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Use of vancomycin chiral stationary phase for the enantiomeric resolution of basic and acidic compounds by nano-liquid chromatography

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Abstract

In this paper we studied the potentiality of nano-liquid chromatography (nano-LC) for the enantiomeric resolution of both basic and acidic compounds of pharmaceutical interest using a vancomycin modified silica stationary phase. Experiments were carried out in a fused silica capillary of 75 μm I.D. packed with chiral modified silica particles of 5 μm diameter, the detection, was done on-line at 195 nm. Enantiomeric resolution of alprenolol, atenolol, metoprolol, oxprenolol, pindolol, propranolol (basic compounds) and some acidic analytes, namely 2-[(5′-benzoyl-2′-hydroxy)phenyl]propionic acid (DF1738Y), 2-[(4′-benzoyloxy-2′-hydroxy)phenyl]propionic acid (DF1770Y), ketoprofen, indoprofen and suprofen was studied by nano-LC utilizing mobile phases containing methanol-acetonitrile-ammonium formate or acetate. The effect of mobile phase composition (buffer type and concentration, organic modifier type and concentration) on chiral resolution (*Rs*), retention factor (*k*) and retention time (t_R) was also investigated. Good enantiomeric resolution was achieved for basic compounds utilizing the mobile phase containing 90% (MeCN–MeOH)/5% water/5% of 100 mM ammonium acetate pH 4.5. Acidic compounds such as DF1738Y and DF1770Y were better resolved at lower pH 3.5 while ketoprofen, indoprofen and suprofen exhibited the highest resolution at pH 4.5; in this case the mobile phase contained MeOH or MeCN (90%), 5% buffer and 5% of water. The nano-LC method was validated using *R*-(+)-propranolol as an internal standard finding good repeatability, detection limit, correlation coefficient and recovery and applied to the assay of a pharmaceutical formulation containing a racemic mixture of metoprolol.

Keywords: Nano-liquid chromatography; Enantiomers; Chiral; Drugs; Vancomycin

1. Introduction

In the last decade the separation and quantification of chiral compounds was one of the most studied subject by several research groups due to the growing interest for this type of molecula in the different fields such as biological, biomedical, ageing, food, agriculture, pharmaceutical, etc. The modern orientation of pharmaceutical industry is the production of the single enantiomer as an active component of a certain drug when it has been demonstrated that the two enantiomers exhibit different pharmacological/biological activity. Additionally the analysis of enantiomers of a drug and of its chiral metabolites can be used as a test in order to discover metabolic disorders [1]. Therefore, due to the increasing interest to the human health, there is a need of analytical methods capable to determine qualitatively and quantitatively enantiomeric compounds.

Different chromatographic techniques including gas chromatography (GC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC) and high performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) have been widely applied to the separation of enantiomeric compounds [2–9]. Among them HPLC resulted to be the most employed tool especially in pharmaceutical routinary

Abbreviations: nano-LC, nano-liquid chromatography

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analysis since offering excellent reproducibility and robustness. In spite of the mentioned features, the use of analytical columns may be unfavourable because the elevated costs of chiral stationary phase, consumption of large volumes of mobile phases with a consequent environmental impact. Therefore in the last decade different miniaturized techniques were developed and investigated for the separation of chiral compounds. Among them capillary liquid chromatography (CLC) and nano-liquid chromatography (nano-LC) have received great attention by several research groups mainly studying theoretical and instrumental solutions at the problem, e.g., generation of μ/nano-flow, column diameter, packing or monolithic material etc [10–13].

The nano-scale LC techniques may offer several benefits when compared to conventional HPLC such as (i) increased mass sensitivity because the reduced chromatographic dilution, (ii) higher efficiency and (iii) lower consumption of solvents [10]. Additionally the nano-LC system can easily be coupled to the mass spectrometer (MS) through a nano-spray interface.

A wide number of chiral selectors possessing different properties and operating with different resolution mechanisms were successfully studied for the enantiomeric resolution of a wide number of compounds in separation science. Among them glycopeptide antibiotics exhibited very high enantioselectivity towards several classes of compounds, e.g., amino acids, peptides, herbicides, drugs etc. [7,14,15].

Armstrong's group firstly demonstrated the usefulness of such class of chiral selectors by using HPLC [14]. Since this time numerous papers were published extending the applicability of these chiral compounds to other separation methods such as capillary zone electrophoresis (CZE) [16–18], capillary electrochromatography (CEC) [7,19–24] and recently nano-liquid chromatography (nano-LC) [25].

In this study we developed a nano-LC method using a vancomycin modified silica stationary phase for the enantiomeric resolution of basic and acidic compounds of pharmaceutical interest. Different experimental parameters such as composition of the mobile phase, namely organic modifier type and concentration, buffer type and pH were studied to optimize the enantioseparation of the studied compounds. The optimized method was validated and applied to the analysis of a pharmaceutical formulation containing metoprolol.

