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ORIGINAL RESEARCH

Enantioselective HPLC analysis of propafenone and of its main metabolites using polysaccharide and protein-based chiral stationary phases

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ABSTRACT: HPLC on chiral stationary phases has been used for the enantioselective assay of propafenone (PPF), 5-hydroxypropafenone (PPF-50H) and *N*-despropylpropafenone (PPF-NOR) enantiomers. The results obtained on Chiralpak AD column showed that it is useful for the resolution of PPF and of its main metabolites, although the peaks obtained for PPF-NOR were not symmetrical under the conditions investigated. This column and circular dichroism-based detection system were used to determine the absolute configuration of the eluates. Furthermore, the influence of the mobile phase composition on the resolution of PPF and of its main metabolites was investigated on cellulose derivatives (Chiralcel OD-H and Chiralcel OD-R) and protein (Chiral AGP and Ultron ES-OVM)-based chiral stationary phases. The enantiomers of PPF were resolved on all the columns, except for the Ultron ES-OVM. This column, the Chiralpak AD and the Chiralcel OD-H columns were suitable for the resolution of the PPF-50H enantiomers. The PPF-NOR enantiomers were resolved on the Chiralpak AD, Chiral AGP and Chiralcel OD-R columns.

INTRODUCTION

Propafenone [2′-(3-(propylamino)-2-(hydroxy)-propoxy)-3-phenylpropiophenone] is a chiral drug with high antiarrhythmic activity and moderate *β*-blocking and calcium channel blocking actions. Both enantiomers of *rac*-propafenone (PPF) equally contribute to the antiarrhythmic activity of the drug through the blockade of sodium channels. However, compared to its antipode, the (*S*)-enantiomer exhibits 100-fold greater affinity for the human *β*-adrenoceptors (Stoschitzky *et al.*, 1990; Kroemer *et al*, 1989). Recently, pharmacokinetics of the individual enantiomers of PPF were investigated (Kroemer *et al.*, 1989, 1991, 1994) showing marked stereoselectivity. PPF is extensively metabolized to 5-hydroxypropafenone (PPF-50H) and *N*-despropylpropafenone (PPF-NOR) (Fig. 1) and the pharmacokinetic profile of the two enantiomers is different, as hydroxylation, which is the major route of metabolism, is stereoselective and favours (*R*)-PPF. The hydroxylation of propafenone is related to debrisoquin oxidation polymorphism, and stereoselectivity seems to be abolished in poor metabolizers (Kroemer *et al.*, 1989, 1991). Furthermore, enantiomer–enantiomer interaction of (*S*)- and (*R*)-PPF has been observed (Kroemer *et al.*, 1991, 1994).

Thus the study of the stereoselectivity phenomena involving PPF and its metabolites deserved the availability of efficient methods for the resolution of the enantiomers. Chiral separation by high-performance liquid chromatography using chiral stationary phases is now a well-established method for the enantioselective determination of chiral drugs. The most commonly used chiral stationary phases are based on polysaccharides such as cellulose and amylose derivatives coated in silica gel and on immobilized proteins such as α_1-acid

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Abbreviations used: α_1-AGP, α_1-acid glycoprotein; BSA, bovine seium albumin; CD, circular dichroism; HSA, human serum albumin; PPF, propafenone; PPF-NOR, *N*-despropylpropafenone; PPF-5OH, 5-hydroxypropafenone.

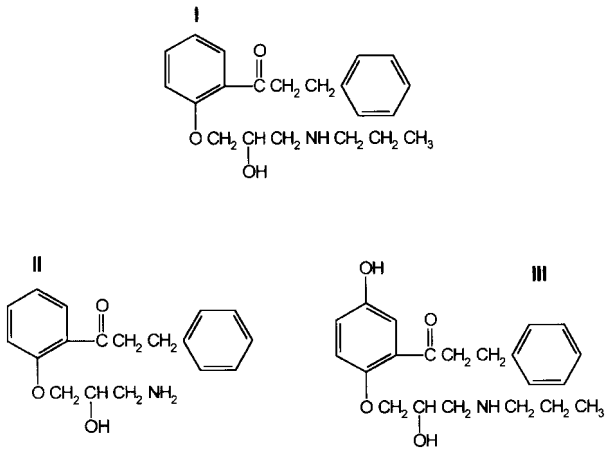

Figure 1. Structures of PPF (**I**), PPF-NOR (**II**) and PPF-50H (**III**).

glycoprotein (α_1-AGP), bovine serum albumin (BSA) and human serum albumin (HSA) (Okamoto and Kaida, 1994; Allenmark and Andersson, 1994).

