surface area of 350 m^2/g. The stationary phase was modified with a 70-mM solution of sodium periodate in water/methanol (80/20 v/v) cooled to 0 °C at 0.5 ml/min for 4 h followed by flushing with water. Immobilization of the chiral selector was carried out by a reductive amination with a 10-mg/ml solution of vancomycin hydrochloride and sodium cyanoborohydride in 50 mM NaH_2PO_4 at pH 7 for 12 h. Finally CSP was flushed by a solution 50 mM ammonium nitrate in water/methanol (60/40, v/v) for 1 h.

Results and discussion

In our experiments, to optimize the enantioseparation, we first evaluated the chromatographic parameters k_1, α and R_s as a function of pH and molarity of ammonium nitrate

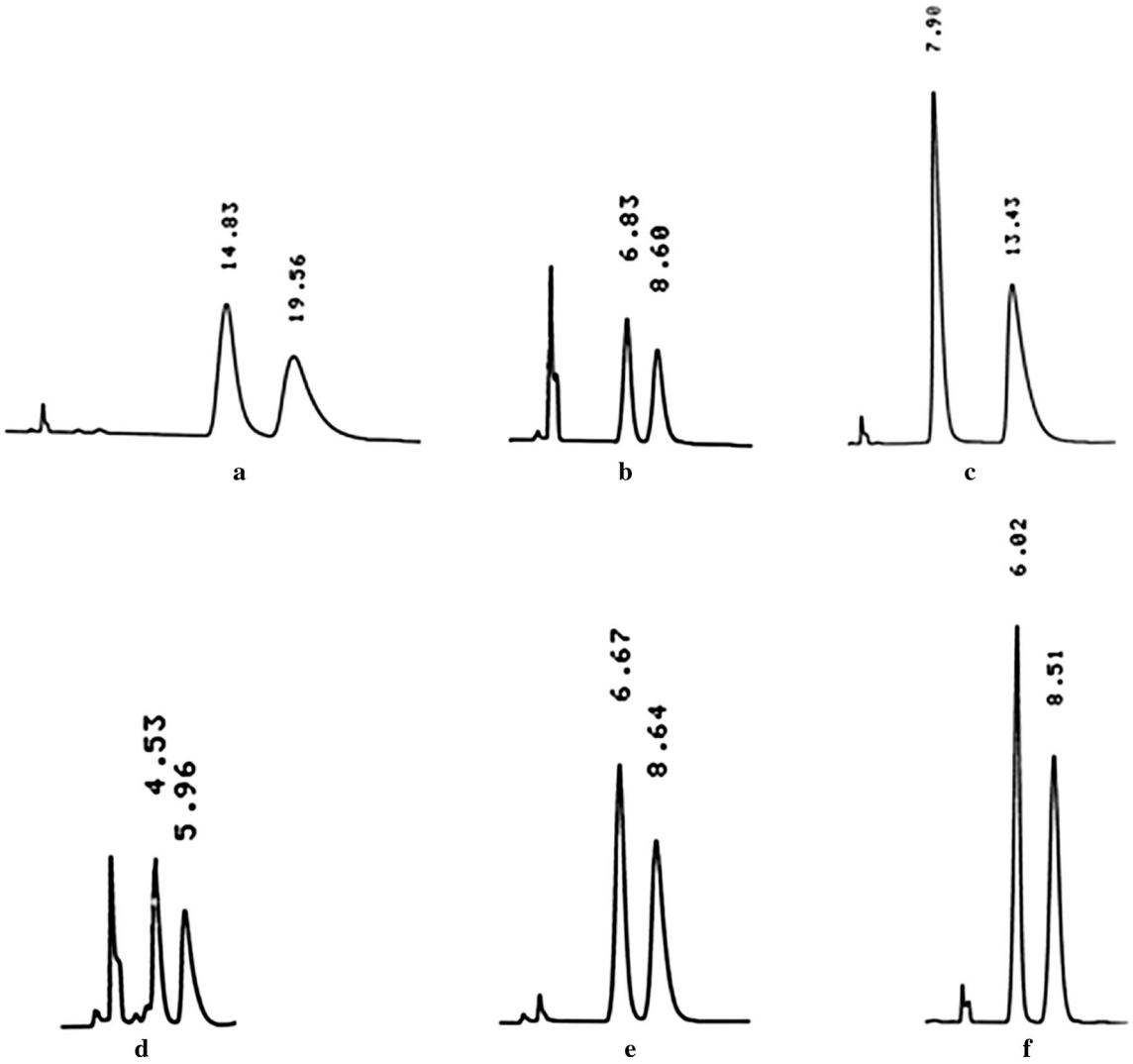

Fig. 3 Chromatograms of the profens in 5 mM, pH 5.0 phosphate buffer/MeOH (50/50, v/v %); **a** carprofen, **b** fenoprofen, **c** flurbiprofen, **d** ibuprofen, **e** indoprofen, **f** ketoprofen

buffer in a mixture with methanol. Using different organic modifiers and optimized pH and molarity, all chromatographic parameters were calculated.

