



Separation of 2-arylpropionic acids on a cellulose based chiral stationary phase by RP-HPLC

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Abstract: The enantiomers of eight 2-arylpropionic acids, a group of chiral non steroidal antiinflammatory drugs, were resolved as their benzylamide derivatives on a high-performance liquid chromatographic chiral stationary phase consisting of a covalently bound tris (4-methylbenzoate) cellulose layer on silica gel. The column was used under reversed-phase conditions using methanol as the main mobile phase component, with a perchlorate buffer pH 2.0. A compromise for derivatization with a water soluble carbodiimide and 1-hydroxybenzotriazole of a group of eight analytes was obtained. The derivatives were identified by IR- and MS-spectroscopy.

Keywords: Tris(4-methylbenzoate)cellulose; non steroidal antiinflammatory drugs; 2-arylpropionic acids; enantiomeric resolution.

Introduction

2-Arylpropionic acids (2-APAs) are a group of non steroidal antiinflammatory drugs (NSAIs) that are characterized by a chiral carbon adjacent to the carboxylic acid moiety (Fig. 1). They are widely used in relief of acute and chronic rheumatoid arthritis and osteoarthritis. They are marketed as racemic compounds with the exceptions of naproxen and flunoxaprofen, which have been commercialized as pure (S)-(+)-enantiomers.

Various stages in the metabolic pathway of the racemates may be enantioselective, such as absorption, plasma and tissue binding and elimination [1, 2]. The pharmacological activity of 2-arylpropionic acids resides mainly, if not exclusively, in the (S)-enantiomer. They exert the majority of their pharmacological and toxicological effects by specific inhibition of the binding of arachidonic acid to the cyclooxygenase subunit of the prostaglandin synthetase. The formation of several prostaglandins is prevented [3, 4]. The (R)-enantiomer (distomer) shows a lack of the anti-inflammatory effect. Its pharmacological activity however, arises indirectly from an *in vivo* inversion mechanism to the therapeutic

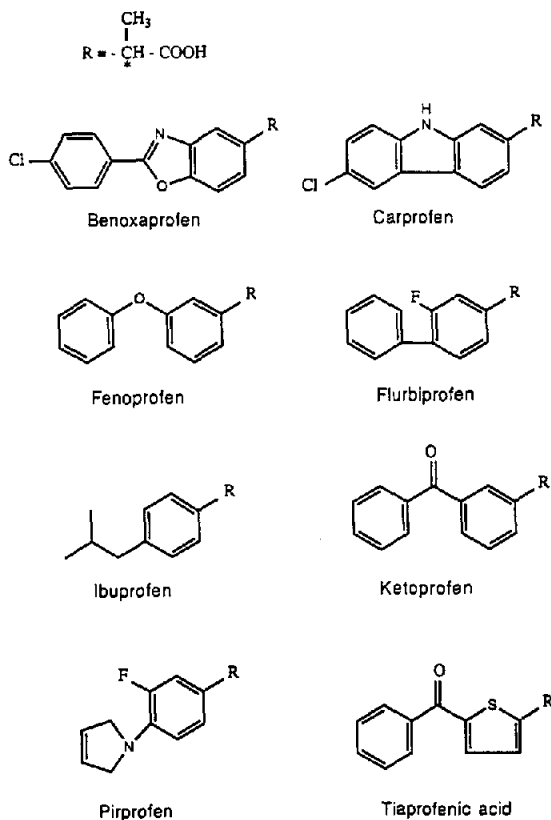


Figure 1
Chemical structures of the NSAIs under investigation.

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active (S)-form (eutomer). The enzymatic formation of a thioester between a 2-APA and Coenzyme A is substrate and (R)-enantiomer specific. The diastereomeric ester may undergo epimerization to yield the thioester in which the 2-arylpropionyl moiety has the (S)-configuration. Subsequent hydrolysis completes the inversion process [2, 5–7]. In humans this unidirectional process is significant for ibuprofen [8–11], benoxaprofen and fenoprofen [12, 13] and negligible for flurbiprofen [14–16], tiaprofenic acid [21] and carprofen. The elimination of conjugated ketoprofen is stereoselective in favour of the (S)-enantiomer [17, 18].