The enantiomers of PPF have already been resolved on Tris-(3,5-dimethylphenyl-carbamate) derivatives of cellulose (Chiralcel OD, Chiralcel OD-R) and amylose (Chiralpak AD) (Hollenhorst and Blaschke, 1991; Aboul-Enein and Bakr, 1993; Bohm *et al.*, 1995; Abreu *et al.*, 1999; Gaitani *et al.*, 1998). Two of these papers were derived from the results obtained in the present work (Abreu *et al.*, 1999; Gaitani *et al.*, 1998). Using the Chiralcel OD column, Hollenhorst and Blaschke (1991) also resolved the enantiomers of PPF-NOR and PPF-50H, but under different conditions. PPF and PPF-5-OH enantiomers were also recently resolved on a Chiral AGP column (Zhong and Chen, 1999). In spite of this, the absolute configuration has been established only for PPF (Blaschke and Walther, 1987).

Thus, in this paper we evaluated the Chiralpak AD column for the resolution of PPF, PPF-50H and PPF-NOR enantiomers and, using a circular dichroism (CD)-based detection system, we were able to assign the absolute configuration for the enantiomers of the two PPF metabolites. In addition, the influence of mobile phase composition was evaluated on this column and on Tris-(3,5-dimethylphenylcarbamate) derivative of celullose under normal and reversed-phase conditions and on protein-based chiral stationary phases.

EXPERIMENTAL

Chemicals. (*RS*)-PPF, (*RS*)-PPF-50H, and (*RS*)-PPF-NOR were kindly supplied by Knoll S.A. (Rio de Janeiro, RJ, Brazil) and Knoll AG (Ludwigshapen-Rhein, Germany). The solvents used as mobile phase were HPLC grade, purchased from Merck (Rio de Janeiro, RJ, Brazil) or EM Science (Gibbstown, NJ, USA). All other chemicals were of analytical grade. Water was purified in a Milli-Q-plus reagent water system (Millipore, São Paulo, SP, Brazil).

Equipment. The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10 AS pump, a Rheodyne model 7125 injector fitted with a 20 or 50 μL sample loop and a SPD-10A variable UV–vis detector. Retention times were obtained by an electronic integrator, Shimadzu model CR-6A interfaced with the detector. Several experiments were also performed with a modular HPLC system that consisted of a Jasco 887-PU pump, a multiwavelength Jasco Multi340 UV multichannel detector (Jasco Instruments, Tokyo, Japan), and a Rheodyne model 7125 injector equipped with a 20 μL loop. The system was interfaced with a personal computer for data recording and analysis. The eluates were also monitored using a Jasco J710 spectropolarimeter (set at 300 nm) equipped with a micro HPLC cell. This detection system allows the absorption and the CD signals to be simultaneously detected. The same apparatus was used to carry out CD spectra of the single enantiomers by trapping the enantiomeric fractions of PPF and of its metabolites in the HPLC cell. The HPLC columns used for the resolution of PPF and of its metabolites were purchased from Chiral Technologies, Exton, PA, USA (Chiralcel OD-H, 150 × 4.6 mm i.d., 5 μm particle size; Chiralcel OD-R, 250 × 4.6 mm i.d., 10 μm particle size; and Chiralpak AD, 250 × 4.6 mm i.d., 10 μm particle size), J. T. Baker, Phillipsburg, NJ, USA (Chiral AGP, 150 × 4.0 mm i.d., 5 μm particle size) and Rockland Technologies, Newport, DE, USA (Ultron ES-OVM, 150 × 4.6 mm i.d., 5 μm particle size).

Chromatographic procedure. Chromatographic data were reported as capacity factor k', which is defined as $(t_R - t_0)/t_0$, where t_R is the retention time of the solute of interest and t_0 is that of an unretained solute. The enantioselectivity (α) was also calculated ($\alpha = k'_2/k'_1$), where k'_2 and k'_1 are the capacity factors of the second and of the first eluted enantiomers.

The solutions were prepared in methanol at the concentration of 100 μg/mL for the three compounds. Immediately before the analysis, 25 μL of the solutions were evaporated to dryness under nitrogen and the residues were dissolved in 100 μL of the mobile phase. The mobile phases used for the evaluation of the chiral columns as well the flow rates are reported in Tables 1–3. All separations were performed at ambient temperature.