2. Experimental

2.1. Reagents and chemicals

Formic acid (90%) and ammonia solution (30%) were purchased from Carlo Erba (Milan, Italy). Mobile phases were daily prepared by mixing the appropriate volumes of organic solvents, water and buffer solutions. LiChrospher diol silica phase 5 μm particle diameter, sodium cyanoborohydride and sodium periodate were from Merck (Darmstadt, Germany). Ammonium acetate, methanol (MeOH), acetonitrile (MeCN), all of HPLC analytical grade, were purchased from BDH (Poole, England). Racemic alprenolol, atenolol, metoprolol, oxprenolol, pindolol, propranolol were purchased from Sigma (St. Louis, MO, USA). 2-[(5′-benzoyl-2′-hydroxy)phenyl]propionic acid (DF1738Y), 2-[(4′-benzoyloxy-2′-hydroxy)phenyl]propionic acid (DF1770Y) is candidate drugs under evaluation and kindly provided by Dompè (L'Aquila, Italy).

Stock standard solutions (1 mg/mL) were prepared by dissolving the appropriate weighted powder of each analyte in methanol and the appropriate volume of each solution was daily mixed and diluted at the desired concentration with the mobile phase for the nano-LC analysis.

2.2. Instrumentation

Nano-LC experiments were carried out using an in house packed capillary with vancomycin modified silica stationary phase where the chiral selector was chemically bonded. A Spectrasystem P2000 HPLC pump (Thermo Separation Product, Fremont, CA, USA) was used to deliver the mobile phase at a flow rate of 300–800 μL/min to the capillary column after splitting. A stainless steel T piece, purchased by Valco (Houston, TX, USA), was connected to the pump through a PEEK capillary 50 cm × 127 μm I.D.; connection to the injector and to waste was done using a stainless steel tube 5 cm (100 μm I.D.) and 40 cm (50 μm I.D.) fused silica capilllary, respectively. The split ratio using 75 μm I.D. packed capillary was about 1/2000. Injection was done using a manual four-port injection valve 60 nL internal loop (Valco Instruments, Houston, TX, USA) while detection was done with a multiwavelength UV–vis spectra focus detector (Thermo Separation Product, Fremont, CA, USA) operated at 195 nm. Data were acquired on line with a Compaq 486 computer with an appropriate software (Thermo Separation Product).

The marker used to determine the dead volume (t_o) was methanol and the perturbance of the baseline observed in the chromatogram. Retention and enantioresolution factors were calculated by using the following equations:

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

$$Rs = \frac{2(t_{R2} - t_{R1})}{(w_2 + w_1)} \quad (2)$$

Where t_R, t_0, w are the retention time of analytes, dead time and base-peak width, respectively of enantiomer 1 and 2 first and second eluted, respectively.

2.3. Capillary column preparation

Fused silica capillary tubing (75 μm I.D. and 375 μm O.D.) purchased by Composite Metal Services (Hallow, UK) was packed in our laboratory with chiral stationary phase (CSP). CSP was diol-silica particles modified with vancomycin (chemically bonded). This CSP was synthesized in our laboratory using a previously reported method [26]. One

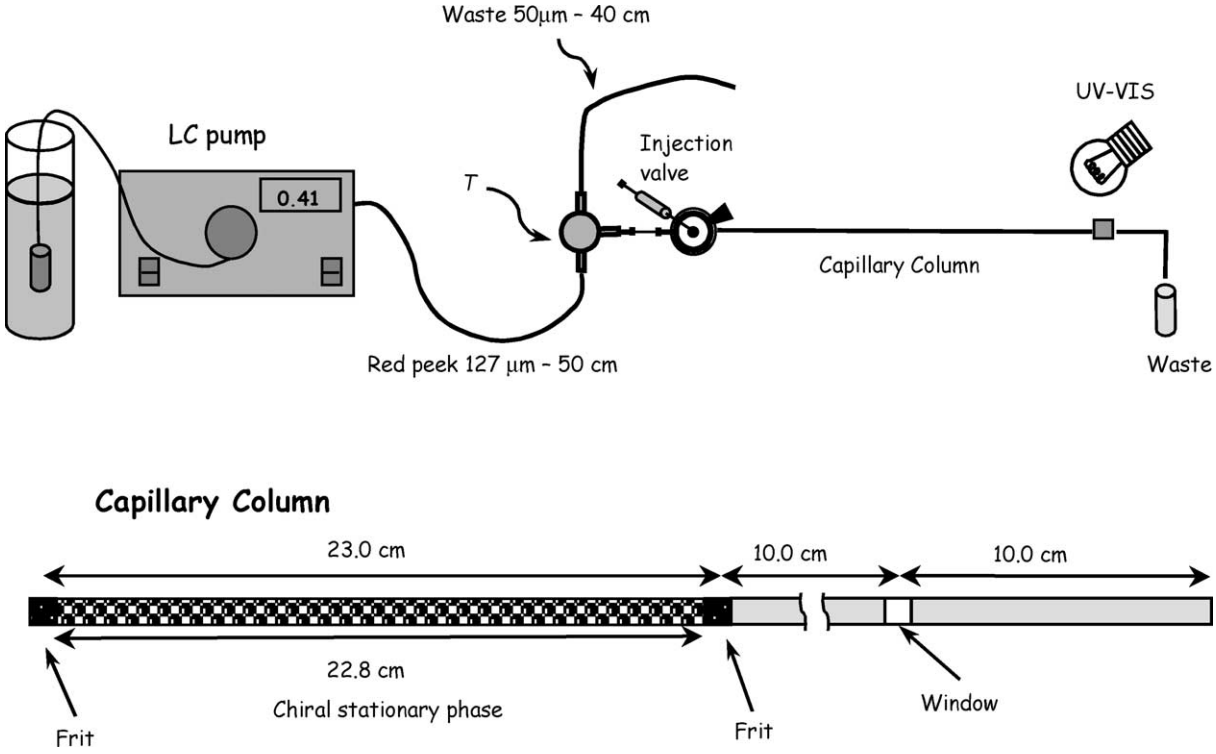

Fig. 1. Scheme of the laboratory made nano-LC system.

