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ORIGINAL RESEARCH

Chiral resolution of flurbiprofen and ketoprofen enantiomers by HPLC on a glycopeptide-type column chiral stationary phase

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ABSTRACT: Vancomycin is an amphoteric, glycopeptide, macrocyclic antibiotic. When attached to 5 μspherical silica gel, vancomycin proved to be an effective chromatographic chiral stationary phase that could be used in the reversed-phase mode. In this study, a bonded vancomycin chiral stationary phase (Chirobiotic V™) was investigated for the chiral liquid chromatography analysis of ketoprofen and flurbiprofen. The selectivity factor (α) and the chiral resolution factor (R_S) of Chirobiotic V™ were evaluated first as a function of the buffer pH and molarity, and second as a function of organic modifier type and composition of the mobile phase. Four organic modifiers (tetrahydrofuran, 2-propanol, 1,4-dioxane and methanol) have been tested for their selectivity. Optimized conditions using 20% of tetrahydrofuran in ammonium nitrate (100 mM, pH 5) were selected for the enantioseparation of flurbiprofen and ketoprofen from their racemic forms. At pH 5, these acidic compounds are almost negatively charged, while the chiral selector possesses a positive charge allowing it to interact electrostatistically with the analytes. Using these chromatographic conditions, the column stability was excellent over several months of experiments. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Many pharmaceuticals contain enantiomeric active principles administered as racemates. The separation of optical isomers is essential since the enantiomers are often distinguished by biological systems. Moreover, they may have different pharmacological, pharmacokinetic and toxicological effects (Williams and Lee, 1985; Berry and Jamali, 1989; Bannwarth *et al.*, 1995).

Flurbiprofen and ketoprofen are potent non-steroidal anti-inflammatory drugs (NSAIDs) of the 2-arylpropionic acid class, currently available in their racemic form. Both enantiomers exert different pharmacodynamic effects, ie the prostaglandin synthetase inhibiting effect is attributable to the *S*-enantiomer (Hutt and Caldwell, 1984; Geisslinger and Schaible, 1996). Consequently, the specific determination of each enantiomer in plasma is of potential clinical importance (Bhushan and Martens, 1998). This can be performed using different methods:

1. a direct separation using chiral HPLC columns (Menzel-Soglowek *et al.*, 1990; Oda *et al.*, 1991; Geisslinger and Menzel-Soglowek, 1992; Haginaka and Murashima, 1993; Castellani *et al.*, 1994; Van Overbeke *et al.*, 1994, 1995; Boisvert *et al.*, 1997);
2. a pre-column derivatization with optically pure chiral reagents (Péhourcq *et al.*, 1995; Al-Kindy *et al.*, 1997; Pecanac *et al.*, 1997);
3. the use of an achiral HPLC method with chiral mobile phase (Ameyribor and Stewart, 1998; Trelli-Seifert and Risley, 1998).

Among these methods, the direct ones (1) appear to be of great applicability for optical purity control in drug production or for pharmacokinetic studies. The direct separation of flurbiprofen and ketoprofen enantiomers has been accomplished on various types of protein columns, such as α1-acid glycoprotein (Menzel-Soglowek *et al.*, 1990; Geisslinger and Menzel-Sloglowek, 1992), avidin (Oda *et al.*, 1991) or ovomucoid (Haginaka and Murashima, 1993). The possibilities offered by different derivatized cellulose columns have also been proposed (Van Overbeke *et al.*, 1994, 1995).

Recently, several macrocyclic antibiotics were shown to be very successful in achieving enantioseparations. For example, avoparcin, ristocetin A and vancomycin contain moieties capable of providing several potential interaction sites for chiral recognition between enantiomers (Armstrong *et al.*, 1995; Ekborg-Ott *et al.*, 1998). Therefore, these antibiotics were covalently bonded to a silica gel, leading to different types of chiral stationary phases useful for HPLC.