The enantiomeric elution order on the Chiralpak AD column was determined by CD detection using hexane–ethanol (85:15) containing 0.1% of diethylamine as mobile phase. The elution order in the other columns was determined by injecting the pure enantiomers previously separated on Chiralpak AD column.

RESULTS AND DISCUSSION

Evaluation of polysaccharide based chiral stationary phases

Table 1 shows the influence of mobile phase composition on the retention of PPF, PPF-50H and PPF-NOR enantiomers and on enantioselectivity using the Chiralpak AD column. As expected, when the polarity of the

Table 1. Effect of mobile phase composition on capacity factor and enantioselectivity of PPF, PPF-50H and PPF-NOR on the Chiralpak AD column

	Chromatographic parameters					
	PPF		PPF-50H		PPF-NOR	
Mobile phase composition	k'	α	k'	α	k'	α
Percentage of IPA						
H–IPA + 0.1% DEA						
90:10	3.49	1.60	5.64	1.00	5.78	1.21
85:15	2.17	1.68	2.41	1.00	3.29	1.19
80:20	1.47	1.68	1.53	1.00	2.05	1.18
Nature of the alcohol						
H–alcohol (85:15) + 0.1% DEA						
IPA	2.17	1.68	2.41	1.00	3.29	1.19
E	2.03	1.89	2.27	1.26	4.13	2.27
Percentage DEA						
H–IPA (85:15) + DEA						
0	2.53	1.63	—	—	4.80	1.14
0.1	2.17	1.68	2.41	1.00	3.29	1.19
0.2	2.09	1.65	2.57	1.00	3.16	1.19
0.4	2.05	1.69	2.47	1.00	3.15	1.20
Percentage TFAA						
H–IPA (85:15) + TFAA						
0	2.53	1.63	—	—	4.80	1.14
0.1	1.90	1.21	—	—	1.17	1.17
0.2	1.57	1.25	—	—	0.97	1.16

Flow rate = 1.0 mL/min; IPA, 2-propanol; H, hexane; E, ethanol; DEA, diethylamine; TFAA, trifluoroacetic acid.

Table 2. Effect of mobile phase composition on enantioselectivity of PPF, PPF-50H and PPF-NOR on the Chiralcel OD-R column

	Chromatographic parameters					
	PPF		PPF-50H		PPF-NOR	
Mobile phase composition	k'	α	k'	α	k'	α
PH						
$NaClO_4$ 0.25 mol/L–acetonitrile (60:40)						
3.0	6.79	1.07	2.97	1.00	2.62	1.11
4.0	6.33	1.07	2.77	1.00	2.50	1.06
5.0	6.29	1.07	2.78	1.00	2.45	1.06
5.5	6.57	1.06	2.99	1.00	2.70	1.04
$NaClO_4$ concentration (mol/L)						
$NaClO_4$ (pH 5.5)–acetonitrile (60:40)						
0.1	5.75	1.04	2.58	1.00	2.42	1.00
0.25	6.57	1.06	2.99	1.00	2.70	1.04
0.5	7.91	1.07	3.44	1.00	3.12	1.02
Percentage of acetonitrile						
$NaClO_4$ 0.25 mol/L (pH 5.0)–acetonitrile						
70:30	29.90	1.10	10.09	1.00	8.09	1.09
65:35	15.17	1.09	5.40	1.00	4.72	1.08
60:40	6.29	1.07	2.78	1.00	2.45	1.06

Flow rate = 0.8 mL/min.

mobile phase was increased, by changing the amount of 2-propanol, the k' values decreased for all compounds studied. By contrast, the selectivity factor α was not significantly influenced by the polarity of the mobile phase. The effect of the structure of the alcohol on retention and enantioselectivity was marked. In particular the resolution of PPF-50H was possible only using hexane-ethanol as the mobile phase (Table 1). It is worth

Table 3. Effect of mobile phase composition on enantioselectivity of PPF, PPF-50H and PPF-NOR on the Ultron ES-OVM column