end of the capillary was connected to a stainless steel HPLC pre-column (10 cm × 4.1 mm I.D.) used as a reservoir of the slurry while the other end was connected at a mechanical HPLC frit (Valco, Houston, Tx, USA). The reservoir was filled with slurry containing LiChrospher 100 RP18 5 μm particles-acetone and the capillary packed for 10 cm pumping with water at 30 MPa. The reservoir was removed, cleaned, filled with water and connected again to the capillary that was flushed for 30 min; the frit was prepared with the heated wire (about 1100 °C × 6 s). The capillary was cut close to the end frit, connected to the pump and flushed with water in order to eliminate the stationary phase. The open end of the capillary was connected to the reservoir containing the chiral stationary phase suspended in acetone–water (1:1) mixture and packed again for a length of 22.8 cm. Finally the capillary was packed with the RP18 particles (5 cm) and after flushing with water the frit prepared and the free stationary phase eliminated.

Table 1
Effect of different organic modifiers on retention times t_{r1}, t_{r2} (min), retention factors k of 1 and 2 first and second eluted enantiomers, respectively and resolution Rs

Compounds	Acetonitrile		Methanol		Ethanol		*n*-Propanol		2-Propanol	
	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2	k_1	k_2
Alprenolol	2.31	2.45	3.35	3.66	3.38	3.69	3.16	3.44	3.10	3.38
Atenolol	10.12	10.77	9.79	10.75	11.21	12.23	11.14	12.09	11.34	12.35
Metoprolol	1.88	2.00	4.08	4.44	4.26	4.64	4.02	4.35	3.97	4.32
Oxprenolol	2.46	2.59	3.55	3.83	3.57	3.85	3.38	3.64	3.34	3.61
Pindolol	3.23	3.42	4.44	4.79	4.46	4.81	4.21	4.53	4.16	4.49
Propranolol	2.72	2.89	4.03	4.41	4.16	4.55	3.83	4.18	3.87	4.21
	Rs		*Rs*		*Rs*		*Rs*		*Rs*	
Alprenolol	0.87		1.33		1.30		1.18		1.20	
Atenolol	1.17		1.66		1.79		1.63		1.68	
Metoprolol	0.62		1.36		1.25		1.07		1.13	
Oxprenolol	0.60		1.20		1.13		1.03		1.07	
Pindolol	0.90		1.15		1.14		1.05		1.08	
Propranolol	0.85		1.12		1.24		1.12		1.11	

Capillary, 75 μm I.D., 43 cm total length, 33 cm effective length, 23 cm packed with vancomycin-modified silica stationary phase; mobile phase, 5% 100 mM ammonium acetate (pH 4.5) containing 80% of acetonitrile, 5% water and 10% of different organic modifiers. Flow rate, 400 μL/min split to 200 nL/min. The samples were injected at the final concentration of 100 μg.

2.4. Analysis of metoprolol in tablets

Twenty tablets of Lopreson® declared to contain racemic metoprolol were weighed, crushed to fine powder and the equivalent of 10 mg of metoprolol, accurately weighed, was transferred into a 5 mL beaker. The powder was treated with 5 mL of methanol, shaken vigorously, sonicated for 15 min and shaken again. The resulting suspension was then filtered through a dry filter into a 5 mL volumetric flask, and the volume was adjusted with methanol passed through the beaker and the filter walls. The obtained solution contained metoprolol at a concentration of about 2 mg/mL. A 50 μL aliquot of supernantant was diluted with methanol in order to achieve a metoprolol test concentration of 0.1 mg/mL. To the methanolic solution was added 50 μL of internal standard stock solution and further diluted with the mobile phase to the final concentration (25 μg/mL) before injection.

3. Results and discussion

In this work we had in mind, as the main goal, to study (i) the potential of nano-LC for the chiral resolution of enantiomers by using a glycopeptide antibiotic (vancomycin) bonded to a silica stationary phase (ii) the possibility to run the experiments employing instrumentation currently present in our laboratory for conventional HPLC and CE.

Fig. 1 shows a scheme of the nano-LC instrumentation used in this study.

Based on our previously published results dealing with the use of vancomycin modified silica stationary phase for the chiral resolution of basic and acidic enantiomers by CEC we selected several of this compounds namely alprenolol, atenolol, metoprolol, oxprenolol, pindolol, propranolol and acidic ones DF 1738Y, DF 1770Y, ketoprofen, indoprofen, suprofen.