In this study, a vancomycin-linked column (Chirobiotic V™) was investigated for the chiral LC-analysis of

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Table 1. Effect of buffer pH on capacity factor (k'_1), selectivity (α) and resolution (R_S)

	Flurbiprofen			Ketoprofen		
pH	k'_1	α	R_S	k'_1	α	R_S
4	2.08	1.27	2.46	1.35	1.13	1.12
4.5	1.57	1.46	3.65	0.94	1.23	1.54
5	0.97	1.65	4.37	0.56	1.35	1.68
5.5	1.11	1.60	3.93	0.79	1.29	1.59
6	0.92	1.56	2.96	0.67	1.30	1.43
6.5	0.82	1.64	2.91	0.52	1.30	1.25

k'_1: capacity factor of the first-eluted enantiomer.
In this assay, the eluent was 20 mM ammonium nitrate buffer–tetrahydrofuran (80:20, v/v).

two NSAIDs drugs, flurbiprofen and ketoprofen. The effects of different experimental parameters on the enantioselective separation were tested in order to define the optimal chromatographic conditions.

EXPERIMENTAL

Materials. Racemic flurbiprofen and ketoprofen were purchased from Sigma (St Quentin Fallavier, France). Pure *R*- and *S*-enantiomers of each drug were kindly supplied by the Boots Company Ltd (Nottingham, England) for flurbiprofen and by Rhône-Poulenc Rorer (Vitry-Alforville, France) for ketoprofen. Methanol, 1,4-dioxane, 2-propanol and tetrahydrofuran were supplied by Prolabo (Paris, France) and were of HPLC reagent grade. Ammonium nitrate (Prolabo) was of analytical grade. Water was deionized and doubly-glass distilled.

Apparatus. The analyses were performed on a (Thermo*Q*uest) chromatographic instrument including an isocratic pump Model P100, a Rheodyne injection valve with a 100 µL loop and an UV150 spectrophotometric detector operated at 275 nm. Chromatograms were recorded with a DataJet integrator (Thermo*Q*uest).

Chromatographic conditions. Stereoselective separations were achieved using a vancomycin-linked column (5 µm, 250 × 4.6 mm i.d.; Chirobiotic V™, CIL, Sainte-Foy-La-Grande, France). The column was operated at room temperature (22°C) and at a flow rate of 1.0 mL/min. This chiral stationary phase was used in the reversed phase mode, and several mobile phase compositions were tested to optimize the separations. These eluents were always an organic modifier/ammonium nitrate buffer mixture. The percentage of organic modifier ranged from 10 to 35%. For preparing the ammomium nitrate buffer solutions, the amount of salt needed to obtain the stated molarity was weighed and the pH value of the solution was adjusted with nitric acid.

The concentration of the sample solutes was 0.2 mM, and a volume of 5 µL was injected. For the evaluation of the enantiomeric separation, the following parameters were measured: k'_1, capacity factor of the first eluted enantiomer—$(t_1 - t_0)/t_0$; k'_2, capacity factor of the second eluted enantiomer—$(t_2 - t_0)/t_0$, where t_0 is the time at which the first baseline disturbance by the solvent peak occurred; α, selectivity factor—k'_2/k'_1; R_S, resolution factor—$R_S = 1.18\ (t_2 - t_1)/(w_2 - w_1)$, where w is the width at half-height of the peak based on the peak area and height.

RESULTS

In reversed-phase systems, the retention and the selectivity are controlled by the buffer concentration, the type and the amount of organic modifier. The manufacturer of Chirobiotic V® (ASTEC, 1999) recommends to using ammonium nitrate as buffer in the mobile phase. In order to optimize the enantiomeric separation, we first evaluated the chromatographic parameters k', α and R_S as a function of pH and molarity of this ammonium nitrate buffer. For these experiments, the organic modifier was tetrahydrofuran, the most commonly used solvent on a glycopeptide-type chiral stationary phase. Then, we tested different organic modifiers, using the optimized conditions in terms of buffer pH and molarity.

Effect of buffer pH on capacity factor (k′), selectivity (α) and resolution R_S

The buffer pH is a very effective experimental parameter in selectivity and chiral recognition since the ionization of both the analyte and chiral selector can be controlled by this variable. Because of the complexities of these interactions, it is necessary to observe the chromatographic behaviour of the enantiomers as a function of pH.