	Chromatographic parameters					
	PPF		PPF-50H		PPF-NOR	
Mobile phase composition	k'	α	k'	α	k'	α
Buffer pH						
Acetate 20 mmol/L–methanol (95:5)						
3.5	3.40	1.00	0.40	1.67	0.47	1.00
Phosphate 20 mmol/L–methanol (95:5)						
4.5	8.80	1.09	3.87	1.48	2.80	1.00
5.0	29.53	1.13	6.13	1.58	6.47	1.17
5.5	—	—	8.95	1.53	10.34	1.05
6.0	—	—	10.48	1.32	22.27	1.05
Buffer concentration (mmol/L)						
Acetate (pH 3.5)–methanol (95:5)						
10	3.60	1.00	0.47	1.85	0.60	1.00
20	3.40	1.00	0.40	1.67	0.47	1.00
50	1.87	1.00	0.40	1.50	0.47	1.00
Percentage acetonitrile						
Acetate 20 mmol/L (pH 3.5)–acetonitrile						
100:0	—	—	3.61	2.30	4.48	1.00
97:3	9.80	1.00	1.00	1.80	1.20	1.00
95:5	5.27	1.00	0.67	1.60	0.80	1.00
Organic modifier						
Acetate 20 mmol/L (pH 3.5)–organic modifier (95:5)						
Methanol	3.40	1.00	0.40	1.67	0.47	1.00
Ethanol	1.80	1.00	0.13	2.53	0.27	1.00
Acetonitrile	5.27	1.00	0.67	1.60	0.80	1.00

Flow rate = 1.0 mL/min.

mentioning that the alcohol in the mobile phase not only competes for chiral binding sites with the solutes, but can also alter the steric environment of the chiral cavities on the chiral stationary phase (Kunath *et al.* 1996; Tang, 1996).

The use of additives of organic acids or organic bases in the mobile phase to enhance chiral selectivity has been proved to be important for the separation of acid and basic drugs by reducing the hydrogen-bonding between the chiral drugs and the silanol groups (Tang, 1996). The addition of 0.1% of diethylamine to the mobile phase decreased the k' values when compared to the mobile phase without additives (Table 1). Further increments of diethylamine up to 0.4% did not change the k' values significantly. The influence of trifluoroacetic acid was to decrease the k' values, probably due to the fact that trifluoroacetic acid masks silanol groups on the stationary phase by H-bonding. The effect was remarkable in the case of PPF-NOR (almost 80% reduction of k'), while PPF-50H enantiomers did not elute. Figure 2 shows the resolution of PPF, PPF-50H and PPF-NOR on a Chiralpak AD column. The PPF-NOR enantiomers eluted as asymmetric peaks, besides the mobile phase used.

Similar results were obtained on the evaluation of a Chiralcel OD-H column. In this case, the enantiomers of PPF and PPF-NOR could only be eluted when the mobile phase was added of diethylamine or trifluoroacetic acid. The elution of PPF-50H was only possible by the addition of diethylamine. In addition, this column proved to be suitable for the resolution of PPF and PPF-50H enantiomers (Fig. 3).

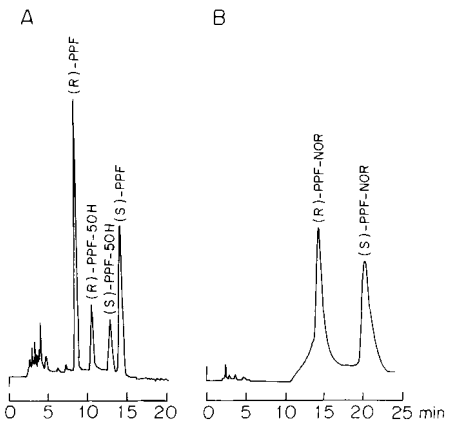

Figure 2. Resolution of PPF and PPF-50H (a) and PPF-NOR (b) enantiomers on the Chiralpak AD column. Mobile phase: (a) hexane–ethanol (88:12) + 0.1% DEA; (b) hexane–ethanol (85:15). Flow rate: 1.3 mL/min. Detection at 300 nm.

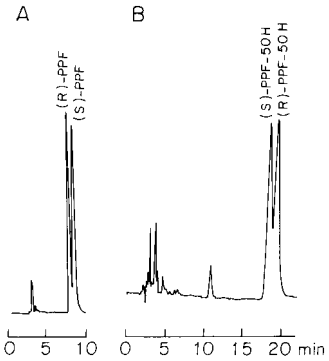

Figure 3. Resolution of PPF (a) and PPF-50H (b) enantiomers on Chiralcel OD-H column. Mobile phase: (a) hexane-2-propanol (90:10) + 0.1% DEA; (b) hexane–ethanol (95:5) + 0.1% DEA. Flow rate: 1.0 mL/min. Detection at 300 nm.