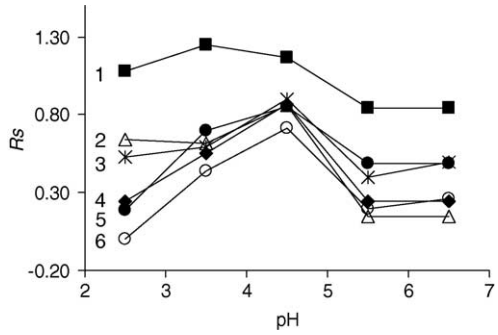

Fig. 2. Effect of buffer pH in the mobile phase on enantioresolution (*Rs*) of (1) atenolol; (2) metoprolol; (3) pindolol; (4) alprenolol; (5) propranolol and (6) oxprenolol. Mobile phase, 100 mM ammonium formate buffer (pH 2.5–3.5), or 100 mM ammonium acetate buffer (pH 4.5–6.5)/90% acetonitrile, and 5% H_2O (v/v/v). Other experimental conditions as reported in Table 1.

Nano-LC experiments were carried out at room temperature and eluting with mobile phases containing volatile buffers (ammonium acetate or formate) at pH 2.5–6.5 and acetonitrile and/or MeOH with the aim to couple the system to a mass spectrometer.

3.1. Enantioresolution of basic compounds of pharmaceutical interest

Preliminary experiments were carried out using a mobile phase that allowed achieving good enantiomeric resolution with vancomycin chiral stationary phase (CSP) by CEC [26].

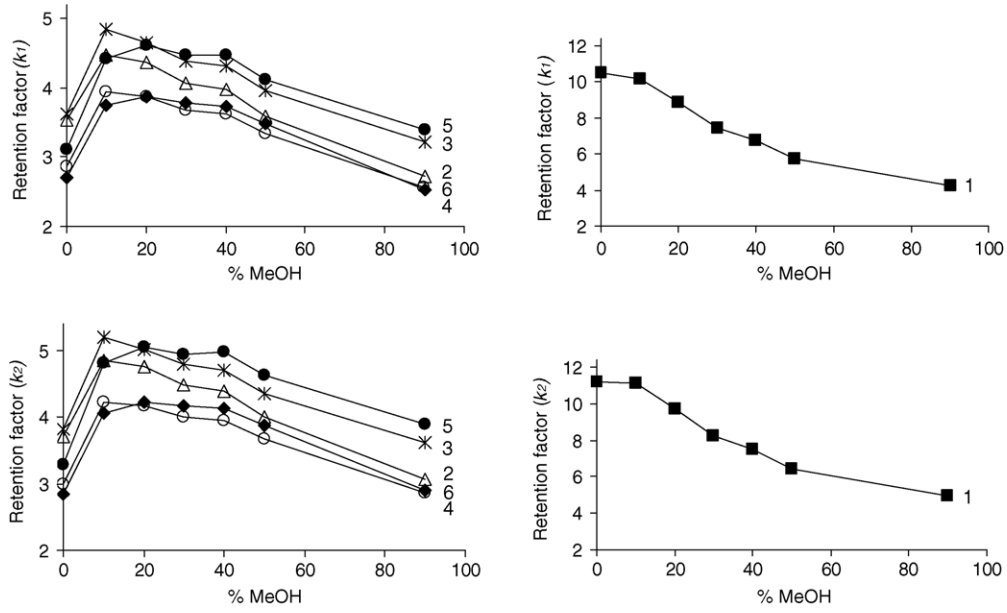

Fig. 3. Effect of methanol concentration in the mobile phase on retention factor (*k*) of studied basic compounds (1) atenolol; (2) metoprolol; (3) pindolol; (4) alprenolol; (5) propranolol and (6) oxprenolol. Mobile phase, 100 mM ammonium acetate (pH 4.5)/ different ratios MeOH/MeCN/ 5% H_2O (5:90:5, v/v/v). For other experimental conditions see Table 1 and text.

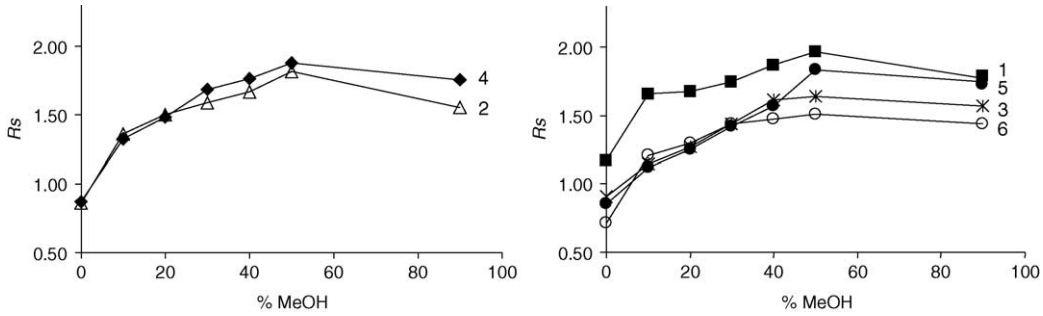

Fig. 4. Effect of methanol concentration in the mobile phase on the enantioresolution (*Rs*). Samples: (1) atenolol; (2) metoprolol; (3) pindolol; (4) alprenolol; (5) propranolol and (6) oxprenolol. Mobile phase, 100 mM ammonium acetate (pH 4.5)/ different ratios MeOH/MeCN/ H_2O (5:90:5, v/v/v). For other experimental conditions see Table 1 and text.