The safest and most stable pH range specified for the Chirobiotic V™ phase is 4.0–7.0 (ASTEC, 1999). According to enantioseparation factor (α) values depicted in Table 1, it appears that chiral recognition operates in the tested pH range. Between 5 and 6.5, α values remain quite constant for flurbiprofen and ketoprofen, with a maximum at pH 5. Moreover, the resolution R_S between *R*- and *S*-forms is also maximum at pH 5. The pK_a values of flurbiprofen and ketoprofen are almost identical (4.2 and 4.0, respectively) (Baselt and Cravey, 1995), leading to 86 and 91% negatively charged species, respectively, at pH 5. At this pH value, the chiral selector still

Table 2. Influence of molar concentration of ammonium nitrate buffer on the stereoselective separation of flurbiprofen and ketoprofen

	Flurbiprofen			Ketoprofen		
Molarity	k'_1	α	R_S	k'_1	α	R_S
10 mM	0.27	2.01	2.61	0.09	2.59	1.33
20 mM	1.39	1.43	3.44	0.88	1.24	1.80
50 mM	1.23	1.52	3.89	0.82	1.28	2.09
70 mM	1.67	1.45	4.14	1.12	1.23	2.17
100 mM	1.50	1.56	4.67	1.03	1.26	2.36

k'_1: capacity factor of the first-eluted enantiomer.
In this assay, the eluent was ammonium nitrate buffer pH 5–tetrahydrofuran (80:20, v/v).

Table 3. Variation of capacity factor (k'_1), selectivity (α) and resolution (R_S) of the enantiomers with percentages of 1,4-dioxane in ammonium nitrate buffer (100 mM, pH 5)

	Flurbiprofen			Ketoprofen		
1,4-dioxane (%)	k'_1	α	R_S	k'_1	α	R_S
10	1.71	1.20	1.94	1.61	1.17	1.40
15	1.34	1.21	1.83	1.19	1.18	1.41
20	0.92	1.23	2.11	0.83	1.25	1.48
25	0.78	1.31	2.01	0.63	1.25	1.31
30	0.57	1.29	1.85	0.50	1.24	1.28
35	0.43	1.35	1.62	0.39	1.31	0.92

k'_1: capacity factor of the first-eluted enantiomer.

Table 4. Variation of capacity factor (k'_1), selectivity (α) and resolution (R_S) of the enantiomers with percentages of 2-propanol in ammonium nitrate buffer (100 mM, pH 5)

	Flurbiprofen			Ketoprofen		
2-Propanol (%)	k'_1	α	R_S	k'_1	α	R_S
10	2.20	1.15	1.36	2.16	1.17	1.46
15	2.00	1.19	1.60	1.79	1.20	1.66
20	1.63	1.21	1.91	1.46	1.21	1.76
25	1.08	1.25	1.81	0.97	1.23	1.64
30	0.59	1.28	1.47	0.64	1.25	1.40
35	0.34	1.32	1.17	0.41	1.26	1.18

k'_1: capacity factor of the first-eluted enantiomer.

Table 5. Variation of capacity factor (k'_1), selectivity (α) and resolution (R_s) of the enantiomers with percentages of methanol in ammonium nitrate buffer (100 mM, pH 5)

	Flurbiprofen			Ketoprofen		
Methanol (%)	k'_1	α	R_s	k'_1	α	R_s
10	2.47	1.13	1.06	2.58	1.18	1.02
15	2.08	1.13	1.14	2.09	1.13	1.15
20	2.03	1.17	1.27	2.02	1.11	1.18
25	1.35	1.16	1.14	1.39	1.14	1.05
30	1.05	1.17	1.16	1.03	1.14	0.93
35	0.76	1.20	1.15	0.77	1.15	0.80

k'_1: capacity factor of the first-eluted enantiomer.

Table 6. Variation of capacity factor (k'_1), selectivity (α) and resolution (R_s) of the enantiomers with percentages of tetrahydrofuran in ammonium nitrate buffer (100 mM, pH 5)

	Flurbiprofen			Ketoprofen		
Tetrahydrofuran (%)	k'_1	α	R_s	k'_1	α	R_s
10	2.55	1.40	3.67	1.93	1.23	2.24
15	2.13	1.47	4.49	1.45	1.24	2.20
20	1.67	1.46	4.67	1.04	1.26	2.36
25	1.19	1.46	3.54	0.88	1.17	1.42
30	0.77	1.38	2.71	0.63	1.17	1.10
35	0.54	1.33	1.76	0.69	1.16	0.66

k'_1: capacity factor of the first-eluted enantiomer.