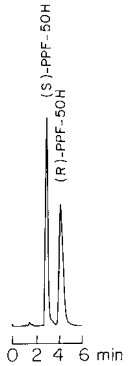

Figure 5. Resolution of PPF-50H enantiomers on Ultron ES-OVM column. Mobile phase: acetate 0.02 mol/L (pH 3.5)–acetonitrile (97:3). Flow rate: 0.9 mL/min. Detection at 254 nm.

The Chiralcel OD-R is also a Tris -3,5-dimethylphenylcarbamate derivative of cellulose, but used under reversed-phase conditions. This column was evaluated using mobile phases consisting of perchlorate solutions added of acetonitrile to control the retention. The pH has a slightly effect on the retention of PPF, PPF-50H and PPF-NOR enantiomers, while the concentration of perchlorate solution has a more pronounced effect on retention, apparently in a mechanism similar to ion-pair chromatography (Ishikawa and Shibata, 1993) (Table 2). Furthermore, the pH or concentration of perchlorate solutions did not significantly affect the enantioselectivity (Table 2).

As expected for reversed-phase systems, the retention and selectivity decreased when the amount of uncharged organic modifier in the mobile phase was increased (Table 2). The use of the Chiralcel OD-R column allowed the resolution of PPF and PPF-NOR, whereas PPF-50H could not be resolved with the mobile phases studied (Fig. 4).

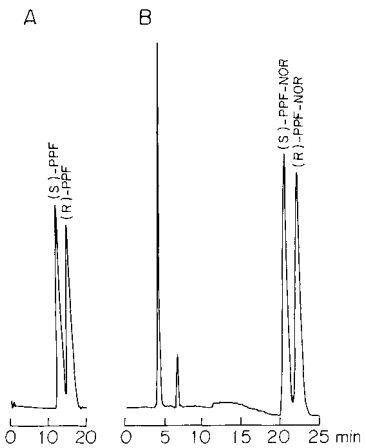

Figure 4. Resolution of PPF (a) and PPF-NOR (b) enantiomers on Chiralcel OD-R column. Mobile phase: (a) $NaClO_4$ 0.25 mol/L (pH 4.0)–acetonitrile (60:40), flow rate: 0.8 mL/min; (b) $NaClO_4$ 0.25 mol/L (pH 5.5)–acetonitrile (70:30). Flow rate: 0.7 mL/min. Detection at 254 nm.

Evaluation of the protein-based chiral stationary phases

The Ultron ES-OVM column has a chicken ovomucoid protein as the chiral selector and it is used for the resolution of acidic, basic and neutral chiral compounds (Kirkland *et al.* 1991). This column was evaluated by changing pH (3.5–6.0), the nature of the buffer and the buffer concentration (10–50 mmol/L). Furthermore the influence of uncharged organic modifier such as methanol, ethanol and acetonitrile was also investigated.

The results are summarized in Table 3. As far as the effect of pH is concerned, the dominant effect of increasing pH was a marked increase of k' values, suggesting binding on cation-exchanging sites in addition to hydrophobic interactions (Table 3). The ion-exchanging effect was also observed when the buffer concentration was changed. The retention of PPF and of its metabolites indeed decreased by increasing the concentration of the buffer from 10 to 50 mmol/L (Table 3).

Uncharged organic modifiers are usually added to the mobile phase to change the retention and selectivity by controlling the hydrophobic interaction (Arvidsson *et al.*, 1992). An increase in the polarity of the mobile phase was obtained by decreasing either the concentration of acetonitrile in the mobile phase or the nature of the organic modifier. As a general trend, an increase either of the capacity factor or of the enantioselectivity was observed (Table 3). This may be due to increased solvatation of the enantiomers in the mobile phase, a process that affects chiral and non-chiral binding. The possibility of a conformational change of the protein also has to be considered, this causing structural changes in the recognition sites.

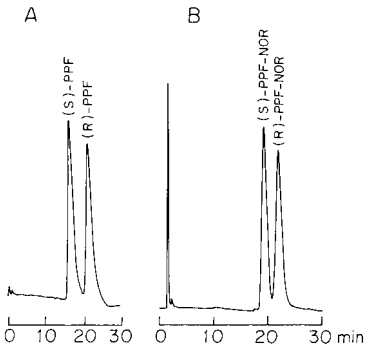

Figure 6. Resolution of PPF (a) and PPF-NOR (b) enantiomers on Chiral AGP column. Mobile phase: (a) phosphate 0.01 mol/L (pH 5.5)–1-propanol (92:8); (b) phosphate 0.1 mol/L (pH 5.5)–methanol (92:8). Flow rate: 0.9 mL/min. Detection at 254 nm.