Various reasons have been given in favour of the use of pure (S)-enantiomers, despite a metabolic activation of the (R)-form of some of the cited drugs. The use of racemic mixtures implies the administration of a metabolic ballast of (R)-enantiomers with their stereospecific pharmacodynamics. The (R)-enantiomers can also be partially responsible for some side-effects, as a contribution to direct gastrointestinal irritation. Another potential toxic effect, which is still under investigation, is the possibility of the CoA-thioesters of the (R)-enantiomers to replace endogenous fatty acids in triacylglycerols forming unnatural glycerolipids and interfere with normal lipid metabolism and membrane function [2, 19].

Because of their different pharmacological properties, the enantiomers should be considered as different drugs and therefore be separated [1, 21, 22]. The racemic 2-arylpropionic acids have been resolved into their enantiomers using different methods, indirectly via formation of diastereomers as well as by direct techniques. Diverse classes of chiral stationary phases (CSPs) are appropriate for the direct chiral resolution by HPLC.

Protein based CSPs are frequently applied as the 2-APAs can be resolved as free acids. Separations have been performed on phases such as human serum albumin [23, 24], α_1 -glycoprotein [16, 25, 26–28], ovomucoid [29–31], bovine serum albumin [32] and avidine [33, 34]. The carboxylic group of the 2-APAs needs to be derivatized with an aromatic amine in order to get proper interactions, e.g. via hydrogen bonding and π - π and dipole stacking interaction with Pirkle type phases [28, 35–42].

As for cellulose polymers, different deriv-

atives have been synthesized since the successful introduction of cellulose triacetate phases. Although they are mostly adsorbed on macroporous silica gel [46–49], various cellulose derivatives have been used in their pure form as beads [43–45]. They have been tested for their enantioselectivity for a large number of drugs and are commercially available from Daicel (Tokyo, Japan) and J.T. Baker (Deventer, The Netherlands) [50]. Carbamate derivatives of cellulose offer many interactive possibilities and especially the substituted triphenylcarbamates have been extensively applied [51–55]. Some 2-APAs have been resolved after masking the carboxylic acid moiety by derivatization into amides [37]. Until the recent commercialization of the Chiralcel OD-R column by Daicel [56, 57], for which acetonitrile is a first choice mobile phase constituent, carbamate cellulose phases were generally applied under normal phase conditions. Hexane and a modifier (e.g. 2-propanol) are also common components of the mobile phase for the ester derivatives of cellulose. Among the cellulose based CSPs the methylbenzoate derivatives have also proved their enantioselective capacities [58–65]. The 4-methylbenzoate ester derivative has been commercialized as the Chiralcel OJ column (Daicel) and is routinely practised under normal phase conditions. The applicability under reversed-phase conditions has been tested for the resolution of neutral compounds using aqueous acetonitrile as mobile phase [57].

In this study a new experimental tris(4-methylbenzoate) cellulose phase (Bio-Rad RSL, Nazareth, Belgium) has been tested on its feasibility to resolve a group of eight 2-arylpropionic acids. Due to the fact that the cellulose layer is bound onto a silica support rather than being adsorbed, a wide range of solvents commonly used in HPLC could be applied. Elution with pH values within a range of 1–8 are allowed. Previous results have revealed its possibility to be used under normal as well as reversed-phase conditions for the resolution of a few representatives of the 2-APAs [66]. Because of poor or no resolution of the free acids, the 2-APAs have been derivatized. A larger group has been resolved using an aqueous methanolic mobile phase following a uniform derivatization procedure into their amides.