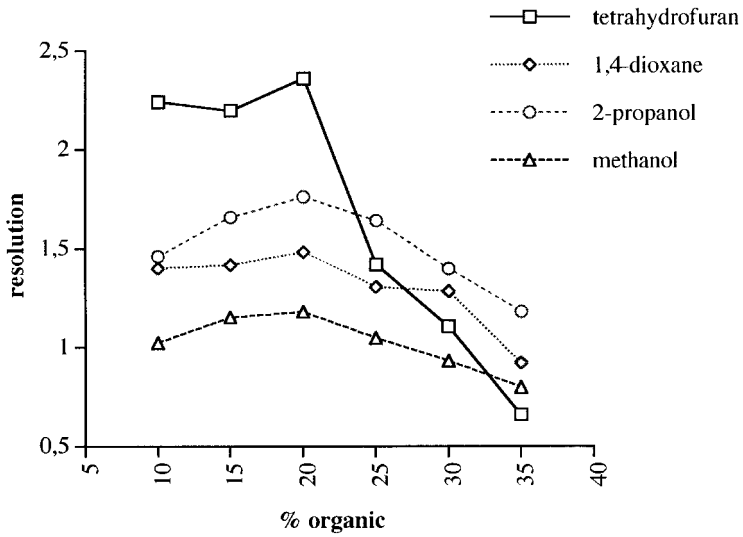

Figure 1. Effect of organic modifiers on resolution of flurbiprofen enantiomers.

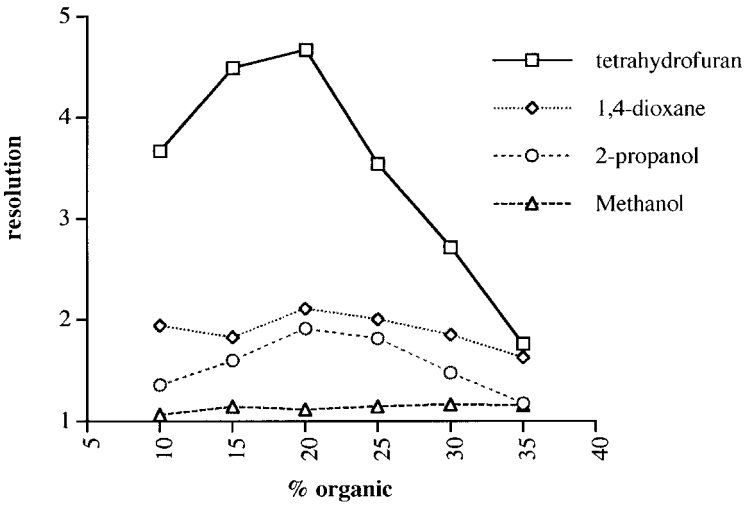

Figure 2. Effect of organic modifiers on resolution of ketoprofen enantiomers.

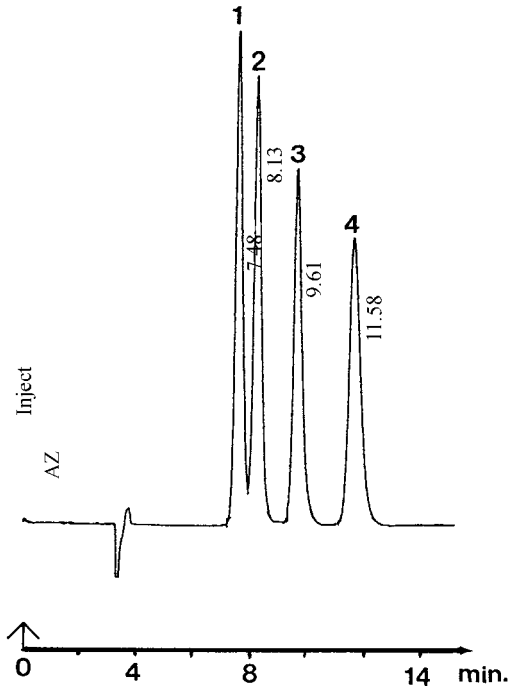

Figure 3. Chiral separation of *S*-ketoprofen (peak 1), *R*-ketoprofen (peak 2), *S*-flurbiprofen (peak 3) and *R*-flurbiprofen (peak 4) enantiomers. Chromatographic conditions: 100 mM ammonium nitrate buffer (pH 5.0)/tetrahydrofuran (80:20, v/v); flow-rate 1 mL/min; λ, 275 nm.