Using this column a baseline resolution of PPF-50H enantiomers was observed, but the others drugs could not be completely resolved (Fig. 5). A partial resolution of PPF and PPF-NOR was observed by increasing the mobile phase pH, but the peaks obtained were not symmetrical. The same asymmetry was observed in the case of the PPF-50H enantiomers at pH higher than 5.0.

The chiral AGP column was recently used by Zhong and Chen (1999) for the resolution of PPF and PPF-50H, using acetate/1-propanol mobile phases. In the present paper the Chiral AGP column was evaluated using phosphate buffer solutions (pH 4.5–6.0, 10–100 mmol/L) added of acetonitrile, 1-propanol, 2-propanol, ethanol or methanol.

Ours results (Fig. 6) indicate that the Chiral AGP column is efficient in the resolution of PPF and PPF-NOR enantiomers, while the PPF-50H enantiomers were not separated, at least in the experimental conditions adopted.

Determination of the elution order

The determination of the elution order is essential in the enantioselective analysis of PPF and of its metabolites. Indeed the monitoring of the enantiomeric ratio of PPF and of its metabolites in body fluids deserves a complete stereochemical characterisation, ie the determination of the enantiomeric excess and the absolute configuration of the prevailing enantiomer. Blaschke and Walther (1987) assigned the absolute configuration to the single enantiomers of PPF but not for the enantiomers of PPF metabolites. Using the Chiralpak AD column, Hollenhorst and Blaschke (1991) reported that (*R*)-PPF was eluted first using hexane/2-propanol/DEA as the mobile phase. This result has been recently confirmed by Bohm *et al.* (1995), by injection of the single enantiomers on the Chiralpack AD column, using hexane/2-propanol/DEA as the mobile phase.

As shown in Table 1, the use of Chiralpack AD and hexane/ethanol/DEA as the mobile phase allowed the resolution of PPF, PPF-NOR, and of PPF-50H. This enantioselective HPLC method and the use of a CD-based detection system allowed the elution order to be tentatively assigned. The (*R*)-PPF, the first eluted enantiomer on Chiralpack AD, either using 2-propanol or ethanol as the alcohol in the mobile phase, showed a negative CD band at about 300 nm. A negative CD signal, in the same spectral range, was observed also in the cases of PPF-NOR and PPF-50H. Taking into account the structural similarity of PPF and of its metabolites, (*R*)-absolute configuration can be tentatively assigned to those fractions which show negative CD at 300 nm. Furthermore the same elution order, the (*S*)-enantiomer, being the more retained one, has been obtained for the three compounds on the Chiralpack AD column.

The recovery of the single enantiomer of PPF and of its metabolites from the Chiralpack AD allowed the elution orders to be determined also in the case of the other stationary phases employed in the present investigation (Figs 3–6). In particular, an inversion of the elution order was observed in all the examined cases with the exception of PPF on Chiralcel OD-H. Actually a reverse elution order was also expected in this case, according to literature data on the enantioselective analysis of PPF on Chiralcel OD column (Aboul-Enein and Bakr, 1993). HPLC resolution of PPF on this column confirmed the elution order obtained on Chiralcel OD-H column. However a very low enantioselectivity was obtained using hexane:2-propanol:TEA (90:10:0.4) as the mobile phase and Chiralcel OD as the column. Indeed only CD detection allowed to observe the resolution, even if very poor.

CONCLUSION

PPF was resolved with all the columns evaluated, except the Ultron ES-OVM-column. This column, the Chiralpak AD and Chiralcel OD-H columns were suitable for the resolution of the PPF-50H enantiomers, whereas the Chiral AGP and Chiralcel OD-R columns were suitable for the resolution of PPF-NOR enantiomers. The Chiralpack AD column was the only efficient one in the enantioselective analysis of PPF and of its metabolites. The control of the mobile phase composition resulted essentialy in the development of the enantioselective HPLC methods with all the chiral stationary phases used. The (*R*)-PPF eluted first in the Chiralpak AD and Chiralcel OD-H columns, whereas it was the more retained one on the other columns. The CD-based detection system also allowed the absolute configuration to be tentatively assigned to the enantiomeric fractions of PPF-50H and PPF-NOR. The metabolites of PPF have the same elution order except for the PPF-50H in the

Chiralcel OD-H column in which the (*S*)-enantiomer elutes first.

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