Experimental

Chemicals

Benzylamine, 1-naphthylmethylamine, 1-ethyl-3-dimethylaminopropyl-carbodiimide (HCl (EDC) and 1-hydroxybenzotriazole (HOBT) were purchased from Sigma-Aldrich (Bornem, Belgium). Carprofen [(RS), (R) and (S)] was a kind gift of Produits Roche (Brussels, Belgium), flurbiprofen of Upjohn Co. (Kalamazoo, MI, USA), pirofenon (\pm , -) of Ciba-Geigy (Groot-Bijgaarden, Belgium), calcium fenoprofen of Eli Lilly Co (Indianapolis, IN, USA) and tiaprofenic acid of Erfa (Brussels, Belgium). Benoxaprofen was obtained from Eli Lilly Co (Windelsham, UK) before withdrawal from the market, ibuprofen [(RS) and (S)] from Profarma (Oud-Turnhout, Belgium), ketoprofen from Sigma-Aldrich (St Louis, MO, USA). Sodium perchlorate, perchloric acid 70% aqueous solution, methanol and dichloromethane, kept on molecular sieve 4 Å, were all of analytical grade. Deionised water was used throughout.

Apparatus

Chromatography was performed with a Varian 9010 SDS pump (Varian Associates Inc., Walnut Creek, CA, USA) using a Rheodyne injector with a 10 μ l loop. Detection was performed at two different wavelengths simultaneously (230 and 254 nm) with a HP series 1050 diode array (Hewlett Packard, Waldbronn, Germany). Integration was made of the most intense chromatogram with the Hewlett Packard software package (1990). 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: $(t_2 - t_0)/t_0$.
- t_0 : time at which the first baseline disturbance by the solvent peak occurred.
- N : plate number: $N = 5.54 (t/w)^2$; (15 cm column).
- R_s : resolution factor: $R_s = 1.18 (t_2 - t_1)/(w_1 + w_2)$; w is the width at half-height of the peak based on peak area and height.
- α : separation factor: $k'2/k'1$.

Infrared spectra of the derivatives were recorded on Perkin-Elmer System 2000 FTIR (Perkin-Elmer Ltd, Beaconsfield, England), equipped with a DTGS detector at a resolution of 4 cm^{-1} .

Mass spectra were taken with a Hewlett-Packard 5988 A mass spectrometer applying a 70 eV electron impact mode.

Chromatographic conditions

The mobile phase consisted of methanol and perchlorate buffer 0.1 M pH 2.0, mixed in varied ratios and ultrasonicated before it was pumped at a flow rate of 1 ml min^{-1} over a short tris(4-methylbenzoate) cellulose column (150 \times 4.6 mm) [EXP B101, Bio-Rad RSL, Nazareth, Belgium]. The cellulose layer (Fig. 2) of this stationary phase is covalently bound onto a 10 μm silica gel with a mean pore size of 300 Å. The coverage is about 10%. Chromatography was carried out at ambient temperature. For preparing the buffer solution, 14.05 g sodium perchlorate was dissolved in water and after adjusting the pH-value with a concentrated perchloric acid solution, water was added up to 1 l.

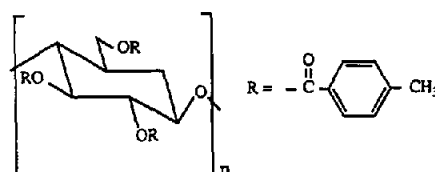


Figure 2
Chemical structure of the tris(4-methylbenzoate) cellulose layer.

Derivatization procedure

To 1 ml of a solution of a 2-arylpropionic acid (0.1 mg ml^{-1} dichloromethane) were added HOBT (300 μ l of a 0.1 mg ml^{-1} dichloromethane solution containing 1% w/v pyridine), EDC (300 μ l of a 1.1 mg ml^{-1} dichloromethane solution) and benzylamine (300 μ l of a 0.3 mg ml^{-1} dichloromethane solution) or 1-naphthylmethylamine (300 μ l of a 0.38 mg ml^{-1} dichloromethane solution). The mixture was vortexed and left for 1.5 h. The dichloromethane layer was evaporated to dryness under a stream of nitrogen and the residue was taken into 0.5 ml of methanol.