Effect of buffer pH on retention and enantioseparation

The effect of the pH of the aqueous mobile phase on analyte retention and enantioseparation is critical since the ionization of acidic analytes and chiral selector can be controlled according to this.

The commercially available column Chirobiotic V containing vancomycin is described as being stable between pH 3.5 and 7. From the values listed in Table 1, it appears that chiral recognition operates in the tested pH range. Most compounds tested exhibit good enantioseparation between pH 4 and 6 with a maximum at pH 5. The pK_a values of profens are between 4 and 4.4 leading to negatively charged species at pH 5. At this pH value, the chiral selector still possesses positive charges, allowing it to interact electrostatically with the analytes.

Effect of buffer molarity on retention and enantioseparation

The concentration of the buffer is a very effective experimental parameter for controlling analyte retention and resolution. A range of ammonium nitrate buffer concentrations between 30 and 100 mM was investigated. Separation of all profens was observed in this range of concentrations. In our studies, an increase of the molarity gave a decrease of analyte retention (Fig. 1a), but the selectivity values remained quite constant. Most of the profens exhibited

maximum values of resolution at the concentration of 50 mM (Fig. 1b).

Effect of organic modifier on retention and enantioselectivity

In the chromatographic conditions, 50 mM ammonium nitrate buffer and pH 5, it was found that the elution strength of methanol is lower than the other organic modifiers, tetrahydrofuran and acetonitrile. In the case of tetrahydrofuran and acetonitrile both solvents were tested at percentages 20, 25 and 30 % v/v, while methanol was investigated at 40 and 50 % v/v. For all solvents tested, the retention of the profens decreased with increasing content of organic modifier. With respect to tetrahydrofuran, all the analytes were completely resolved for all solvent compositions, however, relatively short retention times were observed for 30 % v/v. In the case of 20 and 25 % v/v, the retention times were acceptable (<18 min for both enantiomers), except for carprofen and flurbiprofen which had a relatively long retention times, as can be seen in Table 2.

Adding TEA to the mobile phase of tetrahydrofuran caused a decrease in the retention time of ketoprofen and an increase in retention times for the other profens.

For acetonitrile as mobile phase (25 and 30 % v/v), the resolution of some analytes was not complete. At 20 % v/v acetonitrile, the six profens enantiomers were completely resolved with acceptable retention times (Table 2).

With methanol, analyte retention and resolution values represented in Table 2 show that the optimum conditions were achieved using a mobile phase consisting of 40 % v/v methanol.

The chromatograms of the six profens obtained using optimum conditions of pH and buffer concentration are shown in Fig. 2 with the mobile phase consisting of 40 % v/v methanol.

Effect of phosphate buffer

Although the six profens were successfully separated using the optimum conditions discussed above, the retention times of carprofen and flurbiprofen were relatively long, therefore, phosphate buffers were investigated. Sodium dihydrogen phosphate buffer, disodium hydrogen phosphate buffer and phosphate buffer were tested at different concentrations with a fixed amount of methanol, 50 % v/v. By changing the ammonium nitrate buffer to sodium dihydrogen phosphate buffer, it was found that the elution strength of methanol become weaker. The best retentions and resolutions were obtained with 50 % v/v methanol at pH 5 (Table 3). Almost all analytes showed good separation with the mobile phases tested (Tables 3, 4). The best retention times and resolutions were obtained with the mobile phases consisting of: 50 mM sodium dihydrogen phosphate buffer, pH 5: MeOH (50/50, v/v %), 25 mM disodium hydrogen phosphate buffer, pH 5: MeOH (50/50, v/v %) and 5 mM phosphate buffer, pH 5: MeOH (50/50, v/v %).

Figure 3 shows the chromatograms of the six profens using the mobile phase consisting of 5 mM phosphate buffer and MeOH (50/50, v/v %) at pH 5.