possesses positive charges, allowing it to interact electrostatically with the analytes.

Effect of buffer molarity on capacity factor (k′), selectivity (α) and resolution (R_S)

Once the optimum pH has been defined, the next optimization step was accomplished by investigating a range of buffer molarities between 10 and 100 mM. The results are listed in Table 2. For both NSAIDs, the chromatographic parameters exhibited maximum values at the concentration of 100 mM. On the other hand, at 10 mM, the enantiomers of flurbiprofen and ketoprofen were not resolved from the dead volume (capacity factor $k' < 0.3$). So, increasing the buffer molarity improved resolution R_S, while selectivity was quite unaffected.

Effect of the organic modifier on capacity factor (k′), selectivity (α) and resolution (R_S)

The four organic modifiers, ie 1,4-dioxane, methanol, 2-propanol and tetrahydrofuran, were tested at percentages ranging from 10 to 35% in ammonium nitrate buffer (100 mM, pH 5; Tables 3–6). For all organic solvents tested, the relative retention of enantiomeric compounds (k'_1) increased by increasing the water content of the mobile phase. In all cases, at percentages ranging between 30 and 35%, the enantiomers of flurbiprofen and ketoprofen were not resolved from the dead volume (capacity factor $k' \leq 1.05$). This effect was more marked with 1,4-dioxane, since k'_1 was still inferior to 1 at a 20% percentage. Thus, in our chromatographic conditions, these four organic modifiers can be ranged according to their increasing elution strength: 1,4-dioxane > 2-propanol > tetrahydrofuran > methanol, for flurbiprofen; and 1,4-dioxane > tetrahydrofuran > 2-propanol > methanol, for ketoprofen.

For both NSAIDs, the plots of R_S vs organic modifier content percentages (Figs 1 and 2) show that the best Rs values were obtained with tetrahydrofuran at 20%.

CONCLUSION

In this study, a bonded vancomycin chiral stationary phase (Chirobiotic V™) was investigated for the chiral liquid chromatography analysis of ketoprofen and flurbiprofen. The enantioselectivity and chiral resolution factors were adjusted by varying the buffer pH and the buffer molarity as well as the organic modifier nature and the organic modifier concentration in the mobile phase.

It is well recognized that selectivity (α) is an important parameter capable of influencing chromatographic separation (Snyder *et al.*, 1993; Sandi and Spepezy, 1999). This factor is a complex phenomenon depending on either the type of the stationary phase and the mobile phase parameters, or the chemical nature of the analytes. According to the chromatographic conditions studied in the present work, the enantioselectivity remained quite constant for flurbiprofen and ketoprofen. Consequently, the type and the concentration of mobile phase components did not significantly modulate the enantioselectivity. As a good separation is noticed, the peak shape is appreciated by the resolution factor R_S. Excellent R_S values were obtained for flurbiprofen and ketoprofen enantiomers using an eluent composition consisting of 20% tetrahydrofuran in ammonium nitrate buffer (100 mM, pH 5). The enantiomers were separated from each other within 12 min (Fig. 3). The *S*-enantiomer was eluted before the *R*-form for both drugs. This elution order was observed whatever the chromatographic conditions tested. Moreover, the peak area of the first eluted enantiomer (*S*) was always equal to the second one (*R*), indicating the absence of any racemisation reaction.

At 1-mL/min, the vancomycin chiral stationary phase could be used under reversed-phase conditions without significant deterioration over several months of experiments. So, this glycopeptide type chiral stationary phase proved to be a powerful tool to separate arylpropionic enantiomers.

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