Employing this mobile phase containing ammonium acetate pH 4.5/MeCN (10:90, v/v) we achieved enantiomeric resolution of all studied basic racemic mixtures, however their baseline enantioseparation was not observed. Therefore in order to find the optimum experimental conditions we investigated the use of different mobile phases containing other organic modifiers in addition to MeCN. Consequently 10% (v/v) of MeOH or EtOH or PrOH or iso-PrOH were added to the above-described mobile phase reducing the content of MeCN at 80% (v/v) and the experiments run in order to investigate their effect on retention time, retention factor and enantioresolution.

As can be seen in Table 1 the *k* and *Rs* values increased for all studied basic enantiomers by replacing MeCN with 10% of the above mentioned organic modifiers. Therefore, from these data it can be observed that the addition of less polar organic solvent to the mobile phase (EtOH, PrOH, iso-PrOH) is helpful in improving both selectivity and stereoselectivity; the behavior of MeOH is very similar to that of the other organic solvents besides this modifier is more polar than MeCN.

To explain the enantioresolution mechanism when using glycopeptide antibiotics is not an easy task and cannot be done only considering the competition of the organic solvent with the analytes but other interactions such as electrostatic or hydrogen bonding has to be taken into account.

In the affinity interaction process, typical of chiral selectors such as vancomycin, the pH of the mobile phase is a very important parameter that has to be carefully controlled because can influence the charge of both analyte and chiral selector. For that reason the pH of buffer present in the mobile phase was selected in the range 2.5–6.5 using ammonium formate (pH 2.5 and 3.5) or ammonium acetate (pH 4.5, 5.5, 6.5).

A general increase of *Rs* of all analytes was observed by raising the buffer pH reaching a maximum and then decreased. As can be seen in Fig. 2 the maximum value of the enantioresolution factor was achieved at pH 4.5 for all analytes with the exception of atenolol that was observed at pH 3.5.

The effect of the mobile phase allowing the highest resolution for most of the analyzed compounds (buffer pH 4.5) was further investigated modifying the MeCN/MeOH ratio keeping under observation t_R, *k* and *Rs*; the results are shown in Figs. 3 and 4.

The increase of MeOH concentration in the mobile phase from 0 to 90% (lowering proportionally MeCN) caused a general increase of *k* value at 10% of MeOH and then a decrease

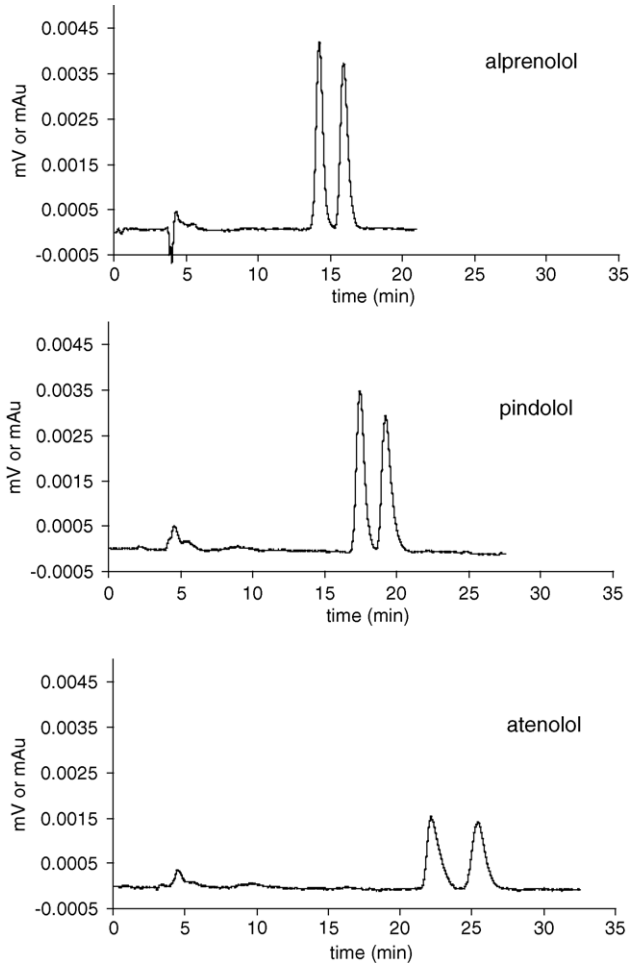

Fig. 5. Chromatograms of the enantiomeric separation of alprenolol, pindolol and propranolol by nano-LC in a 75 μm I.D. capillary packed with a silica modified vancomycin stationary phase. Mobile phase MeOH/water/100 mM ammonium acetate pH 4.5 (90:5:5, v/v/v). For other experimental conditions see Table 1 and text.