Conclusion

In this work, a column based on vancomycin immobilized by reductive amination to aldehyde functionalized silica was prepared in house and investigated for the chiral liquid chromatographic analyses of six NSAIDs. The effects of key experimental parameters on the enantioseparation were investigated. The selectivity, resolution and retention factors were studied for variation in buffer pH, solvent composition and nature of organic modifier. Good enantioseparations were obtained at pH 5 for all profens investigated with tetrahydrofuran, acetonitrile and methanol and 50 mM of ammonium nitrate buffer. Replacement of the ammonium nitrate buffer by a phosphate buffer gave good separations with shorter retention times. From the results obtained, the vancomycin stationary phase showed high enantioselectivity and stability over the pH range investigated (pH 4–6). From these results it is suggested that the chiral recognition mechanism for vancomycin is due to electrostatic interactions (charge–charge) between the negatively charged acidic compounds and the positively charged chiral selector. In conclusion, the results obtained for all profens show good separatory performance for the prepared column without deterioration of the vancomycin stationary phase incorporated into the silica. The results were reproducible and therefore the column can be considered robust and suitable for routine analysis.

References

1. S.S. Adams, P. Bresloff, C.G. Mason, J. Pharm. Pharmacol. **28**, 256 (1976)
2. A.J. Hutt, J. Caldwell, J. Pharm. Pharmacol. **35**, 693 (1983)
3. A.M. Evans, Eur. J. Clin. Pharmacol. **42**, 237 (1992)
4. N.M. Davies, Clin. Pharmacokinet. **34**, 101 (1998)
5. M.E. Anderson, D. Aslan, A. Clarke, J. Roeraade, G. Hagman, J. Chromatogr. A **1005**, 83 (2003)
6. J. Szymura-Oleksiak, M. Walczak, J. Bojarski, H.Y. Aboul-Enein, Chirality **11**, 267 (1999)
7. Y. Okamoto, R. Aburatani, Y. Kaida, K. Hatada, N. Inotsume, M. Nakano, Chirality **1**, 239 (1989)
8. A. Ducret, M. Trani, P. Pepin, R. Lortie, J. Pharm. Biomed. Anal. **16**, 1225 (1998)

9. X.W. Teng, S.W.J. Wang, N.M. Davies, J. Pharm. Biomed. Anal. **33**, 95 (2003)
10. E. Ameyibor, J.T. Stewart, J. Liq. Chromatogr. Relat. Technol. **20**, 855 (1997)
11. M. Pawlowska, S. Chen, D.W. Armstrong, J. Chromatogr. A **641**, 257 (1993)
12. B. Mistry, J.L. Leslie, D.N. Eddington, J. Chromatogr. B **758**, 153 (2001)
13. K.H. Ekborg-Ott, J.P. Kullman, X. Wang, K. Gahm, L. He, D.W. Armstrong, Chirality **10**, 627 (1998)
14. F. Pehourcq, C. Jarry, B. Bannwarth, Biomed. Chromatogr. **15**, 217 (2001)
15. D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.-R. Chen, Anal. Chem. **66**, 1473 (1994)
16. Z. Bosáková, E. Cuřínová, E. Tesařová, J. Chromatogr. A **1088**, 94 (2005)
17. A. Berthod, T. Yu, J.P. Kullman, D.W. Armstrong, F. Gasparrini, I. D'Acquarica, D. Misiti, A. Carotti, J. Chromatogr. A **897**, 113 (2000)
18. W.H. Pirkle, J.A. Burke, J. Chromatogr. A **557**, 173 (1991)
19. R. Cirilli, F. La Torre, J. Chromatogr. A **818**, 53 (1998)
20. F. Barbato, C. Carpentiero, L. Grumetto, M.I. La Rotonda, Eur. J. Pharm. Sci. **38**, 472 (2009)
21. E. Calleri, G. Massolini, D. Lubda, C. Temporini, F. Loiodice, G. Caccialanza, J. Chromatogr. A **1031**, 93 (2004)
22. F.C. Normand, D.M. Goudall, S.B. Duckett, M.F.H. Van-Tol, Chromatographia **55**, 307 (2002)
23. B. Kafková, Z. Bosáková, E. Tesařová, P. Coufal, A. Messina, M. Sinibaldi, Chirality **18**, 531 (2006)
24. L.A. Svensson, K.-E. Karlsson, J. Microcol. Sep. **7**, 231 (1995)
25. L.A. Svensson, K.-E. Karlsson, A. Karlsson, J. Vessman, Chirality **10**, 273 (1998)
26. K. Takács-Novák, B. Noszál, M. Tókés-Kövesdi, G. Szász, Int. J. Pharm. **89**, 261 (1993)
27. J.L.H. Johnson, S.H. Yalkowsky, AAPS PharmSciTech. **7**, 33 (2006)
28. I. Ilisz, R. Berkecz, A. Péter, J. Chromatogr. A **1216**, 1845 (2009)