Results and Discussion

Derivatization procedure

The free 2-arylpropionic acids under investigation could not be resolved enantiomerically to an acceptable extent. Only four racemates were poorly resolved into their enantiomers as free acids, using methanol-perchlorate buffer 0.1 M pH 2.0 as mobile phase (Table 1).

Table 1
Resolution of underivatized acids

Analyte	<i>k'</i> 1	<i>N</i> 1	<i>k'</i> 2	<i>N</i> 2	<i>R</i> s	α
Ibuprofen	2.30	1103	2.46	946	0.38	1.07
Benoxaprofen	14.90	906	17.23	727	0.97	1.16
Flurbiprofen	9.69	846	10.95	620	0.75	1.13
Tiaprofenic acid	3.27	973	3.66	734	0.63	1.12

Chromatographic conditions: see Experimental. Mobile Phase: methanol-perchlorate buffer 0.1 M pH 2.0 (65:35, v/v).

Increasing the aqueous buffer content improved slightly the α -value at the expense of longer retention times.

To improve the stereoselective interactions of the analytes with the chiral layer of tris(4-methylbenzoate) cellulose their amide derivatives were formed using the carbodiimide reagent EDC as coupling agent and 1-hydroxybenzotriazole as a catalyst. These agents were originally reported for their use in peptide synthesis [67, 68]. The EDC-HOBT combination has been profoundly tested and applied to obtain diastereomeric amides of 2-arylpropionic acids [11, 12, 15, 69, 70]. A general reaction scheme is depicted in Fig. 3. The amines of choice were benzylamine and 1-naphthylmethylamine [37, 41, 64].

An optimization of the former applied method [66] has been carried out for the benzylamide formation of ibuprofen and fenoprofen, considering as relevant parameters the variations of AUC of underivatized acid and of the first eluting amide peak. As the mobile phase contained an aqueous fraction with low pH-value (perchlorate buffer 0.1 M pH 2.0), interfering peaks of the catalyst HOBT and of smaller disturbances due to the coupling agent, eluted before the free acid. The underivatized acids were chromatographed before their amide derivatives.

The influence of the *benzylamine* concentration was most pronounced. Figure 4a clearly shows that an excess of benzylamine had an unfavourable influence both on the amount of underivatized acid and of formed amide derivatives. An optimum revealed at about 1.5 times molar equivalents amine versus acid. When the amine was added in deficient equivalents, the influence on the yield of derivatization was clearly seen for the amide area. The expected increase of a relative excess of free acid was not detected, probably because it was still bound as an intermediate product of reaction.

Lowering the *EDC* amount gave rise to a quick increase of underivatized acid and a