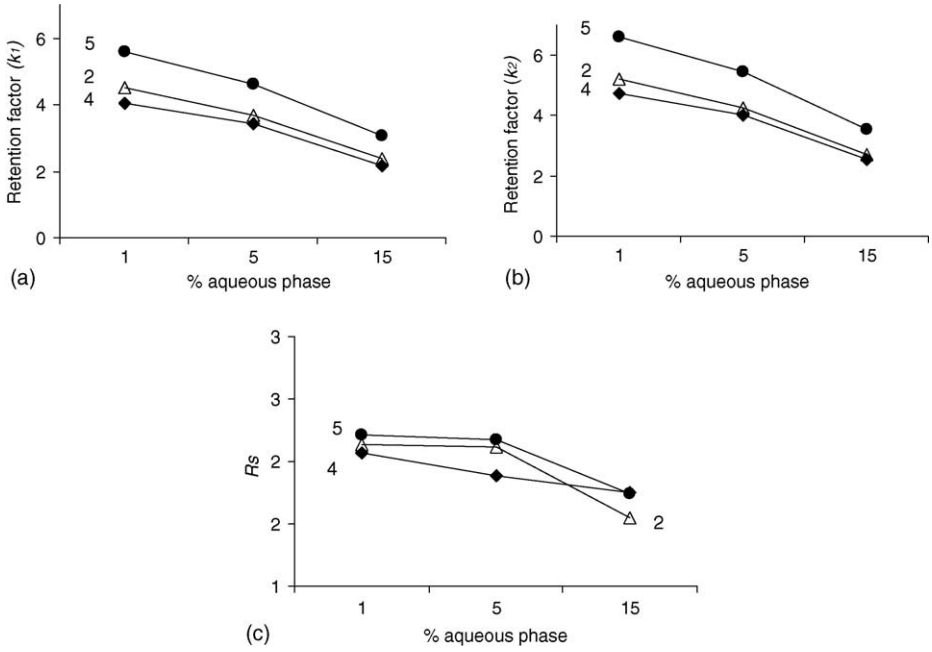

Fig. 6. Effect of the water concentration in the mobile phase on retention factor (a and b) and enantioresolution factor (c) of (2) metoprolol, (4) alprenolol, 5 – propranolol. Mobile phase: MeOH/water/500 mM ammonium acetate pH 4.5 (99/0/1; 95/4/1; 85/14/1, v/v/v).

for alprenolol, metoprolol, oxprenolol and pindolol. In the case of propranolol a similar behavior was noticed but with a maximum at 20% of MeOH. Atenolol exhibited the highest *k* at 0% of MeOH (90% MeCN) that lowered by increasing the content of this solvent (see Fig. 3).

A general increase of *Rs* values was observed by raising the MeOH concentration (see Fig. 4) until 50% with a decrease at higher content of this organic modifier. Considering the above discussed results we can conclude that the presence of high concentration of MeOH in the mobile phase is of great importance in achieving good enantioresolution for all studied basic analytes in a reasonable time.

As an example of the good performance of the nano-LC method, Fig. 5 shows the baseline enantiomeric separation of some studied basic analytes.

Therefore nano-LC experiments were carried out maintaining constant the buffer concentration and modifying the water/MeOH ratio, e.g., 0, 1, 5 and 15% of water studying

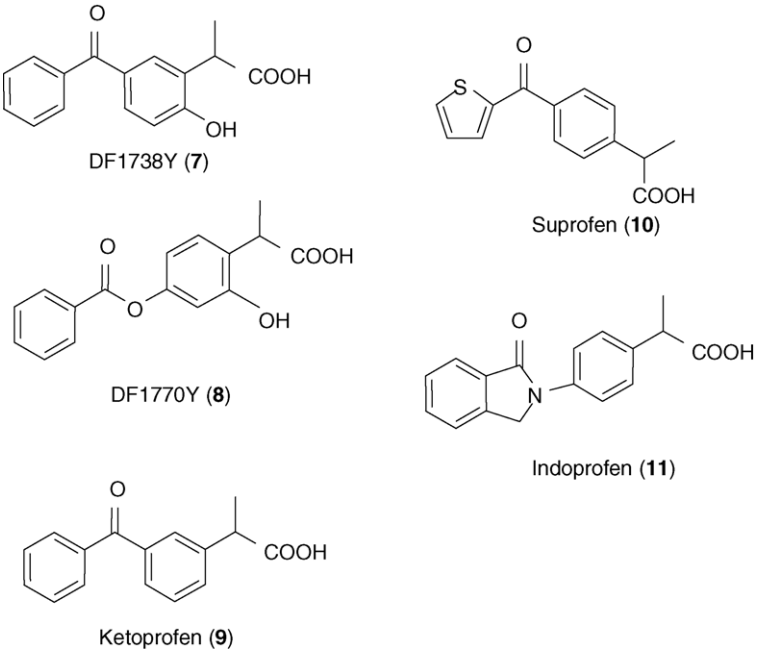

Fig. 7. Chemical structures of studied acidic compounds of pharmaceutical interest.

only selected basic enantiomers of alprenolol, metoprolol and propranolol.

Raising the water concentration caused an increase of t_R for all three studied couples of enantiomers (results not shown). A very similar trend was observed for k_1 and k_2 (see Fig. 6a and b). When the water was not present (absence of the buffer) peak were not detected at all. The presence of 1% water (corresponding to 1% buffer) caused the achievement of the highest enantioresolution factor for the three racemic analytes while the increase of water resulted in a decrease of *Rs* with the lowest values at 15%.

Based on the above discussed results it seems that the mobile phase containing 95% MeOH/4% water/1% ammonium acetate pH 4.5 was the most appropriate for allowing good enantiomeric resolution in a reasonable analysis time for all analyzed compounds (the final concentration of ammonium acetate in the mobile phase was 5 mM). Further experiments done by changing the type of positive ion (buffer), e.g., K^+, Na^+, NH_4^+ revealed that the use of ammonium allowed achieving the highest enantioresolution for the three studied basic compounds (alprenolol, metoprolol and propranolol). This effect can be explained considering the affinity separation mechanism where among the different interactions between analytes-vancomycin we have to consider the electrostatic one. Consequently the co-ion type present in the mobile phase influences the ion-exchange process.

3.2. Enantioresolution of acidic compounds of pharmaceutical interest

Some acidic compounds of pharmaceutical interest belonging to the family of non steroidal anti-inflammatory drugs namely indoprofen, suprofen, ketoprofen and two related drugs to the last compounds (DF 1738Y and DF 1770Y) were selected as model analytes in order to test the nano-LC system for their chiral resolution.

Based on our previous results achieved in CEC for the enantiomeric resolution of acidic compounds by using silica capillaries packed with vancomycin–silica stationary phase we selected MeCN–ammonium formate or ammonium acetate in the pH range 2.5–6.5 in order to separate the five racemic acidic compounds in their enantiomers.

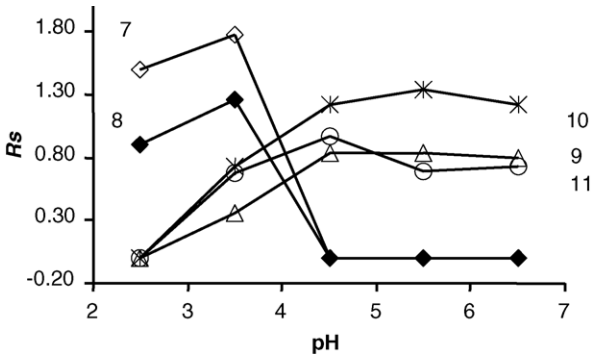

Fig. 8. Effect of buffer pH in the mobile phase on enantioresolution of acidic compounds. Mobile phase: 100 mM ammonium formiate pH 2.5, 3.5 or 100 mM ammonium acetate pH 4.5, 5.5, 6.5/water/MeCN (5/5/90, v/v/v): Flow rate about 100 nL/min. Injection 60 nL of 10 μg/mL of each racemic compound. For other experimental conditions see Table 1 and text.

The three acidic compounds with a related chemical structure (see Fig. 7) ketoprofen, DF 1738Y and DF 1770Y exhibited a very different behavior changing the buffer pH of the mobile phase. At pH 2.5 DF 1738Y enantiomers were the most retained followed by DF 1770Y and then ketoprofen; similar behavior was noticed for retention factor (results not shown).

The change of the buffer pH present in the mobile phase markedly influenced the enantiomeric resolution of the studied acidic analytes and the effect was strongly dependent by the chemical structure of analytes. As can be observed in Fig. 8 at pH 2.5 DF 1738Y was baseline resolved in its enantiomer, DF 1770Y was partly resolved while ketoprofen showed $Rs = 0$; no enantioresolution was observed for indoprofen and suprofen. The increase of buffer pH at 3.5 caused an increase of enantioresolution for all studied racemic compounds and then a further increase of pH resulted in the lost of *Rs* for DF 17738Y and DF 1770Y while in the case of ketoprofen enantioresolution increased reaching a maximum at pH 5.5. Indoprofen and suprofen exhibit a maximum value of *Rs* at pH 4.5; at higher pH the resolution decreased. Based

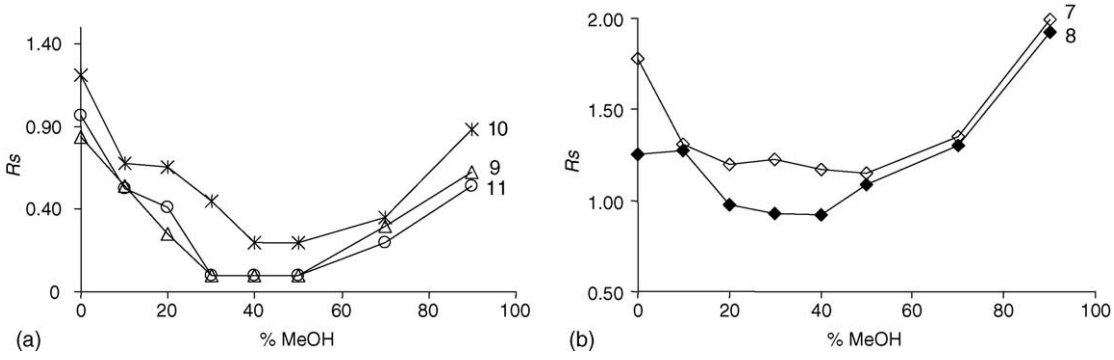

Fig. 9. Effect of MeOH concentration in the mobile phase on enantioresolution factor of (a) ketoprofen, indoprofen and suprofen and (b) DF1738Y and DF1770Y. Experimental conditions: mobile phase (a) 100 mM ammonium acetate pH 4.5 (b) 100 mM ammonium formiate pH 3.5/water/MeOH–MeCN at different ratio (5:5:90, v/v/v). For other conditions see text.