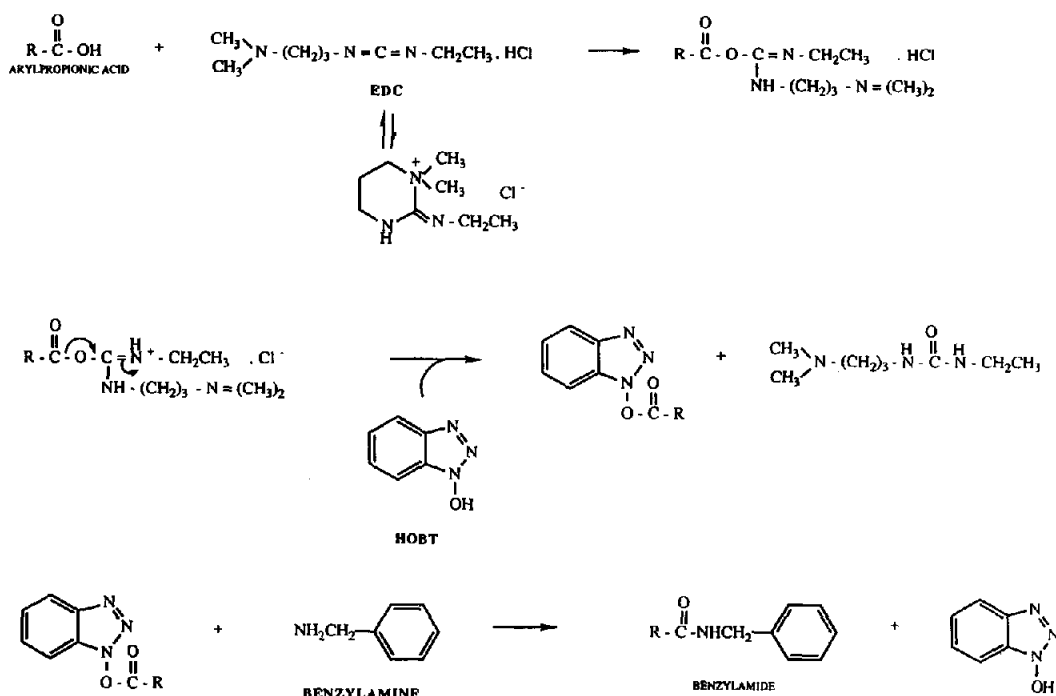


Figure 3
General reaction scheme of benzylamide derivatization of 2-arylpropionic acids.

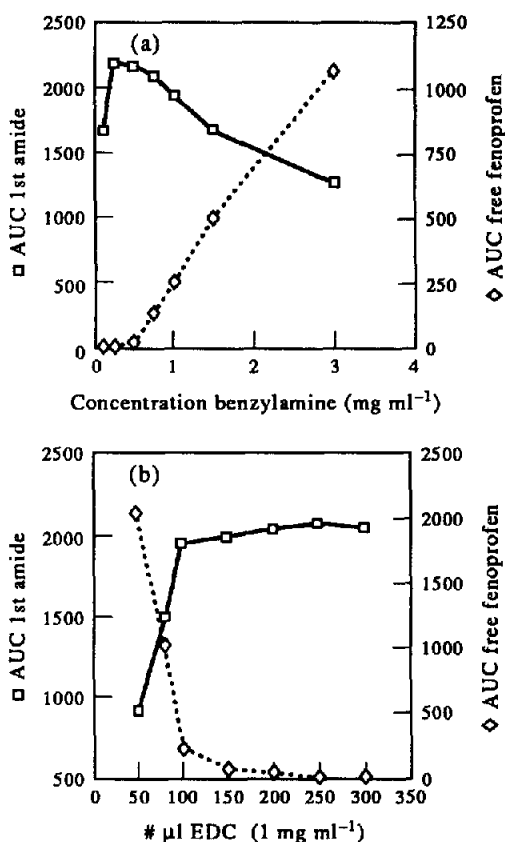


Figure 4

(a) Influence of the benzylamine concentration on the reaction yield of benzylamide derivative of fenoprofen. (Fenoprofen: 1 ml of a 0.1 mg ml⁻¹ solution; EDC: 300 μl of a 1.0 mg ml⁻¹ solution; HOBT: 300 μl of a 1.0 mg ml⁻¹ solution). (b) Influence of the EDC concentration on the reaction yield of benzylamide derivative of fenoprofen. (Fenoprofen: 1 ml of a 0.1 mg ml⁻¹ solution; benzylamine: 300 μl of a 0.5 mg ml⁻¹ solution; HOBT: 300 μl of a 1.0 mg ml⁻¹ solution).

complementary decrease of formed amide (Fig. 4b). Addition of 250 μl of a 1 mg ml⁻¹ EDC solution to 0.1 mg of acid was considered minimum, meaning that at least three times the molar equivalent of coupling agent versus acid was added. Addition of more EDC lead to constant parameters. Lower amide yields were obtained when about 2 mg HOBT was added to 0.1 mg of acid. Higher amounts gave merely constant values. Hence relative excess of HOBT was added. After a reaction time of about 75 min up to 120 min, the AUC of the amides was virtually constant.

These results generally coincided well with literature data [69]. The concentration of carbodiimide was found to have no significant effect above 200 μg per 100 μg of acid, whereas increasing the quantity of amine decreased the formation of the amide. Ad-

dition of HOBT increased the reaction yield in quantities as small as 5 μg per tube of 100 μg pirprofen and at concentrations of 10 μg per tube or above, the reaction was essentially quantitative.

For the acids under investigation in this study, the given derivatization procedure (see Experimental) was assumed to be an acceptable compromise for the formation of benzylamide derivatives of benoxaprofen with the highest and ibuprofen with the smallest molecular weight. As the derivatization of a single enantiomer gave rise to only one peak and the area under the curve (AUC) of the derivatives was virtually the same for both enantiomers, this procedure was thought to cause no stereospecific derivatization.

The reproducibility of derivatization was tested for the benzylamide derivative of ibuprofen. The RSD of the AUC of the first eluting amide peak measured 1.75%, while the fault on injection ($n = 10$) was 1.49%, using no internal standard. Hence the derivatization showed good reproducibility. Linearity was tested by injecting benzylamide derivatives obtained from 0.5 to 20 μg ibuprofen; a good linear plot ($r > 0.999$) was observed.

Purification and identification of the benzylamides

Prior to confirmation of the identity of the formed derivatives, a purification of the reaction mixture was carried out. The reaction was performed on 20 mg acid per sample. Following the reaction period, the dichloromethane solution was extracted with an aqueous dilution of HCl at pH 1. Injection of the acid layer in the described chromatographic conditions revealed no detectable presence of amides. The washing step enabled removal of HOBT and coupling product. The dichloromethane layer was filtered over a phase separating filter and evaporated under a nitrogen stream. TLC experiments were performed to determine the appropriate hexane-ethyl acetate ratio for elution on a small silica column. A narrow glass tube was filled with about 3 g silica (70–230 mesh, Merck) and the sample was fractionally collected. The proper fractions were combined and dried.

Depending on the consistency of the residues, *infrared spectra* were recorded either as KBr pellets (13 mm) or as cast films of a concentrated dichloromethane solution on KBr. The spectra were ratioed against a KBr

Table 2
Separation of amide derivatives of 2-arylpropionic acids with an increasing percentage of perchlorate buffer 0.1 M pH 2.0 in methanol (MP)

MP	Analyte	$k'1$	$N1$	$k'2$	$N2$	R_s	α
20%	Naphthylmethyamides						
	Carprofen	17.74	694	22.14	693	1.38	1.25
	Fenoprofen	9.46	1054	11.40	851	1.30	1.21
	Pirprofen	6.10	672	7.69	587	1.26	1.26
	Ibuprofen	2.18	704	3.61	624	2.35	1.65
	Ketoprofen	5.87	472	5.87	472	0	1.0
	Flurbiprofen	14.08	1663	15.11	746	0.54	1.07
	Tiaprofenic acid	6.24	589	9.14	422	1.84	1.47
10%	Benoxaprofen	8.61	487	23.89	518	4.91	2.77
20%	Benzylamides						
	Carprofen	5.83	765	7.11	575	1.09	1.22
25%		13.60	614	17.21	492	1.28	1.27
30%		28.22	778	36.55	595	1.67	1.30
20%	Fenoprofen	1.80	884	2.43	750	1.44	1.35
25%		3.67	708	5.18	610	1.79	1.41
30%		6.82	633	9.98	554	2.04	1.46
20%	Pirprofen	1.78	889	2.17	628	0.90	1.22
25%		3.20	667	4.02	666	1.16	1.26
30%		5.15	615	6.62	580	1.31	1.29
20%	Ibuprofen	0.82	1067	1.02	943	0.85	1.25
25%		1.53	776	2.01	703	1.20	1.32
30%		2.77	671	3.75	625	1.45	1.35
20%	Ketoprofen	1.34	879	1.71	655	1.00	1.28
25%		2.54	711	3.42	513	1.35	1.35
30%		4.06	623	5.69	455	1.58	1.40
20%	Flurbiprofen	3.12	780	3.82	655	1.05	1.21
25%		7.06	1012	8.83	830	1.49	1.25
30%		13.11	1006	16.60	846	1.67	1.27
20%	Tiaprofenic acid	1.73	992	2.04	765	0.78	1.18
25%		3.23	862	3.85	686	0.95	1.19
30%		5.36	763	6.45	549	1.00	1.20
10%	Benoxaprofen	2.56	825	6.36	538	4.31	2.49
20%		8.43	599	25.11	661	5.97	2.98