on the above-illustrated results the mobile phase was modified adding different concentrations of MeOH and decreasing proportionally those of MeCN. Fig. 9a and b show the effect of MeOH concentration on enantioresolution of the studied analytes using the buffer at pH 3.5 for DF compounds and 4.5 for ketoprofen, indoprofen and suprofen. As can be observed in both figures the resolution decreased by increasing the concentration of MeOH and at 40–50% increased. The highest *Rs* values were recorded both at the highest and at the lowest concentration of MeOH. Comparing the results achieved at pH 3.5 that allowed the enantioresolution of ketoprofen and its related DF compounds, it is noteworthy to mention that the enantiorecognition capability of the nano-LC system was as follow: DF1738Y > DF1770Y > ketoprofen that can be explained by the differences in the chemical structures of analytes. DF1738Y differs from ketoprofen by the presence of a hydroxy group in ortho position that can strongly contribute to the chiral recognition mechanism, e.g., on forming hydrogen bonds with the vancomycin stationary phase. A similar trend was observed analyzing the same compounds in CEC using vancomycin stationary phase [27].

4. Validation and assay of metoprolol in tablets

With the aim to apply the optimized nano-LC method to the analysis of β-blockers in pharmaceutical preparations we studied several validation parameters for the analysis of metoprolol enantiomers such as intra- and inter-day repeatability, limits of detection (LOD), limits of quantitation (LOQ), linearity. 25 μg/mL standard solution of *R*-(+)-Propranolol was used as internal standard. Analytes were eluted using the mobile phase containing: 500 mM ammonium acetate pH 4.5/water/MeOH (1:4:95, v/v/v) allowing obtaining the optimum enantioresolution.

LOD and LOQ were determined from three and ten times the signal-to-noise ratio (*S*/*N*) values by analyzing a standard mixture containing racemic metoprolol and assuming that the two separated enantiomers were at 50% concentration finding 1.5 and 4.0 μg/mL for limits of detection and limits of quantification, respectively.

The standard mixture 50 μg/mL of each metoprolol enantiomer was analysed seven times in order to verify the repeatability (within-day precision) of the method in terms of retention times and peak area ratios. The same mixture was analysed over three days to evaluate intermediate precision (between-day precision).

A very good within-day precision was observed (seven runs of the same standard mixture), e.g., RSD of 0.4 and 0.5% for retention time, 0.8 and 0.9 for retention factor of the first and second eluted enantiomer, respectively; 1.2% was recorded for enantioresolution factor. As expected the intermediate precision was less satisfactory, RSD 3.1, 4.8 and 1.8% for retention times, retention factors and resolution, respectively.

Linearity of the method was studied analyzing six standard mixtures at different concentration of racemic metoprolol in the range 5–100 μg/mL and 25 μg/mL of *R*-(+)-propranolol (I.S.). The plots of concentration of metoprolol versus peak area ratios (A_s/A_{st}) of the first and second eluting metoprolol enantiomers were linear in the studied concentration ranges with a good correlation coefficient. First eluting enantiomer: $R^2 = 0.9998$, $y = 0.016x - 0.0085$; second eluting enantiomer: $R^2 = 0.9997$, $y = 0.0156x - 0.0064$.

The accuracy of the method was verified spiking the drug product with standard metoprolol at three concentrations covering the linearity range, namely 40, 60, 80 mg for tablet obtaining the following recovery: 97.7 ± 1.2, 97.3 ± 2.2 and 97.1 ± 1.2, respectively.

The optimized nano-LC method was applied to the assay of a pharmaceutical formulation (Lopreson®) containing 100 mg for each tablet (with a mass of 336.11 mg) of racemic metoprolol. *R*(+)-propranolol was used as the internal standard. The recovery of metoprolol (racemic mixture) was in agreement with the 100 mg declared content (recovery $98.2 \pm 0.6\%$, RSD 0.2%).

5. Conclusions

Enantiomeric separation of basic and acidic compounds of pharmaceutical interest was achieved by using nano-liquid chromatography utilizing laboratory-assembled instrumentation. Analyses were carried out in a capillary column packed by us with vancomycin modified silica stationary phase (5 μm particles) and eluting with stationary phases containing ammonium formate or acetate (pH 2.5–6.5)/MeCN and/or MeOH. The vancomycin chiral stationary phase exhibited good enantiorecognition capability towards all studied compounds by selecting the appropriate mobile phase taking into account the pH of the buffer, the type and concentration of the organic modifier. Acidic compounds were better resolved at lower pH than basic one. The nano-LC method was validated finding good repeatability of retention time and good LOD and LOQ; the method was successful applied to the assay of metoprolol enantiomers present in a pharmaceutical formulation. Compared to other related analytical methods, e.g., HPLC, nano-LC allows to achieve good results at lower costs and minor environmental impact due to the minute amount of chiral stationary phases and lower volumes of mobile phases, respectively. The limitation of this nano-technique is the low injected volume samples that may represent a disadvantage due to the sensitivity necessary for practical applications, e.g., in biological fluids, however this problem can be resolved using different sampling approaches of sample pre-treatment. In order to apply the nano-LC method to practical analysis, we are currying out in our laboratory studies dealing with focusing effect or sample pre-treatment (liquid–liquid extraction).

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