Chromatographic conditions: see Experimental.

background (200 scans). The C=O stretch band of the underivatized acid (1730–1700 cm^{-1}) shifted to lower wavenumbers (amide I band 1660–1630 cm^{-1}). A single νNH absorption band appeared in the region of 3300–3215 cm^{-1} and the amide II band was found within 1550–1535 cm^{-1} .

The *mass spectra* all contained the molecular ion and fragmentation ions of the amide binding.

Liquid chromatography

Considering the data in Table 2, it is clear that, except for ibuprofen and tiaprofenic acid, the benzylamide derivatives were resolved to a higher extent than their corresponding naphthylmethyamides (Fig. 5). As expected for chiral polymer columns, plate numbers were quite low. As for benoxaprofen, the second eluting enantiomer amide derivative was retained so long on the column, that an adaptation of the mobile phase towards a lower

buffer fraction was preferred so as to detect it within a reasonable time limit. Piroprofen (Almirall, Barcelona, Spain), an amide of ketoprofen and 2-amino, 4-methylpyridine, was not resolved. Protizinic acid (99.2%, Rhône-Poulenc Rocer, Brussels, Belgium) could not be resolved as benzylamide derivatives and was only poorly resolved as naphthylmethyamide ($\alpha = 1.15$, $R_s = 0.87$).

For ibuprofen and carprofen the elution order was (R) before (S) as determined with authentic reference samples. For pirprofen it was assumed to be similar [69]. Figure 6 depicts a chromatogram of a mixture of the benzylamide derivatives of three compounds: ibuprofen, fenoprofen and flurbiprofen.

Increasing the buffer fraction had a similar effect on the benzylamide derivatives as on the free acids: the analytes eluted slower, resulting in an improved α -value. No significant improvement of the separation was obtained increasing the buffer molarity up to 0.5 M. The

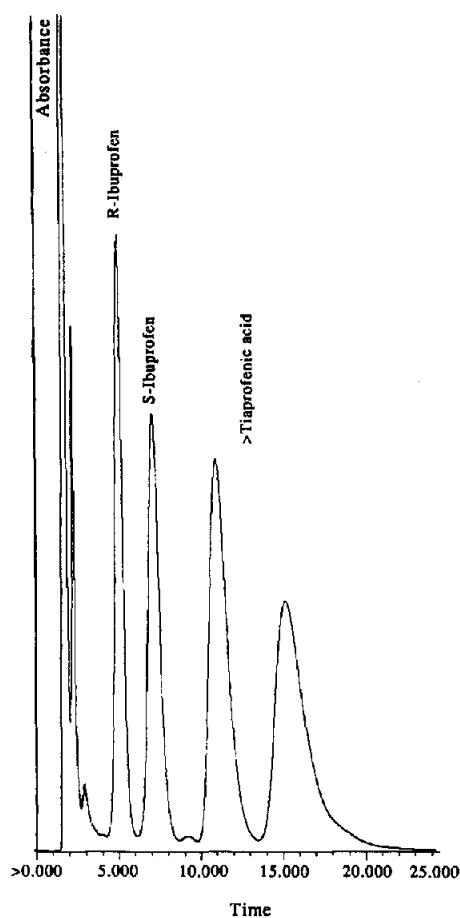


Figure 5

Naphthylmethylamine derivatives of ibuprofen and tiaprofenic acid (1:2:5, v/v). Mobile phase: methanol-perchlorate buffer 0.1 M pH 2.0 (80:20, v/v), 1.0 ml min⁻¹. Detection at 230 nm.

use of acetonitrile instead of methanol caused a faster elution of the analytes. Increasing the perchlorate buffer portion up to 60% as often applied for the Chiralcel OD-R column [56, 57] could not compensate for the loss in resolution. A partial resolution was only obtained for the benzylamide derivatives of ibuprofen ($\alpha = 1.10$), tiaprofenic acid ($\alpha = 1.06$) and benoxaprofen ($\alpha = 1.44$). Benoxaprofen was resolved as free acid into its enantiomers ($\alpha = 1.13$).

The chiral recognition mechanisms of cellulose based CSPs have not been fully explained so far in the specific literature. Cellulose derivatives have been classified as chiral phases containing cavities. Derivatization of the carboxylic acids into amides has provided structures with possibilities of dipole, H-bonding and π - π interactions, which were more likely to interact with the ester derivative of cellulose. The mobile phase also played a

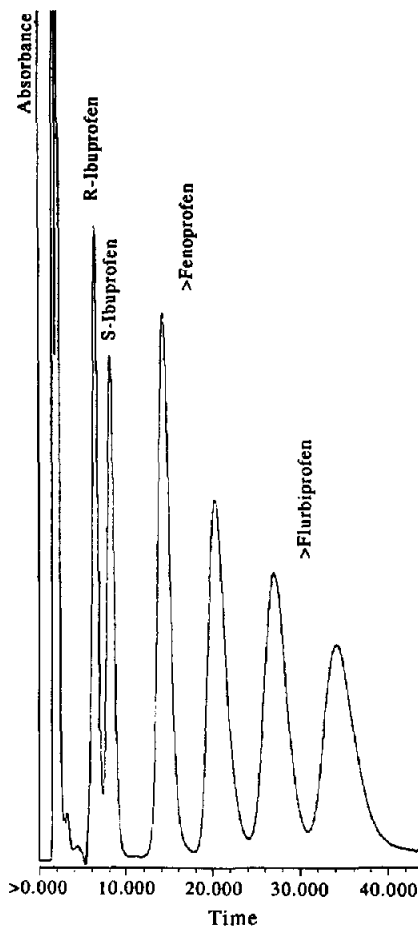


Figure 6

Benzylamide derivatives of ibuprofen, fenoprofen and flurbiprofen (mixture of equal volumina of amide solutions, see Experimental, *Derivatization procedure*). Mobile phase: methanol-perchlorate buffer 0.1 M pH 2.0 (70:30, v/v), 1.0 ml min⁻¹. Detection at 230 nm.

crucial role in the stereoselective character of the cellulose. Methanol was to be preferred to acetonitrile. Wainer and Alembik have studied the chiral mechanism of amides interacting with a tribenzoate cellulose phase [64]. In their study, they concluded that a stereospecific interaction was the result of three interrelated aspects. A diastereomeric complex was temporarily formed between the analyte and the cellulose layer through attractive interactions. These interactions involved H-bonding π - π and dipole interactions between the amide bond of the analyte and the ester bond of the CSP. Within this complex, the amide is positioned thanks to at least two of the possible interactions. Finally, the relative stability of the diastereomeric complexes and thus the extent of chiral resolution is determined by the steric fit of the asymmetric portion of the analyte in a chiral cavity of the CSP.

Conclusion

The investigated tris(4-methylbenzoate)-cellulose column has proved to be suitable for the enantiomeric resolution of a representative group of non steroidal antiinflammatory drugs under reversed-phase conditions. Methanol gave better results than acetonitrile. Derivatization of the carboxylic group into an amide enhanced the stereospecific resolution. The use of a carbodiimide coupling agent and HOBt as catalyst in convenient conditions was found to be a good reproducible method.

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